Progesterone Receptor Isoforms : functional Selectivity and Pharmacological Targeting

Junaid Ali Khan

UNIVERSITE PARIS-SUD 11
FACULTE DE MEDECINE PARIS-SUD

Année 2011

N° attribué par la bibliothèque

I_I_I_I_I_I_I_I_I_I_I

THESE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITE PARIS-SUD 11

Champ disciplinaire : Endocrinologie Moléculaire et Cellulaire
Ecole Doctorale : Signalisation et Réseaux Intégratifs en Biologie

Présentée et soutenue publiquement par

Junaid Ali KHAN

Le 6 Octobre 2011

Titre :

Progesterone Receptor Isoforms :
Functional Selectivity and Pharmacological Targeting

Directeur de thèse: Dr Hugues LOOSFELT

JURY

Président :	**Monsieur Jack-Michel RENOIR**
Rapporteur :	**Monsieur Vincent CAVAILLES**
Rapporteur :	**Monsieur Philippe-Claude LEFEBVRE**
Examinateur :	**Madame Michèle RESCHE-RIGON**
Examinateur :	**Monsieur Fabrice ANDRE**
Directeur de thèse :	**Monsieur Hugues LOOSFELT**

ACKNOWLEDGEMENTS

It was a long journey finally ending to a thesis defense. And I would like to thank the people who supported me during this important period of my life. First of all I would like to thank members of the jury who accepted to evaluate my thesis. **Drs Vicent Cavaillès** and **Philippe Lefebvre** who accepted to analyze and judge the work presented in this thesis. I address them my heartiest thanks. I am thankful to **Dr Jack-Michel Renoir** for honoring me to be the president of the jury and **Drs Michel Resche-Rigon** and **Fabrice André** for accepting to be the examiners of my thesis.

Hugues, je voudrais te remercier pour beaucoup des choses, d'abord de m'accepter pour réaliser ce travail dans ton laboratoire. J'ai pu profiter de ton expérience dans le domaine des récepteurs des hormones stéroïdes surtout celui de la progestérone, dont tu es l'un des premiers chercheurs à avoir dévoiler ses secrets. Grâce à ton aide et la liberté que tu m'as accordée pour explorer les mécanismes de la diversité fonctionnelle des isoformes du PR, je crois que je suis devenu un chercheur bien entrainé et indépedant. Tu as été très patient avec moi pendant toutes ces longues périodes. Tu m'as encouragé et motivé quand j'étais bloqué à un moment donné. Pendant tes vacances, je ne souviens pas une seule fois quand je ne t'ai pas contacté par téléphone et emails pour discuter de mes résultats. Tu étais toujours disponible et à mon écoute pour me donner tes conseils précieux. Je te remercie pour toutes ces raisons et on restera bien sûr en contact.

Marc, je manque de mots pour pouvoir te remercier, tellement c'est grand. C'était un honneur pour moi de travailler avec toi. Tu m'as appris beaucoup des choses. J'ai fait plus de manips avec toi qu'avec n'importe quelle autre personne. J'ai eu vraiment de la chance. Tu m'as donné la confiance et la maturité dans les idées scientifiques. J'ai beaucoup apprécié ta façon de regarder les choses et pour le maintien d'une ambiance amicale dans le labo. Tu étais disponible quand j'ai eu besoin de ton aide que ce soit pour les manips, pour discuter de mes résultats, pour écrire/corriger mes articles ou pour résoudre des problèmes administratifs. Merci encore pour la relecture de ma thèse. J'espère que tu comprendras mes sentiments respectueux et amicaux. Je pars avec de nombreux beaux souvenirs de toi. Merci pour tout.

Merci beaucoup **Marie-Edith** pour ton aide précieuse dans la partie pharmacologique de ma thèse. Tu m'as beaucoup aidé surtout dans ces derniers jours. J'ai appris pas mal des choses par toi pendant cette courte durée de travailler à tes coté. Je tiens également à remercier nos chers collaborateurs **Mouad** et **Monsieur Jean-Daniel Brion,** et **Michel** pour ces nombreuses réunions qui étaient très intéressantes et enrichissantes pour l'avancement de nos travaux collectifs.

Merci **Nathalie,** en dehors de notre collaboration qui continue à ce jour, tu étais la première personne à m'apprendre la culture cellulaire. Je tiens à remercier **Nadine** et **Anne Mantel** pour leurs soutients et encouragements. **Larbi,** thanks buddy for your precious help on so many occasions related to both professional and personal life. Your company will remain a highly valuable and memorable asset for me. **Catherine,** We have shared the ups and downs of being a PhD student. Long discussions on music, films, culture and of course science were very interesting. It was a lot of fun playing tennis or going to cinema. Thank you for your help, company and being a great co-worker. Bonne chance pour ta thèse!

Say, I remember you helped me a lot particularly in the beginning, provided me company and your friendship, many many thanks. Thank you **Damien,** I always enjoyed talking with you. I took the advantage of working with you in cell culture lab and learnt a lot of things. You were available whenever I consulted you. Dear **Géri**, your delicious cakes and sweet hug will remain in my memory for a long time. Thanks for your help in reading and correcting my English from time to time and particularly for your help in immunocytochemistry. I would like to thank my current lab fellows; **Nadège, Justine, Julien, Audrey, Jérôme F, Jérôme B, Jérôme N, Séverine, Claudia, Mériem, Claudia, Luc, Lavinia, Ségolène, Charlotte, Luigi,** and **Simon**. Working with you had been a pleasure.

I cannot go without thanking to my former colleagues and friends.

Bruno, Doctor Love, merci pour tes conseils et disponibilité pour les réunions 'sex steroide'. Tu as de très bonnes idées scientifiques. Je n'ai pas eu l'occasion de travailler avec toi mais j'ai toujours apprécié de parler avec toi. **Laetitia,** thanks for your company. I appreciated your letter writing skills ;-) that I will never forget. You were the key to many extracurricular activities in the laboratory. To name a few, hiding chocolate eggs in L1 and elsewhere at Pâques, organizing grand déjeuner de noël et celui de l'été. We missed you. Thanks for all the fun and nonsense conversations. **Adéline,** Merci pour maintenir l'ambiance amicale dans le labo et pour ton organisation extraordinaire dans les affaires quotidiennes du laboratoire. Merci pour partager tes expertises scientifiques. **Mathilde,** I shall remember you for a long period, thanks for all the discussions during travelling in RER and in 125. Merci à **Emmanuelle, Bruno, Frédéric, Vixra, Morwenna, Adela, Céline** and **Cécile,** qui ont été de bons copains et dont l'amitié m'a été précieuse au cours de ces années. On reste en contact. Dear **Kahina,** our friendship oscillated between good and bad periods. The same continues till today. I enjoyed a lot talking with you. Thanks for your company. **Peter,** the time passes very quickly. Thanks for your help in my initial days in lab.

Special thanks to **my parents and family**, who always supported me. Thanks for believing in me.

LIST OF PUBLICATIONS

This thesis is based on following publications which are referred to in the text by the Roman numerals :

I. **Khan JA**, Amazit L, Bellance C, Guiochon-Mantel A, Lombès M and Loosfelt H. p38 and p42/44 MAPKs differentially regulate progesterone receptor A and B isoform stabilization. *Mol Endocrinol*, 2011, 25(10):0000-0000 Epub ahead of print Aug 4, 2011 as doi:10.1210/me.2011-1042

II. Amazit L, Roseau A, **Khan JA**, Chauchereau A, Tyagi RK, Loosfelt H, Leclerc P, Lombès M and Guiochon-Mantel A. Ligand-dependent degradation of SRC-1 is pivotal for progesterone receptor transcriptional activity. *Mol Endocrinol*, 2011 25(3):394-408

III. **Khan JA**, Bellance C, Guiochon-Mantel A, Lombès M and Loosfelt H. Cell proliferation genes differentially regulated by progesterone receptor isoforms PRA and PRB in a new bi-inducible breast cancer cell line. *Submitted manuscript*.

IV. **Khan JA**, Tiked A, Fay M, Hamze A, Fagart J, Chabbert-Buffet N, Brion JD, Alami M, Lombès M, Loosfelt H and Rafestin-Oblin ME. A new rationally designed homosteroid ligand with high progesterone receptor selectivity and full antagonist properties. *Submitted manuscript.*

INTERNATIONAL PATENT

Marie-Edith Rafestin-Oblin, Mouad Alami, Hugues Loosfelt, Abdallah Hamze, **Junaid A Khan**, Abdellatif Tikad, Marc Lombès, Jean-Daniel Brion. Progesterone receptor antagonists and uses thereof Patent INSERM-CNRS-UPS; EP N°10305484.7, May 7, 2010, International PCT/EP2011/057387, May 9, 2011.

RELATED PUBLICATION

During PhD, the author has also contributed in other related projects. The following publications are under preparation and are not included in the thesis.

Hamze A, **Khan JA**, Tiked A, Fay M, Fagart J, Chabbert-Buffet N, Lombès M, Loosfelt H, Brion JD, Rafestin-Oblin ME, Alami M. Structural basis of progesterone receptor inactivation with new class antagonists. *Manuscript in preparation.*

Bellance C, **Khan JA**, Lombès M and Loosfelt H. Conditional expression of progesterone receptor isoforms PRA and/or PRB impacts breast cancer cell migration via urokinase system and focal adhesion kinase activities. *Manuscript in preparation*.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

aa	Amino acids
AF	Activation function
AP1	Activating protein 1
AR	Androgen receptor
ATP	Adenosine triphosphate
BUS	B-upstream segment
CBP	CREB-binding protein
cDNA	Complementary DNA
CREB	CRE-binding protein
DBD	DNA-binding domain
DHT	Dihydrotestosterone
EGF	Epidermal growth factor
ER	Estrogen receptor
ERE	Estrogen response element
FCS	Fetal calf serum
FKBP5	FK506 binding protein 5
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HB	Heparin binding
HB-EGF	HB-EGF-like growth factor
HRE	Hormone response element
Hsp	Heat shock protein
IF	Inhibition function
JNK	c-Jun-N-terminal kinase
kDa	Kilo Dalton
LBD	Ligand-binding domain
MEK	MAPK kinase
MEKK	MEK kinase
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
N	Amino
NCoR	Nuclear receptor corepressor
NES	Nuclear export signal
NF-κB	Nuclear factor kappa B
NLS	Nuclear localization signal
NTD	N-terminal domain
N-ter	N-terminal domain
PCR	Polymerase chain reaction
PR	Progesterone receptor
PRA	Progesterone receptor isoform A
PRB	Progesterone receptor isoform B
PRE	Progesterone response element
RAR	Retinoic acid receptor
RID	Receptor interacting domain
RNA	Ribonucleic acid
Sgk1	Serum and glucocorticoid regulated kinase 1
SMRT	Silencing mediator of retinoid and thyroid receptors
SP1	Specificity protein 1
SRC	Steroid receptor coactivator
STAT	Signal transducers and activators of transcription
SUMO	Small ubiquitin related modifier
TBP	TATA binding protein
TFII	Transcription factor for polymerase II
TR	Thyroid hormone receptor

TABLE OF ILLUSTRATIONS

PREFACE

In the beginning of twentieth century, it was established that corpus luteum is an endocrine gland, necessary for the continuation of pregnancy (Fraenkel, 1903). Three decades later progesterone was purified from corpus luteum (Fels and Slotta, 1931) and its biological role was elaborated (Allen and Doisy, 1923; Corner and Allen, 1929). The concept of steroid hormone "receptors" originated in 1962 (Jensen and Jacobsen, 1962) and sooner it was established that steroid hormone binds to specific cytoplasmic receptor protein, steroid-receptor complex translocates to nucleus and binds to acceptor sites on the genome to activate the transcriptional apparatus and produce target-tissue specific increase in intracellular amounts of specific mRNA molecules (O'Malley and Means, 1974). Progesterone receptor (PR) was identified independently by teams of B.W. O'Malley, E.E. Baulieu and E. Milgrom in 1970 (Milgrom et al., 1970; O'Malley et al., 1970). The first hormone receptor (glucocorticoid receptor) was cloned by Evans group in 1985 (Hollenberg et al., 1985) and almost simultaneously the complete amino acid sequence of human PR was established from cloning of complementary DNA by E. Milgrom's group (Loosfelt et al., 1986). Soon after, P. Chambon's group identified that two functionally different human progesterone receptor forms A and B (PRA and PRB) are transcribed from two distinct estrogen-regulated promoters (Kastner et al., 1990). Implication of PR in reproductive functions was demonstrated by PR knock-out mice (Lydon et al., 1995) and functional distinction/target tissue specificity of PR isoforms was validated in mice models (Conneely et al., 2003; Mulac-Jericevic et al., 2003; Mulac-Jericevic et al., 2000). Almost simultaneously but independently, studies in transgenic mice demonstrated the importance of balanced PR isoforms ratio in normal reproductive physiology (Shyamala et al., 2000; Shyamala et al., 1998). However, the mechanisms underlying the alteration in PR isoforms ratio and its consequence in human pathophysiology are yet to be elaborated.

In 1995, the concept that steroid receptors modulate gene expression via recruitment of transcriptional coregulators was established with the discovery of SRC1, the first transcriptional coactivator of steroid receptors (Onate et al., 1995). To-date more than 200 transcriptional coregulators (coactivators and corepressors) are known (O'Malley, 2007) and cellular response to a given hormonal stimulus is in part determined by coactivator/corepressor expression ratio (Liu et al., 2002) and cellular as well as promoter context (Smith and O'Malley, 2004). Therapeutic use of progesterone for the treatment of female reproductive disorders started in early 1940's (Allen, 1941). Thus studying the effects of progesterone on various aspects of pathophysiology using mouse model gained interest and the first report on the role of progesterone in mice mammary cancer development dates back to 1946 (Burrows and Hoch-Ligeti, 1946). Since then both

beneficial and adverse effects of progestins/progesterone in mammary and endometrial carcinogenesis have been reported (Africander et al., 2011; Howell and Evans, 2011; Yang et al., 2011a). In 2002, it was first shown that progestins along with estrogen in hormone replacement therapy increased breast cancer risk in postmenopausal women (Rossouw et al., 2002). Recently, various independent studies have demonstrated an important role of progesterone signaling in mammary carcinogenesis via paracrine mechanism (Beleut et al., 2010; Gonzalez-Suarez et al., 2010; Schramek et al., 2010). These observations have renewed interest in understanding the role PR in carcinogenesis as well as in the development of PR antagonists.

Since the discovery of the first PR antagonist mifepristone (RU486) in 1981 by Philibert and Baulieu (Herrmann et al., 1982), several steroidal or non steroidal molecules have been synthesized. These molecules display PR agonistic (levonorgestrel) or antagonistic (onapristone) or mixed agonistic/antagonistic (RU486, asoprisnil) properties. Some of these compounds are under pre-clinical or clinical development phase for being used in contraception, hormone replacement therapy, uterine fibrosis, endometriosis and certain hormone-dependent cancers (Bungay et al., 2011; Chabbert-Buffet et al., 2005). However, side effects associated by the long term use of the currently available antiprogestins including RU486 are explained by their lack of PR specificity and partial agonist effects. Currently, several pharmaceutical companies and research laboratories are focusing on the development of PR selective antiprogestins devoid of partial agonist effects for several therapeutic applications.

INTRODUCTION

1. Progesterone Receptor

1.1 Progesterone

Progesterone also known as P4 (pregn-4-ene-3,20-dione) is a C-21 steroid hormone essentially synthesized by ovaries, to a lesser extent by testes and adrenal glands, from second trimester of pregnancy by placenta, and to some extent from neurons (Lu et al., 2006; Schumacher et al., 2008). Progesterone is synthesized from cholesterol molecule characterized by cholestane nucleus or cyclo-pentano-phenanthrene skeleton comprising of three hexagonal cycles, A, B, C and one pentagonal cycle D **(Figure 1)**. Desmolase enzyme converts cholesterol into pregnenolone which is then converted to progesterone by isomerase/dehydrogenase enzymes.

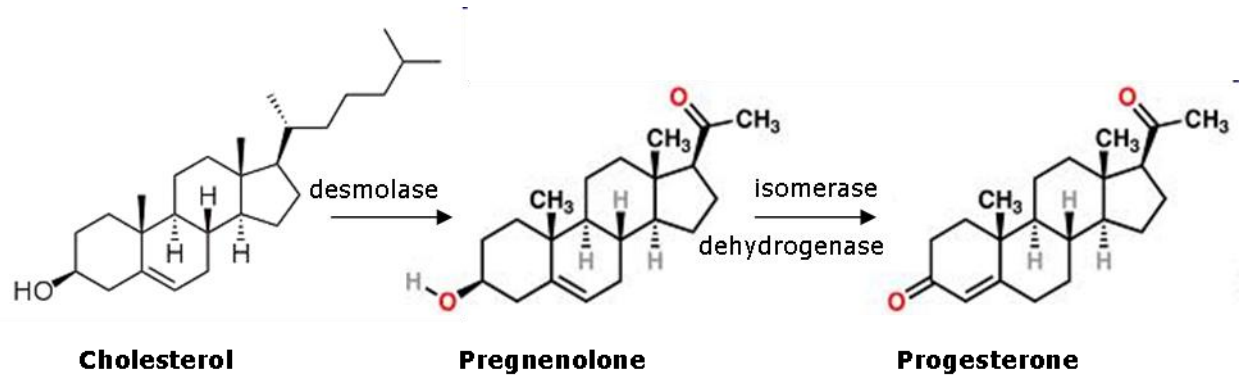


Figure 1. Biosynthesis of progesterone hormone.
Progesterone is synthesized from cholesterol by action of desmolase enzyme which converts cholesterol into pregnenolone which is then converted into progesterone by the action of isomerase/dehydrogenase enzymes.

Progesterone secretion is not constant during menstrual cycle. Just before ovulation, progesterone secretion is induced by luteinizing hormone (LH) secreted by pituitary gland and is maintained till second part of menstrual cycle. Progesterone level diminishes near the end of cycle resulting in menstruation. In case of fertilization, chorionic hormone secreted by trophoblast cells maintains corpus luteum which then continues to secrete progesterone during gestation. After first trimester, placenta becomes major source of progesterone till delivery. In plasma, progesterone exists both in free form as well as bound to albumin or transcortin or sex hormone binding protein. Progesterone is catabolized into pregnanediol mainly in liver by several enzymes and is also a precursor of testosterone, aldosterone and cortisol synthesis (Lu et al., 2006).

1.2 Mammary gland development-Role of progesterone and its receptor

Progesterone plays pivotal role in normal female reproductive functions in the uterus, ovary, mammary gland and brain. Progesterone is also important for normal functioning of several non-reproductive tissues such as cardiovascular system, bone and central nervous system (Graham and Clarke, 1997; Li et al., 2004; Mote et al., 2007; Rocha and Soares, 2009).

During pregnancy, progesterone is important for uterine quiescence and mammary gland development. Physiological actions of progesterone are mediated via intracellular progesterone receptor (PR) expressed in various tissues including mammary glands, endometrium, myometrium, fallopian tube, vagina, hypophysis, hypothalamus, cerebral cortex, testes, thymus, kidney and prostate (Scarpin et al., 2009). It is generally believed that until puberty, mammary gland development occurs independently of hormone. However, hormone receptors are expressed before puberty (Brisken and O'Malley, 2010; Grimm et al., 2002; Hovey et al., 2001; Stumpf et al., 1980) and perinatal exposure to exogenous hormones or endocrine disruptors results in developmental abnormalities (Bern et al., 1983). This suggests that even in the absence of hormone, receptors play a role in mammary gland development since exposure to endocrine disruptors leads to abnormal development due probably to divergence of the physiological role played by ligand-free hormone receptors. At puberty, mammary gland development i.e., ductal elongation, side branching (during recurrent estrous cycles) and alveologenesis (during pregnancy) occurs under the major influence of ovarian hormones estrogen and progesterone, pituitary hormones prolactin and growth hormone, and to some extent adrenal hormone cortisol. To decipher the role of individual hormone in mammary gland development, specific cognate receptor knock-out mice have been generated. Mice deficient in either estrogen receptor (ERα or ERβ) or PR or prolactin receptor (Antal et al., 2008; Dupont et al., 2000; Lydon et al., 1995; Ormandy et al., 1997) are viable but sterile whereas sexual maturation is delayed in growth hormone receptor knock-out mice (Spencer et al., 1997). PR null mice display pleiotropic reproductive abnormalities including inability to ovulate, uterine hyperplasia and inflammation, severely limited mammary gland development, and impaired thymic function and sexual behavior (Lydon et al., 1995). In mouse mammary gland, PR is expressed as two isoforms (PRA and PRB) in both epithelial and stromal compartments (Haslam, 1989; Haslam and Shyamala, 1981). Mammary transplants lacking PR in the stromal compartment did not affect mammary gland development while side branching and alveologenesis was perturbed in PR-/- epithelial grafts (Brisken et al., 1998; Lydon et al., 1995). This indicates that progesterone acts by paracrine mechanism on a subset of epithelial cells for normal mammary gland development. Furthermore, mouse strains selectively invalidated for PRA or PRB showed that only PRB is required for mammary gland development while PRA is crucial for ovarian and uterine functios (Mulac-Jericevic et al., 2003; Mulac-Jericevic et al., 2000).

1.3 Progesterone receptor – a member of nuclear receptor family

Progesterone receptor (PR), also known as NR3C3, is a ligand-induced transcription factor which belongs to Class III of nuclear receptor (NR) superfamily (Mangelsdorf et al., 1995; McEwan, 2009). Such nuclear receptors are functionally composed of following main domains **(Figure 2)**: a less conserved N-terminal region (A/B) harboring inhibition function (IF) and hormone-independent activation function 1 (AF1), a central highly conserved DNA binding domain (C) composed of two zinc-containing motifs also known as zinc fingers. The first zinc finger is involved in target site recognition and the second zinc finger plays a role in stabilization of NR-DNA interactions and receptor dimerization.

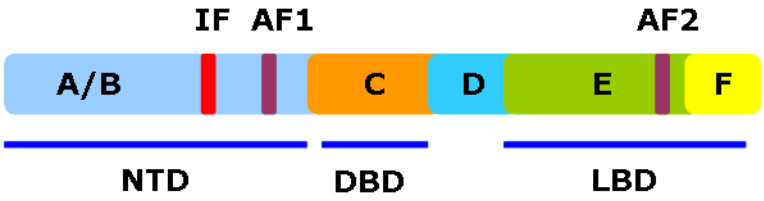


Figure 2. Classical structure of nuclear receptors.
The nuclear receptors are comprised of three main domains, N-terminal domain (NTD), DNA-binding domain (DBD) and ligand-binding domain (LBD). Location of Activation Function 1 (AF1), Activation Function 2 (AF2), Inhibitory Function (IF) are indicated.

NR are also composed of a variable hinge region (D), a variable C-terminal region (E/F) harboring ligand binding domain and hormone-dependent activation function 2 (AF2). Activation function 3 (AF3) is encoded by the full length PR transcript (PRB) and is present at the N-terminal extremity. In addition to AF, inhibitory function (IF) elements are also present in N-terminal region (Hovland et al., 1998). Deletion of all or part of this autoinhibitory region in PR increases its transcriptional activity. Indeed, AF and IF enhance or repress transcriptional activity by association of these regions with transcriptional coregulators (Brisken et al., 1998; Clemm et al., 2000; Lanz et al., 1999; McEwan, 2009; McKenna and O'Malley, 2002; Sartorius et al., 1994; Vegeto et al., 1993).

1.4 PR isoforms

Human PR gene, located on chromosome 11 (11q2.2), codes for two receptor isoforms (PRA and PRB) by alternate initiation of transcription at internal translational start sites from two distinct estrogen regulated promoters (Kastner et al., 1990; Tetel et al., 1999). Majority of PR positive cells express equal amounts of PRA and PRB isoforms under physiological conditions. PRB is full length protein comprising of 933 amino acids (114 kDa). PRA is truncated in the N-terminal region and lacks the first 164 amino acids (94 kDa) harboring activation function 3

(AF3) which is present in PRB unique sequence (also called as BUS domain) **(Figure 3)**. Another PR isoform (PRC) has also been described which lacks first 594 amino acids. PRC lacks the N-terminal region and one zinc finger of DBD, thus unable to bind DNA and is transcriptionally inactive. PRC is nevertheless able to bind hormone and heterodimerize with full length PR, localizes in nucleus (Wei et al., 1990; Wei et al., 1996) and seems to be implicated in initiation of labor (Condon et al., 2006). Some *in vitro* studies show that PRC may either enhance or repress the activities of PRA and PRB (Wei et al., 1996; Wei et al., 1997), however, recent studies have questioned if any physiological role is played by PRC in progesterone signaling (Samalecos and Gellersen, 2008).

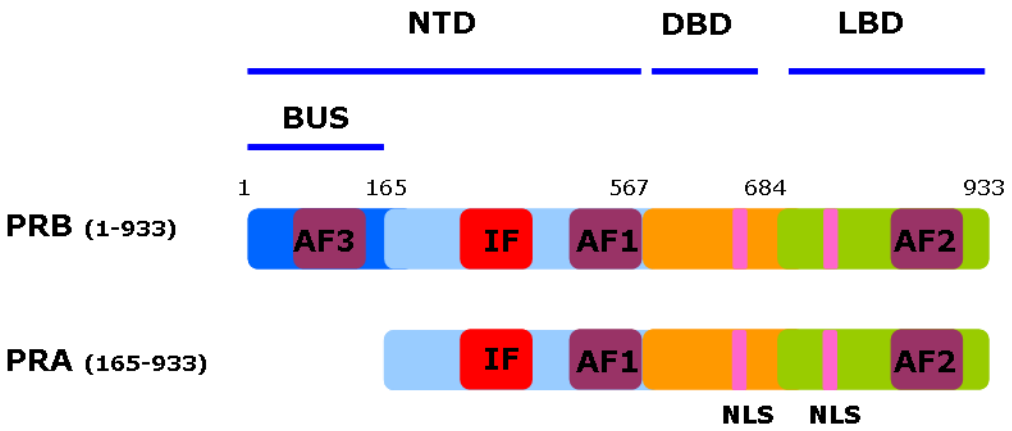


Figure 3. Structure of progesterone receptor isoforms PRB and PRA.
PRB and PRA share identical amino acids sequence except that PRB is full length receptor and PRA is truncated in N-terminal domain and lacks first 164 amino acids also called as PRB-unique sequence (BUS) which harbors Activation Function 3 (AF3). Like other nuclear receptors, PR isoforms are comprised of three main domains, N-terminal domain (NTD), DNA-binding domain (DBD) and ligand-binding domain (LBD). Activation Function 1 (AF1), Activation Function 2 (AF2), Inhibitory Function (IF), nuclear localization signals (NLS) are shown.

2. Mechanism of action of PR

2.1 PR a nucleo-cytoplasmic shuttling protein

Neo-synthesized PR, like other steroid hormone receptors such as glucocorticoid receptor (GR), mineralocorticoid receptor (MR), androgen receptor (AR) and estrogen receptor (ER), is assembled in an inactive multi-protein chaperone complex comprising of heat-shock protein of 90 kDa (hsp90) and hsp70 (Catelli et al., 1985; Picard, 2006). One role of these chaperone proteins is to maintain the steroid receptor in a conformation suitable for ligand binding (Picard, 2006; Pratt et al., 2004; Smith, 1993). Such complexes also protect the receptor from proteolytic damages in addition to keeping it in an inactive structure. In the absence of ligand, intracellular location of such complexes depends on the receptor type, for example, GR (DeFranco et al., 1991; Picard et al., 1990) and AR

(Kemppainen et al., 1992) are predominantly found in the cytoplasm while PR (Guiochon-Mantel et al., 1991) is predominantly present in the nucleus. However, cytonuclear localization of these receptors is also influenced by the cell type. Steroid receptors shuttle between the cytoplasm and nucleus due to presence of nuclear localization signals (NLS) (Baumann et al., 1999; Dauvois et al., 1993; DeFranco et al., 1991; Guiochon-Mantel et al., 1994; Guiochon-Mantel et al., 1991; Guiochon-Mantel et al., 1989). In PR, one highly conserved constitutive NLS, comprising of 638-642 amino acids, is localized in hinge region and is responsible for hormone-independent nuclear localization since its deletion leads to preferential cytoplasmic localization of the receptor in the absence of hormone (Guiochon-Mantel et al., 1989). A second NLS is located in second zinc finger of DBD between amino acids 593-638. This NLS is ligand-dependent since ligand binding unmasks this NLS region and leads to nuclear localization of PR following its dissociation from the heat-shock proteins/chaperone complexes. Ligand binding also induces various post-translational modifications in PR (Weigel and Moore, 2007). Classically, PR like other steroid hormone receptors binds as a dimer to a specific hormone response element (HRE) located upstream of target gene promoter (Hard et al., 1990; Luisi et al., 1991; Picard and Yamamoto, 1987) **(Figure 4)**. The DNA-bound receptor can then exert a positive or negative effect on gene transcription by recruiting either coactivators or corepressors.

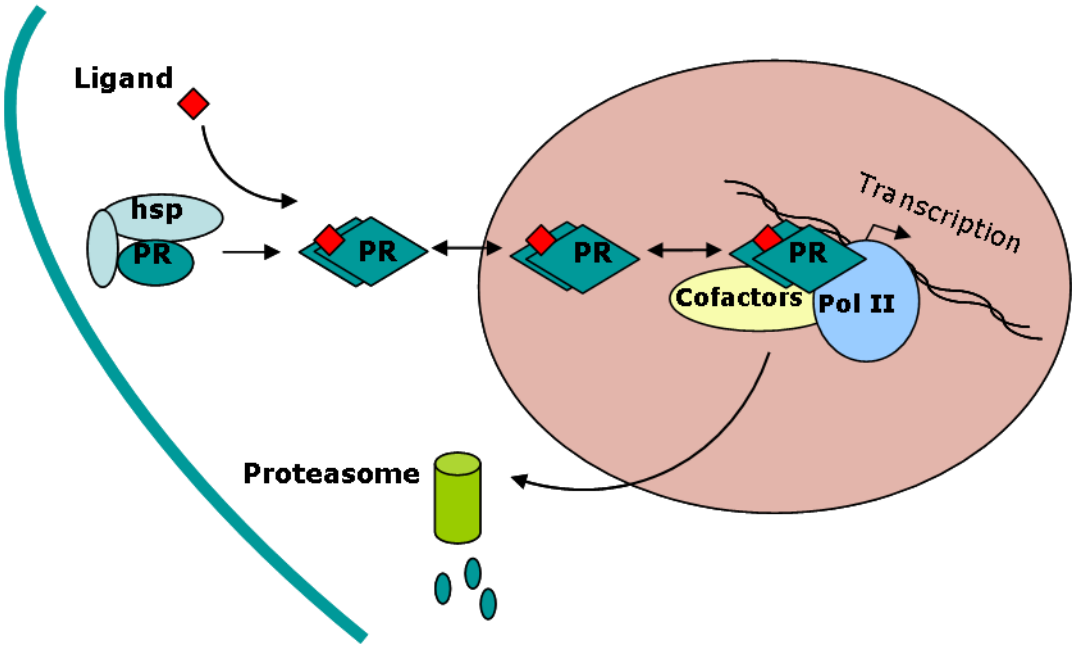


Figure 4. Classical mode of action of PR
In the absence of ligand, PR is associated with heat-shock protein (hsp) chaperone complexes in the cytoplasm. Upon ligand binding, conformational changes in PR allows it to dissociate from chaperone complexes, to dimerize and translocate in the nucleus. Inside nucleus, PR binds to its response elements present in target gene promoters, recruits transcriptional cofactors and modulates the transcription. Alongwith its transcriptional activities, PR undergoes to ligand-induced proteasome-mediated rapid turnover.

Coactivators positively regulate transcriptional efficacy by recruiting multiprotein complexes to DNA leading to chromatin remodelling and interaction with general transcription factors. Corepressors recruited to the DNA-bound receptor facilitate chromatin condensation and silence transcription. Numerous transcriptional coactivators and corepressors have been identified whose relative and absolute expression levels vary among different cell types. During the course of transcriptional activities, PR undergoes rapid hormone-induced proteasome-mediated degradation (Lange et al., 2000). Interestingly, PR turnover is important for its transcriptional properties (Shen et al., 2001) and both of these phenomena seems to be interconnected since blocking of proteasome-mediated PR degradation impedes PR transcriptional properties (Amazit et al., 2011; Dennis et al., 2005). Likewise, inhibition of protein neosynthesis abolishes proteasome-dependent PR turnover (Kahmann et al., 1998; Khan et al., 2011) ***[See also Results-PAPER I)*** **(Figure 5)** suggesting involvement of neosynthesized co-regulatory proteins in these processes. Whether the same molecular partners are implicated in ligand-induced PR transcriptional activity and proteasome-mediated degradation remains to be demonstrated.

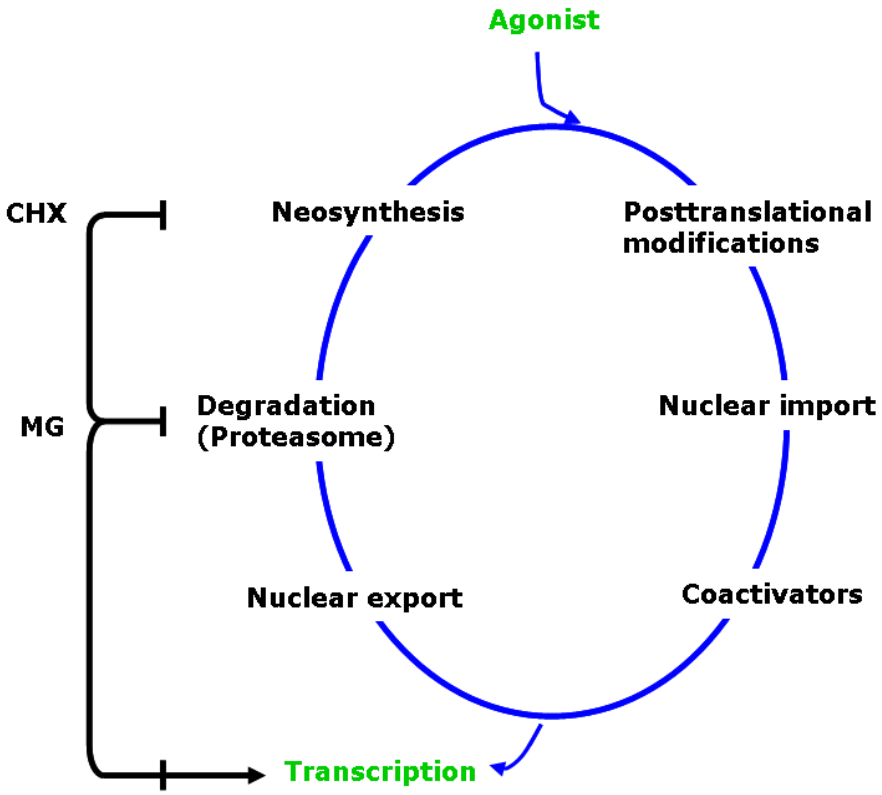


Figure 5. Coupling of PRB transcriptional activity and its down-regulation
Agonist ligand binding induces post-translational modifications in the receptor which then translocates to the nucleus and recuits transcriptional coactivators to enhance gene transcription. Receptor is thereafter degraded in the cytoplasm by proteasomes. Blockage of protein neosynthesis by cycloheximide (CHX) or proteasome-dependent protein turnover by using MG132 (MG) blocks the transcriptional activity of PR.

2.2 Non genomic actions of PR

Since PR is known to actively shuttle between cytoplasm and nucleus by active import and export mechanisms, recently it has been demonstrated that PR is capable of interacting with molecular partners in the cytoplasm (Boonyaratanakornkit et al., 2007). Therefore, beside classical genomic functions, PR isoforms are also capable of interacting with major cytoplasmic signaling kinases (p42/44 MAPK, c-Src/p21ras, PI3K/Akt pathways) (Carnevale et al., 2007) **(Figure 6)** frequently activated during carcinogenesis and resulting in progestin-inducible cell proliferation and metastasis.

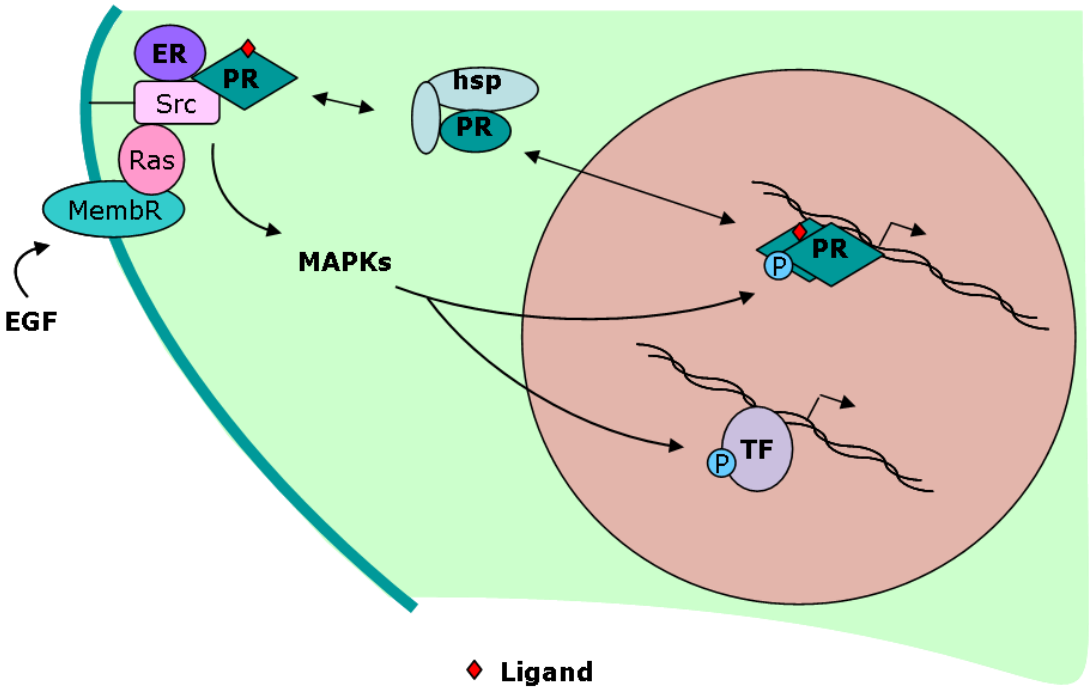


Figure 6. Non-genomic actions of PR
PR is a nucleo-cytoplasmic shuttling protein. Apart from its genomic effects, PR is known to interact with membrane associated receptors (MembR) and cytoplasmic proteins invloving Src-MAPKs pathways in a ligand-sensitive manner. Such interactions lead to activation of MAPKs which then phosphorylate PR and other transcription factors to modulate the transcription of both PR-regulated as well as PR-independent genes regulated by other transcription factors (TF).

The proline rich motif (PxxP) in the N-terminal domain of PR mediates interaction with Src homology 3 (SH3) domain of Src tyrosine kinase (Boonyaratanakornkit et al., 2001; Wardell et al., 2002). This explains molecular mechanism for some of the rapid non-genomic actions of progesterone. In some other studies, it has been shown that agonist-activated PRB but not PRA interacts with ER to activate Src/ras/MAPK pathway in breast cancer cells and hence could play an important role in growth-promoting effects in cancer cells (Migliaccio et al., 1998). This shows that PR isoforms might have differential impact on cytoplasmic signaling pathways. Such non-genomic pathways activated by PR influence transcription of not only PR-regulated but also PR-independent genes regulated by other transcription factors.

2.3 Genomic actions of PR

The genomic actions of PR are mediated either through its direct binding to specific DNA sequences or indirectly via association with other transcription factors. HRE is a consensus nucleotide sequence comprising of an inverted repeat sequence separated by three nucleotides i.e., AGAACAnnnTGTTCT called historically as glucocorticoid response element (GRE) since it was initially shown as a binding site for GR. Later on, it was found that GRE are also recognized by PR, AR and MR (Beato et al., 1989). However, transcriptional regulation by steroid receptors is not that simple as it appeared in early 1990's since recent thermodynamic studies have questioned this long accepted dogma, and probably rightly so knowing the fact that diverse physiological roles are played by these receptors which generally regulate different target genes. Most of our knowledge on DNA binding sites of steroid hormone receptors comes from two widely used promoters i) rat tyrosine amino transferase (TAT) promoter which harbors perfect palindromic GRE (Jantzen et al., 1987) ii) mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter which contains imperfect palindromic GRE (Hager, 1988; Lefebvre et al., 1991). These synthetic promoters were useful in understanding several aspects of transcriptional regulation by steroid hormone receptors. However, these sequences are not representative of endogenous progesterone responsive gene promoters since only 565 exact consensus GRE are present in whole human genome of which only 26 are located within 10 kb of the transcription start site of known genes (Horie-Inoue et al., 2006). Furthermore, binding of a steroid receptor to these GRE was confirmed only on 14 out of 26 perfect GRE (Horie-Inoue et al., 2006). Indeed, GRE are as abundant in PR regulated as in PR non-regulated gene promoters **(Figure 7)** (Jacobsen et al., 2009).

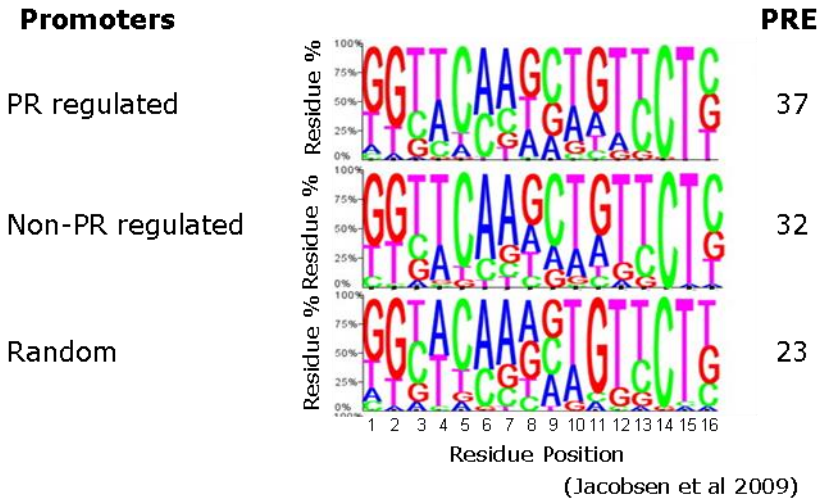


Figure 7. PRE abundance in PR regulated, non-regulated and random promoters. *In silico analysis showing PRE abundance in 12 PR-regulated or non-regulated or random promoter sequences retrieved from TRANSFAC database and compared with known PRE consensus sequence.*

In contrast to scarcely present GRE upstream of known genes, several half-sites are present in natural PR-regulated promoters (Jacobsen et al., 2009). It has now been shown that PR monomers are competent to bind progesterone response element (PRE) half-sites and show strong cooperativity in transcriptional regulation (Connaghan-Jones et al., 2008). Another level of complexity arises from the fact that PR regulates transcription without directly binding to DNA. This indirect transcriptional regulation is achieved by PR tethering to other DNA bound transcriptional factors such as specificity protein 1 (SP1), SP4, activating protein 1 (AP1), the RelA (p65) subunit of nuclear factor κB (NF-κB), signal transducers and activators of transcription (STAT) (Bamberger et al., 1996; Kalkhoven et al., 1996; Owen et al., 1998; Proietti et al., 2005; Richer et al., 1998; Shatnawi et al., 2007; Tseng et al., 2003)**(Figure 8)**. Thus PR regulates target gene transcription both directly as well as indirectly by association with other transcription factors without binding directly to promoters.

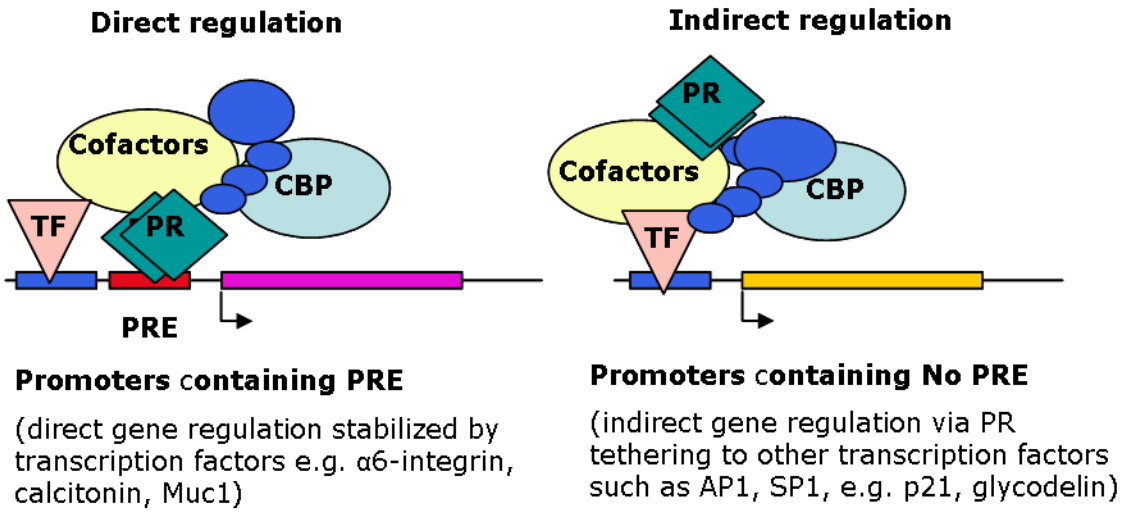


Figure 8 : Direct and indirect transcriptional regulation by PR
A schema representing transcriptional regulation by PR from promoters harboring or not PRE. Few examples of genes regulated by such mechanisms are also shown

3. PR-associated Transcriptional coregulators

Initial understanding of transcriptional regulation by NR was that NRs recruit general transcription factors and RNA polymerase II at gene promoters to start gene transcription (O'Malley and Means, 1974). However, *in vitro* transcriptional systems consisting of purified NRs and general transcription factors did not produce efficient transcriptional activation (Klein-Hitpass et al., 1990) supporting the idea that additional factors might also be required for full ligand-induced transcriptional activation by NRs. The indirect inhibition of transcriptional properties of one NR by the activation of another overexpressed NR (Halachmi et al., 1994; Meyer et al., 1989) suggested for the existence of common

coregulatory proteins that might be squelched by the overexpressed receptor. Finally, the concept of a new type of transcription factors, that do not bind to DNA, but control transcription by binding to NRs either directly or indirectly, was validated in 1995 with the discovery of the first NR coactivator, steroid receptor coactivator 1 (SRC1) (Onate et al., 1995). The following criteria were thus proposed for a protein to be categorized as a coactivator. Coactivators bind to NRs (directly or indirectly) but not directly to DNA, agonist ligands promote while antagonist ligands inhibit coactivators binding to NRs, coactivators are able to reverse receptor-receptor squelching and overexpression of coactivators greatly enhances while the expression of dominant negative mutant inhibit ligand-dependent NR function (Onate et al., 1995).

Gene transcription by NR oscillates between on and off states and is finely regulated be transcriptional coregulators (coactivators and corepressors) which play a major role in chromatin remodeling through histone amino-terminal modifications such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation (Bhaumik et al., 2007; Kouzarides, 2007; Suganuma and Workman, 2008). The term 'histone code' is employed to represent a specific combination of these modifications. While coactivators enhance NR-mediated transcription mostly due to associated histone acetylase activity, the primary function of corepressors is to attenuate the transcription by chromatin condensation mostly due to histone deacetylation and methylation **(Figure 9)**.

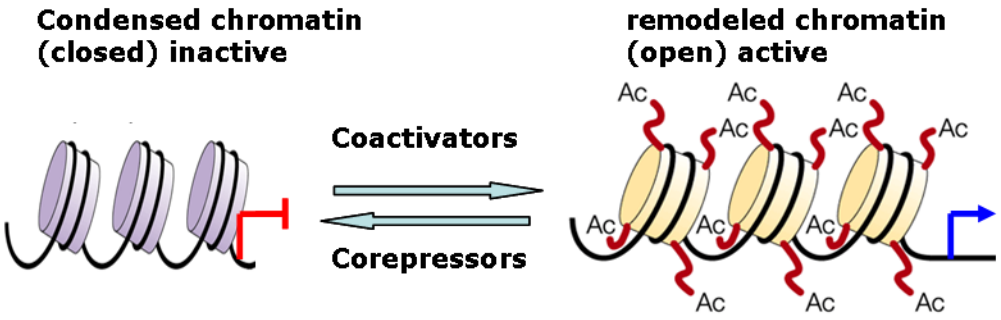


Figure 9: Chromatin remodeling by coregulators
Corepressors by their associated enzymatic activities enhance chromatin condensation leading to gene silencing whereas enzymatic activities associated with coactivators reverse the condensed state in such a way that transcriptional initiation becomes possible. Histones are indicated by round boxes encircled by chromatin threads. Histone modification by acetylation (Ac) is shown as an example.

Although the initial identification of coregulators became possible due to their interacting abilities with a given nuclear receptor, it soon became clear that most of the coregulators are not specific for a particular receptor, although preferential interaction with a particular steroid receptor depending on cell type and promoter context occurs through unclear mechanisms. Moreover, a coactivator can have highly variable effects depending on not only the cellular and promoter contexts but also the receptor subtype. For instance, a

given coactivator (HBO1) can increase the transcriptional response for one receptor (PR) (Georgiakaki et al., 2006) while inhibiting transactivation properties of another receptor (AR) (Sharma et al., 2000). Depending on cell type, opposite effects of the coactivator SRC1 in regulating PR transcriptional properties was demonstrated in mouse model where SRC1 increased PR transcriptional activity in response to estrogen and progesterone in uterine stroma and myometrium while decreased PR target gene transcription in the luminal and glandular epithelial compartments (Han et al., 2005). Mechanism of action of coactivators is also variable, for example, they can stabilize the receptor or transcriptional machinery on promoter (McKenna et al., 1999; Rowan and O'Malley, 2000), participate in chromatin remodeling by covalent modifications of not only histones (Glass and Rosenfeld, 2000) but also of other coregulators (Chen et al., 1999b) or regulate the stability/turnover of regulatory proteins (Imhof and McDonnell, 1996; Nawaz et al., 1999b; Rowan and O'Malley, 2000). Furthermore, optimal transcriptional activation by specific steroid hormone receptor can employ interplay of distinct coregulators depending on the nature of ligand, promoter environment, activation/inhibition of various signaling pathways and cell type (Hermanson et al., 2002).

3.1 PR-interacting coactivators

Coactivators serve as a bridge between steroid receptor and the general transcriptional machinery, and might be considered as scaffolding proteins able to recruit other regulatory partners. These are the protein molecules which influence transcriptional response of steroid receptors on target gene transcription without binding directly to DNA. The most thoroughly characterized PR interacting transcriptional coactivators belong to p160 SRC family which comprises of three homologous proteins namely, SRC1, SRC2 and SRC3. These interact with not only several steroid receptors but also to other NR, specific and general transcription factors, and provide platform for other enzymes to facilitate the transcription by chromatin remodeling. The physiological relevance of SRC1 and SRC3 for PR signaling was demonstrated in mice by O'Malley's group. SRC1 knock-out mice presented severe reproductive abnormalities particularly in the uterus (Xu et al., 1998) whereas selective ablation of SRC3 resulted in impaired mammary gland development and function (Xu et al., 2000). In agreement with the above studies, it has been recently demonstrated that physiological functions of PR in the uterus are modulated primarily by SRC1 whereas SRC3 acts as a major coactivator for normal PR functioning in mammary gland (Han et al., 2006).

3.1.1 SRC1

The SRC1, also known as NCOA1, was isolated using the PR-LBD as bait in yeast two hybrid screen of a human B-cell cDNA library and was shown to interact and enhance the transcriptional activity of several steroid hormone receptors in a ligand-dependent manner

(Onate et al., 1995). SRC1 exerts its action on a large number of nuclear receptors since it is capable of co-activating transcription induced by PR, GR, ER, thyroid receptor (TR), retinoic X receptor (RXR) (Onate et al., 1995), hepatocyte nuclear factor 4 (HNF4) (Wang et al., 1998), steroidogenic factor (SF1) (Crawford et al., 1997) and peroxisome proliferator-activated receptor gamma (PPARγ) (Zhu et al., 1996). Association of SRC1 with PR LBD is strictly dependent on the nature of the ligand and antagonist RU486 partially abolishes this interaction (Onate et al., 1995). Some studies have also shown the role of SRC1 in transcriptional activation of other transcription factors such as AP1 (Lee et al., 1998), serum response factor (Kim et al., 1998), NF-κB (Na et al., 1998) and p53 (Lee et al., 1999). SRC1 is capable of counterbalancing the squelching phenomenon observed during transactivation by PR in the presence of ER suggesting that SRC1 is independently recruited by both of these receptors and that SRC1 recruitment by PR and ER for optimal transactivation is a limiting factor (Onate et al., 1995). Together, these studies show an important role for SRC1 in multiple intracellular signaling pathways.

Principal interacting domain of SRC1 with PR is situated in the extreme C-terminus since *in vitro* transient transfection experiments have demonstrated that a dominant negative mutant inhibits PR co-activation by SRC1 (Jenster et al., 1997; Onate et al., 1995). It has been shown that SRC1 interacts mainly with AF2 domain of steroid receptors, however, interaction with AF1 region has also been suggested (Ikonen et al., 1997; McInerney et al., 1996; Onate et al., 1998). Such interactions involve different domains of SRC1 and are necessary so that AF1 and AF2 can act in synergy for transcriptional activation (Benecke et al., 2000; Onate et al., 1998). Given that interaction of SRC1 with steroid receptors requires presence of AF2 amphipathic helix 12 (H12), SRC1 is considered to be interacting with AF2 (McKenna et al., 1999), in contrast to other coactivators such as steroid RNA activator (SRA) which interact more specifically with AF1 domain (Lanz et al., 1999). SRC1 has also been shown to bind the N-terminal domain of PR and AR in inverted position. Deletion of the PRB LBD led to generate a constitutive receptor which is able to recruit SRC1 suggesting an important role of PRB AF3 domain in SRC1-mediated transcriptional co-activation (Georgiakaki et al., 2006). SRC1 contains two activation domains AD1 and AD2 which activate the transcription of a reporter gene when fused to DNA binding domain of yeast Gal4 protein. Some *in vitro* studies have shown the interaction of SRC1 with general transcription factors such as TATA-binding protein (TBP) and transcription factor IIB (TFIIB) suggesting a role of SRC1 in stabilization of transcriptional machinery (Ikeda et al., 1999; Takeshita et al., 1996). We have recently shown that proteasome-mediated proteolysis of SRC1 is pivotal for PR transcriptional activity. Two degradation motifs, PEST (amino acids 2-16) and basic helix loop helix domain (amino acids 41-136) were identified and shown to control the basal expression levels as well as hormone-dependent down-regulation of SRC1. ***[See Results-PAPER II]***

3.1.2 SRC2 and SRC3

Two other homologous proteins, acting as NR coactivators have been characterized namely SRC2 (TIF2, GRIP1, NCOA2) (Hong et al., 1997; Voegel et al., 1996) and SRC3 (p/CIP, RAC3, AIB1, ACTR, TRAM1, NCOA3) (Anzick et al., 1997; Chen et al., 1997; Li et al., 1997; Takeshita et al., 1997; Torchia et al., 1997). SRC2, like SRC1, is capable of interacting with LBD of numerous steroid receptors in a hormone-dependent manner (Hong et al., 1997; Hong et al., 1996; Voegel et al., 1996). It contains two constitutive activation domains capable of stimulating transcription when fused to DBD of heterologous proteins (Hong et al., 1996; Voegel et al., 1998; Voegel et al., 1996) . Like SRC1, SRC2 restores squelching phenomenon of PR induced by ER (Voegel et al., 1996). SRC3 was first isolated from mice as a molecular partner of CREB binding protein (CBP) during transcriptional activation of CRE binding protein (CREB) (p300/CBP cointegrator-associated protein, p/CIP) (Torchia et al., 1997). Almost simultaneously, different groups characterized this transcriptional coregulator as coactivator of TR (ACTR, activator of thyroid receptor) (Chen et al., 1997) or thyroid receptor molecule 1 (TRAM1) (Takeshita et al., 1997), a coactivator overexpressed in mammary and ovarian cancers (amplified in breast cancer , AIB1) (Anzick et al., 1997), a coactivator of nuclear receptors (Receptor-associated coactivator 3, RAC-3) (Li et al., 1997) or steroid receptor coactivator 3 (SRC3) (Suen et al., 1998). In human, SRC3 acts as a transcriptional coactivator of several NRs such as PR, ER, GR, RAR, RXR, TR (Anzick et al., 1997; Chen et al., 1997; Li et al., 1997) while in mice mSRC3 presents a broader spectrum of activity since it can enhance transactivation of other transcriptional activators such as STATs and CRE-binding protein (CREB) (Torchia et al., 1997).

3.1.3 NR-coactivator interaction regions

There are three structural domains in SRCs, the most conserved amino-terminal basic helix-loop-helix-Per/ARNT/Sim (bHLH-PAS) domain which is involved in interactions with numerous other coactivators such as coiled-coil coactivator A (CoCoA) (Kim et al., 2003), GRIP1 associated coactivator 63 (GAC63) (Chen et al., 2005) and coactivator associated arginine methyltransferase 1 (CARM1) (Chen et al., 1999a; Lee et al., 2004), a central region containing three NR interacting LxxLL motifs (Darimont et al., 1998; Heery et al., 1997; Voegel et al., 1998) and a carboxyl terminus harboring two transcriptional activation domains (AD1 and AD2). AD1 is involved in interaction with CREB-binding protein (CBP) and the histone acetyltransferase p300 while the interaction with histone methyltransferases such as CARM1 and protein arginine N-methyltransferase 1 (PRMT1) is mediated via AD2 (Xu et al., 2009). The C-terminal regions of SRC1 and SRC3 also possess domains responsible for their histone acetyltransferase (HAT) activity.

3.2 PR-interacting corepressors

The two most widely studied transcriptional corepressors are nuclear receptor corepressor (NCoR also known as NCOR1) and silencing mediator of retinoid acid and thyroid hormone receptor (SMRT, also known as NCOR2). NCoR and SMRT were simultaneously cloned in 1995 by Rosenfeld (Horlein et al., 1995) and Evans (Chen and Evans, 1995) respectively. Using a similar approach that led to the identification of the first steroid receptor coactivator, NRs were used as a bait to isolate the interacting proteins which demonstrated transcription repressing properties. Like coactivators, corepressors such as NCoR and SMRT target not only NR but also diverse transcription factors such as TFIIB, AP1, and NF-κB (Battaglia et al., 2010) reflecting multiple roles played by corepressors in pathophysiology. Altered expression and localization of NCoR and SMRT is reported in various cancers including breast, bladder and prostate cancer (Abedin et al., 2009; Banwell et al., 2006; Girault et al., 2003; Khanim et al., 2004; Kim et al., 2009; Zhang et al., 2006).

In contrast to ligand dependency for coactivator's recruitment, initial biochemical studies elaborating the mechanisms of interaction between NR and various corepressors proposed a simple but attractive model suggesting that the open conformation of unliganded receptor allows constitutive interaction with corepressors leading to repression of target gene transcription. Specifically the hydrophobic groove in the LBD region is involved in interaction with LxxH/IIxxxI/L motif or corepressor nuclear receptor (CoRNR) box present in the corepressors (Hu and Lazar, 1999; Perissi et al., 1999). During gene activation process, these corepressors are replaced by coactivators **(Figure 10)**.

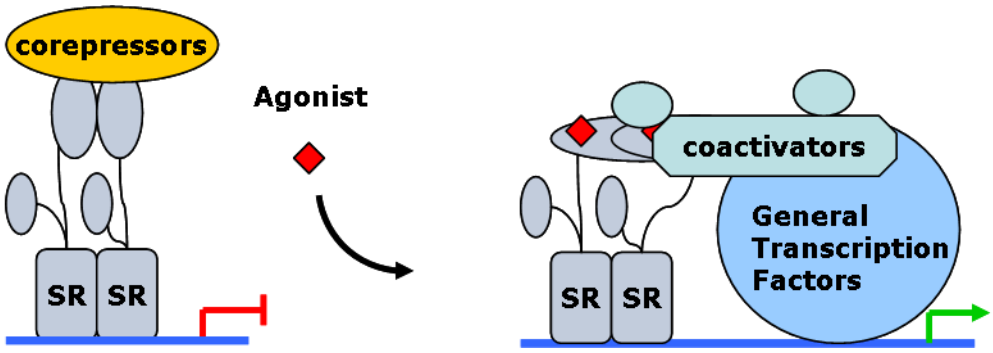


Figure 10. Classical mechanism of coregulator recruitment by steroid receptors.
Corepressor recruited by unliganded steroid receptor maintain chromatin in condensed conformation leading to silencing of transcription. In the presence of agonist ligand, conformational change in the receptor provides platform for the recruitment of transcriptional coactivators which leads to chromatin remodelling/decondensation. Coactivators also serve as the bridge between the receptor and the general transcription machinery and serve as scaffolding protein for other coregualtors binding, ultimately activating the gene transcription.

Agonist ligand binding induces a conformational change in the receptor i.e. repositioning of the helix 12 of LBD in such a way that the size of hydrophobic groove is reduced which can now accommodate shorter LxxLL motif (also known as NR box) present in coactivators but not the larger CoRNR box of corepressors (Glass and Rosenfeld, 2000). This model suggests that agonist-dependent NR transactivation is due to an upshift in NR interaction with coactivators instead of corepressors which preferably interact with unliganded receptor. While agonist ligand binding to NR, facilitates recruitment of coactivators, antagonist ligand-bound NR preferably interact with corepressors. For example, in the presence of PR antagonist asoprisnil, preferential interaction of PR LBD with SMRT corepressor has been shown (Madauss et al., 2007). Although, asoprisnil-bound PR LBD preferentially recruited corepressors, interaction with coactivators SRC1, SRC2 and SRC3 was also observed (Madauss et al., 2007). On the other hand, although corepressor binding is facilitated by NR conformation in the absence of ligand, agonist ligands do not always enhance the transcriptional properties of NR and almost as many genes are repressed as activated by PR following agonist ligand treatment (Tung et al., 2006). Recent studies have revealed that certain corepressors are specifically recruited by ligand activated transcription factors leading to active silencing of target gene transcription. For example, interaction of PR and ERα with ligand-dependent corepressor (LCoR) requires hormone binding to the receptor (Palijan et al., 2009b). Indeed, LCoR is recruited to promoters of genes whose transcription is repressed following hormone treatment (Palijan et al., 2009b). Similarly, transrepression of PPARgamma on certain genes is mediated by ligand-dependent recruitment of NCoR and HDAC3 by PPARg LBD to promoter sequences (Tiefenbach et al., 2006).

Other examples of ligand-dependent corepressors include nuclear receptor-interacting protein 1 (NRIP1, also know as RIP140), preferentially expressed antigen in melanoma (PRAME), prohibitin 2 (PHB2) and transcription intermediary factor 1a (TIF1a, also known as TRIM24) which all contain LxxLL motifs responsible for their interaction with liganded NR bound to the promoter sequences. Transcriptional repression is facilitated by subsequent recruitment of other classical corepressors such as C-terminal binding protein (CtBP) and histone deacetylases (HDACs) (Augereau et al., 2006; Epping et al., 2005; Fernandes et al., 2003; Montano et al., 1999; Palijan et al., 2009a; Wei et al., 2000).

These studies suggest that transcriptional regulation by NR and coregulators is much more complex than initially thought and highly depends on the cell type, the receptor, the coregulator and the promoter context. Any model explaining such mechanisms in a generalized fashion risks resembling an iceberg and also with substantial lacunae.

4. PR Post-translational modifications

PR like other steroid hormone receptors undergo important post-translational modifications (PTM) including phosphorylation, acetylation, sumoylation and ubiquitylation (Abdel-Hafiz et al., 2002; Daniel et al., 2010; Lange et al., 2000; Ward and Weigel, 2009; Weigel, 1996). Several aspects of PR signaling including hormone responsiveness, subcellular localization (Qiu et al., 2003), dimerization, DNA binding (Takimoto et al., 1996), promoter selectivity (Daniel et al., 2007b), interaction with molecular partners (Wardell et al., 2010), transcriptional activity (Pierson-Mullany and Lange, 2004; Shen et al., 2001) and receptor turnover (Lange et al., 2000) are controlled by phosphorylation(s).

4.1 Phosphorylation

In PR, there are at least 14 phosphorylation sites (thirteen serine residues and one threonine residue) mostly within Serine-Proline motifs in the N-terminal domain (Beck et al., 1996a; Beck et al., 1996b; Knotts et al., 2001; Zhang et al., 1997; Zhang et al., 1994; Zhang et al., 1995) **(Figure 11)**. Among these, six (Ser 20, 25, 81, 102, 130, 162) are only present in PRB upstream segment (BUS). In the absence of hormone, PR is basally phosphorylated at Ser 81, 162, 190 and 400 (Zhang et al., 1997), whereas ligand binding induces phosphorylation of Ser 102, 294, 345 and 400 (Zhang et al., 1995).

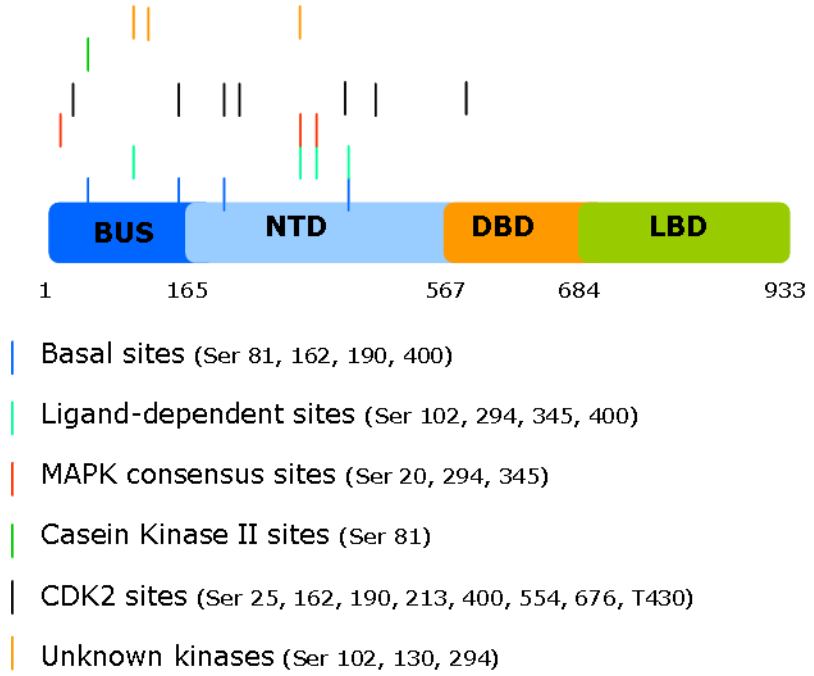


Figure 11. Phosphorylation sites in PR
Phosphorylation sites are indicated with colored bars.

These residues are targeted by multiple kinases including proline-directed kinases such as cyclin dependent kinase 2 (CDK2) (Ser 25, 162, 190, 213, 400, 554, 676 and Thr 430)

(Knotts et al., 2001; Zhang et al., 1997), MAPK (Ser 20, 294, 345) (Lange et al., 2000) and casein kinase II (Ser 81) (Zhang et al., 1994). Moreover, an individual residue might also be targeted by multiple kinases *in vivo*. Ser 102 and 130 and ligand-dependent Ser 294 phosphorylation is induced by unknown kinases (Lange, 2004; Qiu et al., 2003). Functional role of each of these phosphorylation sites is yet to be known. PRB serine 294 phosphorylation (pS294-PRB) has been studied extensively and shown to play a critical role in cross talk with growth factor signaling pathways. Ser 294 acts as an important sensor for growth factor inputs that affects PR functions such as nuclear translocation, DNA binding, transcriptional activity and proliferation (Daniel et al., 2007b; Dressing et al., 2009). Its role in PR degradation has also been studied by mutagenesis experiments showing that substitution of serine 294 by an alanine (S294A) led to PRB stabilization suggesting that PRB down-regulation is mainly addressed by the Ser 294 site (Lange et al., 2000). However, in stably transfected T47D cells, PRB-S294A mutant underwent ligand-induced turnover, though to lesser extent as compared to wild type PRB (Skildum et al., 2005). Furthermore, antiprogestin RU486 also induces strong Ser 294 phosphorylation. However, PRB turnover is markedly slowed down upon PR binding to RU486. This shows that mechanisms independent from S294 phosphorylation might also play a role in PR stability/turnover. ***[See also Results-PAPER I]***.

The other relatively better studied PR phosphorylation sites include Ser 345 and 400. It has been shown that phosphorylation of S345, a MAPK consensus site, is mediated by activation of EGFR-c-Src-MAPK by progestins. Furthermore, Ser 345 phosphorylated PRB associates with SP1 to regulate transcription of selected PR target genes such as p21 and EGFR whose promoters lack canonical PRE but harbor SP1 sites (Faivre et al., 2008). The significance of Ser 400 phosphorylation in PR signaling has also been demonstrated. Ser 400 is phosphorylated in the presence of progestins or growth factors and requires CDK2 activity. Indeed, CDK2 induces Ser 400 phosphorylation even in the absence of ligand and regulates nuclear localization and transcriptional activity of both unliganded and liganded PR (Pierson-Mullany and Lange, 2004).

It has been reported that PR isoforms undergo differential phosphorylations at Ser 294 and Ser 345. PRB is rapidly and highly phosphorylated at Ser 294 residue following agonist ligand or EGF treatment while PRA undergo very weak and delayed S130 phosphorylation in response to agonist (Clemm et al., 2000) or EGF (Daniel et al., 2007a). Also basal levels of PRA S130 phosphorylation is much lower as compared to PRB S294 in cells coexpressing both PR isoforms (Clemm et al., 2000). In contrast, PRA is rapidly and more intensely phosphorylated at Ser345 residue as compared to PRB following progestin treatment (Daniel et al., 2007a).

This shows that in addition to intracellular levels of the receptor, hormone and PR-interacting proteins such as transcriptional coregulators, PR signaling is also finely controlled

by the activation of specific kinases and phosphatases in progesterone target cells. Functional differences in PRA and PRB might in part be related to differential PR isoforms phosphorylation pattern and kinetics ***[See also Results-PAPER I]***

4.2 Ubiquitylation

The ubiquitin/proteasome system is known for regulated protein turnover in a highly specific manner. The ubiquitin, a small protein (76 amino acids), is specifically conjugated to the target protein destined for degradation (Pickart, 2001) and also acts as an acceptor for another ubiquitin molecule resulting in a polyubiquitin chain (Hoppe, 2005). Protein ubiquitylation is a multistep process mainly involving E1-activating enzyme, E2 conjugating enzymes, and E3 ligases **(Figure 12)**. To date, only one E1 enzyme is known while a dozen of E2 and over 200 E3 family members, responsible for substrate specificity, exist in humans (Faus and Haendler, 2006). Ubiquitin molecule is covalently attached to E1 enzyme in an ATP-dependent process. Sequential action of E2 and E3 enzymes results in the formation of an isopeptide bond between the carboxyl terminal glycine of ubiquitin and the amino group of a lysine residue in the target protein. Once ubiquitinated, the protein is recognized and degraded by a large multi-subunit protease complex called as 26S proteasome comprising of 20S core subunit and the 19S regulatory subunit (Voges et al., 1999). Similar to other post-translational modifications, ubiquitylation is a reversible process involving deubiquitylating enzymes (Nijman et al., 2005).

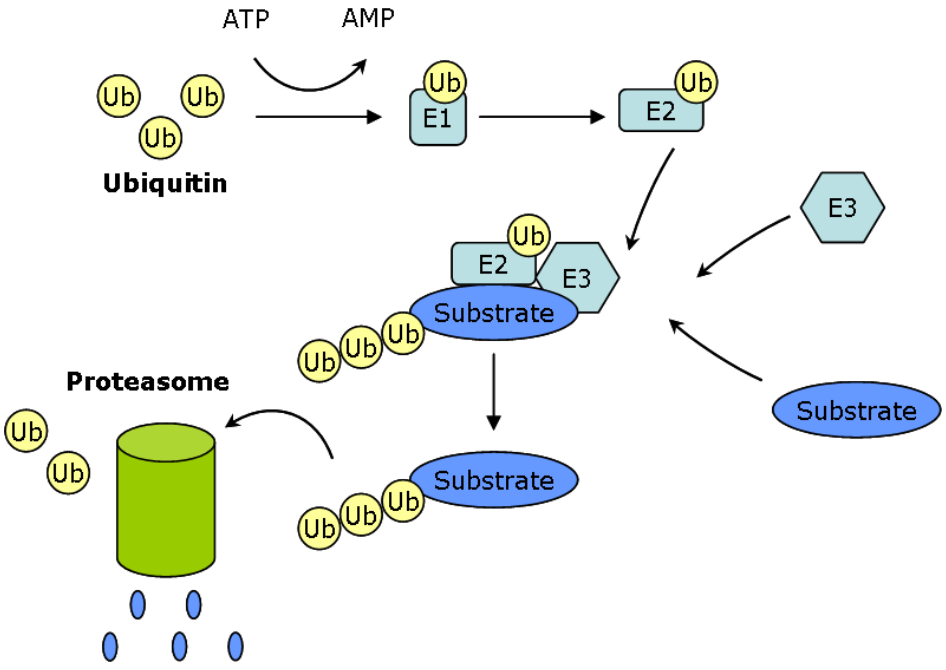


Figure 12. Ubiquitin-proteasome mediated protein turnover
Ubiquitin molecule is covalently attached to E1 enzyme in an ATP-dependent process. Ubiquitin is taken up by E2 conjugating enzyme and is transferred to the protein substrate to be degraded with or without assistance of E3 ligase enzyme which is responsible for protein specificity. Polyubiquitinated proteins are then recognized and degraded by 26S proteasomes.

Protein synthesis and degradation go side by side and the balance between these processes is critical for normal cell functioning. Despite the important role of protein degradation in cellular homeostasis, interest in understanding ubiquitin proteasome pathway is further increased by the fact that the enzymes involved in ubiquitin proteasome pathway are recruited to nuclear receptor target genes and proteolysis of nuclear receptors and coregulators is required for efficient transcriptional outcome (Nawaz and O'Malley, 2004). This coupling of the receptor transcriptional activity with its turnover was initially seamed to be puzzling and paradoxical. It was shown that recruitment of E3-ubiquitin ligase, E6-associated protein (E6-AP) and the regulatory subunits of the proteasome (Rpt6 and S1) on hormone responsive promoter is cyclical and can occur either in the absence or the presence of ligand (Kang et al., 2002). In support of these studies, several nuclear receptors (ER, PR, GR, TR, RXR, RAR) were found to be ubiquitinated and degraded in the course of their transcriptional activities (Dace et al., 2000; Deroo et al., 2002; Lange et al., 2000; Masuyama and MacDonald, 1998; Nawaz et al., 1999a; Tanaka et al., 2001; Wallace and Cidlowski, 2001; Zhu et al., 1999) ***[See also Results-PAPER II]***. Who dictates the terms and what is the signal for determining the fate of the receptor? Neosynthesized receptors are stabilized and protected by association with chaperone heat shock protein complexes and undergo minor degradation in the absence of hormone. Dissociation of the receptor from such complexes by the use of geldamycin, a benzoquinone that blocks the addition of the chaperone assembly factors to PR-hsp complexes, results in rapid proteasome-mediated turnover even in the absence of the hormone (Lange et al., 2000). Most of the steroid hormone receptors (with the exception of AR) undergo accelerated degradation via proteasomes following hormone binding. As described above, ligand-bound receptor conformation helps it dissociate from these chaperone complexes and undergo different post-translational modifications which influence the receptor turnover. For example, in ER, a role of ligand-induced acetylation in receptor turnover has been shown (Wang et al., 2001). Similarly, PR S294A mutation reduces hormone-induced PR degradation (Lange et al., 2000).

4.3 Sumoylation

Conjugation of small ubiquitin-like modifier (SUMO) protein to steroid hormone receptors has been shown to strongly influence their transcriptional properties (Chauchereau et al., 2003; Daniel and Lange, 2009; Holmstrom et al., 2008; Le Romancer et al., 2011; Tallec et al., 2003; Yang et al., 2011b). Like ubiquitylation, sumoylation is also a three step process involving E1 activating enzyme, E2 conjugating enzyme and E3 SUMO ligases. Interplay of these enzymes leads to covalent attachment of a SUMO chain to lysine residues in target proteins. Sumoylation affects several biological functions of transcription factors ranging from subcellular localization, DNA binding or

transcriptional properties (Faus and Haendler, 2006). Reversal of sumoylation is mediated by SUMO specific proteases (Seeler and Dejean, 2003). Like other steroid receptors, PR post-translational modification by sumoylation plays a major role in transcriptional activity and stability of the receptor (Chauchereau et al., 2003; Daniel et al., 2007a). The N-terminal domain (165-456 amino acids), common to both PRA and PRB, also known as inhibitory function (IF) located upstream of AF1, harbors a single consensus ψKXE SUMO-conjugation motif centered at lysine 388 (Abdel-Hafiz et al., 2002).

An important role of PR Lys388 in controlling ligand-independent PR transcriptional activity has been recently demonstrated (Daniel and Lange, 2009). Ligand-independent proliferation and growth in soft agar was highly increased by T47D cells expressing PRB K388R mutant as compared to those expressing wild type or S294A phospho-mutant PRB. Basal PRB K388R transcriptional activity on selected target genes i.e. heparin-binding (HB) EGF-like growth factor (HB-EGF), insulin receptor substrate 1 (IRS-1) and stanniocalcin (STC1) was increased by activated CDK2. STC1, a peptide hormone, is involved in cell metabolism and tumor growth, and is overexpressed in breast cancers (Chang et al., 2003). On STC1 promoter, ligand-independent K388R PRB recruited SRC1 and displaced HDAC3 showing a role of PR Lys388 sumoylation in negatively regulating STC1 gene transcription by modifying chromatin remodeling/coregulator recruitment (Daniel and Lange, 2009).

Upon ligand-binding, both PRA and PRB undergo Lys388 sumoylation which represses PR transcriptional activity. A single point K388R mutation is sufficient to reverse the autoinhibitory and transrepressor functions of ligand-bound full length PRA and PRB (Abdel-Hafiz et al., 2002). PRA, which is less phosphorylated at the Ser294 site, is sumoylated more at Lys388 than PRB (Daniel et al., 2007a). Based on this finding, it was proposed that differential PR isoforms sumoylation might be due to PR isoforms differences in Ser294 phosphorylation. However, recent studies have shown that S294/344/345A triple mutant is as well sumoylated as the wild type PRB in the presence of agonist ligands suggesting the absence of connection between S294 phosphorylation and Lys388 sumoylation (Abdel-Hafiz et al., 2009). The K388R mutation strongly enhances agonist-bound wild type PRB transcriptional activity on exogenous promoters as well as plays a critical role in endogenous genes selection and PRB turnover (Daniel et al., 2007a). As described above PRB S294A mutant displays reduced transcriptional activity and turnover. Mutation K388R induces transcriptional hypersensitivity to progesterone and rescues turnover of S294A mutant (Daniel et al., 2007a). Indeed, it has been shown recently that Lys388 acts as a target for CUE domain containing 2 protein (CUEDC2) which decreases PRB sumoylation at this residue while promoting ubiquitination and proteasome-mediated turnover (Zhang et al., 2007). Ligands play important role in both PR ubiquitylation and sumoylation, and the role of different co-regulatory proteins has

been reported. Both agonist R5020 and antagonist RU486 induce PR Lys388 sumolyation and PR Ser294 phosphorylation. However, R5020- but not RU486-bound PR undergoes rapid proteasome-mediated turnover. Importantly the differences also exist between PR isoforms for these post-translational modifications and their role in stability as well as transactivation properties. It thus seems likely that the co-regulatory proteins implicated in these processes might interact/function differentially depending not only on the nature of the ligand but also on PR isoforms **(Figure 13)**.

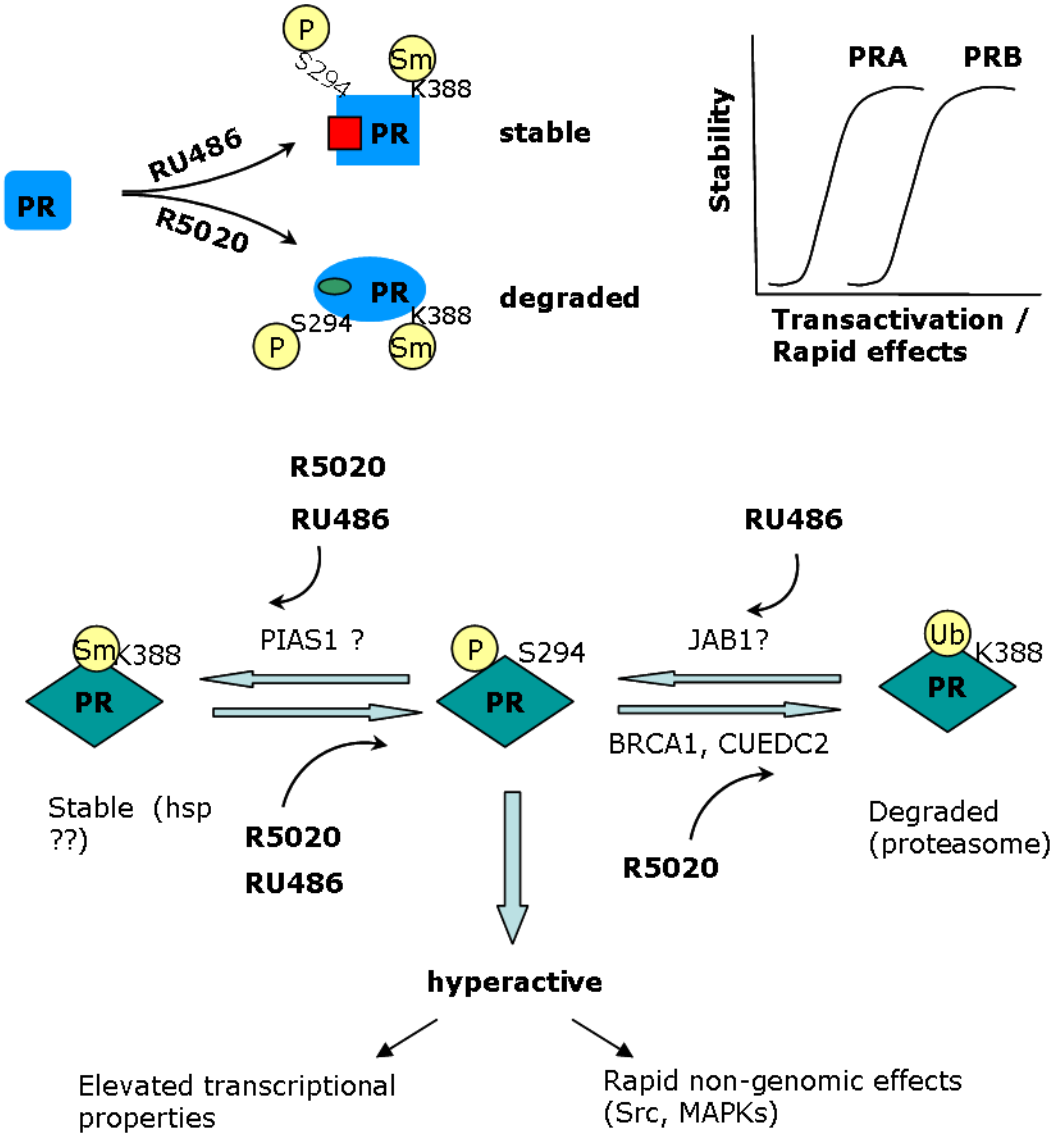


Figure 13. Interconnection between PR post-translational modifications, role of co-regulatory proteins and functional consequences.
R5020- and RU486-induced PR Ser294 phosphorylation and PR Lys388 sumoylation are shown. Please note conformational differences in RU486- vs R5020-bound PR. Isoform differences in stability and transactivation properties are shown in graphs. Ligand sensitive interplay between PR post-translational modifications such as phosphorylation on serine 294 residue, ubiquitination and sumoylation on the lysine 388 residue is shown. Also the role of different coregulatory proteins and functional consequences on PR signaling are suggested.

4.4 Acetylation

Steroid receptors also serve as targets for acetylation on lysine residues present in conserved acetylation motifs (Daniel et al., 2010; Faus and Haendler, 2006; Kino and Chrousos, 2011; Wang et al., 2011). Various aspects of steroid receptor functioning such as transcriptional properties, DNA binding, hormone responsiveness and coregulator recruitment are highly influenced by acetylation depending on receptor subtype and cellular context (Fu et al., 2003; Kim et al., 2006; Wang et al., 2001). In PR, a recent report has highlighted the role of ligand-induced PR acetylation at an acetylation motif, KxKK (638-641 amino acids), embedded in the PR hinge region (Daniel et al., 2010). It has been demonstrated that PR acetylation at these lysine residues influences PR phosphorylation pattern, nuclear retention and transcriptional properties (Daniel et al., 2010).

5. Functional diversity of PR isoforms

Despite the high degree of sequence similarity, accumulating evidence from several studies indicate that PRB and PRA isoforms are functionally distinct transcriptional factors. Importantly, PRA and PRB have distinct physiological role in progesterone target tissues as demonstrated by selective ablation of PRA or PRB in mouse models. Mice lacking PRB exhibit defective mammary gland development (Mulac-Jericevic et al., 2003) while mice that endogenously express PRB but lack PRA exhibit normal mammary gland development (Mulac-Jericevic et al., 2000). Although, mammary gland development is normal in PRA knock-out mice, severe reproductive tract abnormalities have been observed such as marked hyperplasia in response to hormone stimulation suggesting a role of PRA in opposing proliferative effects mediated by PRB and ER in the uterus (Mulac-Jericevic et al., 2000). From the above studies, we can conclude that PRA is essential for normal uterine development and reproductive functions while PRB is required for normal mammary gland development.

Differential role for PR isoforms has been demonstrated by *in vitro* studies showing that PRA and PRB differ not only in ligand response (Meyer et al., 1990) but also differentially regulate gene transcription from the same promoter as well as recognize entirely different target gene promoters (Brayman et al., 2006; Jacobsen et al., 2002; Richer et al., 2002; Tung et al., 2006) ***[See also Results - PAPER III]***. On most artificial or viral promoters, PRB is a much stronger transactivator than PRA which also behaves as a dominant repressor of transcription mediated by PRB and other steroid receptors (Hovland et al., 1998). Although the precise mechanism behind differential transcriptional activities of PR isoforms is yet to be fully known, studies based on structure-function activities suggest that AF3 present in PRB masks the inhibitory function common in PRA and PRB and thus contributes to higher PRB transcriptional activity (Abdel-Hafiz et al., 2002; Hovland et al., 1998; Tung et al., 2006). However, endogenous genes harboring complex response

elements are also differentially and distinctly regulated by A or B isoform of PR. Certain endogenous genes in breast cancer cells (Richer et al., 2002; Salama et al., 2007; Tung et al., 2006) or in hypothalamus (White et al., 2007) are uniquely up-regulated by PRA. Biochemical and biophysical studies suggest that unique conformation of PRA and PRB allows PRA to interact with a set of coregulators which are different from those recruited by PRB (Bain et al., 2001; Tetel et al., 1999). This might explain to some extent the functional differences exhibited by PR isoforms.

5.1 Differential coregulator recruitment by PR isoforms

PR isoforms differ in their ability to interact with transcriptional coregulators due to conformational differences associated with differential interaction between the carboxyl and amino termini of PRB and PRA. It has been shown that PRB but not PRA associates efficiently with the coactivators SRC1 and GRIP1 due to presence of AF3 (absent in PRA) which masks inhibitory domain in the N-terminus (Dong et al., 2004; Tung et al., 2006). However, PRA specific up-regulation of certain genes (Tung et al., 2006) suggests the recruitment of specific coactivators by ligand-bound PRA. In the presence of agonist ligand, PRA does not interact directly or with as high affinity as PRB to the coactivator SRC1 or SRC2 (Heneghan et al., 2007; Molenda-Figueira et al., 2008). In the presence of an antagonist (RU486), PRA shows higher affinity for the corepressors SMRT (Giangrande et al., 2000). Moreover, the partial agonist effects associated with the antagonist RU486 on certain cellular and promoter context are explained by the ability of PRB but not PRA to interact with coactivators (Beck et al., 1993). This high variability in recruitment of coregulators by PRA and PRB may account for the differential transcriptional regulation by PR isoforms in various progesterone target tissues.

5.2 PR isoform ratio and target gene selectivity

PR isoforms PRA and PRB are capable of forming homo- and hetero-dimers, produce differential transcriptional response from the same promoter sequence as well as recognize distinct target gene promoters. One PR isoform influences the transcriptional properties of the other isoform when co-expressed through mechanism(s) that might involve both the competition between two isoforms as well as heterodimerization which might either enhance or repress target gene transcription **(Figure 14)**. Given that several aspects of progesterone signaling are differentially influenced by PR isoforms, PRA/PRB expression ratio should be considered as an important determinant of functional consequences of progesterone signaling. It has been demonstrated that the gene subsets regulated by liganded PR isoforms are largely non-overlapping. In the presence of hormone, only few genes are regulated by PRA alone while a larger number of genes are regulated by liganded-PRB (Richer et al., 2002). Interestingly, in another study using separate cell lines expressing

either inducible PRA or PRB, it has been shown that unliganded PRA regulates much larger unique gene population as compared to PRB (Jacobsen et al., 2005). This suggests that target gene transcription is differentially regulated by PR isoforms depending on the absence or presence of ligand.

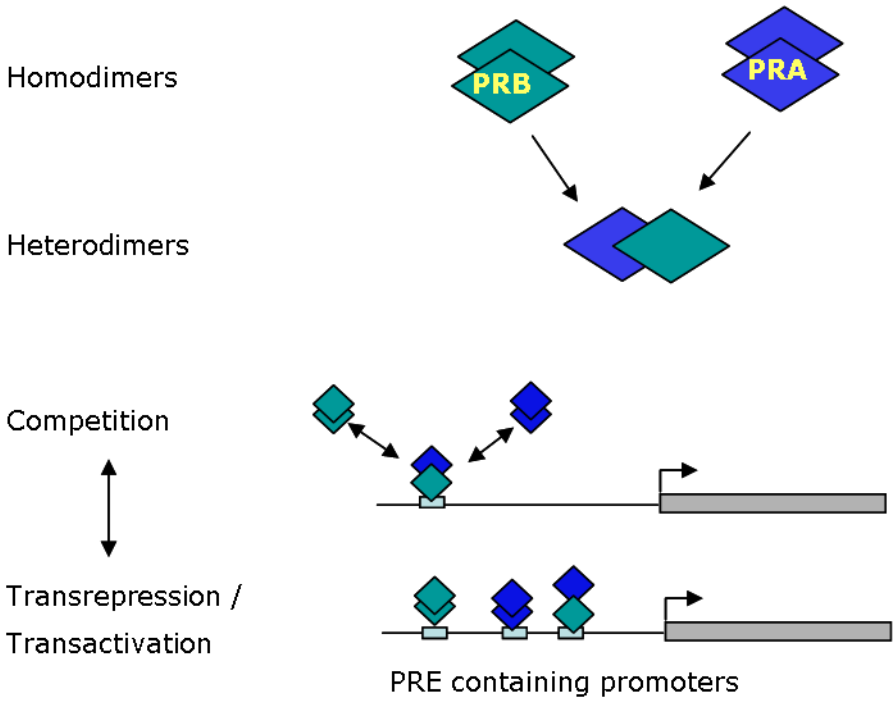


Figure 14. Mechanism of transcriptional regulation by PRA:PRB heterodimers
PR isoforms PRA and PRB are capable of forming homo- and hetero-dimers, produce differential transcriptional response from the same promoter sequence as well as recognize distinct target gene promoters. PRA and PRB can enhance or repress the target gene transcription by mechanisms involving either or both the competition between PR isoform homo- as well as hetero-dimerization.

Other teams have studied transcriptomic profile following progesterone stimulation in MDA-MB-231 cells expressing both PR isoforms at similar levels (Leo et al., 2005). Under physiological conditions, PRA and PRB are expressed at equimolar level and majority of PR positive cells express both PR isoforms (Mote et al., 1999; Mote et al., 2002). However, PRA/PRB expression ratio is often associated with pathological conditions (Hopp et al., 2004; Jacobsen et al., 2003; Mote et al., 2002; Mote et al., 2007; Mote et al., 2004). Theoretically, three molecular species might exist (PRA homodimer, PRB homodimer and PRA-PRB heterodimer) in PR positive cells. It is highly likely that functions of PRA-PRB heterodimer may differ from that of the homodimers since PRA is known to transrepress the activity of PRB (Hovland et al., 1998). Therefore, the ratio of PRA/PRB in a target tissue seems to highly influence the response to progesterone. One drawback of most of the previous *in vitro* studies showing distinct target gene regulation by PR isoforms is that such studies were conducted in T47D cells expressing only single PR isoform (PRA or PRB) where PR

homodimers are the only molecular species and PR signaling under varied PRA/PRB expression could not be studied. This demands the generation of bi-inducible cell systems allowing controllable PRA/PRB expression ratio in order to get better insights into PR signaling not only in the absence of hormone but also in response to various agonist/antagonist ligands and cross talk with growth factor stimulated pathways. ***[See also Results-PAPER III]***.

5.3 PR isoform ratio in pathophysiology

Although, majority of PR positive mammary epithelial cells express both PR isoforms at equimolar levels (Mote et al., 2002), PRA/PRB ratio fluctuates in reproductive tissues as a consequence of developmental (Shyamala et al., 1990), hormonal status (Duffy et al., 1997) and menstrual cycle (Mangal et al., 1997; Mote et al., 1999). Variable expression and function of these isoforms under certain physiological and pathological conditions is of high clinical importance as various studies have shown a link between disruption in PR isoforms expression and progression to malignancy in the breast and endometrium (Arnett-Mansfield et al., 2004; Arnett-Mansfield et al., 2001; McGowan et al., 2004; Mote et al., 2000; Mote et al., 2002).

Alteration of PRA/PRB ratio in transgenic mice overexpressing PRA transgene led to abnormal mammary gland development characterized by extensive lateral branching, ductal hyperplasia and disorganized basement membrane with decreased cell to cell adhesion (Shyamala et al., 1998). Likewise, transgenic mice overexpressing PRB transgene also presented abnormal mammary gland development with inappropriate alveolar growth, a phenomenon not observed with PRA transgenic mice (Shyamala et al., 2000). Two points are to be noted from these above studies i) both increase and decrease in PRA/PRB ratio led to abnormal mammary gland development with distinct phenotypic outcome ii) these studies were conducted in intact mice with presumably physiological progesterone levels. This indicates that progesterone signaling is highly altered by disruption of normal PRA/PRB ratio strengthening the distinct role played by individual PR isoforms in progesterone target tissues. A possible explanation for this abnormal PR functioning might be the predominance of homodimer species on the detriment of heterodimer PR species having differential conformation and thus interaction with coregulatory proteins.

Importantly, PRA/PRB ratio is also often altered in breast tumors resulting in a predominance of one isoform over the other (Graham et al., 1995b; Mote et al., 2002). PR isoform specific differences in growth of estrogen-dependent human breast tumor xenografts expressing either PRA or PRB have been reported (Sartorius et al., 2003). Similarly, in endometrial cancers, disrupted PRA/PRB expression is also observed and cancers with elevated PRA/PRB ratio are correlated with poor prognosis (Arnett-Mansfield et al., 2001). Differential cell invasion and matrix metalloproteinase 2 expression in human trophoblasts

was associated with variation in PRA/PRB expression ratio between early and late first trimester (Goldman and Shalev, 2006). These studies suggest that PR signaling is highly altered due to imbalance in PR isoforms expression. It is to be noted that genetic predisposition to cancer development due to mutations in BRCA1 or BRCA2 leads to PRA overexpression which may play a role in disease progression (Mote et al., 2004). Indeed, it has been shown in mouse model that Brca1/p53 mutation leads to elevated PRA expression which was associated with mammary tumorigenesis (Poole et al., 2006). Involvement of PR in such tumorigenic processes is highly likely since antiprogestin RU486 prevented mammary tumorigenesis suggesting important therapeutic perspectives for antiprogestins in breast cancer prevention in individuals with BRCA1 mutations (Poole et al., 2006). *In vivo* relationship between PR isoforms expression ratio and antiprogestin responsiveness has been studied showing that decreased PRA/PRB ratio is associated with antiprogestin resistance (Wargon et al., 2009). Restoration of balanced PRA/PRB ratio using methyltransferase inhibitors re-established antiprogestin sensitivity in mouse mammary carcinomas (Wargon et al., 2011). These studies highlight the importance of understanding the mechanism of variation in PRA/PRB ratio and its functional consequences in cell signaling. ***[See also Results - PAPER III].***

6. PR a therapeutic target

6.1 PR and breast cancer

Genomic targets of PRA and PRB include the key mediators of various cell signaling pathways (cell cycle, apoptosis, adhesion, growth factors) implicated in cancer (Jacobsen et al., 2002; Richer et al., 2002). Apart from classical genomic functions, PR isoforms are also capable of interacting with major cytoplasmic signaling pathways (p42/44 MAPK, EGF, Src) frequently activated in cancer cells (Faivre and Lange, 2007). Together, these targets play essential role in cell proliferation.

PR isoforms are considered as prognostic markers of breast cancer development and metastasis independently of the progestational status (Hopp et al., 2004; Punglia et al., 2006). Progesterone exerts antiproliferative effects in PR expressing cells (Beleut et al., 2010; Li et al., 2011) and few proliferating mammary epithelial cells express PR (Clarke et al., 1997; Russo et al., 1999). Mammary transplants lacking PR in the stromal compartment did not affect mammary gland development while side branching and alveologenesis is perturbed in PR-/- epithelial grafts (Brisken et al., 1998; Lydon et al., 1995). This indicates that progesterone acts by a paracrine mechanism on a subset of mammary epithelial cells for normal mammary gland development. Recently, the role of progesterone signaling in mammary carcinogenesis via paracrine mechanism gained strength since various independent studies have demonstrated that progesterone drives proliferation of PR negative mammary epithelial cells leading to carcinogenesis via receptor activator of NF-κB

ligand (RANKL), a tumor necrosis factor family member, and its receptor (Beleut et al., 2010; Gonzalez-Suarez et al., 2010; Schramek et al., 2010). Involvement of RANKL and its receptor (RANKLR) in progestin (medroxy progesterone acetate)-induced mammary epithelial cell proliferation and mammary carcinogenesis was demonstrated by genetic inactivation of RANKR in these cells (Schramek et al., 2010). Furthermore, mammary stem cells (MaSC) which lack PR are highly responsive to progesterone stimulation and several (11 to 14) fold increase in MaSC pool was observed during peak circulating levels of progesterone under physiological conditions such as luteal diestrus and pregnancy or by exogenous progesterone administration (Asselin-Labat et al., 2010). Given the lack of PR in MaSC, it is highly likely that such effects of progesterone signaling are mediated through paracrine mechanism.

PR is now known as an important marker of responsiveness to endocrine therapy in ER positive breast tumors. Breast cancer occurs in 10 % of occidental women, and represents 23 % of women's cancers. It is one of the leading causes of death in women suffering from cancer in Europe (11000 deaths per year in France), and one of the major prognostic factors is its metastasis to axillary lymph nodes. ER+ cancers are treated by anti-estrogens in premenopausal women and anti-aromatases in postmenopausal women to interrupt the pro-proliferative effect of estrogen. Acquired resistances to anti-estrogens are of considerable clinical significance and are due to various escape mechanisms (loss of ER gene expression, alteration in cofactors expression and/or MAPK pathways). As highlighted above, progestins also seem to favor breast cancer development (70 % of sporadic cancers are estrogen and progesterone-dependent). Hormone replacement therapy using estrogen and progestins substantially increased the risk of incident and fatal breast cancer in postmenopausal women as compared to estrogen alone therapy (Anderson et al., 2004; Bakken et al., 2010; Rossouw et al., 2002). A higher number of menstrual cycles correlate with an increased risk of developing breast cancer (Bernstein, 2002). Recent studies also suggest a major impact of progesterone and progestins in malignant transformation of mammary adult stem cells (Horwitz et al., 2008; Horwitz and Sartorius, 2008; Joshi et al., 2010). Accumulating evidence based on various studies in cellular and mouse models also identify PR as a key factor involved in mammary carcinogenesis (Lanari et al., 2009; Liang et al., 2007).

6.2 PR antagonists in breast cancer

The above observations have renewed interest for the use of PR antagonists in mammary oncology. In both humans and mice, mutations in the breast cancer gene BRCA1 predisposes to breast cancer development (Mote et al., 2004; Poole et al., 2006). In mice, the progesterone antagonist mifepristone (RU486) prevented mammary tumorigenesis in Brca1/p53-deficient mice suggesting therapeutic perspectives for antiprogestins in breast cancer prevention in individuals with BRCA1 mutations (Poole et al., 2006). Mifepristone

(RU486) in combination with antiestrogen or aromatase inhibitors has been shown to control the evolution of metastatic breast cancer (Klijn et al., 2000; Vanzulli et al., 2005). The same molecule and its derivatives have been suggested for the prevention of some genetic forms of breast cancers affecting young women (Busia et al., 2011; Luo et al., 2010; Mote et al., 2004; Poole et al., 2006; Wiehle et al., 2011; Wiehle et al., 2007). Taken together, these studies strongly indicate that PR constitutes a major pharmacological target for mammary cancer therapeutics, through utilization of progesterone antagonist ligands.

Apart from potential use in breast cancer therapy, PR antagonists provide interesting perspectives in other reproductive tract abnormalities. Uterine myomas are present in about 25% of 40-50 years old women (AFSSAPS 10/2004). They are responsible for pelvic pain and heavy bleeding leading to anemia. Treatments currently available are progestins, and gonadorelin (synthetic gonadotropin-releasing hormone, GnRH) analogues. Progestins are well tolerated and inexpensive and when used in high doses, they can control bleeding, but do not allow significant tumor shrinkage or even increase tumor size (Joshi et al., 2010). GnRH analogues correct bleeding and control the size of myomas, facilitating a subsequent surgical procedure. PR antagonists currently under development reach the same goal. However, their long-term use produces endometrial changes (Steinauer et al., 2004), recently defined as a class specific effect (Mutter et al., 2008). Reassuring clinical data have shown the absence of hyperplasia and proliferation in endometrial samples obtained during treatment with PR antagonists (Wilkens et al., 2009; Williams et al., 2007).

Mifepristone has been proposed as a palliative therapy in people suffering from widely metastatic and chemoresistance human cancer (Check et al., 2010a). Some recent studies in mouse model have highlighted an important therapeutic potential for PR antagonists not only in spontaneous lung cancers (Check et al., 2010c) but also in cancers restricted to males such as testicular and prostate cancers (Check et al., 2010b). However, further studies are needed to define the mechanisms underlying such effects and to establish a role of antiprogestins in such cancers of human origin.

6.3 Need for the PR specific antagonists

Since the discovery of the first PR antagonist, mifepristone (RU486) in 1981, several molecules which display PR antagonistic or mixed agonistic/antagonistic activity (SPRM) have been synthesized. There is strong preclinical and clinical evidence for the value of progestin antagonists in treatment of endometriosis, uterine fibroids, dysfunctional uterine bleeding and breast cancer (Cadepond et al., 1997; Kettel et al., 1998; Mahajan and London, 1997; Murphy et al., 1995; Spitz, 2003). Steroidal PR antagonists available to date have been derived from either testosterone or progesterone and possess a conserved ketone function at C3 and methyl group at C11 which are present in natural PR ligand progesterone. The antagonistic property of these compounds is conferred by a bulky group substitution at

C11 position **(Figure 15)** (Garcia et al., 1992). This creates steric interference which impedes the correct positioning of the c-terminal α helix 12 of LBD on the liganded hydrophobic pocket. In contrast to agonist ligand-bound receptors conformation which favors recruitment of coactivators, the antagonist-bound PR LBD adopts a stable conformation permitting the recruitment of transcriptional corepressors (Madauss et al., 2007).

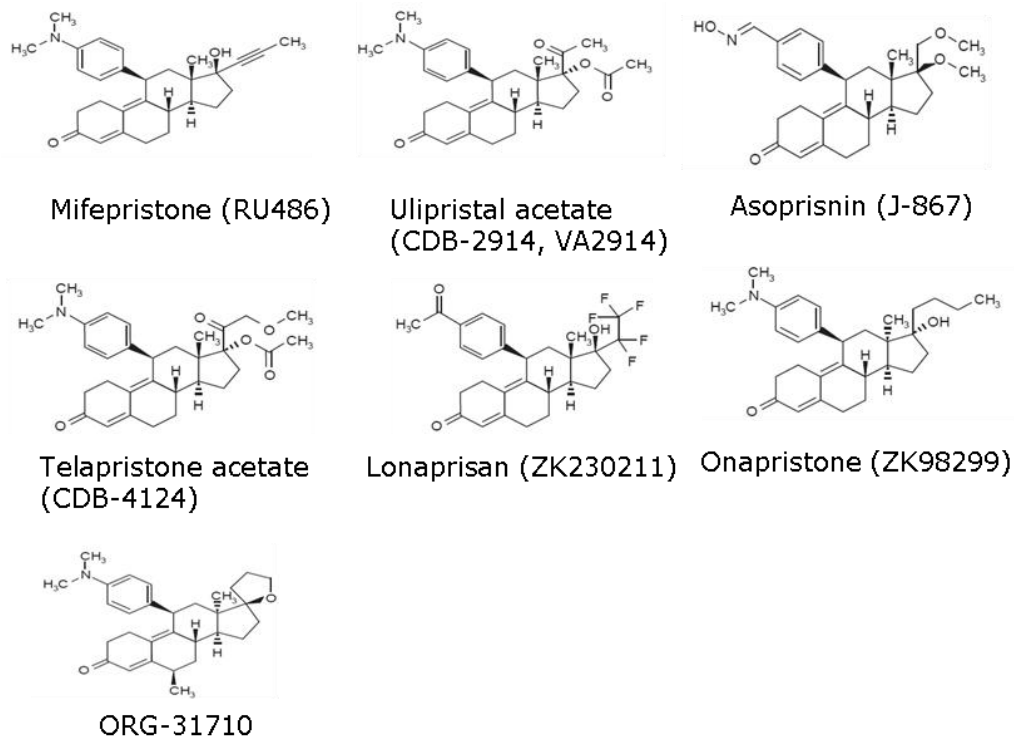


Figure 15. Structural formulae of principle PR antagonist ligands
The antagonist ligands of PR currently under development phase or in clinical use are steroidal molecules which conserved certain structures of progestins such as a ketone function at C3 and methyl group at C11 which are present in natural PR ligand progesterone. The antagonistic property of these compounds is conferred by substiutution of a bulky group at C11 position as indicated by red circles.

Although antagonist-bound PR LBD preferentially recruited corepressors, interactions with coactivators are also observed (Madauss et al., 2007) leading to partial agonist character of such antagonists. Indeed it has been shown that the transcriptional outcome following bulky antagonists binding is highly influenced by coactivator/corepressor expression ratio **(Figure 16)** (Liu et al., 2002). Based on their mode of action these ligands are classified as *active antagonists*. Furthermore, despite high efficacy to antagonize PR, most of the C11-aryl substituted molecules are not PR selective and interact with AR and GR (Lu et al., 2006). These ligand-associated variations in coregulator recruitment, along with the evidence for variable PR isoform expression and function under different physiological and pathological conditions, demand the development of PR specific antagonists to address more targeted treatments of PR associated diseases including breast cancer. A new concept of

inactivating steroid receptors by passive mechanism is emerging. Passive antagonists are usually small molecules, lacking bulky substituents, that dissociate quickly from the receptor and are unable to establish the contacts which are critical to maintain the ligand binding domain in its active conformation (Shiau et al., 2002). They form with the receptor unstable complexes which are unable to recruit transcriptional coregulators. Such passive antagonists have been developed for AR (Soderholm et al., 2005), ER (Shiau et al., 2002) and MR (Fagart et al., 2010; Fagart et al., 1998). Based on this strategy, highly specific progesterone antagonist ligands able to by-pass PR interactions with co-regulating proteins should be of choice to prevent deleterious effects on transcription regulation often observed with classical antagonists. ***[See also Results - PAPER IV].***

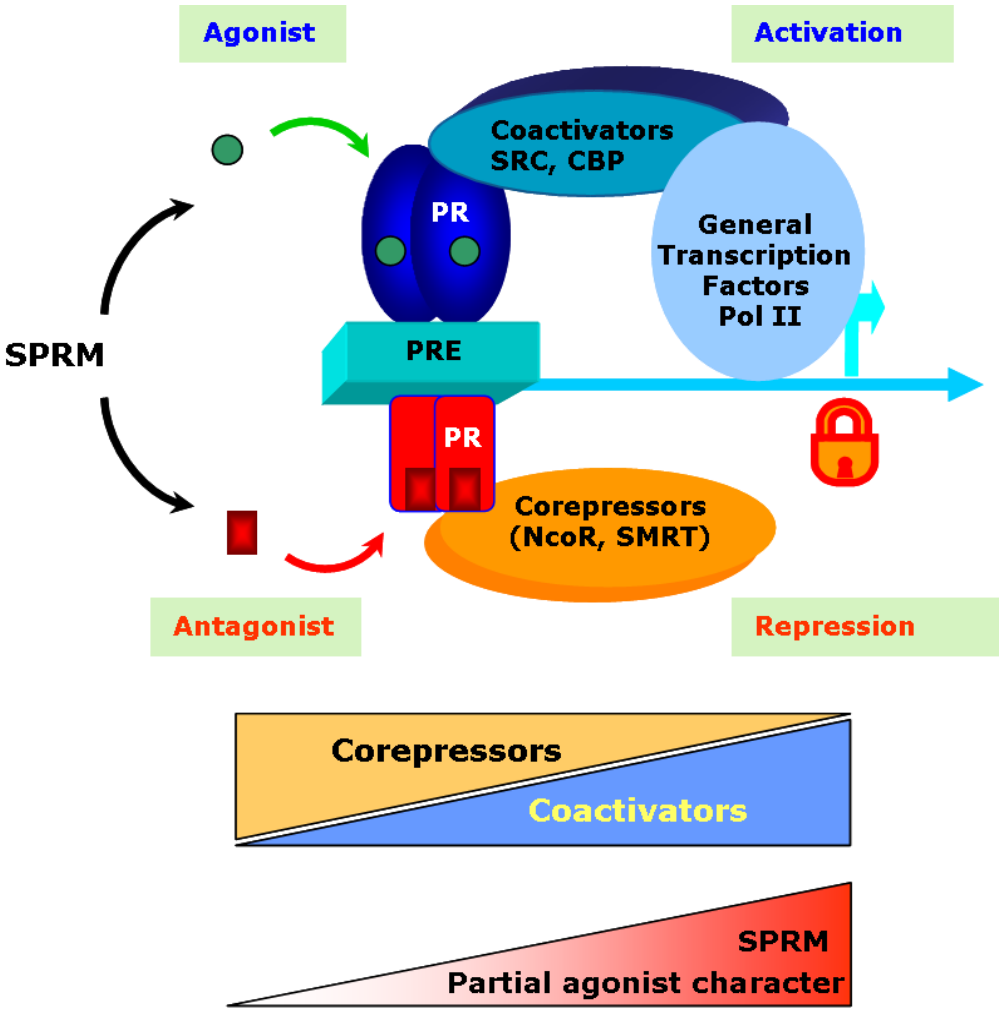


Figure 16. Mechanism of action of SPRM
The classical mode of action of PR ligands with mixed agonist/antagonist character or Selective Progesterone Receptor Modulators (SPRM) is shown. Agonist effects of SPRM are mediated via recruitment of coactivators which lead to activation of gene transcription whereas corepressors recruitment leads to gene silencing. However, the expression levels of transcriptional coregulators (coactivators and corepressors) are highly variable depending on cell type and influences the agonist/antagonist character of SPRM. Partial agonist properties of SPRM increases with increase in expression levels of coactivators and vice versa. Agonist character of SPRM is inversely proportional to expression levels of corepressors.

AIMS OF THE THESIS

The above mentioned studies highlighting an important role of progesterone receptor (PR) signaling in mammary carcinogenesis have renewed interest in understanding the role PR in carcinogenesis as well as in the development of PR antagonists. Human PR exists as two main isoforms PRA and PRB which behave as distinct transcription factors. Variation in their expression ratio might significantly influence signalling in progesterone target cells. Given that PRA/PRB ration is often altered under various pathological conditions due to unknown mechanisms, it becomes important to explore the mechanisms leading to such variations and their impact on various aspects of cell signalling such as transcriptional regulation/selection of target genes, cell proliferation and cross talk with growth factors signalling pathways. One of the main reasons why such important aspects of PR signalling remain unexplored till today was the lack of appropriate cellular models allowing controllable PRA/PRB ratio. While understanding the contribution of PR in several pathophysiological scenarios is pivotal, a possible therapeutic approach in PR-dependent pathological conditions constitutes the use of PR antagonists. However, most of the currently available antiprogestins present partial agonist activity and are not selective to PR since they also antagonize other steroid receptors such as GR and AR. The development of PR specific antagonists devoid of partial agonist character might provide important therapeutic perspectives for various reproductive tract abnormalities and certain hormone-dependent uterine and breast cancers.

Therefore, the objectives of this thesis mainly focus on three aspects of PR signaling i.e:

i) Understanding the mechanism of alteration in PRA/PRB expression ratio
ii) Defining the significance of variation in PRA/PRB ratio in target gene selection/transcriptional regulation, hormonal responsiveness and cross talk with growth factor signaling pathways
iii) Pharmacological targeting of PR by antagonist ligands inactivating PR through unusually passive mechanism of action

The results section is divided into 3 parts:

In the **Part 1**, we studied the ligand-induced PR isoforms and coactivator SRC1 turnover and its functional coupling with PR transcriptional activity. The main findings are as under:

- Defined a role of ligand-induced PR post-translational modifications in governing PR isoforms stability/turnover ***(Paper I)***
- Identification of p38 and p42/44 MAPK as critical regulators of PRA/PRB expression ratio ***(Paper I)***
- Demonstration of a critical role of ligand-induced PR-SRC1 turnover in controlling PR transcriptional activity ***(Paper II)***.

In **Part 2**, we describe the strategy of bi-inducible promoter system for selective PR-isoform expression. To validate our newly established breast cancer cellular model, we studied several aspects of PR signaling such as transcriptional regulation/selection of targets and cross talk with growth factor signaling pathways. This part includes one submitted reseach article and the results obtained by transcriptomic studies.

- Establishment and characterization of an original bi-inducible breast cancer cellular model allowing controllable PRA/PRB expression ratio ***(Paper III)***
- Demonstration of a critical role of PRA/PRB ratio in target gene selection, transcriptional regulation and in cross talk with growth factors ***(Paper III)***

Part 3 deals with the molecular characterization of several new class steroidal and non steroidal PR ligands synthesized in a collaborative project. This part includes the results obtained by *in vitro* characterization of these ligands and a proof-of-concept study is presented in the form of an article on a prototype homosteroid ligand inactivating PR through passive mechanism of action.

- *In vitro* and *in vivo* characterization of new class steroidal and non steroidal PR antagonists and elaboration of their mechanism of action ***(Patent)***
- A new rationally designed homosteroid ligand with high progesterone receptor selectivity and full antagonist properties. *(**Paper IV)***

RESULTS

Part 1. Regulation of PR isoforms and coactivator turnover

A. Identification of distinct MAPK-dependent mechanisms controlling PRA/PRB ratio at post-translational level (*PAPER I*)

Background

Under physiological conditions, progesterone receptor isoforms (PRA and PRB) are expressed at equimolar level in progesterone target cells. An imbalanced PRA/PRB expression ratio is often associated with aggressive breast and endometrial cancers (Arnett-Mansfield et al., 2001; Mote et al., 2002) through unknown mechanisms. Any differential regulation of PR isoform transcripts has never been reported. The presence of estrogen response element (ERE) in exon sequence of PRB (Savouret et al., 1991) might be a potential mechanism for such differential regulation, however, it remains to be demonstrated. Nevertheless, such regulations might also occur through mechanisms independent of estrogens and may involve proteasome-mediated downregulation and/or stabilization. It is now well accepted that PR isoforms have differential role not only in target genes selection but also contribute distinctly towards pathophysiological conditions depending on progesterone target tissues (Mulac-Jericevic et al., 2003; Mulac-Jericevic et al., 2000; Poole et al., 2006; Richer et al., 2002). Knowing the fact that PRA/PRB ratio is altered in pathological conditions (breast and endometrial cells) it was important to explore the underlying mechanisms and its impact on PR signaling.

When we started this work, we had a collection of several cellular models (MDA-MB-231, Ishikawa, HEK293) expressing either PRA or PRB or both. The mechanisms behind alteration in PRA/PRB ratio are not well understood. Beside alternate transcription of PR isoforms, only few studies reported the preferential regulation of one isoform at post-transcriptional level (Graham et al., 1995a). It was initially shown by Clemm and colleagues (Clemm et al., 2000) and later on confirmed by other laboratories (Faivre and Lange, 2007) that in T47D cells, co-expressed PR isoforms undergo simultaneous ligand-induced down-regulation due probably to PRA:PRB heterodimerization. However, when PR isoforms are expressed alone, PRB but not PRA is rapidly degraded following progestin treatment (Faivre and Lange, 2007). In cells expressing either of the PR isoforms, the accelerated PRB turnover as compared to PRA is conferred by the down-regulatory properties of AF3 present in BUS domain of PRB (Tung et al., 2006). The BUS domain is involved in N-C terminal intramolecular interactions resulting in distinct PRB conformation as compared to PRA (Dong et al., 2004). It has been shown that PR-interacting proteins having associated ubiquitin E3-ligase activity such as BRCA1 (Poole et

al., 2006) and E6-AP (Ramamoorthy et al., 2010) selectively control PRA or PRB turnover indicating that differential regulatory proteins are involved in PR isoform down-regulation.

Results

The first study included in the thesis aimed at defining a role of agonist and antagonist ligands in proteasome-mediated turnover of progesterone receptor. Using endometrial and mammary cancer cells stably expressing PRA and/or PRB, we discovered that the antiprogestin RU486 inhibited the agonist-induced turnover of PR isoforms through active mechanism(s) involving distinct MAPK-dependent phosphorylations. p42/44 MAPK activity inhibited proteasome-mediated degradation of RU486-bound PRB but not PRA in both cell lines. Furthermore, ligand-induced PRB turnover required neosynthesis of a mandatory down-regulating partner whose interaction/function is negatively controlled by p42/44 MAPK. Such mechanisms are also functional with natural PR ligand progesterone and might play fundamental role in determining 'fate' of PR and thus be a key in functional dynamics of PR isoforms. Interestingly, in contrast to PRB, PRA stability was specifically increased by MEKK1-induced p38 MAPK activation showing that MAPK-mediated cell signaling differentially controls PRA/PRB expression ratio at post-translational level. Imbalance in PRA/PRB ratio frequently associated with carcinogenesis might be a direct consequence of disorders in MAPK signaling that might switch cellular responses to hormonal stimuli and contribute towards pathogenesis.

Perspectives

Nevertheless, one limitation of our study is that unknown additional mechanisms at transcriptional and even post transcriptional levels might also affect PR expression. The combination of which might influence PRA/PRB ratio and probably not in the same direction. Remains to be identified are the MAPK-dependent PR isoforms phosphorylation site(s) which contribute towards PR stabilization. Such studies will involve mutagenesis of multiple potential sites found in the N-terminal domain of PRB to discriminate their differential role on MAPK-dependent stabilization, and will require stable transfection experiments. Similarly, since we did not identify the putative PR partner(s) involved in PR stabilization that might also be regulated by MAPK, experiments using siRNA against several potential candidates such as E6-AP and BRCA1 should be performed. Such identifications could be of broad concerns for understanding the dysregulation of PR isoform expression resulting in drastic change in PRA/PRB ratio in cancer cells.

PAPER I

p38 and p42/44 MAPKs differentially regulate progesterone receptor A and B isoform stabilization

Khan JA, Amazit L, Bellance C, Guiochon-Mantel A, Lombès M and Loosfelt H

Mol Endocrinol, 2011, 25(10):0000-0000 Epub ahead of print Aug 4, 2011 as doi:10.1210/me.2011-1042

Molecular Endocrinology. First published ahead of print August 4, 2011 as doi:10.1210/me.2011-1042

p38 and p42/44 MAPKs Differentially Regulate Progesterone Receptor A and B Isoform Stabilization

Junaid A. Khan, Larbi Amazit, Catherine Bellance, Anne Guiochon-Mantel, Marc Lombès, and Hugues Loosfelt

Institut National de la Santé et de la Recherche Médicale Unité 693 (J.A.K., L.A., C.B., A.G.-M., M.L., H.L.) and Université Paris-Sud (J.A.K., L.A., C.B., A.G.-M., M.L., H.L.), Faculté de Médecine Paris-Sud, Unité Mixte de Recherche-S693, Le Kremlin-Bicêtre F-94276, France; and Assistance Publique-Hôpitaux de Paris (A.G.-M.), Hôpital Bicêtre, Service de Génétique Moléculaire, Pharmacogénétique et Hormonologie, and Assistance Publique-Hôpitaux de Paris (M.L.), Hôpital Bicêtre, Service d'Endocrinologie et Maladies de la Reproduction, Le Kremlin-Bicêtre F-94275, France

Progesterone receptor (PR) isoforms (PRA and PRB) are implicated in the progression of breast cancers frequently associated with imbalanced PRA/PRB expression ratio. Antiprogestins represent potential antitumorigenic agents for such hormone-dependent cancers. To investigate the mechanism(s) controlling PR isoforms degradation/stability in the context of agonist and antagonist ligands, we used endometrial and mammary cancer cells stably expressing PRA and/or PRB. We found that the antiprogestin RU486 inhibited the agonist-induced turnover of PR isoforms through active mechanism(s) involving distinct MAPK-dependent phosphorylations. p42/44 MAPK activity inhibited proteasome-mediated degradation of RU486-bound PRB but not PRA in both cell lines. Ligand-induced PRB turnover required neosynthesis of a mandatory down-regulating partner whose interaction/function is negatively controlled by p42/44 MAPK. Such regulation strongly influenced expression of various endogenous PRB target genes in a selective manner, supporting functional relevance of the mechanism. Interestingly, in contrast to PRB, PRA stability was specifically increased by MAPK kinase kinase 1-induced p38 MAPK activation. Selective inhibition of p42/p44 or p38 activity resulted in opposite variations of the PRA/PRB expression ratio. Moreover, MAPK-dependent PR isoforms stability was independent of PR serine-294 phosphorylation previously proposed as a major sensor of PR down-regulation. In sum, we demonstrate that MAPK-mediated cell signaling differentially controls PRA/PRB expression ratio at posttranslational level through ligand-sensitive processes. Imbalance in PRA/PRB ratio frequently associated with carcinogenesis might be a direct consequence of disorders in MAPK signaling that might switch cellular responses to hormonal stimuli and contribute towards pathogenesis. **(*Molecular Endocrinology* 25: 0000–0000, 2011)**

Progesterone receptor (PR), a steroid-activated transcription factor, is an important pharmacological target for contraception, female reproductive disorders, as well as for hormone-dependent breast and uterine cancers. Alternative transcription of PR gene results in equal expression of two major isoforms PRA and PRB (1, 2). PRA lacks the 164 N-terminal amino acids, also called the B-upstream segment (BUS) present in PRB (3). Each isoform having distinct genomic targets (4) and exerting tissue-specific effects (5), PRA/PRB expression ratio is a key biological determinant selecting tissue responsiveness to hormone and growth factors stimuli. Neosynthesized PR is stabilized by interacting with heat shock protein 90-containing complexes (6). Upon ligand binding, PR

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

doi: 10.1210/me.2011-1042 Received April 18, 2011. Accepted July 12, 2011.

Abbreviations: AR, Androgen receptor; BUS, B-upstream segment; cMEKK1, constitutively active MEKK1; EGF, epidermal growth factor; FCS, fetal calf serum; FKBP5, FK506 binding protein 5; GR, glucocorticoid receptor; HB, heparin binding; HB-EGF, HB EGF-like growth factor; JNK, c-Jun-N-terminal kinase; MEK, MAPK kinase; MEKK, MEK kinase; P-JNK, phosphorylated JNK MAPK; P-p38, phosphorylated p38 MAPK; P-p42/44, p42/44 MAPK phosphorylation; PR, progesterone receptor; pS294, serine-294 phosphorylation; siRNA, small interfering RNA; SMRT, silencing mediator of retinoid and thyroid receptor.

dissociates from these chaperones and undergoes conformational changes leading to its homo- and heterodimerization and sequential interactions with transcriptional coregulators (coactivators and corepressors). Ligand also induces posttranslational modifications, notably phosphorylations, ubiquitination, and sumoylation, and regulates PR functions at multiple levels as well as its down-regulation via proteasomes (7–11). Beside alternative transcription of PR isoforms, only few studies reported the preferential regulation of one isoform at the posttranscriptional level (12). However, aberrant PRA/PRB expression is frequently observed in breast and endometrial cancers (2, 13), suggesting potential alterations in down-regulation mechanisms affecting PR isoforms stabilities via posttranslational modifications.

In PR, at least 14 phosphorylation sites are targeted by multiple kinases, mostly within serine-proline motifs in N-terminal domain affecting PR transcriptional activity and turnover (7, 14–17). Among these phosphorylation events, PRB serine-294 phosphorylation (pS294) (pS294-PRB) has been shown to act as an important sensor for growth factor inputs that affects PR function and plays a critical role in cross talk with growth factor signaling pathways (17, 18). Blocking of progestin-induced receptor turnover by proteasome inhibitors blocks PR transcriptional activities (9). The underlying mechanisms of this paradoxical link between PR stabilization and transcriptional inactivation are yet to be fully understood but likely involve direct coupling of proteasomes with transcriptional machinery as already demonstrated for estrogen receptor (19). RU486 (Mifepristone), a widely used PR antagonist, has been proposed for hormone-dependent breast cancer treatment (20). Although RU486 blocks PR transcriptional activity by favoring corepressors recruitment, it was found that PR turnover was highly reduced after RU486 treatment (8, 21, 22). Like progesterone, RU486 stimulates similar early cascade of events, including chaperone dissociation, dimerization, and posttranslational modifications, such as sumoylation (10) and phosphorylation (8, 22). Mutation of breast cancer 1, a PR-interacting protein, leads to deregulated PRA/PRB ratio, resulting in mammary tumorigenesis that was prevented by RU486 (23). It thus becomes of major importance to explore the mechanisms regulating posttranslational modifications of PR isoforms and their respective turnover.

In this study, we investigated the effects of RU486 on PR isoforms turnover in endometrial and mammary cancer cells stably expressing PRA or PRB or both. We report that, in contrast to other antagonists and progestin R5020, RU486 strongly inhibits PRB and PRA degradation. Further investigations revealed that down-regulations of PRB and PRA are negatively controlled by key phosphorylation events involving distinct MAPK, resulting in selective PR isoform stabilization. Furthermore, these phosphorylation events are differentially controlled by ligands and antagonize PRB degradation via proteasome. Our data support the existence of a switching mechanism differentially regulating PR isoform expression ratio via MAPK-dependent phosphorylations, which might have important consequences in progression of hormone-dependent cancers.

Results

Antagonist RU486 inhibits agonist-induced down-regulation of PRA and PRB

Both PR isoforms when coexpressed undergo agonist-induced degradation to similar extent (22). However, PRB is degraded much more rapidly as compared with PRA in cells expressing either of PR isoforms (24). Given that PR transcriptional activity is coupled to its proteasome-mediated down-regulation, we wondered whether antagonist RU486 that inhibits PR target gene transcription could impair agonist-induced PR protein degradation. To investigate the mechanisms controlling differential PR isoforms protein stability/degradation independently of transcriptional contributions from endogenous PR promoters, we used endometrial (Ishikawa) and mammary cancer cells (MDA-MB-231) stably expressing recombinant PRA or PRB under the control of same promoter (25, 26). In these models, PR isoform expression was comparable with that of endogenous expression levels detected in wild-type breast cancer cells T47D (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). As expected, in both cell types, agonist R5020 (10^{-8} M)-induced PRA- or PRB-mediated up-regulation of FK506 binding protein 5 (*FKBP5*) gene expression was abrogated by 100-fold excess of RU486 (10^{-6} M) (Fig. 1, *lower panels*). Under similar hormonal conditions, RU486 was found to abolish agonist-induced PRA or PRB turnover and led to both PR isoforms accumulation with an electrophoretic upshift, characteristic of phosphorylated PR species (Fig. 1, *upper panels*). Therefore, in both endometrial and mammary cancer cells, silencing of agonist-induced PR isoforms-mediated target gene transcription by RU486 is accompanied with PR isoforms accumulation through unknown mechanisms.

RU486 stabilizes pS294-PRB

To understand the mechanisms by which RU486 stabilizes PR isoforms, we first hypothesized that RU486

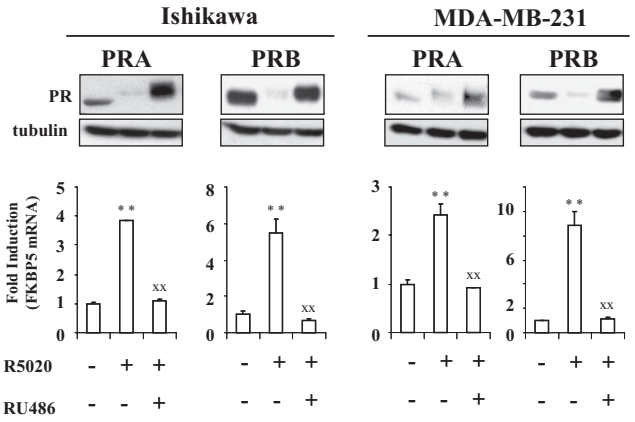


FIG. 1. RU486 abrogates agonist-dependent PRA and PRB down-regulation and transcriptional activity. Ishikawa or MDA-MB-231 cells stably expressing PRA or PRB were treated either by vehicle or R5020 (10^{-8} M) alone or in combination with RU486 (10^{-6} M) during either 6 h for the analysis of FKBP5 gene transcripts by real time quantitative PCR (*lower panel*) or 24 h for immunoblot detection of PR isoforms using anti-PR antibody (*upper panel*). Statistical significance is shown for agonist-induced transactivation as compared with vehicle-treated cells (*asterisks*) or for comparison between agonist alone or with antagonist-treated cells (XX).

might inhibit agonist-induced PRB pS294, which has been described as a major signal for PRB turnover and hypertranscriptional activity (14). To test this possibility, Ishikawa PRB or Ishikawa PRA cells were treated by R5020 (10^{-8} M) alone or in combination with equal concentration of RU486 for 6 h or 14 h. pS294-PRB and total PRB levels (Fig. 2A) or pS130-PRA (equivalent residue of PRB S294) and total PRA levels (Supplemental Fig. 2) were analyzed by Western blotting. We found that RU486 was unable to inhibit the agonist-induced pS294-PRB (Fig. 2A) or pS130-PRA (Supplemental Fig. 2) after 6 h, whereas down-regulation of pS294-PRB in RU486-treated cells occurred to a much lesser extent than with agonist alone after 14 h (Fig. 2A). Moreover, RU486 markedly slowed down the agonist-induced degradation of PRB (Fig. 2A), indicating that RU486 did not inhibit agonist-induced pS294-PRB but instead strongly stabilized it. We next examined whether RU486 and ZK98299, another PR antagonist, could induce pS294-PRB and impact PRB turnover. After 6 h, pS294-PRB levels were higher accompanied by lack of PRB turnover in RU486-treated cells, as compared with R5020 (Fig. 2B, *inset*). In contrast, ZK98299, as expected (8, 22), resulted in a weak overall PRB phosphorylation (lack of electrophoretic upshift), including pS294-PRB, and still provoked intermediary PRB degradation (Fig. 2B, *inset*). This suggested that pS294-PRB turnover might be interrupted by RU486 binding. Therefore, we next compared R5020- or RU486-induced pS294-PRB kinetics in Ishikawa PRB cells under similar ligand concentration (10^{-8} M) (Fig. 2C). Quantification of electrophoretic bands (Fig. 2C, *graphs*) allowed analyzing the time course of ligand-induced pS294-PRB and PRB degradation. R5020 induced a robust early pS294-PRB (Fig. 2C, *left graph*) reaching a peak at 1 h and then decreased concomitantly to PRB degradation (Fig. 2C, *left and middle graphs*). RU486 also induced pS294-PRB but with slower kinetics reaching a plateau at 12–14 h, which remained stable thereafter (Fig. 2C, *right graph*), parallel to PRB accumulation profile (Fig. 2C, *middle graph*). As expected (22), analysis of PRA phosphorylation on S130 in Ishikawa PRA cells showed that PRA also undergoes agonist-induced pS130-PRA but with much slower kinetics and to a lesser extent as compared with PRB (Supplemental Fig. 3). Although agonist R5020 or antagonist RU486 induced pS294-PRB as early as 15 min, ligand-induced pS130-PRA is detectable only after 1 h of hormonal treatment (Supplemental Fig. 3). Although both R5020 and RU486 induced pS294-PRB (and pS130-PRA), only the agonist-bound PR isoform is signaled toward degradation, whereas antagonist-bound PR failed to undergo expected pS294- or pS130-driven PR isoform down-regulation. Therefore, we asked whether RU486-bound PRB might be insensitive to ubiquitination. Parental Ishikawa cells were transiently transfected with HA-tagged ubiquitin and PRB expression vectors, pretreated with proteasome inhibitor MG132, and incubated or not by ligands during 4 h. Immunoprecipitated PRB was analyzed by Western blotting using anti-HA antibody. RU486 markedly reduced basal PRB ubiquitination (Fig. 2D). Taken together, our results indicate that RU486, despite inducing pS294, stabilizes PRB in part by inhibiting ubiquitination processes. Thus, turnover of R5020- or RU486-bound PR isoform is inversely correlated, irrespective of the pS294 status.

Phosphorylated p42/p44 are pivotal for PRB but not PRA stability

MAPK were reported to enhance PRB transcriptional activity and turnover through PRB phosphorylation at S294 in the presence of agonist ligands (27). Therefore, we wondered whether PRB or PRA stabilization by RU486 could be related to alterations in p42/44 MAPK-dependent phosphorylation events. Ishikawa cells stably expressing either PRB or PRA were incubated with vehicle or R5020 or RU486 for 1, 6, or 24 h in the absence or presence of U0126, a specific MAPK kinase (MEK)1/2 inhibitor that prevents p42/44 MAPK phosphorylation (P-p42/44). PRB and its S294 phosphorylated moiety were examined to determine P-p42/44-dependent early and late events that might affect PR isoform down-regulating mechanisms. As expected, U0126 inhibited P-p42/44 to similar extent in both PRB and PRA cell lines (Fig. 3A). After 1 h, the agonist-induced as well as antagonist-induced pS294-

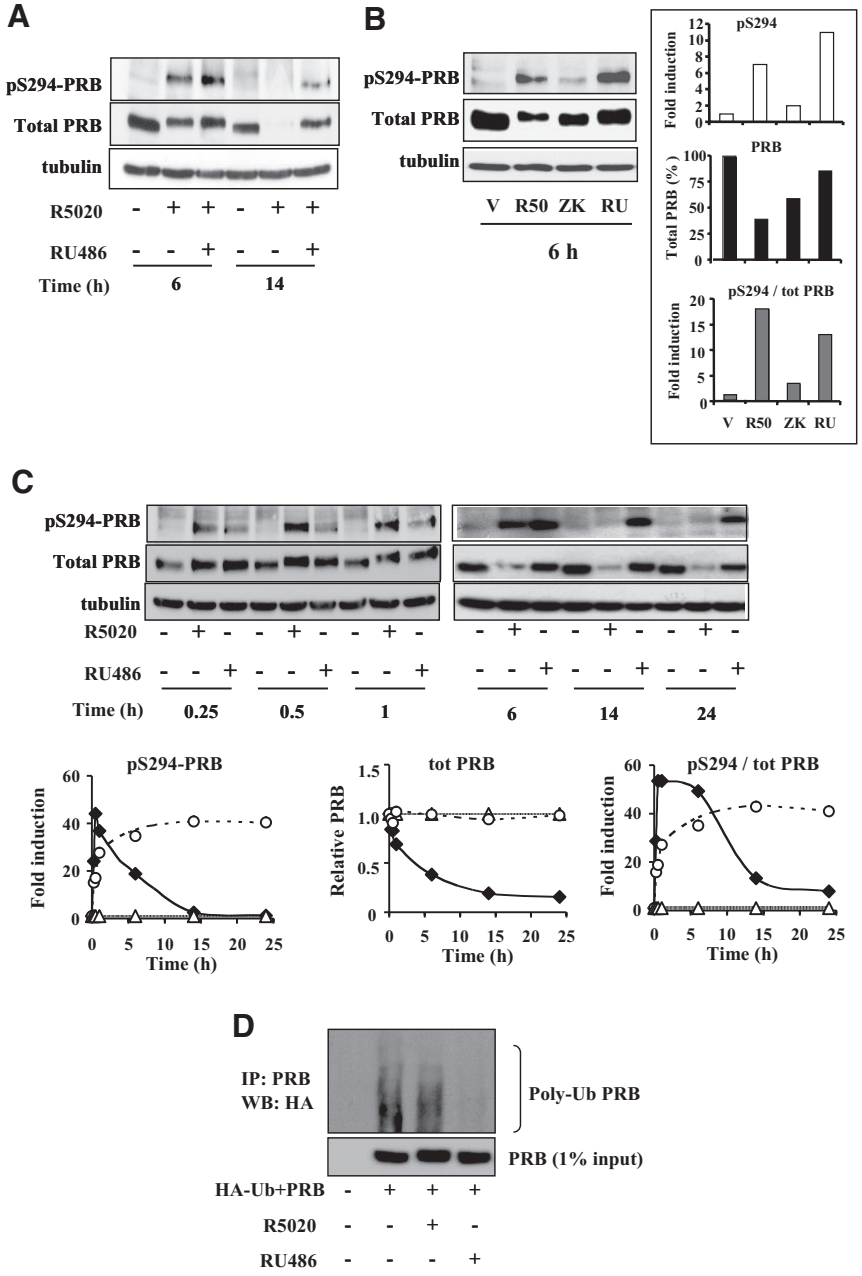


FIG. 2. Unlike R5020 and ZK98299, RU486 induces stable PRB- pS294. A, Ishikawa PRB cells were treated by ligands as in Fig. 1 during 6 or 14 h, and whole cells extracts were immunoblotted using PRB serine 294 phospho-specific antibody, anti-PRB, or antitubulin antibody. B, Ishikawa PRB cells were treated with vehicle (V) or R5020 (R50) (10^{-8} M) or ZK98299 (ZK) (10^{-6} M) or RU486 (RU) (10^{-8} M) during 6 h, and whole cell extracts were immunoblotted as in A. Numerized band densities corresponding to pS294-PRB (*upper inset*) or total PRB (*middle inset*) are normalized to vehicle or tubulin controls and plotted as fold induction or percentage of total PRB in ligand-free condition. Ligand-induced pS294/PRB (*lower inset*) is presented as fold induction of vehicle-treated cells. C, Ishikawa PRB cells were treated without or with R5020 (10^{-8} M) or RU486 (10^{-8} M) during the indicated time periods, and whole cell extracts were immunoblotted as in A. pS294-PRB and PRB band densities were normalized to vehicle or tubulin controls and plotted as fold induction of ligand-free species for each time point (*left and middle panels*), and the corresponding ratio is shown in the *right panel* (*white triangle*, vehicle; *black diamond*, R5020; and *white circle*, RU486). D, Parental Ishikawa cells lacking PRB expression were transiently transfected with HA-ubiquitin and PRB expression vectors during 48 h, pretreated with MG132 (5 μM) during 30 min, and then incubated without or with R5020 (10^{-8} M) or RU486 (10^{-8} M) during 4 h. After PRB immunoprecipitation (IP) using monoclonal anti-PR antibody, ubiquitinated-PRB was analyzed by Western blotting (WB) using anti-HA antibody (*upper panel*). PRB levels corresponding to 1% input were detected by anti-PR antibody (*lower panel*).

PRB remained unchanged in the presence of U0126 (Fig. 3A, *upper panels*), indicating that an unknown kinase distinct from p42/44 targets S294, as previously suggested for agonist ligand (28, 29). Similar results were obtained for PRA and its pS130 species (Fig. 3, *lower panels*). Surprisingly, after 6 h, inhibition of P-p42/44 specifically triggered degradation of RU486-bound PRB with a parallel decrease in pS294-PRB without altering basal or agonist-bound PRB levels (Fig. 3A, *middle panels*). In sharp contrast, U0126 did not affect RU486-bound PRA level. At longer time period (24 h), although P-p42/44 inhibition further enhanced RU486-bound PRB degradation, small decrease in ligand-free as well as R5020-bound PRB was also observed (Fig. 3A, *right panels*), indicating that P-p42/44 also enhances the agonist-bound PRB stability but to a lesser extent as compared with RU486. Quantitative RT-PCR analysis showed that PRB mRNA levels were unchanged after U0126 treatment (Supplemental Fig. 4), indicating that p42/44 control PRB stability at posttranslational levels. Similar P-p42/44-dependent stabilization of RU486-bound PRB was observed when Ishikawa PRB cells were cultured in serum-free medium (Supplemental Fig. 5). Surprisingly, U0126 treatment did not affect basal or ligand-bound PRA levels even after 24 h, indicating that PRB but not PRA turnover is negatively controlled by P-p42/44 in a ligand-sensitive manner (Fig. 3A, *right panels*). To substantiate P-p42/44 as well as ligand specificity for PRB stabilization at shorter time points (6 h), Ishikawa PRB cells were exposed to increasing concentrations of U0126 under constant amounts of R5020 or RU486 (Fig. 3B). In contrast to vehicle or R5020 treatment, degradation of RU486-bound PRB (and pS294-PRB species) occurred as a function of P-p42/44 inhibition with a decrease in overall PRB upshift. This strongly indicates that RU486-induced PRB stabilization is controlled by p42/44 activity in a

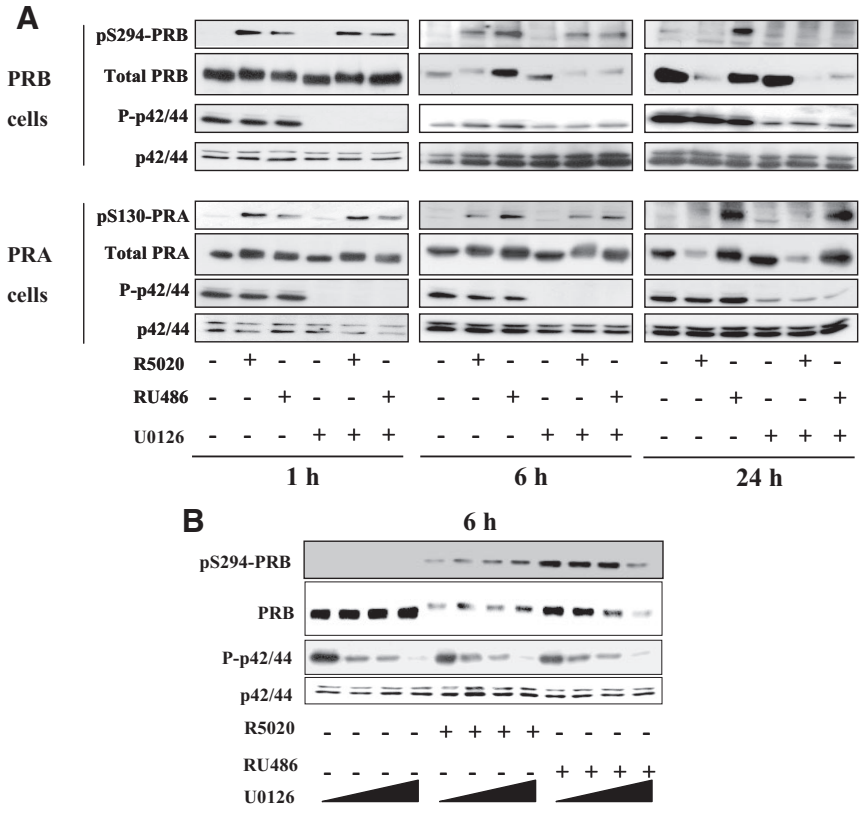


FIG. 3. Phosphorylated p42/44 MAPK stabilize PRB but not PRA in a ligand-dependent manner. A, Ishikawa PRB or PRA cells were pretreated with dimethylsulfoxide (DMSO) or U0126 (10 μM) during 30 min and then incubated without or with R5020 (10^{-8} M) or RU486 (10^{-8} M) during 1, 6, or 24 h. Whole cell extracts were immunoblotted using either phospho-specific (pS294-PRB, pS130-PRA) or anti-PR antibody (PRB, PRA). From the same immunoblot, either total p42/p44 or their phosphorylated species (P-p42/44) were analyzed using the corresponding antibodies. B, Ishikawa PRB cells were pretreated without or with U0126 (5, 10, or 20 μM) during 30 min and then treated without or with R5020 (10^{-8} M) or RU486 (10^{-8} M) during 6 h. Whole cell extracts were immunoblotted as in A.

dose-dependent manner. These results demonstrate that RU486, when compared with R5020 or vehicle, strongly facilitates P-p42/44-dependent phosphorylation of PRB on a residue other than S294 resulting in slower PRB degradation.

P42/44 MAPK control proteasome-dependent turnover of ligand-bound PRB in endometrial and mammary cancer cells

To further analyze the p42/44-dependent mechanism of PRB stabilization, we asked whether this mechanism could be also functional in breast cancer cells. For this, MDA-MB-231 PRB cells were treated or not by RU486 and U0126 for 24 h. In contrast to Ishikawa cells, basal PRB level increased after inhibition of p42/44 activity (Fig. 4A). However, RU486-bound PRB was degraded after U0126 treatment as in Ishikawa cells, indicating that ligand-specific p42/44-dependent mechanism controlling PRB stability is conserved in both cell types.

Our previous results in Fig. 3A showed that at delayed time points, P-p42/44 inhibition also accelerated R5020-bound PRB degradation. Therefore, we asked whether ligand-specific p42/44 control of PRB stability is relevant for the natural ligand progesterone known to induce slower PRB turnover than synthetic progestin R5020. As shown in Fig. 4B, progesterone-bound PRB degradation was enhanced by U0126 in both MDA and Ishikawa cells, thus indicating that p42/44 activity also slows down progesterone-induced PRB turnover.

To verify whether association of RU486 and U0126 had provoked any change in subcellular localization of PRB that might intervene in PRB stabilization, immunofluorescence studies in MDA-MB-231 PRB cells demonstrated that PRB remained mainly localized in the nuclei in all conditions (Fig. 4C). As expected, the agonist stimulated PRB degradation, whereas RU486 provoked a strong PRB nuclear retention. Specific inhibition of p42/44 resulted in RU486-bound PRB degradation consistent with Western blot analyses (Fig. 4A), thus strengthening the important role of P-p42/44 signaling cascade in ligand-bound PRB stabilization.

Given that PR is degraded via proteasomes, we wondered whether inhibition of RU486-bound PRB ubiquitination could be reversed by ubiquitin overexpression. Ishikawa PRB cells were transiently transfected with control or HA-ubiquitin encoding vector during 24 h and treated with vehicle or R5020 or RU486 during 6 h. As expected, ubiquitin overexpression decreased basal PRB levels. However, RU486-bound PRB (and pS294-PRB species) underwent much slower degradation as compared with R5020-induced PRB turnover (Supplemental Fig. 6). We next examined the contribution of P-p42/44 in the control of such processes by using proteasome inhibitors. We found that MG132, as well as lactacystin (data not shown), strongly enhanced P-p42/44 in Ishikawa cells without affecting total p42/44 levels (Fig. 4D), as was already reported for other cell lines (30). As expected, MG132 exposure resulted in PRB accumulation in vehicle as well as in hormonal conditions. Interestingly, however, P-p42/44 inhibition partially impaired PRB accumulation under MG132 (Fig. 4D, lanes 3 *vs.* 4, 7 *vs.* 8, and 11 *vs.* 12) and lactacystin exposure (data not shown), indicating that proteasome inhibitors stabilize PRB by activating p42/44 in addition to the blockade of proteolytic functions of

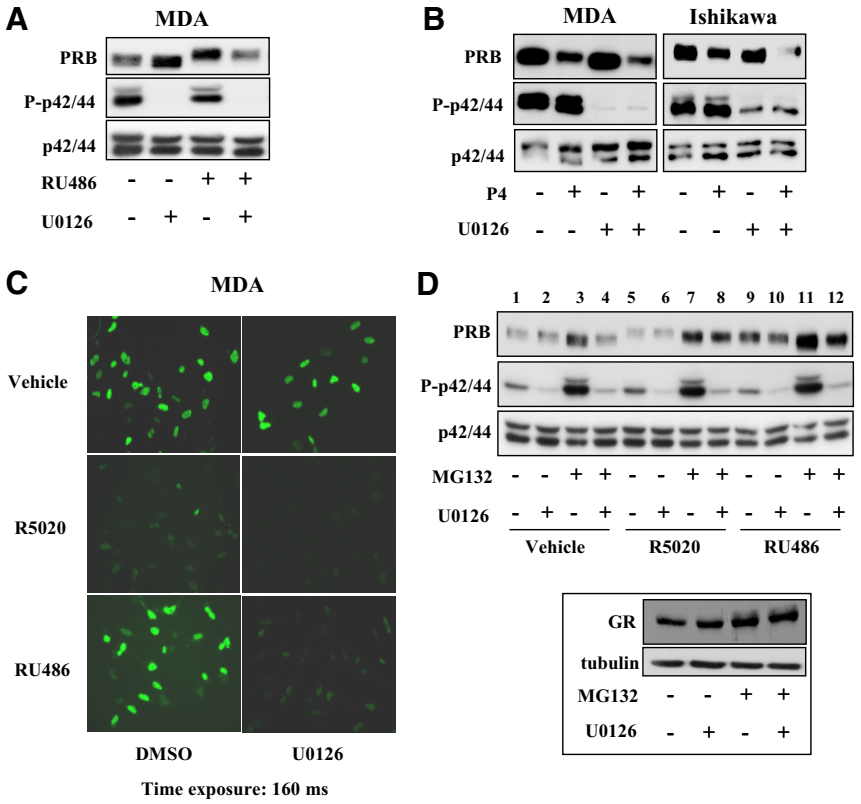


FIG. 4. P42/44 MAPK control proteasome-dependent turnover of ligand-bound PRB. A, MDA-MB-231 cells (MDA) stably expressing PRB were treated with vehicle or RU486 (10^{-8} M) during 24 h, and whole cell extracts were immunoblotted as in Fig. 3. B, MDA-MB-231-PRB or Ishikawa-PRB cells were pretreated with dimethylsulfoxide (DMSO) or U0126 (10 μM) during 30 min and then incubated without or with progesterone (P4) (10^{-8} M) during 24 h. Whole cell extracts were immunoblotted as in A. C, MDA-MB-231 PRB cells were pretreated with DMSO or U0126 (10 μM) during 30 min and then incubated with vehicle or R5020 (10^{-8} M) or RU486 (10^{-8} M) during 24 h. Immunofluorescence analysis was performed as described in *Materials and Methods* using anti-PR antibody, and images were obtained for an identical time exposure. D, Ishikawa PRB cells were pretreated without or with MG132 (5 μM) and/or U0126 (10 μM) during 30 min and then treated without or with R5020 (10^{-8} M) or RU486 (10^{-8} M) during 6 h. Immunoblot analysis was performed as in A. Cell lysates from the same RU486-treated cells were also immunoblotted for either GR detection using anti-GR antibody or tubulin as loading control (*inset*).

proteasome. To rule out the possibility that U0126 might interfere with proteasome activity, we examined the expression of glucocorticoid receptor (GR), another nuclear receptor belonging to the same nuclear receptor subfamily as PR and also degraded by proteasomes. In the presence of RU486, also a powerful antagonist of GR, U0126 treatment did not induce degradation of RU486-bound GR, nor inhibited GR accumulation by MG132 (Fig. 4D, *inset*), demonstrating that P-p42/44 selectively controls PRB stability without affecting general proteasome activity.

Collectively, these results demonstrate that P-p42/44-dependent mechanism slows down the proteasome-dependent turnover rate of ligand-bound PRB in mammary as well as in endometrial cells. This stabilizing mechanism is potentiated by RU486 as compared with progestins and is also functional with the natural ligand progesterone. Therefore, p42/44 MAPK act as brakes for proteasome-dependent turnover of PRB in a ligand-sensitive manner.

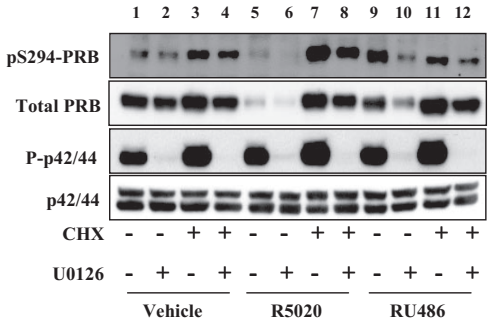


FIG. 5. Ligand-induced PRB degradation requires protein neosynthesis. Ishikawa PRB cells were pretreated without or with 100 μg/ml cycloheximide (CHX) and/or U0126 (10 μM) during 30 min and then treated without or with R5020 (10^{-8} M) or RU486 (10^{-8} M) during 24 h. Immunoblot analyses were performed as in Fig. 3A.

Phosphorylated p42/p44 inhibit function of a down-regulating protein partner

To analyze the impact of P-p42/44-dependent phosphorylation on PRB turnover independently of transcriptional and translational events, we preincubated Ishikawa-PRB cells with cycloheximide alone or in combination with U0126 and then treated with vehicle or R5020 or RU486 (Fig. 5). Surprisingly, we found that presynthesized PRB was highly stabilized after a 24-h treatment by cycloheximide, to a level similar for each ligand condition (lanes 3, 7, and 11). Of note, the strong impact of progestin on PRB degradation as well as RU486-bound PRB degradation in the presence of U0126 was fully abolished when neosynthesis was turned off. This strongly suggested that both agonist-induced as well as antagonist-induced PRB down-regulation requires *de novo* synthesis of down-regulating protein partner(s). Intermediary patterns were also analyzed at shorter time points (data not shown), mainly showing that agonist-induced PRB down-regulation was inhibited as early as 6 h after cycloheximide treatment. Cycloheximide abrogated the degradation of agonist-bound pS294-PRB, which is known to be directed to the proteasome pathway (lane 7 *vs.* 5). This indicates that the putative down-regulating factor might preferentially target the pS294-PRB species. We noted that

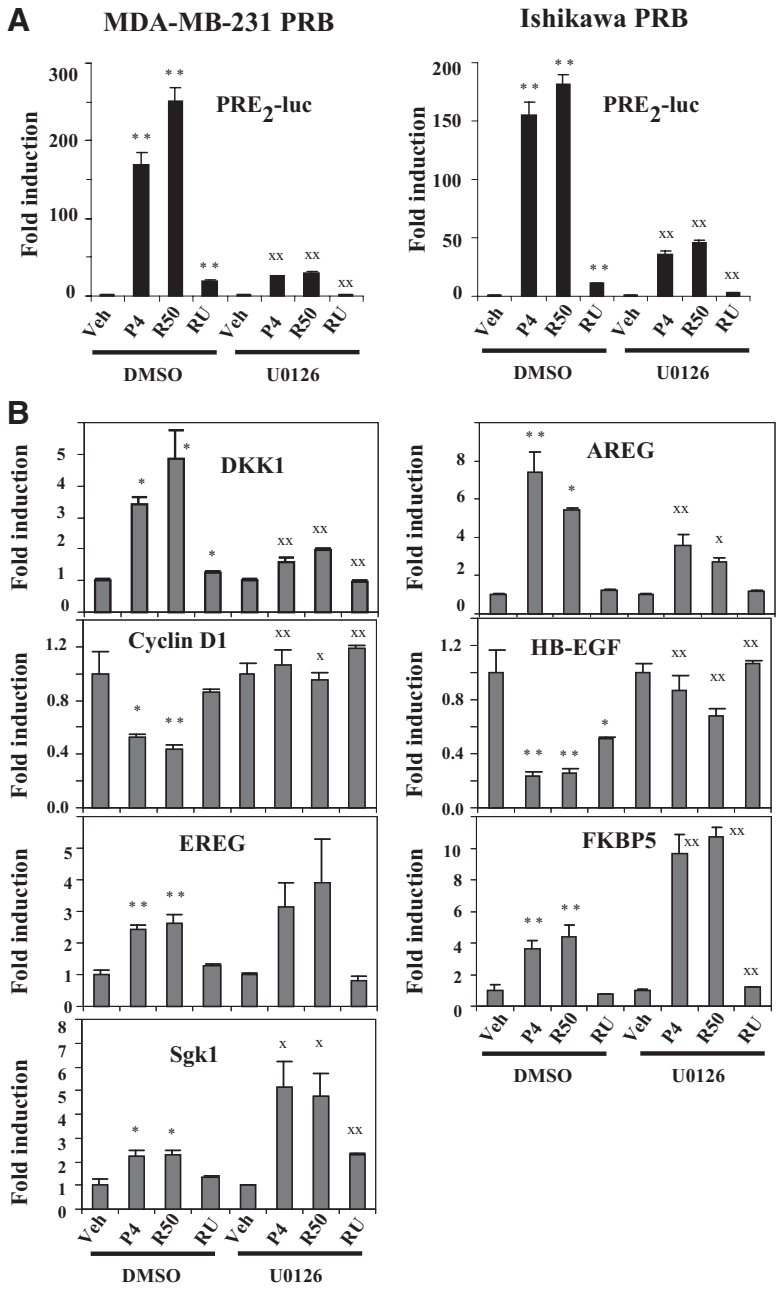


FIG. 6. Phosphorylated p42/p44 differentially influence PRB transcriptional activity. A, MDA-MB-231-PRB or Ishikawa PRB cells were transiently transfected with PRE_2-luciferase vector during 24 h, pretreated with dimethylsulfoxide (DMSO) or U0126 (10 μM) during 30 min, and then incubated with vehicle (Veh) or progesterone (P4) (10^{-8} M) or R5020 (R50) (10^{-8} M) or RU486 (RU) (10^{-8} M) during 24 h. Luciferase activity was determined and normalized to total protein concentration. The data (mean ± SEM) from six independent cell cultures are set to 1 for ligand-free condition from DMSO or U0126-treated cells, and fold induction by hormone is presented. Statistical significance is shown by *asterisks* for ligand-induced transactivation as compared with vehicle or by XX when similar ligand condition is compared between DMSO or U0126-pretreated cells. B, MDA-MB-231 PRB cells were incubated with U0126 and hormones as above during 6 h, and quantitative real time PCR analysis was performed for indicated gene transcripts. The data (mean ± SEM) from three independent cell cultures measured in duplicate are set to 1 for ligand-free condition from DMSO or U0126-treated cells, and fold induction by hormone is presented. Statistical significance is shown as in A. DKK1, Dickkopf homolog 1; AREG, amphiregulin; EREG, epiregulin; Sgk1, serum and glucocorticoid-regulated kinase 1. *, $P \leq 0.05$; X, $P \leq 0.05$; **, $P \leq 0.01$; XX, $P \leq 0.01$.

cycloheximide decreased the level of RU486-induced pS294-PRB (lane 11 *vs.* 9). By blocking neosynthesis of the ligand-specific kinase targeting S294, cycloheximide might interrupt the delayed pS294 processes (6–24 h) induced by RU486 without affecting early processes (1–2 h) initiated by agonist as shown in Fig. 2C. We may thus hypothesize that agonist ligand induces interaction of pS294-PRB with down-regulating factor(s) and that RU486 might specifically inhibit this step. Furthermore, we observed that cycloheximide led to increased P-p42/44 levels (but not total p42/44) that might contribute toward PRB protein stabilization (lanes 3, 7, and 11) consistent with our previous findings showing that P-p42/44 stabilizes PRB. Cotreatment of cells with U0126 partially restored degradation of presynthesized PRB (lanes 3 *vs.* 4, 7 *vs.* 8, and 11 *vs.* 12), supporting that P-p42/44 might inhibit interaction with a protein partner required for PRB turnover. Differential effects of ligands on PRB stability might result from their respective ability to control kinetics of at least two phosphorylation events having opposite effects on PR stability, one targeting S294 of PRB independently of MAPK (accelerating turnover) and the other involving a p42/44-dependent kinase activity targeting phosphorylation site other than S294 that inhibits pS294-PRB degradation.

p42/44 MAPK differentially impact PRB transcriptional activity

Because proteasome-dependent turnover of PRB has been shown to be coupled to its transcriptional activity, we asked whether p42/44-dependent stabilization of ligand-bound PRB could impact transcription of progesterone-responsive genes. In both MDA-MB-231 PRB cells and Ishikawa PRB cells, inhibition of p42/44 activity dramatically decreased PRB-mediated reporter gene transcription in response to progesterone and R5020 (Fig. 6A). The partial agonistic effect of RU486 was similarly diminished after U0126 treatment. This shows that p42/44 facilitates PRB transcriptional activity from synthetic promoters.

Given that PRB-mediated transcription of endogenous genes involves promoter-dependent recruitment of coregulators, we examined the impact of MAPK signaling on ligand-dependent transcription of various PRB target

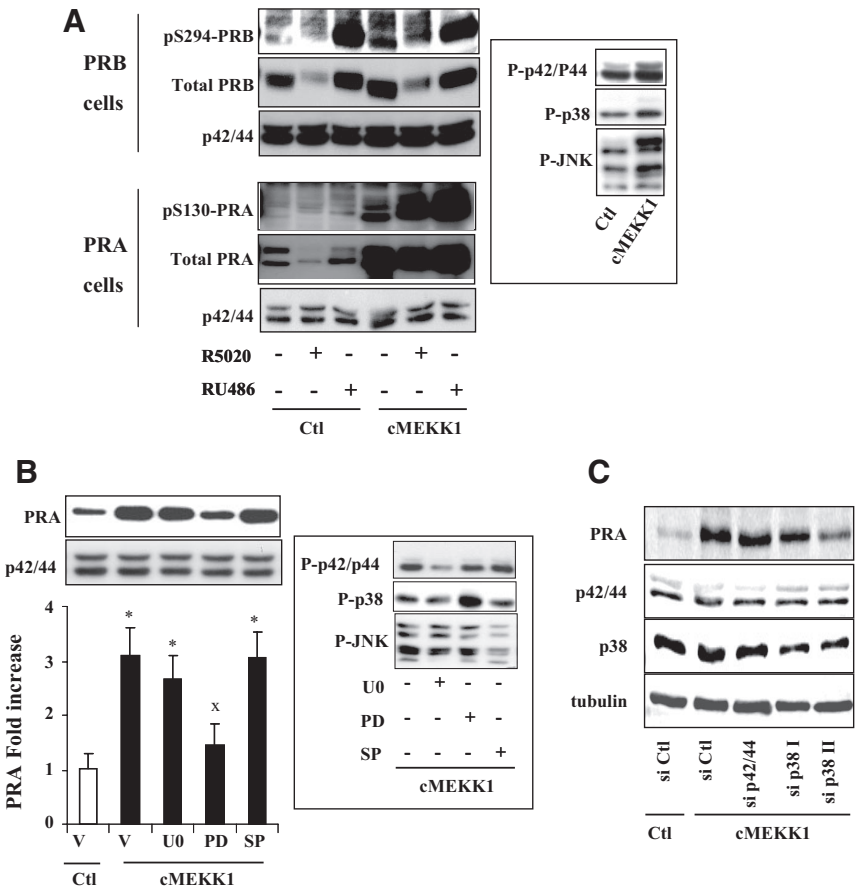


FIG. 7. MEKK1-induced PRA stabilization is impaired by p38 inhibition. A, Ishikawa PRB or PRA cells were transiently transfected with either empty control (Ctl) vector or cMEKK1 expression vector during 24 h and then treated without or with R5020 (10^{-8} M) or RU486 (10^{-8} M) during 24 h. Whole cell extracts were immunoblotted, and P-p42/44, P-p38, and P-JNK MAPK levels were detected by specific antibodies (*inset*). B, Ishikawa PRA cells were pretreated with vehicle (V) or U0126 (U0) (10 μM) or PD169316 (PD) (10 μM) or SP600125 (SP) (10 μM) during 30 min and then transfected with empty or MEKK1 expression vector during 24 h. Immunoblot analysis was performed as above, and normalized PRA band intensities are presented as fold increase as compared with PRA levels in control cells (*inset*). Statistical significance is represented by *asterisks* when comparison is done between control or cMEKK1 condition and by XX when selective MAPK inhibition is compared with nontreated MEKK1-transfected cells. C, Ishikawa PRA cells were cotransfected with control or cMEKK1 vector along with either control or specific siRNA against both p42 and p44 or p38 MAPK during 24 h as described in *Materials and Methods*. Cells were then incubated in 5% fetal calf serum containing medium for another 48 h before performing immunoblot analysis.

genes. MDA-MB-231 cells were preincubated with U0126 and treated with vehicle, progesterone, R5020, or RU486 during 6 h. As shown in Fig. 6B, P-p42/44 inhibition differentially influenced ligand-regulated transcription of PRB target genes. Similar to inhibitory effect of U0126 on reporter gene transcription, agonist-induced *Dickkopf homolog 1* and *amphiregulin* gene transcription was dramatically reduced after U0126 treatment. Likewise, U0126 reversed the agonist ligand-dependent transcriptional repression of *cyclin D1* and heparin-binding (HB) epidermal growth factor (EGF)-like growth factor (*HB-EGF*) genes. Moreover, RU486 also decreased HB-EGF gene transcription that was inhibited by U0126. However, U0126 did not alter PRB-mediated *epiregulin* gene transcription. Interestingly, P-p42/44 inhibition strongly enhanced ligand-induced transcription of *FKBP5* and *serum* and *glucocorticoid-regulated kinase 1* genes. These results show that p42/44 MAPK fine tune PRB mediated transcription depending on target gene promoter context and influence transcription of both up-regulated as well as down-regulated PRB target genes. Thus, p42/44 not only stabilize ligand-bound PRB but also play a major role in modulating as well as selecting PRB-mediated transcriptional response to ligands.

MEK kinase (MEKK)1 stabilizes PRA through phosphorylated-p38 MAPK

Our findings that P-p42/44 stabilizes PRB but not PRA suggest that distinct MAPK cascades could selectively control PR isoforms stabilities. To test this hypothesis, we transiently transfected PRB or PRA expressing Ishikawa cells by a vector encoding constitutively active MEKK1 (cMEKK1) and treated with agonist or antagonist ligands for 6 or 24 h. cMEKK1 primarily phosphorylates p38 and c-Jun-N-terminal kinase (JNK)/stress-activated protein kinase and to a lesser extent p42/44 MAPK (Fig. 7A, *inset*). cMEKK1 increased basal PRB levels and concomitantly S294 phosphorylated species after 6 h (Supplemental Fig. 7) as well as 24 h (Fig. 7A). However, a much more pronounced increase in total PRA and pS130-PRA levels was observed under both vehicle as well as ligand conditions (Fig. 7A and Supplemental Fig. 7), suggesting that high MEKK1 activity preferentially stabilized PRA in Ishikawa cells. Such stabilizing effect was observed at lower extent by decreasing cMEKK1 amount, showing dose dependency of the mechanism (Supplemental Fig. 8). We then aimed at identifying the specific MEKK1-downstream MAPK, possibly involved in the regulation of PRA stability. Ishikawa PRA cells were pretreated with specific inhibitors of P-p42/p44 (U0126), phosphorylated p38 MAPK (P-p38) (PD169316), or phosphorylated JNK MAPK (P-JNK) (SP600125) and transfected with cMEKK1 expression vector. After 24 h, the phosphorylation status of MAPK was examined (Fig. 7B, *inset*). Although U0126 and SP600125

inhibited P-p42/44 and P-JNK, respectively, it is not surprising that PD169316 did not inhibit MEKK1-induced p38 phosphorylation. Indeed, in contrast to U0126 or SP600125 that selectively inhibit the phosphorylation of p-42/44 or JNK, respectively, PD169316 is known to selectively inhibit the kinase activity of the phosphorylated p38 without hindering upstream kinases to phosphorylate p38 (31, 32). Increased phospho p-38 levels in the presence of PD169316 (Fig. 7B, *inset*) are most likely due to blockade of negative feedback loop of dephosphorylation of p38 MAPK by MAPK phosphatases (33, 34). As shown in Fig. 7B, MEKK1-dependent increase in PRA stability was clearly impaired in cells treated with PD169316 but not by U0126 or SP600125, suggesting that P-p38 pathway is implicated in the regulation of PRA stability. To strengthen our argument for p38-dependent stabilization of PRA, Ishikawa PRA cells were cotransfected with control or cMEKK1 vector along with specific small interfering RNA (siRNA) against both p42 and p44 or p38 MAPK. Results presented in Fig. 7C demonstrate that p38 but not p42/44 siRNA clearly inhibited increase in PRA stability by cMEKK. These observations along with our previous findings provided first evidence that distinct MAPK differentially regulate PR isoforms stability.

PRA/PRB expression ratio is controlled by distinct MAPK

The selective MAPK control of PR isoforms stabilities prompted us to examine the impact of MAPK on PRA/PRB expression ratio when both isoforms are coexpressed in the same cells, *i.e.* in conditions where ligand-bound PRA and PRB can interact as heterodimers and can compete for their proteasome-mediated turnover. In Ishikawa PRAB cells coexpressing both PR isoforms, MEKK1 stabilized basal PRA at much higher level than PRB (Fig. 8A), indicating that basal PRA turnover is selectively and highly sensitive to p38 MAPK activities even in the presence of PRB. Such effect led to a strong increase of ligand-free PRA/PRB ratio from 0.3 to 1. As expected (22, 29), basal as well as MEKK1-induced pS294-PRB levels were higher as compared with pS130-PRA levels in cells coexpressing both PR isoforms. This cell-based model enabled us to investigate the relative contribution of P-p42/44 and P-p38 MAPK in regulating PRB or PRA stabilities under MEKK1 stimulation and thus in controlling PRA/PRB expression ratio at posttranslational level. P-p42/44 inhibition using U0126 (Fig. 8B) or p42/44 knockdown by specific siRNA (Supplemental Fig. 9) selectively but not exclusively decreased PRB stability. Such preferential decrease in PRB levels after p42/44 inhibition resulted in increased PRA/PRB ratio under vehicle and R5020 exposure but not in RU486-treated cells (Fig. 8B). In contrast,

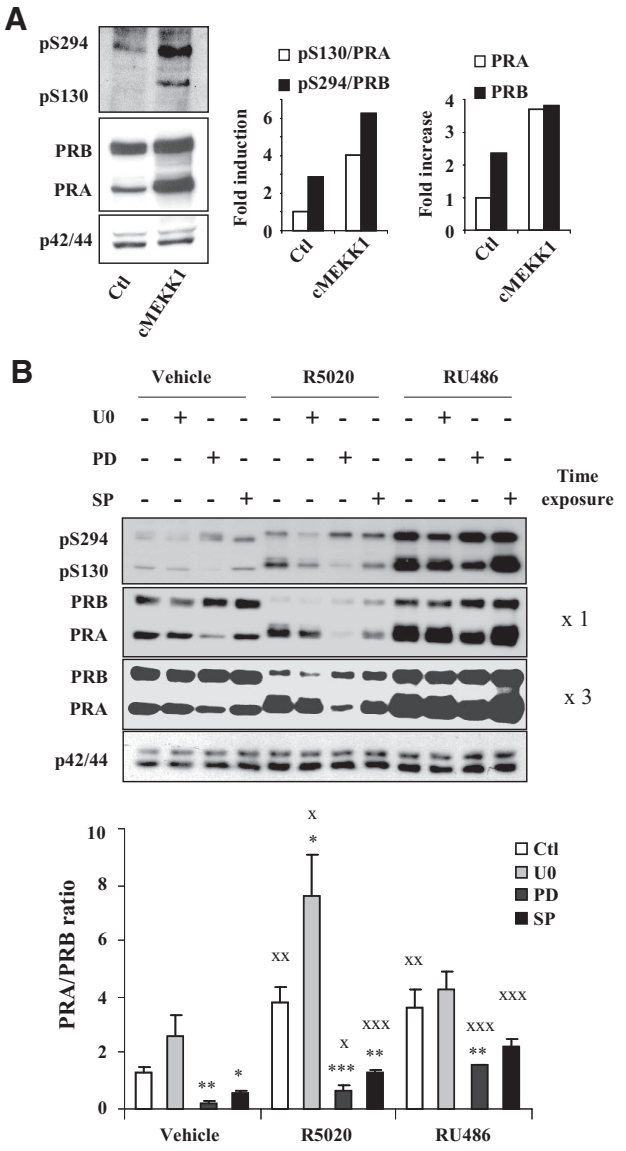


FIG. 8. Distinct MAPK control PRA/PRB expression ratio. A, Ishikawa cells stably coexpressing PRA and PRB (Ishikawa PRAB) were transiently transfected with empty control (Ctl) vector or cMEKK1 expression vector during 24 h, and pS294-PRB or pS130-PRA levels or total PRB or total PRA levels were detected by immunoblotting whole cell extracts using phospho-specific or total PR or p42/44 antibodies. Band intensities were quantified, and pS130/PRA or pS294/PRB (*middle panel*) as well as total PRA and PRB levels (*right panel*) under basal and cMEKK1 conditions are presented. B, Ishikawa PRAB cells were pretreated with vehicle control (Ctl) or selective inhibitors of p42/44 (U0126) (UO) or p38 (PD169316) (PD) or JNK (SP600125) (SP) activity. Cells were then transfected with MEKK1 expression vector in the presence of vehicle or R5020 (10^{-8} M) or RU486 (10^{-8} M) during 24 h. Whole cell extracts were immunoblotted as in Fig. 7B. Band densities corresponding to PRA and PRB were quantified from at least two nonsaturating exposures of the same immunoblot (two film exposures are shown). PRA/PRB expression ratio was calculated for each ligand and inhibitor condition from three independent cell cultures and presented as mean ± SEM. Under a given ligand condition, the effect of selective MAPK inhibition as compared with nontreated cells is shown by *asterisks*. Statistical significance is shown by XX when the effect of ligand is compared with vehicle under a given MAPK inhibition. *, $P \leq 0.05$; X, $P \leq 0.05$; **, $P \leq 0.01$; XX, $P \leq 0.01$; ***, $P \leq 0.001$; XXX, $P \leq 0.001$.

PRA/PRB ratio drastically decreased after PD169316 treatment, irrespective of ligand conditions, consistent with impaired PRA stabilization upon P-p38 inhibition. Moreover, inhibition of P-JNK by SP600125 enhanced PRB stability, thus decreasing PRA/PRB ratio. However, PRA expression was also slightly decreased by U0126, particularly in vehicle and R5020-treated cells, suggesting that p42/44 specificity of PRB might be conferred to the PRA:PRB heterodimer. Furthermore, variation of pS294-PRB and pS130-PRA levels was correlated with ligand-induced changes in total PRB and total PRA levels under selective inhibition of MAPK. These results indicate that S294-PRB and S130-PRA are targeted by a kinase distinct from p42/44, p38, or JNK MAPK. Of interest, the differential impact of distinct MAPK pathways on PR isoforms stability, *i.e.* P-p42/44 for PRB and P-p38 for PRA, also varies to different extent depending on the nature of PR ligand (agonist or antagonist). For a given status of MAPK activities, ligand treatment led to higher PRA stability as compared with PRB, resulting in increased PRA/PRB ratio. In contrast, for a given ligand condition, p38 or p42/44 MAPK selectively controlled PRA or PRB stabilities, resulting in overall upshift or downshift in PRA/PRB ratio. Such mechanisms controlling PRA/PRB expression ratio might play a crucial role in hormonal responsiveness in progesterone target tissues.

Discussion

The putative functional link between agonist-induced PRB phosphorylation and down-regulation has been extensively analyzed by other laboratories (11). Agonist ligands induce PRB phosphorylation at multiple sites in the N-terminal region, notably at serine residues 102, 294, and 345 (8), whereas other residues are phosphorylated in the ligand-free PRB (35). Although RU486 induces phosphorylation of identical sites as compared with agonist (8), it was shown that RU486 has either no effect on PRB down-regulation (36) or induces PR down-regulation through much slower kinetics than agonist (21). We have recently reported that steroid receptor coactivator 1 was degraded by the proteasome in a PRB-dependent manner that was also inhibited by RU486 (37). To explore the role of PR phosphorylation on its degradation, mutagenesis experiments revealed that substitution of S294 by an alanine (S294A) led to PRB stabilization, suggesting that PRB down-regulation is mainly addressed by the Ser294 site (11). However, in stably transfected T47D cells, PRB-S294A mutant underwent ligand-induced turnover, although to a lesser extent as compared with wild-type PRB (38). We have thus considered that PR stability/turnover might also be governed by pS294-independent mechanisms. Here, we demonstrate that RU486 promotes PRB or PRA protein stabilization despite inducing pS294 or pS130 (equivalent serine residue on PRA), respectively, indicating that RU486 interferes in downstream events of pS294- or pS130-signaled PR isoforms down-regulation. Our data do not correlate with previous reports using T47D-YB cells (stably expressing PRB) or in HeLa cells transiently transfected with PRB expression vector showing that P-p42/44 MAPK accelerate PRB degradation (11, 28). Furthermore, it was reported that EGF- but not progestin-induced pS294 requires p42/44 MAPK activity (14). However, in the same study, it was shown that EGF, despite inducing pS294, increased PRB stability in T47D-YB cells, consistent with our observations. Increased pS294-PRB levels observed after EGF in this previous report might in part be due to PRB accumulation by p42/44 activation. Similarly, enhancing p42/44 activity by MEKK1 was reported to induce pS294 and accelerated PRB turnover in transiently transfected HeLa cells (14). Our results in cells stably expressing PRB show that p42/44 MAPK increase PRB stability that might in part account for increased pS294-PRB species. In support of our results, it has been recently described that degradation of androgen receptor (AR) is enhanced after p42/p44 inhibition by U0126 in prostate cancer LNCaP cells (39). It thus seems very likely that such p42/44 MAPK-dependent-stabilizing effect might be conserved for this nuclear receptor subfamily.

The N-terminal BUS domain of PRB, containing several PEST-like sequences (enriched in proline, glutamate, serine, and threonine) that might initiate turnover process as degron signals (40), accounts for increased turnover rate of PRB than PRA. BUS domain can also confer ligand-dependent down-regulating properties to other nuclear receptors, such as estrogen receptor and AR (41). This property corresponds to the N-end rule for protein degradation as defined by Varshavsky (42). Furthermore, the BUS domain is involved in N-C-terminal intramolecular interactions via two LXXLL motifs similar to nuclear receptor boxes present in coactivator sequences that interact with nuclear receptors (43), accounting for native PRB conformation that is distinct from PRA. Mutations of these sequences abolish the agonist-induced PRB turnover (41) and decrease the reporter gene transcriptional activity similar to that exhibited by PRA. It has been shown that PR-interacting proteins having associated ubiquitin E3-ligase activity, such as breast cancer 1 (23) and E6-associated protein (44), selectively control PRA or PRB turnover, indicating that differential regulatory proteins are involved in PR isoform down-regulation. Involvement of such molecular partners is very likely, be-

cause we found that agonist-induced PRB down-regulation was completely abrogated by blocking protein neosynthesis. Recently, it was demonstrated that RU486-bound PRB conformation, in conjunction with PR co-regulatory protein Jun dimerization protein-2, exposes protein interaction surfaces that are distinct from those presented by agonist ligand (45). In agreement with these studies, our results indicate that unique conformation of RU486-bound PRB might strongly facilitate stabilizing effects of p42/44-dependent phosphorylation (on a residue other than S294), which impedes interaction with coregulatory proteins implicated in PRB turnover. This p42/44-dependent phosphorylation also occurs upon agonist binding but with a more discrete stabilizing effect as compared with RU486. Such differences might be due to distinct conformations induced by ligands in PRB N-terminal domain. Although agonist ligand might strongly favor interaction of pS294-PRB with putative ubiquitin-ligase(s), RU486-bound PRB might be refractory to such interactions by favoring the stabilizing effect of the p42/44-dependent phosphorylation. As shown by the surprising effect of U0126 in restoring fast RU486-PRB turnover, this interaction is directly inhibited by p42/44-dependent phosphorylation(s). Whether p42/p44 target a PRB-interacting down-regulatory protein is less likely given the ligand sensitivity of the mechanism and the lack of PRB electrophoretic upshift under P-p42/44 inhibition. Nevertheless, we could not rule out the possibility that MAPK-dependent phosphorylation(s) of PR molecular partner(s) may also play a role in determining PR stability.

We have studied the impact of p42/44 on PRB stabilization and its transcriptional activity. Although p42/44 MAPK inhibition dramatically reduced transcriptional activity from exogenous promoter, differential effects were observed on endogenous gene transcription. Inhibition of P-p42/44 reversed the ligand-induced transcriptional activation (*Dickkopf homolog 1* and *amphiregulin* genes) or repression (*cyclin D1* and *HB-EGF* genes). Certain genes might be insensitive to MAPK inhibition (*epiregulin*), whereas transcription of a gene subset (*FKBP5* and *serum* and *glucocorticoid-regulated kinase 1*) was highly potentiated by inhibition of p42/44 activity. This shows that p42/44 MAPK fine tune PRB-mediated transcription depending on target gene promoter context and influence transcription of both up-regulated as well as down-regulated PRB target genes. It was shown previously that *HB-EGF* and *cyclin D1* expression increased after progestin treatment in T47D cells, (38, 46). However, in MDA-MB-231 cells, we found that both agonist (progesterone or R5020) and antagonist (RU486) ligands decreased *cyclin D1* and *HB-EGF* expression similar to antiproliferative effects of these ligands (data not shown). It has been reported that progesterone decreases *HB-EGF* transcription in epithelial cells, whereas in stromal cells, *HB-EGF* transcription is increased by progesterone (47). Consistent with our results, it was shown that progestin inhibits proliferation of MDA-MB-231 cells stably expressing both PR isoforms (48) and that progesterone decreased *cyclin D1* expression as early as 4 h after hormonal treatment (49). The differences in T47D (luminal) and MDA-MB-231 (basal epithelial) cells for these PR target genes regulations might result from differential PR signaling and/or differential expression of coregulatory proteins. Diverse transcriptional effects after p42/44 inhibition does not support that U0126 could artifactually shut down PRB activity through nonspecific effects. MAPK-dependent extracellular signaling might thus selectively influence PRB-mediated transcription depending on various parameters linked to both target gene promoter context and dynamics of proteasome-dependent PRB turnover. MAPK inhibitors have been recently shown to promote the interaction of corepressor silencing mediator of retinoid and thyroid receptors (SMRT) with antagonist-bound AR (50). Moreover, combined treatment of LNCaP prostate cancer cells by p42/p44 inhibitor and AR antagonist cyproterone acetate inhibits AR-mediated transcription as well as agonist-induced cell proliferation. These features are similar to what we obtained with RU486 and U0126 in MDA-MB231 cells. Because SMRT corepressor also mediates RU486-bound PRB transcriptional repression (51), enhancement of SMRT interaction for PRB after p42/44 inhibition remains to be proven.

Although p42/p44 stabilizes PRB, p38 MAPK selectively enhances PRA stabilization, irrespective of ligand, through an unidentified site other than S130. PR upshift after ligand exposure is mainly attributed to PR phosphorylation at S345, a MAPK consensus residue (52). Given that PRB degradation was enhanced after U0126 treatment, a role of S345 in such stabilizing mechanism seems likely. Lack of BUS domain in PRA structure might allow p38-dependent phosphorylation that might be inaccessible in PRB due to conformational differences in PR isoforms. In Ishikawa cells coexpressing PRA and PRB, MEKK1 stimulation increased basal PRA/PRB expression ratio that was further enhanced by agonist as well as antagonist ligands. Although PRB was able to confer p42/p44 sensitivity to PRA:PRB heterodimer, PRB remained refractory to p38-dependent PRA-stabilizing effect. These observations highly support that distinct MAPK-mediated extracellular signaling can highly influence PRA/PRB expression ratio. PRA and PRB regulate common as well as distinct target gene subsets (4, 41), and

disruption of relative PR isoforms expression is reported in both breast and endometrial cancers (2, 13). Variations in PRA/PRB expression ratio leading to a change in PR isoforms homo- and heterodimers balance might thus be a critical determinant of PR target gene selection and/or disordered transcriptional regulation resulting in altered cellular response to hormonal stimuli that might contribute toward pathogenesis. Our results highlight that imbalance in PRA/PRB ratio frequently associated with carcinogenesis might be a direct consequence of disorders in MAPK signaling. Using p42/44 selective inhibitors in mammary oncotherapy, because was previously proposed to decrease PRB transcriptional activity (24), might indirectly favor PRA stability/signaling to the detriment of PRB. In contrast, we propose that p38 inhibitors might help to rescue normal PRA/PRB balance in cancer cells overexpressing PRA.

In sum, our results, summarized in Fig. 9, reveal that p38 and p42/44 MAPK selectively control PRA and PRB stabilities. We propose that the BUS domain encompasses a down-regulation tag conferring to PRB a fast agonist-inducible turnover that is negatively controlled by p42/44 MAPK targeting PRB on a residue distinct from S294. PRB stabilization by RU486 might be due to enhancement of this p42/p44 control resulting in downstream inhibition of interaction with (or function of) mandatory down-regulating partner(s). Given the conformational differences between PRA and PRB, p38 MAPK selectively targets PRA, leading to its stabilization. Extracellular stimuli, such as EGF or proinflammatory cytokines, which preferentially activate p42/44 or p38 MAPK, respectively, may lead to opposite variations in PRA/PRB expression ratio at posttranslational level. Changes in extracellular signaling in these cells might strongly influence PRA/PRB ratio and lead to dramatic shift in selection of PR target gene subsets, thus switching cellular responses to hormonal/growth factor stimuli. This might be of broad concern for designing pharmacological intervention in breast cancers regarding combination of selective MAPK inhibitors along with antiprogestins.

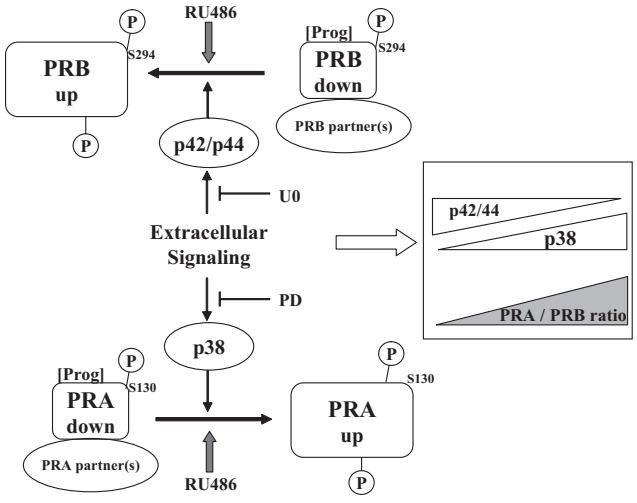


FIG. 9. MAPK-dependent control of PRA/PRB expression ratio. Control of PRA and PRB stabilities and PRA/PRB ratio by p38 and p42/44 MAPK activities is schematically summarized. Progesterone (Prog) and specific MAPK inhibitors are indicated (U0, U0126; PD, PD169316).

Materials and Methods

Cell culture and reagents

Human endometrial cancer cell lines Ishikawa PRA, Ishikawa PRB, Ishikawa PRAB engineered to stably express either, or both PR isoforms (PRA, PRB, PRA, and PRB) were kindly provided by L. J. Blok (Erasmus University, Rotterdam, The Netherlands) (25). Human breast cancer cells MDA-MB-231 stably expressing PRB were kindly provided by A. Gompel (Université Paris Descartes, Paris, France) (26). All cell lines were routinely cultured in DMEM with glutamine, enriched with 10% fetal calf serum (FCS) (BioWest, Denver, CO), and supplemented with antibiotics (penicillin 100 UI/ml, streptomycine 100 μg/ml) (PAA Laboratories GmbH, Cölbe, Germany). For each experiment, cells were preincubated in steroid-free medium containing 5% dextran-coated charcoal-treated serum without antibiotics for at least 24 h before hormonal treatment. Progesterone, R5020, RU486 and inhibitors for MEK1/2 (U0126), phospho-p38 (PD169316) and phospho-JNK (SP600125) MAPK, proteasome (MG132), and protein neosynthesis (cycloheximide) were purchased from Sigma (St. Louis, MO).

Immunoblotting

For whole cell protein extraction, cells were rinsed twice with PBS and lysed by scrapping in extraction buffer [0.1% (vol/vol) Triton X-100, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.2% (wt/vol) NaF, and 1.3% (wt/vol) sodium pyrophosphate] containing phosphatases and proteases inhibitors mixture (Sigma). Lysates were clarified by centrifugation at 16,000 $\times$ *g* for 15 min in a refrigerated microfuge. Soluble proteins were quantified using the bicinchoninic acid assay kit (Interchim, Montluçon, France), and equal amounts of protein were mixed with 1/3 volume of 3$\times$ Laemmli sample buffer [187.5 mM Tris-HCl (pH 6.8), 15% (vol/vol) β mercapto-ethanol, 30% (vol/vol) glycerol, 6% (vol/vol) sodium dodecyl sulfate, and 0.03% (wt/vol) bromophenol blue] and heated at 95 C for 5 min for denaturation. Equal amounts of protein were resolved by SDS-PAGE (7.5 or 10% acrylamide) and transferred on polyvinylidene fluoride membrane. Primary antibody solutions were prepared in Tris-buffered saline with Tween 20 containing 5% fat skimmed dry milk at the final dilution of 1:3000 for PRA and PRB phospho S294-specific antibody (Affinity BioReagent, Golden, CO), 1:500 for anti-PRB-specific mouse monoclonal antibody Let 126 (53), 1:10,000 for mouse monoclonal anti-PRA and anti-PRB antibody (NCL-L-PGR-312/2; Novocastra Laboratories, Newcastle upon Tyne, UK), 1:3000 for phospho-specific or total p38, p42/p44, or JNK MAPK antibodies (Cell Signaling Technology, Beverly, MA), 1:250 for anti-GR antibody (AbC10-G015; AbCys S.A., Paris, France), or

1:10,000 for anti-α-tubulin antibody (Sigma). The membranes were immersed in primary antibody solution on a rotator either at 4 C overnight or at room temperature during 1 h. Incubation with horseradish peroxidase-conjugated goat antimouse or antirabbit secondary antibody solution (Vector Laboratories, Burlingame, CA) was prepared in Tris-buffered saline with Tween 20 5% skimmed dry milk at 1:15,000 dilutions. Membranes were then incubated for 1 h at room temperature. Target proteins were detected using ECL Plus reagent (GE Healthcare, Princeton, NJ) and visualized by chemiluminescence. Bands corresponding to target proteins were quantified by scanning films obtained for several nonsaturating time exposures, using MacBiophotonics ImageJ 1.43s software and were normalized to either tubulin or total p42/p44 loading control.

Immunoprecipitation assays

Parental Ishikawa cells were transfected in a 100-mm plate with HA-ubiquitin and PRB expression vectors (54) during 48 h in steroid-free medium. Cells were treated with MG132 (5 μM) during 30 min before treatment with vehicle or R5020 (10^{-8} M) or RU486 (10^{-8} M) during 4 h in 5% steroid-free FCS-containing medium. Cells were lysed at 4 C in 500 μl lysis buffer, cell debris was pelleted by centrifugation (14,000 × *g*, 15 min, 4 C), and the supernatant was obtained. One milligram of total protein was immunoprecipitated using anti-PR antibody (C-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and Protein G Magnetic Beads (Millipore, Bedford, MA) according to the manufacturer's instructions. Bound immunocomplexes were boiled in Laemmli buffer and resolved on 7.5% acrylamide gel as described above. Anti-HA (12CA5; Roche Diagnostics, Indianapolis, IN) or anti-PR antibody (NCL-L-PGR-312/2; Novocastra Laboratories) was used for the detection of ubiquitinated or total PRB, respectively.

Real-time quantitative RT-PCR

Hormone-treated cells were rinsed twice with PBS, and total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) as described previously (54). One microgram of total RNA was treated with DNase I Amplification Grade (Invitrogen) and reverse transcribed using cDNA RT kit from Applied Biosystems (Foster City, CA). cDNA thus obtained was diluted 10-fold, and 1/20th fraction of the cDNA preparation was amplified by PCR using the Power SYBR Green PCR Master Mix (Applied Biosystems). Primers (300 nM) sequences are presented in Supplemental Table 1. Reaction parameters were set to 95 C for 10 min, 40 cycles at 95 C for 15 sec, and 60 C for 1 min on ABI 7300 Sequence Detector (Applied Biosystems). A dissociation curve was also obtained to verify primer pair specificity. For standards preparation, amplicons were purified after agarose gel electrophoresis, subloned in pGEMT-easy (Promega, Madison, WI), and then sequenced for verification of the amplification product. These plasmid-amplicons were linearized and used for standardization of real-time quantitative PCR. All samples were analyzed in duplicate from at least three independent cell cultures. The relative expression level of each gene transcript was normalized with 18S RNA level of the corresponding sample.

Transient transfection

cMEKK1 expression vector (55) was kindly provided by M. H. Cobb (University of Texas, Southwestern Medical Center, Dallas, TX). Transfections were performed using LipofectAMINE 2000 according to the manufacturer's recommendations (Invitrogen). The cells were plated at 1.2×10^6 per well in six-well plates and then transiently transfected with control or HA-ubiquitin or MEKK1 expression vector during indicated time periods in phenol red-free medium containing 2.5% steroid-depleted FCS. The cells were then treated with ethanol (vehicle) or R5020 (10^{-8} M) or RU486 (10^{-8} M) for indicated durations in steroid-free medium. For siRNA transfection experiments, cells were cotransfected with control or cMEKK1 expression vector (1 μg) along with either of the following siRNA (SignalSilence; Cell Signaling Technology): control (no. 6568), p42 and p44 (no. 6560), or p38 (nos. 6564 or 6243) MAPK (100 nM) using Lipofectamine 2000.

Luciferase reporter gene assays

MDA-MB-231 PRB or Ishikawa PRB cells were cultured in steroid-free medium and transfected with PRE2-TATA-luciferase reporter gene (100 ng) and β-galactosidase (10 ng) plasmids in 96-well plates. After 24 h of transfection, cells were incubated with vehicle or progesterone (10^{-8} M) or R5020 (10^{-8} M) or RU486 (10^{-8} M) for 24 h. Cells were collected with the Passive Lysis Buffer (Promega). Luciferase activity was measured with a luminometer (Victor; PerkinElmer, Waltham, MA) and normalized with either β-galactosidase activity or total protein concentration. The data are presented as means ± SE of six independent cell cultures (n = 6).

Immunocytochemical assays

Cells were seeded in 24-well plates, fixed with 4% paraformaldehyde, and permeabilized 30 min with PBS containing 0.5% Triton X-100. Cells were then incubated with primary anti-PR antibody (Novocastra Laboratories) overnight at 4 C and for 30 min with an Alexa Fluor 488-coupled antimouse IgG secondary antibody. Fluorescent cells were analyzed with an Olympus Provis AX70 microscope (Olympus, Tokyo, Japan). Pictures acquisition was performed at ×20 magnitude for 160 msec with imaging Qcapture Pro version 5.1 (QImaging, Inc., Surrey, Canada).

Statistical analysis

Data are expressed as mean ± SEM. Nonparametric Mann-Whitney test for transactivation studies or unpaired *t* test for quantitative analysis of Western blotting images was used to determine significant differences between groups using the computer software Prism 4 (GraphPad Software, San Diego, CA). Statistical significance is indicated at $P \leq 0.05$, 0.01, and 0.001.

Acknowledgments

We thank Dr. M. H. Cobb (University of Texas, Southwestern Medical Center, Dallas, TX) for providing MEKK1 expression vector, Dr. L. J. Blok (Erasmus University, Rotterdam, The Netherlands) for Ishikawa cells lines, Dr. A Gompel (Université Paris Descartes, Paris, France) for MDA-MB-231 cell lines, Dr. Say Viengchareun for useful discussions and technical assistance, Meriem Messina for plasmid preparations, and Luc Outin for image quantifications.

Address all correspondence and requests for reprints to: Dr. Hugues Loosfelt, M.S., Ph.D., Institut National de la Santé et de la Recherche Médicale Unité 693, Faculté de Médecine Paris-Sud, 63 Rue Gabriel Péri, Le Kremlin-Bicêtre F-94276, France. E-mail: hugues.loosfelt@u-psud.fr.

This work was supported by grants from Institut National de la Santé et de la Recherche Médicale, the Université Paris-Sud 11, and Association pour la Recherche sur le Cancer. J.A.K. is on study leave from the Department of Physiology and Pharmacology, University of Agriculture, Faisalabad, Pakistan, and is a recipient of doctoral scholarship from Higher Education Commission, Islamabad, Pakistan, and a fellowship from La Ligue Contre le Cancer, France. C.B. is recipient of a fellowship from the Conseil Régional de la Martinique.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P** 1990 Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 9:1603–1614
2. **Mote PA, Bartow S, Tran N, Clarke CL** 2002 Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis. Breast Cancer Res Treat 72:163–172
3. **Sartorius CA, Melville MY, Hovland AR, Tung L, Takimoto GS, Horwitz KB** 1994 A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. Mol Endocrinol 8:1347–1360
4. **Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB** 2002 Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J Biol Chem 277: 5209–5218
5. **Conneely OM, Mulac-Jericevic B, Lydon JP, De Mayo FJ** 2001 Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. Mol Cell Endocrinol 179:97–103
6. **Pratt WB, Toft DO** 1997 Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev 18:306–360
7. **Beck CA, Zhang Y, Altmann M, Weigel NL, Edwards DP** 1996 Stoichiometry and site-specific phosphorylation of human progesterone receptor in native target cells and in the baculovirus expression system. J Biol Chem 271:19546–19555
8. **Beck CA, Zhang Y, Weigel NL, Edwards DP** 1996 Two types of anti-progestins have distinct effects on site-specific phosphorylation of human progesterone receptor. J Biol Chem 271:1209–1217
9. **Dennis AP, Lonard DM, Nawaz Z, O'Malley BW** 2005 Inhibition of the 26S proteasome blocks progesterone receptor-dependent transcription through failed recruitment of RNA polymerase II. J Steroid Biochem Mol Biol 94:337–346
10. **Daniel AR, Faivre EJ, Lange CA** 2007 Phosphorylation-dependent antagonism of sumoylation derepresses progesterone receptor action in breast cancer cells. Mol Endocrinol 21:2890–2906
11. **Lange CA, Shen T, Horwitz KB** 2000 Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci USA 97:1032–1037
12. **Graham JD, Roman SD, McGowan E, Sutherland RL, Clarke CL** 1995 Preferential stimulation of human progesterone receptor B expression by estrogen in T-47D human breast cancer cells. J Biol Chem 270:30693–30700
13. **Arnett-Mansfield RL, deFazio A, Wain GV, Jaworski RC, Byth K, Mote PA, Clarke CL** 2001 Relative expression of progesterone receptors A and B in endometrioid cancers of the endometrium. Cancer Res 61:4576–4582
14. **Shen T, Horwitz KB, Lange CA** 2001 Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294. Mol Cell Biol 21:6122–6131
15. **Pierson-Mullany LK, Lange CA** 2004 Phosphorylation of progesterone receptor serine 400 mediates ligand-independent transcriptional activity in response to activation of cyclin-dependent protein kinase 2. Mol Cell Biol 24:10542–10557
16. **Weigel NL, Moore NL** 2007 Kinases and protein phosphorylation as regulators of steroid hormone action. Nucl Recept Signal 5:e005
17. **Dressing GE, Hagan CR, Knutson TP, Daniel AR, Lange CA** 2009 Progesterone receptors act as sensors for mitogenic protein kinases in breast cancer models. Endocr Relat Cancer 16:351–361
18. **Lange CA** 2008 Challenges to defining a role for progesterone in breast cancer. Steroids 73:914–921
19. **Reid G, Hübner MR, Métivier R, Brand H, Denger S, Manu D, Beaudouin J, Ellenberg J, Gannon F** 2003 Cyclic, proteasome-mediated turnover of unliganded and liganded ERα on responsive promoters is an integral feature of estrogen signaling. Mol Cell 11:695–707
20. **Klijn JG, Setyono-Han B, Foekens JA** 2000 Progesterone antagonists and progesterone receptor modulators in the treatment of breast cancer. Steroids 65:825–830
21. **el-Ashry D, Oñate SA, Nordeen SK, Edwards DP** 1989 Human progesterone receptor complexed with the antagonist RU 486 binds to hormone response elements in a structurally altered form. Mol Endocrinol 3:1545–1558
22. **Clemm DL, Sherman L, Boonyaratanakornkit V, Schrader WT, Weigel NL, Edwards DP** 2000 Differential hormone-dependent phosphorylation of progesterone receptor A and B forms revealed by a phosphoserine site-specific monoclonal antibody. Mol Endocrinol 14:52–65
23. **Poole AJ, Li Y, Kim Y, Lin SC, Lee WH, Lee EY** 2006 Prevention of Brca1-mediated mammary tumorigenesis in mice by a progesterone antagonist. Science 314:1467–1470
24. **Faivre EJ, Lange CA** 2007 Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. Mol Cell Biol 27:466–480
25. **Smid-Koopman E, Blok LJ, Kühne LC, Burger CW, Helmerhorst TJ, Brinkmann AO, Huikeshoven FJ** 2003 Distinct functional differences of human progesterone receptors A and B on gene expression and growth regulation in two endometrial carcinoma cell lines. J Soc Gynecol Investig 10:49–57
26. **Petit E, Courtin A, Kloosterboer HJ, Rostène W, Forgez P, Gompel A** 2009 Progestins induce catalase activities in breast cancer cells through PRB isoform: correlation with cell growth inhibition. J Steroid Biochem Mol Biol 115:153–160
27. **Qiu M, Lange CA** 2003 MAP kinases couple multiple functions of human progesterone receptors: degradation, transcriptional synergy, and nuclear association. J Steroid Biochem Mol Biol 85:147–157
28. **Qiu M, Olsen A, Faivre E, Horwitz KB, Lange CA** 2003 Mitogen-activated protein kinase regulates nuclear association of human progesterone receptors. Mol Endocrinol 17:628–642
29. **Narayanan R, Edwards DP, Weigel NL** 2005 Human progesterone receptor displays cell cycle-dependent changes in transcriptional activity. Mol Cell Biol 25:2885–2898
30. **Chen JJ, Huang WC, Chen CC** 2005 Transcriptional regulation of cyclooxygenase-2 in response to proteasome inhibitors involves reactive oxygen species-mediated signaling pathway and recruitment of CCAAT/enhancer-binding protein δ and CREB-binding protein. Mol Biol Cell 16:5579–5591
31. **Mathay C, Giltaire S, Minner F, Bera E, Hérin M, Poumay Y** 2008 Heparin-binding EGF-like growth factor is induced by disruption of lipid rafts and oxidative stress in keratinocytes and participates in

the epidermal response to cutaneous wounds. J Invest Dermatol 128:717–727
32. **Samuvel DJ, Jayanthi LD, Bhat NR, Ramamoorthy S** 2005 A role for p38 mitogen-activated protein kinase in the regulation of the serotonin transporter: evidence for distinct cellular mechanisms involved in transporter surface expression. J Neurosci 25:29–41
33. **Patterson KI, Brummer T, O'Brien PM, Daly RJ** 2009 Dual-specificity phosphatases: critical regulators with diverse cellular targets. Biochem J 418:475–489
34. **Farooq A, Zhou MM** 2004 Structure and regulation of MAPK phosphatases. Cell Signal 16:769–779
35. **Ward RD, Weigel NL** 2009 Steroid receptor phosphorylation: assigning function to site-specific phosphorylation. Biofactors 35: 528–536
36. **Kahmann S, Vassen L, Klein-Hitpass L** 1998 Synergistic enhancement of PRB-mediated RU486 and R5020 agonist activities through cyclic adenosine 3′,5′-monophosphate represents a delayed primary response. Mol Endocrinol 12:278–289
37. **Amazit L, Roseau A, Khan JA, Chauchereau A, Tyagi RK, Loosfelt H, Leclerc P, Lombès M, Guiochon-Mantel A** 2011 Ligand-dependent degradation of SRC-1 is pivotal for progesterone receptor transcriptional activity. Mol Endocrinol 25:394–408
38. **Skildum A, Faivre E, Lange CA** 2005 Progesterone receptors induce cell cycle progression via activation of mitogen-activated protein kinases. Mol Endocrinol 19:327–339
39. **Agoulnik IU, Bingman 3rd WE, Nakka M, Li W, Wang Q, Liu XS, Brown M, Weigel NL** 2008 Target gene-specific regulation of androgen receptor activity by p42/p44 mitogen-activated protein kinase. Mol Endocrinol 22:2420–2432
40. **Varshavsky A** 1997 The ubiquitin system. Trends Biochem Sci 22: 383–387
41. **Tung L, Abdel-Hafiz H, Shen T, Harvell DM, Nitao LK, Richer JK, Sartorius CA, Takimoto GS, Horwitz KB** 2006 Progesterone receptors (PR)-B and -A regulate transcription by different mechanisms: AF-3 exerts regulatory control over coactivator binding to PR-B. Mol Endocrinol 20:2656–2670
42. **Varshavsky A** 1997 The N-end rule pathway of protein degradation. Genes Cells 2:13–28
43. **Dong X, Challis JR, Lye SJ** 2004 Intramolecular interactions between the AF3 domain and the C-terminus of the human progesterone receptor are mediated through two LXXLL motifs. J Mol Endocrinol 32:843–857
44. **Ramamoorthy S, Dhananjayan SC, Demayo FJ, Nawaz Z** 2010 Isoform-specific degradation of PR-B by E6-AP is critical for normal mammary gland development. Mol Endocrinol 24:2099–2113
45. **Wardell SE, Narayanan R, Weigel NL, Edwards DP** 2010 Partial agonist activity of the progesterone receptor antagonist RU486 mediated by an amino-terminal domain coactivator and phosphorylation of serine400. Mol Endocrinol 24:335–345
46. **Daniel AR, Qiu M, Faivre EJ, Ostrander JH, Skildum A, Lange CA** 2007 Linkage of progestin and epidermal growth factor signaling: phosphorylation of progesterone receptors mediates transcriptional hypersensitivity and increased ligand-independent breast cancer cell growth. Steroids 72:188–201
47. **Zhang Z, Funk C, Roy D, Glasser S, Mulholland J** 1994 Heparin-binding epidermal growth factor-like growth factor is differentially regulated by progesterone and estradiol in rat uterine epithelial and stromal cells. Endocrinology 134:1089–1094
48. **Lin VC, Ng EH, Aw SE, Tan MG, Ng EH, Chan VS, Ho GH** 1999 Progestins inhibit the growth of MDA-MB-231 cells transfected with progesterone receptor complementary DNA. Clin Cancer Res 5:395–403
49. **Lin VC, Woon CT, Aw SE, Guo C** 2003 Distinct molecular pathways mediate progesterone-induced growth inhibition and focal adhesion. Endocrinology 144:5650–5657
50. **Eisold M, Asim M, Eskelinen H, Linke T, Baniahmad A** 2009 Inhibition of MAPK-signaling pathway promotes the interaction of the corepressor SMRT with the human androgen receptor and mediates repression of prostate cancer cell growth in the presence of antiandrogens. J Mol Endocrinol 42:429–435
51. **Liu Z, Auboeuf D, Wong J, Chen JD, Tsai SY, Tsai MJ, O'Malley BW** 2002 Coactivator/corepressor ratios modulate PR-mediated transcription by the selective receptor modulator RU486. Proc Natl Acad Sci USA 99:7940–7944
52. **Zhang Y, Beck CA, Poletti A, Edwards DP, Weigel NL** 1995 Identification of a group of Ser-Pro motif hormone-inducible phosphorylation sites in the human progesterone receptor. Mol Endocrinol 9:1029–1040
53. **Lorenzo F, Jolivet A, Loosfelt H, Thu vu Hai M, Brailly S, Perrot-Applanat M, Milgrom E** 1988 A rapid method of epitope mapping. Application to the study of immunogenic domains and to the characterization of various forms of rabbit progesterone receptor. Eur J Biochem 176:53–60
54. **Georgiakaki M, Chabbert-Buffet N, Dasen B, Meduri G, Wenk S, Rajhi L, Amazit L, Chauchereau A, Burger CW, Blok LJ, Milgrom E, Lombès M, Guiochon-Mantel A, Loosfelt H** 2006 Ligand-controlled interaction of HBO1 with the N-terminal transactivating domain of progesterone receptor induces SRC-1-dependent co-activation of transcription. Mol Endocrinol 20:2122–2140
55. **Xu S, Robbins DJ, Christerson LB, English JM, Vanderbilt CA, Cobb MH** 1996 Cloning of rat MEK kinase 1 cDNA reveals an endogenous membrane-associated 195-kDa protein with a large regulatory domain. Proc Natl Acad Sci USA 93:5291–5295

SUPPLEMENTAL FIGURES LEGENDS

Fig S1. PR isoforms expression in T47D, MDA-MB-231 and Ishikawa cells.

Whole cell extracts were prepared from wild type T47D cells expressing endogenous PR isoforms and Ishikawa or MDA-MB-231 cells expressing recombinant PRA or PRB. Immunoblot detection of PR isoforms and loading control tubulin was done using anti-PR and anti-tubulin antibody.

Fig S2. RU486 induces stable S130-PRA phosphorylation.

Ishikawa PRA cells were treated by vehicle or R5020 (10 nM) or RU486 (10 nM) during 6 h and whole cells extracts were immunoblotted using phospho-specific S130-PRA, anti-PRA or anti-tubulin antibody.

Fig S3. R5020-induced slower and lesser pS130-PRA than pS294-PRB.

Ishikawa PRA or PRB cells were incubated without or with R5020 (10^{-8} M) during indicated durations and whole cell extracts were immunoblotted using either phospho-specific (pS130-PRA, pS294-PRB) or anti-PR (PRA, PRB) or anti-tubulin antibody. Band densities corresponding to target protein were quantified as in Fig 2C and ligand-induced pS130-PRA or pS294-PRB is presented as fold induction of vehicle treated cells.

Fig S4. U0126 did not affect PRB mRNA levels

MDA-MB-231 PRB cells were treated with DMSO (vehicle) or U0126 during 6 h and qRT-PCR analysis was performed for determining PRB transcript levels as described in Materials and Methods.

Fig S5. Phosphorylated p42/44 MAPK stabilize PRB in a ligand-sensitive manner.

Ishikawa PRB cells were cultured in serum free medium and pre-treated with DMSO or U0126 (10 µM) during 30 min. Cells were then treated without or with R5020 (10 nM) or RU486 (10 nM) during 1, 6 or 24 h in serum free medium. Whole cell extracts were immunoblotted using either phospho-specific S294-

PRB, or anti-PR antibody. From the same immunoblot, either total p42/p44 or their phosphorylated species (P-p42/44) were analyzed using the corresponding antibodies.

Fig S6. Ubiquitin over-expression does not accelerate RU486-bound pS294-PRB turnover

Ishikawa PRB cells were transfected with control or HA-ubiquitin expression vector during 24 h and then treated with vehicle or R5020 (10 nM) or RU486 (10 nM) during 6 h. Whole cell extracts were immunoblotted for pS294-PRB or total PRB or tubulin expression as described above.

Fig S7. Stabilizing effect of cMEKK1 is more pronounced on PRA than on PRB

Ishikawa PRB or PRA cells were transiently transfected with 1 µg of either empty or constitutively active MEKK1 expression vector (cMEKK1) during 24 h and then treated without or with R5020 (10 nM) or RU486 (10 nM) during 6 h. Whole cell extracts were immunoblotted as in Fig 7A.

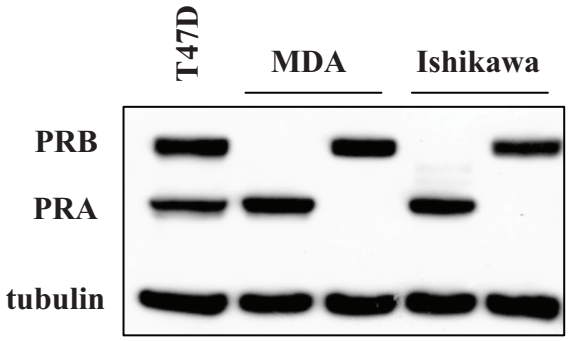
Fig S8. cMEKK1-dependent PRA stabilization is less pronounced by decreasing cMEKK1

Ishikawa PRA cells were transiently transfected with 0.5 µg of either empty or constitutively active MEKK1 expression vector (cMEKK1) during 24 h and then treated without or with R5020 (10 nM) or RU486 (10 nM) during 24 h. Whole cell extracts were immunoblotted as in Fig 7A.

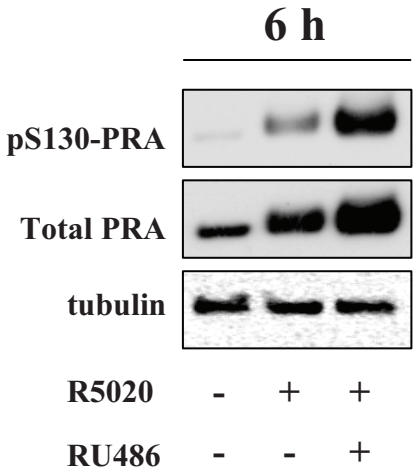
Fig S9. p42/44 MAPK knock-down selectively inhibited PRB stability

Ishikawa PRAB cells were co-transfected with control or cMEKK1 vector along with either control or specific siRNA against both p42 and p44 MAPK during 24 h. Cells were maintained in 5 % FBS containing medium for another 48 h before immunoblot analysis of whole cell extracts.

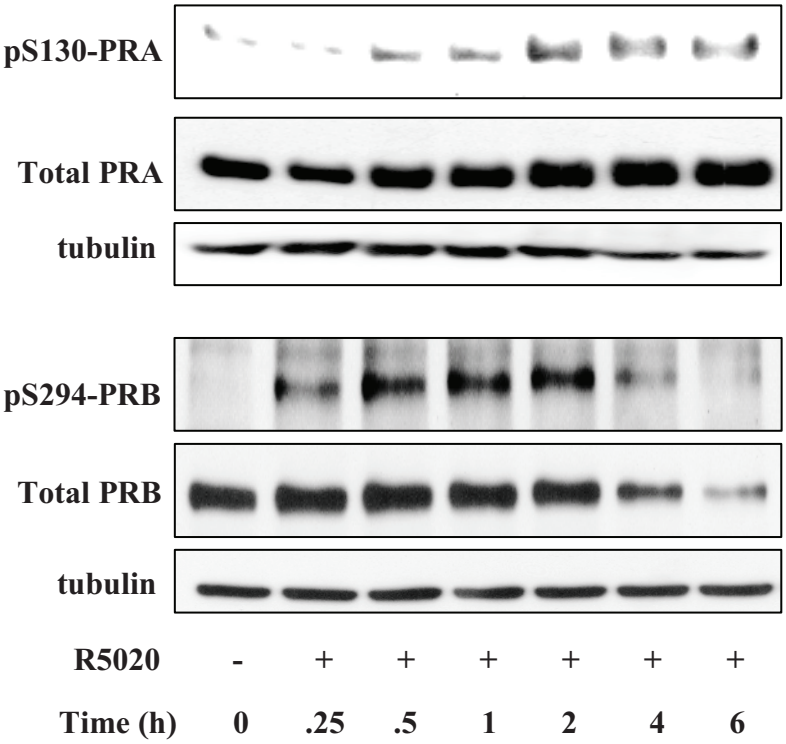
Supplemental Fig. S1

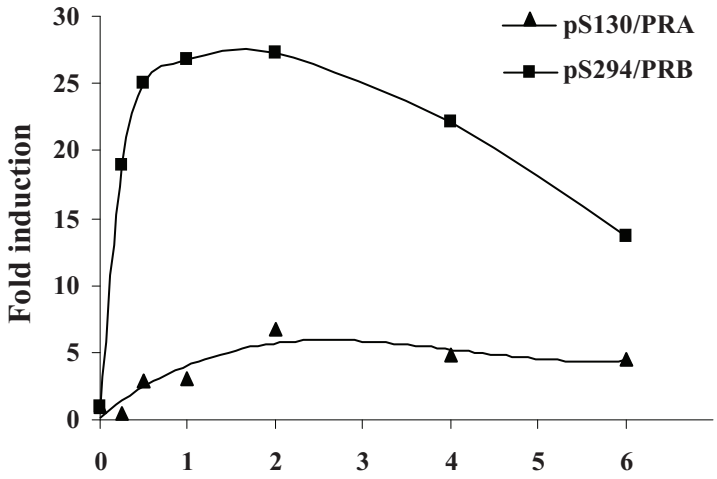


Supplemental Fig. S2

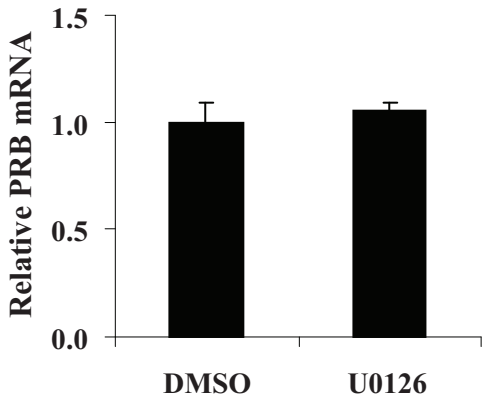


Supplemental Fig. S3

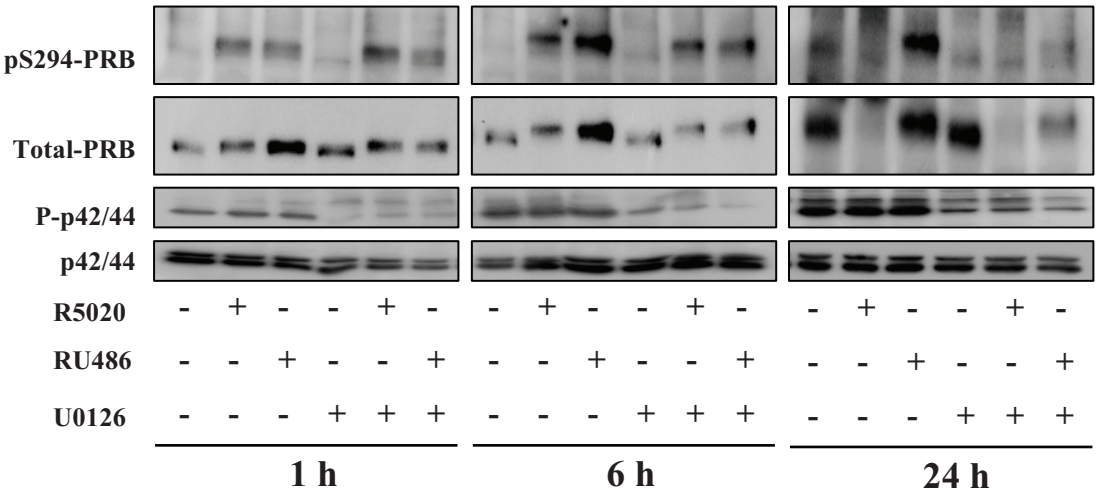




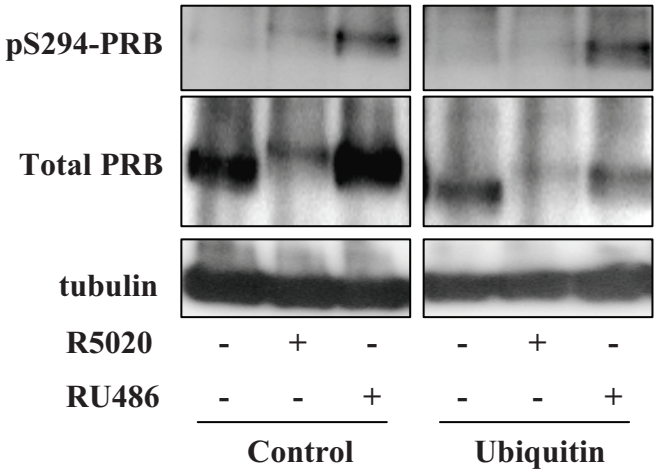
Supplemental Fig. S4



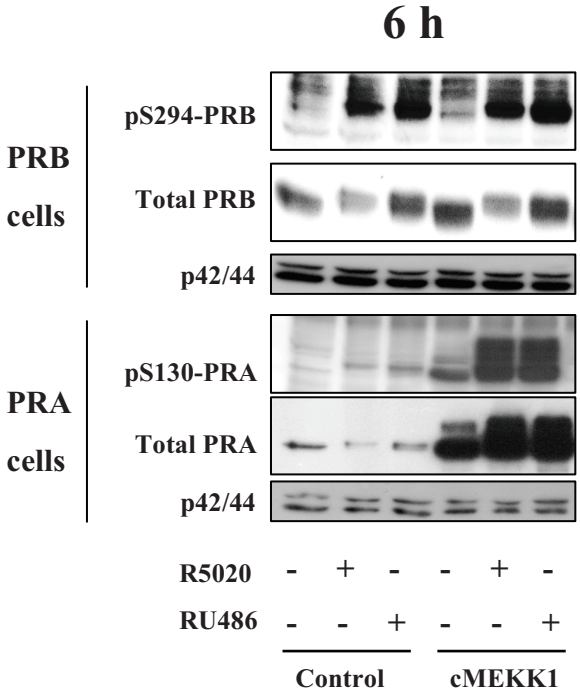
Supplemental Fig. S5



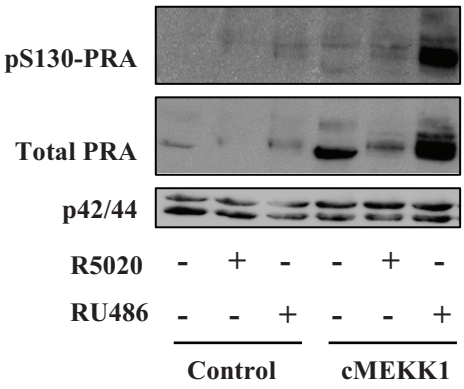
Supplemental Fig. S6



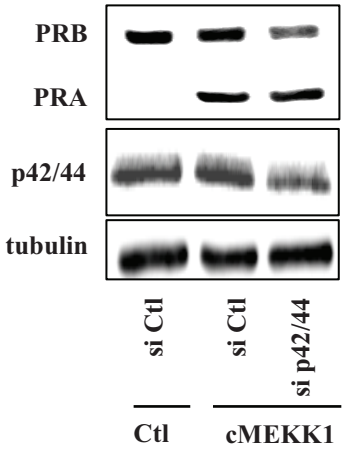
Supplemental Fig. S7



Supplemental Fig. S8



Supplemental Fig. S9



SUPPLEMENTAL TABLE 1. PRIMERS SEQUENCES

Primer	Sequence (5' to 3')	Gene
FKBP5, F	CCGGAGAACCAAACGGAAA	FK506-binding protein 5
FKBP5, R	TGAATGCCACATCTCTGCAGT	
DKK1, F	CCAGCGTTGTTACTGTGGAGAA	Dickkopf-related protein 1
DKK1, R	TTACTGGCTTGATGGTGATCTTTC	
AREG, F	ACTCTGGGAAGCGTGAACCAT	Amphiregulin
AREG, R	TAGTCATAGTCGGCTCCCGAG	
Cyclin D1, F	ACAGATCATCCGCAAACACG	Cyclin D1
Cyclin D1, R	TCTGGAGAGGAAGCGTGTGA	
HB-EGF, F	TGAGCCTCCCAGTGGAAAAT	Heparin-binding EGF-like growth factor
HB-EGF, R	AACATGAGAAGCCCCACGAT	
EREG, F	TGCCTGGGTTTCCATCTTCT	Epiregulin
EREG, R	TTGAGCCACACGTGGATTGT	
Sgk1, F	GTGGCAATTCTCATCGCTTTC	Serum- and glucocorticoid-regulated kinase 1
Sgk1, R	CTTCAGGGTGTTTGCATGCAT	
PRB, F	AAAGGGGAGTCCAGTCGTCAT	Progesterone receptor isoform B
PRB, R	GGAGATAGGTATGGCCGAAACTT	
18S-rRNA, F	GTGCATGGCCGTTCTTAGTTG	18S rRNA
18S-rRNA, R	CATGCCAGAGTCTCGTTCGTT	

B. Impact of proteasome-mediated PR-SRC1 turnover on PR transcriptional properties (*PAPER II*)

Background

Previous studies have revealed a tight association between the turnover rate of several nuclear receptors (NR) and their transcriptional activity, showing that both aspects of NR function appear to be inversely related (Lipford and Deshaies, 2003; Lonard et al., 2000; Reid et al., 2003; Shen et al., 2001; Tirard et al., 2007; Wijayaratne and McDonnell, 2001; Yokota et al., 2004). For example, blocking of progestin-induced PR turnover by several means, such as proteasome inhibitors (Dennis et al., 2005), nuclear export inhibitors (Qiu and Lange, 2003) or mutation of PRB S294 (Lange et al., 2000), abolishes PR transcriptional activity. The underlying mechanisms of this paradoxical link between PR stabilization and transcriptional inactivation are yet to be fully understood. It appears that complex interactions between regulatory molecules governing both transcription and ubiquitination/degradation exist (Masuyama and MacDonald, 1998; Nawaz et al., 1999a; Verma et al., 2004) as already demonstrated for ER (Reid et al., 2003). However, little is known concerning the fate of coregulators during ligand-dependent NR down-regulation (Gianni et al., 2006; Han et al., 2007).

Results

In this part of thesis, we demonstrated that SRC1 expression level is hormonally regulated. Agonist ligand induced proteolysis of PR/SRC1 complex to attain transcription whereas PR antagonist RU486 impaired the ligand-dependent degradation of PR/SRC1 and consequently the transactivation process. We identified two degradation motifs, PEST (amino acids 2-16) and basic helix loop helix domain (amino acids 41-136) which control the basal expression levels as well as hormone-dependent down-regulation of SRC1.

Perspectives

It would be important to see what happens to PR target genes transcription which is not influenced by SRC1. We showed that SRC2 and SRC3 are not degraded in the course of transcriptional activation by PR. Knowing the fact that PR transcriptional activity is controlled by distinct coregulators depending on cell type, an intriguing question would be whether other PR interacting transcriptional coactivators are also degraded in other cell types notably in breast cancer cells in which the principal coactivator for PR activity is SRC3. Also the role of PRA isoform in ligand-dependent SRC1 proteolysis remains to be defined.

PAPER II

Ligand-dependent degradation of SRC-1 is pivotal for progesterone receptor transcriptional activity

Amazit L, Roseau A, **Khan JA,** Chauchereau A, Tyagi RK, Loosfelt H, Leclerc P, Lombès M and Guiochon-Mantel A

Mol Endocrinol, 2011 25(3):394-408

Ligand-Dependent Degradation of SRC-1 Is Pivotal for Progesterone Receptor Transcriptional Activity

Larbi Amazit, Audrey Roseau, Junaid A. Khan, Anne Chauchereau, Rakesh K. Tyagi, Hugues Loosfelt, Philippe Leclerc, Marc Lombès, and Anne Guiochon-Mantel

Institut National de la Santé et de la Recherche Médicale Unité 693 (L.A., A.R., J.A.K., A.C., R.K.T., H.L., M.L., A.G.-M.), Unité Mixte de Recherche S693 (L.A., A.R., J.A.K., H.L., M.L., A.G.-M.), Faculté de Médecine Paris-Sud, Univ Paris-Sud, and Institut Biomédical de Bicêtre (P.L.), Le Kremlin Bicêtre F-94276, France; and Service d'Endocrinologie et Maladies de la Reproduction (M.L.) and Service de Génétique Moléculaire, Pharmacogénétique, et Hormonologie (A.G.-M.), Assistance Publique-Hôpitaux de Paris, Hôpital Bicêtre, Le Kremlin Bicêtre F-94275, France

The progesterone receptor (PR), a ligand-activated transcription factor, recruits the primary co-activator steroid receptor coactivator-1 (SRC-1) gene promoters. It is known that PR transcriptional activity is paradoxically coupled to its ligand-dependent down-regulation. However, despite its importance in PR function, the regulation of SRC-1 expression level during hormonal exposure is poorly understood. Here we report that SRC-1 expression level (but not other p160 family members) is down-regulated by the agonist ligand R5020 in a PR-dependent manner. In contrast, the antagonist RU486 fails to induce down-regulation of the coactivator and impairs PR agonist-dependent degradation of SRC-1. We show that SRC-1 proteolysis is a proteasome- and ubiquitin-mediated process that, predominantly but not exclusively, occurs in the cytoplasmic compartment in which SRC-1 colocalizes with proteasome antigens as demonstrated by confocal imaging. Moreover, SRC-1 was stabilized in the presence of leptomycin B or several proteasomal inhibitors. Two degradation motifs, amino-acids 2–16 corresponding to a PEST motif and amino acids 41–136 located in the basic helix loop helix domain of the coactivator, were identified and shown to control the stability as well as the hormone-dependent down-regulation of the coactivator. SRC-1 degradation is of physiological importance because the two nondegradable mutants that still interacted with PR as demonstrated by coimmunoprecipitation failed to stimulate transcription of exogenous and endogenous target genes, suggesting that concomitant PR/SRC-1 ligand-dependent degradation is a necessary step for PR transactivation activity. Collectively our findings are consistent with the emerging role of proteasome-mediated proteolysis in the gene-regulating process and indicate that the ligand-dependent down-regulation of SRC-1 is critical for PR transcriptional activity. **(*Molecular Endocrinology* 25: 394–408, 2011)**

The progesterone receptor (PR), also known as NR3C3, plays a crucial role in the coordination of several aspects of female reproductive development and function (1). Invalidation of the *PR* gene in mice leads to pleiotropic reproductive abnormalities and demonstrates that PR orchestrates key events associated with the establishment and maintenance of pregnancy. From a pathophysiological perspective, accumulating evidence indicates that PR is involved in breast cancer cells proliferation and is implicated in the development and progression of breast cancer (2). Coregulators (coactivators or corepressors) are important nuclear receptor

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

doi: 10.1210/me.2010-0458 Received November 8, 2010. Accepted December 13, 2010.
First Published Online January 27, 2011

Abbreviations: AIB1, Amplified in breast cancer 1; AU, arbitary unit; bHLH, basic helix-loop-helix; CBP, cAMP response element-binding protein; ER, estrogen receptor; HEK, human embryonic kidney; Jab1, Jun-activation domain-binding protein 1; LB, leptomycin B; NES, nuclear export signal; NR, nuclear receptor; PR, progesterone receptor; SRC-1, steroid receptor coactivator-1; wt, wild type.

(NR)-recruited cofactors modulating NR-mediated transcription and leading to activation or repression of target specific genes (3). Steroid receptor coactivator-1 (SRC-1) is a PR coactivator belonging to the p160 gene family, which contains three homologous members (SRC-1, -2, and -3) serving as NR transcriptional coactivators (4). This family of coactivators is characterized by the presence of several conserved functional domains: a basic helix-loop-helix (bHLH)-Per-ARNT-Sim N-terminal domain, a cAMP response element-binding protein (CBP) interacting domain (AD1), a glutamine-rich region, a C-terminal activation domain (AD2), and several LXXLL boxes involved in NR binding. The p160 coactivators are defined as primary coactivators whose activity is regulated by posttranslational modifications (5–10). The current models indicate that p160 coactivators serve as a recruitment platform for other coactivator complexes carrying intrinsic enzymatic activities to specific enhancers/promoters resulting in the covalent modification of specific histones and/or other coregulators involved in the transcriptional machinery (11, 12).

Several experiments have revealed a tight association between the turnover rate of several NR and their transcriptional activity, showing that both aspects of NR function appear to be inversely related (13–18). Among the factors regulating PR levels are its ligands. It was initially shown that administration of progesterone to ovariectomized guinea pigs provoked a rapid fall in uterine receptor concentration (19). Hormone-dependent down-regulation of PR has been finally confirmed by several groups (20–22), but its biological significance is still unclear. Phosphorylation of PR on a key serine residue (Ser294) by MAPKs was shown to couple multiple receptor functions, including ligand-dependent PR down-regulation by the ubiquitin-proteasome pathway (13). The concept that transcriptional activation and ubiquitin-mediated proteolysis are interdependent processes is emerging as a potentially important control mechanism of transcription (16, 23). Although their significance remains to be defined, it appears that complex interactions between regulatory molecules governing both transcription and ubiquitination/degradation exist (24–26). However, little is known concerning the fate of coregulators during ligand-dependent NR down-regulation (27, 28).

In a previous study, we have shown that SRC-1 is exported from the nucleus to the cytoplasm and speculated that this export might be a regulatory mechanism controlling the termination of hormone action, possibly through its degradation (29). To establish a link between SRC-1 proteolysis and the PR-mediated transcription process, we studied the mechanism governing SRC-1 proteolysis at the steady-state level and questioned whether the ligand could modulate its turnover. In this study, we demonstrate that SRC-1 undergoes covalent modifications by ubiquitin, which targets the coactivator to the proteasome at the steady-state level. We identify two critical degron domains directly linked to the coactivator proteolysis. Aside from this ligand-independent stability regulation, we show that SRC-1 undergoes accelerated agonist-dependent and PR-mediated down-regulation via the ubiquitin-proteasome pathway. SRC-1 proteolysis occurs concomitantly of ligand-dependent PR degradation. Of note, the nature of the ligand is shown to be critical for this process because both PR and SRC-1 ligand-dependent proteolysis was inhibited in the presence of RU486, leading to dramatic loss of PR-transactivating capability.

Results

SRC-1 mainly colocalizes with cytoplasmic proteasome antigens

In our previous report about the regulatory mechanisms of SRC-1 subcellular trafficking, we have shown that SRC-1 localizes both in nuclear and cytoplasmic corpuscular structures (29). Several studies have reported coregulators localization in organelles (30, 31). We tried to identify the nature of these cytoplasmic and nuclear speckles by colocalization studies with various antigens and with fluorescent organelles markers. Because several NRs and coactivators such as SRC-3 have been shown to interact with the proteasome (32, 33), we used confocal microscopy to investigate whether proteasome components might also accumulate in SRC-1 speckles. By using antibodies directed against the human S7 subunit of the 19S (Rpt1) and the α/β-subunits of the 20S proteasome, we found that SRC-1 colocalized with both 26S proteasome antigens (Fig. 1A and Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). The fluorescence intensity profile indicates that colocalization was predominant in SRC-1 speckles: simultaneous fluorescence intensity increase was observed in cytoplasmic speckles but also in lesser extent in nuclear speckles (Fig. 1B), suggesting that SRC-1 is mainly but not exclusively proteolyzed in the cytoplasm. Similar intensity profiles were obtained for cells immunolabeled for SRC-1 and the 20S proteasome (data not shown). A partial colocalization of SRC-1 was also found with the promyelocytic leukemia protein in the typical nuclear domain (nuclear domain 10) (Supplemental Fig. 2A). Such an association has been already described (34). In contrast, nuclear speckles did not overlap with transcription sites as evidenced by the absence of colocalization with the SC-35/SRp30 spliceo-

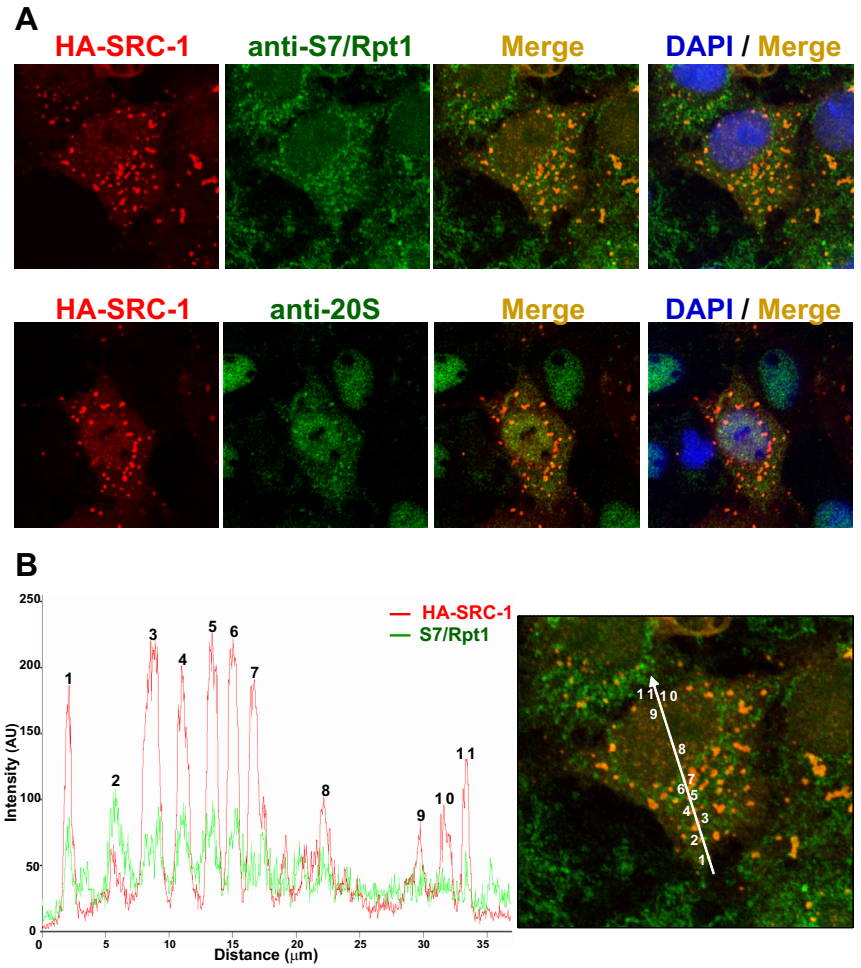


FIG. 1. Colocalization of SRC-1 with the 26S proteasome by confocal microscopy. A, Colocalization analysis between HA-SRC-1 and endogenous proteasome antigens S7/Rpt1 and 20S subunits. COS-7 cells were transiently transfected with the expression vector encoding HA-SRC-1. Cells were fixed after 40 h, immunolabeled with anti-HA and either anti-S7/Rpt1 or anti-20S antibodies, and then observed by confocal microscopy. B, Validation of colocalization by scan of intensity profiles of a representative cell [expressed as arbitrary units (AU)]. Fluorescence intensity was calculated and plotted by drawing a line through the middle of the cell image in a distance covering several cytosolic and nuclear foci. *Green lines* represent the intensity profile for the proteasome antigen S7/Rpt1 signal, and the *red lines* represent the intensity profile for SRC-1 signal. *Indicated numbers* refer to identified speckles: cytoplasmic (1 to 7) or nuclear (8 to 11). Note that although the fluorescence intensity from the two channels is different, the peaks of both signals are overlapping.

some component (Supplemental Fig. 2B). Similarly, no colocalization of SRC-1 with organelles such as mitochondria, lysosomes, peroxisomes, or the Golgi apparatus could be observed (Supplemental Fig. 2, C–E, and data not shown).

SRC-1 is ubiquitinylated *in vivo* and is degraded by the proteasome

We next studied the mechanism of SRC-1 down-regulation. First, we investigated whether the coactivator was ubiquitinylated and targeted to the proteasome. COS-7 cells were transfected with the expression vector encoding the full-length SRC-1 and incubated in the presence of proteasome inhibitors, MG132, or epoxomicin. Consistent with previous reports (14, 35), both inhibitors increased SRC-1 protein level in comparison with cells treated with vehicle (Fig. 2A and Supplemental Fig. 3). To demonstrate that SRC-1 is polyubiquitinylated, COS-7 cells were transfected with SRC-1 expression vector in the presence or absence of a vector encoding His-tagged ubiquitin (His 6-Ub) and analyzed by Western blot. In the absence of His 6-Ub, the anti-SRC-1 antibody detected a major band of approximately 160 kDa (Fig. 2B, *left panel*, lane 1). In cells cotransfected with His 6-Ub expression vector, a moderate decrease in band intensity was observed with a slightly higher molecular weight smear, indicative of ubiquitinylated moieties (Fig. 2B, *left panel*, lane 2). His-tagged proteins were purified by chromatography on nickel-charged agarose beads (Ni-NTA) and analyzed by Western blot with an anti-SRC-1 antibody to show that these bands correspond to ubiquitinylated SRC-1 (Fig. 2B, *right panel*, lane 2).

Several cytoplasmic proteasome substrates have been shown to relocalize into the nucleus upon stabilization by proteasome inhibitors (36–38). We thus examined whether SRC-1 subcellular distribution was similarly modified in such conditions. Indeed, overnight treatment of cells with MG132 induces an obvious shift of the coactivator into the nucleus (Fig. 2C). This result suggests that escape from cytoplasmic proteolytic degradation stimulates the nuclear accumulation of SRC-1 (36). If our hypothesis is true, then inhibition of SRC-1 nuclear export should induce SRC-1 expression level stabilization. To verify this point, we followed the turnover rate of SRC-1Δ(NES), a mutant deleted of its nuclear export signal (NES) (29). The result shows a better stability of this mutant compared with the wild-type (wt) SRC-1 (Supplemental Fig. 4). In a similar approach, we used the nuclear export inhibitor leptomycin B (LB) to impede wt SRC-1 access to cytoplasm. In the presence of LB, SRC-1 not only relocalized into the nucleus (data not shown and Ref. 29), but its expression level also increased approximately 2.5 fold (Fig. 2D). However, SRC-1 stabilization with LB

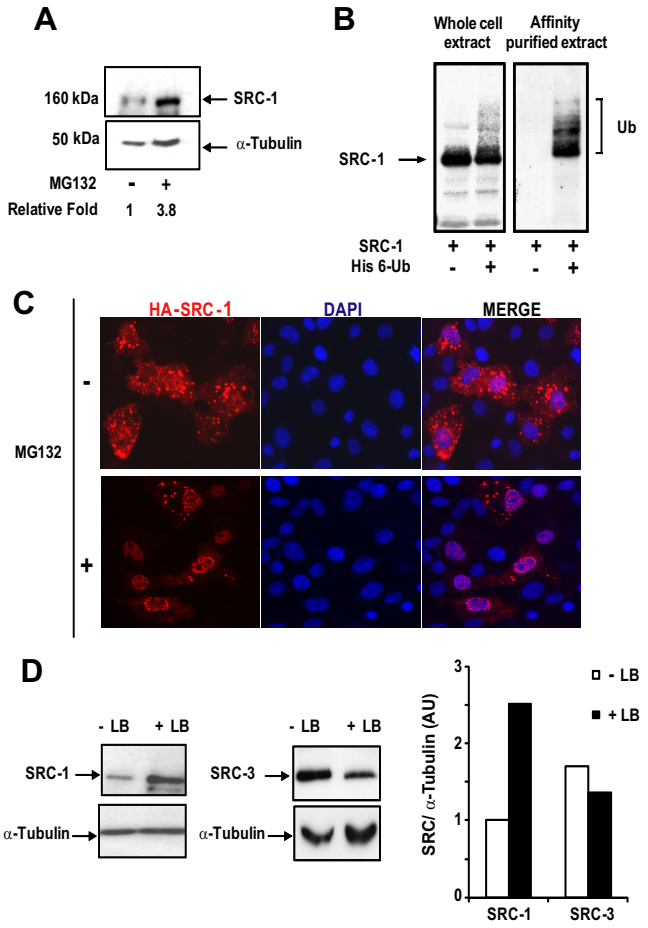


FIG. 2. SRC-1 is proteolyzed by the 26S proteasome in a ubiquitin-dependent manner. A, COS-7 cells were transfected with the expression vector encoding SRC-1 and incubated in the absence or presence of MG132 (5 μM) during 15 h. Expression of SRC-1 was analyzed by Western blot using anti-SRC-1 and anti-α-tubulin antibodies. Band intensities corresponding to SRC-1 were quantified as described in *Materials and Methods*. B, CV-1 cells were transfected with the expression vector encoding HA-SRC-1 in the presence of the His6-tagged ubiquitin expression vector (His 6-Ub). Whole-cell extracts were analyzed by electrophoresis on 6.4% SDS-PAGE and immunoblotted with anti-HA monoclonal antibody. Alternatively, the same cotransfected CV-1 cells were lysed in buffer containing guanidium-HCl (Ni-NTA). The ubiquitin-modified proteins were purified using Ni-NTA agarose beads as described in *Materials and Methods*. Affinity-purified proteins were separated by electrophoresis, and His6-SRC-1 conjugates were detected by Western blot using the anti-HA monoclonal antibody. The ubiquitin conjugates of SRC-1 are indicated with *brackets*. C, COS-7 cells were transfected with the expression vector encoding HA-SRC-1. Twenty hours after transfection, cells were incubated during 24 h with MG132 (1 μM) or treated with vehicle (dimethylsulfoxide). Cells were then fixed and immunolabeled with anti-HA antibody. D, COS-7 cells were transfected with the expression vector encoding SRC-1 or SRC-3 and treated similarly than in C except that MG132 was replaced by LB treatment (20 ng/ml). Expression levels of SRC-1 and SRC-3 were analyzed by Western blot using anti-SRC-1 or anti-SRC-3 monoclonal antibodies as indicated. Band intensities representing the mean of at least two independent experiments were quantified as described in *Materials and Methods*.

did not reach the level obtained with MG132 (data not shown; and compare quantification in Fig. 2A with Fig. 2D). Thus, the nuclear accumulation of the coactivator indicates a possibility of a partial degradation of SRC-1 in the nuclear compartment. Interestingly, similar experiments with the p160 coactivator SRC-3, which has been shown to be degraded mainly in the nucleus (39), showed no significant increase of SRC-3 expression level under LB treatment (Fig. 2D). Overall, our data show that SRC-1 turnover is a proteasome- and ubiquitin-mediated process that takes place, predominantly but not exclusively, in the cytoplasm.

Agonist ligand enhances concomitant proteolysis of PR and SRC-1

We next studied SRC-1 degradation in the context of PR activation. Progestins are known to induce PR proteolysis by the proteasome (22, 40). In addition, Li *et al.* (41) have shown that upon ligand treatment, PR preferentially interacts with SRC-1. We thus investigated whether SRC-1 down-regulation might be also modulated by PR ligands. As previously reported (22), immunocytochemical studies (Fig. 3A) and Western blot experiments (Supplemental Fig. 5) showed that the agonist ligand R5020 stimulates stably expressed endogenous PR proteolysis after 24 h treatment, whereas the antagonist ligand RU486 prevents PR proteolysis in Ishikawa cells stably expressing PR-B (Ishi-PR-B). To test the impact of ligands on SRC-1 expression level, Ishi-PR-B cells were transiently transfected with a SRC-1 expression vector and incubated overnight with R5020 or RU486. Western blot analyses revealed that SRC-1 and PR are concomitantly degraded in the presence of agonist R5020 and that RU486 prevents the degradation of both proteins (Fig. 3B). Similar results were obtained using different Ishi-PR-B subclones (data not shown). Real-time quantitative RT-PCR excluded the possibility of any ligand-dependent down-regulation of SRC-1 mRNA levels (Supplemental Fig. 6). MG132 exposure inhibited the agonist-dependent proteolysis of SRC-1 (Fig. 3B, lane 4), indicating that this stimulated down-regulation is mediated by the proteasome. Importantly, using antibodies specifically detecting endogenous SRC-1, we similarly observed agonist-dependent degradation of endogenous SRC-1 in Ishi-PR-B cells (Fig. 3, C and D). Of note, a 10-fold excess of antiprogestin RU486 abrogated the R5020-dependent degradation of endogenous SRC-1 and PR as shown in Fig. 3D (third lane), suggesting that SRC-1 degradation is tightly linked to the ligand-dependent PR activation. To further verify this hypothesis, we tested whether SRC-1 proteolysis could be stimulated in the absence of PR. We used the Ishikawa parental cell line (Ishi-PR-0) initially used to establish the Ishi-PR-B cell line and that lacks PR-B expression (42). Ishi-PR-0 cells were transfected with SRC-1 expression vector and incubated 24 h with R5020 or RU486. Under these conditions, both ligands did not af-

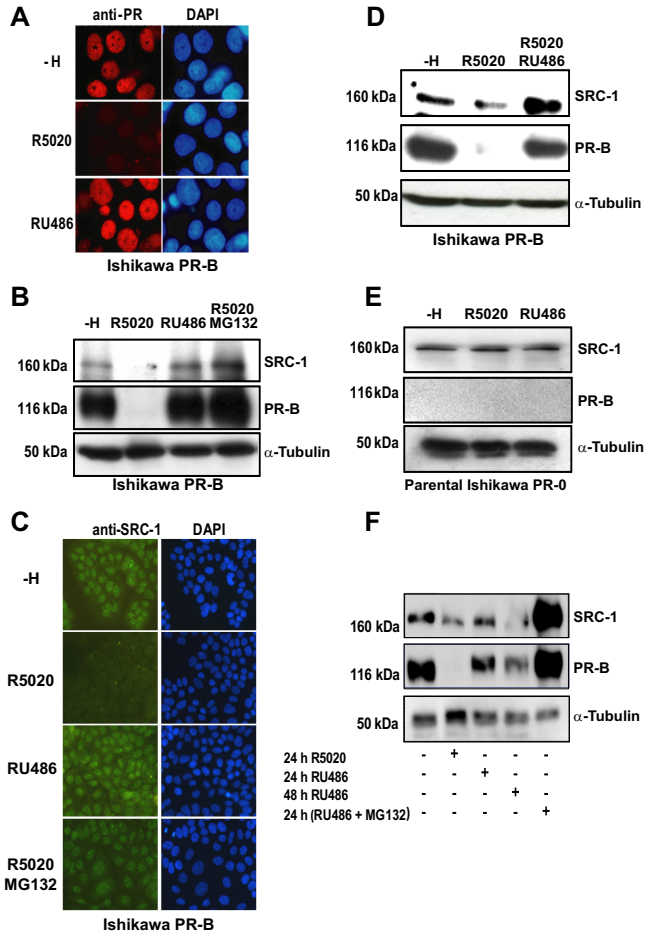


FIG. 3. Ligand- and PR-dependent SRC-1 proteolysis. A, Ishi PR-B cells, a cell line stably expressing PR-B, were cultured 24 h in the absence or in the presence of either the agonist R5020 (10 nM) or the antagonist RU486 (10 nM). Cells were then treated for immunocytochemistry with anti-PR antibody (Let 126) and observed by fluorescence microscopy. DAPI, 4′,6′-Diamino-2-phenylindole. B, Ishi PR-B cells were transfected with the SRC-1 encoding vector. After 48 h, cells were cultured 15 h as indicated, either in the absence of ligand (control vehicle, −H), in the presence of R5020 (10 nM) or RU486 (10 nM), or in the presence of both R5020 (10 nM) and MG132 (5 μM). Whole-cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. C, Nontransfected Ishi PR-B cells were treated as in B. Cells were immunolabeled for endogenous SRC-1 using an anti-SRC-1 antibody. Note the agonist-ligand-dependent down-regulation of endogenous SRC-1. DAPI, 4′,6′-Diamino-2-phenylindole. D, Nontransfected Ishi PR-B cells were cultured 24 h in the absence of ligand (vehicle, −H) or in the presence of either the agonist R5020 (10 nM) alone or in combination with a 100× excess of the antagonist RU486 (1 μM). Whole-cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted to detect endogenous SRC-1 and PR with the indicated antibodies. E, Ishi PR-0 cells (parental cell line, devoid of PR) were treated as in A. Cells were then analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. F, Ishi PR-B cells were transfected with the SRC-1 encoding vector. After 24 h, cells were cultured in the absence of ligand (vehicle, −H), treated with R5020 (10 nM, 24 h) or RU486 (10 nM, 24 or 48 h) or RU486 (10 nM, 24 h) along with MG132 (1 μM). Whole-cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies.

fect the SRC-1 expression level, indicating that SRC-1 down-regulation requires the presence of PR-B (Fig. 3E). Finally, we determined whether other p160 coactivators such as SRC-2/transcription intermediary factor-2/glucocorticoid receptor-interacting protein-1 or SRC-3/ amplified in breast cancer 1 (AIB1), which are also known proteasome targets (14), could be degraded in response to R5020. None of these coactivators was significantly degraded under similar experimental conditions (Supplemental Fig. 7), suggesting a target-specific coactivator effect of PR.

It has been initially proposed that antiprogestins are capable of inducing PR down-regulation but with much slower kinetics than agonists (22). We therefore tried a longer time point to check whether SRC-1 degradation was occurring in the presence of RU486. The result shows that, in contrast to 24 h incubation (Fig. 3F, lane 3), 48 h treatment with RU486 induced a significant reduction of both SRC-1 and PR (Fig. 3F, lane 4). More importantly, in the presence of MG132, RU486 treatment resulted in a dramatic accumulation of PR and SRC-1 (Fig. 3F, lane 5), showing that RU486-induced down-regulation is mediated by the proteasome. Thus, these results not only indicate that RU486 impairs the ligand-dependent down-regulation of PR and SRC-1 by slowing down their degradation but also confirm the concomitance of their ligand-dependent proteolysis. Collectively our results indicate that specific SRC-1 turnover is modulated in a ligand-dependent manner and requires PR expression.

Identification of SRC-1 domains involved in its degradation

To elucidate the mechanisms driving SRC-1 to the proteasome under basal conditions, we identified the domains involved in SRC-1 turnover. *In silico* analysis of SRC-1 primary sequence was carried out in search for putative PEST degradation motifs. The result indicated that the amino acids 2-16 of SRC-1 had a high score (+9.63) for this type of motif. We therefore focused our investigation on the N-terminal subdomain of the coactivator. A critical importance of the bHLH domain for AIB1/SRC-3 mediated proteolysis has been previously reported by Li *et al.* (39). Thus, we also explored the role of this domain in SRC-1 down-regulation. Two deletion mutants were generated, lacking either the PEST sequence or the bHLH domain, encompassing amino acids 2-16 [Δ(PEST)] and amino acids 41-136 [Δ(bHLH)], respectively (Fig. 4A).

To investigate whether these two motifs were involved in SRC-1 degradation, wt SRC-1, Δ(PEST), or Δ(bHLH) mutants were expressed in COS-7 cells, and cycloheximide was added to block protein neosynthesis. The decay of wt SRC-1 and mutant proteins was monitored and quantified by Western blot as a function of time. SRC-1 expression levels decreased after 1 h and almost disappeared after 6 h (Fig. 4B, *left panel*), indicating of a half-

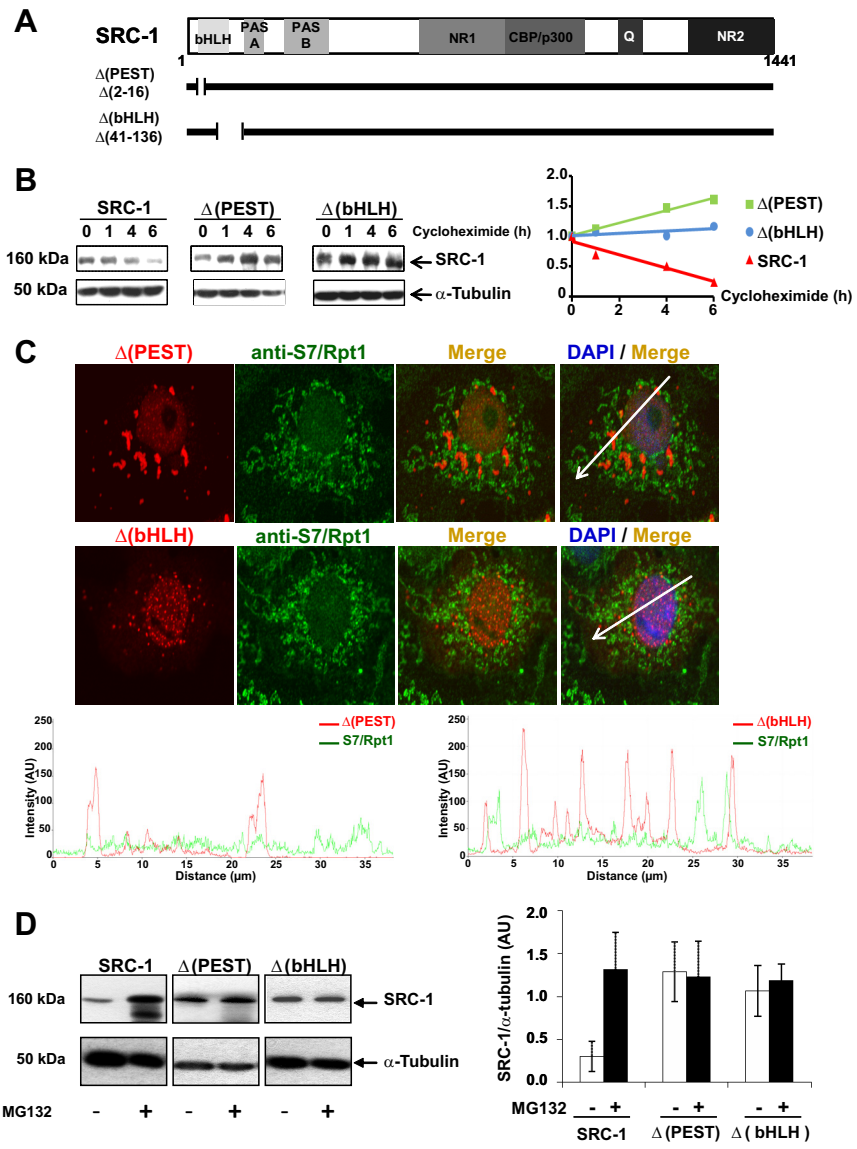


FIG. 4. The N-terminal region of SRC-1 targets the coactivator to degradation. A, Schematic representation of the wild-type coactivator SRC-1 (1441 amino acids in length) with *boxes* corresponding to major functional domains: PAS, Per-ARNT-Sim motif; NR1 and NR2, NR-interacting domains 1 and 2, CBP/p300 interacting domain; Q, glutamine-rich domain. SRC-1 deletion mutants Δ(PEST) and Δ(bHLH) are represented below with a *thick line interrupted by a gap* corresponding to the deleted amino acids. B, COS-7 cells were transfected as indicated with SRC-1, Δ(PEST), or Δ(bHLH) encoding vectors. Seventy-two hours after transfection, cells were treated with cycloheximide (100 μg/ml) during 1, 4, or 6 h. Whole-cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. Band intensities (*right panel*) representing the mean of at least two independent experiments were quantified as described in *Materials and Methods*. C, *Upper panel*, Colocalization analysis of SRC-1 deletion mutants and S7/Rpt1. COS-7 cells were transiently transfected with Δ(PEST)- or Δ(bHLH)-encoding vectors. Cells were fixed after 40 h and immunolabeled with anti-HA and anti-Rpt1/S7 antibodies prior to analysis by confocal microscopy. *Lower panel*, Scan of intensity profiles expressed as arbitrary units (AU). Fluorescence intensity was calculated and plotted by drawing a line through the middle of the cell image in a distance covering several cytosolic and nuclear foci. *Green lines* represent the intensity profile for the proteasome antigen S7/Rpt1 signal, and the *red lines* represent the intensity profile for Δ(PEST) or Δ(bHLH) signals. Note the absence of significant peaks with overlapping signals. D, COS-7 cells were transfected with HA-SRC-1-, Δ(PEST)-, or Δ(bHLH)-encoding vectors. After 48 h, cells were incubated during 15 h with MG132 (5 μM) or vehicle. Whole-cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. The band intensities (*right panel*) were quantified as described in *Materials and Methods*.

life of approximately 3 h. In contrast, both Δ(PEST) and Δ (bHLH) expression levels showed no decrease under the same experimental conditions (Fig. 4B, *middle* and *right panels*), showing that Δ(PEST) and Δ(bHLH) mutants are more stable than wt SRC-1.

To confirm that these motifs were involved in proteasome-mediated SRC-1 degradation, we compared both mutants and wt SRC-1 localization by immunocytochemistry and found that in contrast to the wild-type coactivator (Fig. 1) and the Δ(PEST) mutant, the Δ(bHLH) mutant localized predominantly in the nucleus (Fig. 4C). In contrast to the wild-type coactivator (Fig. 1), colocalization studies of both mutants with 19S proteasome antigens S7/Rpt1 and with the α/β-proteasome 20S subunits showed no significant overlap (Fig. 4C and data not shown).

Moreover, to investigate the involvement of these domains on SRC-1 protein stability, we compared the impact of MG132 on both mutants with wt SRC-1. Whereas SRC-1 protein levels were increased approximately 3-fold under 15 h MG132 treatment (Fig. 4D, *left panel*), the expression level of either Δ(PEST) or Δ(bHLH) remained unchanged under the same conditions (Fig. 4D, *middle* and *right panels*). Similarly, expression levels of both mutants were not increased in presence of epoxomicin (Supplemental Fig. 8). Of note, quantification comparison of band intensity (Fig. 4D, histograms) showed that both mutants were expressed to a greater extent than the wild-type coactivator, suggesting that the deletions may have indeed a stabilizing effect on these mutants. Taken together, our observations show that amino acids 2-16 and 41-136 are involved in SRC-1 down-regulation by targeting SRC-1 to proteasome degradation at the steady-state.

N-terminal degradation motifs of SRC-1 are necessary for its ligand-dependent down-regulation

To evaluate the contribution of the two degradation domains in the con-

text of the hormonal activation, we transiently transfected Ishi-PR-B cells with wt SRC-1 or Δ(PEST) or Δ(bHLH) mutants. We hypothesized that if the two degradation motifs are also involved in hormone-stimulated down-regulation of SRC-1, then both mutants should not undergo proteolysis under hormone stimulation. As expected, after 24 h of R5020 treatment, wt SRC-1 was significantly down-regulated, whereas the expression level of both mutants showed no significant variation (Fig. 5A). Interestingly, the ligand-dependent down-regulation of PR still occurred in each condition, showing that the receptor down-regulation does not require SRC-1 degradation (Fig. 5A). To exclude the possibility that the two deletions may have impaired the interaction between the SRC-1 and PR, we conducted reciprocal coimmunoprecipitation experiments in cells transiently expressing PR and either wt SRC-1 or the deletion mutants. The result shown in Fig. 5B indicates that PR reciprocally coimmunoprecipitates with wt SRC-1 as well as with Δ(PEST) and Δ(bHLH) mutants. Taken together, these results indicate that under hormonal stimulation, SRC-1 ligand-dependent proteolysis requires both degradation signals.

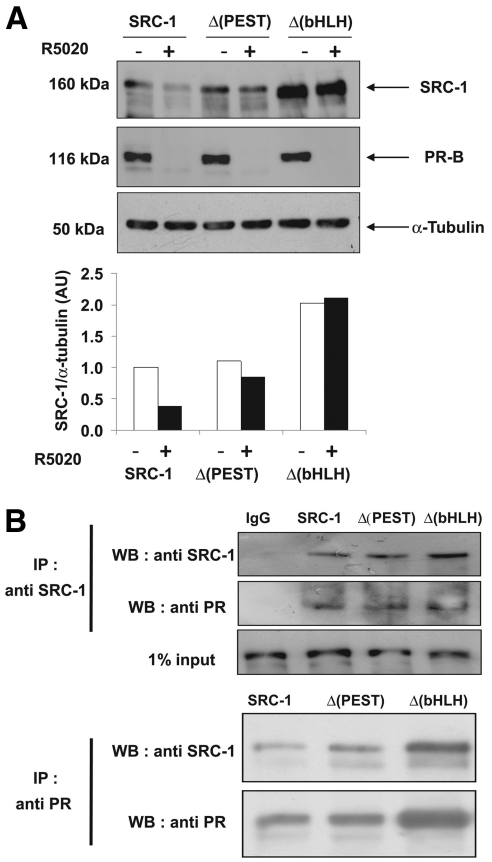


FIG. 5. Ligand-dependent down-regulation of SRC-1 requires both degradation motifs of the coactivator. A, Ishi PR-B cells were transfected as indicated with HA-SRC-1-, Δ(PEST)-, or Δ(bHLH)-encoding vectors. After 48 h, cells were cultured in the absence of ligand (vehicle, −) or presence of the agonist R5020 (10 nM) during 24 h. The corresponding whole-cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. The band intensities (*lower panel*) were quantified as described in *Materials and Methods*. B, HEK 293 cells were cotransfected with PR and the SRC-1-, Δ(PEST)-, or Δ(bHLH)-encoding vectors. Twenty four hours after transfection, cells were treated during 24 h with the agonist R5020 (10 nM). A coimmunoprecipitation assay was performed using the anti-SRC-1, the anti-PR, or the IgG1 control antibodies (IgG1). Purified proteins were separated on 7.5% SDS-PAGE. Coprecipitated complexes were identified with the indicated antibodies. IP, Immunoprecipitation; WB, Western blot.

Because we showed that SRC-1 could be partially proteolyzed in the cytoplasm in which it colocalized in speckles with the proteasome (Fig. 1), we next wondered whether PR will colocalize in the same cytoplasmic speckles. This may specially be the case if we consider the work of Qiu *et al.* (43), who have shown that PR down-regulation under hormone treatment occurs in the cytoplasm. Our result shows that in the absence of hormone, SRC-1 is expectedly cytonuclear and does not colocalize with PR (Fig. 6A). Eight hours of hormonal treatment (in the presence of cycloheximide) induces the nuclear accumulation of both PR and SRC-1, indicative of their interaction during the nuclear import (29). Interestingly, the ligand also induces the colocalization of PR and its coactivator in cytoplasmic speckles (Fig. 6A), suggesting that PR/SRC-1 complexes might be exported back to the cytoplasm. In contrast, in the presence of R5020, Δ(PEST), and Δ(bHLH) mutants were efficiently accumulated in the nucleus, consistent with our coimmunoprecipitation data showing that they do interact with PR in the presence of ligand, but did not colocalize with PR in cytoplasmic speckles (Fig. 6, B–D). Overall, this experiment suggests that PR and SRC-1 could be proteolyzed as a PR/SRC-1 complex through the same proteasome.

Ligand-dependent proteolysis of SRC-1 is necessary for PR-mediated gene transactivation

To examine the functional link between SRC-1 degradation and its coactivating function, we investigated the impact of coactivator proteolysis on PR-mediated transcription. To this aim, we first analyzed whether the proteasome function was required for efficient PR transcriptional activation. Cotransfection of PRE2-TATA-luc reporter gene with the PR encoding vector was performed in parental Ishi-PR-0 cells (devoid of PR), either alone or in combination with the vector encoding SRC-1. Twenty-four hours after transfection, cells were treated for 24 h with R5020 alone or in combination with MG132. To exclude the possibility that the cellular toxicity of MG132 might affect general transcription in Ishikawa cells, we used a 500-nM concentration of the inhibitor, a dose compatible with cell survival of endometrial carcinoma cell

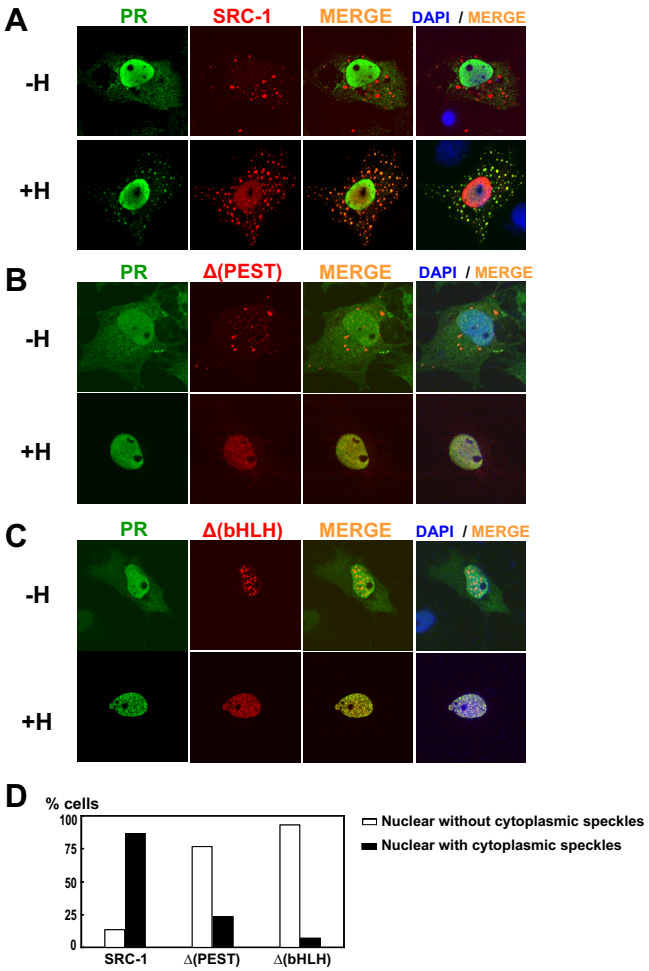


FIG. 6. Colocalization of PR and SRC-1 in cytoplasmic speckles. A, COS-7 cells were transiently transfected with the expression vector encoding HA-SRC-1 and PR. Twenty-four hours after transfection, cells were incubated or not for 8 h with R5020 in presence of cycloheximide (100 μg/ml) prior to fixation. Cells were immunolabeled with anti-PR (Let 126) and anti-HA antibodies. DAPI, 4′,6′-Diamino-2-phenylindole. B and C, cells were treated as in A, except that PR was transfected as indicated with Δ(PEST) and Δ(bHLH), respectively. D, Quantification of cells treated as described in A–C. Percent of cells treated with R5020 shows nuclear localization with or without cytoplasmic speckles. At least 100 cells were counted.

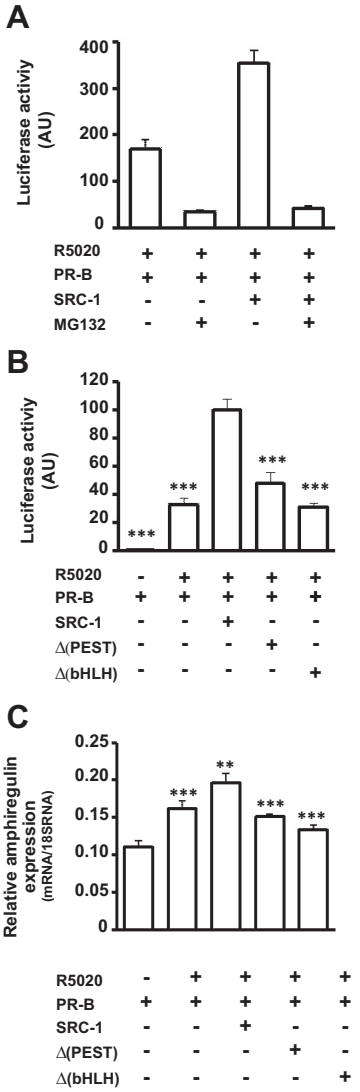


FIG. 7. SRC-1 degradation is necessary for PR transcriptional activity. A, Ishi PR-0 cells were cotransfected as indicated with expression vectors encoding PR and SRC-1 together with the reporter gene *PRE2-TATA-luc* and the internal control pRS-β-gal. Cells were incubated with R5020 (10 nM) and treated or not with MG132 (500 nM) during 24 h. Luciferase activity was quantified and normalized by β-galactosidase activity. Data represent means ± SEM of at least three independent determinations. B, COS-7 cells were cotransfected as indicated with HA-SRC1-, Δ(PEST)-, or Δ(bHLH)-encoding vectors together with expression vector encoding PR, the reporter gene *PRE2-TATA-luc*, and the internal control pRS-β-gal. Cells were treated during 24 h with R5020 (10 nM) or vehicle (control, −). Luciferase activity was quantified and normalized by β-galactosidase activity. Data represent means ± SEM of four independent determinations performed in triplicate. C, Ishi PR-0 cells were cotransfected as indicated with HA-SRC-1-, Δ(PEST)-, or Δ(bHLH)-encoding vectors together with PR encoding vector and were treated with the agonist R5020 10 nM for 3 h. Total RNAs were extracted and relative expression of amphiregulin gene was quantified by quantitative RT-PCR. Results, normalized by the amplification of 18S RNA, are mean ± SEM of three independent determinations. ***, Statistical significance, $P < 0.001$ *vs.* wild-type SRC-1 used as reference.

lines (44). We show that MG132 drastically attenuates ligand-dependent PR transactivation (Fig. 7A), confirming previous observations made by Dennis *et al.* (45). Interestingly, SRC-1-potentiated PR-mediated transcription was also abolished by the proteasome inhibitor (Fig. 7A). This result suggests that the proteasome-mediated degradation is required not only for PR transcriptional activity but also for SRC-1 potentiation of PR. To further explore the relationship between coactivator degradation and the functional consequences on PR-mediated transcription, we used the two nondegradable mutants Δ(PEST) and Δ(bHLH) in cotransfection experiments with PR (Fig. 7B). Because these two mutants are not efficiently degraded by the proteasome (see Fig. 4, B and D), we predicted that they might not exert efficient potentiation of PR transactivation. Indeed, in the presence of R5020, SRC-1 strongly coactivated PR, whereas both Δ(PEST) and Δ(bHLH) mutants were unable to enhance

PR-mediated transactivation as compared with wt SRC-1 (Fig. 7B). These results suggest that the concomitant degradation of SRC-1 and PR is necessary for efficient transcriptional activity of the receptor. Finally, to determine whether the functional link between SRC-1 proteolysis and its coactivating properties were also relevant for human endogenous gene activation, we quantified the level of the progesterone-induced amphiregulin gene that we have previously studied (46). Parental Ishi-PR-0 cells were transfected with PR alone or in combination with wt or SRC-1 mutants. Amphiregulin mRNA levels were significantly increased upon R5020-dependent PR activation and were further enhanced in the presence of SRC-1 (Fig. 7C). Conversely, coexpression of PR with either Δ(PEST) or Δ(bHLH) mutant significantly reduced amphiregulin expression ($P < 0.001$). Taken together, our results demonstrate that hormone-induced degradation of SRC-1 is physiologically relevant for potentiation of PR-mediated transcriptional events.

Discussion

In this study, we investigated the impact of SRC-1 proteolysis on PR-mediated transcription. We provided evidence that the agonist-dependent degradation of SRC-1 is pivotal for PR-mediated transcription. We have established that agonist ligand R5020, but not antagonist RU486, induces the concomitant degradation of endogenous or ectopic PR and SRC-1. Interestingly, SRC-1 turnover requires the presence of PR. Both basal and induced SRC-1 down-regulation are mediated through the proteasome pathway and seem to occur at least in part, in the cytoplasmic compartment. Two regions located in the N-terminal part of SRC-1 (*i.e.* a PEST motif and amino acids 41-136 of the bHLH domain) were identified as two degron motifs. Both signals were shown to be responsible for basal- and hormone-induced degradation of SRC-1. Deletion of each of these domains [Δ(PEST) and Δ(bHLH) mutants] leads to nondegradable SRC-1 mutants insensitive to proteasome inhibitors. By comparing the biological functions of these two mutants with wt SRC-1, we found that they were incapable of potentiating PR-mediated transactivation on a synthetic PR response-element but also on amphiregulin, an endogenous PR target gene. The HAT motif and the CBP-interacting domain of SRC-1 are known to regulate the transcriptional activity of SRC-1 (47, 48). Both regions are present in Δ(PEST) and Δ(bHLH) mutants (Fig. 4A), and therefore, the reduced PR-dependent transactivation of the mutants is not due to an alteration of these regulatory domains but rather to a defect in down-regulation. Thus, our results are indicative of a functional link between proteasome-mediated down-regulation of SRC-1 and its coactivating property.

We have previously shown that SRC-1 is a transcriptional coactivator whose localization is hormonally regulated in the presence of PR (29). Mainly functioning in the nuclear compartment, this coactivator may also be present in the cytoplasm, predominantly concentrated in cytoplasmic speckles (29). Several studies have also demonstrated that p160 coregulators might be localized in the cytoplasm (7, 30, 31). Although the concentration of SRC-1 in cytoplasmic speckles was initially reported to be linked to overexpression (49), it has been also observed for endogenous p160 coactivators (50), and more importantly, a recent study correlated this archetypical distribution with the cytoplasmic sequestration of SRC-1 by SRC-interacting protein (51). During our primary search to identify the nature of these speckles, we initially observed a colocalization between SRC-1 and proteasome antigens, indicating that SRC-1 cytoplasmic speckles are enriched of proteasome components (Fig. 1A). Similar subcellular distribution studies already reported SRC-2 colocalization with proteasome antigens but specifically at the nuclear level (34, 52). Coactivator/proteasome interaction also have been described at the biochemical level for the p160 coactivators (33, 53), as well as NR such as the thyroid receptor, the retinoic acid receptor-α and retinoid X receptor, the estrogen receptor (ER)-α, or the vitamin D receptor (32). We detected a strong colocalization in the cytoplasmic compartment, although a weaker colocalization in speckles was also observed in the nuclear compartment (Fig. 1B) indicative of a predominant but not exclusive proteolysis of the coactivator in the cytoplasmic compartment. Interestingly, nuclear export of SRC-3 has been shown to be required for its proteasomal degradation (54). However, our finding is not consistent with the work of Li *et al.* (39), who recently showed that proteasome-dependent turnover of SRC-3 occurs specifically in the nucleus. Although we could not completely exclude that nuclear degradation also occurs for SRC-1 (see colocalization profiles in Fig. 1B), this discrepancy between SRC-1 and SRC-3 argues for the fact that each SRC family member has different and specific physiological functions (55).

We have shown that the ubiquitin-proteasome pathway mediates selective degradation of SRC-1 and regulates the steady-state expression level of the coactivator. Similarly, Yan *et al.* (35) have shown that several SR coactivators were degraded through the ubiquitin-proteasome dependent pathway and that SRC-1 proteolysis occurs specifically through the ubiquitin-conjugating enzyme 2. The half-life regulation of p160 coactivators has been extensively investigated since the discovery of their

prototype SRC-1, and several studies have demonstrated the physiological and pathophysiological importance of regulating SRC-1 expression levels (56–58). SRC-1 is an important modulator of PR-mediated gene transcription, and to accurately exert its physiological function, its level must be therefore tightly regulated *in vivo*. In this context, Han *et al.* (59) used an original transgenic mouse model in which SRC-1 levels were shown to influence the compartment specific corepressor to coactivator ratio to modulate PR activity in the uterus. Cell regulation of SRC-1 levels seems to be also critical for tumorigenesis, and studies have demonstrated that SRC-1 expression is significantly increased in breast tumors and positively correlates with disease recurrence and poor disease-free survival (55). Consistent with this finding, SRC-1 level is up-regulated during mammary tumor progression (60), and the role of this coactivator in promoting mammary tumor cell invasion was recently demonstrated *in vivo* (57, 58).

Besides the regulation of SRC-1 proteolysis at basal level, the present study also analyzes ligand-stimulated down-regulation of the coactivator. Similarly to other rapidly turned over transcription factors, engagement of PR in transactivation has been shown to be coupled to PR degradation by the ubiquitin-proteasome pathway (13). However, the functional impact of the SRC-1 coactivator on PR-mediated transactivation has never been clearly established. We demonstrate for the first time that concomitantly to PR degradation, SRC-1 proteolysis is dramatically increased in the presence of the agonist ligand R5020 and that this process is mediated through the proteasome. Similarly to PR (22), this down-regulation is necessary for PR-mediated transcription. Recent advances in molecular biology have redefined the role of proteasome as a regulatory system that influences the fate of many cellular processes, such as cell proliferation, apoptosis, and more recently gene transcription. Despite the disparate nature of the later process, a growing body of evidences indicates that ubiquitin and the proteasome are intimately involved in NR-mediated gene control (45, 61, 62). Steroid hormone receptors and their coactivators cycle onto and off steroid-responsive promoters in a ligand-dependent manner, and it is now believed that the ubiquitin-proteasome functions in promoting the turnover of transcription complexes, thereby facilitating proper gene transcription (16, 63). Dennis *et al.* (45) have proposed the existence of a transcriptional mechanism that links the proteasome function with the continued recruitment of RNA polymerase II to sustain the transcriptional response. Consistent with these observations is the fact that a number of ubiquitin pathway enzymes and components of the proteasome have been found to act as modulators of NR function (24, 26, 64) and that enzymes and components of the proteasome are recruited to the promoters of NR-responsive genes (16, 63).

Despite this, it is difficult to conceive how a coactivator will be paradoxically part of a coactivating complex positively modulating gene activation and at the same time a specific target of the ubiquitin-proteasome pathway. Thus, the coupling of PR/SRC-1 proteolysis and efficient transcriptional activation is counterintuitive and rather puzzling but could be a general phenomenon occurring during transcription (65). Consistent with this, is the fact that neither PR nor its coactivator was down-regulated in presence of the antagonist RU486. This result may suggest that RU486 indirectly prevents recruitment of the proteasome machinery, thereby inhibiting transcription. The same observation was made with ERα and the partial antagonist tamoxifen, although it may not be considered as a general phenomenon for steroid receptor because the pure antagonist faslodex dramatically stabilize ER in similar conditions (49). It is not the first example of a hormonal regulatory mechanism implicated in specific coregulators proteolysis: indeed, SRC-2 is down-regulated through the activation of the cAMP-dependent protein kinase pathway (52). More importantly, Gianni *et al.* (27) showed that SRC-3, but not SRC-1 or SRC-2, is phosphorylated by p38MAPK in a retinoic acid-dependent manner and then degraded by the proteasome pathway. In this case, phosphorylation of SRC-3 has a biphasic effect on retinoic acid receptor-α transactivation with facilitation followed by restriction of transcription.

Because the presence of PR is required for SRC-1 degradation, two important remaining questions concern the identification of the key player responsible for SRC-1 degradation and whether this factor is involved in both basal and ligand-induced SRC-1 down-regulation. Shao *et al.* used RNA interference to knock-down SRC-3 that consequently abolishes ERα ligand-dependent degradation, suggesting that the coactivator itself regulates ERα degradation (66). Conversely, because the two nondegradable mutants did not impede the ligand-induce PR down-regulation (Fig. 5A), our results do not converge toward a link between the recruitment of a common E3-ligase by SRC-1 which will in turn induce the ligand-dependent degradation of the PR/SRC-1 complex. The signal that targets PR and SRC-1 to progress from transcription to degradation may also involve post-translational modifications operating like a molecular signature such as phosphorylation, ubiquitinylation or sumoylation (9, 67, 68). Alternatively, direct recruitment of ligase in the vicinity of the coactivator complex or directly at the enhancer level may be also implicated in SRC-1 turnover along with PR. A good candidate would be the PR-B coactivator/ubiquitin ligase E6-AP because this coactivator plays a major

role in controlling the regulated degradation of SRC-3 and PR-B isoform (54, 69). Alternatively, the colocalization with proteasome antigens observed in our study (Fig. 1) might also be linked to the direct interaction observed between SRC-1 and the proteasome through the low molecular mass polypeptide 2 proteasome subunit (53). Such a direct ligand-dependent interaction may drive the coactivator to proteolysis. Another potential candidate for PR and SRC-1 degradation might be Jun-activation domain-binding protein 1 (Jab1), a coactivator involved in ER degradation (70). We are currently investigating this hypothesis because we have shown in a previous study that Jab1 is a coactivator of PR, inducing the formation of a PR/SRC-1/Jab1 ternary complex during the transcription process (71).

In summary, we demonstrate in the present study that SRC-1 expression level is hormonally regulated by the ligand. Whereas in the presence of an agonist, the PR/SRC-1 complex is proteolyzed to achieve transcription, an antagonist such as RU486 impairs the ligand-dependent degradation of PR/SRC-1 and consequently the transactivation process. Our data indicate that the expression level of SRC-1 coactivator is critical for PR transcriptional activity. These findings are consistent with the emerging role of the 26S proteasome in the gene regulation process (72). P160 family members are certainly not the only coactivators implicated in such processes, and it will be interesting to elucidate the sequential progression of each coregulator degradation during gene regulation.

Materials and Methods

Hormone and inhibitors

Cycloheximide, epoxomicin, MG132, and LB were purchased from Sigma (St. Louis, MO). Agonist R5020 (17,21-dimethyl-19-norpregna-4,9-dien-3,20-dione) and antagonist RU486 (Sigma) were used at a concentration of 10 nM, except where indicated.

Plasmids

Nomenclature

Derivatives denoted with a Δ lack the protein segment delineated by the numbered amino acids. Plasmids encoding the wild-type human progesterone receptor (pSG5-PR) and coactivator SRC-1 (pSG5-SRC-1, pSG5-HA-SRC-1, pSG5-HA-GFP-SRC-1) have previously been described (29). PCR-based site-directed mutagenesis of pSG5-HA-SRC-1 was used to create deletion mutants: pSG5-HA-SRC-1-Δ (2–16) [named Δ(PEST)], pSG5-SRC-1-Δ (41-136) [named Δ(bHLH)], and pSG5-HA-SRC-1Δ(990-1060) [named Δ(NES), (29)]. The plasmid pPRE2-TATA-Luc has been previously described (71). Plasmid pSG5-His6-Ub is a gift of D. Bohmann (Laboratory EMBL, Heidelberg, Germany). Plasmids pSG5-SRC-2 and pCR3.1-SRC-3 have been described previously (7, 73), and GFP-peroxisome targeting signal expression vector was purchased (CLONTECH, Mountain View, CA).

Cell culture and DNA transfection

Human endometrial Ishikawa cells (parental cell line, Ishi-PR-0, and stable, Ishi-PR-B) were provided by Dr. L. J. Blok (Erasmus University, Rotterdam, The Netherlands) (74). COS-7, human embryonic kidney (HEK) 293, Ishi-PR-0, and Ishi-PR-B were grown in DMEM containing 10% fetal bovine serum (Biowest, Miami, FL) and supplemented with L-glutamine and antibiotics (penicillin/streptomycin; PAA Laboratories Les Mureaux, France). For hormonal regulation experiments, cells were grown in the presence of 10% steroid-depleted fetal bovine serum prior (24 h) and during transfection experiments. Transfections were performed with the indicated expression vectors using LipofectAMINE 2000 according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA).

Antibodies

Monoclonal anti-PR antibodies used in the study were the Let126 (0.5 μg/ml) (75), the monoclonal anti-PR from Novocastra (NCL-L-PGR-312/2; Newcastle-upon-Tyne, UK) or the rabbit polyclonal anti-PR (sc-538) from Santa Cruz Biotechnology (Santa Cruz, CA), used for immunoprecipitation. Anti-SRC-1 mouse monoclonal antibody (Millipore, Billerica, MA) was used for Western blot and immunocytochemistry (1 μg/ml). Endogenous SRC-1 was detected with anti-SRC-1 (sc-6096) purchased from Santa Cruz Biotechnology. Anti-HA 3F10 (200 ng/ml) was from Roche Applied Science (Indianapolis, IN). Rabbit polyclonal antibody directed against human S7/Rpt1 and 20S proteasome subunits and KAT13C/nuclear receptor coactivator 2/SRC-2 were purchased from Abcam (Cambridge, MA) and used at 1:1000 dilution. Anti-α-tubulin (1:10,000) and anti-SC-35 (1 μg/ml) were purchased from Sigma. Antipromyelocytic leukemia protein was provided by H. de Thé (Institut Universitaire d'Hématologie, Paris, France). Anti-SRC3/AIB1 antibody was purchased from BD Biosciences (San Diego, CA) and was used at 0.5 μg/ml. Secondary antibodies (1:4000) included the following: antimouse, antirat, and antirabbit antibodies conjugated to Alexa 488 (green) or 595 (red) or Dylight 549 (red) were from Invitrogen and Jackson ImmunoResearch Laboratories (West Grove, PA). Secondary peroxidase-conjugated antimouse (Calbiochem, San Diego, CA) and antirabbit (Vector Laboratories Inc., Burlingame, CA) antibodies were used at 1:15,000 dilution.

Luciferase reporter gene assays

COS-7 cells were cultured in free steroid medium and reverse transfected in 96-well plates with 4 ng PR, 100 ng PRE2-TATA-Luciferase, 100 ng SRC-1 (wild type or mutants), and 5 ng β-galactosidase (internal control). The pBlue-Script plasmid was used to equally adjust DNA quantity. After 24 h transfection, cells were incubated with or without 10 nM R5020 for 24 h. Cells were collected with the passive lysis buffer (Promega, Madison, WI), and luciferase activity was measured with a luminometer (Victor; PerkinElmer, Waltham, MA). Luciferase activity was normalized with β-galactosidase activity. The results are means ± SE of four independent experiments.

Immunocytochemistry

Cells were seeded in 24-well plates and processed as previously described (7). Briefly, cells were fixed with 4% parafor-

maldehyde and permeabilized for 30 min with a 0.5% solution of Triton X-100 diluted in PBS. Cells were then incubated with primary antibody overnight at 4 C, followed by the appropriate fluorochrome-coupled secondary antibody (Alexa 488 or 595; Invitrogen; or Dylight 549, Jackson ImmunoResearch Laboratories) for 30 min. Nuclear counterstaining was performed with 0.5 μg/ml 4′,6′-diamidino-2-phenylindole, and coverslips were mounted on slides with ProLong Gold mounting medium (Invitrogen). For standard microscopy (see Figs. 2 and 3), fluorescent cells were observed with an Olympus Provis AX70 (Rungis, France), and images were acquired with Qcapture Pro version 5.1 (Q Imaging Inc., Surrey, British Columbia, Canada) using an Evolution VF Monochrome camera (Media Cybernetics Inc., Bethesda, MD).

Confocal microscopy

For some of the figures (see Figs. 1, 4, and 6), a Zeiss LSM-510 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY) was used for fluorescence acquisition. Images of fixed cells were collected from equatorial planes of cells with a pinhole setting of approximately 1.0 airy unit (optical thickness of 0.8 μm) using a ×63:1.4NA oil immersion plan-apochromat objective with ×8 frame averaging accumulation. To exclude cross talk artifacts, both red and green fluorescence emission were acquired sequentially in separated channels. The confocal microscope settings were kept the same for all scans. To validate colocalization of proteins (see Figs. 1 and 4), line scans of intensity profiles across the cells were generated with the LSM browser software (76). This function associates the merge images with an intensity profile of each channel, measured along a freely positioned line. To obtain an average representative intensity profile expressed as arbitrary units (AU), lines were drawn through the middle of each cell images in a distance covering the cytosol and the nucleus. Green lines represent the intensity profile for the proteasome antigen S7/Rpt1 signal and the red lines represent the intensity profile for SRC-1 signal.

Western blot and immunoprecipitation

Cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.2 mM NaF, 0.2 mM Na_3VO_4, protease inhibitor cocktail] for 15 min, and the debris was cleared by centrifugation at 14,000 × *g* for 15 min at 4 C. Samples were resolved by 7.5% sodium dodecyl sulfate gel electrophoresis and transferred onto nitrocellulose membranes. The indicated antibodies were diluted in Tris-buffered saline and 0.1% Tween 20 buffer supplemented with 5% nonfat milk and added to the membranes for 1 h 30 min at room temperature or overnight at 4 C followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature. All proteins were detected with ECL Plus detection reagents (Amersham Biosciences Corp., Piscataway, NJ) and visualized by chemiluminescence. For the normalization, the membrane was stripped, probed with anti-α-tubulin antibody diluted to 1:1000 (Sigma). The bands were quantified after digitalization on a gel scanner using Image J software (National Institutes of Health, Bethesda, MD). Results, mean of three independent experiments (except Fig. 5A), are presented as the ratio SRC-1 (or PR) to α-tubulin and are expressed as fold induction above the value measured for wild-type SRC1 in the absence of MG132 arbitrarily set at 1. For coimmunoprecipitation, HEK 293 cells were transfected in a 100-mm plate with wild-type SRC-1, Δ(PEST), or Δ(bHLH) plasmids and cultured in presence of 10^{-8} M R5020 for 24 h. Cells were lysed at 4 C in 500 μl lysis buffer, and cell debris was pelleted by centrifugation (14,000 rpm, 15 min, 4 C). Immunoprecipitation of the supernatant with anti-SRC-1 or with the rabbit polyclonal anti-PR or with IgG control were performed with Protein G magnetics beads (Millipore) according to the manufacturer's instructions. Bound immunocomplexes were boiled in Laemmli buffer, separated by 7.5% SDS-PAGE, blotted nitrocellulose membranes with anti-1 μg/ml SRC-1 (Millipore) and anti-PR-B (Let 126, 0.5 μg/ml) antibodies, detected with ECL Plus detection reagents (Amersham Biosciences), and visualized by chemiluminescence.

Real-time RT-PCR

The Ishikawa cell line expressing PR-B or not was transfected by the indicated plasmids by Polyfect reagent (QIAGEN, Valencia, CA) in six-well plates (six wells per condition). After a 2 h-treatment by R5020 10 nM, cells were washed and lysed by Trizol reagent (Life Technologies, Gaithersburg, MD). Total RNA was extracted as described by the manufacturer. One microgram of each sample was treated by deoxyribonuclease I and was reverse transcribed using random primers as previously described (77). Real-time quantitative PCR of the amphiregulin gene was performed as described (46) using the Power SYBR Green master mix (Applied Biosystems, Carlsbad, CA) in duplicate with 1:20 fraction of each cDNA sample and the corresponding primers, using an ABI Prism 7300 apparatus(Applied Biosystems). For each sample, the mRNA concentration was extrapolated from standard curve and averaged cycle threshold value was divided by that of the corresponding reverse-transcribed 18S RNA (relative mRNA).

Statistical analysis

Data are expressed as the mean ± SEM. The Mann-Whitney *U* test was used to determine significant differences between the two groups. For multiple comparisons, the Kruskal-Wallis test followed by Dunn's posttest was performed using the computer software Prism 4 (GraphPad Software, San Diego, CA). Statistical significance is indicated at $P < 0.05$, 0.01, and 0.001.

Acknowledgments

The authors are indebted to Youssef Alj for the construction of the Δ(PEST) and Δ(bHLH) SRC-1 mutants. We thank C. Massaad and B. W. O'Malley for kindly providing SRC-2 and SRC-3 plasmids, respectively. We gratefully acknowledge Luc Outin for excellent technical support and Geri Meduri for critical and thorough reading of the manuscript. We are thankful to Meriem Messina for her help in plasmid preparation.

Address all correspondence and requests for reprints to: Professor Anne Guiochon-Mantel, M.D., Ph.D., Institut National de la Santé et de la Recherche Médicale Unité 693, 63 Rue Gabriel Péri, Le Kremlin-Bicêtre F-94276, France. E-mail: anne.mantel@bct.aphp.fr.

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, the Université Paris-Sud 11. L.A. and R.K.T. were recipients of a fellowship from the Fondation pour la Recherche Médicale. A.R. is a recipient of a fellowship from the Université Paris-Sud 11. J.A.K.

is on study leave from the University of Agriculture, Faisalabad, Pakistan, and is a recipient of a doctoral scholarship from the Higher Education Commission, Pakistan.

This work was supported by grants from Inserm, the Université Paris-Sud 11 and the Académie Nationale de Médecine.

Present address for A.C.: Institut National de la Santé et de la Recherche Médicale Unité 981, Institut de Cancérologie Gustave Roussy, 114 Rue Edouard Vaillant, Villejuif F-94805, France.

Present address for R.K.T.: Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi 110067, India.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Li X, O'Malley BW** 2003 Unfolding the action of progesterone receptors. J Biol Chem 278:39261–39264
2. **Lange CA** 2008 Challenges to defining a role for progesterone in breast cancer. Steroids 73:914–921
3. **Lonard DM, O'Malley BW** 2006 The expanding cosmos of nuclear receptor coactivators. Cell 125:411–414
4. **Xu J, Li Q** 2003 Review of the *in vivo* functions of the p160 steroid receptor coactivator family. Mol Endocrinol 17:1681–1692
5. **Chen D, Ma H, Hong H, Koh SS, Huang SM, Schurter BT, Aswad DW, Stallcup MR** 1999 Regulation of transcription by a protein methyltransferase. Science 284:2174–2177
6. **Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG** 1997 The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature 387:677–684
7. **Amazit L, Pasini L, Szafran AT, Berno V, Wu RC, Mielke M, Jones ED, Mancini MG, Hinojos CA, O'Malley BW, Mancini MA** 2007 Regulation of SRC-3 intercompartmental dynamics by estrogen receptor and phosphorylation. Mol Cell Biol 27:6913–6932
8. **Naeem H, Cheng D, Zhao Q, Underhill C, Tini M, Bedford MT, Torchia J** 2007 The activity and stability of the transcriptional coactivator p/CIP/SRC-3 are regulated by CARM1-dependent methylation. Mol Cell Biol 27:120–134
9. **Rowan BG, Weigel NL, O'Malley BW** 2000 Phosphorylation of steroid receptor coactivator-1. Identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. J Biol Chem 275:4475–4483
10. **Wu RC, Qin J, Hashimoto Y, Wong J, Xu J, Tsai SY, Tsai MJ, O'Malley BW** 2002 Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) Coactivator activity by IκB kinase. Mol Cell Biol 22:3549–3561
11. **McKenna NJ, O'Malley BW** 2002 Minireview: nuclear receptor coactivators—an update. Endocrinology 143:2461–2465
12. **Rosenfeld MG, Lunyak VV, Glass CK** 2006 Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes Dev 20:1405–1428
13. **Shen T, Horwitz KB, Lange CA** 2001 Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294. Mol Cell Biol 21:6122–6131
14. **Lonard DM, Nawaz Z, Smith CL, O'Malley BW** 2000 The 26S proteasome is required for estrogen receptor-α and coactivator turnover and for efficient estrogen receptor-α transactivation. Mol Cell 5:939–948
15. **Wijayaratne AL, McDonnell DP** 2001 The human estrogen receptor-α is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. J Biol Chem 276:35684–35692
16. **Reid G, Hübner MR, Métivier R, Brand H, Denger S, Manu D, Beaudouin J, Ellenberg J, Gannon F** 2003 Cyclic, proteasome-mediated turnover of unliganded and liganded ERα on responsive promoters is an integral feature of estrogen signaling. Mol Cell 11:695–707
17. **Yokota K, Shibata H, Kobayashi S, Suda N, Murai A, Kurihara I, Saito I, Saruta T** 2004 Proteasome-mediated mineralocorticoid receptor degradation attenuates transcriptional response to aldosterone. Endocr Res 30:611–616
18. **Tirard M, Almeida OF, Hutzler P, Melchior F, Michaelidis TM** 2007 Sumoylation and proteasomal activity determine the transactivation properties of the mineralocorticoid receptor. Mol Cell Endocrinol 268:20–29
19. **Milgrom E, Thi L, Atger M, Baulieu EE** 1973 Mechanisms regulating the concentration and the conformation of progesterone receptor(s) in the uterus. J Biol Chem 248:6366–6374
20. **Mullick A, Katzenellenbogen BS** 1986 Progesterone receptor synthesis and degradation in MCF-7 human breast cancer cells as studied by dense amino acid incorporation. Evidence for a non-hormone binding receptor precursor. J Biol Chem 261:13236–13246
21. **Wei LL, Krett NL, Francis MD, Gordon DF, Wood WM, O'Malley BW, Horwitz KB** 1988 Multiple human progesterone receptor messenger ribonucleic acids and their autoregulation by progestin agonists and antagonists in breast cancer cells. Mol Endocrinol 2:62–72
22. **Lange CA, Shen T, Horwitz KB** 2000 Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci USA 97:1032–1037
23. **Lipford JR, Deshaies RJ** 2003 Diverse roles for ubiquitin-dependent proteolysis in transcriptional activation. Nat Cell Biol 5:845–850
24. **Masuyama H, MacDonald PN** 1998 Proteasome-mediated degradation of the vitamin D receptor (VDR) and a putative role for SUG1 interaction with the AF-2 domain of VDR. J Cell Biochem 71:429–440
25. **Nawaz Z, Lonard DM, Dennis AP, Smith CL, O'Malley BW** 1999 Proteasome-dependent degradation of the human estrogen receptor. Proc Natl Acad Sci USA 96:1858–1862
26. **Verma S, Ismail A, Gao X, Fu G, Li X, O'Malley BW, Nawaz Z** 2004 The ubiquitin-conjugating enzyme UBCH7 acts as a coactivator for steroid hormone receptors. Mol Cell Biol 24:8716–8726
27. **Gianni M, Parrella E, Raska Jr I, Gaillard E, Nigro EA, Gaudon C, Garattini E, Rochette-Egly C** 2006 P38MAPK-dependent phosphorylation and degradation of SRC-3/AIB1 and RARα-mediated transcription. EMBO J 25:739–751
28. **Han SJ, Tsai SY, Tsai MJ, O'Malley BW** 2007 Distinct temporal and spatial activities of RU486 on progesterone receptor function in reproductive organs of ovariectomized mice. Endocrinology 148:2471–2486
29. **Amazit L, Alj Y, Tyagi RK, Chauchereau A, Loosfelt H, Pichon C, Pantel J, Foulon-Guinchard E, Leclerc P, Milgrom E, Guiochon-Mantel A** 2003 Subcellular localization and mechanisms of nucleocytoplasmic trafficking of steroid receptor coactivator-1. J Biol Chem 278:32195–32203
30. **Chen Y, Chen PL, Chen CF, Sharp ZD, Lee WH** 1999 Thyroid hormone, T_3-dependent phosphorylation and translocation of Trip230 from the Golgi complex to the nucleus. Proc Natl Acad Sci USA 96:4443–4448
31. **Grenier J, Trousson A, Chauchereau A, Cartaud J, Schumacher M, Massaad C** 2006 Differential recruitment of p160 coactivators by glucocorticoid receptor between Schwann cells and astrocytes. Mol Endocrinol 20:254–267
32. **Ferrell K, Wilkinson CR, Dubiel W, Gordon C** 2000 Regulatory subunit interactions of the 26S proteasome, a complex problem. Trends Biochem Sci 25:83–88
33. **Yi P, Feng Q, Amazit L, Lonard DM, Tsai SY, Tsai MJ, O'Malley BW** 2008 Atypical protein kinase C regulates dual pathways for degradation of the oncogenic coactivator SRC-3/AIB1. Mol Cell 29:465–476
34. **Baumann CT, Ma H, Wolford R, Reyes JC, Maruvada P, Lim C,**

Yen PM, Stallcup MR, Hager GL 2001 The glucocorticoid receptor interacting protein 1 (GRIP1) localizes in discrete nuclear foci that associate with ND10 bodies and are enriched in components of the 26S proteasome. Mol Endocrinol 15:485–500
35. **Yan F, Gao X, Lonard DM, Nawaz Z** 2003 Specific ubiquitin-conjugating enzymes promote degradation of specific nuclear receptor coactivators. Mol Endocrinol 17:1315–1331
36. **Chen F, Chang D, Goh M, Klibanov SA, Ljungman M** 2000 Role of p53 in cell cycle regulation and apoptosis following exposure to proteasome inhibitors. Cell Growth Differ 11:239–246
37. **Davarinos NA, Pollenz RS** 1999 Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytoplasmic proteasome following nuclear export. J Biol Chem 274:28708–28715
38. **Tomoda K, Kubota Y, Kato J** 1999 Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1. Nature 398:160–165
39. **Li C, Wu RC, Amazit L, Tsai SY, Tsai MJ, O'Malley BW** 2007 Specific amino acid residues in the basic helix-loop-helix domain of SRC-3 are essential for its nuclear localization and proteasome-dependent turnover. Mol Cell Biol 27:1296–1308
40. **Syvälä H, Vienonen A, Zhuang YH, Kivineva M, Ylikomi T, Tuohimaa P** 1998 Evidence for enhanced ubiquitin-mediated proteolysis of the chicken progesterone receptor by progesterone. Life Sci 63:1505–1512
41. **Li X, Wong J, Tsai SY, Tsai MJ, O'Malley BW** 2003 Progesterone and glucocorticoid receptors recruit distinct coactivator complexes and promote distinct patterns of local chromatin modification. Mol Cell Biol 23:3763–3773
42. **Smid-Koopman E, Blok LJ, Kühne LC, Burger CW, Helmerhorst TJ, Brinkmann AO, Huikeshoven FJ** 2003 Distinct functional differences of human progesterone receptors A and B on gene expression and growth regulation in two endometrial carcinoma cell lines. J Soc Gynecol Investig 10:49–57
43. **Qiu M, Olsen A, Faivre E, Horwitz KB, Lange CA** 2003 Mitogen-activated protein kinase regulates nuclear association of human progesterone receptors. Mol Endocrinol 17:628–642
44. **Dolcet X, Llobet D, Encinas M, Pallares J, Cabero A, Schoenenberger JA, Comella JX, Matias-Guiu X** 2006 Proteasome inhibitors induce death but activate NF-κB on endometrial carcinoma cell lines and primary culture explants. J Biol Chem 281:22118–22130
45. **Dennis AP, Lonard DM, Nawaz Z, O'Malley BW** 2005 Inhibition of the 26S proteasome blocks progesterone receptor-dependent transcription through failed recruitment of RNA polymerase II. J Steroid Biochem Mol Biol 94:337–346
46. **Georgiakaki M, Chabbert-Buffet N, Dasen B, Meduri G, Wenk S, Rajhi L, Amazit L, Chauchereau A, Burger CW, Blok LJ, Milgrom E, Lombès M, Guiochon-Mantel A, Loosfelt H** 2006 Ligand-controlled interaction of histone acetyltransferase binding to ORC-1 (HBO1) with the N-terminal transactivating domain of progesterone receptor induces steroid receptor coactivator 1-dependent coactivation of transcription. Mol Endocrinol 20:2122–2140
47. **Liu Z, Wong J, Tsai SY, Tsai MJ, O'Malley BW** 1999 Steroid receptor coactivator-1 (SRC-1) enhances ligand-dependent and receptor-dependent cell-free transcription of chromatin. Proc Natl Acad Sci USA 96:9485–9490
48. **Smith CL, Oñate SA, Tsai MJ, O'Malley BW** 1996 CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. Proc Natl Acad Sci USA 93:8884–8888
49. **Stenoien DL, Patel K, Mancini MG, Dutertre M, Smith CL, O'Malley BW, Mancini MA** 2001 FRAP reveals that mobility of oestrogen receptor-α is ligand- and proteasome-dependent. Nat Cell Biol 3:15–23
50. **Chen SL, Wang SC, Hosking B, Muscat GE** 2001 Subcellular localization of the steroid receptor coactivators (SRCs) and MEF2 in muscle and rhabdomyosarcoma cells. Mol Endocrinol 15:783–796
51. **Zhang Y, Zhang H, Liang J, Yu W, Shang Y** 2007 SIP, a novel ankyrin repeat containing protein, sequesters steroid receptor coactivators in the cytoplasm. EMBO J 26:2645–2657
52. **Hoang T, Fenne IS, Cook C, Borud B, Bakke M, Lien EA, Mellgren G** 2004 cAMP-dependent protein kinase regulates ubiquitin-proteasome-mediated degradation and subcellular localization of the nuclear receptor coactivator GRIP1. J Biol Chem 279:49120–49130
53. **Zhang H, Sun L, Liang J, Yu W, Zhang Y, Wang Y, Chen Y, Li R, Sun X, Shang Y** 2006 The catalytic subunit of the proteasome is engaged in the entire process of estrogen receptor-regulated transcription. EMBO J 25:4223–4233
54. **Mani A, Oh AS, Bowden ET, Lahusen T, Lorick KL, Weissman AM, Schlegel R, Wellstein A, Riegel AT** 2006 E6AP mediates regulated proteasomal degradation of the nuclear receptor coactivator amplified in breast cancer 1 in immortalized cells. Cancer Res 66:8680–8686
55. **Xu J, Wu RC, O'Malley BW** 2009 Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer 9:615–630
56. **Fleming FJ, Myers E, Kelly G, Crotty TB, McDermott EW, O'Higgins NJ, Hill AD, Young LS** 2004 Expression of SRC-1, AIB1, and PEA3 in HER2 mediated endocrine resistant breast cancer: a predictive role for SRC-1. J Clin Pathol 57:1069–1074
57. **Wang S, Yuan Y, Liao L, Kuang SQ, Tien JC, O'Malley BW, Xu J** 2009 Disruption of the SRC-1 gene in mice suppresses breast cancer metastasis without affecting primary tumor formation. Proc Natl Acad Sci USA 106:151–156
58. **Qin L, Liu Z, Chen H, Xu J** 2009 The steroid receptor coactivator-1 regulates twist expression and promotes breast cancer metastasis. Cancer Res 69:3819–3827
59. **Han SJ, Jeong J, Demayo FJ, Xu J, Tsai SY, Tsai MJ, O'Malley BW** 2005 Dynamic cell type specificity of SRC-1 coactivator in modulating uterine progesterone receptor function in mice. Mol Cell Biol 25:8150–8165
60. **Hudelist G, Czerwenka K, Kubista E, Marton E, Pischinger K, Singer CF** 2003 Expression of sex steroid receptors and their cofactors in normal and malignant breast tissue: AIB1 is a carcinoma-specific co-activator. Breast Cancer Res Treat 78:193–204
61. **Muratani M, Tansey WP** 2003 How the ubiquitin-proteasome system controls transcription. Nat Rev Mol Cell Biol 4:192–201
62. **Collins GA, Tansey WP** 2006 The proteasome: a utility tool for transcription? Curr Opin Genet Dev 16:197–202
63. **Perissi V, Aggarwal A, Glass CK, Rose DW, Rosenfeld MG** 2004 A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. Cell 116:511–526
64. **Nawaz Z, Lonard DM, Smith CL, Lev-Lehman E, Tsai SY, Tsai MJ, O'Malley BW** 1999 The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. Mol Cell Biol 19:1182–1189
65. **Molinari E, Gilman M, Natesan S** 1999 Proteasome-mediated degradation of transcriptional activators correlates with activation domain potency *in vivo*. EMBO J 18:6439–6447
66. **Shao W, Keeton EK, McDonnell DP, Brown M** 2004 Coactivator AIB1 links estrogen receptor transcriptional activity and stability. Proc Natl Acad Sci USA 101:11599–11604
67. **Salghetti SE, Caudy AA, Chenoweth JG, Tansey WP** 2001 Regulation of transcriptional activation domain function by ubiquitin. Science 293:1651–1653
68. **Chauchereau A, Amazit L, Quesne M, Guiochon-Mantel A, Milgrom E** 2003 Sumoylation of the progesterone receptor and of the steroid receptor coactivator SRC-1. J Biol Chem 278:12335–12343
69. **Ramamoorthy S, Dhananjayan SC, Demayo FJ, Nawaz Z** 2010 Isoform-specific degradation of PR-B by E6-AP is critical for normal mammary gland development. Mol Endocrinol 24:2099–2113
70. **Calligé M, Kieffer I, Richard-Foy H** 2005 CSN5/Jab1 is involved in

ligand-dependent degradation of estrogen receptor α by the proteasome. Mol Cell Biol 25:4349–4358
71. **Chauchereau A, Georgiakaki M, Perrin-Wolff M, Milgrom E, Loosfelt H** 2000 JAB1 interacts with both the progesterone receptor and SRC-1. J Biol Chem 275:8540–8548
72. **Kwak J, Workman JL, Lee D** 17 August 2010 The proteasome and its regulatory roles in gene expression. Biochim Biophys Acta 10.1016/j.bbagrm.2010.08.001
73. **Grenier J, Trousson A, Chauchereau A, Amazit L, Lamirand A, Leclerc P, Guiochon-Mantel A, Schumacher M, Massaad C** 2004 Selective recruitment of p160 coactivators on glucocorticoid-regulated promoters in Schwann cells. Mol Endocrinol 18:2866–2879
74. **Smid-Koopman E, Kuhne LC, Hanekamp EE, Gielen SC, De Ruiter PE, Grootegoed JA, Helmerhorst TJ, Burger CW, Brinkmann AO, Huikeshoven FJ, Blok LJ** 2005 Progesterone-induced inhibition of growth and differential regulation of gene expression in PRA- and/or PRB-expressing endometrial cancer cell lines. J Soc Gynecol Investig 12:285–292
75. **Lorenzo F, Jolivet A, Loosfelt H, Thu vu Hai M, Brailly S, Perrot-Applanat M, Milgrom E** 1988 A rapid method of epitope mapping. Application to the study of immunogenic domains and to the characterization of various forms of rabbit progesterone receptor. Eur J Biochem 176:53–60
76. **Potokar M, Kreft M, Chowdhury HH, Vardjan N, Zorec R** 2006 Subcellular localization of Apaf-1 in apoptotic rat pituitary cells. Am J Physiol Cell Physiol 290:C672–C677
77. **Viengchareun S, Kamenicky P, Teixeira M, Butlen D, Meduri G, Blanchard-Gutton N, Kurschat C, Lanel A, Martinerie L, Sztal-Mazer S, Blot-Chabaud M, Ferrary E, Cherradi N, Lombès M** 2009 Osmotic stress regulates mineralocorticoid receptor expression in a novel aldosterone-sensitive cortical collecting duct cell line. Mol Endocrinol 23:1948–1962

SUPPLEMENTAL DATA

Fig S1 : Control analysis of reciprocal bleed-through fluorescence from the red and green channels.

COS-7 cells were transiently transfected with the expression vector encoding HA-SRC-1. Cells were fixed after 40 h and immunolabeled overnight at 4°C with either the primary rat anti-HA (upper panel) or the rabbit anti-S7/Rpt1antibodies (lower panel). First antibody was followed by a 30 min incubation with a mix of the two corresponding secondary antibodies Dylight 549 anti-rat (red) and alexa 488 anti-rabbit (green). Cells were analyzed by confocal microscopy as described in Methods section. Note the lack of overlap of both fluorescences. DIC: Differential Interferential Contrast.

Fig S2 : Colocalization analyses of SRC-1 with antigens and organelles.

A and B, COS-7 cells were transiently transfected with vectors encoding HA-SRC-1. Cells were fixed after 40 h and immunolabeled with the anti-PML (A) or anti-SC-35/SRp30 spliceosome component (B) antibodies. Colocalization analysis was performed by confocal microscopy. Note the partial colocalization of SRC-1 with PML nuclear bodies and the absence of colocalisation with SC-35.

C and D, COS-7 cells were transiently transfected with vector encoding GFP-SRC-1. After 40 h, cells were incubated with a mitochondrial (C) or lysosomial tracker (D) (Invitrogen), followed by fixation and observation by confocal microscopy. No significant colocalization was observed.

E, COS-7 cells were cotransfected with the vector encoding HA-SRC-1 and the pEGFP-Peroxy vector (Clontech). Immunodetection was performed with the anti-HA antibody and cells were analyzed by confocal microscopy. No significant colocalization was observed.

Fig S3: **Epoxomicin increases SRC-1 expression level.**

COS-7 cells were transfected with the vector encoding SRC-1 and incubated during 15 h in the presence of epoxomicin (500 nM) or vehicle (DMSO, -). Expression of SRC-1 was analyzed by Western blot using anti-HA monoclonal antibody. The bands were quantified as described in "Materials and Methods".

Fig S4: The Nuclear Export Signal deletion of SRC-1 decreases its turnover rate.

COS-7 cells were transfected with the wt SRC-1 or the Δ(NES) encoding vector (deleted of the Nuclear Export Signal). Seventy-two hours after transfection, cells were treated with cycloheximide (100 µg/ml) during 1, 4 or 6 h. Whole cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. Band intensities (right panel) were quantified as described in "Material and Methods". Note the decrease in kinetics degradation rate of the nuclear mutant Δ(NES) compared to wt SRC-1.

Fig S5 : PRB is degraded in the presence of R5020 but not RU486.

Ishi PR-B cells were treated during 24 h with vehicule (control, -), the agonist R5020 (10 nM) or the antagonist RU486 (10 nM) and western blot analysis was performed with the corresponding whole cell extracts as described in "Materials and Methods". PR-B was detected using monoclonal antibody Let126 and loading control protein α-tubulin was detected using anti-α-tubulin antibody.

Fig S6 : R5020 or RU486 does not modify endogenous SRC-1 mRNA levels.

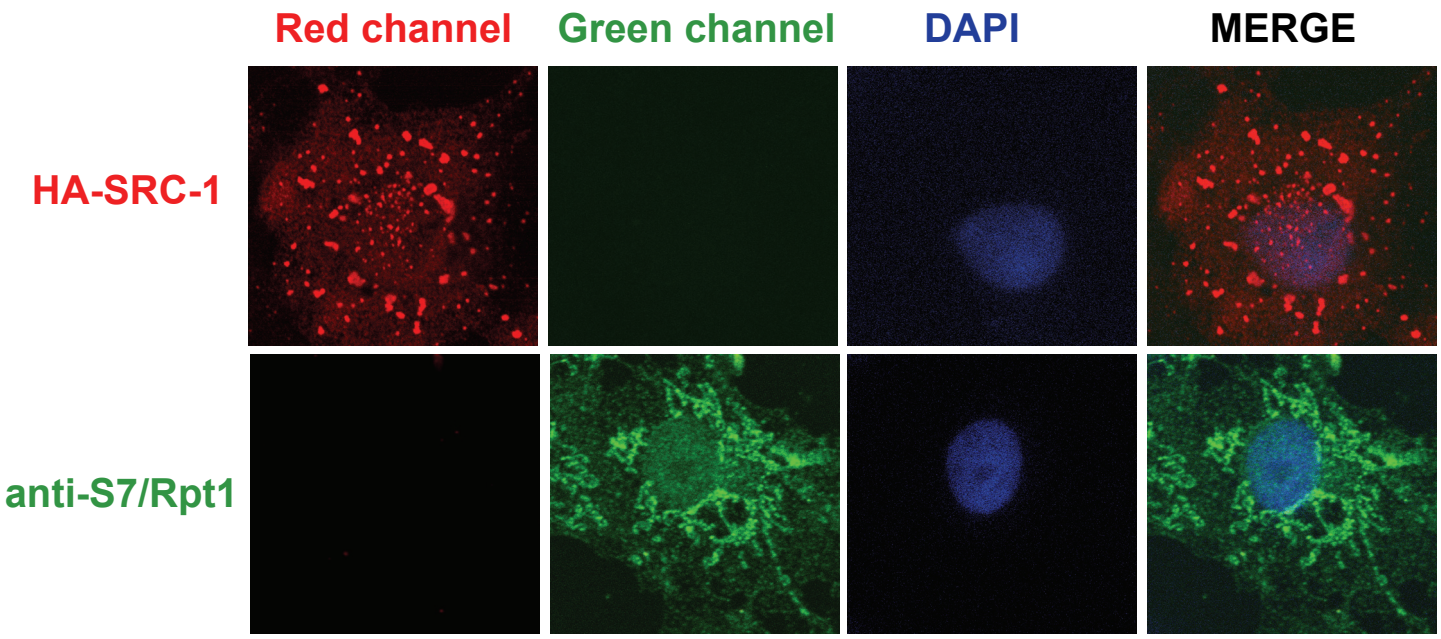
Ishi PR-B cells were treated during 24 h with vehicle (EtOH, -), R5020 (10 nM) or RU486 (10 nM). Total RNA was extracted and relative expression of SRC-1 mRNA levels were quantified by quantitative RT-PCR as described in "Materials and Methods". Results were normalized by the 18S RNA and are expressed as mean ± SEM of six independent determinations.

Fig S7 : Coactivator specificity in the ligand-induced down-regulation.

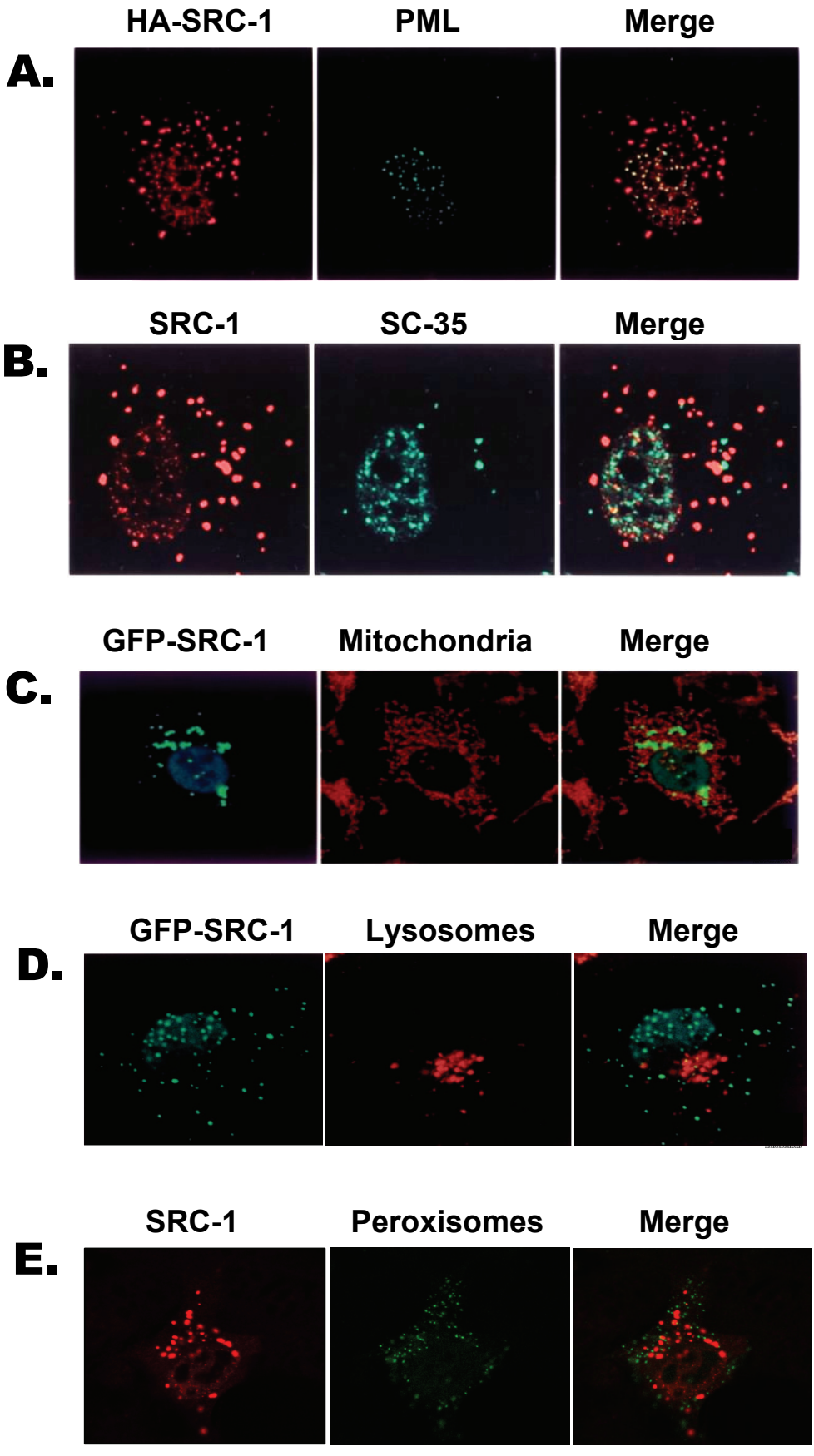
Ishi PR-B cells were transfected as indicated, with either the SRC-1, SRC-2 or SRC-3 encoding vector. Forty-eight h after transfection, cells were cultured for 24 h either in the absence of ligand (control vehicule, -H) or in the presence of R5020 (10 nM). Whole cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies.

<u>Fig S8</u> : Epoxomicin and MG132 do not increase expression levels of the two non degradable Δ(PEST) and Δ(bHLH) mutants.

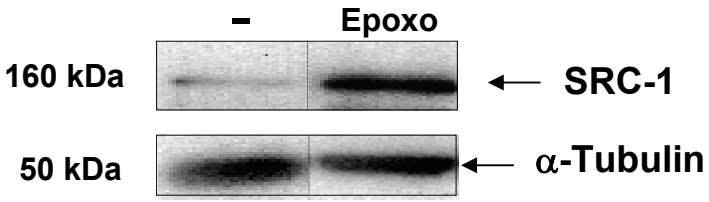
COS-7 cells were transfected with Δ(PEST) and Δ(bHLH) encoding vectors and incubated in the absence (vehicule, -) or presence of either epoxomixin (500 nM) or MG132 (5 μM) for 15 h. Expression level of SRC-1 mutants was analyzed by Western blot using the indicated antibodies. Note the absence of accumulation of both mutants in the presence of both proteasome inhibitors.

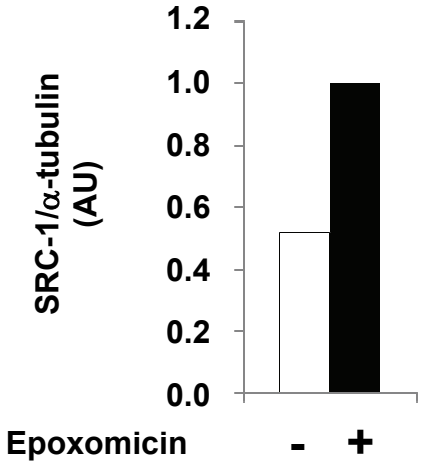


Supplemental Fig S1

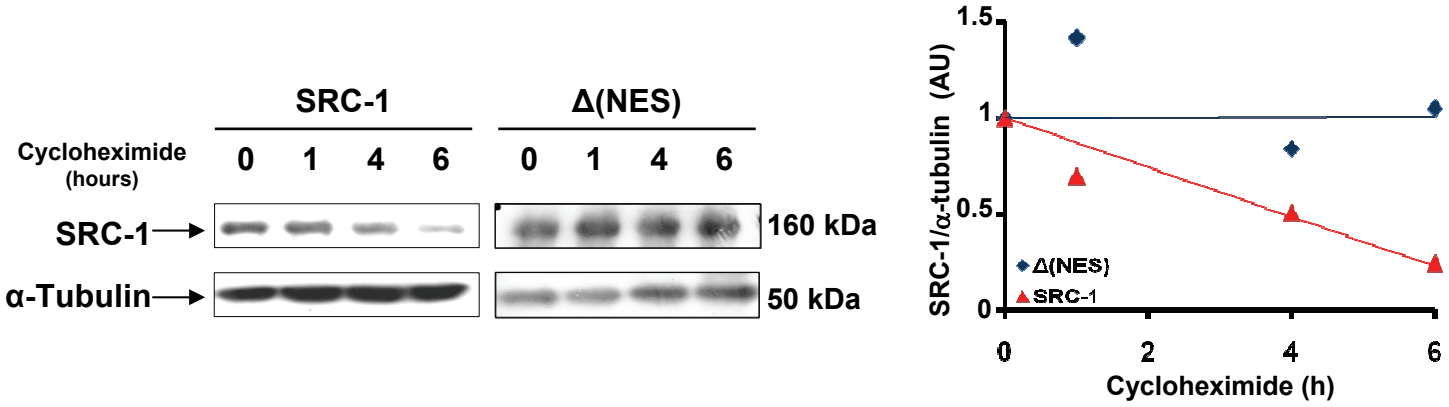


Supplemental Fig S2

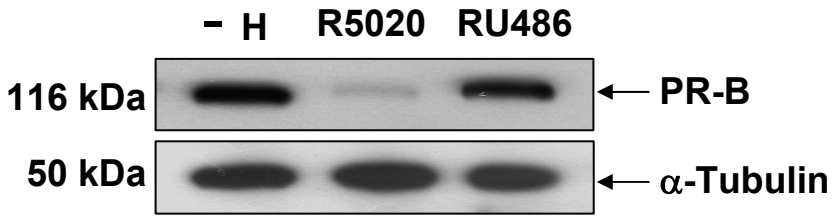




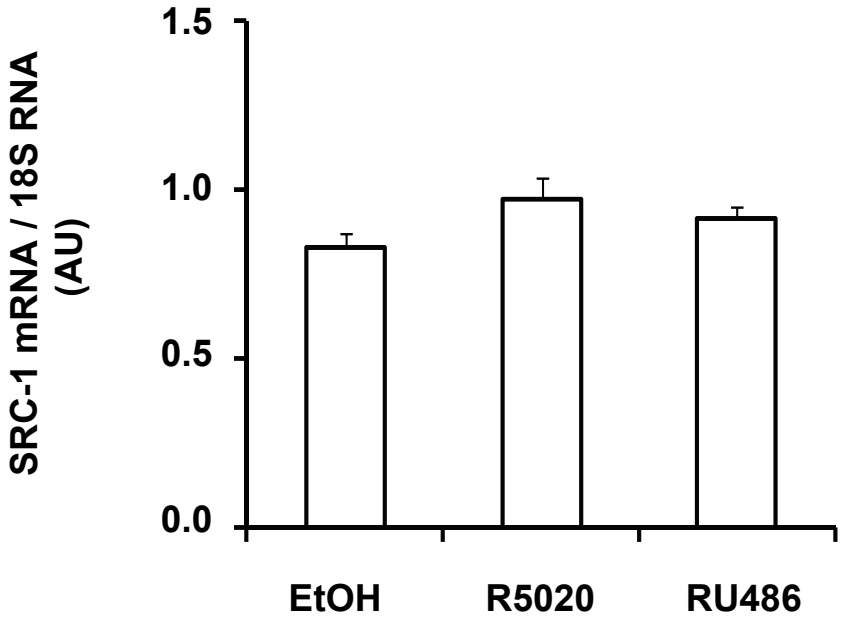
Supplemental Fig S3



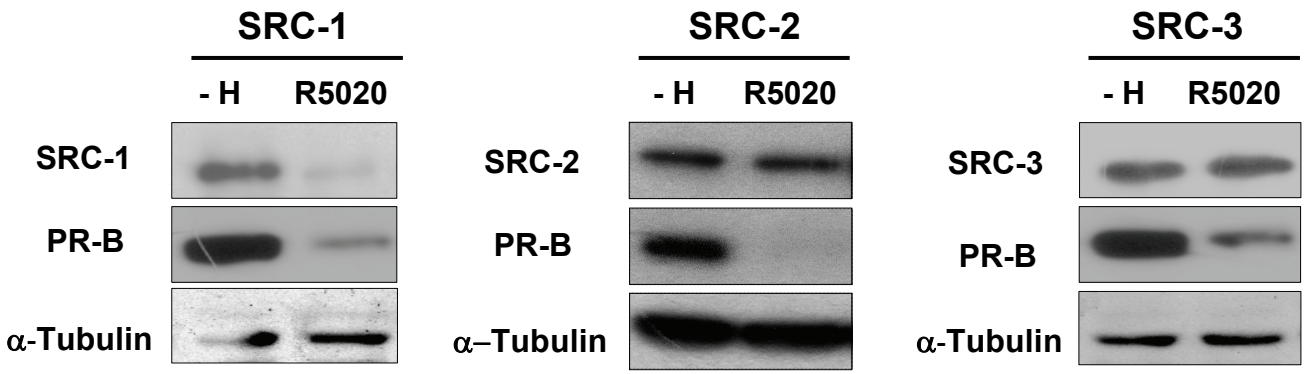
Supplemental Fig S4



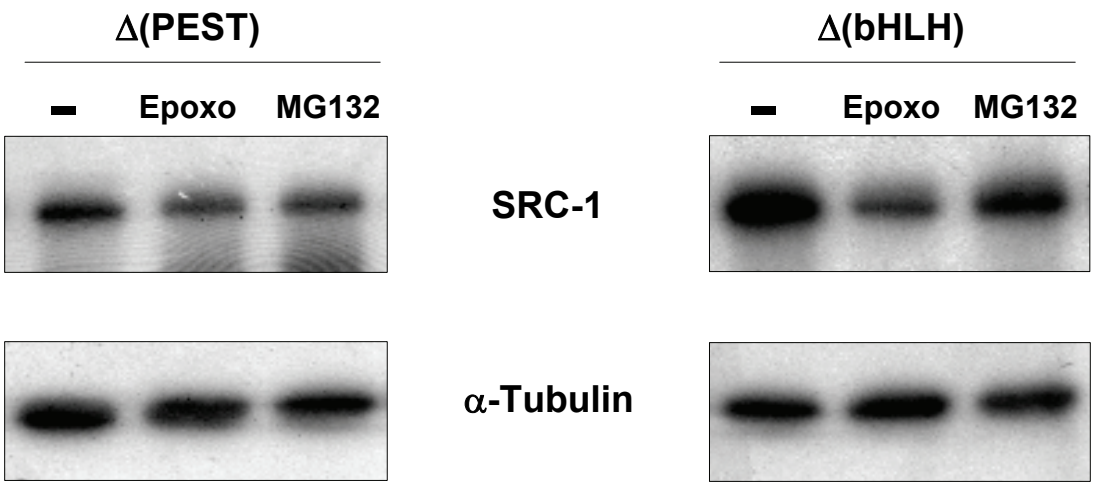
Supplemental Fig S5



Supplemental Fig S6



Supplemental Fig S7



Supplemental Fig S8

Part 2: Functional diversity of PR isoforms

A. New cellular model to study the role of PRA/PRB ratio in pathophysiology (*PAPER III*)

Background

Despite the high sequence similarity, accumulating evidence indicates that PRB and PRA are functionally distinct transcriptional factors (Giangrande and McDonnell, 1999) and exhibit differential physiological responses in target tissues (Mani et al., 2006; Mulac-Jericevic et al., 2003; Mulac-Jericevic et al., 2000). Previous studies (Graham et al., 2005; Jacobsen et al., 2005; Richer et al., 2002) on transcriptional regulation by PR isoforms were conducted in separate T47D cell lines (ER+) expressing either PRA or PRB or both. Genetic background related effects as well as uncontrolled overexpression coming from separate cell lines expressing PRA or PRB makes the comparison difficult for distinct PR isoforms transcriptional properties and particularly the role of variation in PRA/PRB ratio in target gene selection and transcriptional regulation could not be studied.

Results

To better understand the genomic effects of varied PRA/PRB expression, we established a bi-inducible PR-isoform breast cancer cellular model from MDA-MB-231 cells (ER-, PR-) using RheoSwitch and TRex systems. PRA expression was placed under the control of RheoSwitch system and is regulated by RheoSwitch ligand (RSL1) whereas PRB expression is controlled by TRex system using inducer ligand Dox. In these cells, PR isoform expression can be tightly controlled by the respective inducer with undetectable PR expression in uninduced cells. Inducibly expressed PR isoforms are functional by regulating transcriptional from synthetic as well as endogenous promoters. Importantly, the ratio of PRA/PRB expression could be tightly controlled by the dose of inducer ligand(s). Removal of inducer after 24h led to gradual decrease in PR isoform expression indicating that PRA and/or PRB expression could be easily switch-on or -off when desired. Like wild type PRs, inducible PRA and PRB underwent progestin-induced turnover phosphorylation at S294-PRB and S130-PRA residues by both agonist (progesterone) and antagonist (RU486) ligands. The advantage of such cell-based models is that they serve as their own internal control to investigate the differential effect of PRA and PRB under both liganded as well as unliganded conditions. Such models allow comparing the effects of PR isoforms in the same cells at physiological levels thus circumventing any artifactual observation linked to random variations in genomic recombinations as well as uncontrolled over-expression. The newly established bi-inducible cell line allowed us to demonstrate that PRA/PRB ratio is a critical determinant of target genes selectivity and response to hormonal/growth factor stimuli. Using this model we were able to show that functional outcome of epidermal growth factor (EGF) and progesterone

signaling on responsive gene transcription is dependent on PR isoforms expression. Also we were able to demonstrate for the first time that EGF and progesterone exert synergetic or antagonistic effect on PR transactivation depending not only on target genes but more importantly on PRA/PRB expression ratio. Interestingly, unliganded PRA increases amphiregulin (AREG) expression independently of estrogen receptor that can be inhibited by antiprogestins.

Perspectives

Further studies are needed to in vestigate the impact of varied PRA/PRB ratio on biological processes such as cell migration, adhesion and invasion. Remain to be elaborated is the PRA-dependent increase in AREG expression at protein level by using western blot and/or ELISA for detection of secreted AREG in conditioned medium. It might be very interesting to clearly establish the role of AREG, if any, in promoting proliferation of adjacent PR (-) cells. For this, PRA expressing cells should be cultured with PR (-) cells in the absence of hormone and the proliferation should be determined after few days of co-culture compared with conditions where all cells express PRA or not. If the proliferation is higher in co-culture condition, the same experiment needs to be repeated in the presence of small interfering RNA against AREG or with AREG antibody to neutralize the proliferating effect of AREG secreted by PRA expressing cells. Furthermore, histological screening should be conducted to see whether PRA-rich breast tumors express elevated AREG protein as compared to PRB rich tumors or tumors which do not express PR at all. The mechanism of AREG expression by unliganded PRA remains to be further explored. For example, chromatin immunoprecipitation experiments should be performed to explore the promoter region(s) involved in PRA-dependent AREG transcription.

PAPER III

Cell proliferation genes differentially regulated by progesterone receptor isoforms PRA and PRB in a new bi-inducible breast cancer cell line

Khan JA, Bellance C, Guiochon-Mantel A, Lombès M and Loosfelt H

Submitted manuscript

Cell proliferation genes differentially regulated by progesterone receptor isoforms PRA and PRB in a new bi-inducible breast cancer cell line

Authors: Junaid A Khan[1,2], Catherine Bellance[1,2], Anne Guiochon-Mantel[1,2,3], Marc Lombès[1,2,4], and Hugues Loosfelt[1,2*]

Affiliations:

[1]Inserm Unité 693, 63 rue Gabriel Péri, Le Kremlin-Bicêtre, F-94276, France;

[2]Univ Paris-Sud, Faculté de Médecine Paris-Sud, UMR-S693, Le Kremlin-Bicêtre, F-94276, France;

[3]Assistance Publique-Hôpitaux de Paris, Hôpital Bicêtre, Service de Génétique moléculaire, Pharmacogénétique et Hormonologie, Le Kremlin-Bicêtre, F-94275, France ;

[4]Assistance Publique-Hôpitaux de Paris, Hôpital Bicêtre, Service d'Endocrinologie et Maladies de la Reproduction, Le Kremlin-Bicêtre, F-94275, France.

Email adresses :

Junaid A Khan (junaidali.khan@ymail.com)

Catherine Bellance (catherine.bellance@u-psud.fr)

Anne Guiochon-Mantel (anne.mantel@bct.aphp.fr)

Marc Lombès (marc.lombes@u-psud.fr)

Hugues Loosfelt (hugues.loosfelt@u-psud.fr)

***Corresponding Author:** Dr Hugues Loosfelt, PharmD, PhD, Inserm U693, Faculté de Médecine Paris Sud, 63 rue Gabriel Péri, Le Kremlin-Bicêtre, F-94276, France.

Tel: 33 1 49 59 67 10; Fax: 33 1 49 59 67 32; E-mail: hugues.loosfelt@u-psud.fr

Abstract

Introduction

Progesterone receptor isoforms (PRA and PRB) are expressed at equimolar level in normal mammary cells. However, alteration in PRA/PRB expression is often observed under pathological conditions. Previous *in vitro* and *in vivo* studies have reported aggressive breast cancer phenotype of PRA-rich tumors suggesting differential contribution of PR isoforms in tumor pathogenesis. The mechanisms underlying such processes remain to be established mainly due to lack of appropriate cellular models.

Methods

To investigate the role of individual PR isoform and particularly the impact of varied PRA/PRB ratio in transcriptional regulation and selection of target genes, we have generated an original human breast cancer cell model harboring a bi-inducible promoter system allowing selective, reversible and dose-dependent expression of PRA and/or PRB. Using this model, we performed genome-wide transcriptomic studies to identify gene subsets differentially regulated by ligand-free and liganded PR isoforms that might be potential candidates involved in PR-dependent breast carcinogenesis.

Results

We demonstrate that PRA/PRB ratio is a critical determinant of target gene selectivity and response to hormonal/growth factor stimuli. We have identified a large number of novel PR-regulated candidates implicated in carcinogenesis and found that gene subsets associated with the cellular processes involved in breast cancer development and metastasis are differentially regulated by ligand-free and hormone-treated PR isoform(s). The transcriptional regulation of amphiregulin, a paracrine growth factor, is highly influenced by hormone, growth factors stimuli

and selective PR isoform expression. Functional outcome of epidermal growth factor (EGF) and progesterone signaling on responsive gene transcription is dependent not only on target genes but more importantly on PRA/PRB ratio. Unliganded PRA increases amphiregulin expression independently of estrogen receptor and can be inhibited by antiprogestins.

Conclusion

We have established an original bi-inducible breast cancer cell line that allowed us to identify key PR-regulated candidate genes implicated in cell growth and tumor progression. We demonstrate that elevated PRA/PRB ratio increases amphiregulin expression that may constitute continuous paracrine growth stimulus thereby accelerating tumor development and facilitating metastasis. Our results provide further mechanistic support for the aggressive phenotype of breast cancers with high PRA/PRB ratio.

Keywords: Progesterone receptor isoforms, PRA/PRB ratio, transcription, progesterone, amphiregulin, proliferation, antiprogestins

Introduction

Progesterone receptor (PR) is a ligand-induced transcription factor belonging to the nuclear receptor family of steroid hormone receptors [1]. PR exists as two isoforms, PRB and PRA, transcribed by alternate initiation of transcription from two distinct estrogen-regulated promoters [2]. PRB is a full length 114 kDa protein comprising of 933 amino acids while PRA is truncated in the N-terminal region (94 kDa) and lacks the first 164 amino acids. Despite the high sequence similarity, accumulating evidence indicates that PRB and PRA are functionally distinct transcriptional factors [3] and exhibit differential physiological responses in target tissues [4-6].

Biochemical and biophysical studies suggest that unique conformation of PRA and PRB [7] allows interaction with distinct coregulators [8]. Under physiological conditions, the majority of PR positive mammary epithelial cells express both PR isoforms at equimolar levels [9, 10]. However, altered PRA/PRB ratio is often associated with breast carcinogenesis [9, 11, 12]. Metastasis associated protein 1 overexpression in transgenic mice led to tumorigenesis concomitant to elevated PRA/PRB ratio [13]. Genetic predisposition to cancer development due to mutations in *BRCA1* or *BRCA2* leads to PRA overexpression which may play a role in disease progression [14, 15]. Likewise, in endometrial cancers, disrupted PRA/PRB expression is observed and cancers with elevated PRA/PRB ratio are also correlated with poor prognosis [16]. Moreover, transgenic mice overexpressing PRA exhibit abnormal mammary gland development characterized by extensive lateral branching, ductal hyperplasia and disorganized basement membrane with decreased cell to cell adhesion [17]. *In vivo* relationship between PRA/PRB ratio and antiprogestin responsiveness revealed that decreased PRA/PRB ratio is associated with antiprogestin resistance [18]. Furthermore, restoration of balanced PRA/PRB ratio using methyltransferase inhibitors re-established antiprogestin sensitivity in mouse mammary carcinomas [19].

Recent studies show that PR may by-pass progesterone (P4) requirement for transcriptional activation of certain gene subsets [20, 21] and sexual behavior induced by dopamine in mice requires unoccupied PR [22]. Therefore, it seems plausible to define PR as a sensitive transcription factor capable of responding not only to its cognate ligand but also to diverse cellular stimuli/growth factors independently of hormonal status. Given that several aspects of P4 signaling are differentially influenced by PR isoforms, PRA/PRB ratio should be considered as an important determinant of functional consequences of P4 signaling. Most of

previous studies [23-25] on transcriptional regulation by PR isoforms were conducted in T47D cells (ER+) expressing either PRA or PRB where PR homodimers are the only molecular species or in separate cell lines expressing both PR isoforms. Although these studies provided insights in transcriptional regulation by PRs, however, genetic background related effects coming from separate cell lines expressing PRA or PRB makes the comparison difficult for distinct PR isoforms transcriptional properties. Given that three molecular species (PRA, PRB homodimers, PRA-PRB heterodimers), with likely different functions, might exist in PR positive cells, changes in PRA/PRB ratio might strongly influence signaling of ligand-free as well as liganded PRs [26].

To better understand the genomic effects of varied PRA/PRB expression, we have generated a bi-inducible breast cancer cellular model allowing controllable PRA/PRB ratio. Using this original model, we performed genome wide transcriptomic studies and identified that distinct gene subsets are regulated by ligand-free and hormone-bound PR isoforms. We show that target gene selection is highly influenced not only by the presence of hormone but also by selective PR isoform expression. Moreover, functional outcome of epidermal growth factor (EGF) and P4 signaling is dependent on target gene promoter context as well as on PRA/PRB ratio. Furthermore, PR isoforms distinctly impact the transcriptional regulation of heparin-binding growth factor (*HB-EGF*) and amphiregulin (*AREG*), paracrine growth factors implicated in carcinogenesis. Finally, our results demonstrate that unliganded PRA continuously increases *AREG* transcription independently of estrogen receptor that can be inhibited by antiprogestin RU486. Increased *AREG* expression by elevated PRA/PRB ratio may provide paracrine growth stimulus for the proliferation of nearby PR negative cells. This newly established cellular model

thus constitutes a power tool to discriminate the differential impact of PR isoforms in cancer progression.

Materials and methods

Plasmid construction

The plasmid pZX-Luc and pZX-LR [27], containing Gal4DBD-EcRLBD (Rheoreceptor) and VP16-RXRLBD (Rheoactivator) of the RheoSwitch system was kindly provided by Dr. Claude Labrie. The [NotI]-XbaI fragment of pZX-luc containing additional Gal4uas-luciferase and GFP-coding sequences was replaced by the [MluI]-XbaI of pcDNA6-TR (TRex system, Invitrogen) containing the Tet-repressor gene (TetR) to generate pZX-TR (Zeocin resistance) constitutively expressing Rheoreceptor, Rheoactivator and TetR. Human PRA and PRB cDNAs were obtained from pSG5-hPRA and pSG5-hPRB as described previously [28]. The fragment MluI-XbaI of pcDNA3 containing the constitutive CMV promoter was replaced by the fragment AscI-XbaI of pZX-LR containing Gal4uas(x6)-CMV minimal promoter, and then the fragment EcoRI-[BglII] of pSG5-hPRA was inserted in the EcoRI-[XbaI] polylinker to generate pGalp-hPRA vector (neomycin resistance) responding to RheoSwitch system. The fragment MunI-XbaI of pcDNA6-TR containing the TetR coding gene was replaced by the fragment MunI-XbaI of pcDNA4-TO (TRex system) containing 2xtetR binding elements downstream of the constitutive CMV promoter, and then the fragment BspEI-BglII of pSG5-hPRB was inserted in BspEI-BamHI polylinker generating pTO-hPRB (blasticidin resistance) responding to TRex system. The pGal4p-luc reporter gene vector was constructed by replacing the fragment MluI-XbaI of pGL3 (Promega) by the fragment AscI-XbaI of pZX-LR expressing luciferase under the control of 6xGaluas elements and CMV minimal promoter. The pTO-luc reporter gene vector was

constructed by inserting the fragment BglII-XbaI of pGL3 containing the luciferase coding sequence into the BamHI-XbaI polylinker of pcDNA4-TO.

Cell culture and reagents

Human breast cancer cells MDA-MB-231 (ER-, PR-) were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) as described previously [29]. Stable cell lines were grown in the presence of with zeocin (100 µg/ml, Invitrogen) for selection of pZX-TR, neomycin (200 µg/ml, Sigma) for pcDNA3-Galp-hPRA (iPRA), blasticidin (2 µg/ml, Euromedex) for pcDNA6-TO-hPRB (iPRB) and neomycin (200 µg/ml) along with blasticidin (2 µg/ml) for pcDNA3-Galp-hPRA and pcDNA6-TO-hPRB (iPRAB). The non-steroidal RheoSwitch Ligand 1 (RSL1) (New England BioLabs) and/or doxycycline (Dox) (Sigma) were added for induction of PRA and/or PRB. Progesterone, R5020, RU486 and EGF were purchased from Sigma.

Transfection and selection of stable cell lines

Approximately, $2x10^6$ MDA-MB-231 cells were transfected with 2 µg of pZX-TR plasmid using LipofectAMINE 2000 (Invitrogen). For functional analyzing of inducible systems, zeocin resistant clones were transfected with either pGal4p-luc or pTO-luc vectors and incubated with or without RSL1 (0.5 µM) or Dox (2 µM) during 24 h. Clones having the best induced luciferase to background ratio were characterized. Clone 250 was selected to generate secondary stable cells following transfection with pGal4p-PRA/neomycin (iPRA) or pTO-PRB/blasticidin (iPRB) or both of them (iPRAB). After 48 h, cells were plated in medium containing respective selection antibiotic(s) and surviving clones were screened by western blot for inducible PR isoforms(s) expression following 24 h incubation without or with RSL1 and/or Dox.

Luciferase activity assays

PRA and/or PRB expression was induced using RSL1 (0.5 µM) and/or Dox (2 µM) during 24 h in steroid free medium. Cells were transfected with indicated reporter gene (100 ng) and β-galactosidase (10 ng) plasmids. After 24 h, cells were incubated with indicated ligands and cells were collected with the Passive Lysis Buffer (Promega). Luciferase activity was measured with luminometer (Victor, Perkin Elmer) and normalized with either β-galactosidase activity or total protein concentration. The data are presented as means ± SE of six independent cell cultures (n = 6).

Immunoblotting

Western blot analysis was performed as previously described [29]. Antibodies used were, anti-PRA and anti-PRB (NCL-L-PGR-312/2, Novocastra Laboratories), anti-α-tubulin antibody (Sigma), horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA). Target proteins were detected using ECL Plus reagent (GE Healthcare) and visualized by chemiluminescence.

PR quantification by ligand binding assays

The iPRAB cells were incubated with RSL1 (0.25 µM) or Dox (1 µM) during 24 h in steroid free medium, cytosols were prepared and labeled with 10 nM ^{3}H R5020 (Promegestone) in the absence or presence of unlabeled R5020 (1 µM). Bound and unbound steroids were separated by the dextran-charcoal technique and radioactivity was counted.

Immunocytochemical assays

Cells were fixed with 4 % paraformaldehyde and permeabilized 30 min with PBS containing 0.5 % Triton X100. Cells were incubated with primary anti-PR antibody (Novocastra) overnight at 4°C and for 30 min with an Alexa 488-coupled anti-mouse IgG secondary antibody. After

analysing the cells with fluorescent microscopy, pictures acquisition was performed at 20x magnitude for 160 ms with imaging Qcapture Pro version 5.1 (Q Imaging Inc.).

Quantitative real-time RT-PCR

Total RNAs extraction and real-time qRT-PCR procedures have been previously described [28]. Primers sequences are presented in Additional file 1. All samples were analyzed in duplicate from at least three independent cell cultures. The relative expression level of each gene transcript was measured as compared to the corresponding cloned amplicon and normalized with 18S RNA level.

Proliferation assays

Approximately 5000 iPRAB-38 cells per well were plated in 96-well plates for 24 h. On Day 0, medium was replaced with 5% DCC medium with inducer RSL1 (0.5 µM) and/or Dox (2 µM) for PRA and/or PRB expression along with indicated steroid(s). Same treatment was repeated on Day 2 and 4. Cell proliferation was determined on Day 1, 3 and 5, by adding 20 µl of Cell Titer96 AqueousOne solution (Promega) into each well containing 100 µl of culture medium. The plates were incubated at 37°C for 1 h and absorbance value at A490 nm was measured using photometer (Victor 378, Perkin Elmer).

RNA isolation and gene expression profiling

RNA extraction was done using Trizol protocol and purified with Qiagen column (Rneasy micro). The quantity and purity of the extracted RNA was evaluated using a NanoDrop spectrophotometer and its integrity measured using an Agilent Bioanalyzer. For microarray hybridizations, 500 ng of total RNA from each RNA sample was amplified and labeled with two fluorescent dyes (Cy5 and Cy3) using the Low RNA Input Linear Amplification Labeling kit (Agilent Technologies, Palo Alto, CA, USA) following the manufacturer's protocol. Cy3- and

Cy5-labeled cRNA were hybridized in swapped experiments to the Agilent Human Whole Genome Oligo Microarray format 4x44 K(GPL4133-5646, Agilent Technologies), prior to washing and scanning. The data were extracted from scanned images using Feature Extraction software (v 10.5.1.1 Agilent) with default settings. Data from all hybridizations were analyzed with Rosetta Resolver software and statistical analysis for quality test was performed to control homogeneity of hybridization signals. Dye-swapped arrays were combined using an error-weighted average. A list of differentially expressed transcripts was extracted for each hybridization with the following criteria, an absolute fold change (FC) > 1.5, p-value calculated by the software Rosetta Resolver < $1x10^{-5}$, a log10 (mean of intensities Red and Green) > 1.69. Several probes (less than 10) significantly positive with RNA sample obtained from either hormone-treated cells in the absence of PR inducters or from inducer-treated parental cells were eliminated from the list. Similarly, all probes corresponding to false annotations or pseudogens were eliminated after control by blast analyses. Redundant positive probes corresponding to the same gene were grouped to control homogeneity of ratio and p-value and were scored at upper level depending on their count in the whole microarray. Among them, only one representative response was conserved for each gene for the final classification allowing counting the real number of genes. All positive genes were then compared in Microsoft Excel software using Visual Basic algorithms. For a given gene, each condition corresponding to either ratio < 1.3 or p-value $>10^{-3}$ were masked by setting the ratio to 1. Clusters of genes corresponding to each combination of responses are shown in Venn diagrams indicating the number of included genes. For each conditional clusters, a complete hierarchical clustering was performed from $\log_2$ (ratio) values using Genesis software to generate the final heatmaps. Colorization of down-regulated

genes (green) and up-regulated genes (red) were saturated at $\log_2$ (ratio) of ± 2 corresponding to fold change FC ± 4.

Statistical analysis

Data are expressed as mean ± SEM. Non parametric Mann-Whitney test was used to determine significant differences between groups using the computer software Prism 4 (GraphPad Software, San Diego, CA). Statistical significance is indicated at $P \leq 0.05$ or ≤ 0.05.

Results

Establishment and characterization of stable cells lines expressing inducible PR isoforms

We used MDA-MB-231 cells (ER-, PR-) to establish three cell lines, conditionally expressing PRA (iPRA) or PRB (iPRB) or both PRA and PRB (iPRAB) using RheoSwitch and TRex systems which require non-steroidal inducer ligands RheoSwitch Ligand (RSL1) and doxycycline (Dox). RSL1 binding induces heterodimerization of both Rheoreceptor and Rheoactivator hybrids to generate a transcriptionally active complex on Gal4-responsive promoter. The TRex system consists of a mutant Tet repressor (TetR) constitutively recruited by tetO elements downstream of constitutive CMV promoter thus silencing the transcription to very low levels depending on TetR expression. Following Dox binding, the TetR cannot bind to its responsive elements resulting in de-repression of transcription from constitutively active CMV-TO promoter. The regulatory proteins required for functioning of both of these systems were inserted into the same plasmid pZX-TR (zeocin) (Additional file 2 Fig. S1) allowing coordinated and balanced expression of the corresponding regulatory factors. Cells were stably transfected with pZX-TR plasmid and approximately 150 zeocin resistant clones were screened for functioning of both inducible systems by transactivation assays using luciferase reporter gene

linked to promoter containing either Gal4- or TO-responsive elements. Ten dually positive clones having highest inducible expression and minimum background for both systems were selected and tested for the expression of the three regulator proteins (not shown). A representative clone (named Cl250) was then stably transfected with either Gal4uas-PRA (iPRA) or TO-PRB (iPRB) or both Gal4uas-PRA and TO-PRB (iPRAB) expression vectors. Forty five clones resistant to selection antibiotics (neomycin for iPRA, blasticidin for iPRB, neomycin and blasticidin for iPRAB clones) were amplified and screened for PR isoforms expression by immunobloting since the functional screening using PRE^2-luc reporter gene assay did not allow the selection of PRA expressing clones due to much lower transactivation properties of PRA as compared to PRB on such synthetic promoters. From each of three transfection strategies (iPRA, iPRB, iPRAB), at least five clones with minimum background and maximum PR isoform expression following inducer(s) exposure were characterized. Western blot analysis of a representative clone for iPRA, iPRB or iPRAB cell lines is presented in Fig. 1A. PR isoform expression can be tightly controlled by the respective inducer with undetectable PR expression in uninduced cells. The PRA and PRB expression levels were comparable to T47D cells endogenously expressing both PR isoforms. Clonal expression of iPRAB cells was verified by immunocytochemical analysis showing that all cells express either or both of PR isoforms in the presence of respective inducer(s) (Fig 1B). In this study, we focused on iPRAB cells to validate the functional integrity of inducibly expressed PR isoforms.

One of the objectives of establishing bi-inducible cell lines (iPRAB) was to precisely control the amount of each PR isoform in the same cells to allow the adjustment of PRA/PRB expression ratio at will. We firstly determined the time for optimal PR expression levels following inducer exposure. Kinetics experiments showed that PR expression was detectable as

early as 3 h and increased continuously thereafter reaching a peak at 24 h (Fig. 1C) and maintained for up to 72 h (Fig. 1D, left panels) following single exposure of RSL1 or Dox. Removal of inducer after 24h led to gradual decrease in PR isoform expression (Fig. 1D, right panels). This indicated that PRA and/or PRB expression could be easily switch-on or -off when desired. We next asked whether PR isoforms expression levels could be adjusted by altering inducer concentrations. PRA levels were detectable from 0.05 µM RSL1 and reached a plateau at 0.5 µM concentration (Fig. 1E). As shown in Fig. 1F, PRA/PRB ratio could be increased up to 10-fold depending on inducer concentrations. We then quantified PR expression levels by ligand binding assays following 24 h of RSL1 (0.5 µM) or Dox (1 µM) exposure. The data presented in Table. 1 shows that in uninduced cells, basal PR expression levels were negligible as compared to PR negative MDA-MB-231 parental cells and exposure to RSL1- or Dox-induced PR isoforms expression up to physiological levels.

Wild type PRs undergo progestin-induced turnover concomitant to ligand-induced PR phosphorylation at serine 294 [30] which is known to play critical roles in PR signaling at multiple levels including PR transcriptional activity and turnover [31]. Therefore, we analyzed whether inducible PR isoforms are capable of responding to such events. As shown in Additional file 2 Fig. S2, the inducibly expressed PRs underwent expected agonist ligand-induced degradation and were phosphorylated at S294-PRB and S130-PRA residues by both agonist (P4) and antagonist (RU486) ligands [32, 33]. The stable bi-inducible character of iPRAB-38 cells for conditional PRA and/or PRB expression remained preserved after several passages as verified by western blot analysis at passage 22. The results show undetectable PR isoform expression in the absence of inducer(s) and PRA and/or PRB expression upon RSL1 and/or Dox exposure (Additional file 2 Fig. S3). These results demonstrate that PR isoform expression levels are

tightly controlled and could be adjusted by the amount of inducer(s). PRA/PRB ratio can thus be increased or decreased to desired extent, more than 100-fold range, in the same cells as a function of inducer RSL1 or Dox concentration.

PRA and PRB homo- and hetero-dimers exhibit distinct transcriptional properties

PRA and PRB regulate transcription to different extent from exogenous promoters comprising of consensus PRE sequences where PRA acts as a transrepressor of PRB activity [26]. Therefore, we examined whether inducibly expressed PR isoforms are transcriptionally functional as ligand-induced homo- and heterodimers. Following induction of either or both PR isoforms, cells were transiently transfected with plasmid encoding PRE_2-luciferase reporter gene during 24 h and then treated with vehicle or P4 for another 24 h. In uninduced cells, transactivation by P4 was negligible confirming the low background expression of PR isoforms in the absence of inducer(s) (Fig. 2A). As expected, in PRA-induced cells, agonist-induced transcriptional activity was much lower as compared to that in PRB-induced cells when each isoform was expressed alone (Fig. 2A). Co-expression of both PRA and PRB led to decreased luciferase activity due to known transrepressive effects of PRA on PRB-mediated transcription from exogenous promoters.

Given that both common as well as distinct target genes are regulated by PRA or PRB, ligand-induced PRA-PRB heterodimerization in cells co-expressing both PR isoforms might influence target genes regulation. Therefore, we studied the expression of classical PR target genes; FK506 binding protein 5 (*FKBP5*) and serum and glucocorticoid-regulated kinase 1 (*SGK1*) under conditions when either or both PR isoforms are expressed in the same cells (Fig. 2B). Both of these genes are known to contain hormone response elements in the promoter regions and are also regulated by other steroid receptors such as glucocorticoid (GR) and

mineralocorticoid (MR) receptors. As shown in Fig. 2B, both PR isoforms exert distinct effects on *FKBP5* and *SGK1* genes expression. P4 induced transcription of *FKBP5* specifically by PRB and co-expression of PRA led to decreased PRB transcriptional response in a similar way as the transrepressive effect of PRA on PRB-mediated transcription from exogenous promoter (Fig. 2A). Interestingly, *SGK1* expression was highly induced by PRA as compared to PRB alone showing that PRA can also behave as a stronger transcriptional factor as compared to PRB depending on the promoter context. Co-expression of both isoforms resulted in intermediate levels of *SGK1* transcripts showing that PRB can also exert transrepressive effects on PRA-mediated transcription of *SGK1* gene. These results show that PR isoforms homo- and hetero-dimers differentially impact P4-dependent transcription of *FKBP5* and *SGK1* genes and the transcriptional activity of one isoform is compromised when the other isoform is co-expressed.

PR isoforms differentially contribute to antiproliferative effects of antiprogestin RU486

P4 exerts either growth stimulatory or inhibitory effects depending on target cells [34-38]. We thus examined the relative contribution of PR isoforms on cell proliferation in the presence of vehicle or P4 and/or antiprogestin RU486. The proliferation of cells expressing ligand-free PRA was significantly decreased as compared to PRB-expressing cells and co-expressing PRB did not modify the antiproliferative effect of unliganded PRA (Fig. 3A). We next studied the contribution of individual PR isoform in controlling cell proliferation following agonist and/or antagonist ligand treatment. In uninduced (PR-) cells, neither P4 nor RU486 modified cell proliferation (Fig. 3B) whereas both P4 and RU486 exerted antiproliferative effects when either or both PR isoforms were expressed. Interestingly, while P4 exerted antiproliferative effects to similar extent via PRA or PRB, the antiproliferative efficacy of RU486 was stronger in PRA- as compared to PRB-expressing cells. Such exarcerbated antiproliferative effects of RU486 were

also observed when both PR isoforms were co-expressed. These results indicate that the antiproliferative responses of P4 or RU486 in iPRAB cells are specifically mediated via PR and importantly, the antiprogestin exerts differential antiproliferative efficacy depending on selective PR isoforms expression.

PRA/PRB expression ratio determines target gene selectivity depending on ligand status

Recent *in vivo* studies have shown that long term P4 treatment elicits proliferation of PR(-) mammary cells by paracrine mechanims whereas PR(+) cells remain insensitive to such effects [39]. In order to identify the potential candidates for PR-dependent carcinogenesis and to gain insights on the global changes in gene expression by hormone-free as well as liganded PR isoforms, we performed genome-wide transcriptomic studies. Following 24 h induction of PRA and/or PRB, cells were treated with vehicle or P4 and RNA expression was profiled using gene expression chips. A comparison of PR isoform(s) expression under similar experimental condition is presented in Fig. 4 (inset). We found that a total of 1014 genes were regulated by PR independently of hormonal treatment and isoform expression (Fig. 4). A comparison of these genes with previously identified PR-regulated genes following transcriptomic studies in several cell lines showed that 178 genes were previously at least once reported to be PR-regulated whereas 836 genes were newly identified PR-targets (Additional file 3). Complete list of genes along with the fold change in expression levels, corresponding to hormonal condition and PR isoform expression is provided in Additional file 4 and the detailed description of each gene is given in Additional file 5. Venn diagrams quantifying the distinct as well as common genes regulated by unliganded and liganded PR isoforms showed that in the absence of hormone, PRA and PRB regulated the transcription of distinct gene populations and only a few genes were commonly regulated by PRA, PRB and PRA-PRB. Moreover the dominant gene population was

that responding to co-expressed isoforms suggesting that PRA and PRB might transcriptionally cooperate in absence of ligand through unknown mechanism. It should be noted that unliganded PRA regulated expression of a majority of genes whose transcription was not influenced by PRB co-expression. In contrast, in the presence of P4, the majority of genes (156 genes) were commonly regulated by PRA, PRB and PRA-PRB whereas in term of isoform specificity a dominant population was regulated by PRA (134 genes) but insensitive to PRB. Exclusive genes were also found that only respond to one isoform expressed alone, i.e. are shifted to basal level by the other isoform independent to their activation or repression. These genes are therefore targets of isoform-specific transrepression mechanism, and are rather equally distributed between PRA and PRB (28 versus 37 genes). Genes responding only when PRA and PRB are equally co-expressed are likely to be heterodimer-specific (52 genes). Only 4 genes were sensitive to either PRA or PRB homodimer but insensitive to heterodimer. This has to be compared with the absence of genes responding to unliganded PRA or PRB but not to their co-expression. Interestingly, although 235 genes are commonly regulated by unliganded and liganded PR isoforms (mixed regulation) in a diversified manner (right panel), the majority of genes are highly sensitive to hormonal status (Fig. 4). Of note, the global level of transcriptional responses to PRA and PRB were similar in sharp contrast to much lower transcriptional properties of PRA as compared to PRB on synthetic promoter-driven reporter gene transactivation assays (Fig. 2A). These results provide first evidence that PR target genes are selected not only by the presence or absence of hormone but more importantly also by the expression of individual PR isoform. Any variation in PRA/PRB ratio would thus lead to a significant shift in PR transcriptional selectivity.

Genes implicated in breast cancer development and metastasis are differentially regulated PR isoforms and hormonal condition

We next employed the Ingenuity Pathway Analysis (IPA) to investigate the key biological functions linked to the identified genes and classified them into functionally related subgroups. Interestingly, cellular functions implicated in breast cancer development and metastasis such as cellular growth and proliferation, cancer, cell death, cellular movement and cellular development were among the top cellular functions that were highlighted in at least one of the experimental conditions (Fig. 5). Functional significance is represented in the form of negative log of p-values which are indicative of the number of genes differentially expressed in a given experimental condition *vs* the total number of genes representative of a particular cellular process. Complete list of genes along with the corresponding p-values for each experimental condition and the top cellular processes activated are presented in Table 2 and Additional file 6. As shown in Fig. 5, genes regulated by unliganded PRA strongly influenced cellular processes involved in cancer development and metastasis as compared to unliganded PRB. Progesterone treatment further increased the number of PRA- as compared to PRB-regulated genes involved in such processes. Co-expression of PRA and PRB gave almost similar results as those obtained by PRA alone suggesting that the major impact of progestin signaling on tumorigenesis might preferentially involve PRA isoform. Although we did not identify distinct pathways specifically regulated by one isoform or ligand condition, our results, nevertheless, show that PR isoforms differentially contribute to carcinogenesis-associated cellular processes in a ligand-sensitive manner with a predominant role of PRA as compared to PRB.

HB-EGF and *AREG* transcription is differentially influenced by ligand, growth factors and PR isoforms

To validate our microarray studies, we selected two paracrine growth factors, heparin-binding epidermal growth factor (*HB-EGF*) and amphiregulin (*AREG*), both belonging to EGF-like growth factor family. *HB-EGF* and *AREG* play an important role in breast tumorigenesis [40-43] and are proposed as therapeutic targets for various human cancers including breast cancer [44]. Previous studies have identified *AREG* to be a PRB- but not PRA-regulated gene whose transcription is increased following hormonal treatment in Ishikawa cells stably expressing either of the PR isoforms [45]. PR-dependent transcriptional regulation of *HB-EGF* gene has also been reported [46]. As shown in Table 2, we found that *AREG* was present not only among the genes regulated by liganded PRB but interestingly also by unliganded PRA and PRA-PRB. *HB-EGF* expression was detected under all experimental conditions except with unliganded PRB. Since previous transcriptomic studies did not allow identification such differential effects, we selected these genes to verify our gene classification. Quantitative RT-PCR analyses shown in Fig. 6A were performed in independent experiments under varied conditions of PR isoforms expression to confirm that PRA and PRB exerted distinct effects on the transcription of *HB-EGF* and *AREG* genes. While *HB-EGF* transcription was significantly down-regulated by unliganded PRA and PRA-PRB, P4 exposure decreased *HB-EGF* transcription irrespective to PR isoforms expression. Interestingly, PRA increased *AREG* transcript levels irrespective of hormone (Fig. 5A) whereas PRB up-regulated *AREG* transcription in the presence of P4. Co-expression of PRA and PRB led to elevated *AREG* mRNA levels which were further increased by P4. Regulation of other genes was also analyzed by qRT-PCR (not shown) confirming our microarray data. These results

validated the observations from microarray studies and indicated that elevated PRA/PRB ratio alone has a significant impact on AREG expression.

It is known that PR isoforms differentially interact with growth factors-stimulated signaling partners [47]. Moreover epidermal growth factor (EGF) signaling has been shown to highly enhance the transcriptional activity of both ligand-free as well as liganded PRB [48]. Since breast cancer cells often harbor overactivation of growth factor signaling, we asked whether our transcriptomic studies in MDA-MB-231 cells could reflect a saturated response to growth factor stimuli. To this aim, we explored the impact of EGF on PR isoform-mediated transcriptional regulation of *HB-EGF* and *AREG* genes. In order to highlight the contribution of individual PR isoform in synergism/antagonism between P4 and EGF, expression levels of PRA and/PRB were decreased by reducing inducer concentrations similar to that used in our transcriptomic study. Transcriptional regulation by unliganded PR isoform was arbitrarily set to 1 for vehicle-treated conditions to determine the influence of P4 and/or EGF. In the absence of PR (uninduced cells), EGF did not significantly modify *HB-EGF* expression (Fig. 6B) while in conditions where PRA or PRB was expressed in the absence of ligand, EGF increased *HB-EGF* transcript levels, although non-significantly. Co-expression of both ligand-free PR isoforms led to a significant increase in *HB-EGF* mRNA levels following EGF stimulation supporting the synergetic role of both PR isoforms on EGF-induced *HB-EGF* transcription. This shows that growth factor stimuli are capable of eliciting transcriptional responses of unliganded PR isoforms. While P4 treatment decreased *HB-EGF* expression irrespective to PR isoforms, surprisingly, P4 counteracted EGF-stimulated *HB-EGF* expression when either or both PR isoforms were expressed indicating that EGF and P4 exert opposite effects on *HB-EGF* transcription via PR. As shown in Fig. 6B, EGF alone increased *AREG* transcription irrespective

to PR isoforms expression. Simultaneous treatment with EGF and P4 did not enhance *AREG* transcription in the absence of PR or when only PRA was expressed. As expected, decreasing PR expression led to lowered P4-induced PRB-mediated *AREG* expression as compared to that observed in Fig. 6A. Interestingly, simultaneous treatment with P4 and EGF led to synergetic increase in *AREG* transcription in cells expressing uniquely PRB isoform. Moreover, such synergetic effect compensated for decreased PRB expression highlighting the important role of EGF signaling in governing PR transcriptional properties. This potentiating effect of EGF on PRB-mediated *AREG* transcription was compromised by co-expression of PRA consistent with lack of synergism between P4 and EGF in PRA expressing cells for *AREG* expression. This indicates that selective PR isoform expression strongly influences synergetic responses to EGF and P4 on *AREG* transcription. Furthermore, these results demonstrate that functional outcome of EGF and P4 signaling on a given target gene expression is dependent on PR isoforms expression ratio. EGF and P4 exert synergetic or antagonistic effect on PR transactivation depending on target genes. Collectively, our data underscore the pivotal role of P4 and EGF signaling cross-talks for controlling PR-dependent transcriptional regulation of key target genes such as *HB-EGF* and *AREG*.

Unliganded PRA increases *AREG* mRNA levels by stimulating transcriptional events

Owing to the functional significance of *AREG* as a paracrine growth factor in cell proliferation, we examined the kinetics of *AREG* expression along with cell cycle regulatory gene cyclin D1, following PRA induction. As shown in Fig. 7A, *AREG* mRNA levels increased as early as 3 h post PRA expression and remained elevated up to 48 h as compared to *AREG* levels in uninduced PR(-) cells indicating that PRA induces continuous expression of this paracrine factor. Under similar conditions, cyclin D1 transcript levels significantly increased following 3 h of

PRA expression and then continuously decreased as compared to PR negative cells consistent with antiproliferative effect of unliganded PRA (Fig. 3A). This suggests that PRA expressing cells might be insensitive to continuously increased *AREG* expression which, nevertheless, might influence proliferation of nearby PR negative cells through its well known paracrine growth stimulatory effects.

We next examined the molecular mechanisms by which unliganded PRA transcriptionally or post-transcriptionally increased *AREG* mRNA levels. PRA expression was induced during 24 h and cells were then incubated with actinomycin D, an inhibitor of transcription, during 1 to 6 h. As shown in Fig. 7B, elevated *AREG* levels in PRA expressing cells gradually decreased following actinomycin D treatment and reached similar levels as in uninduced cells providing strong evidence that unliganded PRA regulates *AREG* expression at transcriptional level. The possibility that increase in *AREG* expression could be artifactually related to PRA inducer RSL1, was excluded since RSL1 treatment did not enhance *AREG* expression in neither parental MDA nor clone-250 cells (Additional file 2 Fig. S4). We also confirmed that unliganded PRA increases *AREG* expression in other cell models such as MDA and endometrial cancer cells Ishikawa both stably expressing PRA (Additional file 2 Fig. S5) [33]. We finally demonstrated that antiprogestin RU486 completely blocked PRA-mediated increase in *AREG* transcription, confirming the role of unliganded PRA in *AREG* transcriptional activation. Taken together, these results indicate that elevated PRA/PRB ratio increases *AREG* expression irrespective of P4 and can be controlled by PR antagonist RU486. Up-regulation of such paracrine growth factors by unliganded PRA might contribute towards aggressive phenotype of PRA-dominant breast tumors.

Discussion

The presence of PR and more importantly the PRA/PRB ratio directly influences breast cancer phenotype, even in the absence of P4 [49] and breast cancer patients with PRA dominant tumors have poorer disease-free survival rates [50]. If PR isoforms contribute towards pathogenesis, aggressive phenotype of PRA- as compared to PRB-rich tumors might be due to major alterations in P4 signaling via PRA vs PRB isoform. The mechanisms by which PR contributes to such tumorigenic processes are not fully known in part due to lack of appropriate cellular models. *In vitro* transcriptomic studies performed in separate cell lines conditionally expressing either PRA or PRB or both [23-25, 51-53] have highlighted distinct transcriptional properties of PR isoforms depending on the presence or absence of P4. Upon P4 exposure, PRB behaves as a much stronger transcriptional factor than PRA alone [24]. Conversely, in the absence of P4, PRA plays a prominent role since it regulates a much larger distinct gene population as compared to PRB alone [24]. Whether such target gene selection and transcriptional regulation is also influenced by PRA-PRB heterodimers or PRA/PRB ratio could not be investigated due to expression of PRA or PRB in two separate cell lines. The strategy of conditional expression of steroid hormone receptors (SR) including PR by the use of inducible systems has been reported [27, 54-57] providing new insights in transcriptional regulation by SR. However, none of the currently available cellular models allows PRA and/or PRB expression in the same cells and thus could not permit studying the role of PRA/PRB ratio in cell signaling in the absence or presence of P4.

To circumvent these draw backs, it was necessary to develop a new strategy in the form of bi-inducible system for generating suitable cellular models to investigate the relative contribution of each PR isoform in cell signaling. In this study, we established an original breast

cancer cellular model conditionally expressing either or both PRA and PRB in the same cells allowing fine-tuned adjustment of PRA/PRB ratio. Importantly, the contribution of PRA, PRB homo- and hetero-dimers in P4 signaling could be investigated with more confidence since such cells serve as their own controls when cultured in the absence of inducer(s). Indeed, such cell-based system eliminates genetic background related effects coming from separate cell lines transfected by distinct expression or control vectors. We placed the coding sequences for the three regulatory proteins required for the functioning of both inducible systems in the same plasmid allowing their constitutive expression at similar levels after genomic recombination. TRex system was chosen instead of alternate Tet-on/Tet-off systems in order to minimize the background expression in the absence of inducer. Mammary breast cancer MDA-MB-231 (PR-) cells were selected since they do not express ER and hence enabling investigation of PR specific events independently of estrogen. Analysis of primary stable cell line (clone 250) expressing functional regulatory proteins for both RheoSwitch and TREx systems demonstrates for this first time that these systems are compatible when expressed in the same cells from a single vector. Using clone 250, we established three cell lines iPRA, iPRB and iPRAB. Although we focused on iPRAB cells in this study, iPRA or iPRB cells could be exploited to study the functional interaction of one PR isoform with its molecular partners such as SRC1 and SRC3 expressed under the control of appropriate inducible promoter. Since regulatory proteins of both inducible systems are of non-mammalian origin and inducers are non-steroidal ligands, transcriptional drawbacks in human cells are highly limited. Indeed, we did not observe any impact of RSL1 or Dox in regulating transcription of PRE^2-driven reporter gene or endogenous PR target genes that we studied. TRex system has been extensively used due to its low artifactual transcriptional effects. Genome-wide transcriptomic studies in prostate cancer cell line LNCaP have identified

only four transcripts to be significantly regulated by RSL1-activated RheoSwitch heterodimer [27].

To validate our strategy of bi-inducible PR isoforms expression, we focused on several aspects of PR signaling highlighting the critical role of PRA/PRB ratio in transcriptional regulation and cross-talk with growth factor stimuli. PR isoforms are considered as prognostic markers of breast cancer development and metastasis independently of the progestational status [50, 58]. P4 exerts antiproliferative effects in PR expressing cells [39] and few proliferating mammary epithelial cells (MEC) express PR [59, 60]. Recently, the role of P4 signaling in mammary carcinogenesis via paracrine mechanism gained strength since various independent studies have demonstrated that P4 drives proliferation of PR(-) MEC leading to carcinogenesis via receptor activator of NF-kB ligand (RANKL), a tumor necrosis factor family member, and its receptor [39, 61, 62]. Consistent with previous studies [63], we found that both P4 and antiprogestin RU486 exerted antiproliferative effects when either or both PR isoforms were expressed. Interestingly RU486 but not P4 exhibited stronger antiproliferative effects in PRA- as compared to PRB-expressing cells suggesting that PRA/PRB expression ratio influences the antiproliferative efficacy of antiprogestins.

Cross talk of PR with EGF-initiated pathways has been extensively studied [64]. It has been shown that PR action is linked to EGF stimulations at various levels such as subcellular PRB localization, DNA binding and target gene regulation [65]. We have recently reported a major role played by p38 and p42/44 mitogen-activated protein kinases (MAPKs) in selectively enhancing PRA or PRB protein stability in cells co-expressing both PR isoforms resulting in robust, rapid and opposite variations in PRA/PRB expression ratio [33]. Paracrine EGF-like growth factors *HB-EGF* and *AREG* are highly implicated in mammary gland carcinogenesis and

metastasis [66, 67]. The differential role of PR isoforms in regulating *HB-EGF* and *AREG* expression under P4 and EGF stimulation is not known. Therefore, using iPRAB cells, we explored the transcriptional regulation of *HB-EGF* and *AREG* by PR isoforms in the context of P4 and EGF stimulations. We show that PR isoforms distinctly impact the transcription of *HB-EGF* and *AREG* genes not only in the absence of hormone but also under P4 and EGF stimulations independently of contributions from ER. Studies on PRB transactivation from exogenous promoters have shown that EGF strongly enhances P4-induced transcriptional response [48]. Whether EGF also potentiates P4-induced PRA-mediated transactivation from similar exogenous promoters has not been described. Our results on *AREG* transcription revealed that EGF strongly potentiates P4-induced *AREG* expression uniquely via PRB similar to synergetic effect of P4 and EGF on PRB-mediated transactivation from exogenous promoters. Such PR isoforms-dependent synergetic response of EGF and P4 on *AREG* transcription are supported by the finding that co-expression of PRA compromised such potentialization effect of EGF. Interestingly, P4 and EGF exerted opposing effects on *HB-EGF* transcription, P4 decreased while EGF increased the *HB-EGF* expression. These opposing effects of P4 and EGF involve PR since such effects are not observed in uninduced (PR -) cells. Although P4 exerted antiproliferative effect in PR expressing cells, a noteworthy finding of our study is that unliganded PRA increased *AREG* expression that may provide continuous paracrine growth stimuus to adjacent PR (-) cells. Based on our results, it is tempting to speculate that PRA controls cell proliferation by two mechanisms, a cell-intrinsic mechanism i.e., by decreasing cyclin D1 expression and by paracrine mechanism by increasing *AREG* expression. The latter possiblity could not be verified in the current study since all cells homogeneously expressed PR

in our model. However, it might be interesting to establish the role of *AREG* in promoting proliferation of adjacent cells following co-culture of PRA-expressing cells with PR(-) cells.

Conclusions

Our newly established bi-inducible breast cancer cellular model is of broad interest since it allows mechanistic understanding of how target cells behave with PR ligand and with other PR activators such as EGF under normal and pathological contexts. Importantly the role of varied PRA/PRB expression ratio in breast cancer development and metastasis can be explored in mouse models xenografted with iPRAB cells and exposed to varying amounts of doxycycline and/or RSL1 to control PRA/PRB ratio. This might be of particular pharmacological interest. Moreover, since both PR isoforms are selectively expressed within the same cells, this cell-based system is also highly suitable for screening and characterization of new PR antagonists for their PR isoform selective inhibitory properties. Using genome-wide transcriptomic studies, we demonstrate that gene subsets are differentially regulated by PR isoforms under ligand-free and hormonal conditions. We have identified new potential candidates that might be involved in PR-dependent carcinogenic processes. We show that cellular processes linked with breast cancer development and metastasis are differentially regulated by PR isoforms in a ligand-sensitive manner. The iPRAB cells allowed us to show that antiproliferative efficacy of antiprogestins is highly influenced by PRA/PRB ratio that thus should be taken into consideration during therapeutic applications involving antiprogestins. Our results show that elevated PRA/PRB ratio is associated with increased *AREG* expression that was inhibited by antiprogestin RU486. Given the involvement of *AREG* in cancer development/metastasis as a paracrine growth stimulus, aggressive phenotype of PRA rich tumors might at least in part be attributed to elevated *AREG* levels which may enhance the proliferation of nearby PR negative cells.

Abbreviations: PR, progesterone receptor; PRA, progesterone receptor isoform A; PRB, progesterone receptor isoform B; P4, progesterone; R5020 (17,21-dimethyl-19-norpregna-4,9-dien-3,20-dione), RU486 (11β-(4-Dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one).

Competing interests: The authors declare that they have no competing interest.

Author's contributions

JAK, ML and HL designed the study, established cells lines, performed the experiments, analyzed microarray data and wrote the manuscript. CB assisted with qRT-PCR analysis and experiments. AGM participated in discussions and data interpretation. All authors have read and approved the manuscript for publication.

Acknowledgements

The authors are grateful to Dr. Claude Labrie (Laval University Medical Research Center, Quebec) for pZX vectors and technical support, Dr. Sylvie Brailly-Tabard (Hôpital Bicêtre, Le Kremlin-Bicêtre, France) for PR quantification by ligand binding assays, Dr Philip Dessen and Justine Guégan (Institut Gustave Roussy, Villejuif, France) for help in microarray studies. This work was supported by grants from INSERM, the Université Paris-Sud 11 and Association pour la Recherche sur le Cancer (ARC). JAK is on study leave from the department of Physiology and Pharmacology, University of Agriculture, Faisalabad and is a recipient of doctoral scholarship from Higher Education Commission, Islamabad, Pakistan and a fellowship from La Ligue Contre le Cancer, France. CB is recipient of a fellowship from the Conseil Régional de la Martinique.

References

1. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P *et al*: **The nuclear receptor superfamily: the second decade**. *Cell* 1995, **83**(6):835-839.
2. Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P: **Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B**. *Embo J* 1990, **9**(5):1603-1614.
3. Giangrande PH, McDonnell DP: **The A and B isoforms of the human progesterone receptor: two functionally different transcription factors encoded by a single gene**. *Recent Prog Horm Res* 1999, **54**:291-313; discussion 313-294.
4. Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM: **Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform**. *Science* 2000, **289**(5485):1751-1754.
5. Mulac-Jericevic B, Lydon JP, DeMayo FJ, Conneely OM: **Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform**. *Proc Natl Acad Sci U S A* 2003, **100**(17):9744-9749.
6. Mani SK, Reyna AM, Chen JZ, Mulac-Jericevic B, Conneely OM: **Differential response of progesterone receptor isoforms in hormone-dependent and -independent facilitation of female sexual receptivity**. *Mol Endocrinol* 2006, **20**(6):1322-1332.
7. Bain DL, Franden MA, McManaman JL, Takimoto GS, Horwitz KB: **The N-terminal region of human progesterone B-receptors: biophysical and biochemical comparison to A-receptors**. *J Biol Chem* 2001, **276**(26):23825-23831.

8. Tetel MJ, Giangrande PH, Leonhardt SA, McDonnell DP, Edwards DP: **Hormone-dependent interaction between the amino- and carboxyl-terminal domains of progesterone receptor in vitro and in vivo**. *Mol Endocrinol* 1999, **13**(6):910-924.

9. Mote PA, Bartow S, Tran N, Clarke CL: **Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis**. *Breast Cancer Res Treat* 2002, **72**(2):163-172.

10. Mote PA, Graham JD, Clarke CL: **Progesterone receptor isoforms in normal and malignant breast**. *Ernst Schering Found Symp Proc* 2007(1):77-107.

11. Graham JD, Yeates C, Balleine RL, Harvey SS, Milliken JS, Bilous AM, Clarke CL: **Characterization of progesterone receptor A and B expression in human breast cancer**. *Cancer Res* 1995, **55**(21):5063-5068.

12. McGowan EM, Saad S, Bendall LJ, Bradstock KF, Clarke CL: **Effect of progesterone receptor a predominance on breast cancer cell migration into bone marrow fibroblasts**. *Breast Cancer Res Treat* 2004, **83**(3):211-220.

13. Bagheri-Yarmand R, Talukder AH, Wang RA, Vadlamudi RK, Kumar R: **Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis**. *Development* 2004, **131**(14):3469-3479.

14. Mote PA, Leary JA, Avery KA, Sandelin K, Chenevix-Trench G, Kirk JA, Clarke CL: **Germ-line mutations in BRCA1 or BRCA2 in the normal breast are associated with altered expression of estrogen-responsive proteins and the predominance of progesterone receptor A**. *Genes Chromosomes Cancer* 2004, **39**(3):236-248.

15. Poole AJ, Li Y, Kim Y, Lin SC, Lee WH, Lee EY: **Prevention of Brca1-mediated mammary tumorigenesis in mice by a progesterone antagonist**. *Science* 2006, **314**(5804):1467-1470.

16. Arnett-Mansfield RL, deFazio A, Wain GV, Jaworski RC, Byth K, Mote PA, Clarke CL: **Relative expression of progesterone receptors A and B in endometrioid cancers of the endometrium**. *Cancer Res* 2001, **61**(11):4576-4582.

17. Shyamala G, Yang X, Silberstein G, Barcellos-Hoff MH, Dale E: **Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor exhibit developmental abnormalities in mammary glands**. *Proc Natl Acad Sci U S A* 1998, **95**(2):696-701.

18. Wargon V, Helguero LA, Bolado J, Rojas P, Novaro V, Molinolo A, Lanari C: **Reversal of antiprogestin resistance and progesterone receptor isoform ratio in acquired resistant mammary carcinomas**. *Breast Cancer Res Treat* 2009, **116**(3):449-460.

19. Wargon V, Fernandez SV, Goin M, Giulianelli S, Russo J, Lanari C: **Hypermethylation of the progesterone receptor A in constitutive antiprogestin-resistant mouse mammary carcinomas**. *Breast Cancer Res Treat* 2011, **126**(2):319-332.

20. Faivre EJ, Daniel AR, Hillard CJ, Lange CA: **Progesterone receptor rapid signaling mediates serine 345 phosphorylation and tethering to specificity protein 1 transcription factors**. *Mol Endocrinol* 2008, **22**(4):823-837.

21. Pierson-Mullany LK, Lange CA: **Phosphorylation of progesterone receptor serine 400 mediates ligand-independent transcriptional activity in response to activation of cyclin-dependent protein kinase 2**. *Mol Cell Biol* 2004, **24**(24):10542-10557.

22. Mani SK, Allen JM, Lydon JP, Mulac-Jericevic B, Blaustein JD, DeMayo FJ, Conneely O, O'Malley BW: **Dopamine requires the unoccupied progesterone receptor to induce sexual behavior in mice**. *Mol Endocrinol* 1996, **10**(12):1728-1737.

23. Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB: **Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells**. *J Biol Chem* 2002, **277**(7):5209-5218.

24. Jacobsen BM, Schittone SA, Richer JK, Horwitz KB: **Progesterone-independent effects of human progesterone receptors (PRs) in estrogen receptor-positive breast cancer: PR isoform-specific gene regulation and tumor biology**. *Mol Endocrinol* 2005, **19**(3):574-587.

25. Graham JD, Yager ML, Hill HD, Byth K, O'Neill GM, Clarke CL: **Altered progesterone receptor isoform expression remodels progestin responsiveness of breast cancer cells**. *Mol Endocrinol* 2005, **19**(11):2713-2735.

26. Hovland AR, Powell RL, Takimoto GS, Tung L, Horwitz KB: **An N-terminal inhibitory function, IF, suppresses transcription by the A-isoform but not the B-isoform of human progesterone receptors**. *J Biol Chem* 1998, **273**(10):5455-5460.

27. Lessard J, Aicha SB, Fournier A, Calvo E, Lavergne E, Pelletier M, Labrie C: **Characterization of the RSL1-dependent conditional expression system in LNCaP prostate cancer cells and development of a single vector format**. *Prostate* 2007, **67**(8):808-819.

28. Georgiakaki M, Chabbert-Buffet N, Dasen B, Meduri G, Wenk S, Rajhi L, Amazit L, Chauchereau A, Burger CW, Blok LJ *et al*: **Ligand-controlled Interaction of HBO1**

with the N-terminal Transactivating Domain of Progesterone Receptor Induces SRC-1-dependent Co-activation of Transcription. *Mol Endocrinol* 2006, **20**:2122-2140.

29. Amazit L, Roseau A, Khan JA, Chauchereau A, Tyagi RK, Loosfelt H, Leclerc P, Lombes M, Guiochon-Mantel A: **Ligand-dependent degradation of SRC-1 is pivotal for progesterone receptor transcriptional activity**. *Mol Endocrinol* 2011, **25**(3):394-408.

30. Lange CA, Shen T, Horwitz KB: **Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome**. *Proc Natl Acad Sci U S A* 2000, **97**(3):1032-1037.

31. Shen T, Horwitz KB, Lange CA: **Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294**. *Mol Cell Biol* 2001, **21**(18):6122-6131.

32. Beck CA, Zhang Y, Weigel NL, Edwards DP: **Two types of anti-progestins have distinct effects on site-specific phosphorylation of human progesterone receptor**. *J Biol Chem* 1996, **271**(2):1209-1217.

33. Khan JA, Amazit L, Bellance C, Guiochon-Mantel A, Lombes M, Loosfelt H: **p38 and p42/44 MAPKs Differentially Regulate Progesterone Receptor A and B Isoform Stabilization**. *Mol Endocrinol* 2011, **doi:10.1210/me.2011-1042**.

34. Musgrove EA, Lee CS, Sutherland RL: **Progestins both stimulate and inhibit breast cancer cell cycle progression while increasing expression of transforming growth factor alpha, epidermal growth factor receptor, c-fos, and c-myc genes**. *Mol Cell Biol* 1991, **11**(10):5032-5043.

35. Groshong SD, Owen GI, Grimison B, Schauer IE, Todd MC, Langan TA, Sclafani RA, Lange CA, Horwitz KB: **Biphasic regulation of breast cancer cell growth by progesterone: role of the cyclin-dependent kinase inhibitors, p21 and p27(Kip1)**. *Mol Endocrinol* 1997, **11**(11):1593-1607.

36. Clarke CL, Sutherland RL: **Progestin regulation of cellular proliferation**. *Endocr Rev* 1990, **11**(2):266-301.

37. Horwitz KB, Sartorius CA: **Progestins in hormone replacement therapies reactivate cancer stem cells in women with preexisting breast cancers: a hypothesis**. *J Clin Endocrinol Metab* 2008, **93**(9):3295-3298.

38. Horwitz KB: **The molecular biology of RU486. Is there a role for antiprogestins in the treatment of breast cancer?** *Endocr Rev* 1992, **13**(2):146-163.

39. Beleut M, Rajaram RD, Caikovski M, Ayyanan A, Germano D, Choi Y, Schneider P, Brisken C: **Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland**. *Proc Natl Acad Sci U S A* 2010, **107**(7):2989-2994.

40. Bordoli MR, Stiehl DP, Borsig L, Kristiansen G, Hausladen S, Schraml P, Wenger RH, Camenisch G: **Prolyl-4-hydroxylase PHD2- and hypoxia-inducible factor 2-dependent regulation of amphiregulin contributes to breast tumorigenesis**. *Oncogene* 2011, **30**(5):548-560.

41. Busser B, Sancey L, Brambilla E, Coll JL, Hurbin A: **The multiple roles of amphiregulin in human cancer**. *Biochim Biophys Acta* 2011, **1816**(2):119-131.

42. Higginbotham JN, Demory Beckler M, Gephart JD, Franklin JL, Bogatcheva G, Kremers GJ, Piston DW, Ayers GD, McConnell RE, Tyska MJ *et al*: **Amphiregulin exosomes increase cancer cell invasion**. *Curr Biol* 2011, **21**(9):779-786.

43. Yotsumoto F, Oki E, Tokunaga E, Maehara Y, Kuroki M, Miyamoto S: **HB-EGF orchestrates the complex signals involved in triple-negative and trastuzumab-resistant breast cancer**. *Int J Cancer* 2010, **127**(11):2707-2717.

44. Yotsumoto F, Yagi H, Suzuki SO, Oki E, Tsujioka H, Hachisuga T, Sonoda K, Kawarabayashi T, Mekada E, Miyamoto S: **Validation of HB-EGF and amphiregulin as targets for human cancer therapy**. *Biochem Biophys Res Commun* 2008, **365**(3):555-561.

45. Smid-Koopman E, Kuhne LC, Hanekamp EE, Gielen SC, De Ruiter PE, Grootegoed JA, Helmerhorst TJ, Burger CW, Brinkmann AO, Huikeshoven FJ *et al*: **Progesterone-induced inhibition of growth and differential regulation of gene expression in PRA- and/or PRB-expressing endometrial cancer cell lines**. *J Soc Gynecol Investig* 2005, **12**(4):285-292.

46. Zhang Z, Funk C, Roy D, Glasser S, Mulholland J: **Heparin-binding epidermal growth factor-like growth factor is differentially regulated by progesterone and estradiol in rat uterine epithelial and stromal cells**. *Endocrinology* 1994, **134**(3):1089-1094.

47. Faivre EJ, Lange CA: **Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells**. *Mol Cell Biol* 2007, **27**(2):466-480.

48. Daniel AR, Qiu M, Faivre EJ, Ostrander JH, Skildum A, Lange CA: **Linkage of progestin and epidermal growth factor signaling: phosphorylation of progesterone receptors mediates transcriptional hypersensitivity and increased ligand-independent breast cancer cell growth**. *Steroids* 2007, **72**(2):188-201.

49. Jacobsen BM, Richer JK, Sartorius CA, Horwitz KB: **Expression profiling of human breast cancers and gene regulation by progesterone receptors**. *J Mammary Gland Biol Neoplasia* 2003, **8**(3):257-268.

50. Hopp TA, Weiss HL, Hilsenbeck SG, Cui Y, Allred DC, Horwitz KB, Fuqua SA: **Breast cancer patients with progesterone receptor PR-A-rich tumors have poorer disease-free survival rates**. *Clin Cancer Res* 2004, **10**(8):2751-2760.

51. Leo JC, Wang SM, Guo CH, Aw SE, Zhao Y, Li JM, Hui KM, Lin VC: **Gene regulation profile reveals consistent anticancer properties of progesterone in hormone-independent breast cancer cells transfected with progesterone receptor**. *Int J Cancer* 2005, **117**(4):561-568.

52. Tung L, Abdel-Hafiz H, Shen T, Harvell DM, Nitao LK, Richer JK, Sartorius CA, Takimoto GS, Horwitz KB: **Progesterone receptors (PR)-B and -A regulate transcription by different mechanisms: AF-3 exerts regulatory control over coactivator binding to PR-B**. *Mol Endocrinol* 2006, **20**(11):2656-2670.

53. Afhuppe W, Sommer A, Muller J, Schwede W, Fuhrmann U, Moller C: **Global gene expression profiling of progesterone receptor modulators in T47D cells provides a new classification system**. *J Steroid Biochem Mol Biol* 2009, **113**(1-2):105-115.

54. McGowan EM, Clarke CL: **Effect of overexpression of progesterone receptor A on endogenous progestin-sensitive endpoints in breast cancer cells**. *Mol Endocrinol* 1999, **13**(10):1657-1671.

55. Jacobsen BM, Richer JK, Schittone SA, Horwitz KB: **New human breast cancer cells to study progesterone receptor isoform ratio effects and ligand-independent gene regulation**. *J Biol Chem* 2002, **277**(31):27793-27800.

56. Peng J, Jordan VC: **Expression of estrogen receptor alpha with a Tet-off adenoviral system induces G0/G1 cell cycle arrest in SKBr3 breast cancer cells**. *Int J Oncol* 2010, **36**(2):451-458.

57. Bake S, Ma L, Sohrabji F: **Estrogen receptor-alpha overexpression suppresses 17beta-estradiol-mediated vascular endothelial growth factor expression and activation of survival kinases**. *Endocrinology* 2008, **149**(8):3881-3889.

58. Punglia RS, Kuntz KM, Winer EP, Weeks JC, Burstein HJ: **The impact of tumor progesterone receptor status on optimal adjuvant endocrine therapy for postmenopausal patients with early-stage breast cancer: a decision analysis**. *Cancer* 2006, **106**(12):2576-2582.

59. Clarke RB, Howell A, Potten CS, Anderson E: **Dissociation between steroid receptor expression and cell proliferation in the human breast**. *Cancer Res* 1997, **57**(22):4987-4991.

60. Russo J, Ao X, Grill C, Russo IH: **Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland**. *Breast Cancer Res Treat* 1999, **53**(3):217-227.

61. Gonzalez-Suarez E, Jacob AP, Jones J, Miller R, Roudier-Meyer MP, Erwert R, Pinkas J, Branstetter D, Dougall WC: **RANK ligand mediates progestin-induced mammary epithelial proliferation and carcinogenesis**. *Nature* 2010, **468**(7320):103-107.

62. Schramek D, Leibbrandt A, Sigl V, Kenner L, Pospisilik JA, Lee HJ, Hanada R, Joshi PA, Aliprantis A, Glimcher L *et al*: **Osteoclast differentiation factor RANKL controls development of progestin-driven mammary cancer**. *Nature* 2010, **468**(7320):98-102.

63. Lin VC, Ng EH, Aw SE, Tan MG, Ng EH, Chan VS, Ho GH: **Progestins inhibit the growth of MDA-MB-231 cells transfected with progesterone receptor complementary DNA**. *Clin Cancer Res* 1999, **5**(2):395-403.

64. Faivre E, Skildum A, Pierson-Mullany L, Lange CA: **Integration of progesterone receptor mediated rapid signaling and nuclear actions in breast cancer cell models: role of mitogen-activated protein kinases and cell cycle regulators**. *Steroids* 2005, **70**(5-7):418-426.

65. Daniel AR, Faivre EJ, Lange CA: **Phosphorylation-dependent antagonism of sumoylation derepresses progesterone receptor action in breast cancer cells**. *Mol Endocrinol* 2007, **21**(12):2890-2906.

66. Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, Minn AJ, van de Vijver MJ, Gerald WL, Foekens JA *et al*: **Genes that mediate breast cancer metastasis to the brain**. *Nature* 2009, **459**(7249):1005-1009.

67. Desruisseau S, Palmari J, Giusti C, Romain S, Martin PM, Berthois Y: **Clinical relevance of amphiregulin and VEGF in primary breast cancers**. *Int J Cancer* 2004, **111**(5):733-740.

Figures legends:

Figure 1. Inducible expression of PRA and/or PRB in iPRA, iPRB or iPRAB cell lines.

(A) The iPRA or iPRB or iPRAB cells were incubated or not with inducer(s), RheoSwitch Ligand (RSL1) (0.25 µM) and/or doxycycline (Dox, 1 µM) during 24 h and immunoblot analysis of whole cell extracts was performed using Novocastra (NCL-L-PGR-312/2) anti-PR antibody recognizing both PR isoforms or anti-tubulin antibody for sample loading control tubulin. Inducible PRA and/or PRB electrophoretic bands were compared with endogenously expressed PR isoforms levels in wild-type T47D. **(B)** The iPRAB cells were incubated with DMSO vehicle or RSL1 (0.5 µM) or Dox (2 µM) during 24 h and immunocytochemical analysis for PR isoforms detection was performed using Novocastra anti-PR antibody as described in *Materials and Methods*. **(C)** Cells were incubated with RSL1 (0.5 µM) or Dox (2 µM) during indicated time intervals and whole cell extracts were immunoblotted using antibodies against PR or loading control tubulin. **(D)** Cells were incubated with RSL1 (0.5 µM) or Dox (2 µM) continuously during indicated time intervals (left panels) or after 24 h of inducer exposure (right panels), cells were rinced twice with PBS and then incubated with fresh medium without inducer. Before harvesting, cells were rinced twice with PBS and whole cell extracts were immunoblotted as above. **(E)** Cells were incubated with increasing concentrations of RSL1 as indicated and immunoblot detection of PRA or loading control tubulin from whole cell extracts was performed. **(F)** The iPRAB cells were incubated with RSL1 (1 µM) and Dox (1 µM) during 24 h and PR isoforms expression was compared with T47D cells treated or not with estradiol (E2, 10 nM) during 24 h.

Figure 2. Inducibly expressed PR isoforms are functional by regulating reporter and endogenous gene transcription.

(A) Cells were cultured in 96-well plates. Following 24 h induction of PRA and/or PRB using RSL1 (0.5 µM) and/or Dox (2 µM) in the medium containing 5% dextran-coated charcoal treated serum, cells were transiently transfected with vector encoding PRE^2-luciferase reporter gene (100 ng) as described in *Materials and Methods*. Following 6 h of transfection, cells were treated with vehicle (ethanol) or 10 nM progesterone (P4) during 24 h in steroid free medium. Cells were then rinced twice with PBS, luciferase activity was determined and normalized with total protein concentration. Results (mean ± SEM) from six independent cell cultures are presented (graphs). Western blot analysis of whole cell extract showing PR isoforms expression under similar induction and treatment conditions is presented (lower panel). **(B)** The iPRAB cells were incubated with RSL1 (0.5 µM) and/or Dox (2 µM) during 24 in steroid-free medium for PRA and/or PRB expression. Cells were then treated with vehicle (ethanol) or P4 (10 nM) during 6 h in steroid-free medium and quantitative real-time RT-PCR analysis was performed for determining *FKBP5* or *SGK1* transcripts levels as described in *Materials and methods*. Data (mean ± SEM) from three independent cell cultures measured in duplicate are presented as fold increase in transcript levels as compared to that in vehicle-treated uninduced (PR-) cells. Star (*) represents statistical difference at $p \leq 0.05$.

Figure 3. Antiproliferative efficacy of antiprogestin RU486 depends on PRA or PRB isoform expression

(A) Approximately 5,000 iPRAB cells per well were cultured in 96-well plates in steroid-free medium during 24 h. On Day 0, 2 and 4, fresh steroid-free medium was replaced containing or not RSL1 (0.5 µM) and/or Dox (2 µM). Cell proliferation was determined using CellTiter 96®

Aqueous One Solution Cell Proliferation Assay as described in *Materials and Methods* and absorbance at 490 nm, representative of total number of living cells was determined. Data (mean ± SEM) from six independent cell cultures (n = 6) are presented. Star (**) represents statistical difference at $p \leq 0.01$ between uninduced and PR isoforms(s) expressing cells in the absence of ligand. **(B)** PR isoforms(s) expression was induced as above and cell proliferation was determined on day 1, 3 and 5. Data (mean ± SEM) from six independent cell cultures (n = 6) are presented. Star (**) represents statistical difference at $p \leq 0.01$ when comparison is made between P4- *vs* vehicle-treated cells whereas cross (xx) represents statistical difference at $p \leq 0.01$ when comparison is made between P4- and RU486-treated cells.

Figure 4. Gene expression profiles following PR isoform expression and hormonal treatment

The iPRAB cells were treated for 24 h by RSL1 and/or Dox for PRA and/or PRB expression and then incubated with vehicle or progesterone (10^{-8}M) for 6 h. PR isoforms expression were analyzed by western-blot (inset). The corresponding total RNA were extracted by the Trizol method and gene expression profiling was done as described in *Materials and Methods*. Venn diagrams show the total number of genes regulated and clustering analysis of differentially expressed genes (up- or down-regulated) in each experimental condition was performed as described in *Materials and Methods*.

Figure 5. Cellular functions associated with the genes differentially regulated by unliganded and liganded PR isoform(s).

IPA generated cellular functions as resulting from the analysis of genes regulated by selective PR isoform(s) expression and hormonal condition are shown.

Figure 6. PR isoforms distinctly regulate transcription of *HB-EGF* and *AREG* genes

(A) Following 24 h induction of PRA and/or PRB expression using RSL1 (0.5 µM) and/or Dox (2 µM) in steroid-free medium, cells were treated with vehicle or P4 (10 nM) during 6 h and qRT-PCR analysis was performed for determining *HB-EGF* and *AREG* mRNA levels as described in *Materials and Methods*. Data (mean ± SEM) from three independent cell cultures measured in duplicate are presented as fold change in transcript levels as compared to that in vehicle-treated uninduced (PR-) cells. Star (*) represents statistical difference at $p \leq 0.05$ when transcript levels are compared between vehicle-treated uninduced (PR-) vs vehicle-treated PRA and/or PRB expressing cells. Cross (x) represents statistical significance at $p \leq 0.05$ when comparison is made between vehicle-treated vs P4-treated PRA and/or PRB expressing cells. **(B)** Cells were incubated with RSL1 (0.25 µM) and/or Dox (1 µM) during 24 h in steroid-free medium and then treated with vehicle or P4 (10 nM) or EGF (30 ng/ml) or both during 6 h. qRT-PCR analysis was performed as above and data (mean ± SEM) are presented as fold change with respect to vehicle-treated uninduced (PR-) cells. Star (*) represents statistical difference at $p \leq 0.05$ between indicated treatment groups. Cross (x) represents statistical difference at $p \leq 0.05$ between P4-treated vs P4+EGF-treated cells for *HB-EGF* or *AREG* mRNA levels.

Figure 7. Un-liganded PRA induces continuously elevated *AREG* mRNA levels

(A) Cells were incubated with DMSO vehicle or RSL1 (0.5 µM) in steroid-free medium for indicated time periods and qRT-PCR analysis was performed for *AREG* or cyclin D1 transcript levels. Data (mean ± SEM) from three independent cell cultures measured in duplicate are presented as fold change with respect to transcript levels at 1 h time period in uninduced cells. Star (*) represents statistical difference at $p \leq 0.05$ between *AREG* or cyclin D1 levels in uninduced (PR-) and PRA-induced cells at indicated time point. **(B)** Following 24 h exposure to

DMSO vehicle or RSL1 (0.5 µM), cells were incubated with actinomycin D (4 µM) during indicated time periods and qRT-PCR analysis was performed. Star (*) represents statistical difference at $p \leq 0.05$ between *AREG* transcript levels in uninduced (PR-) vs PRA-induced cells at indicated time point. **(C)** Cells were incubated or not with RSL1 (0.5 µM) during 24 h and then treated with vehicle (ethanol) or antiprogestin RU486 (10 nM) during 6 h. qRT-PCR analysis was performed and data (mean ± SEM) from three independent cell cultures measured in duplicate are presented as fold change with respect to transcript levels in vehicle-treated uninduced cells (PR-). Star (*) represents statistical difference at $p \leq 0.05$ between *AREG* transcript levels in uninduced *vs* PRA-induced cells whereas cross (x) represents statistical difference at $p \leq 0.05$ when comparison is made between vehicle-treated *vs* RU486-treated PRA-induced cells.

Figure 1

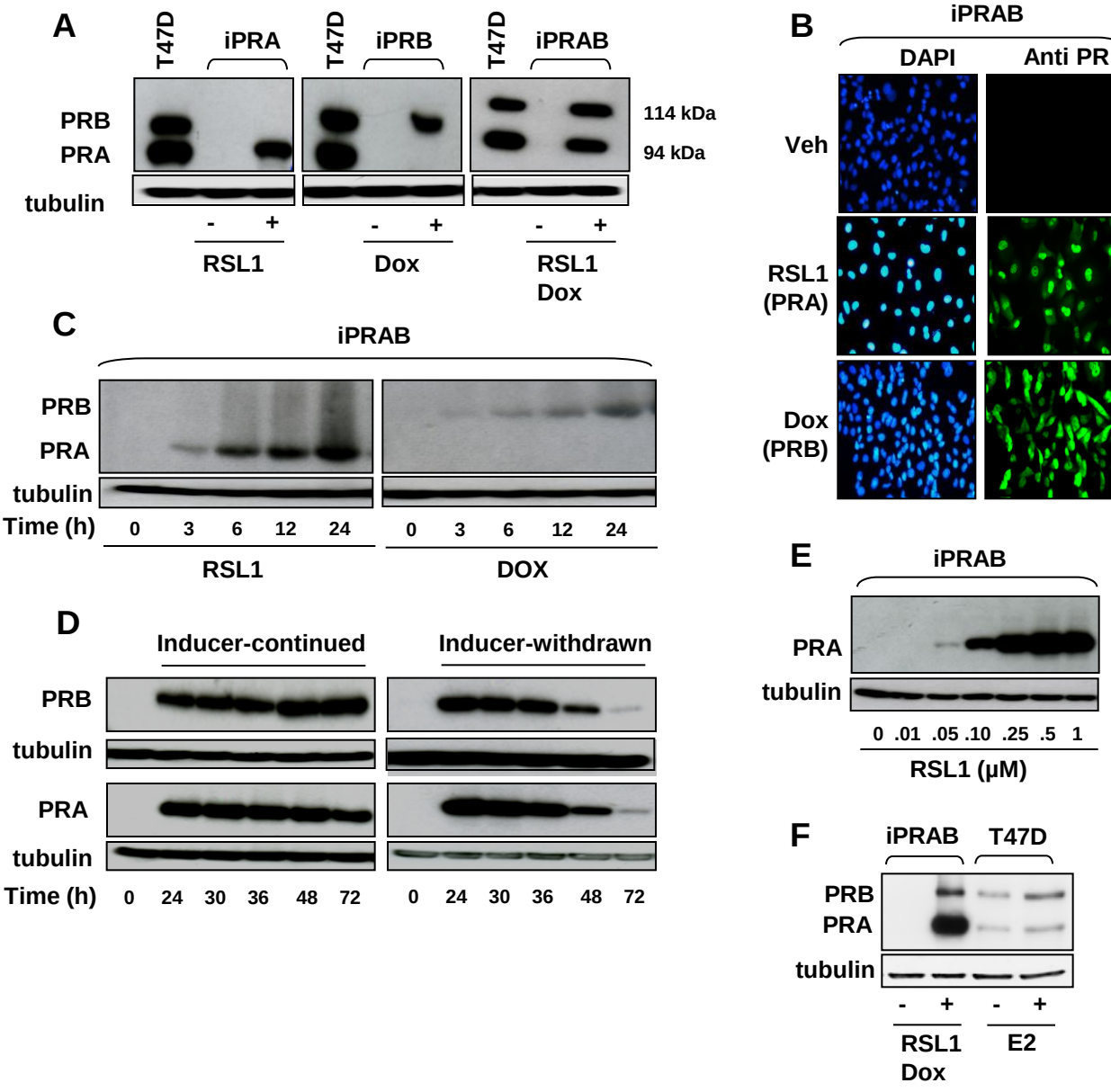

A
T47D
iPRA
T47D
iPRB
T47D
iPRAB
PRB
PRA
tubulin
114 kDa
94 kDa
- + - + - +
RSL1
Dox
RSL1
Dox
B
iPRAB
DAPI
Anti PR
Veh
RSL1
(PRA)
Dox
(PRB)
C
iPRAB
PRB
PRA
tubulin
Time (h)
0 3 6 12 24 0 3 6 12 24
RSL1
DOX
D
Inducer-continued
Inducer-withdrawn
PRB
tubulin
PRA
tubulin
Time (h)
0 24 30 36 48 72 0 24 30 36 48 72
E
iPRAB
PRA
tubulin
0 .01 .05 .10 .25 .5 1
RSL1 (μM)
F
iPRAB
T47D
PRB
PRA
tubulin
- + - +
RSL1
Dox
E2

Figure 2

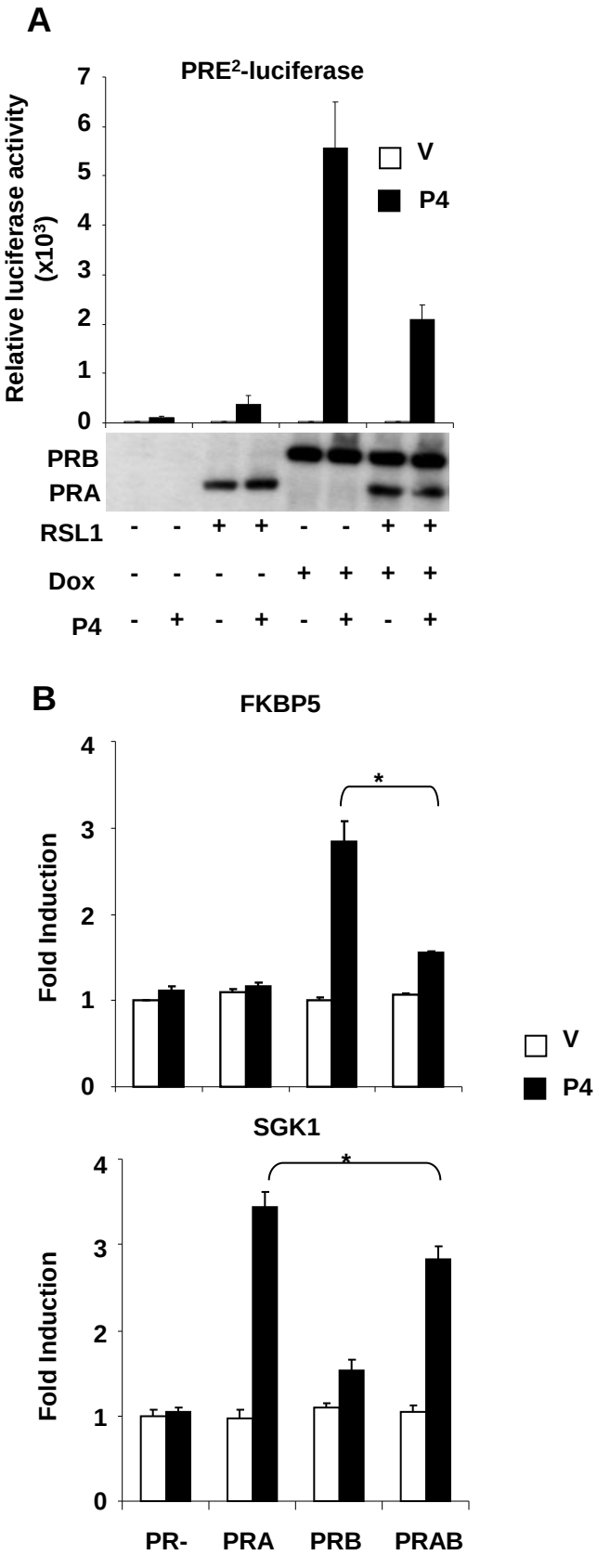

A
PRE2-luciferase
Relative luciferase activity (x10³)
7
6
5
4
3
2
1
0
V
P4
PRB
PRA
RSL1 - - + + - - + +
Dox - - - - + + + +
P4 - + - + - + - +
B
FKBP5
Fold Induction
4
3
2
1
0
*
V
P4
SGK1
Fold Induction
4
3
2
1
0
*
PR-
PRA
PRB
PRAB

Figure 3

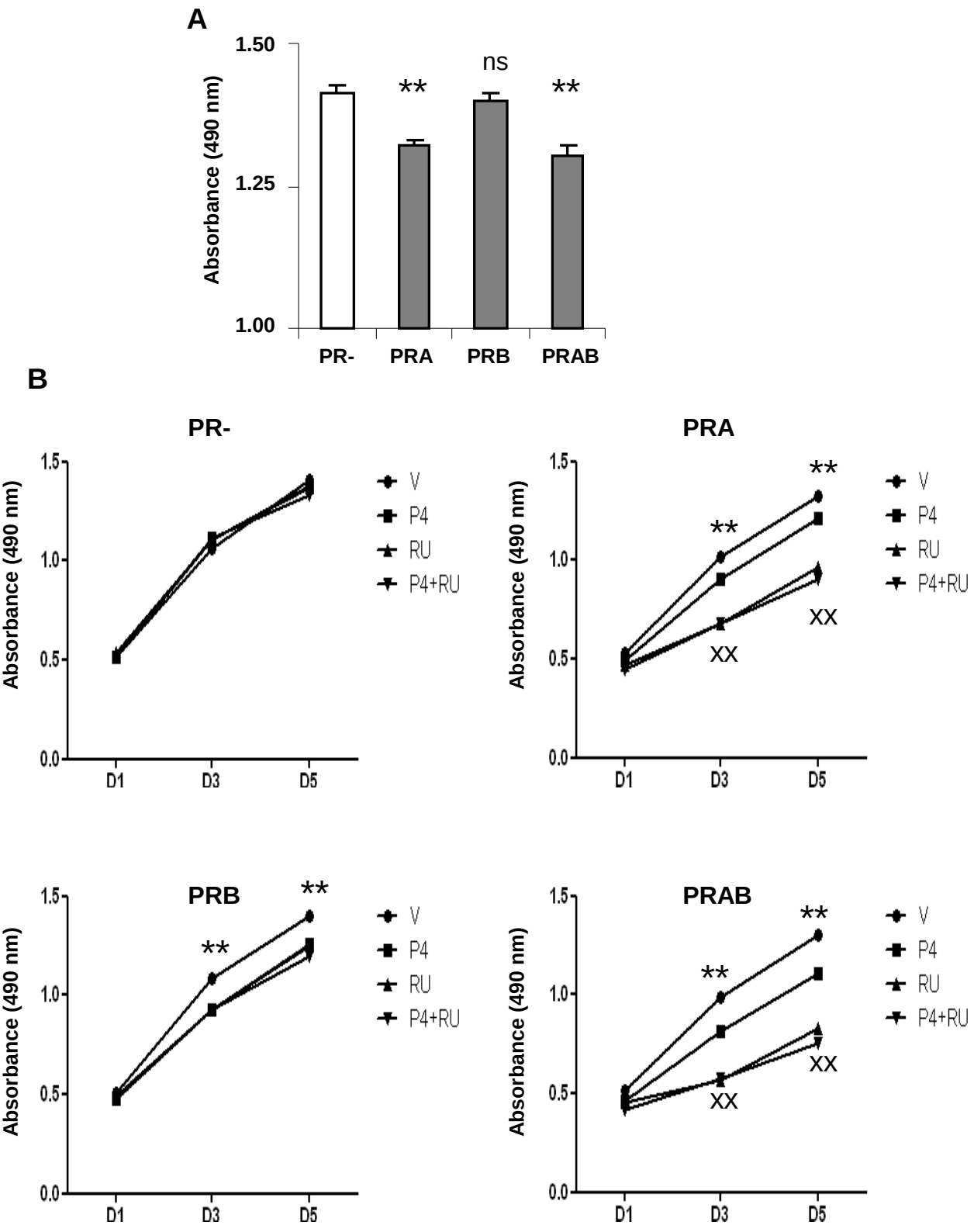

A
Absorbance (490 nm)
1.50
1.25
1.00
**
ns
**
PR-
PRA
PRB
PRAB
B
PR-
PRA
PRB
PRAB
Absorbance (490 nm)
1.5
1.0
0.5
0.0
D1
D3
D5
V
P4
RU
P4+RU
**
XX

Figure 4

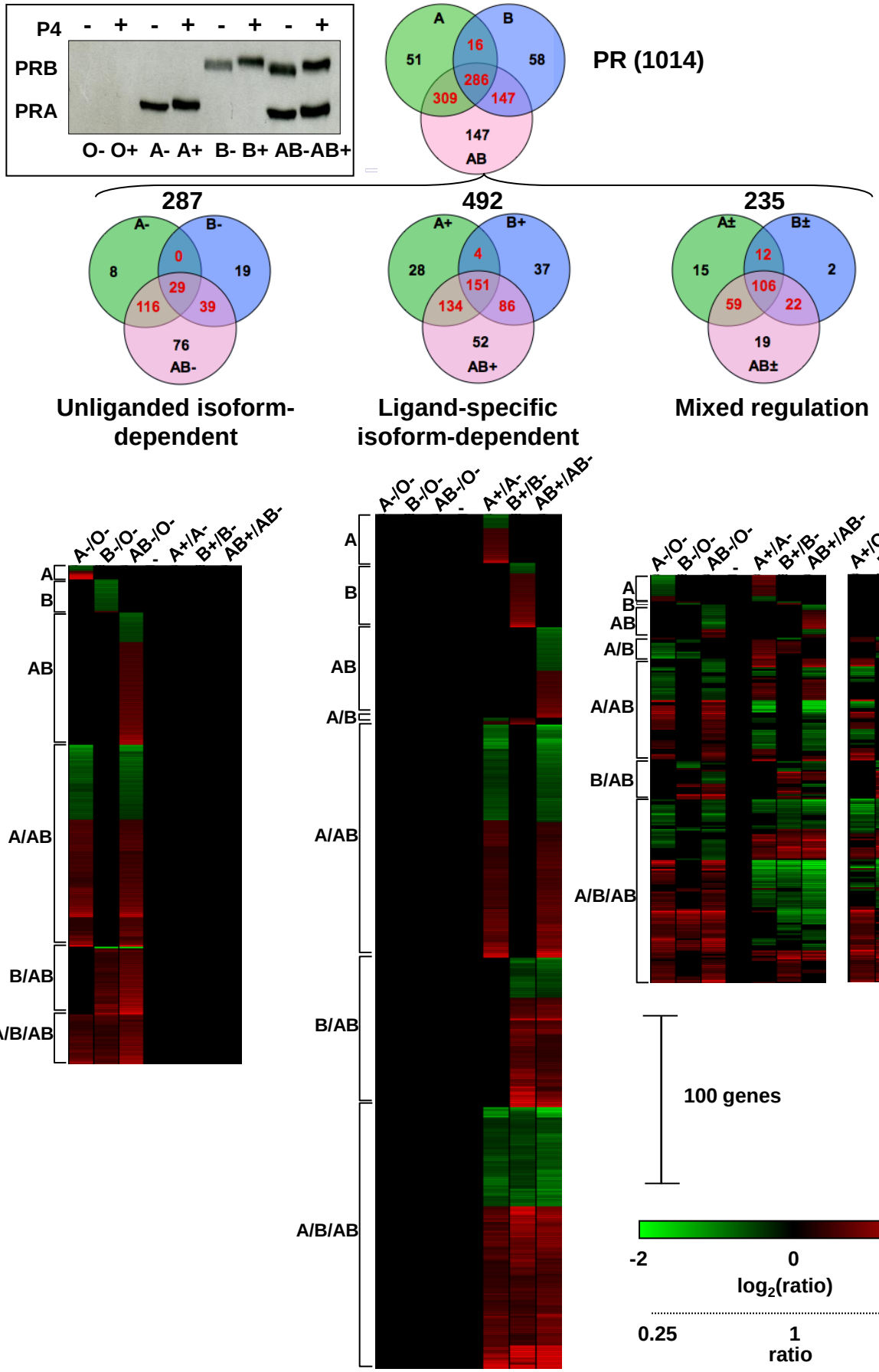

P4 - + - + - + - +
PRB
PRA
O- O+ A- A+ B- B+ AB- AB+
A
B
51
16
58
286
309
147
147
AB
PR (1014)
287
A-
B-
8
0
19
29
116
39
76
AB-
Unliganded isoform-dependent
492
A+
B+
28
4
37
151
134
86
52
AB+
Ligand-specific isoform-dependent
235
A±
B±
15
12
2
106
59
22
19
AB±
Mixed regulation
A-/O-
B-/O-
AB-/O-
A+/A-
B+/B-
AB+/AB-
A+/O-
B+/O-
AB+/O-
A
B
AB
A/B
A/AB
B/AB
A/B/AB
100 genes
-2
0
+2
log2(ratio)
0.25
1
4
ratio

Figure 5

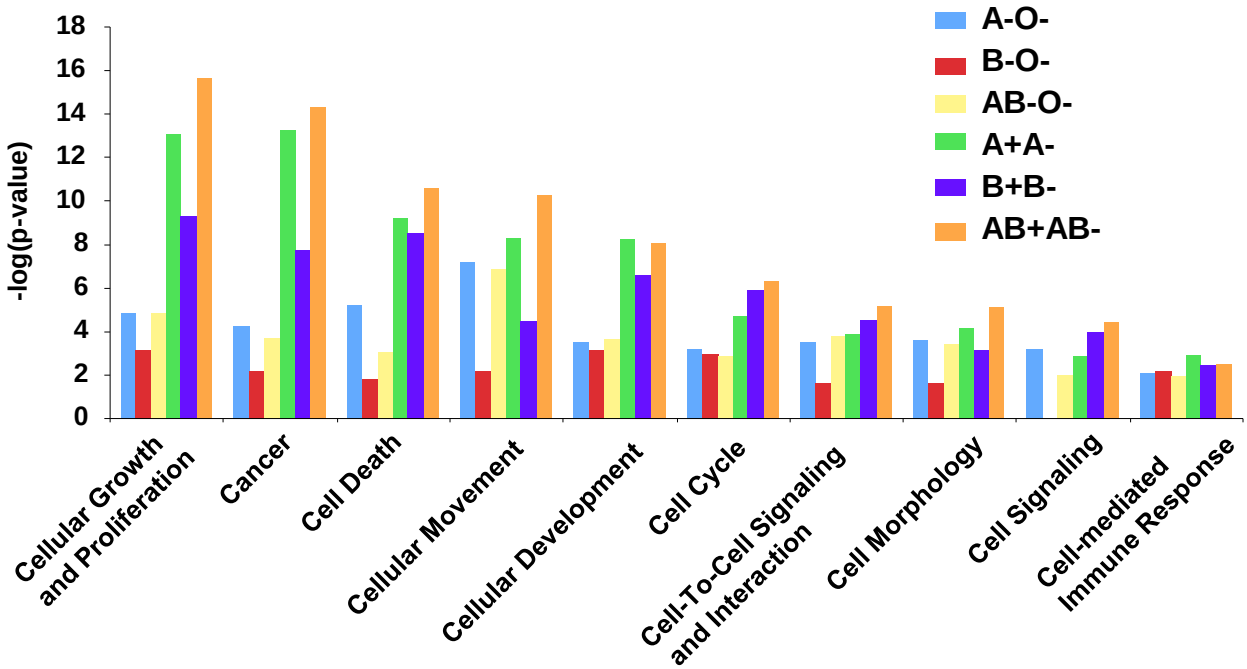

-log(p-value)
18
16
14
12
10
8
6
4
2
0
A-O-
B-O-
AB-O-
A+A-
B+B-
AB+AB-
Cellular Growth and Proliferation
Cancer
Cell Death
Cellular Movement
Cellular Development
Cell Cycle
Cell-To-Cell Signaling and Interaction
Cell Morphology
Cell Signaling
Cell-mediated Immune Response

Figure 6

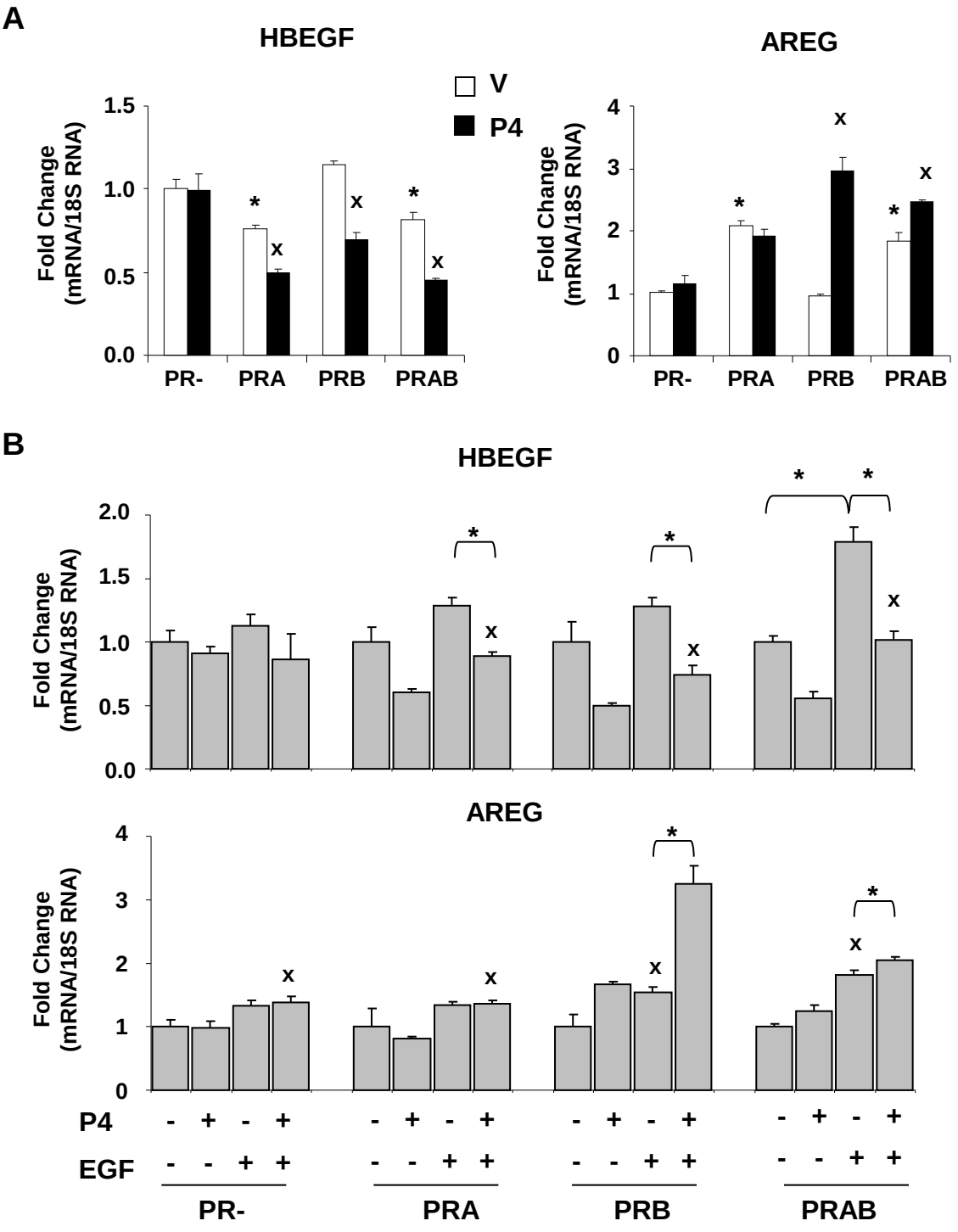

A
HBEGF
V
P4
Fold Change (mRNA/18S RNA)
1.5
1.0
0.5
0.0
PR-
PRA
PRB
PRAB
AREG
4
3
2
1
0
B
HBEGF
2.0
AREG
P4
EGF

Figure 7

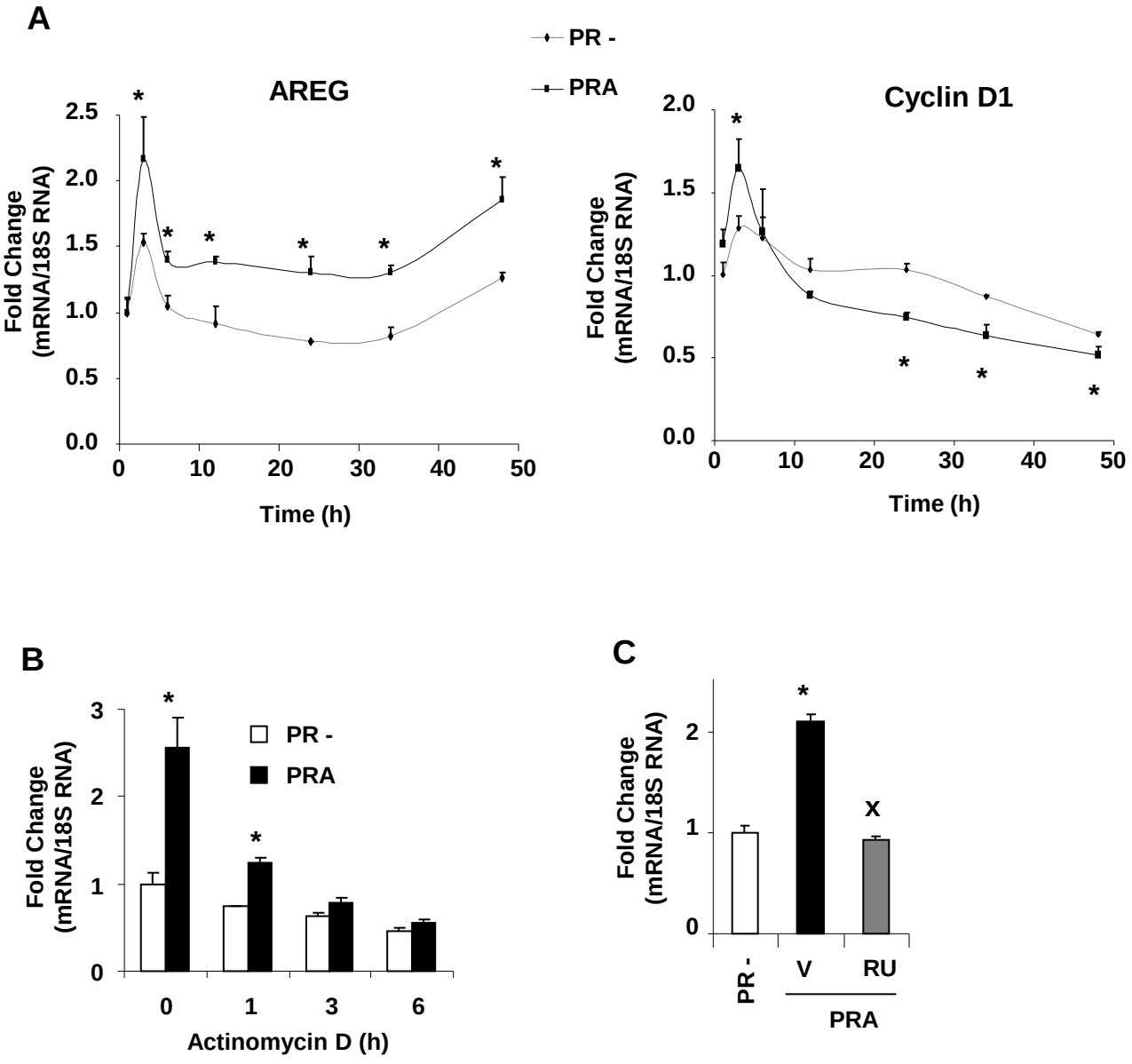

A
PR -
PRA
AREG
Fold Change (mRNA/18S RNA)
Time (h)
Cyclin D1
Fold Change (mRNA/18S RNA)
Time (h)
B
PR -
PRA
Fold Change (mRNA/18S RNA)
Actinomycin D (h)
C
Fold Change (mRNA/18S RNA)
X
PR -
V
RU
PRA

Table 1. Total PR levels measured by ligand binding assay

Cells	PR femtomole/mg total protein
Parental MDA-MB-231	7.22
iPRAB-38, un-induced	14.48
iPRAB-38, PRA induced	318.66
iPRAB-38, PRB induced	186.93

TABLE 2: Ligand-free and liganded PR isoforms-dependent genes present in the cellular growth and proliferation functional group

	A-O-	B-O-	AB-O-	A+A-	B+B-	AB+AB-
p-val range	**1.42E-05-3.12E-02**	**7.58E-04-4.97E-02**	**1.41E-05-2.43E-02**	**8.26E-14-1.3E-02**	**4.7E-10-2.4E-02**	**2.24E-16-9.78E-03**
-log(highest p-val)	**4.85**	**3.12**	**4.85**	**13.08**	**9.33**	**15.65**
Cellular Growth and Proliferation	JAK1, GLI2, CXCL1, HES1, POLM, CD63, ODC1, SERPINB2, IL7R, TBRG1, EZR, DLST, ITGAV, LAMA1, SAA1, ATM, PTX3, PDE2A, ADAMTS1, THBS1, RGS4, **HBEGF**, ANXA2, NFKB2, ATP8A2, MLL, **AREG**, MYL9 (includes EG:10398), CAPN1, BCL2A1, JAG1, LOX, IL1A, ICAM1, BMPR2, GPC4, SYNM, TRA2A, COL6A1, JUN, ARNTL2, EDN1, VIPR1, FASN, TGFB2, CASP1, HEXB, CASP8, OXTR, TPMT, LAMA5, RELB, BCL3, PDAP1, EMP1, GPR56, PMP22, ARF1, ESM1, CAT, NR4A1, IL1B, MST1R, CTSC, PTPN22, MMP9, IL11	TRA2A, PFKFB3, ARNTL2, KIF20B, BRCA2, MCTS1, CTNNB1, MLL, ATM	DBF4B, GLI2, CXCL1, CD63, POLM, SERPINB2, TBRG1, EGR2, ITGAV, LAMA1, SMYD4, FOSL1, PRLR, SPOCK1, SAA1, PDGFRB, ATM, PTX3, PDE2A, PTPRG, METTL3, ADAMTS1, THBS1, CABLES1, **HBEGF**, RGS4, ANXA2, NFKB2, INHBB, ATP8A2, MLL, **AREG**, MYL9 (includes EG:10398), TNS4, ANKRD1, ADAM12, CAPN1, HAS2, RBBP6 (includes EG:5930), JAG1, BCL2A1, VHL, S100P, BUB3, LOX, ICAM1, MCTS1, BCAR1, SYNM, TRA2A, COL6A1, ARNTL2, EDN1, MYBL1, VIPR1, FASN, TGFB2, CASP1, HEXB, FABP3, CTNNB1, GJB2, OXTR, TPMT, PPP2R5C, IL3RA, RELB, BCL3, EMP1, GPR56, ARF1, SSTR2, CAT, ESM1, NR4A1, IL1B, MST1R, CTSC, CSF2, PTPN22, MMP9, IL11	NF2, IL6, SERPINB2, LAMC1, MT2A, EZR, FOSL1, CD274, IGFBP1, SPOCK1, ICOSLG, PDE2A, PTCH1, **HBEGF**, IER3, TFPI2, IRS1, ITPKC, DACH1, HAS2, RPS6KA1, BCL2A1, ENC1, NRP1, DUSP6, BMPR2, GPC4, SYNM, TGM2, JUN, PTPRJ, F2RL1, MYBL1, FGF18, HNRNPA0, PRKCE, GJB2, PLAT, TACSTD2, OXTR, HEY1, CYP1A1, PPP1R15A, TOB1, F3, CYP1B1, SPRY2, CDKN1A, SEC14L2, S1PR1, NR4A1, CSF2, PHLDA1, SOCS3, PER1, JAK1, LIF, PTHLH, CXCL1, BCL6, IL7R, DLST, LAMA1, ITGAV, SMYD4, PRLR, CDKN1C, KLF2, TNFRSF11B, PTX3, IL8, ADAMTS1, EPOR, CABLES1, LAMA2, IL6R, FOXC1, HAS3, RCAN1, INHBA, CBLB, TLR2, BCL2L1, ADAM12, CEBPD, IGFBP3, JAG1, CST6, EREG, IL1A, ID2, MMP14, SOX9, DKK3, EDN2, STEAP2, HSD11B2, NAMPT, TPMT, TFAP2C, EPAS1, CXCR4, TNFRSF11A, MT1A, PDAP1, COL1A1, PMP22, LPAR1, PUS7L, ESM1, CALCR, IL1B, DKK1, PTPRR, IL11, WNT5A	SOCS1, NF2, HES1, SERPINB2, MT2A, EZR, CD274, FOSL1, ICOSLG, METTL3, **HBEGF**, IER3, **AREG**, IRS1, ITPKC, HAS2, CTDSPL, ENC1, NRP1, GPC4, PTPRJ, FGF18, MYBL1, PRKCE, SERPINA1, RASSF5, CASP8, FKBP5, OXTR, BMP1, PLAT, RGS16, PPP1R15A, TOB1, FRK, CYP1B1, F11R, SPRY2, CDKN1A, SEC14L2, S1PR1, CSF2, PHLDA1, SOCS3, PER1, JAK1, PTHLH, CXCL1, BCL6, CD163, CDKN1C, KLF2, ADRA1B, TNFRSF11B, EIF5A2, PTX3, IL8, ADAMTS1, IL6R, CDK6, MALT1, FOXC1, INHBA, RCAN1, RASD1, TLR2, CBLB, BCL2L1, CEBPD, MAPRE2, SLC7A7, RBBP6 (includes EG:5930), FGF5, LOX, ID2, IL1A, SOX9, COL6A1, EDN1, ARNTL2, STEAP2, HSD11B2, NAMPT, PTGER4, TPMT, TFAP2C, RELB, MT1A, PDAP1, TLR4, FOXO1, LPAR1, ESM1, CALCR, IL1B, DKK1, PTPRR, IL11, PIM2 (includes EG:11040)	SOCS1, NF2, HES1, SERPINB2, LAMC1, MT2A, CD274, FOSL1, IGFBP1, SPOCK1, AFAP1, ICOSLG, ATM, PDGFRB, ETS1, PDE2A, TNFRSF10B, **HBEGF**, IER3, TFPI2, TNS4, IRS1, ITPKC, DACH1, HAS2, CTDSPL, RPS6KA1, CDC14A, BCL2A1, ENC1, NRP1, DUSP6, SOCS2, GPC4, SYNM, TGM2, JUN, PTPRJ, MYBL1, FGF18, PRKCE, SERPINA1, RASSF5, FABP3, GJB2, PLAT, HEY1, TACSTD2, OXTR, CYP1A1, ITGA2, RGS16, PPP1R15A, TOB1, F3, FRK, CYP1B1, F11R, SPRY2, CDKN1A, SEC14L2, NR4A1, S1PR1, CSF2, PTPN22, PER1, PHLDA1, SOCS3, LIF, PTHLH, CXCL1, CD163, BCL6, IL7R, ITGAV, LAMA1, PRLR, CDKN1C, KLF2, ADRA1B, TNFRSF11B, EIF5A2, PTX3, IL8, PFKFB3, ADAMTS1, EPOR, CABLES1, IL6R, LAMA2, CDK6, MALT1, FOXC1, INHBB, RCAN1, RASD1, HAS3, INHBA, CBLB, TLR2, BCL2L1, ADAM12, CEBPD, IGFBP3, JAG1, CST6, EREG, FGF5, DCBLD2, LOX, IL1A, ID2, IL22RA1, BCAR1, SOX9, COL6A1, ARNTL2, EDN1, DKK3, EDN2, STEAP2, TGFB2, NAMPT, PTGER4, TPMT, TFAP2C, EPAS1, CXCR4, TNFRSF11A, MT1A, EMP1, COL1A1, TLR4, PMP22, GHR, FOXO1, SSTR2, LPAR1, PUS7L, ESM1, CALCR, IL1B, DKK1, CXCL2, CTSC, PTPRR, WNT5A, IL11

Additional file 1:

SUPPLEMENTAL TABLE 1. PRIMERS SEQUENCES

Primer	Sequence (5' to 3')	Gene
FKBP5, F	CCGGAGAACCAAACGGAAA	FK506-binding protein 5
FKBP5, R	TGAATGCCACATCTCTGCAGT	
SGK1, F	GTGGCAATTCTCATCGCTTTC	Serum- and glucocorticoid-regulated kinase 1
SGK1, R	CTTCAGGGTGTTTGCATGCAT	
HB-EGF, F	TGAGCCTCCCAGTGGAAAAT	Heparin-binding EGF-like growth factor
HB-EGF, R	AACATGAGAAGCCCCACGAT	
AREG, F	ACTCTGGGAAGCGTGAACCAT	Amphiregulin
AREG, R	TAGTCATAGTCGGCTCCCGAG	
Cyclin D1, F	ACAGATCATCCGCAAACACG	Cyclin D1
Cyclin D1, R	TCTGGAGAGGAAGCGTGTGA	
18S-rRNA, F	GTGCATGGCCGTTCTTAGTTG	18S rRNA
18S-rRNA, R	CATGCCAGAGTCTCGTTCGTT	

Figure S1: The strategy employed for conditional PR isoforms expression from bi-inducible promoter.

The coding sequences of the regulatory proteins required for RheoSwitch (Rheoreceptor, Rheoactivator) and TRex (Tet Repressor) systems were inserted in the same plasmid named pZX-TR and primary stable cell lines were established as described in *Materials and Methods*. For secondary stable cell lines, PRA or PRB expression was placed under the control of RheoSwitch or TRex system respectively. In uninduced state, PRA or PRB expression is silenced, the addition of RSL1 or Dox selectively induce the transcription of PRA or PRB.

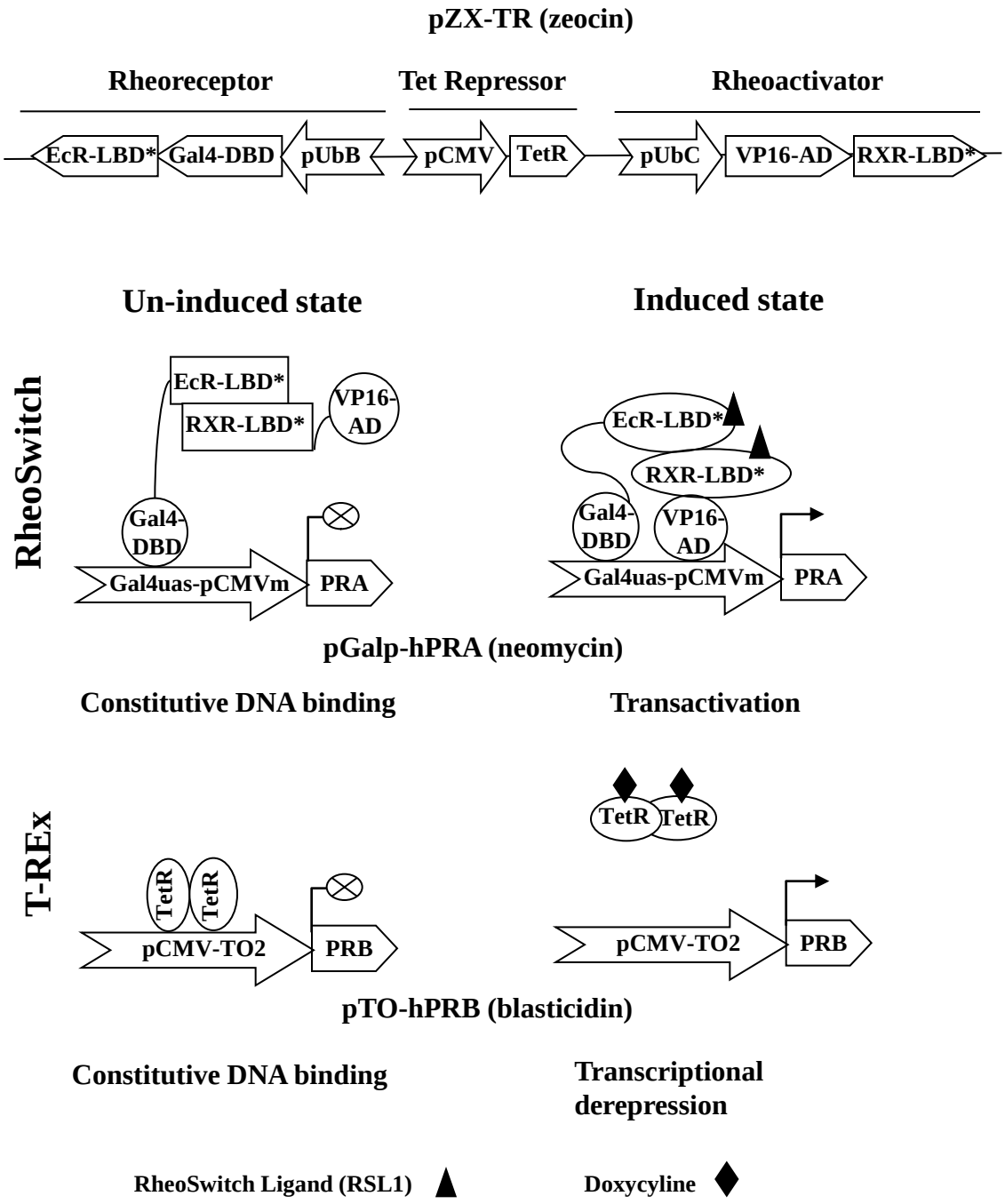


Figure S2 : Inducible PR isoforms undergo agonist-ligand dependent turnover

The iPRAB cells were incubated with indicated inducer during 24 h and then treated with P4 (10 nM) or RU486 (10 nM) for another 24 h. Immunoblot analysis of total PRA and/or PRB or phosphorylated species (pS130-PRA, pS294-PRB) is presented along with tubulin sample loading control.

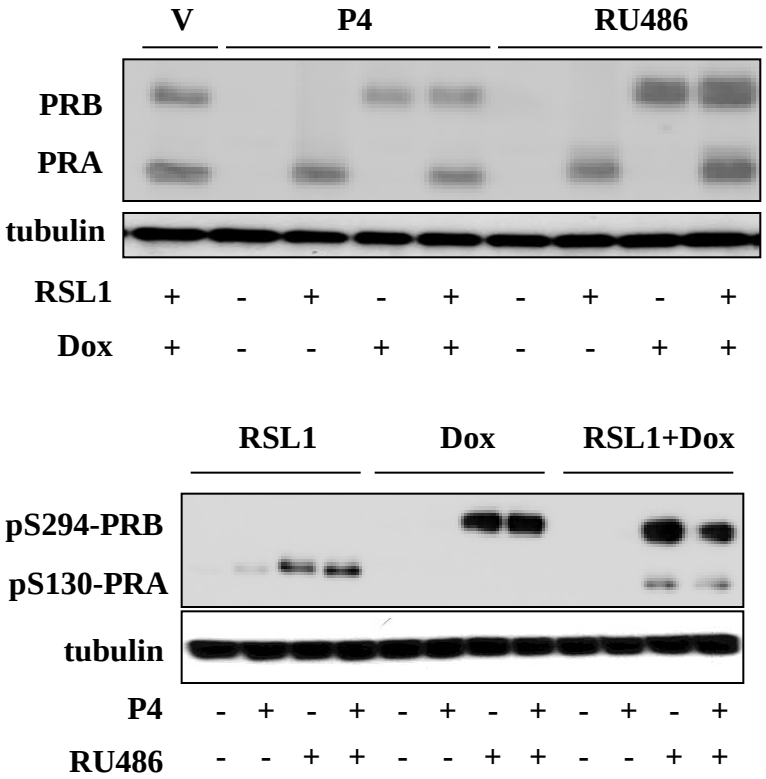


Figure S3 : The character of iPRAB cells for conditionally expressing PR isoforms is conserved following 22 passages.

The iPRAB cells were cultured upto 22 passages and immunoblot analysis was performed following 24 h of exposure to vehicle or indicated inducer(s) as described in *Materials and Methods*.

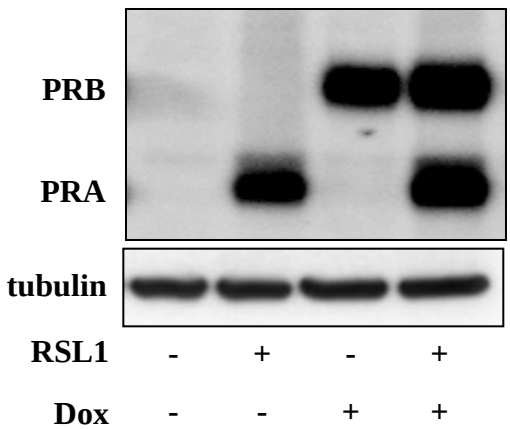


Figure S4 : RSL1 did not induce AREG expression in parental MDA-MB-231 or clone 250 cells.

Parental MDA-MB-231 (PR-) or clone 250 cells were cultured in the presence of vehicle or RSL1 (0.5 µM) during 24 h and qRT-PCR analysis was performed for AREG transcript levels as described in *Materials and Methods*.

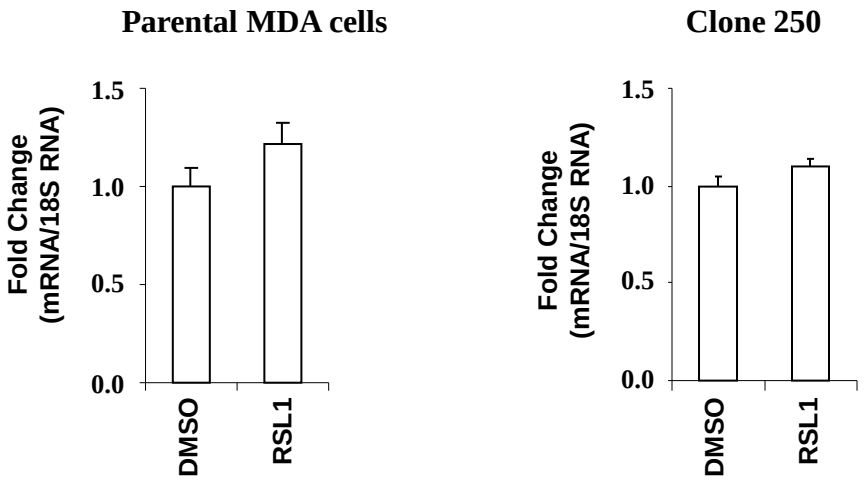
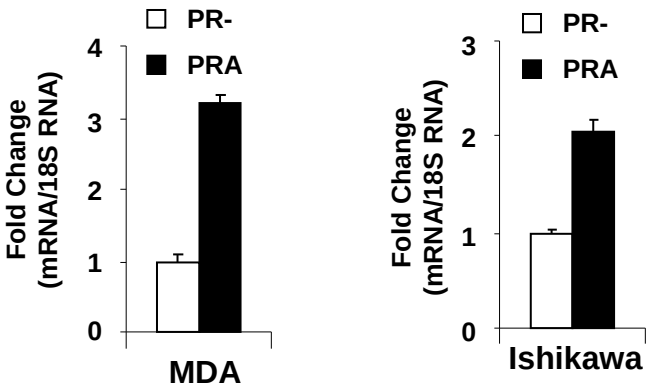


Figure S5 : MDA-MB-231 or Ishikawa cells stably expressing PRA have elevated AREG mRNA levels as compared to PR- cells.

MDA-MB-231 or Ishikawa cells stably expressing PRA or not were cultured under similar conditions and qRT-PCR analysis was performed for AREG transcript levels as described in *Materials and Methods*. The data (mean ± SEM) from three independent cell cultures measured in duplicate is presented as fold change in AREG transcript levels in PRA expressing cells as compared to that in PR- cells.



Additional file 3:
Known PR-regulated genes from previously published microarray studies

publication N°	
1	Richer et al 2002 J Biol Chem 277, 5209-5218
2	Jacobsen et al 2005 Mol Endocrinol 19, 574-587
3	Graham et al 2005 Mol Endocrinol 19, 2713-2735
4	Bray et al 2005 J Steroid Biochem Mol Biol 97, 328-341
5	Leo et al 2005 Int J Cancer 117, 561-568
6	Tung et al 2006 Mol Endocrinol 20, 2656-2670
7	Paulssen et al 2008 Steroid 73, 116-128
8	Afhuppe et al 2009 J Steroid Biochem Mol Biol 113, 105-115

total	1014
published	177
new	837

- = no ligand
+ = progesterone
± = mixed specificities
FC = fold change

publication N°	1	2	2	3	4	5	6	7	8	Khan et al, 2011	Khan et al, 2011
Cells	T47D YA;YA	T47D YA;YA	T47D YA;YA	T47D	T47DC0	MDA-MB-231	T47D	Ishikawa	T47D	MDA-MB-231	MDA-MB-231
PR isoforms	PRA;PRB	iPRA;iPRB	iPRA;iPRB	iPRA/PRB=5	PRA;PRB	PRA+PRB	PRA;PRB;PRB*	PRA+PRB	PRA+PRB	iPRA;iPRB;iPRAB	iPRA;iPRB;iPRAB
ligand	P4	no	P4	ORG2058	P4	P4	P4	P4	R5020	no	P4
total genes	69	7	15	47	4	51	67	33	2		

	Entrez gene ID	Gene symbol	FC	FC	FC	FC	FC	FC	FC	FC	FC	FC	FC	Class
1	22885	ABLIM3	; 4,6						;4,6;			0,0;0,0;0,0	+1,6;+2,2;+1,5	A+&B+&AB+
2	9510	ADAMTS1						-3.3				+1,8;0,0;+1,7	-2,2;-1,9;-3,1	A±&B±&AB±
3	79026	AHNAK	; 1,5				1.71		;1,5;			-1,5;0,0;0,0	+1,5;0,0;0,0	A±
4	347902	AMIGO2	; 1,6					-6.0	;;1,6		−31,8	-1,7;0,0;-1,6	-1,6;-1,7;-1,9	A±&B±&AB±
5	309	ANXA6				-2.02						0,0;0,0;0,0	0,0;+1,6;0,0	B+
6	316	AOX1						3.0				+2,5;0,0;+2,4	0,0;0,0;0,0	A-&AB-

7	360	**AQP3**			1,9;							-1,8;0,0;-1,4	+2,0;0,0;+1,7	A±&AB±
8	374	**AREG**	; 2,0			-2.99	-1.81		;;2,0			+1,3;0,0;+1,4	0,0;+2,0;0,0	A±&B±&AB±
9	84159	**ARID5B**	; 1,8						;;1,8			0,0;0,0;0,0	0,0;-1,6;0,0	B+
10	476	**ATP1A1**	; 2,4			6.76	3.47		;2,4;	1.6		0,0;0,0;0,0	0,0;+1,8;+1,4	B+&AB+
11	5205	**ATP8B1**	; 1,5						;1,5;			0,0;0,0;0,0	-1,6;0,0;-1,6	A+&B+
12	9531	**BAG3**								1.6		0,0;0,0;0,0	0,0;+1,5;+1,6	B+&AB+
13	10458	**BAIAP2**	1,8;						1,8;1,8;1,6			0,0;0,0;0,0	+1,7;+2,0;+2,3	A+&B+&AB+
14	602	**BCL3**	; 1,5						;;1,5			-1,7;0,0;-1,4	0,0;0,0;0,0	A-&AB-
15	604	**BCL6**	2,3; 2,4		2,1; 1,5		2.41		1,6;;			0,0;0,0;0,0	+1,4;+2,6;+2,4	A+&B+&AB+
16	330	**BIRC3**	; ~7,2		; 2.O							-1,8;0,0;-1,8	0,0;0,0;0,0	A-&AB-
17	79686	**C14orf139**						6.5				0,0;0,0;0,0	0,0;+1,6;0,0	B+
18	760	**CA2**								-1.5		+1,7;0,0;+1,9	0,0;+1,4;0,0	A±&B±&AB±
19	799	**CALCR**						5.4				0,0;0,0;0,0	+10,;+16,;+16,	A+&B+&AB+
20	868	**CBLB**	; 2,4						;;2,4			0,0;0,0;0,0	+1,5;+1,6;+1,8	A+&B+&AB+
21	9034	**CCRL2**						-3.3				+1,4;0,0;+1,4	-1,7;-1,4;-1,9	A±&B±&AB±
22	8556	**CDC14A**						2.2				0,0;0,0;0,0	0,0;0,0;+2,6	AB+
23	1026	**CDKN1A**	; 2,0	2,1; 1,4	1,2;				;2,0;1,8			0,0;0,0;0,0	+2,0;+2,6;+3,0	A+&B+&AB+
24	1028	**CDKN1C**	; 6,4				2.48		;6,4;			0,0;0,0;0,0	+1,8;+1,4;+2,0	A+&B+&AB+
25	1052	**CEBPD**	2,0;		; 1,9		4.37	4.0	2,0;4,3;	1.4		0,0;0,0;0,0	+2,8;+5,2;+4,2	A+&B+&AB+
26	1063	**CENPF**				-3.91		-4.0				0,0;-1,6;0,0	0,0;0,0;0,0	B-
27	9076	**CLDN1**					-1.96					0,0;0,0;0,0	0,0;0,0;-2,3	AB+
28	1291	**COL6A1**		2,9; 2,5								-1,6;0,0;-1,4	0,0;+1,4;+1,4	A±&B±&AB±
29	1295	**COL8A1**						4.3				0,0;0,0;0,0	+1,7;0,0;0,0	A+
30	64764	**CREB3L2**					2.46					0,0;0,0;0,0	0,0;+1,8;+1,4	B+&AB+
31	1437	**CSF2**						-5.8				0,0;0,0;-1,6	-1,9;-1,8;-2,7	A±&B±&AB±
32	1545	**CYP1B1**	; 3,0						;;3,0	-6.2		0,0;0,0;0,0	-2,0;-1,4;-2,1	A+&B+&AB+
33	54541	**DDIT4**								1.9		0,0;0,0;0,0	-1,5;0,0;-1,6	A+&B+
34	9732	**DOCK4**						-3.0				+1,6;0,0;+1,7	-1,4;-1,3;-1,6	A±&B±&AB±
35	1808	**DPYSL2**	; 2			2.51						+1,6;0,0;+1,4	0,0;0,0;0,0	A-&AB-
36	667	**DST**	; 1,8						;;1,8			+1,7;0,0;+2,2	0,0;0,0;0,0	A-&AB-
37	1843	**DUSP1**					3.01	7.2				0,0;0,0;0,0	+1,6;+1,3;+1,9	A+&B+&AB+
38	1848	**DUSP6**						3.2		2		0,0;0,0;0,0	+1,8;0,0;+2,1	A+&B+
39	1906	**EDN1**				1.10						-1,7;0,0;-1,4	0,0;-1,6;-1,5	A±&B±&AB±
40	80303	**EFHD1**				1.99						0,0;0,0;-1,7	0,0;0,0;0,0	AB-
41	22936	**ELL2**		1,8;	; 2	2.41		3.3				0,0;0,0;0,0	0,0;+1,6;+1,5	B+&AB+
42	2012	**EMP1**						-3.6				+1,5;+1,4;+2,2	0,0;0,0;-1,5	A±&B±&AB±
43	8507	**ENC1**	; 2,9	2,1;		3.11			;2,9;1,9			0,0;0,0;0,0	-1,4;-1,6;-1,7	A+&B+&AB+

44	2034	**EPAS1**				-1.71						0,0;0,0;0,0	+1,4;0,0;+1,7	A+&B+
45	54566	**EPB41L4B**						4.3				0,0;0,0;0,0	+1,7;+4,8;+3,7	A+&B+&AB+
46	2150	**F2RL1**	2,3;		; 2,3				2,3;;1,8			0,0;0,0;0,0	-1,9;0,0;0,0	A+
47	2152	**F3**	; ~18,1				15.83		;6,2;			0,0;0,0;0,0	-2,5;0,0;-2,4	A+&B+
48	2194	**FASN**				1.36						+1,6;0,0;+1,8	0,0;0,0;0,0	A-&AB-
49	8817	**FGF18**						6.6		-1.3		0,0;0,0;0,0	+2,5;+4,0;+3,6	A+&B+&AB+
50	2289	**FKBP5**	3,3; 9,4		2,6; 6,2	6.78		12.6	;6,4;	1.8		0,0;0,0;0,0	0,0;+2,3;0,0	B+
51	8061	**FOSL1**						-3.4				0,0;0,0;-1,4	-1,5;-1,7;-2,5	A±&B±&AB±
52	3169	**FOXA1**	; 2,2						;2,2;2,1			0,0;0,0;0,0	+1,6;0,0;+1,4	A+&B+
53	2296	**FOXC1**			; 2,5			4.5				0,0;0,0;0,0	+1,7;+1,6;+2,3	A+&B+&AB+
54	2535	**FZD2**	; 1,5						;;1,5			0,0;0,0;0,0	-1,5;0,0;-1,6	A+&B+
55	7855	**FZD5**									3.30	0,0;0,0;0,0	+2,0;0,0;+2,3	A+&B+
56	50486	**G0S2**				-1.84						0,0;0,0;0,0	-2,0;-1,9;-2,1	A+&B+&AB+
57	23710	**GABARAPL1**	; 2,3						;;2,3			0,0;0,0;0,0	+1,8;0,0;+2,0	A+&B+
58	23766	**GABARAPL3**					2.7					0,0;0,0;0,0	+1,6;0,0;+1,7	A+&B+
59	2690	**GHR**				1.52						0,0;0,0;0,0	0,0;0,0;+1,6	AB+
60	2706	**GJB2**				0.11						0,0;0,0;-1,8	+1,8;0,0;+2,1	A±&AB±
61	9289	**GPR56**					-1.67					+1,8;0,0;+1,9	0,0;0,0;0,0	A-&AB-
62	3280	**HES1**	; 2,1						;2,1;1,8			-1,3;0,0;0,0	0,0;-1,4;-1,7	A±&B±&AB±
63	3096	**HIVEP1**	1,9;				-2.14		1,9;1,8;2,0			-1,6;0,0;0,0	0,0;-1,4;-1,4	A±&B±&AB±
64	9953	**HS3ST3B1**									5.70	0,0;0,0;0,0	0,0;+1,8;+2,0	B+&AB+
65	9394	**HS6ST1**								-1.4		0,0;0,0;0,0	0,0;+1,5;+1,4	B+&AB+
66	3291	**HSD11B2**	~6,5; ~22,6			6.72	11.09	6.1	;7,1;			0,0;0,0;0,0	+1,3;+2,2;0,0	A+&B+
67	29094	**HSPC159**						4.1				0,0;0,0;0,0	+1,5;0,0;+1,7	A+&B+
68	3383	**ICAM1**				2.01						-1,4;0,0;-1,6	0,0;0,0;0,0	A-&AB-
69	3398	**ID2**								1.4		0,0;0,0;0,0	+1,4;+2,1;+1,7	A+&B+&AB+
70	9592	**IER2**	; 1,5			1.24			;;1,5			0,0;0,0;0,0	-1,5;-1,5;-2,0	A+&B+&AB+
71	8870	**IER3**	1,7;					-3.3	1,7;;2,1			0,0;0,0;0,0	-1,6;-1,5;-2,3	A+&B+&AB+
72	3576	**IL8**						-6.5				0,0;0,0;0,0	-2,8;-2,7;-5,6	A+&B+&AB+
73	3614	**IMPDH1**	; 1,7						;1,7;			0,0;0,0;0,0	0,0;+1,9;0,0	B+
74	3625	**INHBB**					1.59					0,0;0,0;-1,7	0,0;0,0;+2,0	AB±
75	3667	**IRS1**	; 3,8					-4.4	;;3,8			0,0;0,0;0,0	-1,4;-1,5;-1,8	A+&B+&AB+
76	3706	**ITPKA**						3.7				0,0;0,0;0,0	+1,5;+3,8;+2,1	A+&B+&AB+
77	182	**JAG1**				0.01						-1,4;0,0;-1,3	+1,4;0,0;+1,7	A±&AB±
78	3725	**JUN**				2.32	2.04					-1,3;0,0;0,0	-1,6;0,0;-1,7	A±&AB±
79	9764	**KIAA0513**	; 2,4						;2,4;			0,0;0,0;0,0	+1,5;0,0;+1,5	A+&B+
80	687	**KLF9**	; 3,9						;3,9;2,3			0,0;0,0;0,0	+2,1;+2,9;+2,3	A+&B+&AB+

81	3866	**KRT15**						-6.8				+1,7;0,0;+1,7	0,0;0,0;0,0	A-&AB-
82	3872	**KRT17**								-1.8		+1,7;0,0;+1,4	0,0;0,0;-1,3	A±&AB±
83	8942	**KYNU**	; 2,0			-3.69		-4.3	;;2,0			+2,0;0,0;0,0	0,0;0,0;0,0	A-
84	26468	**LHX6**					2.64					0,0;0,0;0,0	+1,9;+2,3;+2,4	A+&B+&AB+
85	4001	**LMNB1**						-3.2				-2,9;-1,9;0,0	+2,9;0,0;0,0	A±&B±
86	8543	**LMO4**	1,9;			0.05	-1.62		1,9;2,3;1,8	1.6		-1,5;0,0;0,0	+1,6;0,0;0,0	A±
87	23175	**LPIN1**	; 1,6						;;1,6			0,0;0,0;0,0	-1,4;0,0;-1,5	A+&B+
88	4038	**LRP4**						3.7				0,0;0,0;0,0	0,0;+1,8;+2,0	B+&AB+
89	131578	**LRRC15**				1.06						0,0;0,0;0,0	0,0;0,0;+2,1	AB+
90	5606	**MAP2K3**		1,7; 1,9	; -1,7							0,0;0,0;0,0	0,0;-1,6;0,0	B+
91	4286	**MITF**	; 2,1						;;2,1			+1,5;0,0;+1,7	0,0;0,0;-1,4	A±&AB±
92	4297	**MLL**					1.09					+1,6;+1,4;+2,1	0,0;0,0;0,0	A-&B-&AB-
93	4312	**MMP1**						-4.2				+3,5;0,0;+3,4	-1,5;-1,3;-1,6	A±&B±&AB±
94	4323	**MMP14**				-4.44						0,0;0,0;0,0	+1,7;0,0;0,0	A+
95	4486	**MST1R**	; 1,5			-3.14			;;1,5			+1,6;0,0;+1,7	0,0;0,0;0,0	A-&AB-
96	4489	**MT1A**								2.8		0,0;0,0;0,0	+1,6;+1,5;+1,8	A+&B+&AB+
97	4490	**MT1B**				0.14				2.8		0,0;0,0;0,0	+1,7;+1,6;+2,0	A+&B+&AB+
98	4493	**MT1E**	1,6;						1,6;;	2		0,0;0,0;0,0	+1,9;+1,8;+2,1	A+&B+&AB+
99	4495	**MT1G**				0.22				2.7		0,0;0,0;0,0	+1,7;+1,7;+1,9	A+&B+&AB+
100	4496	**MT1H**	1,6;				3.08		1,6;;	3		0,0;0,0;0,0	+1,6;+1,6;+1,9	A+&B+&AB+
101	4501	**MT1X**				0.29	4.16			3		0,0;0,0;0,0	+1,8;+1,6;+1,9	A+&B+&AB+
102	4502	**MT2A**								3.2		0,0;0,0;0,0	+1,7;+1,6;+1,8	A+&B+&AB+
103	10398	**MYL9**				-2.05				-1.3		-1,8;0,0;-1,7	0,0;0,0;0,0	A-&AB-
104	89795	**NAV3**						-3.6				0,0;0,0;0,0	-1,6;-2,2;-2,7	A+&B+&AB+
105	23327	**NEDD4L**					1.91					0,0;0,0;0,0	-2,0;-1,4;-2,3	A+&B+&AB+
106	9520	**NPEPPS**				-1.40						0,0;0,0;0,0	-1,6;0,0;0,0	A+
107	8620	**NPFF**				-1.63						0,0;0,0;+1,7	0,0;0,0;0,0	AB-
108	3164	**NR4A1**								1.6		-1,4;0,0;-1,3	+1,6;0,0;+1,6	A±&AB±
109	4907	**NT5E**						-3.4				0,0;0,0;0,0	-1,3;-1,5;-1,6	A+&B+&AB+
110	4953	**ODC1**				2.12				-1.3		+1,6;0,0;0,0	0,0;0,0;0,0	A-
111	5021	**OXTR**						6.0				-1,4;0,0;-1,4	+1,3;+3,6;+2,1	A±&B±&AB±
112	10606	**PAICS**				-2.91						0,0;+1,3;0,0	0,0;-1,6;0,0	B±
113	5166	**PDK4**				2.11						0,0;0,0;0,0	0,0;+2,0;+1,9	B+&AB+
114	9124	**PDLIM1**	; 2				1.91					0,0;0,0;0,0	0,0;+1,9;+1,3	B+&AB+
115	5187	**PER1**	; 1,7						;1,7;	1.3		0,0;0,0;0,0	+1,7;+2,6;+2,0	A+&B+&AB+
116	5209	**PFKFB3**	2,2;						2,2;;			0,0;-2,0;0,0	0,0;0,0;-1,3	B±&AB±
117	23338	**PHF15**	1,8;			0.19			1,8;;			0,0;0,0;+1,7	0,0;0,0;0,0	AB-

118	22822	**PHLDA1**						-2.9				0,0;0,0;0,0	-2,3;-1,5;-2,8	A+&B+&AB+
119	8503	**PIK3R3**	; 1,9						;1,9;1,7			0,0;0,0;0,0	+2,1;0,0;0,0	A+
120	11040	**PIM2**			; 1,9	0.50						0,0;0,0;0,0	0,0;+1,6;0,0	B+
121	8395	**PIP5K1B**	; 1,5		; 2,2				;1,5;			0,0;0,0;0,0	+1,3;+1,5;+1,3	A+&B+&AB+
122	8544	**PIR**					-1.39					-1,7;0,0;-1,7	0,0;0,0;0,0	A-&AB-
123	5318	**PKP2**						5.2				0,0;0,0;0,0	0,0;+2,0;0,0	B+
124	22874	**PLEKHA6**	; 1,5						;;1,5			0,0;0,0;0,0	0,0;0,0;+1,6	AB+
125	10769	**PLK2**	2,3;					-3.7	2,3;;3,3			0,0;0,0;0,0	0,0;-1,7;-1,6	B+&AB+
126	5409	**PNMT**	; 4,4						1,9;;			0,0;0,0;0,0	+1,5;+3,7;+2,2	A+&B+&AB+
127	23509	**POFUT1**								-1.3		0,0;0,0;0,0	0,0;-2,0;0,0	B+
128	57460	**PPM1H**	; 1,8						;1,8;			0,0;0,0;0,0	0,0;0,0;+1,6	AB+
129	23645	**PPP1R15A**								1.4		0,0;0,0;0,0	-1,3;-1,4;-1,5	A+&B+&AB+
130	5618	**PRLR**	; 1,6	2; 1,3					;1,6;3,3			0,0;0,0;+1,3	-2,1;0,0;-1,7	A±&AB±
131	5744	**PTHLH**						3.5			n,c,	0,0;0,0;0,0	+3,1;+3,5;+4,6	A+&B+&AB+
132	5795	**PTPRJ**				0.17						0,0;0,0;0,0	+1,6;+1,8;+1,8	A+&B+&AB+
133	5801	**PTPRR**						-3.4				0,0;0,0;0,0	-2,1;-2,4;-3,9	A+&B+&AB+
134	5874	**RAB27B**	3,2;		; -1,6				3,2;;3,5			0,0;0,0;0,0	-1,5;0,0;-1,8	A+&B+
135	9770	**RASSF2**	; 10,2		; 3,4			4.8	;8,6;1,7			0,0;0,0;0,0	+1,3;+3,0;+2,0	A+&B+&AB+
136	5971	**RELB**				2.37						-1,5;0,0;-1,5	0,0;-1,4;0,0	A±&B±&AB±
137	5999	**RGS4**						-6.7				+1,6;0,0;+1,6	0,0;0,0;0,0	A-&AB-
138	58480	**RHOU**								1.5		0,0;0,0;-1,3	0,0;+3,2;+1,9	B±&AB±
139	6195	**RPS6KA1**					1.58					0,0;0,0;0,0	+1,3;0,0;+1,6	A+&B+
140	6196	**RPS6KA2**				0.38		4.7				0,0;0,0;0,0	0,0;0,0;+1,6	AB+
141	6286	**S100P**	2,4; 3,6		; 1,6	0.71	10.53		;4,0;			0,0;0,0;-1,5	0,0;0,0;0,0	AB-
142	8436	**SDPR**						3.6				0,0;0,0;0,0	+2,3;+5,2;+4,1	A+&B+&AB+
143	23541	**SEC14L2**					2.07	4.6				0,0;0,0;0,0	+1,8;+3,7;+2,5	A+&B+&AB+
144	9871	**SEC24D**					2					0,0;0,0;0,0	+2,5;0,0;+2,6	A+&B+
145	26168	**SENP3**				0.56						+1,7;+1,6;+2,0	0,0;0,0;0,0	A-&B-&AB-
146	5265	**SERPINA1**		9,8;								0,0;0,0;0,0	0,0;+1,6;+1,4	B+&AB+
147	6478	**SIAH2**	1,5;						1,5;;			+1,6;0,0;+1,5	0,0;0,0;0,0	A-&AB-
148	9121	**SLC16A5**	1,5;						1,5;;			0,0;0,0;0,0	+1,5;0,0;+1,6	A+&B+
149	6584	**SLC22A5**	1,8;						1,8;;			0,0;0,0;0,0	+1,5;+1,7;+1,8	A+&B+&AB+
150	9056	**SLC7A7**						6.5				0,0;0,0;0,0	0,0;+4,6;0,0	B+
151	10924	**SMPDL3A**						3.0				0,0;0,0;0,0	+1,4;+1,4;+1,9	A+&B+&AB+
152	9580	**SOX13**	; 1,8						;;1,8			0,0;0,0;0,0	+1,5;0,0;0,0	A+
153	6662	**SOX9**				0.27	1.52	-3.0				0,0;0,0;0,0	-2,0;-1,4;-2,2	A+&B+&AB+
154	58472	**SQRDL**					1.98					+1,7;0,0;+1,6	0,0;0,0;+1,5	A±&AB±

155	10847	**SRCAP**	; 2,3						;2,3;			0,0;0,0;0,0	+1,9;0,0;+1,7	A+&B+
156	6764	**ST5**						3.3				0,0;0,0;0,0	0,0;+1,5;0,0	B+
157	11037	**STON1**									−8,7	0,0;0,0;0,0	+1,8;0,0;+1,5	A+&B+
158	10579	**TACC2**						2.9		1.4		0,0;0,0;0,0	+1,7;+1,8;+3,0	A+&B+&AB+
159	27097	**TAF5L**	; 1,5				1.55		;1,5;1,5			0,0;0,0;0,0	+1,7;0,0;0,0	A+
160	6876	**TAGLN**								-1.6		-1,6;0,0;-1,6	0,0;0,0;0,0	A-&AB-
161	30851	**TAX1BP3**								-1.3		0,0;-1,7;0,0	0,0;+1,9;0,0	B±
162	55357	**TBC1D2**								-1.4		0,0;0,0;0,0	-1,8;0,0;-2,1	A+&B+
163	7022	**TFAP2C**	; 2,0			0.01			;2,0;			0,0;0,0;0,0	-1,8;-1,4;-2,1	A+&B+&AB+
164	7057	**THBS1**								-1.4		-1,5;0,0;-1,5	0,0;0,0;0,0	A-&AB-
165	25976	**TIPARP**	; 9,7				2.09		;9,7;			0,0;0,0;0,0	0,0;+2,6;+1,3	B+&AB+
166	7099	**TLR4**				2.22						0,0;0,0;0,0	0,0;-1,7;-1,4	B+&AB+
167	8795	**TNFRSF10B**	; 1,6			,d,			;1,6;2,1			0,0;0,0;0,0	0,0;0,0;+1,6	AB+
168	10318	**TNIP1**					2.23					0,0;0,0;0,0	+1,4;+1,4;+1,6	A+&B+&AB+
169	79155	**TNIP2**					1.26					0,0;0,0;0,0	0,0;+1,5;0,0	B+
170	146691	**TOM1L2**	; 1,7						;;1,7			+1,6;0,0;+1,7	0,0;0,0;0,0	A-&AB-
171	1831	**TSC22D3**	3,1;						3,1;3,4;			0,0;0,0;0,0	0,0;+2,0;+1,8	B+&AB+
172	7296	**TXNRD1**				0.01						0,0;0,0;0,0	0,0;-1,6;-1,3	B+&AB+
173	7357	**UGCG**	~2,9; ~5,6									0,0;0,0;0,0	-1,5;0,0;-1,8	A+&B+
174	7433	**VIPR1**	; 1,8			-1.67			;;1,8			-2,1;0,0;-1,5	0,0;0,0;0,0	A-&AB-
175	7538	**ZFP36**	; 2,4					3.6	;2,4;			0,0;0,0;0,0	+4,3;+1,9;+4,4	A+&B+&AB+
176	677	**ZFP36L1**	; 1,8			2.02			;;1,8	1.5		0,0;0,0;0,0	-1,5;+1,5;0,0	A+&B+
177	7743	**ZNF189**	; 1,7						;1,7;			0,0;0,0;0,0	0,0;+2,3;+1,4	B+&AB+

Additional file 4 :

Complete gene list corresponding to Venn diagram

(2x16 colors ranging from dark green (FC-3) to dark red (FC+3). FC range -1.3 to +1.3 or p-value > 10^{-3} gives white color). PR-regulated genes identified from previously published transcriptomic studies are indicated by *.

Genes regulated by unliganded PRA (A-)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	10537	**UBD**	-1.72	1.13	-1.16	1.05	-1.00	-1.02	-1.13
	2	3911	**LAMA5**	-1.67	-1.01	-1.40	1.07	1.18	1.04	1.15
	3	8714	**ABCC3**	-1.58	1.10	-1.17	-1.06	1.04	-1.07	-1.13
*	4	4953	**ODC1**	1.57	-1.03	1.36	1.03	-1.00	1.09	1.18
*	5	8942	**KYNU**	1.98	1.03	1.38	-1.12	-1.55	1.03	-1.18
	6	84824	**FCRLA**	4.19	-1.03	-1.00	-1.31	-1.10	1.02	1.08
	7	140680	**C20ORF96**	6.94	1.11	-1.01	-1.16	1.34	-1.18	-1.15
	8	439916	**C12ORF37**	8.19	1.00	1.00	1.00	1.00	1.00	1.00

Genes regulated by unliganded PRB (B-)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	1062	**CENPE**	1.03	-1.93	-1.16	-1.23	-1.11	1.01	-1.28
	2	9585	**KIF20B**	-1.08	-1.91	-1.11	-1.19	1.05	-1.08	-1.24
	3	675	**BRCA2**	-1.20	-1.88	-1.26	-1.15	-1.02	-1.19	-1.26
	4	9321	**TRIP11**	1.01	-1.87	-1.18	-1.15	-1.10	-1.10	-1.24
	5	23253	**ANKRD12**	1.01	-1.83	-1.27	-1.13	-1.03	-1.09	-1.03
	6	85444	**LRRCC1**	-1.23	-1.80	-1.20	-1.11	1.03	-1.11	-1.20
	7	10142	**AKAP9**	-1.08	-1.79	-1.24	-1.17	-1.00	-1.03	-1.08
	8	2803	**GOLGA4**	-1.20	-1.79	-1.21	-1.18	1.01	-1.12	-1.15
	9	10111	**RAD50**	-1.47	-1.71	-1.24	-1.05	1.42	1.11	-1.46
	10	22852	**ANKRD26**	-1.09	-1.65	-1.22	-1.08	-1.09	-1.08	-1.12
*	11	1063	**CENPF**	-1.01	-1.64	-1.15	-1.08	-1.01	-1.10	-1.12
	12	54809	**SAMD9**	1.14	-1.61	1.10	-1.11	-1.06	-1.14	-1.25
	13	55196	**C12ORF35**	-1.13	-1.58	-1.03	-1.07	-1.04	1.25	-1.49
	14	22832	**KIAA1009**	-1.18	-1.56	-1.21	-1.01	1.01	1.01	-1.05
	15	151246	**SGOL2**	-1.06	-1.55	-1.14	-1.08	1.05	-1.09	-1.10
	16	55704	**CCDC88A**	-1.01	-1.55	-1.10	-1.13	1.01	-1.07	-1.15
	17	2804	**GOLGB1**	-1.08	-1.55	-1.10	-1.10	-1.01	1.05	-1.11
	18	55075	**UACA**	-1.06	-1.54	-1.13	-1.00	1.38	1.21	1.24
	19	79411	**GLB1L**	1.35	1.51	1.22	1.02	1.04	-1.22	-1.29

Genes regulated by unliganded PRA:PRB (AB-)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	1959	**EGR2**	-1.50	-1.00	-1.87	1.20	1.43	-1.20	1.01
	2	7114	**TMSB4X**	-1.58	1.06	-1.83	1.09	-1.26	-1.15	-1.09
*	3	80303	**EFHD1**	-1.52	1.07	-1.71	-1.14	1.26	1.11	1.20
	4	84958	**SYTL1**	-1.49	1.05	-1.70	1.07	1.05	1.01	1.00
	5	284611	**FAM102B**	-1.09	1.16	-1.68	-1.17	-1.23	-1.25	1.02
	6	6578	**SLCO2A1**	-1.45	1.01	-1.60	1.16	1.34	1.23	1.36
	7	84695	**LOXL3**	-1.45	-1.09	-1.56	1.02	-1.17	1.02	-1.01
	8	3563	**IL3RA**	-1.46	1.00	-1.55	-1.01	1.12	-1.23	1.15
	9	168090	**C6ORF118**	-1.42	-1.01	-1.54	1.04	1.23	1.10	1.22

	10	284677	**C1ORF204**	-1.25	-1.18	-1.53	1.19	1.07	1.04	1.13
	11	283518	**KCNRG**	-1.03	1.13	-1.53	-1.02	-1.06	-1.03	1.08
	12	57482	**KIAA1211**	-1.38	1.01	-1.53	-1.04	1.14	-1.10	1.14
*	13	6286	**S100P**	-1.47	-1.06	-1.52	1.12	1.03	1.18	1.11
	14	112399	**EGLN3**	-1.36	1.04	-1.51	1.04	1.24	1.14	1.24
	15	26050	**SLITRK5**	-1.19	-1.25	-1.50	-1.06	1.15	-1.00	1.10
	16	54102	**CLIC6**	-1.07	-1.08	-1.50	-1.04	1.29	1.02	1.19
	17	6843	**VAMP1**	-1.20	1.03	-1.50	-1.13	1.12	1.18	1.42
	18	148137	**C19orf55**	1.10	1.14	1.51	1.00	-1.01	1.05	1.11
	19	84277	**DNAJC30**	1.33	1.25	1.52	-1.02	1.11	-1.05	1.04
	20	3215	**HOXB5**	1.28	1.13	1.52	1.00	1.05	1.06	1.06
	21	57185	**NIPAL3**	1.45	1.09	1.52	-1.08	1.07	1.18	1.27
	22	90332	**EXOC3L2**	1.22	1.24	1.52	1.01	1.04	-1.03	-1.00
	23	130872	**AHSA2**	1.28	1.13	1.53	1.11	-1.00	1.12	-1.02
	24	7428	**VHL**	1.30	1.25	1.54	-1.02	1.03	-1.07	-1.08
	25	10051	**SMC4**	1.25	-1.04	1.55	-1.00	-1.07	-1.06	-1.26
	26	80174	**DBF4B**	1.21	1.24	1.55	1.00	-1.04	-1.03	-1.15
	27	79802	**HHIPL2**	1.41	1.03	1.56	1.03	1.04	1.09	-1.03
	28	84839	**RAX2**	1.28	1.26	1.56	1.00	1.02	-1.02	-1.05
	29	26013	**L3MBTL1**	1.39	1.02	1.57	1.02	1.02	1.05	-1.01
	30	100132247	**LOC100132247**	1.18	1.21	1.57	1.01	1.12	1.13	-1.00
	31	9266	**CYTH2**	1.36	1.25	1.58	1.05	-1.34	-1.09	-1.19
	32	114041	**C21orf88**	1.42	1.29	1.58	1.00	1.04	-1.02	-1.08
	33	378938	**MALAT1**	1.17	1.21	1.59	1.06	1.17	1.26	1.03
	34	100289949	**LOC100289949**	1.31	1.16	1.60	-1.05	-1.04	-1.03	1.02
	35	9852	**EPM2AIP1**	1.15	1.16	1.61	1.13	1.05	1.12	-1.02
	36	5004	**ORM1**	-1.07	1.22	1.61	-1.07	1.24	1.21	1.03
	37	5829	**PXN**	1.27	1.02	1.62	1.06	-1.10	-1.01	1.04
	38	150759	**LOC150759**	1.36	1.17	1.62	1.05	1.17	1.20	1.11
	39	124975	**GGT6**	1.32	1.31	1.64	-1.00	1.05	-1.06	-1.04
	40	221037	**JMJD1C**	1.43	1.21	1.64	-1.01	1.03	-1.08	1.00
	41	9349	**RPL23**	1.30	1.24	1.64	1.04	-1.05	1.02	-1.09
	42	57498	**KIDINS220**	1.31	1.14	1.64	1.01	1.00	1.39	1.11
*	43	23338	**PHF15**	1.24	1.39	1.65	1.09	-1.07	1.20	1.00
	44	728882	**FAM182B**	1.29	1.02	1.66	-1.04	1.11	-1.10	-1.12
	45	10432	**RBM14**	1.50	1.21	1.67	-1.02	-1.05	-1.07	-1.17
	46	8528	**DDO**	1.31	1.13	1.67	1.05	1.06	-1.22	1.12
	47	1232	**CCR3**	1.46	1.16	1.68	1.37	1.10	-1.11	-1.14
	48	728534	**LOC728534**	1.39	1.25	1.70	1.13	-1.01	1.05	-1.08
	49	5143	**PDE4C**	1.36	1.35	1.70	1.01	1.09	-1.02	-1.05
	50	8552	**INE1**	1.34	1.35	1.71	1.00	1.04	-1.06	-1.09
	51	389741	**MGC21881**	1.46	1.26	1.72	-1.02	1.07	-1.05	1.07
*	52	8620	**NPFF**	1.25	1.21	1.72	-1.02	1.24	1.27	1.17
	53	2686	**GGT7**	1.29	1.25	1.78	1.07	1.24	1.03	1.20
	54	55345	**C4ORF21**	1.23	-1.07	1.79	1.08	-1.05	-1.23	-1.41
	55	100131564	**LOC100131564**	1.50	1.27	1.79	1.20	1.00	1.24	1.01
	56	100133299	**LOC100133299**	1.33	1.33	1.79	-1.05	-1.20	1.02	-1.03
	57	57730	**ANKRD36B**	1.47	1.27	1.82	-1.01	1.16	1.21	1.10
	58	51479	**ANKFY1**	1.32	1.42	1.84	1.01	1.11	1.10	1.12
	59	5527	**PPP2R5C**	1.32	1.25	1.85	1.08	-1.11	1.02	-1.02
	60	11322	**TMC6**	1.06	1.21	1.85	1.08	1.03	-1.09	-1.24

61	124923	**SGK494**	1.50	1.29	1.85	1.03	1.01	-1.28	-1.15
62	50839	**TAS2R10**	1.39	1.27	1.86	-1.05	1.31	1.42	1.28
63	8671	**SLC4A4**	1.60	-1.10	1.87	1.03	-1.11	-1.16	-1.09
64	11129	**SFRS16**	1.29	1.23	1.91	1.00	-1.13	-1.08	-1.27
65	280	**AMY2B**	1.52	1.23	1.94	1.20	-1.12	-1.05	-1.03
66	283663	**LOC283663**	1.45	1.15	1.99	1.06	1.25	1.19	1.20
67	284992	**CCDC150**	1.84	1.32	2.00	-1.02	1.04	1.12	1.10
68	89797	**NAV2**	1.16	1.21	2.04	1.06	-1.09	-1.04	1.19
69	7318	**UBA7**	1.56	1.04	2.08	-1.02	1.10	1.14	1.05
70	80045	**GPR157**	1.67	1.39	2.09	1.11	-1.02	-1.00	1.13
71	142937	**LOC142937**	1.27	1.37	2.33	-1.01	1.09	-1.05	1.02
72	100292160	**LOC100292160**	1.48	1.18	2.52	1.11	1.12	1.37	1.04
73	100190986	**LOC100190986**	1.23	1.54	2.58	-1.08	1.72	1.31	-1.19
74	26069	**DKFZP586B0319**	1.75	1.62	2.74	1.01	1.15	1.14	1.11
75	9639	**ARHGEF10**	1.70	1.43	3.00	-1.02	-1.19	-1.18	-1.28
76	121665	**SPPL3**	1.86	2.26	3.04	1.03	-1.07	-1.03	-1.10

Genes regulated by unliganded PRA and PRA:PRB (A-&AB-)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
*	1	7433	**VIPR1**	-2.14	1.04	-1.52	1.15	1.16	1.06	-1.03
	2	2736	**GLI2**	-2.08	-1.11	-2.54	-1.01	1.17	1.08	1.21
	3	6288	**SAA1**	-2.85	1.01	-2.69	-1.02	1.19	1.02	1.05
	4	6289	**SAA2**	-2.76	1.00	-2.55	-1.02	1.16	1.02	1.05
	5	57214	**KIAA1199**	-2.36	1.07	-2.21	1.08	-1.13	-1.17	1.08
	6	57597	**BAHCC1**	-1.85	-1.01	-2.09	-1.05	1.21	-1.15	1.13
	7	147138	**TMC8**	-1.96	-1.05	-1.90	-1.00	-1.12	-1.12	-1.17
	8	1305	**COL13A1**	-1.72	-1.01	-1.86	1.02	1.02	-1.13	1.00
	9	220963	**SLC16A9**	-1.59	1.01	-1.83	1.02	1.07	-1.13	1.19
	10	3855	**KRT7**	-1.84	1.04	-1.81	1.04	1.04	1.26	1.03
*	11	330	**BIRC3**	-1.77	1.01	-1.80	-1.06	-1.02	1.11	1.02
	12	80755	**AARSD1**	-1.67	-1.09	-1.77	1.08	-1.08	-1.02	1.05
	13	64129	**TINAGL1**	-1.76	-1.02	-1.74	-1.07	1.26	-1.16	1.16
*	14	8544	**PIR**	-1.68	1.01	-1.74	-1.02	1.10	-1.05	1.11
	15	1953	**MEGF6**	-1.99	1.09	-1.72	-1.17	-1.12	-1.28	-1.36
	16	4318	**MMP9**	-1.67	1.03	-1.71	1.03	-1.07	-1.08	-1.10
	17	27063	**ANKRD1**	-1.92	1.04	-1.68	-1.06	1.21	-1.16	-1.10
	18	92241	**RCSD1**	-1.69	1.04	-1.68	1.00	-1.21	-1.09	-1.21
	19	3892	**KRT86**	-1.78	1.05	-1.67	1.01	-1.03	-1.13	-1.06
*	20	10398	**MYL9**	-1.77	-1.06	-1.66	-1.04	1.11	1.23	1.21
	21	725	**C4BPB**	-1.45	-1.09	-1.62	-1.13	-1.07	-1.08	-1.03
	22	94031	**HTRA3**	-1.59	1.04	-1.62	1.03	-1.11	-1.04	-1.10
*	23	3383	**ICAM1**	-1.38	1.03	-1.61	1.03	-1.02	-1.25	-1.03
	24	81621	**KAZALD1**	-1.45	-1.06	-1.60	1.08	1.00	-1.04	-1.07
	25	84171	**LOXL4**	-1.71	-1.02	-1.59	-1.02	-1.09	1.13	1.05
	26	4794	**NFKBIE**	-1.57	1.02	-1.57	1.00	1.00	1.02	1.06
	27	9423	**NTN1**	-1.73	-1.04	-1.57	1.01	1.29	1.19	1.24
	28	8111	**GPR68**	-1.48	1.03	-1.55	1.05	-1.18	1.19	-1.14
*	29	6876	**TAGLN**	-1.61	1.02	-1.55	-1.02	-1.07	-1.03	-1.09
	30	339983	**NAT8L**	-1.41	-1.12	-1.54	-1.02	1.17	1.10	1.04
	31	147166	**TRIM16L**	-1.53	1.08	-1.54	1.02	-1.24	1.12	1.03

	32	4237	**MFAP2**	-1.60	-1.03	-1.52	1.02	1.27	1.03	1.15
*	33	7057	**THBS1**	-1.54	1.10	-1.52	-1.03	1.11	1.02	1.11
	34	4791	**NFKB2**	-1.50	1.01	-1.49	-1.02	-1.11	-1.12	-1.14
	35	5361	**PLXNA1**	-1.57	1.10	-1.45	1.04	1.09	1.01	1.15
	36	4919	**ROR1**	-1.56	1.05	-1.45	-1.01	1.09	-1.27	1.03
	37	859	**CAV3**	-1.54	1.02	-1.44	1.01	1.14	1.26	1.32
	38	91409	**CCDC74B**	-1.54	-1.06	-1.42	1.00	1.10	1.03	1.04
	39	1289	**COL5A1**	-1.60	1.06	-1.42	1.01	1.11	-1.05	1.05
	40	59269	**HIVEP3**	-1.58	1.06	-1.42	-1.01	1.06	-1.21	-1.08
*	41	602	**BCL3**	-1.70	1.05	-1.42	-1.06	1.02	-1.23	-1.09
	42	1265	**CNN2**	-1.50	-1.01	-1.38	1.03	1.10	1.13	1.12
	43	80774	**LIMD2**	-1.51	1.00	-1.35	1.01	-1.05	-1.07	-1.30
	44	9123	**SLC16A3**	1.51	1.04	1.36	1.05	-1.15	-1.15	-1.12
*	45	1808	**DPYSL2**	1.56	1.03	1.40	1.06	-1.03	1.03	1.02
	46	392288	**LOC392288**	1.52	1.05	1.46	-1.05	1.03	-1.01	-1.03
	47	80833	**APOL3**	1.67	-1.00	1.47	1.11	-1.26	-1.17	-1.19
	48	728294	**D2HGDH**	1.56	-1.01	1.47	-1.00	-1.06	-1.01	1.01
	49	6307	**SC4MOL**	1.51	-1.01	1.47	1.09	1.04	1.20	1.02
	50	10455	**PECI**	1.53	1.02	1.48	-1.01	-1.09	-1.05	-1.13
	51	81631	**MAP1LC3B**	1.58	1.03	1.48	-1.00	1.06	-1.01	1.00
	52	968	**CD68**	1.58	1.01	1.48	1.06	-1.12	-1.01	-1.05
*	53	6478	**SIAH2**	1.60	1.04	1.49	1.01	-1.24	-1.08	-1.20
	54	404550	**C16orf74**	1.52	1.01	1.49	-1.02	1.07	1.13	-1.00
	55	1288	**COL4A6**	1.63	-1.04	1.50	-1.04	-1.09	-1.01	-1.08
	56	9723	**SEMA3E**	1.38	-1.01	1.50	1.02	-1.26	-1.26	-1.22
	57	9896	**FIG4**	1.54	1.01	1.50	1.00	-1.14	1.07	1.01
	58	5910	**RAP1GDS1**	1.58	-1.00	1.52	-1.01	-1.11	1.07	1.03
	59	838	**CASP5**	1.58	1.02	1.53	-1.02	-1.21	-1.17	-1.30
	60	837	**CASP4**	1.68	1.02	1.54	-1.00	-1.27	-1.18	-1.33
	61	4879	**NPPB**	1.46	-1.06	1.54	-1.13	-1.10	-1.21	-1.30
	62	117144	**CATSPER1**	1.65	1.10	1.55	1.03	1.03	-1.08	-1.11
	63	23643	**LY96**	1.56	1.04	1.56	1.01	-1.02	1.13	-1.06
	64	114769	**CARD16**	1.58	-1.04	1.57	1.09	-1.20	-1.31	-1.28
	65	3320	**HSP90AA1**	1.71	1.11	1.58	1.07	-1.46	-1.24	-1.31
	66	9304	**SNORD22**	1.43	1.27	1.58	1.24	-1.22	1.09	-1.10
	67	646626	**LOC646626**	1.68	1.11	1.59	-1.01	1.03	1.22	1.29
	68	4354	**MPP1**	1.84	-1.05	1.60	1.00	1.03	1.27	1.28
	69	84757	**MGC10814**	1.36	1.33	1.60	-1.00	1.08	-1.08	-1.05
	70	202020	**FLJ39653**	1.39	1.33	1.61	1.04	-1.08	1.08	-1.08
	71	51200	**CPA4**	1.60	-1.02	1.61	-1.08	1.17	-1.13	1.21
	72	88455	**ANKRD13A**	1.52	-1.05	1.61	-1.03	-1.00	-1.04	-1.02
*	73	5999	**RGS4**	1.62	-1.03	1.64	-1.04	1.19	-1.29	-1.01
	74	147700	**KLC3**	1.39	1.29	1.64	-1.01	-1.08	1.02	-1.09
*	75	4486	**MST1R**	1.63	1.02	1.66	1.04	1.13	1.06	1.25
	76	3074	**HEXB**	1.73	-1.01	1.67	1.00	-1.06	1.06	1.04
	77	100131733	**LOC100131733**	1.67	-1.03	1.67	-1.00	1.06	-1.06	1.06
	78	57464	**FAM40B**	1.59	1.45	1.68	1.07	1.26	-1.01	1.12
	79	51761	**ATP8A2**	1.73	-1.03	1.68	-1.03	-1.12	-1.05	-1.13
*	80	146691	**TOM1L2**	1.63	1.26	1.70	1.12	1.02	-1.01	-1.02
	81	51747	**LUC7L3**	1.46	1.10	1.70	1.20	-1.05	1.26	1.03
*	82	3866	**KRT15**	1.72	-1.04	1.72	1.03	-1.14	-1.11	-1.25

	83	3157	**HMGCS1**	1.83	1.02	1.75	1.04	-1.09	-1.01	-1.23
*	84	2194	**FASN**	1.55	-1.05	1.75	-1.04	1.04	1.11	-1.03
	85	164091	**PAQR7**	1.97	1.04	1.76	1.04	-1.09	1.02	-1.01
	86	10818	**FRS2**	1.93	-1.05	1.76	-1.04	-1.14	-1.08	-1.04
	87	847	**CAT**	1.72	1.06	1.77	1.01	1.06	1.03	1.02
	88	333926	**PPM1J**	1.87	-1.07	1.78	1.05	1.07	1.08	1.21
	89	55784	**MCTP2**	1.76	1.25	1.86	1.06	1.25	1.22	1.27
	90	834	**CASP1**	1.97	1.02	1.87	1.06	-1.27	-1.16	-1.27
	91	302	**ANXA2**	1.95	-1.01	1.90	-1.04	-1.12	1.00	-1.04
	92	79674	**VEPH1**	2.15	-1.11	1.94	-1.01	-1.14	-1.24	-1.17
*	93	9289	**GPR56**	1.82	-1.00	1.94	1.02	-1.07	-1.07	-1.21
	94	100288671	**LOC100288671**	2.15	-1.08	2.09	-1.12	-1.25	-1.17	-1.26
	95	967	**CD63**	2.15	-1.02	2.13	1.04	-1.11	1.06	1.01
*	96	316	**AOX1**	2.49	1.00	2.40	-1.00	-1.17	1.22	-1.00
	97	1780	**DYNC1I1**	2.53	-1.01	2.46	-1.03	-1.11	1.02	-1.02
	98	2778	**GNAS**	3.20	-1.03	3.36	-1.02	-1.02	-1.01	-1.07
	99	22979	**EFR3B**	3.48	-1.04	3.43	1.04	1.13	1.10	1.15
	100	79955	**PDZD7**	1.31	1.28	1.59	1.01	1.05	-1.01	-1.01
	101	286676	**ILDR1**	1.32	1.30	1.59	1.03	1.03	-1.04	-1.07
	102	339665	**SLC35E4**	1.32	1.26	1.59	1.05	1.09	1.02	-1.04
	103	401251	**C6orf26**	1.33	1.12	1.62	1.00	-1.02	1.22	1.05
	104	100287593	**LOC100287593**	1.32	1.28	1.63	1.02	1.02	-1.01	1.00
	105	80035	**C15orf28**	1.33	1.28	1.64	1.02	1.09	-1.05	-1.02
	106	84897	**TBRG1**	1.37	1.26	1.71	1.03	1.15	1.23	1.12
	107	25956	**SEC31B**	1.32	1.02	1.75	1.09	1.00	1.27	1.07
	108	27434	**POLM**	1.52	1.37	1.87	1.09	1.18	-1.00	1.09
	109	54487	**DGCR8**	1.43	1.36	1.97	1.01	1.02	1.14	-1.06
*	110	667	**DST**	1.70	1.15	2.17	1.02	1.00	1.17	-1.00
	111	5274	**SERPINI1**	1.99	1.01	1.44	1.06	-1.32	-1.15	-1.05
	112	53840	**TRIM34**	1.76	-1.00	1.47	-1.00	-1.20	-1.18	1.08
	113	1308	**COL17A1**	2.01	-1.04	1.66	1.13	-1.20	1.32	-1.07
	114	1755	**DMBT1**	2.54	-1.17	1.82	1.03	1.17	1.22	-1.09
	115	956	**ENTPD3**	2.42	-1.07	2.05	-1.04	1.41	1.22	1.36
	116	5865	**RAB3B**	4.62	-1.48	3.81	1.04	-1.21	1.45	-1.15

Genes regulated by unliganded PRB and PRA:PRB (B-&AB-)

#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
1	80705	**TSGA10**	-1.08	-6.43	-6.33	-1.04	1.23	-1.21	-1.18
2	284001	**CCDC57**	1.27	1.56	1.34	1.10	-1.27	-1.24	-1.05
3	1499	**CTNNB1**	1.40	1.33	1.56	1.05	-1.05	1.30	1.03
4	390595	**LOC390595**	1.46	1.36	1.62	-1.10	1.17	1.11	1.04
5	84851	**TRIM52**	1.44	1.45	1.63	1.08	-1.03	-1.15	-1.00
6	22796	**COG2**	1.57	1.81	1.77	1.03	-1.07	1.25	1.01
7	56244	**BTNL2**	1.39	1.58	1.87	-1.02	-1.13	-1.10	-1.14
8	28985	**MCTS1**	2.33	2.24	2.44	-1.22	1.37	-1.56	-1.77
9	26993	**AKAP8L**	1.38	1.39	1.67	-1.01	1.10	1.12	1.08
10	80097	**MZT2B**	1.25	1.31	1.68	-1.02	-1.00	1.08	-1.12
11	55744	**C7orf44**	1.20	1.32	1.69	1.01	-1.19	-1.04	-1.28
12	22901	**ARSG**	1.69	1.45	1.77	1.12	1.23	-1.03	-1.07
13	55525	**DKFZp547G183**	1.31	1.41	1.78	-1.01	1.05	-1.03	-1.02

14	653268	**AGAP7**	1.44	1.39	1.80	1.01	-1.02	-1.02	-1.10
15	7978	**MTERF**	1.67	1.33	1.85	1.10	1.17	1.00	1.01
16	85367	**KIAA1652**	1.71	1.50	1.85	1.16	-1.13	1.26	-1.03
17	6672	**SP100**	1.29	1.50	1.87	1.09	-1.14	-1.22	-1.23
18	55768	**NGLY1**	1.52	1.42	1.94	1.07	1.03	-1.03	-1.12
19	1427	**CRYGS**	1.61	1.47	1.95	1.06	1.03	1.32	-1.05
20	9184	**BUB3**	1.52	1.34	1.98	1.00	1.30	-1.27	1.11
21	100292101	**LOC100292101**	1.47	1.53	1.99	1.06	-1.11	1.19	1.07
22	79992	**NCRNA00241**	1.28	1.49	1.99	-1.09	1.04	1.15	1.14
23	112616	**CMTM7**	1.55	1.45	2.02	1.03	1.09	1.11	1.23
24	79932	**KIAA0319L**	1.30	1.41	2.06	1.05	1.04	1.09	1.07
25	55668	**C14ORF118**	1.59	1.60	2.09	1.03	1.13	-1.14	1.05
26	100129406	**LOC100129406**	1.49	1.75	2.11	1.03	1.16	1.08	1.09
27	100169750	**PRINS**	1.48	1.71	2.18	-1.14	-1.25	-1.23	-1.08
28	7756	**ZNF207**	1.63	1.52	2.24	1.08	1.07	1.05	-1.15
29	9440	**MED17**	1.27	1.78	2.29	-1.00	1.07	-1.01	-1.27
30	643314	**KIAA0754**	1.75	1.67	2.30	1.19	1.03	1.09	1.01
31	55697	**VAC14**	1.75	1.51	2.46	1.04	-1.02	-1.17	-1.01
32	55209	**SETD5**	1.49	1.62	2.50	-1.09	1.33	1.07	1.03
33	54441	**STAG3L1**	1.51	1.56	2.58	-1.04	1.09	-1.08	1.03
34	5150	**PDE7A**	1.28	1.48	2.66	1.16	1.02	1.19	-1.14
35	5793	**PTPRG**	2.04	2.28	2.91	1.01	1.06	1.10	1.06
36	54861	**SNRK**	1.92	1.97	2.93	-1.03	1.07	1.38	1.02
37	23314	**SATB2**	1.56	1.79	3.21	-1.10	1.13	-1.42	-1.16
38	26058	**GIGYF2**	2.07	1.96	3.82	-1.03	-1.06	1.34	1.10
39	23269	**MGA**	2.44	2.61	6.25	1.07	1.09	1.07	-1.34

Genes regulated by unliganded PRA and PRB and PRA:PRB (A-&B-&AB-)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	91749	**KIAA1919**	1.32	1.49	1.67	1.02	1.32	-1.05	1.02
	2	375	**ARF1**	1.36	1.51	1.71	-1.00	-1.06	1.07	1.02
	3	647022	**LOC100288583**	1.66	1.80	2.70	-1.04	1.09	-1.07	-1.04
	4	11276	**SYNRG**	1.69	1.96	1.86	1.00	1.35	-1.21	-1.34
	5	255239	**ANKK1**	1.33	1.34	1.62	1.01	1.03	-1.05	-1.05
	6	402483	**FLJ45340**	1.34	1.30	1.67	-1.02	1.09	-1.09	-1.03
	7	80196	**RNF34**	1.38	1.38	1.74	1.04	1.03	1.16	1.05
	8	55096	**EBLN2**	1.43	1.31	1.84	-1.00	1.14	1.06	1.02
*	9	26168	**SENP3**	1.66	1.57	2.00	1.07	-1.10	1.15	-1.01
	10	441951	**NCRNA00275**	1.46	1.56	1.62	1.04	-1.12	1.09	-1.15
	11	2317	**FLNB**	1.54	1.58	1.74	1.12	-1.06	1.15	1.03
	12	165324	**UBXN2A**	1.49	1.68	1.42	-1.12	1.24	1.04	-1.18
	13	9790	**BMS1**	1.46	1.36	1.68	-1.01	-1.03	1.03	1.02
	14	85007	**AGXT2L2**	1.50	1.32	1.73	1.07	-1.09	1.23	1.19
	15	400931	**LOC400931**	1.55	1.35	1.82	-1.03	1.06	1.22	1.01
	16	151194	**FAM119A**	1.58	1.41	2.01	1.00	1.01	1.09	1.05
	17	84062	**DTNBP1**	1.55	1.36	2.02	1.01	1.03	1.13	1.12
	18	55338	**FLJ11292**	1.67	1.38	2.05	1.30	-1.10	-1.17	-1.10
	19	6789	**STK4**	1.76	1.43	2.09	1.02	-1.17	-1.00	-1.04
*	20	4297	**MLL**	1.60	1.42	2.09	1.05	1.00	1.14	-1.04
	21	29896	**TRA2A**	1.83	1.65	2.09	1.10	1.01	-1.23	-1.10

22	10724	**MGEA5**	1.63	1.55	2.16	1.09	1.04	1.14	1.13
23	84268	**RPAIN**	1.58	1.42	2.16	1.06	1.03	1.13	1.08
24	823	**CAPN1**	1.85	1.49	2.21	1.07	1.08	1.08	1.04
25	347240	**KIF24**	1.73	1.58	2.27	-1.06	-1.10	1.10	-1.02
26	100272216	**LOC100272216**	2.02	1.77	2.64	1.01	-1.02	-1.03	1.10
27	57522	**SRGAP1**	2.88	1.58	2.76	-1.13	1.00	1.17	1.03
28	10721	**POLQ**	2.03	1.57	2.82	1.09	-1.05	-1.05	-1.05
29	100130401	**LOC100130401**	2.31	2.10	3.49	-1.14	1.08	-1.08	1.07

Genes regulated by liganded PRA (A+)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	79884	**MAP9**	1.37	-1.21	-1.10	1.20	-1.79	-1.28	-1.18
*	2	2150	**F2RL1**	1.25	1.01	-1.06	1.05	-1.89	-1.14	-1.24
*	3	9520	**NPEPPS**	1.33	1.13	1.14	1.01	-1.56	-1.09	-1.05
	4	7903	**ST8SIA4**	1.08	1.05	-1.02	1.09	-1.54	-1.27	-1.24
	5	259282	**BOD1L**	1.10	-1.25	1.04	1.01	-1.54	-1.33	-1.13
	6	3569	**IL6**	-1.15	1.05	-1.13	-1.03	-1.53	1.06	-1.26
	7	5608	**MAP2K6**	-1.05	-1.09	-1.07	-1.04	-1.52	-1.14	-1.44
	8	54206	**ERRFI1**	-1.01	1.03	-1.00	-1.01	-1.52	1.22	-1.03
	9	10949	**HNRNPA0**	-1.38	-1.23	-1.11	-1.01	1.51	1.14	-1.14
*	10	9580	**SOX13**	-1.11	-1.01	1.02	-1.09	1.51	1.08	1.17
	11	9682	**KDM4A**	-1.26	-1.05	1.05	-1.07	1.53	1.19	1.14
	12	747	**DAGLA**	-1.47	-1.18	-1.31	2.05	1.53	1.06	1.39
	13	8394	**PIP5K1A**	-1.32	-1.03	-1.01	-1.05	1.54	1.06	1.21
	14	1748	**DLX4**	1.04	1.10	-1.06	-1.30	1.54	1.07	1.21
	15	29979	**UBQLN1**	-1.25	1.08	1.09	1.07	1.56	-1.34	-1.21
	16	2010	**EMD**	-1.62	-1.26	-1.28	-1.06	1.58	1.06	-1.13
	17	5727	**PTCH1**	-1.64	-1.29	-1.25	-1.06	1.64	1.28	-1.24
*	18	27097	**TAF5L**	-1.51	-1.09	-1.08	1.00	1.66	1.32	-1.03
*	19	1295	**COL8A1**	1.04	-1.01	1.06	1.03	1.68	-1.12	1.34
*	20	4323	**MMP14**	-1.68	-1.29	-1.32	-1.13	1.68	1.28	-1.74
	21	6493	**SIM2**	1.04	1.09	-1.07	-1.06	1.69	-1.15	1.00
	22	128866	**CHMP4B**	-1.46	-1.22	-1.14	-1.12	1.72	1.08	-1.01
	23	23529	**CLCF1**	-1.09	1.02	-1.06	-1.09	1.85	-1.23	1.29
	24	27125	**AFF4**	-1.46	-1.17	-1.15	-1.01	1.89	1.42	1.02
	25	64839	**FBXL17**	-1.89	-1.33	-1.32	1.06	1.89	1.08	-1.29
*	26	8503	**PIK3R3**	-1.04	1.05	1.06	-1.04	2.08	1.38	1.29
	27	64925	**CCDC71**	-1.65	-1.23	-1.15	-1.07	2.40	1.12	-1.30
	28	348840	**LOC348840**	1.00	1.00	1.00	1.46	12.64	1.00	1.00

Genes regulated by liganded PRB (B+)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
*	1	23509	**POFUT1**	-1.08	-1.02	-1.08	1.00	1.06	-1.97	1.02
	2	122786	**FRMD6**	1.48	1.17	1.06	1.02	-1.68	-1.71	-1.46
*	3	84159	**ARID5B**	1.14	1.00	1.35	-1.02	1.26	-1.65	-1.05
*	4	5606	**MAP2K3**	1.05	-1.00	-1.10	-1.05	-1.21	-1.60	-1.31
	5	9331	**B4GALT6**	1.12	1.11	1.06	-1.03	-1.15	-1.53	-1.19
	6	317671	**RFESD**	-1.04	-1.03	-1.09	-1.02	-1.03	-1.50	-1.19
	7	10982	**MAPRE2**	-1.01	-1.07	1.06	-1.07	1.06	1.51	1.32

*	8	6764	**ST5**	-1.08	-1.02	-1.04	1.00	1.00	1.51	1.30
	9	79683	**ZDHHC14**	-1.01	1.03	1.17	1.02	1.13	1.52	1.17
	10	124359	**CDYL2**	1.12	-1.06	1.08	-1.06	1.11	1.52	1.11
*	11	79155	**TNIP2**	-1.18	-1.13	-1.17	1.03	-1.10	1.54	1.11
	12	7301	**TYRO3**	-1.13	-1.03	-1.11	-1.02	1.13	1.55	1.24
	13	51133	**KCTD3**	-1.02	1.03	1.02	1.00	1.10	1.56	1.28
*	14	79686	**C14orf139**	-1.03	1.06	-1.17	1.10	-1.15	1.57	1.24
	15	54629	**FAM63B**	1.16	1.05	1.29	1.03	1.13	1.57	1.24
*	16	11040	**PIM2**	-1.04	1.01	-1.06	1.02	1.18	1.58	1.23
	17	649	**BMP1**	1.07	-1.11	-1.12	1.02	1.04	1.58	1.25
	18	84002	**B3GNT5**	1.06	-1.04	1.15	1.11	1.17	1.60	1.17
	19	6676	**SPAG4**	-1.09	1.04	-1.02	1.03	1.27	1.62	1.42
*	20	309	**ANXA6**	-1.65	-1.53	-1.38	-1.02	1.76	1.63	-1.51
	21	55686	**MREG**	1.24	1.01	1.21	-1.07	1.09	1.67	1.26
	22	56243	**KIAA1217**	1.11	-1.04	1.00	1.02	1.02	1.70	1.21
	23	147372	**CCBE1**	1.21	-1.02	1.36	-1.09	1.17	1.78	1.16
	24	113457	**TUBA3D**	-1.04	1.00	-1.03	1.00	1.06	1.79	1.17
	25	114800	**CCDC85A**	-1.11	1.01	1.02	1.03	-1.06	1.80	-1.08
	26	256691	**MAMDC2**	-1.22	-1.00	-1.26	1.02	-1.02	1.81	1.14
	27	51559	**NT5DC3**	-1.17	1.01	-1.32	1.01	1.10	1.85	1.20
	28	387914	**SHISA2**	1.26	-1.01	-1.28	1.30	-1.66	1.87	-1.07
*	29	3614	**IMPDH1**	-1.19	-1.12	-1.21	1.02	1.04	1.93	1.20
	30	9957	**HS3ST1**	1.15	-1.01	1.21	1.01	1.00	1.95	1.29
	31	10125	**RASGRP1**	-1.40	-1.00	-1.62	1.37	1.19	1.98	1.46
*	32	5318	**PKP2**	-1.20	-1.13	-1.20	1.01	1.18	2.01	1.46
	33	81553	**FAM49A**	-1.07	-1.01	-1.12	1.11	1.12	2.09	1.25
*	34	2289	**FKBP5**	1.04	1.03	1.01	1.07	1.15	2.26	1.40
	35	29842	**TFCP2L1**	-1.05	-1.15	-1.19	1.10	-1.07	2.38	1.33
	36	28231	**SLCO4A1**	-1.24	1.01	-1.01	1.16	-1.14	3.10	1.30
*	37	9056	**SLC7A7**	1.39	1.01	-1.05	1.78	-1.20	4.57	1.78

Genes regulated by liganded PRA:PRB (AB+)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
*	1	9076	**CLDN1**	-1.42	1.16	-1.46	-1.12	-1.22	-1.87	-2.27
	2	3673	**ITGA2**	-1.14	1.09	1.16	-1.07	-1.23	-1.13	-1.97
	3	6675	**UAP1**	1.13	-1.06	1.22	1.00	-1.36	-1.43	-1.94
	4	10371	**SEMA3A**	1.09	-1.30	1.14	-1.24	-1.03	-1.47	-1.87
	5	399959	**LOC399959**	-1.09	-1.00	-1.00	-1.18	-1.45	-1.44	-1.84
	6	131566	**DCBLD2**	1.09	-1.01	1.14	-1.04	-1.45	-1.27	-1.78
	7	2920	**CXCL2**	-1.01	1.05	1.15	-1.00	-1.43	-1.42	-1.72
	8	26046	**LTN1**	-1.01	-1.10	1.12	-1.03	-1.29	-1.24	-1.69
	9	375033	**PEAR1**	-1.10	-1.08	1.14	-1.12	-1.57	-1.39	-1.69
	10	93663	**ARHGAP18**	1.22	-1.03	1.19	1.02	-1.35	-1.24	-1.69
	11	196792	**FAM24B**	-1.01	-1.03	1.12	-1.11	-1.37	-1.21	-1.69
	12	7546	**ZIC2**	-1.27	-1.04	-1.09	-1.14	1.03	-1.21	-1.66
	13	3821	**KLRC1**	-1.22	1.05	1.04	1.11	-1.34	-1.10	-1.65
	14	572558	**LOC572558**	1.16	1.03	1.04	-1.03	-1.36	-1.05	-1.63
	15	8835	**SOCS2**	1.11	1.00	-1.08	-1.10	-1.29	-1.41	-1.63
	16	2113	**ETS1**	-1.21	-1.02	-1.11	-1.02	-1.28	-1.29	-1.61
	17	644192	**LOC644192**	1.38	-1.03	1.28	1.01	-1.29	-1.32	-1.60

	18	2921	**CXCL3**	-1.01	-1.01	1.08	-1.05	-1.25	-1.47	-1.58
	19	150468	**CKAP2L**	-1.19	-1.29	-1.15	1.05	1.03	-1.04	-1.58
	20	60312	**AFAP1**	1.05	1.07	-1.03	-1.09	-1.56	-1.26	-1.58
	21	80790	**CMIP**	-1.15	-1.01	-1.07	-1.25	-1.31	-1.29	-1.57
	22	55512	**SMPD3**	1.17	-1.08	1.25	1.11	-1.19	-1.23	-1.55
	23	8578	**SCARF1**	1.19	1.01	1.03	1.01	-1.44	-1.25	-1.53
	24	9497	**SLC4A7**	1.20	1.02	1.28	-1.09	-1.25	-1.23	-1.51
	25	441094	**FLJ42709**	-1.09	-1.04	1.00	1.04	-1.21	-1.25	-1.51
	26	115207	**KCTD12**	1.03	1.03	-1.02	1.03	1.31	1.25	1.52
	27	23499	**MACF1**	-1.18	1.01	-1.13	1.13	1.13	1.15	1.52
	28	81606	**LBH**	1.14	1.29	1.04	-1.10	1.10	1.15	1.52
*	29	57460	**PPM1H**	1.24	-1.02	-1.12	1.10	1.53	1.10	1.55
*	30	6196	**RPS6KA2**	1.08	1.17	-1.20	1.05	1.05	1.18	1.56
*	31	2690	**GHR**	1.09	-1.10	-1.15	1.04	1.04	1.24	1.56
	32	221935	**SDK1**	-1.21	1.02	-1.17	1.05	1.21	1.31	1.57
	33	84861	**KLHL22**	1.05	-1.17	-1.18	1.00	1.12	1.33	1.57
*	34	22874	**PLEKHA6**	1.19	-1.02	1.17	-1.02	1.32	-1.12	1.57
	35	80176	**SPSB1**	-1.20	1.01	-1.18	1.00	1.18	1.13	1.58
	36	145781	**GCOM1**	1.00	-1.08	-1.06	-1.03	1.22	1.08	1.59
*	37	8795	**TNFRSF10B**	-1.16	1.02	-1.11	1.02	1.29	1.17	1.60
	38	147906	**DACT3**	-1.03	-1.01	-1.06	-1.04	1.20	1.14	1.61
	39	638	**BIK**	1.01	-1.03	-1.20	1.03	1.13	1.40	1.61
	40	388403	**YPEL2**	1.08	1.09	1.08	1.03	1.24	1.03	1.67
	41	144406	**WDR66**	1.25	1.02	1.12	-1.01	1.23	1.10	1.67
	42	283208	**P4HA3**	1.18	1.03	1.04	1.01	1.37	1.30	1.74
	43	23007	**PLCH1**	1.09	1.11	-1.26	1.16	1.12	1.11	1.81
	44	27253	**PCDH17**	-1.04	-1.18	1.06	1.21	1.38	1.02	1.81
	45	8701	**DNAH11**	1.27	1.07	-1.10	1.13	1.32	1.41	1.85
	46	54508	**FLJ11235**	-1.15	1.02	-1.22	-1.07	1.32	1.41	1.86
	47	7050	**TGIF1**	-1.02	1.05	-1.09	1.01	1.42	1.08	1.94
	48	58985	**IL22RA1**	1.17	-1.02	1.03	1.23	-1.05	1.41	2.00
*	49	131578	**LRRC15**	1.06	-1.07	-1.04	-1.05	1.39	1.19	2.07
	50	9829	**DNAJC6**	1.34	1.05	-1.29	-1.02	1.43	-1.01	2.12
	51	79614	**C5orf23**	-1.37	-1.05	-1.48	1.34	2.35	1.77	2.61
*	52	8556	**CDC14A**	-1.25	1.06	-1.56	1.00	1.58	1.03	2.64

Genes regulated by liganded PRA and PRB (A+&B+)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	343069	**HNRNPCL1**	1.27	-1.08	-1.24	-1.14	-1.58	1.35	1.04
*	2	677	**ZFP36L1**	1.20	1.02	1.08	1.02	-1.45	1.54	-1.22
	3	83874	**TBC1D10A**	-1.31	-1.05	-1.19	1.02	1.49	1.84	1.49
*	4	3291	**HSD11B2**	-1.07	-1.00	-1.15	-1.03	1.32	2.19	1.33

Genes regulated by liganded PRA and PRA:PRB (A+&AB+)

#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
1	386618	**KCTD4**	1.53	1.07	1.51	-1.06	-2.23	-2.90	-5.32
2	338382	**RAB7B**	1.12	-1.13	1.17	-1.05	-2.70	-1.68	-2.97
3	27122	**DKK3**	1.01	-1.00	1.01	1.09	-2.18	-1.67	-2.86
4	80117	**ARL14**	1.26	1.05	1.11	-1.03	-1.95	-1.36	-2.50

	5	2925	**GRPR**	1.32	-1.09	-1.02	-1.02	-1.72	-1.60	-2.49
	6	57574	**MARCH4**	1.14	-1.07	1.15	-1.06	-1.57	-1.32	-2.09
*	7	55357	**TBC1D2**	-1.05	-1.07	-1.09	1.01	-1.81	-1.19	-2.07
	8	2124	**EVI2B**	-1.42	-1.19	-1.18	-1.18	-1.33	-1.25	-2.04
	9	2863	**GPR39**	-1.16	-1.04	-1.19	-1.02	-1.58	-1.22	-2.02
	10	9760	**TOX**	-1.17	1.07	-1.35	1.05	-1.78	-1.61	-1.96
	11	2069	**EREG**	1.19	-1.00	1.12	-1.05	-1.65	-1.04	-1.90
	12	3955	**LFNG**	-1.40	1.01	-1.24	1.02	-1.38	-1.28	-1.87
	13	149111	**CNIH3**	1.04	-1.09	1.02	1.02	-1.66	-1.35	-1.86
	14	8848	**TSC22D1**	1.15	-1.03	1.05	1.00	-1.46	-1.15	-1.86
	15	83448	**PUS7L**	1.16	-1.16	1.05	1.00	-1.60	-1.45	-1.84
*	16	5874	**RAB27B**	1.28	-1.02	1.02	-1.03	-1.54	-1.41	-1.80
*	17	7357	**UGCG**	-1.13	1.04	-1.03	-1.04	-1.50	-1.30	-1.76
	18	9882	**TBC1D4**	1.08	1.03	1.04	-1.00	-1.64	-1.17	-1.70
	19	5923	**RASGRF1**	-1.15	-1.02	-1.23	1.04	-1.54	-1.30	-1.69
	20	10123	**ARL4C**	-1.07	-1.03	-1.07	-1.03	-1.59	-1.30	-1.68
	21	84803	**AGPAT9**	-1.02	1.01	1.06	-1.06	-1.40	-1.17	-1.67
	22	645202	**LOC100132727**	1.18	-1.03	1.15	1.31	-1.40	-1.50	-1.67
	23	2963	**GTF2F2**	-1.03	-1.09	1.06	-1.05	-1.34	-1.22	-1.67
*	24	2535	**FZD2**	-1.07	-1.01	1.01	1.02	-1.47	-1.24	-1.63
*	25	54541	**DDIT4**	-1.09	-1.02	-1.10	1.09	-1.48	1.06	-1.63
	26	64283	**RGNEF**	1.03	-1.02	1.00	1.00	-1.44	-1.16	-1.62
	27	1543	**CYP1A1**	-1.24	-1.06	-1.22	-1.03	-1.32	-1.31	-1.61
	28	8050	**PDHX**	1.25	-1.01	1.26	-1.01	-1.39	-1.26	-1.57
*	29	2152	**F3**	-1.07	1.08	-1.12	-1.04	-2.49	-1.00	-2.45
	30	53405	**CLIC5**	1.22	-1.10	1.09	-1.05	-2.11	-1.23	-2.15
	31	6447	**SCG5**	1.29	-1.02	1.23	-1.08	-1.89	-1.63	-1.86
	32	5142	**PDE4B**	1.23	-1.03	1.13	1.06	-1.90	-1.23	-1.85
	33	9411	**ARHGAP29**	1.32	1.01	1.33	-1.02	-1.84	-1.12	-1.83
	34	92949	**ADAMTSL1**	1.11	-1.00	1.13	1.01	-1.57	-1.15	-1.65
	35	3908	**LAMA2**	1.29	-1.02	1.10	-1.05	-1.54	-1.24	-1.61
*	36	5205	**ATP8B1**	1.14	-1.05	1.03	-1.03	-1.62	-1.27	-1.60
	37	3914	**LAMB3**	-1.20	-1.06	-1.27	1.00	-1.56	-1.26	-1.59
	38	283551	**C14orf182**	1.16	1.08	1.11	1.07	-1.68	1.03	-1.56
	39	8871	**SYNJ2**	-1.16	1.14	-1.14	-1.03	-1.51	-1.27	-1.56
	40	29091	**STXBP6**	1.26	-1.09	1.29	-1.07	-1.41	1.02	-1.56
	41	3484	**IGFBP1**	-1.06	-1.01	1.05	-1.05	-1.50	1.28	-1.56
	42	79083	**MLPH**	-1.27	1.05	-1.15	1.04	-1.48	1.10	-1.55
	43	79628	**SH3TC2**	-1.33	-1.06	-1.27	1.03	-1.69	1.20	-1.54
	44	57539	**WDR35**	1.07	1.02	1.14	-1.00	-1.52	-1.13	-1.54
	45	3486	**IGFBP3**	-1.00	1.01	-1.00	-1.08	-1.40	1.16	-1.54
	46	5137	**PDE1C**	-1.05	-1.06	-1.01	-1.07	-1.66	1.17	-1.53
	47	836	**CASP3**	1.24	1.04	1.24	-1.01	-1.40	-1.15	-1.52
*	48	23175	**LPIN1**	1.09	-1.05	1.21	1.02	-1.36	-1.16	-1.51
	49	197259	**MLKL**	1.12	1.00	1.12	1.03	-1.66	1.05	-1.50
	50	388115	**C15orf52**	-1.03	-1.07	-1.01	-1.03	-1.51	-1.13	-1.49
	51	7052	**TGM2**	1.29	-1.07	1.18	1.01	-1.53	1.04	-1.41
	52	7145	**TNS1**	-1.17	-1.15	-1.23	-1.02	-2.59	1.03	-2.35
	53	7852	**CXCR4**	1.17	-1.05	1.24	-1.01	-2.36	-1.30	-2.05
	54	195828	**ZNF367**	1.27	1.07	-1.11	1.00	-1.72	-1.51	-1.62
	55	79858	**NEK11**	1.26	-1.01	1.16	-1.03	-1.80	-1.04	-1.62

	56	2072	**ERCC4**	-1.06	1.08	1.39	-1.19	1.58	-1.23	-1.42
	57	54532	**USP53**	-1.08	-1.09	1.17	-1.05	1.52	1.20	1.46
	58	29109	**FHOD1**	-1.11	-1.03	-1.06	1.01	1.55	1.18	1.47
	59	8322	**FZD4**	1.04	1.03	1.01	1.03	1.54	1.29	1.47
	60	114991	**ZNF618**	-1.21	1.05	-1.10	-1.00	1.64	1.08	1.53
	61	7461	**CLIP2**	-1.09	1.01	-1.10	-1.02	1.60	1.04	1.53
*	62	9764	**KIAA0513**	-1.01	1.00	-1.04	1.05	1.52	1.20	1.55
	63	8601	**RGS20**	1.10	1.05	1.05	-1.02	1.49	-1.12	1.55
	64	55313	**CPPED1**	1.02	1.00	1.02	-1.02	1.48	1.25	1.56
*	65	9121	**SLC16A5**	-1.05	1.06	-1.12	1.05	1.48	1.14	1.56
	66	2729	**GCLC**	-1.15	1.03	-1.18	1.07	1.48	1.16	1.57
	67	57224	**NHSL1**	-1.15	-1.07	-1.14	1.05	1.58	1.18	1.57
	68	3570	**IL6R**	1.10	1.01	1.20	-1.05	1.44	-1.20	1.58
	69	221662	**RBM24**	-1.29	1.03	-1.19	-1.13	1.54	-1.01	1.59
	70	1277	**COL1A1**	1.02	-1.03	-1.03	1.06	1.57	1.07	1.61
	71	57568	**SIPA1L2**	-1.18	-1.02	-1.21	1.01	1.57	1.12	1.62
	72	4242	**MFNG**	-1.11	1.03	-1.00	-1.04	1.77	1.10	1.75
	73	8997	**KALRN**	-1.02	-1.02	-1.03	-1.03	1.60	1.28	1.75
	74	7474	**WNT5A**	-1.14	-1.01	-1.14	1.03	1.78	1.17	1.78
	75	2354	**FOSB**	-1.34	-1.05	-1.30	-1.14	1.83	-1.00	1.85
	76	55366	**LGR4**	1.15	1.02	1.21	1.00	1.80	1.09	1.85
	77	10560	**SLC19A2**	1.01	1.03	-1.01	1.01	1.77	1.31	1.88
	78	55897	**MESP1**	-1.11	-1.01	-1.06	-1.01	2.17	1.15	2.04
	79	7980	**TFPI2**	1.00	1.06	1.03	-1.04	2.22	1.29	2.19
	80	55359	**STYK1**	1.19	-1.03	1.25	1.00	1.72	-1.12	1.34
	81	2057	**EPOR**	-1.14	-1.03	1.01	1.03	1.52	1.09	1.36
	82	64651	**CSRNP1**	-1.11	1.02	-1.09	-1.00	1.55	-1.18	1.36
	83	162461	**TMEM92**	-1.29	1.00	-1.13	-1.07	1.62	1.03	1.36
*	84	3169	**FOXA1**	1.15	-1.05	1.21	-1.01	1.62	-1.17	1.37
	85	196410	**METTL7B**	-1.13	-1.22	-1.13	-1.14	1.63	1.27	1.40
	86	64132	**XYLT2**	-1.16	-1.08	-1.07	1.00	1.55	1.25	1.43
	87	652995	**UCA1**	1.21	-1.12	1.30	-1.17	1.52	1.45	1.44
*	88	11037	**STON1**	-1.11	1.05	-1.08	-1.01	1.78	1.09	1.48
	89	598	**BCL2L1**	-1.11	1.00	1.09	-1.02	1.68	1.45	1.51
	90	6446	**SGK1**	-1.29	1.03	-1.30	1.02	1.84	1.28	1.73
	91	23462	**HEY1**	1.12	1.01	1.16	1.05	1.87	1.33	1.73
*	92	10847	**SRCAP**	-1.16	-1.04	-1.08	1.07	1.95	1.17	1.74
	93	9050	**PSTPIP2**	-1.00	1.00	-1.16	-1.01	1.97	-1.09	1.83
	94	220441	**RNF152**	-1.03	1.03	1.04	-1.03	2.36	-1.21	1.98
	95	1299	**COL9A3**	-1.09	1.06	-1.12	-1.00	2.77	1.08	2.09
	96	5791	**PTPRE**	1.02	1.07	1.02	-1.09	1.30	-1.11	1.52
	97	388650	**FAM69A**	-1.14	1.02	-1.17	1.05	1.38	-1.03	1.55
	98	100129113	**LOC100129113**	-1.11	1.01	-1.08	-1.00	1.35	1.29	1.57
*	99	6195	**RPS6KA1**	-1.00	-1.04	-1.02	-1.03	1.32	1.19	1.59
	100	24147	**FJX1**	-1.07	1.04	-1.08	-1.04	1.44	1.24	1.61
	101	3976	**LIF**	-1.05	1.01	-1.07	-1.05	1.30	1.18	1.61
	102	6641	**SNTB1**	1.13	-1.01	-1.13	1.02	1.37	-1.19	1.62
	103	83641	**FAM107B**	-1.09	1.04	-1.18	1.01	1.34	1.19	1.63
	104	644596	**NCRNA00087**	1.21	-1.01	1.22	-1.07	1.42	1.09	1.66
	105	56138	**PCDHA11**	-1.19	1.01	-1.09	-1.04	1.52	1.09	1.67
	106	29775	**CARD10**	-1.20	1.03	-1.24	-1.01	1.46	1.15	1.68

*	107	23766	**GABARAPL3**	-1.16	1.02	-1.08	-1.03	1.56	1.07	1.69
	108	3915	**LAMC1**	-1.29	1.11	-1.25	-1.02	1.41	1.07	1.72
	109	9473	**C1orf38**	1.07	1.03	1.03	1.03	1.55	1.03	1.73
*	110	2034	**EPAS1**	1.17	-1.03	1.04	-1.01	1.43	1.16	1.73
	111	1947	**EFNB1**	-1.12	1.02	-1.17	1.01	1.44	1.10	1.73
*	112	29094	**HSPC159**	1.05	-1.02	1.03	1.01	1.48	1.18	1.74
	113	357	**SHROOM2**	-1.27	1.11	-1.16	-1.08	1.48	-1.04	1.75
	114	144404	**TMEM120B**	1.14	1.06	1.11	1.02	1.49	1.24	1.76
	115	219855	**SLC37A2**	-1.08	1.02	-1.13	1.08	1.58	1.20	1.77
	116	3090	**HIC1**	-1.38	-1.11	-1.42	1.02	1.35	1.00	1.78
	117	794	**CALB2**	-1.04	-1.09	-1.29	-1.16	1.52	1.24	1.78
	118	1602	**DACH1**	-1.24	1.03	-1.17	1.02	1.59	-1.26	1.82
	119	8792	**TNFRSF11A**	1.09	1.05	1.06	1.04	1.70	1.25	1.93
	120	4070	**TACSTD2**	1.02	1.02	-1.08	1.02	1.70	-1.27	1.99
	121	57158	**JPH2**	1.02	-1.08	-1.20	-1.03	1.34	1.24	1.99
*	122	23710	**GABARAPL1**	-1.18	1.03	-1.09	-1.00	1.83	1.07	1.99
*	123	1848	**DUSP6**	-1.09	1.07	-1.04	1.05	1.78	1.32	2.05
	124	3038	**HAS3**	1.16	1.01	1.05	1.04	1.66	1.30	2.06
	125	139716	**GAB3**	-1.11	-1.24	1.24	1.04	1.92	1.88	2.18
	126	283537	**SLC46A3**	-1.11	-1.11	1.04	-1.08	1.85	1.66	2.20
	127	78986	**DUSP26**	1.05	-1.11	1.07	-1.02	1.93	1.30	2.21
*	128	7855	**FZD5**	-1.04	-1.00	-1.16	1.03	2.04	1.19	2.31
	129	1474	**CST6**	-1.56	-1.06	-1.66	-1.04	1.61	-1.08	2.36
*	130	9871	**SEC24D**	-1.02	1.06	1.15	-1.04	2.47	1.38	2.62
	131	55793	**FAM63A**	1.22	1.04	1.25	-1.00	2.49	1.11	3.46
	132	1907	**EDN2**	-1.03	1.11	-1.39	-1.22	2.96	1.79	3.79
	133	219699	**UNC5B**	-1.78	1.07	-2.15	1.48	3.82	1.55	4.12
	134	254439	**C11ORF86**	-1.79	-1.15	1.63	-1.13	5.09	1.96	8.61

Genes regulated by liganded PRB and PRA:PRB (B+&AB+)

#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
1	90853	**SPOCD1**	-1.49	-1.06	-1.15	-1.10	-1.26	-1.62	-2.54
2	135398	**C6orf141**	-1.25	1.01	-1.14	1.03	-1.38	-1.46	-1.85
3	201799	**TMEM154**	1.41	-1.07	1.35	-1.02	-1.41	-1.47	-1.85
4	27145	**FILIP1**	1.10	-1.20	1.10	-1.03	-1.38	-1.54	-1.82
5	1021	**CDK6**	1.09	-1.07	1.19	-1.01	-1.29	-1.35	-1.68
6	56256	**SERTAD4**	-1.13	1.00	-1.03	-1.01	-1.29	-1.33	-1.63
7	56648	**EIF5A2**	1.06	1.01	1.01	-1.03	-1.30	-1.31	-1.57
8	25819	**CCRN4L**	-1.23	1.03	-1.25	-1.05	-1.14	-1.30	-1.51
9	650293	**LOC650293**	-1.01	-1.07	-1.03	-1.07	-1.25	-1.86	-1.93
10	118429	**ANTXR2**	-1.07	-1.02	-1.02	-1.02	-1.25	-1.61	-1.59
11	196	**AHR**	-1.15	1.08	-1.01	-1.02	-1.19	-1.41	-1.51
12	448831	**FRG2**	-1.12	1.06	-1.28	-1.08	-1.24	-1.63	-1.51
13	115908	**CTHRC1**	-1.20	1.00	-1.19	1.08	-1.15	-1.64	-1.48
14	342184	**FMN1**	1.03	1.05	1.15	1.03	-1.24	-1.50	-1.48
15	162655	**ZNF519**	1.12	-1.12	-1.09	1.11	-1.24	-1.53	-1.44
16	4071	**TM4SF1**	1.05	1.01	-1.03	-1.00	-1.28	-1.54	-1.41
17	2250	**FGF5**	1.31	-1.04	1.28	-1.02	-1.21	-2.18	-1.80
18	10892	**MALT1**	-1.03	1.08	1.03	-1.14	-1.10	-1.77	-1.58
* 19	10769	**PLK2**	-1.13	1.03	-1.23	-1.02	-1.12	-1.75	-1.57

	20	90102	**PHLDB2**	-1.18	-1.05	-1.19	-1.02	-1.23	-1.71	-1.56
*	21	7099	**TLR4**	1.24	-1.00	1.13	-1.05	-1.23	-1.69	-1.38
	22	92797	**HELB**	1.03	1.19	1.10	-1.02	1.08	-1.52	-1.36
*	23	7296	**TXNRD1**	-1.08	1.03	-1.27	-1.04	-1.25	-1.60	-1.31
	24	1612	**DAPK1**	-1.07	-1.07	-1.02	1.14	1.30	1.37	1.53
	25	85027	**C5orf62**	1.12	-1.02	1.03	1.00	1.19	1.46	1.60
*	26	9531	**BAG3**	-1.12	1.02	-1.05	1.05	1.12	1.54	1.62
	27	201176	**ARHGAP27**	-1.12	-1.01	-1.11	-1.06	1.31	1.51	1.69
	28	79660	**PPP1R3B**	-1.01	1.06	1.05	1.08	1.32	1.62	1.83
	29	1809	**DPYSL3**	-1.28	-1.02	-1.28	1.06	1.38	1.64	1.88
	30	51088	**KLHL5**	-1.09	1.02	-1.26	1.04	1.41	1.61	1.92
*	31	9953	**HS3ST3B1**	-1.05	-1.03	1.09	1.03	1.51	1.80	1.97
	32	50848	**F11R**	1.10	1.07	1.04	1.08	1.49	1.64	1.97
	33	147837	**ZNF563**	1.22	-1.11	-1.02	-1.04	1.09	1.42	2.00
*	34	4038	**LRP4**	-1.11	1.06	-1.08	1.06	1.29	1.85	2.01
	35	83593	**RASSF5**	-1.03	-1.13	-1.22	1.08	1.48	1.98	2.29
	36	55640	**FLVCR2**	1.17	-1.14	-1.15	-1.25	1.81	4.73	9.25
	37	79446	**WDR25**	1.05	-1.09	-1.19	1.05	1.06	1.50	1.58
	38	7849	**PAX8**	1.07	1.03	-1.10	-1.01	1.04	1.60	1.63
	39	6004	**RGS16**	-1.07	1.01	-1.12	-1.02	-1.05	1.62	1.69
	40	200942	**KLHDC8B**	-1.27	-1.04	-1.19	1.04	1.43	1.71	1.74
	41	154810	**AMOTL1**	1.17	-1.01	1.03	-1.25	1.27	1.82	1.81
	42	8794	**TNFRSF10C**	1.00	1.01	-1.20	1.16	1.18	2.06	2.09
	43	50615	**IL21R**	-1.08	-1.00	-1.17	1.16	1.29	2.22	2.10
	44	146227	**BEAN**	-1.49	1.05	-1.16	-1.01	1.72	2.08	2.14
*	45	9124	**PDLIM1**	-1.03	-1.04	-1.04	1.01	1.06	1.87	1.32
	46	26090	**ABHD12**	-1.03	-1.02	-1.07	-1.02	1.00	1.62	1.32
	47	480	**ATP1A4**	-1.10	-1.06	-1.09	1.03	1.06	1.76	1.33
*	48	25976	**TIPARP**	1.04	-1.03	1.08	1.02	1.03	2.59	1.34
	49	473	**RERE**	-1.05	-1.03	-1.07	-1.06	1.21	1.92	1.36
*	50	5265	**SERPINA1**	-1.21	-1.04	-1.28	1.04	1.09	1.59	1.36
	51	152573	**SHISA3**	-1.19	1.00	-1.09	1.04	1.20	1.51	1.37
	52	478	**ATP1A3**	-1.08	-1.07	-1.16	1.05	1.05	1.83	1.38
	53	51655	**RASD1**	-1.55	-1.06	-1.40	1.05	1.39	3.37	1.38
	54	84448	**ABLIM2**	1.20	1.13	-1.32	1.03	1.29	1.59	1.38
*	55	9394	**HS6ST1**	-1.14	-1.03	-1.24	1.03	1.18	1.54	1.39
	56	6322	**SCML1**	1.01	-1.02	1.05	-1.01	1.18	1.55	1.39
	57	23303	**KIF13B**	-1.05	1.02	-1.05	1.08	1.20	1.51	1.39
*	58	64764	**CREB3L2**	1.06	-1.14	-1.03	-1.02	1.16	1.77	1.40
	59	8814	**CDKL1**	1.09	1.04	-1.04	1.11	-1.17	1.68	1.40
*	60	7743	**ZNF189**	1.07	1.04	1.03	1.05	1.06	2.30	1.41
*	61	476	**ATP1A1**	-1.11	-1.09	-1.11	1.01	1.10	1.82	1.41
	62	23102	**TBC1D2B**	-1.10	-1.02	-1.06	1.00	1.12	1.63	1.42
	63	23523	**CABIN1**	-1.12	-1.00	-1.04	-1.03	1.20	1.85	1.43
	64	222643	**UNC5CL**	1.45	1.12	1.30	1.21	1.12	1.63	1.49
	65	128434	**VSTM2L**	-1.15	-1.11	-1.24	1.24	1.30	2.19	1.52
	66	5734	**PTGER4**	-1.18	1.03	-1.21	1.11	1.14	2.67	1.52
	67	27031	**NPHP3**	-1.02	1.15	1.17	1.04	1.11	1.66	1.52
*	68	22936	**ELL2**	1.15	1.06	1.03	-1.01	1.18	1.61	1.53
	69	4524	**MTHFR**	1.22	1.08	1.18	1.07	1.26	1.67	1.55
	70	8651	**SOCS1**	-1.25	-1.02	-1.26	1.11	1.00	2.07	1.57

	71	23396	**PIP5K1C**	1.01	-1.01	-1.10	-1.13	1.30	1.83	1.62
	72	2308	**FOXO1**	-1.05	1.03	1.00	1.02	1.22	1.91	1.62
	73	10217	**CTDSPL**	1.05	1.01	1.08	1.02	1.19	2.71	1.73
*	74	1831	**TSC22D3**	-1.12	1.00	-1.12	1.10	1.29	2.00	1.78
	75	80206	**FHOD3**	-1.01	-1.02	-1.07	1.03	1.30	2.40	1.87
	76	100286909	**LOC100286909**	1.01	1.17	1.54	-1.04	1.45	2.29	1.89
*	77	5166	**PDK4**	-1.13	1.05	1.09	1.13	1.42	2.00	1.89
	78	2444	**FRK**	-1.01	1.02	-1.13	1.18	1.06	3.49	1.95
	79	147	**ADRA1B**	1.06	-1.07	1.01	1.08	1.40	4.11	2.01
	80	151354	**FAM84A**	1.15	-1.02	-1.00	1.27	1.19	2.92	2.04
	81	64333	**ARHGAP9**	-1.13	1.04	-1.18	1.14	1.09	3.08	2.05
	82	126695	**C1ORF172**	1.22	-1.02	-1.27	-1.63	1.33	4.92	2.62
	83	1420	**CRYGC**	1.05	-1.34	-1.50	1.50	1.32	5.06	2.70
	84	339894	**LOC339894**	1.09	-1.09	-1.01	1.03	1.66	6.02	3.17
	85	9332	**CD163**	-1.34	-1.18	1.41	-1.06	1.15	3.80	3.19
	86	153218	**SPINK13**	-1.65	1.14	1.04	2.47	1.84	10.77	5.10

Genes regulated by liganded PRA and PRB and PRA:PRB (A+&B+&AB+)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	2151	**F2RL2**	-1.03	1.03	1.03	1.01	-1.46	-1.78	-2.03
	2	29126	**CD274**	1.12	1.09	-1.05	-1.05	-1.54	-1.71	-2.00
*	3	3667	**IRS1**	-1.06	-1.03	-1.00	1.03	-1.38	-1.53	-1.84
*	4	8507	**ENC1**	-1.01	-1.02	1.10	-1.07	-1.40	-1.58	-1.73
	5	9734	**HDAC9**	1.11	-1.03	1.06	-1.04	-1.54	-1.95	-2.14
	6	4982	**TNFRSF11B**	-1.13	1.09	-1.30	1.03	-1.39	-1.67	-1.61
	7	1591	**CYP24A1**	1.01	-1.01	-1.15	-1.02	-1.47	-2.08	-1.79
	8	55183	**RIF1**	1.13	-1.17	1.01	1.09	-1.39	-1.60	-1.38
*	9	3576	**IL8**	-1.13	1.01	-1.21	1.10	-2.75	-2.69	-5.61
*	10	5801	**PTPRR**	1.11	1.03	1.20	-1.07	-2.06	-2.43	-3.91
	11	137994	**LETM2**	-1.08	-1.03	1.17	-1.19	-1.51	-1.44	-2.34
*	12	8870	**IER3**	-1.19	1.02	-1.04	-1.02	-1.58	-1.46	-2.32
	13	57547	**ZNF624**	1.06	-1.14	-1.14	-1.06	-1.81	-1.83	-2.32
	14	4306	**NR3C2**	1.37	1.01	1.28	-1.03	-1.55	-1.67	-2.09
	15	5581	**PRKCE**	-1.06	1.03	-1.11	-1.03	-1.79	-1.69	-2.07
	16	7026	**NR2F2**	1.09	-1.07	1.30	-1.07	-1.43	-1.54	-2.03
*	17	9592	**IER2**	-1.03	-1.01	-1.08	1.01	-1.48	-1.47	-2.02
	18	27	**ABL2**	1.15	1.02	1.18	-1.02	-1.57	-1.52	-1.97
	19	26579	**MYEOV**	-1.15	-1.00	-1.04	-1.04	-1.62	-1.44	-1.95
	20	3624	**INHBA**	1.16	-1.03	1.10	-1.10	-1.40	-1.55	-1.95
	21	29114	**TAGLN3**	1.17	-1.04	1.18	-1.07	-1.33	-1.53	-1.88
	22	10046	**MAMLD1**	1.02	-1.02	1.04	-1.07	-1.45	-1.44	-1.79
	23	144165	**PRICKLE1**	-1.01	1.01	1.06	-1.00	-1.40	-1.40	-1.73
	24	26511	**CHIC2**	1.16	-1.01	1.16	-1.02	-1.34	-1.42	-1.67
	25	636	**BICD1**	-1.06	1.03	-1.06	-1.06	-1.33	-1.39	-1.57
*	26	4907	**NT5E**	1.08	1.07	1.01	-1.01	-1.33	-1.46	-1.55
*	27	23645	**PPP1R15A**	1.08	1.01	-1.03	1.01	-1.32	-1.41	-1.54
	28	1122	**CHML**	-1.04	-1.29	-1.10	-1.02	-1.32	-1.35	-1.51
	29	4771	**NF2**	1.09	-1.00	1.06	1.01	-1.33	-1.32	-1.51
*	30	50486	**G0S2**	-1.13	-1.04	-1.27	-1.08	-1.99	-1.91	-2.12
	31	390	**RND3**	1.06	1.01	1.00	-1.03	-1.51	-1.46	-1.73

	32	254778	**C8orf46**	-1.23	1.03	-1.29	-1.10	-1.73	-1.67	-1.68
	33	9021	**SOCS3**	-1.14	-1.05	-1.10	-1.06	-1.44	-1.45	-1.67
	34	10135	**NAMPT**	1.10	1.05	1.01	-1.03	-1.44	-1.49	-1.67
	35	8829	**NRP1**	1.20	1.07	-1.02	1.04	-1.75	-1.63	-1.62
	36	22998	**LIMCH1**	1.07	1.05	1.03	1.01	-1.48	-1.47	-1.57
	37	3269	**HRH1**	1.02	1.09	1.02	-1.12	-1.49	-1.44	-1.56
	38	91947	**ARRDC4**	-1.07	1.00	-1.18	-1.02	-1.41	-1.41	-1.52
	39	22943	**DKK1**	1.06	1.03	1.12	-1.06	-2.35	-1.63	-3.86
*	40	7022	**TFAP2C**	-1.17	1.09	-1.08	-1.06	-1.76	-1.35	-2.11
	41	1902	**LPAR1**	1.35	1.01	1.23	-1.01	-2.70	-1.63	-3.17
*	42	22822	**PHLDA1**	1.03	1.05	1.04	-1.03	-2.34	-1.50	-2.75
*	43	23327	**NEDD4L**	1.07	1.01	1.07	1.03	-2.01	-1.45	-2.35
	44	2635	**GBP3**	1.16	-1.05	1.12	-1.05	-1.53	-1.40	-1.79
	45	54453	**RIN2**	1.20	-1.06	1.18	-1.02	-2.70	-1.45	-2.63
*	46	6662	**SOX9**	1.07	-1.02	1.02	-1.04	-1.97	-1.36	-2.17
*	47	1545	**CYP1B1**	-1.17	1.12	-1.14	-1.04	-1.98	-1.45	-2.11
	48	3772	**KCNJ15**	-1.02	1.06	-1.25	-1.14	-2.18	-1.57	-2.04
	49	2131	**EXT1**	1.31	1.17	1.07	1.02	-2.00	-1.51	-2.02
	50	10806	**SDCCAG8**	1.18	-1.09	1.25	-1.03	-1.86	-1.39	-1.90
	51	64859	**OBFC2A**	1.14	1.03	1.11	-1.06	-1.55	-1.34	-1.74
	52	10365	**KLF2**	-1.13	-1.00	-1.10	1.01	-1.62	-1.39	-1.73
	53	10253	**SPRY2**	1.00	1.04	1.06	-1.03	-1.51	-1.37	-1.67
	54	54704	**PDP1**	1.21	1.11	1.21	-1.01	-1.73	-1.40	-1.67
	55	220929	**ZNF438**	1.19	-1.06	1.13	-1.01	-1.48	-1.35	-1.63
*	56	89795	**NAV3**	1.16	1.05	1.18	-1.05	-1.64	-2.19	-2.67
	57	11141	**IL1RAPL1**	1.22	-1.10	1.33	-1.09	-1.56	-1.94	-2.34
	58	6157	**RPL27A**	1.11	1.06	1.03	1.02	1.50	1.82	2.16
	59	163071	**ZNF114**	1.07	1.06	-1.03	1.05	1.83	2.02	2.26
*	60	26468	**LHX6**	-1.05	-1.10	-1.05	-1.02	1.93	2.32	2.36
*	61	1026	**CDKN1A**	-1.14	-1.06	-1.42	1.00	2.04	2.60	3.02
	62	25891	**PAMR1**	-1.31	-1.49	-1.32	-1.42	2.32	2.88	4.80
	63	1901	**S1PR1**	-1.12	1.03	-1.09	1.16	7.56	10.29	13.00
	64	55824	**PAG1**	1.14	-1.00	1.14	1.12	1.34	1.67	1.66
	65	53947	**A4GALT**	-1.17	-1.03	-1.12	1.03	1.36	1.64	1.68
	66	90693	**CCDC126**	1.01	1.05	1.05	1.05	1.44	1.69	1.72
*	67	5795	**PTPRJ**	-1.29	1.07	-1.22	1.05	1.61	1.83	1.78
	68	92370	**ACPL2**	-1.16	1.06	-1.26	1.05	1.54	2.04	1.95
	69	7097	**TLR2**	-1.02	1.02	-1.01	1.05	1.43	1.97	2.04
	70	203260	**CCDC107**	1.18	-1.01	1.23	1.02	1.71	2.31	2.17
	71	1827	**RCAN1**	1.09	-1.02	1.00	1.02	1.66	2.33	2.24
	72	55083	**KIF26B**	-1.16	-1.08	-1.17	1.05	1.74	2.84	2.61
	73	100131646	**LOC100131646**	-1.03	-1.06	1.01	-1.02	1.87	2.54	2.77
*	74	8817	**FGF18**	-1.33	-1.13	-1.18	1.06	2.47	3.96	3.59
	75	121512	**FGD4**	-1.33	-1.10	-1.26	1.08	2.22	3.86	3.76
	76	23767	**FLRT3**	1.37	1.09	1.11	1.32	2.94	6.36	4.11
*	77	799	**CALCR**	-1.55	1.11	-1.22	1.85	10.17	16.27	16.52
*	78	8395	**PIP5K1B**	-1.18	-1.04	-1.14	1.05	1.32	1.52	1.33
*	79	22885	**ABLIM3**	-1.04	-1.06	1.07	1.02	1.59	2.24	1.46
	80	8660	**IRS2**	-1.14	-1.01	-1.08	1.06	1.31	2.10	1.56
	81	10634	**GAS2L1**	-1.23	-1.05	-1.25	1.02	1.34	1.96	1.58
	82	27345	**KCNMB4**	-1.15	1.02	-1.20	1.02	1.36	1.85	1.59

	83	139221	**MUM1L1**	-1.09	-1.05	-1.02	1.02	1.38	1.78	1.62
	84	84815	**MGC12916**	-1.06	-1.20	-1.17	-1.02	1.31	2.29	1.63
*	85	3398	**ID2**	-1.11	1.01	1.01	-1.04	1.36	2.07	1.72
	86	388610	**TRNP1**	-1.20	-1.01	-1.24	-1.00	1.64	1.98	1.85
	87	346887	**LOC346887**	-1.07	-1.03	-1.04	1.04	1.54	2.18	1.89
	88	6482	**ST3GAL1**	1.13	1.03	1.12	-1.03	1.47	2.46	1.95
*	89	9770	**RASSF2**	-1.13	1.04	-1.22	1.07	1.30	3.05	1.97
*	90	5187	**PER1**	-1.13	-1.08	-1.12	1.09	1.66	2.58	2.04
*	91	3706	**ITPKA**	-1.06	-1.03	-1.02	1.07	1.50	3.81	2.08
*	92	5409	**PNMT**	-1.58	1.03	-1.23	1.08	1.50	3.69	2.17
	93	23308	**ICOSLG**	-1.08	1.01	-1.17	-1.02	1.80	2.49	2.27
	94	28984	**C13orf15**	1.03	-1.02	-1.01	1.24	1.66	5.04	2.28
*	95	687	**KLF9**	-1.03	1.02	1.13	1.16	2.11	2.88	2.33
*	96	604	**BCL6**	-1.10	1.07	-1.04	1.08	1.43	2.56	2.35
*	97	23541	**SEC14L2**	-1.15	-1.03	-1.04	1.08	1.79	3.75	2.48
	98	166336	**PRICKLE2**	-1.06	-1.05	1.03	1.10	1.54	2.82	2.48
	99	54626	**HES2**	-1.17	-1.20	-1.14	1.42	1.71	4.82	2.56
*	100	54566	**EPB41L4B**	1.27	1.24	1.38	1.19	1.73	4.84	3.74
	101	23504	**RIMBP2**	1.37	1.05	-1.52	1.12	1.64	6.97	3.76
*	102	8436	**SDPR**	-1.17	-1.02	-1.25	1.14	2.25	5.23	4.10
*	103	1052	**CEBPD**	-1.18	1.01	-1.11	1.13	2.83	5.17	4.25
	104	7005	**TEAD3**	-1.02	-1.03	1.01	1.02	1.36	1.33	1.54
	105	10140	**TOB1**	1.03	1.00	1.02	1.06	1.56	1.35	1.56
	106	27239	**GPR162**	-1.06	1.00	1.03	1.00	1.38	1.38	1.56
	107	51114	**ZDHHC9**	1.03	1.05	1.08	-1.07	1.49	1.38	1.60
	108	115572	**FAM46B**	-1.04	-1.09	1.04	1.06	1.54	1.43	1.62
	109	1945	**EFNA4**	-1.01	1.03	1.06	-1.01	1.39	1.50	1.62
*	110	10318	**TNIP1**	-1.06	1.02	-1.08	1.04	1.38	1.42	1.64
*	111	868	**CBLB**	-1.11	1.05	1.04	-1.01	1.52	1.56	1.77
	112	283991	**FAM100B**	-1.08	1.02	1.00	1.10	1.47	1.49	1.77
*	113	4502	**MT2A**	-1.16	1.02	-1.19	1.04	1.66	1.56	1.79
	114	261729	**STEAP2**	-1.16	-1.03	-1.07	-1.01	1.55	1.62	1.81
*	115	6584	**SLC22A5**	-1.02	1.01	1.06	1.03	1.52	1.70	1.82
*	116	4489	**MT1A**	-1.16	1.03	-1.15	1.03	1.58	1.54	1.83
	117	56935	**C11orf75**	-1.07	1.01	-1.09	1.00	1.45	1.43	1.85
	118	55057	**AIM1L**	1.29	-1.03	1.31	1.02	1.34	1.39	1.88
*	119	10924	**SMPDL3A**	-1.19	1.01	-1.13	-1.06	1.43	1.38	1.89
*	120	4501	**MT1X**	-1.22	1.04	-1.20	1.03	1.76	1.62	1.91
*	121	4495	**MT1G**	-1.21	1.02	-1.16	1.05	1.70	1.65	1.92
*	122	4496	**MT1H**	-1.25	1.03	-1.18	1.03	1.62	1.62	1.93
*	123	4490	**MT1B**	-1.21	1.06	-1.17	-1.01	1.70	1.62	1.99
	124	284996	**RNF149**	-1.12	1.03	-1.09	1.04	1.58	1.63	2.05
*	125	4493	**MT1E**	-1.26	1.06	-1.21	1.03	1.87	1.77	2.12
	126	80271	**ITPKC**	-1.04	1.02	1.00	1.05	1.66	1.75	2.15
*	127	2296	**FOXC1**	-1.03	1.01	-1.07	1.08	1.75	1.62	2.25
*	128	10458	**BAIAP2**	-1.20	-1.05	-1.15	1.00	1.72	1.96	2.29
	129	57530	**CGN**	-1.45	1.00	-1.28	-1.15	1.95	1.87	2.31
	130	402415	**XKRX**	1.48	-1.01	1.02	1.03	1.87	1.86	2.41
	131	56999	**ADAMTS9**	1.02	-1.01	1.06	1.05	1.89	1.95	2.42
*	132	10579	**TACC2**	-1.16	-1.03	-1.23	1.05	1.66	1.83	2.99
*	133	5744	**PTHLH**	-1.06	1.04	-1.02	1.00	3.13	3.55	4.65

	134	55603	**FAM46A**	1.04	1.04	1.20	1.01	1.42	1.37	1.50
	135	65989	**DLK2**	-1.24	-1.13	-1.21	-1.03	1.41	1.53	1.52
	136	339122	**RAB43**	-1.06	1.00	-1.09	1.08	1.42	1.54	1.66
	137	131601	**TPRA1**	-1.08	-1.06	-1.09	1.00	1.68	1.50	1.67
	138	8572	**PDLIM4**	-1.17	1.04	-1.10	-1.01	1.81	1.97	1.99
*	139	1843	**DUSP1**	-1.15	1.03	-1.12	1.02	1.60	1.31	1.91
	140	64762	**FAM59A**	-1.07	-1.01	-1.07	1.03	1.63	1.35	1.94
	141	23239	**PHLPP1**	-1.02	-1.13	-1.07	-1.08	1.73	1.50	2.02
	142	22898	**DENND3**	-1.06	1.03	-1.04	1.01	1.85	1.62	2.09
	143	5327	**PLAT**	1.20	-1.02	1.05	1.02	1.78	1.39	2.15
	144	124976	**SPNS2**	-1.08	-1.06	-1.08	1.02	2.25	1.34	2.61
	145	22996	**TTC39A**	-1.53	-1.05	-1.66	1.21	3.37	2.78	6.25
	146	90362	**FAM110B**	-1.39	-1.03	-1.08	1.03	1.69	1.43	1.72
	147	116039	**OSR2**	1.27	-1.05	1.13	-1.06	1.94	1.46	1.79
	148	9026	**HIP1R**	-1.01	1.03	-1.02	-1.01	1.70	1.40	1.89
*	149	1028	**CDKN1C**	-1.06	-1.02	-1.06	1.05	1.77	1.43	1.95
*	150	7538	**ZFP36**	-1.31	1.02	-1.14	-1.02	4.30	1.90	4.43
	151	118932	**ANKRD22**	1.26	1.37	-1.18	1.36	18.95	10.58	18.69

Genes regulated by unliganded and liganded PRA (A±)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	2802	**GOLGA3**	-2.21	-1.16	-1.16	1.03	1.80	1.07	-1.12
	2	79618	**HMBOX1**	-2.19	1.02	1.11	-1.11	2.29	1.10	-1.07
	3	2070	**EYA4**	-2.11	-1.11	-1.11	-1.04	1.92	-1.13	-1.32
	4	51400	**PPME1**	-1.94	1.16	-1.24	-1.15	1.76	-1.31	-1.12
	5	6599	**SMARCC1**	-1.85	-1.23	-1.15	-1.04	1.80	1.04	-1.16
	6	1743	**DLST**	-1.61	-1.33	-1.19	-1.01	1.85	1.14	-1.15
	7	659	**BMPR2**	-1.53	-1.01	-1.11	-1.16	1.30	-1.04	1.07
	8	60681	**FKBP10**	-1.47	-1.28	-1.23	1.05	1.61	1.00	-1.07
*	9	8543	**LMO4**	-1.47	-1.26	-1.17	1.06	1.57	1.13	-1.30
*	10	79026	**AHNAK**	-1.46	1.01	-1.21	1.08	1.51	1.11	1.29
	11	11228	**RASSF8**	-1.43	-1.37	-1.12	-1.11	1.54	1.07	-1.22
	12	54877	**ZCCHC2**	-1.41	1.07	1.03	-1.10	1.55	-1.20	1.01
	13	220988	**HNRNPA3**	1.41	1.12	1.02	1.00	-1.53	-1.39	-1.12
	14	10640	**EXOC5**	1.49	1.12	1.09	-1.01	-1.61	-1.35	-1.15
	15	79184	**BRCC3**	1.56	1.07	1.06	1.00	-1.52	-1.20	-1.07

Genes regulated by unliganded and liganded PRB (B±)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
*	1	10606	**PAICS**	1.15	1.33	1.28	-1.06	1.05	-1.56	-1.09
*	2	30851	**TAX1BP3**	-1.67	-1.71	-1.55	-1.20	1.35	1.89	-1.17

Genes regulated by unliganded and liganded PRA:PRB (AB±)

#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
1	339512	**C1ORF110**	-1.51	-1.04	-1.54	-1.14	-1.75	-1.63	-2.19
2	3603	**IL16**	-1.29	-1.12	-1.37	-1.01	-1.34	-1.10	-1.70
3	84951	**TNS4**	-1.30	1.04	-1.56	-1.02	-1.40	-1.11	-1.64
4	116372	**LYPD1**	-1.58	-1.07	-1.53	-1.11	1.12	-1.04	1.32

	5	9249	**DHRS3**	-1.27	1.13	-1.56	-1.02	1.20	1.09	1.41
	6	252995	**FNDC5**	-1.50	-1.05	-1.69	1.02	1.18	1.14	1.49
	7	6752	**SSTR2**	-1.41	-1.07	-1.37	-1.04	1.19	1.29	1.65
	8	127544	**RNF19B**	-1.34	1.01	-1.32	-1.03	1.06	1.41	1.67
*	9	3625	**INHBB**	-1.53	-1.25	-1.71	-1.01	1.38	1.45	1.97
	10	84553	**C6orf168**	-1.84	-1.27	-1.70	1.05	1.49	1.13	1.98
	11	151473	**SLC16A14**	-1.49	1.04	-2.39	1.04	1.55	1.29	2.05
	12	57381	**RHOJ**	1.03	1.12	-1.80	1.12	-1.28	1.29	2.41
	13	84777	**MGC11082**	-1.16	1.03	-1.49	-1.06	-1.05	1.19	2.29
	14	5159	**PDGFRB**	-1.41	1.02	-1.51	-1.04	1.25	1.12	2.38
	15	112597	**NCRNA00152**	1.27	1.09	1.74	-1.07	-1.59	-1.06	-1.70
	16	144453	**BEST3**	1.26	1.22	1.94	1.01	-1.25	-1.60	-1.63
	17	152816	**C4orf26**	1.36	-1.09	1.34	1.14	-1.36	-1.28	-1.56
	18	9564	**BCAR1**	1.13	1.22	1.56	1.02	1.18	1.28	1.32
	19	2170	**FABP3**	1.37	1.44	1.64	-1.07	1.19	1.38	1.36

Genes regulated by unliganded and liganded PRA and PRB (A±&B±)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	841	**CASP8**	1.45	1.15	1.31	-1.08	-1.15	-2.00	-1.17
	2	399664	**MEX3D**	-1.43	-1.47	-1.39	-1.06	1.50	1.25	-1.01
	3	2760	**GM2A**	-1.48	-1.41	-1.15	-1.04	1.55	1.45	-1.12
	4	11333	**PDAP1**	-2.05	-1.77	-1.63	1.04	1.90	1.68	-1.53
	5	1174	**AP1S1**	-1.56	-1.41	-1.25	1.01	1.62	1.43	-1.25
	6	7430	**EZR**	-1.40	-1.19	-1.03	-1.15	1.65	1.42	1.01
	7	2909	**GRLF1**	-1.84	-1.39	-1.52	-1.14	1.69	1.75	1.08
	8	3716	**JAK1**	-1.68	-1.05	-1.14	-1.18	1.77	1.39	1.19
	9	26043	**UBXN7**	-1.63	-1.31	-1.32	1.14	1.80	1.34	-1.58
	10	5631	**PRPS1**	-1.66	-1.39	-1.32	1.00	1.75	1.27	-1.45
	11	10471	**PFDN6**	-1.99	-1.47	-1.39	1.03	2.05	1.28	-1.43
*	12	4001	**LMNB1**	-2.93	-1.91	-1.76	1.08	2.88	1.30	-1.42

Genes regulated by unliganded and liganded PRA and PRA:PRB (A±&AB±)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	1767	**DNAH5**	-1.15	-1.03	-1.70	1.06	-1.87	-1.27	-1.35
	2	7851	**MALL**	-1.73	-1.01	-1.78	-1.01	-1.58	-1.32	-1.92
	3	7042	**TGFB2**	-1.85	-1.00	-1.78	-1.06	-1.32	-1.60	-1.84
	4	84419	**C15orf48**	-1.54	1.02	-1.40	1.01	-1.24	-1.29	-1.37
	5	3575	**IL7R**	-1.42	-1.06	-1.24	-1.09	-1.60	-1.16	-2.13
*	6	3725	**JUN**	-1.33	1.03	-1.33	-1.06	-1.56	-1.43	-1.71
	7	80144	**FRAS1**	-1.62	1.11	-1.55	1.16	1.30	1.39	1.75
	8	64759	**TNS3**	-1.54	1.03	-1.58	1.03	1.20	1.27	1.79
	9	59284	**CACNG7**	-1.76	-1.24	-1.74	1.04	1.53	1.06	-1.15
	10	91107	**TRIM47**	-1.78	-1.02	-1.62	-1.03	1.55	-1.01	1.50
	11	23336	**SYNM**	-1.37	1.03	-1.34	1.04	1.35	1.12	1.58
*	12	3164	**NR4A1**	-1.37	-1.08	-1.32	-1.02	1.57	1.20	1.63
	13	399726	**C10orf114**	-1.51	-1.03	-1.59	-1.00	1.75	1.22	1.65
	14	5308	**PITX2**	-1.30	-1.03	-1.33	-1.02	1.87	1.16	1.71
	15	91768	**CABLES1**	-1.22	1.00	-1.32	-1.01	1.51	-1.06	1.75
	16	7481	**WNT11**	-1.66	1.05	-1.81	1.05	2.00	1.41	1.90

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
*	17	2706	**GJB2**	-1.22	-1.04	-1.78	-1.06	1.77	1.01	2.11
	18	91683	**SYT12**	-1.70	1.10	-1.69	-1.20	3.31	1.08	3.12
	19	3685	**ITGAV**	-1.49	1.01	-1.33	1.01	1.56	1.30	1.73
	20	11078	**TRIOBP**	-1.46	-1.09	-1.29	-1.20	1.76	1.32	1.44
	21	79365	**BHLHE41**	-1.45	1.04	-1.18	1.04	1.62	1.12	1.51
	22	5376	**PMP22**	-1.31	-1.05	-1.26	-1.01	1.42	1.24	1.72
*	23	360	**AQP3**	-1.79	-1.16	-1.43	1.01	2.00	1.24	1.69
*	24	182	**JAG1**	-1.43	1.04	-1.32	1.06	1.36	1.14	1.71
	25	85449	**KIAA1755**	1.71	-1.36	1.55	1.24	-2.96	-1.32	-4.00
	26	6000	**RGS7**	1.31	-1.05	1.49	-1.08	-2.16	-1.42	-3.51
	27	168667	**BMPER**	1.70	-1.20	1.71	-1.15	-1.88	-1.30	-2.03
	28	8038	**ADAM12**	1.23	1.06	1.36	-1.17	-1.59	-1.42	-1.90
	29	3884	**KRT33B**	1.71	1.02	1.67	-1.10	-1.80	1.03	-1.87
*	30	5618	**PRLR**	1.43	-1.09	1.34	-1.07	-2.13	-1.34	-1.71
	31	116071	**BATF2**	1.41	-1.11	1.44	1.03	-1.55	-1.30	-1.67
	32	6695	**SPOCK1**	1.62	-1.20	1.56	-1.03	-1.66	1.03	-1.46
	33	728224	**KRTAP4-8**	2.17	1.04	2.38	-1.20	-4.32	-2.00	-6.77
	34	129446	**XIRP2**	2.10	-1.11	1.87	1.14	-3.40	-1.88	-5.24
	35	3885	**KRT34**	2.11	-1.04	1.84	-1.05	-2.16	-1.25	-2.56
	36	284217	**LAMA1**	2.72	1.07	2.30	-1.05	-3.50	-1.49	-3.43
	37	55222	**LRRC20**	1.34	-1.09	1.28	1.01	-1.61	-1.25	-1.69
	38	50617	**ATP6V0A4**	1.50	-1.03	1.34	-1.03	-2.21	-1.04	-1.89
	39	8738	**CRADD**	1.32	1.01	1.26	-1.02	-1.35	-1.11	-1.63
	40	597	**BCL2A1**	12.60	1.02	11.87	1.00	-2.30	-2.76	-4.24
	41	55894	**DEFB103B**	2.67	-1.09	2.86	-1.09	-1.44	-1.50	-1.83
	42	1075	**CTSC**	1.46	-1.00	1.59	-1.04	-1.23	-1.28	-1.71
	43	26191	**PTPN22**	1.60	1.08	1.71	-1.08	-1.40	-1.46	-1.55
	44	79974	**C7orf58**	1.91	-1.06	2.03	-1.00	-1.22	-1.16	-1.46
*	45	4286	**MITF**	1.51	-1.01	1.66	-1.04	-1.21	1.09	-1.37
	46	164781	**WDR69**	2.22	-1.05	2.05	-1.06	-1.21	-1.02	-1.30
	47	58538	**MPP4**	2.07	-1.03	2.08	-1.00	-1.37	-1.26	-1.42
	48	57458	**TMCC3**	1.65	-1.00	1.51	1.03	-1.30	1.21	1.08
	49	440068	**CARD17**	1.59	-1.02	1.60	1.22	-1.30	-1.09	-1.20
*	50	3872	**KRT17**	1.68	-1.12	1.35	1.05	-1.28	-1.14	-1.33
	51	154	**ADRB2**	1.35	-1.04	1.32	-1.08	-1.66	1.04	-1.45
	52	116328	**C8orf34**	1.53	-1.02	1.45	1.08	-1.65	1.15	-1.42
	53	5576	**PRKAR2A**	-1.17	1.15	1.52	-1.23	1.69	-1.29	-1.72
	54	23092	**ARHGAP26**	1.04	1.28	1.47	-1.03	1.54	1.10	-1.12
	55	114826	**SMYD4**	-1.11	1.01	1.99	1.01	1.72	1.30	1.01
*	56	58472	**SQRDL**	1.68	1.01	1.57	-1.00	1.29	1.14	1.53
	57	55344	**PLCXD1**	2.29	1.21	2.18	-1.05	1.39	1.22	1.17
	58	5138	**PDE2A**	1.65	-1.01	1.48	1.06	1.31	1.04	1.40
	59	26207	**PITPNC1**	1.42	-1.15	1.34	-1.09	3.30	1.09	3.02

Genes regulated by unliganded and liganded PRB and PRA:PRB (B±&AB±)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	91624	**NEXN**	1.06	-1.33	1.12	-1.27	-1.06	-1.67	-1.68
	2	51575	**ESF1**	-1.28	-1.63	-1.37	1.00	-1.14	-1.19	-1.44
*	3	5209	**PFKFB3**	1.20	-1.96	-1.20	1.00	-1.18	-1.31	-1.34
	4	80184	**CEP290**	-1.05	-1.89	-1.22	-1.20	-1.17	-1.05	-1.31

	5	8303	**SNN**	-1.17	-1.00	-1.40	1.21	1.15	1.85	1.45
	6	81786	**TRIM7**	-1.19	1.00	-1.33	-1.01	1.10	1.69	1.64
	7	344558	**SH3RF3**	-1.32	-1.02	-1.46	-1.08	1.14	2.33	1.75
	8	10529	**NEBL**	-1.73	-1.09	-2.13	-1.07	1.44	1.50	1.82
*	9	58480	**RHOU**	1.03	-1.01	-1.32	1.15	1.28	3.23	1.87
	10	123920	**CMTM3**	-1.14	-1.15	-1.42	-1.00	1.01	1.50	1.13
	11	25960	**GPR124**	-1.38	1.04	-1.46	1.05	1.33	1.64	1.41
	12	2673	**GFPT1**	1.12	1.38	1.14	1.01	-1.11	-1.63	-1.45
	13	389432	**SAMD5**	1.37	1.21	1.45	1.08	1.02	-1.76	-1.11
	14	54797	**MED18**	1.13	1.59	1.76	-1.20	1.30	-1.70	-1.94
	15	80025	**PANK2**	1.05	1.90	1.84	-1.26	1.30	-1.52	-1.53
	16	253143	**C22orf30**	-1.04	3.03	3.64	1.08	-1.14	-1.34	-1.50
	17	56339	**METTL3**	1.27	1.01	1.54	1.08	1.02	1.35	1.02
	18	5930	**RBBP6**	1.25	1.31	1.64	1.01	1.32	1.40	1.03
	19	23037	**PDZD2**	1.24	1.04	1.34	1.04	-1.07	2.35	1.03
	20	55832	**CAND1**	1.27	1.79	2.02	1.08	1.20	1.83	-1.21
	21	375449	**MAST4**	1.17	1.00	1.38	1.03	1.10	1.83	1.37
	22	100128108	**LOC100128108**	1.97	1.97	2.36	1.19	1.12	1.38	1.54

Genes regulated by unliganded and liganded PRA and PRB and PRA:PRB (A±&B±&AB±)

	#	Entrez gene ID	Gene symbol	A-/O-	B-/O-	AB-/0-	O+/O-	A+/A-	B+/B-	AB+/AB-
	1	4603	**MYBL1**	1.00	1.01	-1.32	-1.03	-1.49	-1.59	-1.32
*	2	1437	**CSF2**	-1.41	1.04	-1.62	-1.11	-1.92	-1.81	-2.68
*	3	8061	**FOSL1**	-1.31	-1.01	-1.36	-1.21	-1.54	-1.65	-2.47
	4	3589	**IL11**	-1.43	1.06	-1.38	-1.14	-2.28	-2.00	-3.59
	5	5806	**PTX3**	-1.51	-1.03	-1.45	-1.08	-1.65	-1.68	-2.18
*	6	347902	**AMIGO2**	-1.70	1.14	-1.59	-1.01	-1.55	-1.68	-1.86
	7	2919	**CXCL1**	-1.34	1.01	-1.30	1.03	-1.64	-1.34	-1.82
	8	144501	**KRT80**	-1.65	1.03	-1.63	-1.05	-1.51	-1.46	-1.81
	9	1839	**HBEGF**	-1.40	-1.00	-1.38	-1.12	-1.43	-1.57	-1.62
*	10	1906	**EDN1**	-1.74	-1.03	-1.42	-1.18	-1.05	-1.60	-1.50
	11	4050	**LTB**	-2.76	1.09	-2.61	1.15	-1.25	-1.31	-1.36
	12	5362	**PLXNA2**	-1.48	1.13	-1.75	-1.08	-1.18	-1.41	-1.20
	13	7185	**TRAF1**	-1.51	1.08	-1.59	-1.06	-1.10	-1.30	-1.16
*	14	5971	**RELB**	-1.51	1.08	-1.46	1.01	1.03	-1.35	-1.20
*	15	3280	**HES1**	-1.34	1.01	-1.23	-1.03	-1.18	-1.38	-1.74
*	16	3096	**HIVEP1**	-1.58	1.04	-1.41	-1.06	-1.19	-1.41	-1.36
	17	374393	**FAM111B**	1.23	-1.37	1.10	1.16	-1.33	-1.51	-1.45
*	18	1291	**COL6A1**	-1.62	1.06	-1.43	1.00	1.32	1.39	1.45
	19	84859	**LRCH3**	-1.92	-1.73	-1.76	-1.15	-1.01	1.73	1.89
	20	7436	**VLDLR**	-1.33	1.02	-1.25	1.04	1.25	1.48	1.67
	21	2239	**GPC4**	-1.57	-1.09	-1.29	-1.02	2.09	1.56	1.75
	22	4499	**MT1M**	-1.31	1.05	-1.27	1.02	2.02	1.77	2.32
	23	5125	**PCSK5**	-1.37	-1.01	-1.18	-1.01	1.76	2.04	2.11
	24	57732	**ZFYVE28**	-1.61	-1.01	-1.45	1.08	1.62	1.69	1.94
	25	169611	**OLFML2A**	-2.11	1.13	-1.68	-1.02	1.81	1.78	2.02
	26	57493	**HEG1**	-1.36	1.05	-1.33	1.08	1.46	1.65	2.04
*	27	5021	**OXTR**	-1.44	-1.17	-1.40	1.10	1.34	3.62	2.11
	28	387763	**C11orf96**	-1.48	-1.05	-1.46	1.04	2.73	2.00	3.74
	29	23508	**TTC9**	-1.31	-1.02	-1.46	1.03	1.63	2.40	3.18

	30	169792	**GLIS3**	-1.22	-1.08	-1.37	-1.05	1.71	2.14	2.02
	31	146760	**RTN4RL1**	-1.39	-1.04	-1.30	-1.01	1.69	1.99	2.10
	32	144100	**PLEKHA7**	-1.43	-1.06	-1.44	1.08	1.62	2.67	2.48
	33	63027	**SLC22A23**	-1.43	-1.06	-1.47	1.07	2.15	2.37	2.52
	34	8609	**KLF7**	-1.48	-1.13	-1.61	1.04	1.61	2.26	1.93
	35	2936	**GSR**	-1.79	-1.35	-1.60	1.02	1.34	1.01	-1.53
	36	5055	**SERPINB2**	3.63	1.01	3.09	1.28	-2.19	-2.25	-2.41
	37	11082	**ESM1**	3.66	-1.00	2.86	-1.16	-2.92	-3.53	-5.43
	38	85294	**KRTAP2-4**	3.50	-1.15	3.40	-1.17	-4.99	-3.30	-12.95
	39	11174	**ADAMTS6**	2.15	-1.11	2.26	-1.14	-2.61	-2.58	-5.66
	40	3553	**IL1B**	1.83	1.01	1.62	-1.02	-2.75	-2.16	-4.38
*	41	9510	**ADAMTS1**	1.85	1.03	1.72	-1.05	-2.24	-1.88	-3.14
	42	117157	**SH2D1B**	1.71	1.02	1.62	-1.34	-2.01	-1.84	-2.49
	43	285704	**RGMB**	1.80	-1.02	1.73	-1.19	-1.80	-1.39	-2.30
*	44	9034	**CCRL2**	1.37	-1.01	1.39	-1.00	-1.69	-1.35	-1.85
	45	2123	**EVI2A**	1.36	-1.13	1.38	1.01	-1.82	-1.76	-2.52
	46	81849	**ST6GALNAC5**	1.39	-1.12	1.37	-1.01	-1.49	-1.51	-1.97
	47	9627	**SNCAIP**	1.62	-1.04	1.45	1.02	-1.60	-1.44	-1.94
	48	100130938	**LOC100130938**	1.34	1.02	1.37	1.00	-1.39	-1.31	-1.70
*	49	9732	**DOCK4**	1.61	1.08	1.66	-1.01	-1.37	-1.35	-1.61
	50	64092	**SAMSN1**	1.64	-1.06	1.52	-1.01	-1.75	-2.04	-2.56
	51	54518	**APBB1IP**	1.52	1.07	1.47	1.23	-1.43	-1.75	-2.02
	52	4314	**MMP3**	1.62	1.06	1.77	-1.15	-1.33	-1.58	-1.64
	53	64750	**SMURF2**	1.63	1.01	1.33	1.05	-2.28	-1.58	-2.16
	54	152110	**NEK10**	1.60	1.04	1.33	1.03	-2.01	-1.62	-2.09
	55	84830	**C6orf105**	1.85	-1.01	1.43	1.07	-1.52	-1.38	-1.70
	56	63827	**BCAN**	1.39	-1.09	1.25	1.13	-1.42	-1.47	-2.19
	57	3552	**IL1A**	1.47	1.00	1.06	-1.03	-2.46	-2.41	-3.08
	58	3037	**HAS2**	1.48	1.01	1.74	-1.11	-3.39	-3.21	-7.18
	59	374987	**C1orf118**	1.35	1.06	1.38	-1.10	-1.61	-1.73	-2.32
	60	9659	**PDE4DIP**	1.27	1.07	1.34	1.01	-2.01	-1.45	-2.48
	61	353088	**ZNF429**	1.60	1.27	1.64	1.02	-1.01	-1.66	-1.38
	62	79814	**AGMAT**	-1.05	1.35	1.25	-1.11	1.51	-1.31	-1.95
	63	414899	**BLID**	1.41	1.63	2.08	-1.04	-1.54	-1.22	-1.75
	64	54941	**RNF125**	1.57	1.98	1.74	-1.04	1.07	-2.25	-2.63
	65	7172	**TPMT**	3.78	3.73	5.69	-1.23	1.58	-2.54	-2.58
*	66	4312	**MMP1**	3.46	-1.02	3.40	-1.03	-1.51	-1.32	-1.63
	67	55691	**FRMD4A**	2.10	1.80	2.59	1.02	-1.57	-1.02	-1.80
	68	80215	**C21orf96**	2.27	1.70	2.68	-1.02	-1.65	1.09	-1.34
	69	23613	**ZMYND8**	1.97	1.60	2.47	-1.01	-1.34	1.12	-1.29
	70	56938	**ARNTL2**	2.65	2.97	2.80	-1.13	-1.10	-2.44	-2.73
	71	162073	**ITPRIPL2**	1.76	2.37	2.22	-1.09	1.22	-1.93	-2.16
	72	54552	**GNL3L**	1.68	1.92	1.84	1.01	1.12	-1.71	-1.89
	73	84296	**GINS4**	2.11	2.25	2.13	-1.09	1.05	-1.69	-2.03
	74	25974	**MMACHC**	2.10	2.16	2.60	-1.24	-1.01	-2.23	-1.97
	75	3077	**HFE**	2.01	2.04	2.08	-1.08	1.27	-1.68	-1.78
	76	84671	**ZNF347**	1.52	1.50	1.58	-1.07	-1.00	-1.49	-1.48
	77	407975	**MIR17HG**	1.53	1.67	2.01	1.04	-1.09	-1.30	-1.34
	78	7029	**TFDP2**	1.41	1.53	1.55	-1.01	1.14	-1.44	-1.27
	79	4512	**MT-COI**	1.47	1.66	1.54	-1.21	1.13	-1.45	-1.33
	80	80830	**APOL6**	1.49	1.53	1.57	1.03	1.12	-1.95	-1.20

	81	348654	**GEN1**	1.90	2.06	1.62	-1.09	1.04	-2.10	-2.14
	82	220869	**CBWD5**	2.01	1.95	2.52	-1.09	1.08	-1.27	-1.54
*	83	2012	**EMP1**	1.48	1.41	2.18	1.02	-1.27	-1.29	-1.48
	84	472	**ATM**	1.61	1.40	1.43	-1.05	1.24	-1.29	-1.42
	85	10380	**BPNT1**	1.95	1.45	1.64	1.06	1.25	-1.26	-1.59
	86	1936	**EEF1D**	1.98	1.60	2.04	-1.12	-1.09	-1.16	-1.34
	87	26155	**NOC2L**	1.80	1.60	2.28	1.09	1.00	-1.18	-1.32
*	88	374	**AREG**	1.35	1.01	1.35	-1.01	1.08	2.04	1.25
	89	654346	**LGALS9C**	1.36	-1.00	1.39	-1.02	-1.03	1.52	1.09
	90	9987	**HNRPDL**	1.42	1.19	1.86	1.17	-1.02	1.33	-1.01
*	91	760	**CA2**	1.68	-1.04	1.95	-1.00	1.09	1.41	-1.00
	92	80820	**EEPD1**	1.59	-1.05	1.68	-1.06	-1.03	1.52	1.10
	93	58504	**ARHGAP22**	1.74	1.02	1.86	-1.01	-1.24	1.76	-1.12
	94	200150	**PLD5**	1.84	-1.05	1.98	1.05	1.15	2.01	1.24
	95	151887	**CCDC80**	1.55	1.01	1.43	-1.00	-1.04	1.43	1.38
	96	4015	**LOX**	1.57	1.04	1.44	1.04	-1.06	1.37	1.41
	97	94234	**FOXQ1**	1.69	-1.04	1.69	1.07	-1.29	2.60	1.71
	98	28999	**KLF15**	1.60	-1.06	1.45	1.07	1.51	1.87	1.67
	99	647024	**C6orf132**	1.98	1.12	1.81	-1.02	2.04	2.24	2.63
	100	26872	**STEAP1**	1.40	1.02	1.31	1.09	1.89	2.22	2.76
	101	114548	**NLRP3**	1.35	1.01	1.15	1.06	1.51	4.28	3.04
	102	9699	**RIMS2**	1.13	-1.02	1.31	-1.10	1.65	1.38	1.63
	103	10984	**KCNQ1OT1**	1.61	1.52	1.99	1.07	1.12	1.30	1.29
	104	51105	**PHF20L1**	1.50	1.31	2.03	1.08	1.09	1.43	1.06
	105	57235	**KIAA0485**	2.15	1.90	3.94	-1.08	-1.11	1.70	1.27
	106	26137	**ZBTB20**	1.99	1.51	2.67	-1.01	1.36	1.43	1.19

Additional file 6:

Supplemental Table S2: Ligand-free and liganded PR isoforms regulated gene categories corresponding to the identified biological processes

	A-O-	B-O-	AB-O-	A+A-	B+B-	AB+AB-
p-val range	5.47E-05-3.28E-02	7.25E-03-4.97E-02	1.86E-04-2.9E-02	5.44E-14-1.18E-02	1.87E-08-2.34E-02	5.06E-15-9.78E-03
-log(highest p-val)	4.26	2.14	3.73	13.26	7.73	14.30
Cancer	DPYSL2, LOXL4, JAK1, HNRPDL, MMP3, KRT7, PFDN6, CXCL1, LTB, HES1, ODC1, SERPINB2, IL7R, STK4, BCAN, EZR, ITGAV, ZBTB20, SAA1, BIRC3, DMBT1, ATM, PTX3, LMO4, ADAMTS1, THBS1, MITF, RGS4, SC4MOL, HBEGF, ANXA2, NFKB2, MLL, AREG, MYL9 (includes EG:10398), CD68, SEC31B, HSP90AA1, JAG1, BCL2A1, CARD16, CA2, LOX, IL1A, ICAM1, COL4A6, BMPR2, CASP4, TRA2A, AQP3, SLC16A3, COL6A1, JUN, EDN1, VIPR1, FASN, DGCR8, TGFB2, CASP1, MAP1LC3B, EYA4, CASP8, TFDP2, MMP1 (includes EG:4312), ADRB2, MT-COI, EBLN2, LAMA5, NLRP3, KRT17, DST, RELB, BCL3, FRMD4A, CPA4, EMP1, GNAS, CAT, TAGLN, NR4A1, LIMD2, IL1B, MST1R, C15ORF48, KIAA1199, CTSC, ABCC3, MMP9	BRCA2, MCTS1, CTNNB1, MLL, ATM	MALAT1, DPYSL2, AKAP8L, LTB, SERPINB2, ZBTB20, FOSL1, ATM, PDGFRB, DMBT1, MITF, HBEGF, SC4MOL, ANXA2, NFKB2, PITX2, AREG, MYL9 (includes EG:10398), TNS4, CD68, KIDINS220, HAS2, BCL2A1, CA2, CARD16, ICAM1, PDE7A, CASP4, MCTS1, AQP3, SLC16A3, MYBL1, VIPR1, FASN, DGCR8, MAP1LC3B, AMY2B (includes EG:280), TFDP2, CTNNB1, GJB2, MMP1 (includes EG:4312), TMSB4X, KRT17, DST, TAGLN, NR4A1, LIMD2, CSF2, SYT12, HOXB5, LOXL4, ARHGAP26, HNRPDL, MMP3, KRT7, CXCL1, SMC4, ORM1, STK4, ITGAV, SMYD4, PRLR, BIRC3, SAA1, PTX3, ADAMTS1, THBS1, RGS4, INHBB, MLL, ADAM12, HSP90AA1, SEC31B, RBBP6 (includes EG:5930), JAG1, VHL, S100P, BUB3, LOX, BLID, COL4A6, EPM2AIP1, BCAR1, TRA2A, COL6A1, EDN1, TGFB2, CASP1, KIAA1211, ADRB2, MT-COI, EBLN2, PXN, PDE4C, IL3RA, PPP2R5C, RELB, BCL3, FRMD4A, CPA4, EMP1, GNAS, SSTR2, CAT, IL1B, MST1R, C15ORF48, KIAA1199, CTSC, MMP9	SGK1, PFDN6, NF2, NPEPPS, IL6, SERPINB2, ATP8B1, HRH1, ZNF367, LHX6, BCAN, MT2A, SCG5, EZR, ZBTB20, CD274, FOSL1, IGFBP1, DDIT4, MT1X, PTCH1, HBEGF, IER3, PITX2, TFPI2, TBC1D4, SLC46A3, IRS1, SLC16A5, DACH1, HAS2, NR3C2, RPS6KA1, BCL2A1, ENC1, NRP1, MAP2K6, ERCC4, ZFP36, FOXA1, DUSP6, BMPR2, STYK1, AQP3, MUM1L1, JUN, PTPRJ, MT1E, FGF18, MYBL1, FGD4, NAV3, PRKCE, LETM2, GJB2, MMP1 (includes EG:4312), TACSTD2, PLAT, HEY1, CYP1A1, EPB41L4B, F3, STXBP6, CYP1B1, ABL2, CDKN1A, RASGRF1, NR4A1, S1PR1, CSF2, SYT12, SOCS3, PER1, CYP24A1, ARHGAP26, JAK1, MMP3, CXCL1, C13ORF15, BCL6, IL7R, MT1B, USP53, SDPR, ITGAV, SIM2, SMYD4, PRLR, CDKN1C, TNFRSF11B, PTX3, MT1G, IL8, LMO4, CASP3, ADAMTS1, IER2, EPOR, IL6R, PDE4B, ADAMTS9, FOXC1, INHBA, MLPH, MEX3D, TLR2, PDLIM4, FOSB, PIK3R3, BCL2L1, ADAM12, DUSP1, CEBPD, CALB2, SLC22A5, IGFBP3, FZD5, CST6, JAG1, EREG, LGR4, BLID, IL1A, ID2, GRPR, MMP14, EXT1, HDAC9 (includes EG:9734), UGCG, DLX4, HIC1, SOX9, ARL4C, EFNB1, HSD11B2, NAMPT, ERRFI1, AGPAT9, EYA4, ADRB2, CLIC5, TFAP2C, EPAS1, CXCR4, EMD, FRMD4A, MT1H, TNFRSF11A, CHIC2, COL1A1, FZD4, TACC2, LPAR1, NT5E, IL1B, DKK1, SLC19A2, WNT5A,	SOCS1, NF2, LTB, HES1, SERPINB2, HRH1, ATP1A1, LHX6, BCAN, MT2A, EZR, CD274, FOSL1, AHR, VSTM2L, DAPK1, MT1X, HBEGF, IER3, AREG, ST5, IRS1, HAS2, CTDSPL, NR3C2, HELB, ENC1, NRP1, CA2, ZFP36, PAX8, MUM1L1, PTPRJ, MT1E, MYBL1, FGF18, FGD4, ANTXR2, NAV3, PRKCE, SERPINA1, LETM2, RASSF5, CASP8, TFDP2, MMP1 (includes EG:4312), PLAT, BMP1, ATP1A3, MREG, EPB41L4B, PAICS, CYP1B1, ABL2, IMPDH1, CDKN1A, S1PR1, CSF2, TM4SF1, SOCS3, PER1, ATP1A4, JAK1, CYP24A1, HNRPDL, MMP3, CXCL1, C13ORF15, CD163, BCL6, MT1B, SDPR, CDKN1C, ADRA1B, PDK4, TNFRSF11B, PTX3, IL8, MT1G, TBC1D2B, ADAMTS1, IER2, IL6R, CDK6, ADAMTS9, FOXC1, INHBA, TLR2, PDLIM4, BCL2L1, DUSP1, CEBPD, SLC22A5, RBBP6 (includes EG:5930), LOX, IL1A, ID2, HDAC9 (includes EG:9734), EXT1, SOX9, COL6A1, EDN1, HSD11B2, NAMPT, TFCP2L1, PTGER4, MT-COI, TFAP2C, MTHFR, RELB, MT1H, CHIC2, TLR4, FOXO1, LPAR1, TACC2, NT5E, IL1B, DKK1, FHOD3 (includes EG:80206), PIM2 (includes EG:11040)	SOCS1, SGK1, NF2, LTB, HES1, SERPINB2, ATP8B1, HRH1, ATP1A1, ZNF367, LHX6, MT2A, BCAN, SCG5, CD274, FOSL1, IGFBP1, AFAP1, AHR, VSTM2L, ATM, PDGFRB, ETS1, DAPK1, DDIT4, MITF, MT1X, TNFRSF10B, HBEGF, IER3, PITX2, TFPI2, TBC1D4, CMIP, TNS4, SLC46A3, IRS1, SLC16A5, DACH1, HAS2, NR3C2, CTDSPL, RPS6KA1, RNF160, HELB, BCL2A1, ENC1, NRP1, ERCC4, ZFP36, FOXA1, DUSP6, PAX8, SOCS2, SDK1, STYK1, AQP3, MUM1L1, JUN, PTPRJ, MT1E, FGF18, MYBL1, ANTXR2, FGD4, NAV3, PRKCE, SERPINA1, RASSF5, LETM2, GJB2, MMP1 (includes EG:4312), TACSTD2, PLAT, HEY1, ATP1A3, CYP1A1, KRT17, EPB41L4B, ITGA2, STXBP6, F3, CYP1B1, ABL2, CDKN1A, RASGRF1, NR4A1, S1PR1, CSF2, TM4SF1, SYT12, PER1, SOCS3, ATP1A4, CYP24A1, MMP3, CXCL1, C13ORF15, BCL6, CD163, IL7R, MT1B, USP53, SDPR, ITGAV, PRLR, CDKN1C, ADRA1B, TNFRSF11B, PDK4, PTX3, IL8, MT1G, TBC1D2B, ADAMTS1, CASP3, IER2, EPOR, IL6R, CDK6, PDE4B, ADAMTS9, FOXC1, INHBA, INHBB, MLPH, TLR2, PDLIM4, FOSB, BCL2L1, ARHGAP18, ADAM12, DUSP1, CEBPD, CALB2, SLC22A5, IGFBP3, FZD5, CST6, JAG1, EREG, LGR4, BLID, LOX, IL1A, ID2, GRPR, UGCG, HDAC9 (includes EG:9734), EXT1, IL22RA1, BCAR1, HIC1, SPSB1, SOX9, ARL4C, COL6A1, EDN1, EFNB1, TGFB2, NAMPT, AGPAT9, PTGER4, ADRB2, CLIC5, TFAP2C, EPAS1, MTHFR, CXCR4, FRMD4A, MT1H, TNFRSF11A, CHIC2, EMP1, TLR4, COL1A1, FZD4, GHR, TACC2, SSTR2, LPAR1, FOXO1, NT5E, CLDN1, IL1B, C15ORF48, DKK1, SLC19A2, CXCL2, CTSC, FHOD3 (includes EG:80206), WNT5A

Supplemental Table S2 - continued

	A-O-	B-O-	AB-O-	A+A-	B+B-	AB+AB-
p-val range	**5.67E-06-3.3E-02**	**1.45E-02-4.27E-02**	**8.03E-04-2.96E-02**	**5.81E-10-1.34E-02**	**2.83E-09-2.4E-02**	**2.5E-11-9.61E-03**
-log(highest p-val)	**5.25**	**1.84**	**3.10**	**9.24**	**8.55**	**10.60**
Cell Death	JAK1, CXCL1, LTB, HES1, BRCC3, LMNB1, ODC1, SERPINB2, IL7R, STK4, CRADD, EZR, DLST, ITGAV, SAA1, BIRC3, ATM, THBS1, HBEGF, NFKB2, ROR1, MLL, AREG, ANKRD1, HSP90AA1, BCL2A1, JAG1, CASP5, CNN2, CA2, IL1A, ICAM1, CASP4, AQP3, JUN, EDN1, FASN, CASP1, TGFB2, CASP8, TRAF1, NLRP3, LUC7L3, RELB, BCL3, EMP1, NOC2L, PMP22, RNF34, CAT, NR4A1, IL1B, MST1R, ABCC3, SNCAIP, PTPN22, MMP9	AKAP8L, BLID, PTPRG, BRCA2, CTNNB1, ATM	AKAP8L, CXCL1, LTB, SERPINB2, STK4, ITGAV, PRLR, BIRC3, SAA1, PDGFRB, ATM, PTPRG, THBS1, HBEGF, NFKB2, ROR1, MLL, AREG, ANKRD1, ADAM12, HAS2, HSP90AA1, RBBP6 (includes EG:5930), BCL2A1, JAG1, CASP5, S100P, CNN2, CA2, BLID, ICAM1, PDE7A, CASP4, MCTS1, BCAR1, DEFB103B, AQP3, EDN1, MYBL1, FASN, TGFB2, CASP1, EGLN3, CTNNB1, TRAF1, PPP2R5C, LUC7L3, PDE4C, RELB, BCL3, EMP1, NOC2L, SSTR2, RNF34, CAT, NR4A1, IL1B, MST1R, SNCAIP, CSF2, PTPN22, WNT11, MMP9	SOCS3, PHLDA1, LIF, JAK1, SGK1, UNC5B, CXCL1, GCLC, IL6, BRCC3, BCL6, LMNB1, SERPINB2, IL7R, G0S2, CRADD, MT2A, A4GALT, EZR, DLST, SIM2, ITGAV, IRS2, CD274, PRLR, CDKN1C, KLF2, TNFRSF11B, IL8, CASP3, IL6R, HBEGF, PDE4B, IER3, INHBA, RCAN1, TLR2, CBLB, BCL2L1, ADAM12, RND3, DUSP1, CALB2, CEBPD, IGFBP3, HAS2, RPS6KA1, JAG1, BCL2A1, NRP1, MAP2K6, ERCC4, BLID, ID2, IL1A, GTF2F2, UBQLN1, DUSP6, HDAC9 (includes EG:9734), UGCG, TGM2, AQP3, SOX9, JUN, MYBL1, DKK3, PRKCE, NAMPT, PDP1, PLAT, HEY1, EPAS1, NLRP3, CXCR4, OBFC2A, PPP1R15A, CLCF1, F3, CYP1B1, PMP22, ABL2, LPAR1, RASSF2, SPRY2, CDKN1A, S1PR1, NR4A1, IL1B, DKK1, SNCAIP, CSF2, WNT11	SOCS1, PHLDA1, SOCS3, JAK1, CXCL1, BAG3, LTB, HES1, BCL6, CABIN1, SERPINB2, G0S2, A4GALT, MT2A, EZR, IRS2, TNFRSF10C, CD274, CDKN1C, KLF2, AHR, TNFRSF11B, IL8, DAPK1, IL6R, CDK6, HBEGF, MALT1, IER3, TXNRD1, INHBA, RCAN1, AREG, CREB3L2, CBLB, TLR2, BCL2L1, RND3, DUSP1, CEBPD, HAS2, RBBP6 (includes EG:5930), NRP1, CA2, ID2, IL1A, HDAC9 (includes EG:9734), SOX9, EDN1, MYBL1, TNIP2, PLK2, ANTXR2, PRKCE, NAMPT, FKBP5, PDP1, CASP8, PLAT, TRAF1, UNC5CL, NLRP3, RELB, RGS16, OBFC2A, PPP1R15A, CYP1B1, TLR4, FOXO1, LPAR1, ABL2, SPRY2, RASSF2, CDKN1A, S1PR1, IL1B, DKK1, CSF2, SNCAIP	SOCS1, SGK1, GCLC, LTB, HES1, CABIN1, SERPINB2, CRADD, MT2A, A4GALT, IRS2, TNFRSF10C, CD274, AHR, ATM, KLRC1, PDGFRB, ETS1, DAPK1, TNFRSF10B, HBEGF, IER3, HAS2, RPS6KA1, BCL2A1, NRP1, ERCC4, DUSP6, SOCS2, TGM2, AQP3, JUN, MYBL1, ANTXR2, PRKCE, HEY1, PLAT, UNC5CL, ITGA2, OBFC2A, RGS16, PPP1R15A, F3, CYP1B1, SEMA3A, ABL2, SPRY2, CDKN1A, S1PR1, NR4A1, CSF2, PTPN22, SOCS3, PHLDA1, LIF, UNC5B, BAG3, CXCL1, BCL6, IL7R, G0S2, ITGAV, PRLR, CDKN1C, KLF2, TNFRSF11B, IL8, CASP3, IL6R, CDK6, MALT1, PDE4B, RCAN1, INHBA, TXNRD1, TLR2, CREB3L2, CBLB, BCL2L1, ADAM12, RND3, DUSP1, CALB2, CEBPD, IGFBP3, JAG1, BLID, IL1A, ID2, GTF2F2, UGCG, HDAC9 (includes EG:9734), BCAR1, DEFB103B, SOX9, EDN1, DKK3, PLK2, TGFB2, NAMPT, PDP1, NLRP3, EPAS1, CXCR4, EMP1, TLR4, NOC2L, PMP22, SSTR2, LPAR1, FOXO1, RASSF2, BIK, IL1B, DKK1, SNCAIP, WNT11

Supplemental Table S2 - continued

	A-O-	B-O-	AB-O-	A+A-	B+B-	AB+AB-
p-val range	**6.71E-08-3E-02**	**7.25E-03-3.57E-02**	**1.28E-07-2.88E-02**	**4.95E-09-1.32E-02**	**3.21E-05-2.4E-02**	**5.69E-11-9.42E-03**
-log(highest p-val)	**7.17**	**2.14**	**6.89**	**8.31**	**4.49**	**10.24**
Cellular Movement	ATP6V0A4, CNN2, DPYSL2, LOX, IL1A, ICAM1, JAK1, MMP3, CXCL1, NPPB, BMPR2, SYNM, DEFB103B, JUN, CCRL2, EDN1, VIPR1, BCAN, EZR, TGFB2, ITGAV, SAA1, MMP1 (includes EG:4312), ADRB2, PDE2A, ADAMTS1, THBS1, ST6GALNAC5, RGS4, HBEGF, ANXA2, MLL, AREG, GNAS, PMP22, CAPN1, CAT, TAGLN, GRLF1, IL1B, MST1R, MMP9, IL11	KIF20B, CTNNB1, AKAP9	DPYSL2, CCR3, MMP3, CXCL1, NPPB, ORM1, L3MBTL1, ITGAV, FOSL1, SPOCK1, SAA1, PDGFRB, PDE2A, ADAMTS1, THBS1, HBEGF, RGS4, ANXA2, MLL, INHBB, AREG, TNS4, CAPN1, HAS2, VHL, S100P, CNN2, ATP6V0A4, LOX, ICAM1, BCAR1, SYNM, DEFB103B, CCRL2, EDN1, VIPR1, TGFB2, CTNNB1, MMP1 (includes EG:4312), ADRB2, PXN, ST6GALNAC5, IL16, GNAS, SSTR2, CAT, TAGLN, SP100, IL1B, MST1R, CSF2, MMP9, IL11	SOCS3, JAK1, MMP3, PTHLH, CXCL1, NF2, IL6, HRH1, BCAN, EZR, ITGAV, IRS2, FOSL1, IGFBP1, SPOCK1, KLF2, IL8, PDE2A, CASP3, ADAMTS1, EPOR, HBEGF, TFPI2, INHBA, HAS3, TLR2, PIP5K1A, RND3, IGFBP3, GRLF1, HAS2, CST6, TNS1, EREG, LGR4, NRP1, ATP6V0A4, IL1A, ID2, MMP14, BMPR2, SYNM, TGM2, JUN, CCRL2, F2RL1, DKK3, EDN2, EFNB1, PRKCE, MMP1 (includes EG:4312), PLAT, ADRB2, HEY1, TFAP2C, CXCR4, ST6GALNAC5, F3, CYP1B1, PMP22, LPAR1, LAMB3, SPRY2, CDKN1A, S1PR1, IL1B, DKK1, CSF2, IL11, WNT5A	SOCS3, LOX, IL1A, ID2, JAK1, MMP3, PTHLH, CXCL1, NF2, NEXN, HRH1, EDN1, BCAN, EZR, PRKCE, IRS2, SERPINA1, FOSL1, KLF2, MMP1 (includes EG:4312), PLAT, IL8, TFAP2C, ADAMTS1, ST6GALNAC5, HBEGF, CYP1B1, INHBA, AREG, TLR2, TLR4, F11R, RND3, FOXO1, LPAR1, RASGRP1, SPRY2, CDKN1A, S1PR1, HAS2, GRLF1, IL1B, MAP2K3, DKK1, CSF2, IL11, NRP1	SOCS3, ATP1A4, MMP3, PTHLH, NF2, CXCL1, NEXN, HRH1, CXCL3, BCAN, ITGAV, IRS2, FOSL1, IGFBP1, SPOCK1, AFAP1, KLF2, PDGFRB, ETS1, IL8, PDE2A, CASP3, ADAMTS1, EPOR, HBEGF, TFPI2, INHBB, INHBA, HAS3, TNS4, TLR2, RND3, IGFBP3, HAS2, CST6, EREG, TNS1, LGR4, NRP1, ATP6V0A4, DCBLD2, LOX, ID2, IL1A, BCAR1, SYNM, TGM2, DEFB103B, CCRL2, JUN, EDN1, DKK3, EDN2, EFNB1, TGFB2, PRKCE, SERPINA1, MMP1 (includes EG:4312), PLAT, ADRB2, HEY1, TFAP2C, CXCR4, ITGA2, ST6GALNAC5, LRRC15, F3, CYP1B1, IL16, SEMA3A, TLR4, F11R, PMP22, SSTR2, LPAR1, FOXO1, LAMB3, SPRY2, CDKN1A, S1PR1, IL1B, DKK1, CSF2, CXCL2, WNT5A, IL11
p-val range	**3.09E-04-3.39E-02**	**7.58E-04-4.97E-02**	**2.12E-04-2.81E-02**	**5.2E-09-1.21E-02**	**2.37E-07-2.4E-02**	**7.81E-09-9.42E-03**
-log(highest p-val)	**3.51**	**3.12**	**3.67**	**8.28**	**6.63**	**8.11**
Cellular Development	DPYSL2, IL1A, ICAM1, CXCL1, BMPR2, HES1, SYNM, IL7R, STK4, EDN1, ARNTL2, EZR, DLST, ITGAV, TGFB2, CASP8, SAA1, DMBT1, OXTR, THBS1, RELB, RGS4, HBEGF, NFKB2, MLL, AREG, PMP22, CAPN1, NR4A1, IL1B, MST1R, CCDC80, MMP9, IL11	TRA2A, CCDC88A, CAPN1, CTNNB1, MLL	DPYSL2, CCR3, CXCL1, STK4, EGR2, ITGAV, FOSL1, SMYD4, FRS2, PDGFRB, ATM, DMBT1, PTPRG, THBS1, CABLES1, HBEGF, RGS4, SNRK, NFKB2, MLL, INHBB, AREG, CAND1, TNS4, MYL9 (includes EG:10398), CAPN1, HAS2, RBBP6 (includes EG:5930), JAG1, CCDC80, VHL, ICAM1, BCAR1, SYNM, TRA2A, EDN1, MYBL1, FASN, TGFB2, CTNNB1, PXN, IL3RA, RELB, IL1B, MST1R, CSF2, MMP9, PTPN22, IL11	SOCS3, LIF, PTHLH, CXCL1, NF2, IL6, BCL6, IL7R, ST8SIA4, EZR, DLST, ITGAV, CD274, FOSL1, IGFBP1, PRLR, KLF2, ICOSLG, TNFRSF11B, IL8, CASP3, EPOR, CABLES1, IL6R, HBEGF, IER3, FOXC1, HAS3, INHBA, CBLB, TLR2, BCL2L1, DUSP1, IRS1, CEBPD, DACH1, IGFBP3, HAS2, RPS6KA1, JAG1, ENC1, EREG, IL1A, ID2, MMP14, BMPR2, HDAC9 (includes EG:9734), SYNM, SOX9, JUN, F2RL1, DKK3, MYBL1, FGF18, STEAP2, EFNB1, PRKCE, NAMPT, OXTR, HEY1, TFAP2C, CYP1A1, CXCR4, PPP1R15A, PMP22, LPAR1, PUS7L, SPRY2, CDKN1A, SEC14L2, CALCR, NR4A1, S1PR1, IL1B, DKK1, CSF2, IL11	SOCS3, PTHLH, CXCL1, NF2, HES1, BCL6, CD163, EZR, CD274, FOSL1, KLF2, ICOSLG, TNFRSF11B, IL8, IL6R, CDK6, HBEGF, IER3, FOXC1, INHBA, AREG, TLR2, CAND1, CBLB, BCL2L1, DUSP1, CEBPD, IRS1, HAS2, RBBP6 (includes EG:5930), CCDC80, ENC1, FGF5, IL1A, ID2, HDAC9 (includes EG:9734), SOX9, EDN1, ARNTL2, MYBL1, FGF18, STEAP2, PRKCE, NAMPT, RASSF5, FKBP5, CASP8, OXTR, TFAP2C, RGS16, PPP1R15A, TLR4, LPAR1, FOXO1, SPRY2, CDKN1A, SEC14L2, CALCR, S1PR1, IL1B, DKK1, CSF2, IL11	SOCS1, NF2, HES1, CD274, FOSL1, IGFBP1, ICOSLG, ATM, KLRC1, PDGFRB, ETS1, TNFRSF10B, HBEGF, IER3, TNS4, IRS1, DACH1, HAS2, RPS6KA1, ENC1, SYNM, JUN, MYBL1, FGF18, PRKCE, RASSF5, HEY1, OXTR, CYP1A1, ITGA2, RGS16, PPP1R15A, SEMA3A, SPRY2, CDKN1A, NR4A1, S1PR1, SEC14L2, CSF2, PTPN22, SOCS3, LIF, PTHLH, CXCL1, BCL6, CD163, IL7R, ITGAV, PRLR, KLF2, TNFRSF11B, IL8, CASP3, EPOR, CABLES1, IL6R, CDK6, FOXC1, INHBA, INHBB, HAS3, TLR2, CBLB, BCL2L1, DUSP1, CEBPD, IGFBP3, JAG1, EREG, CCDC80, FGF5, ID2, IL1A, HDAC9 (includes EG:9734), IL22RA1, BCAR1, SOX9, ARNTL2, EDN1, DKK3, EFNB1, STEAP2, TGFB2, NAMPT, TFAP2C, CXCR4, TLR4, PMP22, GHR, LPAR1, FOXO1, SSTR2, PUS7L, CALCR, IL1B, DKK1, IL11

Supplemental Table S2 - continued

	A-O-	B-O-	AB-O-	A+A-	B+B-	AB+AB-
p-val range	**6.62E-04-3.39E-02**	**1.06E-03-4.97E-02**	**1.49E-03-2.79E-02**	**1.88E-05-1.08E-02**	**1.24E-06-2.4E-02**	**4.97E-07-9.78E-03**
-log(highest p-val)	**3.18**	**2.97**	**2.83**	**4.73**	**5.91**	**6.30**
Cell Cycle	TBRG1, EDN1, THBS1, FASN, TGFB2, HBEGF, IL1B, FRS2, MLL, MMP9, ATM, IL11	CENPF, BLID, ARNTL2, CENPE, KIF20B, BRCA2, AKAP9, MLL, RAD50, BUB3, ATM	BLID, DBF4B, SMC4, TBRG1, EDN1, ARNTL2, MYBL1, L3MBTL1, FASN, TGFB2, FOSL1, FRS2, PDGFRB, ATM, PPP2R5C, THBS1, HBEGF, RPL23, BCL3, MLL, IL16, SSTR2, CAT, NR4A1, HAS2, IL1B, CSF2, MMP9, VHL, IL11, BUB3	MAP2K6, BLID, IL1A, ID2, LIF, PTHLH, NF2, C13ORF15, IL6, BRCC3, BCL6, SOX9, MYBL1, CARD10, PRKCE, FOSL1, NAMPT, CDKN1C, NEDD4L, MAP9, IL8, PPP1R15A, HBEGF, MT1A, FOXC1, CYP1B1, INHBA, COL1A1, NEK11, DUSP1, CEBPD, IRS1, RASSF2, SPRY2, CDKN1A, CALCR, NR4A1, IGFBP3, HAS2, IL1B, CSF2, EREG, IL11	ID2, IL1A, PTHLH, NF2, C13ORF15, HES1, BCL6, SOX9, EDN1, ARNTL2, MYBL1, PRKCE, NAMPT, FOSL1, CDKN1C, NEDD4L, ADRA1B, AHR, IL8, CDK6, PPP1R15A, HBEGF, MT1A, FOXC1, CYP1B1, INHBA, DUSP1, CEBPD, RASSF2, IRS1, SPRY2, CDKN1A, CALCR, HAS2, IL1B, CSF2, TM4SF1, PIM2 (includes EG:11040), IL11	LIF, PTHLH, C13ORF15, NF2, HES1, BCL6, FOSL1, CDKN1C, NEDD4L, AHR, ADRA1B, ATM, PDGFRB, IL8, CDK6, HBEGF, FOXC1, INHBA, DUSP1, IRS1, CEBPD, IGFBP3, HAS2, CDC14A, EREG, BLID, IL1A, ID2, SOX9, EDN1, ARNTL2, MYBL1, CARD10, TGFB2, PRKCE, NAMPT, ITGA2, PPP1R15A, MT1A, CYP1B1, IL16, COL1A1, NEK11, SSTR2, SPRY2, RASSF2, CDKN1A, BIK, CALCR, NR4A1, IL1B, CSF2, TM4SF1, IL11
p-val range	**2.92E-04-3.39E-02**	**2.16E-02-4.97E-02**	**1.47E-04-2.86E-02**	**1.39E-04-1.11E-02**	**2.79E-05-2.4E-02**	**6.29E-06-9.78E-03**
-log(highest p-val)	**3.53**	**1.67**	**3.83**	**3.86**	**4.55**	**5.20**
Cell-To-Cell Signaling and Interaction	IL1A, ICAM1, JAK1, DOCK4, NPPB, LTB, VIPR1, BCAN, FASN, ITGAV, TGFB2, SAA1, MMP1 (includes EG:4312), OXTR, DMBT1, ADRB2, LAMA5, THBS1, ST6GALNAC5, HBEGF, ANXA2, AREG, LY96, CD68, GRLF1, IL1B, BCL2A1, IL11	BRCA2, CTNNB1	CCR3, ICAM1, DOCK4, LTB, NPPB, BCAR1, ORM1, VIPR1, FASN, TGFB2, ITGAV, CTNNB1, SAA1, MMP1 (includes EG:4312), DMBT1, ADRB2, OXTR, PDGFRB, PXN, THBS1, ST6GALNAC5, HBEGF, ANXA2, INHBB, AREG, IL16, LY96, CD68, ADAM12, IL1B, HAS2, BCL2A1, CSF2, VHL, IL11	PHLDA1, C1ORF38, PTHLH, NF2, DOCK4, IL6, LAMC1, HRH1, BCAN, ITGAV, SPOCK1, ICOSLG, TNFRSF11B, IL8, PCSK5, LAMA2, IL6R, HBEGF, HAS3, INHBA, TLR2, ADAM12, RND3, IGFBP3, HAS2, BCL2A1, CST6, EREG, LGR4, NRP1, IL1A, MMP14, TGM2, SOX9, PCDHA11, PTPRJ, EDN2, FGF18, DKK3, EFNB1, PRKCE, NAMPT, MMP1 (includes EG:4312), GJB2, PLAT, OXTR, ADRB2, TFAP2C, CXCR4, ST6GALNAC5, TNFRSF11A, F3, EFNA4, ABL2, NT5E, SPRY2, S1PR1, CALCR, IL1B, CSF2, IL11, WNT5A	IL1A, PTHLH, DOCK4, LTB, CD163, HRH1, SOX9, PTPRJ, EDN1, BCAN, FGF18, PRKCE, NAMPT, CASP8, MMP1 (includes EG:4312), ICOSLG, ADRA1B, PLAT, TNFRSF11B, IL8, TFAP2C, PCSK5, IL6R, ST6GALNAC5, HBEGF, EFNA4, INHBA, AREG, TLR2, F11R, TLR4, ABL2, RASGRP1, SPRY2, S1PR1, GRLF1, IL1B, CSF2, IL11, NRP1, FGF5	PHLDA1, C1ORF38, PTHLH, NF2, DOCK4, LTB, CD163, LAMC1, HRH1, BCAN, ITGAV, SPOCK1, AFAP1, ICOSLG, ADRA1B, KLRC1, TNFRSF11B, PDGFRB, ETS1, IL8, PCSK5, TNFRSF10B, IL6R, LAMA2, HBEGF, TFPI2, INHBA, INHBB, HAS3, TLR2, RND3, ADAM12, IGFBP3, HAS2, BCL2A1, CST6, EREG, LGR4, FGF5, NRP1, IL1A, SOCS2, TGM2, SOX9, PCDHA11, EDN1, PTPRJ, DKK3, FGF18, EDN2, EFNB1, TGFB2, PRKCE, NAMPT, MMP1 (includes EG:4312), GJB2, PLAT, ADRB2, OXTR, TFAP2C, CXCR4, ITGA2, ST6GALNAC5, TNFRSF11A, F3, EFNA4, IL16, F11R, TLR4, SEMA3A, ABL2, SSTR2, CLDN1, NT5E, SPRY2, S1PR1, CALCR, IL1B, CSF2, IL11, WNT5A

Supplemental Table S2 - continued

	A-O-	B-O-	AB-O-	A+A-	B+B-	AB+AB-
p-val range	2.57E-04-3.35E-02	2.16E-02-4.97E-02	3.96E-04-2.42E-02	6.53E-05-5.2E-03	7.04E-04-2.4E-02	7.42E-06-7.89E-03
-log(highest p-val)	3.59	1.67	3.40	4.19	3.15	5.13
Cell Morphology	DPYSL2, IL1A, ICAM1, CXCL1, ODC1, SYNM, AQP3, STK4, EDN1, EZR, FASN, ITGAV, TGFB2, SAA1, ADRB2, PTX3, THBS1, DST, RGS4, ANXA2, MLL, PMP22, NR4A1, IL1B, MST1R, MMP9	CCDC88A, VAC14, BRCA2, MLL	DPYSL2, CCR3, ICAM1, CXCL1, SYNM, STK4, AQP3, EDN1, FASN, ITGAV, TGFB2, SAA1, ADRB2, PTX3, PXN, THBS1, DST, RGS4, ANXA2, INHBB, MLL, NR4A1, CSF2, VHL, MMP9	PHLDA1, IL1A, F2RL2, LIF, CXCL1, AQP3, SOX9, DKK3, EZR, PRKCE, CDKN1C, KLF2, ADRB2, PTX3, IL8, CASP3, CXCR4, INHBA, HAS3, TLR2, PMP22, TACC2, NT5E, CDKN1A, S1PR1, NR4A1, IGFBP3, RASGRF1, GRLF1, IL1B	PTX3, IL8, PHLDA1, IL1A, CXCL1, INHBA, TLR2, F11R, SOX9, EDN1, FOXO1, TACC2, NT5E, SPRY2, EZR, CDKN1A, S1PR1, GRLF1, IL1B, PRKCE, CDKN1C, KLF2	PHLDA1, IL1A, LIF, CXCL1, SYNM, AQP3, SOX9, EDN1, DKK3, EFNB1, ITGAV, PRKCE, CDKN1C, KLF2, ADRB2, KLRC1, PTX3, IL8, CASP3, CXCR4, TNFRSF10B, ITGA2, INHBB, HAS3, INHBA, TLR2, SEMA3A, F11R, PMP22, LPAR1, FOXO1, TACC2, NT5E, SPRY2, CDKN1A, S1PR1, NR4A1, IGFBP3, RASGRF1, IL1B, CSF2
p-val range	6.48E-04-2.28E-02		1.03E-02-2.96E-02	1.4E-03-9.33E-03	1.04E-04-2.09E-02	3.53E-05-7.73E-03
-log(highest p-val)	3.19		1.99	2.85	3.98	4.45
Cell Signaling	IL1A, GLI2, THBS1, BMPR2, HBEGF, ROR1, IL7R, GNAS, LY96, EDN1, VIPR1, IL1B, BIRC3, FRS2, ADRB2		GNAS, CCR3, EDN1, VIPR1, THBS1, CAT, IL1B, HBEGF, CSF2, PTPN22, ADRB2, PDGFRB	IL1A, LIF, PTHLH, CXCL1, NF2, BMPR2, IL6, IL7R, PTPRJ, F2RL1, EDN2, A4GALT, STEAP2, CD274, GPR39, OXTR, TNFRSF11B, ADRB2, TACSTD2, IL8, CXCR4, IL6R, CLCF1, F3, STXBP6, INHBA, TLR2, BCL2L1, IRS1, PAG1, S1PR1, CALCR, IL1B, CSF2, EREG	SOCS1, IL8, IL1A, PTHLH, CXCL1, TLR2, TLR4, BCL2L1, EDN1, PTPRJ, A4GALT, IRS1, S1PR1, CALCR, IL1B, CSF2, AHR, PTGER4, TNFRSF11B, OXTR	SOCS1, IL1A, PTHLH, CXCL1, DEFB103B, EDN1, EDN2, A4GALT, GPR39, PTGER4, AHR, ADRB2, IL8, CXCR4, ITGA2, IL6R, F3, INHBA, TLR2, TLR4, IRS1, CALCR, S1PR1, IL1B, CSF2, EREG
p-val range	8.08E-03-3.35E-02	7.25E-03-7.25E-03	1.14E-02-2.88E-02	1.22E-03-4.61E-03	3.35E-03-2.36E-02	3.32E-03-7.76E-03
-log(highest p-val)	2.09	2.14	1.94	2.91	2.47	2.48
Cell-mediated Immune Response	DEFB103B, IL7R, ICAM1, THBS1, EZR, TGFB2, IL1B, CASP8	CTNNB1	DPYSL2, IL16, DEFB103B, ICAM1, THBS1, IL1B, CTNNB1	IL7R, TLR2, BCL2L1, CASP3, CXCR4, EZR, IL6R, IL1B, CD274, IL6, ICOSLG	TLR2, BCL2L1, EZR, IL6R, IL1B, CD274, CASP8, ICOSLG	SOCS3, IL8, CASP3, CXCR4, IL6R, TLR2, IL7R, TGM2, DEFB103B, IL16, BCL2L1, SEMA3A, S1PR1, TGFB2, IL1B, CD274, ICOSLG, KLRC1

Part 3: New class of PR antagonists with passive mechanism of action

Background

Progesterone receptor (PR) antagonists have potential applications for leiomyoma, endometriosis and certain hormone dependent breast cancers. However, partial agonistic properties and the lack of PR selectivity of the currently available antiprogestins such as RU486 demands for the development of PR selective antagonists devoid of partial agonist effects. Based on recent crystallographic studies identifying the key contacts involved in PR-progestins interactions, we in a collaborative project designed a new approach to inactivate PR. We have thus synthesized and screened several steroid and non-steroid compounds for their agonist and antagonist properties. The APRn molecules (APR-01 to APR-55) were synthesized in the laboratory of Dr Mouad Alami and Prof. Jean-Daniel Brion (Univ Paris-Sud, CNRS, BioCIS-UMR 8076, Faculté de Pharmacie, Châtenay-Malabry, France) in collaboration with Dr Marie-Edith Rafestin-Oblin (Univ. Paris7-Denis Diderot, France) (Progesterone Receptor Antagonists And Uses Thereof. Patent INSERM-CNRS-UPS; EP N°10305484.7, International PCT/EP2011/057387).

Results

A. Structure-activity relation of APRn molecules *(Patent)*

The APRn molecules were classified in 4 groups depending on the nature of their C3 substituent. The first group includes APRn with no C3 substituent whereas groups 2, 3 and 4 are related to APRn bearing a methoxyl, a hydroxyl or a fluorine atom at C3 position, respectively. The agonist/antagonist activities of APRn were determined in human MDA-MB-231 cells stably expressing PRB (MDA-PRB) by using a luciferase reporter gene placed under the control of synthetic promoter comprising of progesterone response element (PRE). Agonist and antagonist efficacy of APRn molecules were determined at 10^{-6} M and/or 10^{-5} M alone or in combination with progesterone (10^{-9} M). The selected APRn were then tested for their antiprogesterone efficacy on endogenous gene transcription. The results are presented as under:

APRn lacking C3-substituent (group 1)

APR-01 (Steraloids, Newport, RI USA) was the first molecule tested for its PRB agonist/antagonist activity **(Figure 17)**. APR-01 is lacking any double bond within the A and D rings and differs from progesterone by the absence of ketone at the C3 position. APR-01 displayed a strong antagonist character since it inhibited progesterone-induced PR transactivation but presented partial agonist effects when cells were treated with APR-01 alone. Thus, APR-01 is a partial PRB agonist. APR-12, which has the same C17 substituent as APR-01 and progesterone, also behaved as partial PR antagonist.

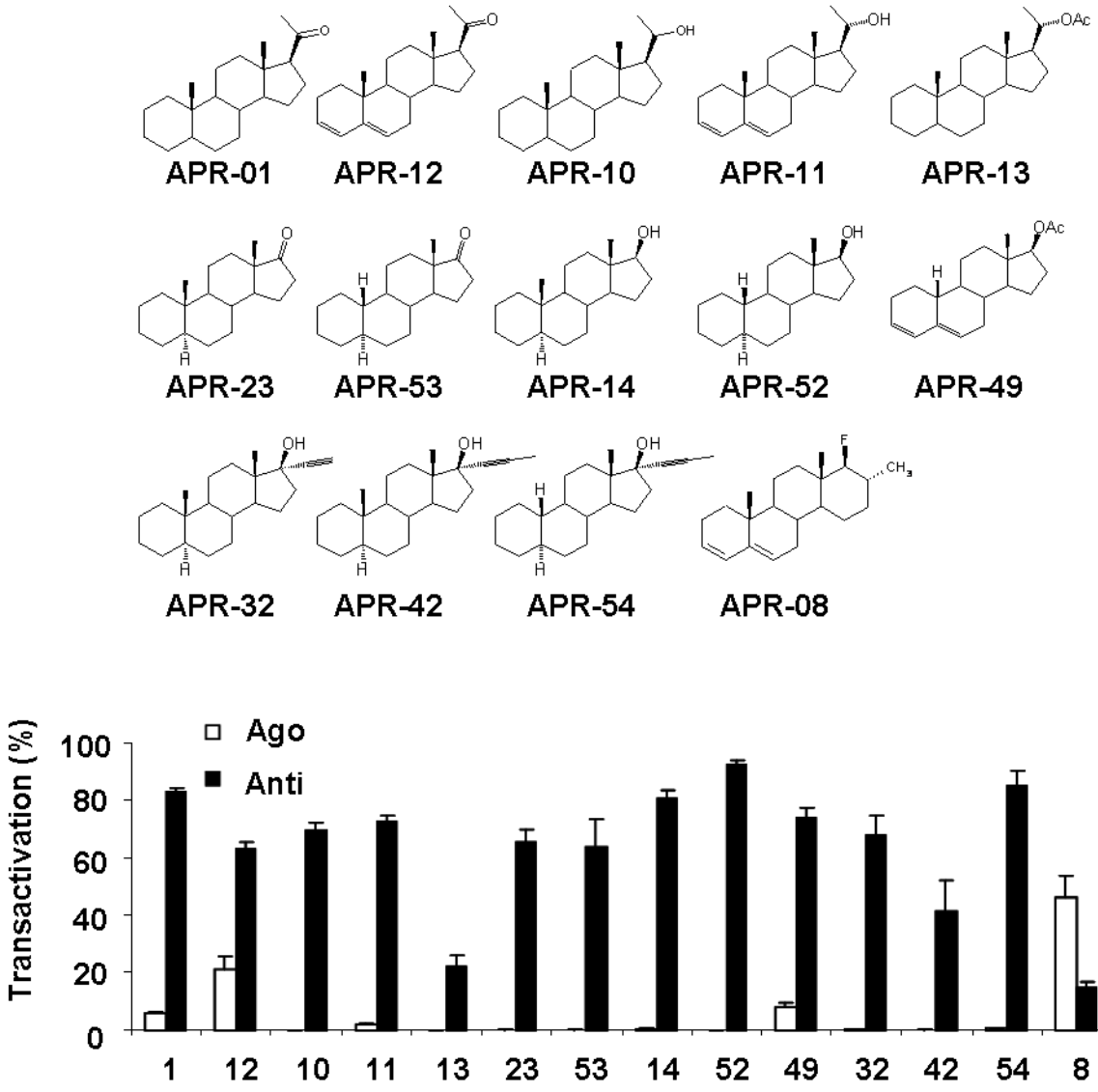


Figure 17. Agonist/antagonist properties of APRn lacking C3 substitution
MDA-PRB cells were transiently transfected with PRE2-luciferase expression vector during 24 h in steroid depleted medium and then treated during 24 h with vehicle or progesterone (10^{-9}M) or indicated APRn molecule (10^{-6}M) or both. Luciferase activity was determined and data (mean ± sem) from six independent cell cultures are presented. Agonist or antagonist efficacy was determined as percentage of maximum transactivation (100 %) obtained following progesterone treatment as compared to vehicle treated cells.

Except the homosteroid APR-08 and APR-49 all the other APRn of group 1 (APR-10, APR-11, APR-13, APR-23, APR-53, APR-14, APR-52, APR-32, APR-42 and APR-54) displayed undetectable to negligible agonist properties with highly variable antagonist efficacies, depending on the nature of the D-ring substituent. While replacing the 20-carbonyl group (APR-01, APR-12) by a 20-hydroxyl group (APR-10, APR-11) had little effect, acetylation of the 20-hydroxyl (APR-13) drastically reduced the antagonist efficacy. The presence of a 17β-hydroxyl (APR-14, APR-52) improved the antagonist properties as compared to a 17-carbonyl (APR-23, APR-53). Acetylation of the 17-hydroxyl group (APR-49) conferred partial agonist character to these molecules without affecting the antagonist properties. The

molecules characterized by a 17β-hydroxyl and a 17α-alkyl substituent (APR-32, APR-42 and APR-54) have variable antagonistic properties, and APR-54 characterized by a 17α-propynyl has the highest antagonist activity. Interestingly, the efficacies of the 19-norsteroids are higher than those of the parent molecules (APR-52 *vs* APR-14, , APR-54 *vs* APR-42).

APRn with 3-methoxy substituent (group 2)

All the APRn molecules characterized by the presence of a 3-methoxy group (APR-02, APR-22, APR-27, APR-28, APR-30, APR-31, APR-38, APR-39) displayed an antagonist character with negligible to no agonist activity. As shown in **Figure 18**, APR-02 and APR-22 are the most potent molecules, indicating that the presence of a 20-keto group (APR-02 and APR-22) is more favorable than a 20-hydroxyl (APR-27, APR-28) or 20-hydroxyl and 20-ethynyl substituents (APR-30, APR-31). The two homosteroids APR-38 and APR-39 with 6-C D ring substituted by a fluorine and a methyl group presented very weak antagonistic effect. It is noteworthy that when APR-22 (group 2) and APR-01 (group 1) were compared, the presence of the C3 methoxy group completely abolished the agonist character without affecting the antagonist efficacy.

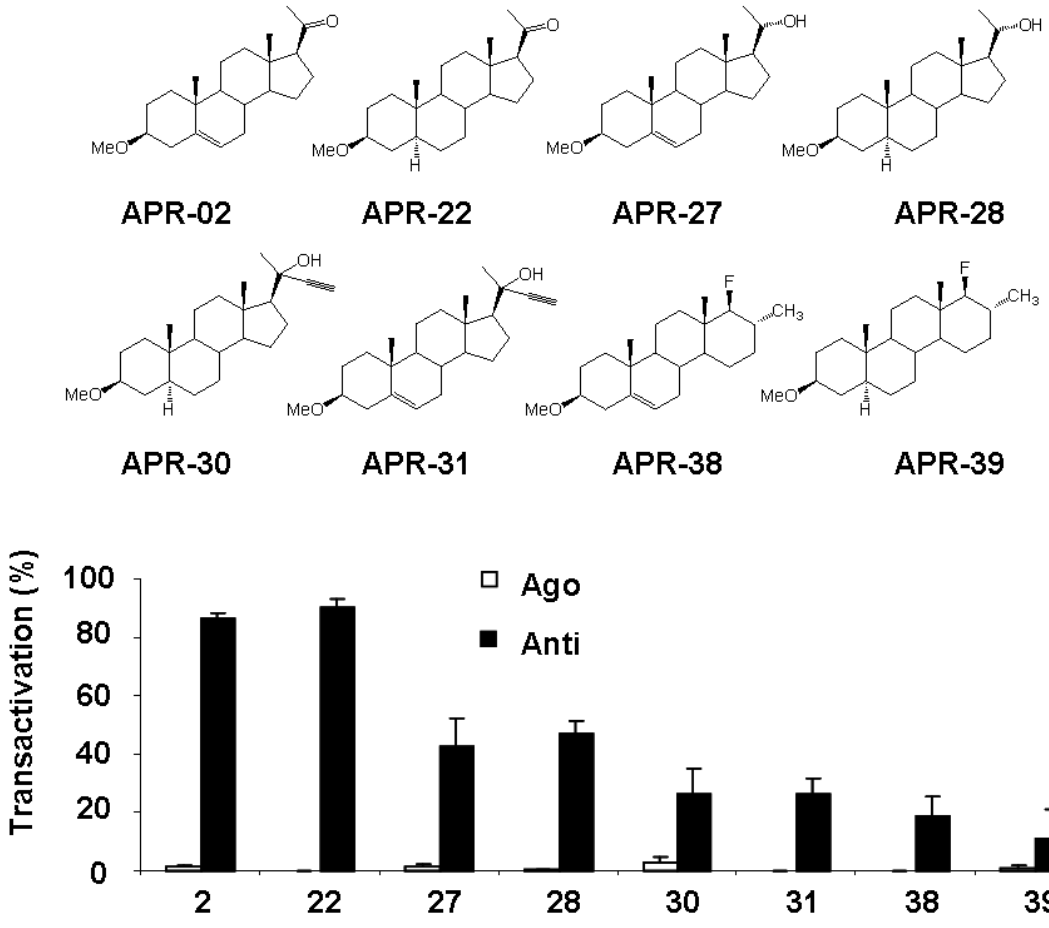


Figure 18. Agonist/antagonist properties of APRn with C3 methoxy substitution
MDA-PRB cells were transiently transfected with PRE2-luciferase expression vector and treated with indicated ligands as in Fig 17. Data (mean ± sem) from six independent cell cultures are presented. Agonist or antagonist efficacy was determined as in Fig. 17.

APRn with a 3-hydroxy substituent (group 3)

Group 3 comprises of APR-15, APR-20, APR-9, APR-18, APR-29, APR-55, APR-48 and APR-07. Most of these molecules presented comparable antagonistic properties except APR-48 which had very weak antagonistic effect **(Figure 19)**. This shows that antagonistic efficacies of these molecules are not modulated by the nature of the C17 substituents. In contrast, agonistic properties of these molecules varied highly, except for APR-18 which did not present agonist properties. The presence of a $\Delta^{5,6}$ insaturation in APR-15 decreased the agonist and increased the antagonist efficiency (compare APR-15 and APR-20). This $\Delta^{5,6}$ insaturation also decreased the agonist efficacy of 20-hydroxylated APRn, however, a slight decrease in antagonist potency was observed (compare APR-18 and APR-29). The $\Delta^{5,6}$ insaturation within 17-hydroxy APRn drastically reduced the antagonistic character (APR-48 vs APR-55). Interestingly, the fluorinated homosteroid APR-07 presented similar antagonistic properties as APR-18, however, small agonist effect was observed with APR-07.

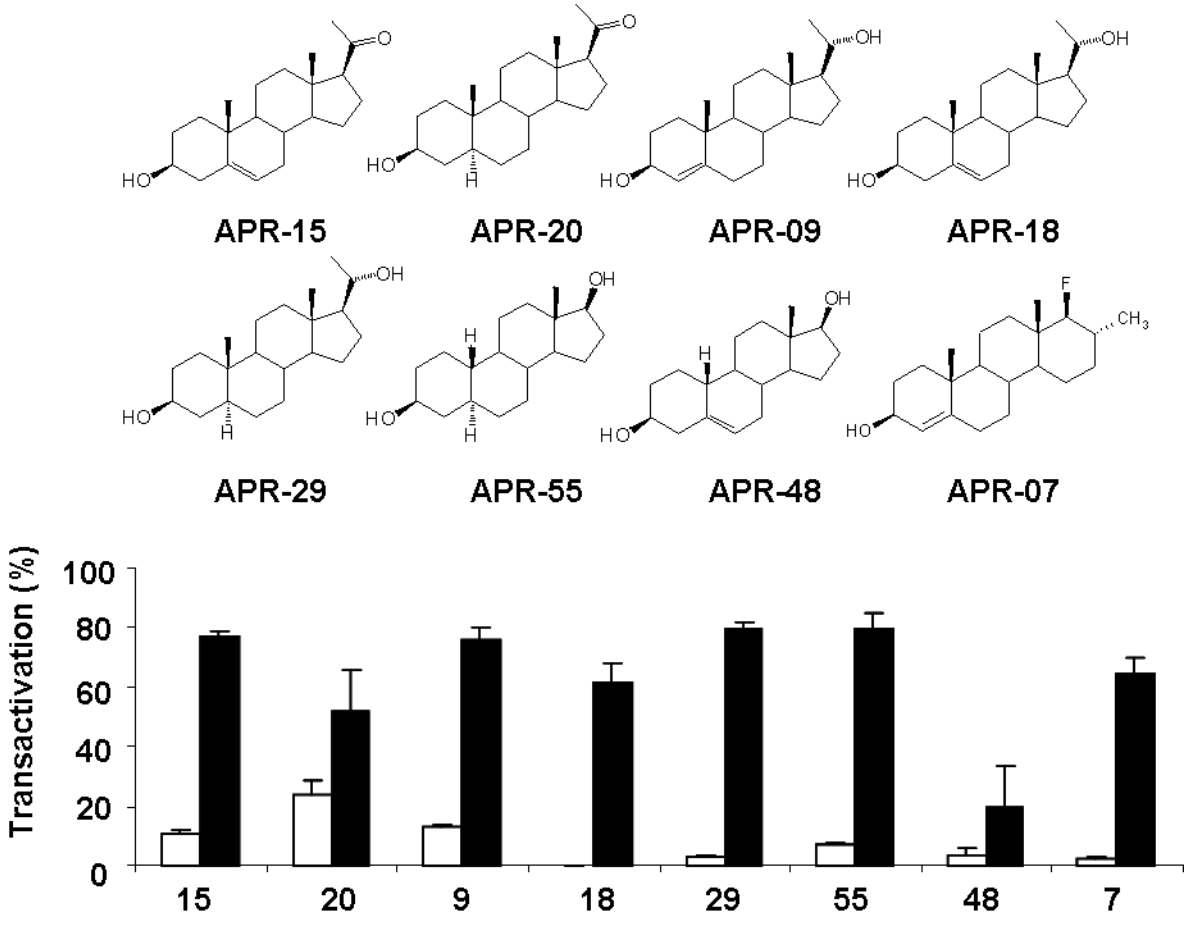


Figure 19. Agonist/antagonist properties of APRn with C3 hydroxy substitution

MDA-PRB cells were transiently transfected with PRE2-luciferase expression vector and treated with indicated ligands as in Fig 17. Data (mean ± sem) from six independent cell cultures are presented. Agonist or antagonist efficacy was determined as in Fig. 17.

APRn with a C3-fluorine atom (group 4)

A C3 fluorine atom was introduced with the aim to maximally reduce the agonist activity of the molecules and to prevent their metabolism. All the fluoro-APRn (APR-16, APR-17, APR-24, APR-25, APR-21, APR-26, APR-35, APR-33, APR-47, APR-40, APR-41, APR-46, APR-45, APR-36, APR-34, APR-50, APR-43, APR-44, APR-51, APR-19, APR-37) were found to be efficient PR antagonists, irrespective to the nature of C17 substituent, with negligible agonist effects except APR-17 and APR-37 **(Figure 20)**. It is interesting to note that the agonist activity of these molecules is completely abolished by introducing a $\Delta^{5,6}$ insaturation (APR-16 vs APR-17, APR-19 vs APR-37). The presence of a six-carbon D-ring (APR-19) instead of five-carbon (APR-16) only slightly decreased PR antagonist activity. These results suggest the dramatic influence of the C3 fluorine atom on the PR antagonist activity.

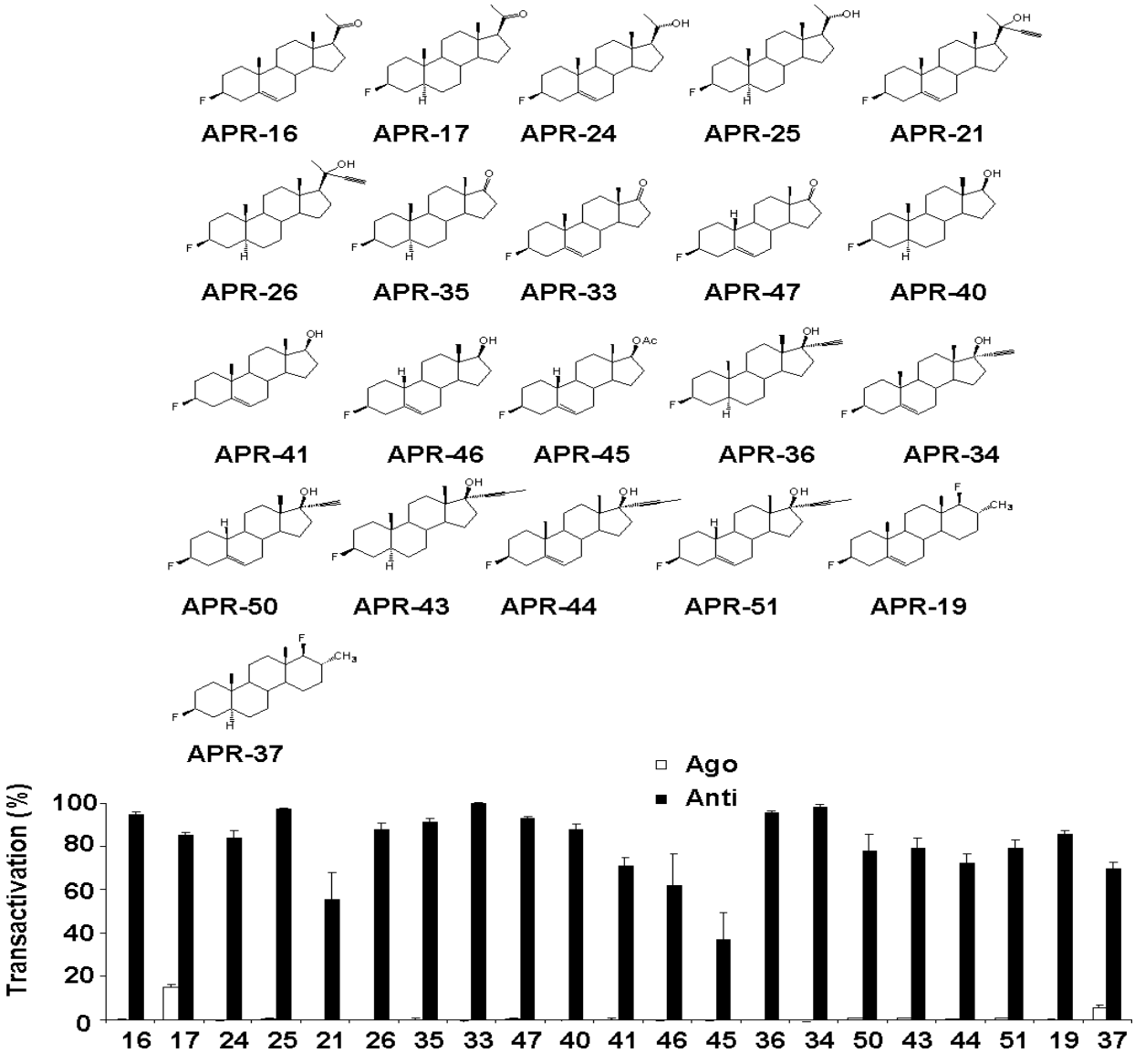


Figure 20. Agonist/antagonist properties of APRn with C3 fluorine substitution
MDA-PRB cells were transiently transfected with PRE2-luciferase expression vector and treated with indicated ligands as in Fig 17. Data (mean ± sem) from six independent cell cultures are presented. Agonist or antagonist efficacy was determined as in Fig. 17.

APRn efficiency on endogenous gene transcription

Based on the PR selective antagonistic properties, APRn molecules (APR-16, APR-19, APR-43, APR-47, APR-51 and APR-54) were chosen for further studies. Firstly, the affinity of these selected molecules to bind PR was determined by binding experiments as shown in **Figure 21A**. It was found that the IC_{50} values of these molecules are about $5x10^{-7}$M. Dose-dependent inhibition of PR transcriptional activity on PRE-driven reporter gene transcription is presented in **Figure 21B**. Finally, these selected APRn molecules were verified for their agonist and antagonistic properties on endogenous amphiregulin gene transcription by quantitative RT-PCR analysis **(Figure 22)**. While the selected APRn were nearly unable to exert agonistic effect on amphiregulin gene transcription, their antagonistic properties varied from 30 to 90 %.

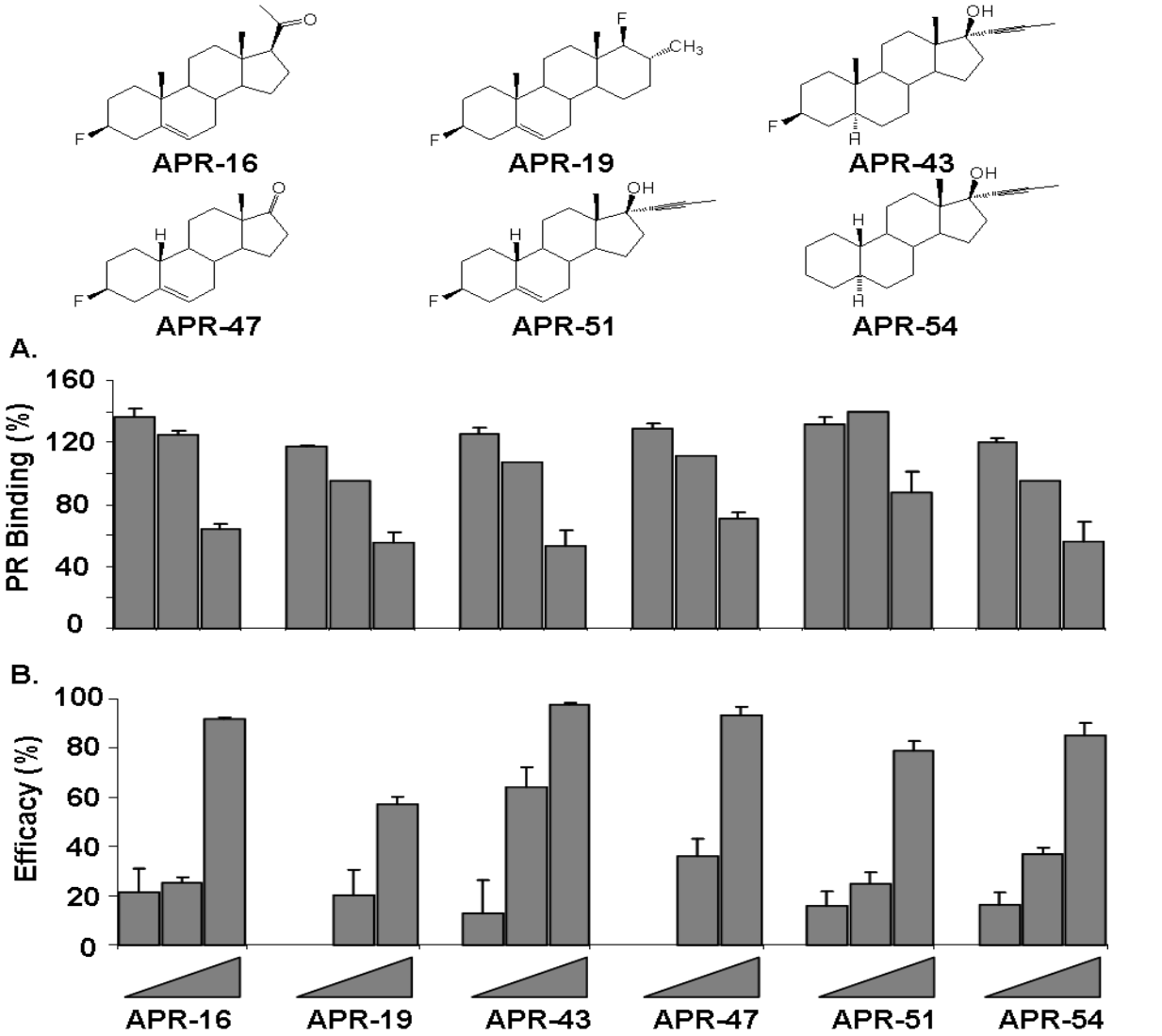


Figure 21. PR binding efficacy and antagonistic properties of selected APRn
A. PR binding of APRn molecules (10^{-8} to 10^{-6} M) as calculated considering 100 % binding by progesterone (10^{-9}M) from cytosols following 4h binding at 4°C.
B. MDA-PRB cells were transiently transfected as in Fig. 17 and treated during 24 h with vehicle or progesterone (10^{-9}M) alone or in combination with APRn (10^{-8} to 10^{-6} M). Luciferase activity was determined and data (mean ± sem) from six independent cell cultures are presented.

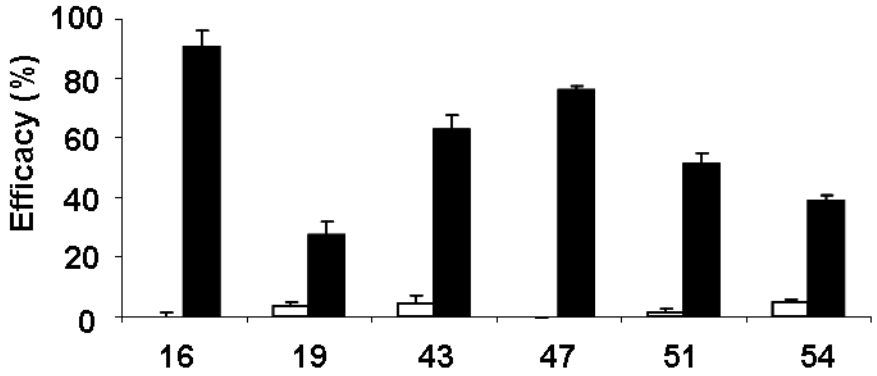


Figure 22. Agonist and antagonist efficacy of selected APRn on amphiregulin gene transcription.
MDA-PRB cells were culutred in steroid free medium during 24 h and then treated with vehicle or progesterone (10^{-9}M) or indicated APRn molecule (10^{-6}M) or both during 6 h. Quantitative RT-PCR analysis was performed for the analysis of amphiregulin gene transcripts and data (mean ± sem) from triplicate independent cell cultures is presented.

B. A rationally designed homosteroid PR antagonist (*PAPER IV*)

In this part of thesis, we undertook a proof-of-concept study on APR19 molecule, a prototype homosteroid ligand belong to new class of passive PR antagonists. We demonstrate that C3 substitution is a key determinant for full PR antagonist character of homosteroid molecules, since APR6 with 3-ketone presented partial agonist features while APR19 characterized by 3-fluorine behaves as a full antagonist. APR19 inhibits progesterone-induced PR transactivation from synthetic as well as endogenous gene promoters in a dose-dependent manner. APR19 is selective to PR and displays high antiprogesterone efficacy *in vivo*. Biochemical and immunofluorescence experiments reveal that upon APR19 binding, PR is unable to recruit transcriptional coactivators (SRC1, SCR2) and corepressors (NCoR, SMRT) as opposed to RU486-PR complexes. *In silico* docking experiments based on crystal structure of the PR ligand-binding domain show that, in contrast to progesterone, APR19 only partially occupies the ligand-binding cavity, preventing PR-APR19 complex to acquire a stable conformation which is a pre-requisite for PR interaction with transcriptional coregulators. Collectively, we demonstrated that APR19 belongs to a new class of PR antagonists that inactivates PR through a passive mechanism.

Perspectives

Given the diverse functional consequences of PRA vs PRB activation, it seems important to investigate the isoform-specific antagonistic properties of the selected APRn molecules. In the current study, we screened these molecules on the basis of PRB-mediated reporter gene transcription since one limitation in screening these molecules for PRA-specific antagonistic properties was the low transcriptional activation by PRA from classical PRE-harboring promoters

linked to reporter gene luciferase. Therefore, it will be of great interest to explore the antagonistic properties of the APRn molecules for their impact on transcriptional regulation of gene subsets identified by transcriptomic studies under varied PR isoforms expression conditions. This might lead to identify antagonist molecules capable of selective inhibition of PRA- or PRB-regulated genes involved in cancer progression. In addition, *in vivo* studies are required to investigate anti-tumoral properties of APR19 and other lead APRn molecules in mouse models xenografted with PR expressing human breast tumors or cancer cell lines expressing PRA and/or PRB. Our newly established bi-inducible breast cancer cells might of particular interest since PR isoform-specific antagonistic properties can be investigated under both ligand-free and progesterone-treated conditions as well as under similar cellular and genetic background of xenografted cells.

PAPER IV

A new rationally designed homosteroid ligand with high progesterone receptor selectivity and full antagonist properties

Khan JA, Tiked A, Fay M, Hamze A, Fagart J, Chabbert-Buffet N, Brion JD, Alami M, Lombès M, Loosfelt H and Rafestin-Oblin ME

Submitted manuscript

A NEW RATIONALLY DESIGNED HOMOSTEROID LIGAND WITH HIGH PROGESTERONE RECEPTOR SELECTIVITY AND FULL ANTAGONIST PROPERTIES

Junaid A Khan[a,b], Abdellatif Tiked[c], Michel Fay[d,e], Abdallah Hamze[c], Jérôme Fagart[a,b], Nathalie Chabbert-Buffet[f,g], Jean-Daniel Brion[c], Mouad Alami[c], Marc Lombès[a,b,h], Hugues Loosfelt[a,b], Marie-Edith Rafestin-Oblin[d,e,1]

[a]Inserm U693, 63 rue Gabriel Péri, Le Kremlin-Bicêtre, F-94276, France;
[b]Univ Paris-Sud, Faculté de Médecine Paris-Sud, UMR-S693, Le Kremlin-Bicêtre, F-94276, France;
[c]Univ Paris-Sud, CNRS, BioCIS-UMR 8076, Faculté de Pharmacie, Châtenay-Malabry, F-92296, France;
[d]Inserm U773, Centre de Recherche Biomédicale Bichat-Beaujon, CRB3, 75870 Paris, France;
[e]Univ Paris 7-Denis Diderot, Site Bichat, 75018 Paris, France;
[f]Obstetrics and Gynecology Unit, AP-HP Hôpital Tenon;
[g]UMR S_ 938 CdR St Antoine, Univ Pierre et Marie Curie, Paris, France;
[h]Assistance Publique-Hôpitaux de Paris, Hôpital Bicêtre, Service d'Endocrinologie et Maladies de la Reproduction, Le Kremlin-Bicêtre, F-94275, France.

Classification: Major: BIOLOGICAL SCIENCES, Minor category: Biochemistry

[1]Corresponding author: Marie-Edith Rafestin-Oblin, Inserm U773, Centre de Recherche Biomédicale Bichat-Beaujon, CRB3, 75870 Paris, France. Email : marie-edith.oblin@inserm.fr

Disclaimer: The authors have no conflict of interest.

Abbreviations used are:
PR, progesterone receptor; GR, glucocorticoid receptor; AR, androgen receptor; MR, mineralocorticoid receptor; LBD, ligand-binding domain; DBD, DNA binding domain, N-ter, N-terminal domain; RID, receptor interacting domain; SRC, steroid receptor co-activator; NCoR, nuclear receptor co-repressor; SMRT, silencing mediator of retinoid and thyroid receptors; P4, progesterone; DHT, dihydrotestosterone.

ABSTRACT

Progesterone receptor (PR) antagonists are used for emergency contraception, and are proposed as effective therapy for patients suffering from endometriosis, leiomyoma and hormone-related breast cancers. However, currently available PR antagonists such as mifepristone lack specificity and display partial agonistic properties, leading to undesirable side effects. Recent crystallographic studies have identified key contacts involved in PR-progestin interaction, allowing a new rational strategy to inactivate PR. Herein we show that the homosteroid APR19 displays *in vivo* endometrial antiprogesterone activity. APR19 inhibits progesterone-induced PR transactivation from synthetic as well as endogenous gene promoters in a dose-dependent manner and lacks agonist activity. It displays high PR selectivity with respect to other 3oxo-steroid hormone receptors. Biochemical experiments and immunofluorescence imaging reveal that upon APR19 binding, PR is unable to recruit neither coactivators (SRC1, SCR2) nor corepressors (NCoR, SMRT) as opposed to RU486-PR complexes. *In silico* docking experiments based on crystal structure of the PR ligand-binding domain show that, in contrast to progesterone, APR19 only partially occupies the ligand-binding cavity, preventing PR-APR19 complex to acquire a stable conformation. Collectively, we demonstrate that APR19 belongs to a new class of PR antagonists that inactivates PR through a passive mechanism. Such specific PR antagonists provide new therapeutic perspectives particularly in hormone-dependent breast cancers.

Key words: Progesterone receptor, antiprogestin, homosteroid, transcription, coregulators

INTRODUCTION

The essential role of progesterone (P4) in mammalian reproductive functions led to the development of synthetic ligands of the progesterone receptor (PR) with either agonist (progestins) or antagonist properties. Progestins have been developed for contraception, menopausal hormone therapy and treatment of gynecological diseases (1-4). Like P4, progestin binding to PR induces a major conformation change within the ligand-binding domain (LBD) which is thought to promote receptor dimerization and its interaction with DNA at specific response elements located in the promoter region of target gene. The agonist-induced PR conformation change also triggers the recruitment of transcriptional coactivators and the ordered assembly of multiprotein complexes with chromatin-modifying activities (5, 6).

The first PR antagonist reported was mifepristone (RU486), also characterized as a potent glucocorticoid receptor (GR) antagonist (7). Since then numerous ligands have been synthesized with the aim to increase their PR selectivity. Most of them are steroidal molecules, derived from testosterone or progesterone, and characterized by a 11-aryl substituent responsible for their antagonistic property (7, 8). They exhibit a spectrum of activity ranging from pure antagonist to mixed agonist/antagonist activity. These latter compounds are classified as selective PR modulators (SPRM) (9-12). While pure antagonists trigger the recruitment of corepressors exclusively, SPRM permit the binding of both coactivators and corepresssors (7-12). The relative coactivator and corepressor expression within a target cell determines their relative agonist *vs* antagonist activity (13). Although the available molecules showed potential use in the treatment of various gynecological disorders, none of them have been developed for therapeutical indications other than termination of pregnancy and postcoital contraception because of undesirable effects (3).

We propose a new strategy for PR inactivation which relies on the formation of an unstable ligand-PR complex unable to recruit both coactivators and corepressors. Such antagonism mode, called passive antagonism (14), has already been described for other steroid receptors, namely androgen receptor (AR) (15), estrogen receptor (ER) (14) and mineralocorticoid receptor (MR) (16, 17). We based the design of this new class of PR antagonists on recent crystal structures of the PR LBD complexed with either agonist or antagonist ligand (8, 18, 19) and on the identification of the PR-ligand contacts responsible for PR activation and inactivation (20). We synthesized homosteroid molecules having a 6-carbon D ring and demonstrate that the APR19 molecule characterized by C3 fluorine is a selective PR antagonist which lacks any agonist activity. We show that, in contrast to RU486, APR19 binding to PR impairs coactivator or corepressor recruitment. *In silico* docking experiments using the crystal structure of the PR ligand-binding domain revealed that stabilizing contacts required for a stable and productive PR

conformation were not observed upon APR19 binding. Collectively, we show that APR19 belongs to a new class of PR antagonists that inactivates PR through a passive mechanism. Such specific PR antagonists provide new therapeutic perspectives, particularly in hormone-dependent breast cancers.

RESULTS

C3 substitution determines agonist character of homosteroid molecules. We aimed at generating passive PR antagonists i.e. molecules lacking bulky substituent at C11 position and able to form unstable complexes upon PR binding. The crystal structures of PR LBD complex bound to either progesterone or testosterone-related progestins have revealed that the A ring is involved in a network of stabilizing contacts through their C3 ketone (8, 18, 19). They also show critical involvement of the D ring substituents in stabilizing contacts. It involved either a direct contact between the 13-methyl group and the Met909 of the H12 helix or, for the 13 ethyl progestins, a network of contacts in which the Met909 plays a critical role (8). Additional contacts between the ligand-binding cavity and the D ring through the C17 hydroxyl group have been identified for the testosterone-related progestin (8). Based on these observations, we synthesized homosteroids in which the steroidal D ring was replaced by a 6-carbon ring with no polar substituent to prevent stabilizing contact with PR. Several homosteroids were synthesized differing by their C3 substituent. Here we report the synthesis and the properties of two homosteroids characterized by a ketone (APR6) or fluorine (APR19) at the C3 position.

Fluorinated APR19 was obtained from readily available pregnenolone as outlined in the Fig. S1. The introduction of fluorine atoms into the steroid skeleton constitutes the key step of this synthetic route. Reduction of the 20-keto group of pregnenolone followed by reaction with diethylaminosulfur trifluoride (DAST) causes a simultaneous fluorination of the C-3 alcohol function together with an unprecedented D-ring expansion, furnishing exclusively difluorinated homosteroid APR19 with 57% overall yield. In addition to its MS and NMR spectra, the X-ray crystallography pattern of APR19 clearly indicated the α-stereochemistry of 17-methyl group as well as the β-stereochemistry of the 3- and 17-fluoro atoms substituents. This DAST-induced D-ring rearrangement was successfully exploited for the synthesis of APR6 from steroid 2 (Fig. S1).

We evaluated, by competition experiments, the ability of APR6 and APR19 to bind to PR, in cytosol prepared from the human breast cancer cell MDA-MB-231 stably expressing PRB (MDA-PRB). The two compounds inhibit ^{3}[H]progesterone binding to PRB with the same efficiency which is lower than that of progesterone (Fig. S2). These results indicate that the two homosteroid molecules are able to bind PR and that their relative affinity for PR is in the same order of magnitude whether the C3 substituent is a ketone (APR6) or a fluorine (APR19).

We then determined the agonist/antagonist properties of APR6 and APR19 on PRB-mediated reporter gene transcription in MDA-PRB (Fig. 1) and HEK293T cells transiently expressing PRB (Fig. S3). Both homosteroid molecules inhibited progesterone-induced PRB transactivation in a dose-dependent manner (Fig. 1A and Fig. S3) with APR19 being more efficient than APR6. Interestingly, in the absence of progesterone, APR6, but not APR19, induced PRB transactivation indicating that the agonist character of homosteroid molecules highly depends on the C3 substituent and is observed with a ketone (APR6) but not with fluorine (APR19). We next evaluated the efficacy of APR19 on inhibiting PRB-mediated endogenous gene transcription in MDA-PRB cells by quantitative RT-PCR. APR19 treatment led to dose-dependent inhibition of classical P4 responsive genes FKBP5, AREG, SGK1 expression while it did not induce any substantial agonist effect (Fig. 1B). Thus, APR19 with its 3-fluorine behaves as a full PR antagonist, while the 3-ketone in APR6 accounts for its partial agonist feature.

The homosteroid APR19 is a selective PR antagonist. The major drawback of 11-aryl substituted PR antagonists, such as RU486, is their low PR specificity, since they also behave as strong GR and AR antagonists and to lower extent MR (7). Therefore, we evaluated by reporter gene assays, the capacity of APR19 to activate and/or inactivate the receptors of other 3oxo-steroids, i.e. GR, AR and MR having high LBD sequence homology with PR. Antagonist property of APR19 for these steroid receptors was compared with RU486, a strong GR and AR antagonist or spironolactone, a MR antagonist (Fig. S4). As expected, RU486 inhibited dexamethasone-induced GR and dihydrotestosterone-induced AR transactivation in a dose-dependent manner and

spironolactone inhibited aldosterone-induced MR transactivation. Interestingly, under similar conditions, APR19 did not antagonize the ligand-induced transactivation via GR, AR or MR and exerted no agonist effect via these receptors. These results demonstrate that APR19, in contrast to the bulky antagonist RU486, is a selective PR antagonist.

The homosteroid APR19 is a potent PR antagonist *in vivo.* We next investigated *in vivo* anti-progesterone efficacy of APR19 in a mouse model for evaluating PR antagonist properties. Immature 4-5 wk old B6D2 female mice were treated with estradiol (E2) administered intraperitoneally to induce endometrial proliferation. In this model, progesterone administration prevents the estrogen-dependent endometrial cells proliferation as assessed by uterine weight gain. APR19 inhibited this anti-proliferative effect of progesterone on E2-induced endometrial proliferation and exerted no significant effect when injected alone (Fig. 2), consistent with a full, antagonist property with no agonist feature of APR19 *in vivo*.

Mechanism of PR inactivation by APR19 binding. Given that P4-induced PR phosphorylation at serine 294 controls multiple aspects of PR functioning including DNA binding and transcriptional activity which is coupled to proteasome-dependent PR degradation (21, 22), we first examined PR S294 phosphorylation status and PR turnover in the presence of P4 and/or APR19. Immunoblot analysis showed that APR19 treatment inhibited P4-induced PR degradation (Fig. S5A) while APR19 alone was unable to induce S294 phosphorylation (Fig. S5B) as opposed to RU486 which concomitantly induced a robust upshift in PR electrophoretic band, indicative of PR phosphorylation at multiple residues (23). This suggests that following APR19 binding, PR interaction with down-regulatory protein partner(s) and unknown kinase(s) targeting S294 is impaired.

Since PR transcriptional activation involves a cascade of events including DNA binding and recruitment of transcriptional coregulators, we next examined by electromobility shift assays whether APR19 binding to PR modified its DNA-binding properties. P4 but not APR19 enhanced PR binding to radiolabelled progesterone response element (PRE) probe while combination of both APR19 and P4 reduced PR binding to PRE to background level (Fig. S6A). Western blot analysis of the same extracts showed similar PR levels under all conditions (Fig. S6B). This demonstrates that APR19-PR complexes are unable to bind classical PRE and that APR19 prevented P4-induced PR binding to DNA.

Both agonist- and antagonist-bound PR regulate gene transcription with the assistance of transcriptional coregulators (24). Several studies have pointed out that coactivator overexpression in cellular models enhanced the agonist feature of selective PR modulators (SPRM) (13). We thus designed several experiments in which we overexpressed SRC1 in MDA-PRB. We first explored whether APR19 could interfere with the SRC1-mediated increase in agonist-induced PR transactivation. In control conditions, a high APR19 concentration completely blocked P4-induced PR transcriptional activity similarly to RU486 (Fig. 3A). Upon SRC1 co-expression, P4 treatment led to 5-fold increase in PR transcriptional response that was inhibited to similar extent by both APR19 and RU486 (Fig. 3A). However, while SRC1 expression strongly potentiated the agonistic properties of RU486 it did no alter APR19 effects (Fig. 3B). These results strongly suggest that APR19 and RU486 act through distinct mechanisms where RU486-PR complex favors functional interaction with SRC1 while APR19-bound PR remains refractory to such interactions.

The PR-SRC1 interaction was further examined using a mutant SRC1 that lacks nuclear localization signal (ΔNLS) and is predominantly located in the cytoplasm. In the presence of P4, ΔNLS SRC1 translocates to the nucleus along with PR, due to P4-dependent interaction between PR and ΔNLS SRC1 (25). APR19 inhibited P4-dependent PR-mediated nuclear translocation of ΔNLS SRC1 (Fig. S7) supporting our above findings that APR19 binding to PR impairs its interaction with SRC1.

To further strengthen our findings, we examined by a mammalian double hybrid approach whether APR19 binding also impedes PR to bind transcriptional coactivator SRC2. The N-terminal region (N-ter) and receptor interacting domain (RID) of SRC2 interact differently with agonist-PR complexes (26). Therefore, we used both SRC2 sequences encompassing either the N-ter region alone (SRC2-Nter) or the N-ter region and RID regions (SRC2-NterRID). As expected, P4 promoted dose-dependent binding of both SRC2 sequences (Fig. 3C and Fig. S8). In the presence of APR19, PR was unable to functionally interact with the two SRC2

sequences and APR19 inhibited P4-induced PR binding to both SRC-2 sequences in a dose-dependent manner thus supporting our previous findings.

We asked whether APR19 binding might trigger recruitment of transcriptional corepressors as observed with other PR antagonists (27). Two hybrid assays revealed that APR19-bound PR was unable to recruit corepressors NCoR (Fig. 3D) and SMRT (Fig. 3E) in contrast to RU486-bound PR that promoted a dose-dependent PR interaction with these corepressors. These results demonstrate that APR19 strongly inhibits P4-induced PR interaction with coactivators and also prevents corepressor recruitment to PR in contrast to RU486.

Taken together, our results demonstrate that the mechanism of PR inactivation by APR19 is clearly distinct from RU486 since APR19, in contrast to RU486, does enhance neither PR S294 phosphorylation nor PR binding to DNA. Similarly it did not allow PR interaction with both coactivators and corepressors. Such properties indicate that APR19 is unable to promote any classical LBD-dependent PR activation step that makes it a true passive PR antagonist.

Accommodation mode of APR19 within PR ligand binding cavity. The antagonist feature of APR19 and the partial agonist character of APR6 raised the question of their accommodation mode within the PR ligand-binding cavity. Manual docking of both compounds within the ligand–binding pocket of the PR LBD (PDB ID: 1A28) (18) revealed that they adopt the same orientation as that of P4, with the A ring directed towards Gln725 and Arg766 residues and the D ring towards Cys891 and Thr894 residues (Fig. 4). The A, B and C rings of both homosteroids occupy well the binding pocket similar to P4 except the D ring. The 17-acetyl substituent of P4 D ring establishes a van der Waals contacts with the Val903 residue of the loop connecting the H11 and H12 helices. In contrast, the two homosteroids devoid of this substituent are unable to form such interactions. In addition, the C20-ketone function of P4 forms a CHO hydrogen bond with the Cα of the Cys891 residue whereas the aprotic nature of the APR6 and APR19 6-carbon D-ring impairs the establishment of any hydrogen bond. The striking difference between APR6 and APR19 is the anchoring of the A-ring C3-ketone of APR6 to the Gln725 and Arg766 residues whereas the 3-fluorine of APR19 does not form similar anchoring. Thus, in contrast to P4, both APR6 and APR19 do not allow stabilizing contacts with the binding cavity at the D-ring. Moreover, APR19 lacks the anchoring of the A-ring observed for APR6 and P4.

DISCUSSION

We report here the synthesis and the *in vivo* and *in vitro* properties of the homosteroid ligand APR19 with a high PR selectivity and which inactivates PR through a passive mechanism by preventing both coactivator and corepressor binding. By competition assays we show that two homosteroid ligands APR6 and APR19, having a common 6-carbon D ring but differing by their C3 substituents, display an IC50 value of $5x10^{-7}$M for PR. Transactivation assays performed in various cell lines further revealed that APR19, with a C3 fluorine, behaves as a full antagonist, while APR6 characterized by a C3 ketone displays a partial agonist activity. Crystallographic and *in vitro* studies of nuclear receptors have suggested that the LBD is rather dynamic in the absence of ligand and that ligand binding compacts it by establishing many polar and hydrophobic contacts. Some of them are involved in the ligand-receptor complex stability and others are critical to stabilize the receptor in an active or inactive conformation (28). From our docking experiments, it can be proposed that the low affinity of the two homosteroid molecules compared to progesterone is due to the inability of their 6-carbone D ring to establish contacts with the ligand binding cavity, as it is the case for C17-acetyl of progesterone which makes both hydrogen bond and hydrophobic contacts with residues of the ligand-binding cavity. The low affinity of the APRn molecules compared to the 11-aryl substituted molecules was expected from the numerous contacts identified in the PR LBB asoprisnil complex, namely through the 11β- and 17-substituent.

Recent crystallographic data of the PR LBD complexed with either a progestin (18, 20, 29) or with antiprogestin asoprisnil (8) have shown a distinct H12 helix positioning in the agonist or antagonist conformation, with the H12 helix adopting an antiparallel position to the H11 helix in the antagonist conformation (8). Biochemical studies further identified the network of contacts responsible for triggering the agonist or antagonist conformation. Met909 in the H12 helix plays a major role in the switch towards the agonist or antagonist position H12 conformation. By establishing direct or indirect contacts between Met909 and the 13-methyl or ethyl group in the D ring respectively, PR is stabilized in an active

conformation (20). In the case of the antagonist asoprisnil the structure reveals that the 11β-benzaldoxime substituent protrudes in the direction of the H12 helix and occupies the same space as the side chain of Met909. To accommodate the asoprisnil pendant group within the ligand-binding cavity the helix 12 was pushed out from its agonist conformation and adopted an alternate position. Our study reveal that the inability of the 6-carbone D ring to fully occupy the ligand-binding activity together with the lack of anchoring of A ring 3-fluorine atom plays a critical role in the full antagonist feature of the APR19. The ability of the 3-ketone of APR6 to be anchored by Gln725 and Arg766 is responsible for its partial agonist character.

Importantly, APR19, in contrast to RU486, is a highly selective PR antagonist since it does not inhibit transcriptional activity of the three other 3oxo-steroid receptors AR, GR and MR. The X-ray crystal structure of the AR, GR and MR LBD complexed with natural or synthetic agonist ligands have revealed a common anchoring mode of their 3 ketone function to glutamine (within the Helix 3) and an arginine (within the Helix 5) which are highly conserved residues in AR (Gln711/Arg752)(30), GR (Gln570/Arg611)(31) and MR (Gln776/Arg817) (32, 33). Since APR19 binds to PR but not to AR, GR and MR, it is likely that the anchoring of the A ring is not a prerequisite for the binding of homosteroid ligand to PR whereas it is for the other oxo-steroid receptors. This is a promising observation for the design of selective PR ligands.

A noteworthy feature of the homosteroid APR19 is that PR, upon APR19 binding, is unable to recruit transcriptional coregulators (corepressors and coactivators). This is in contrast to recruitment of transcriptional coregulators by PR in the presence of classical antiprogestins such as RU486 and asoprisnil (8). The bulky 11beta-benzaldoxime substituent of asoprisnil protrudes in the direction of the H12 helix which is pushed away thus preventing it from adopting the agonist conformation. This steric interference makes room for interaction with corepressor and displaces Glu911 (8), a residue critical for coactivator binding (31). Furthermore, high potency of such bulky antagonist might be explained by the formation of direct stabilizing contacts with corepressors (8). Although asoprisnil-bound PR LBD preferentially recruited corepressors, interaction with coactivators SRC1, SRC2 and SRC3 was also observed (8) leading to partial agonist character of such antagonists. Indeed it has been shown that the transcriptional outcome following bulky antagonists binding is highly influenced by coactivator/corepressor expression ratio (13). Lack of C11 aryl substitution together with C3 ketone function in APR19 allows full PR inactivation due to absence of stable H12 conformation and lack of stabilizing contacts at A ring. Thus APR19-bound PR is unable to interact with corepressors as well as coactivators and therefore, it could be classified as a prototype molecule belonging to a new class of passive PR antagonists. Moreover, the fact that the APR19 fails to promote PR phosphorylations and DNA binding also strongly argues for such non-classical antagonist properties leading to maintain PR in a transient unstable form.

Although APR19 is the first example of passive PR antagonists, there are numerous steroid receptor ligands, lacking any bulky substituent, and acting as antagonists even though they are smaller than endogenous ligands. This is namely the case for spironolactones which are smaller than aldosterone and display high potency to inhibit the aldosterone-induced activity (33). Due to their inability to establish the stabilizing contact with Asn770, these molecules form unstable complexes which are unable to recruit transcriptional coregulator, either coactivator or corepressor (17). The AR antagonist flutamide with a size comparable to testosterone is another example of passive antagonist (15).

In sum, we report the first example of selective PR antagonist inactivating PR by passive mechanism which does not allow partial agonist effects. The lead molecule may open new pharmacological strategies for long-term utilization of PR antagonists, circumventing deleterious side effects of current antiprogestins. This may be particularly promising for treatment or prevention of hormone-dependent breast cancers.

MATERIAL AND METHODS

Synthesis APR6 and APR19. The detailed protocols for the chemical synthesis are described in *SI Materials and Methods*.

Cell Culture and Transient Transfection. MDA-PRB or HEK293 cells were cultured in DMEM as described previously (20, 22). The expression vectors pchGR, pchMR, pchPR, pchAR code for human GR, MR, PR and AR respectively and pcβgal control or GRE2-luciferase vectors are described in *SI Materials and Methods*.

Mammalian double hybrid assays. The vectors used are pVPPR, pGALSMRT, pGALNCoR, pGALSRC2-RID, pGALSRC2-NterRID and pG5luc. The experimental protocol is detailed in *SI Materials and Methods*.

RT-qPCR. Total RNA was extracted using TRIZOL reagent and reverse-transcription and quantitative real-time PCR was performed as described previously (22). See *SI Materials and Methods* for details.

Model of *in vivo* antiprogesterone efficacy of PR antagonists. A previously established model of screening PR antagonists for their *in vivo* antiprogesterone efficacy (34) was modified and is described in detail in *SI Materials and Methods*.

PR-LBD modeling. APR6 was constructed using the X-ray coordinates of APR19 and the Discovery Studio package (Accelrys, Orsay, France) and minimized with the CHARMm forcefield. APR6 and APR19 were manually docked with the PR LBD (PDB ID 1A28) using as guide the probe-occupied volume of the PR ligand-binding cavity calculated with the voidoo package (35).

ACKNOWLEDGEMENTS

The authors are grateful to A. Gompel (Univ. Paris Descartes, France) for MDA-PRB cells, G.A. Coetzee (University of Southern California, Los Angeles) for human AR expression vector, A. Biola-Vidammente and M. Pallardy (Univ. Paris Sud, Châtenay-Malabry, France) for luciferase reporter gene expression vector, P. Balaguer (Univ. Montpellier, France) for SMRT and NCoR hybrid expression vectors, G. Pinon for SRC2 hybrid vectors, P. Fuller for GAL4-responsive promoter driven luciferase reporter. This work was supported by grants from INSERM, the Université Paris-Sud and Association pour la Recherche sur le Cancer. JAK got doctoral scholarship from Higher Education Commission Islamabad, Pakistan and la ligue contre le cancer, France.

References

1. **Schindler AE, Campagnoli C, Druckmann R, Huber J, Pasqualini JR, Schweppe KW, Thijssen JH** 2003 Classification and pharmacology of progestins. Maturitas 46 Suppl 1:S7-S16
2. **Sitruk-Ware R** 2008 Pharmacological profile of progestins. Maturitas 61:151-157
3. **Sitruk-Ware R, Bossemeyer R, Bouchard P** 2007 Preclinical and clinical properties of trimegestone: a potent and selective progestin. Gynecol Endocrinol 23:310-319
4. **Stanczyk FZ** 2003 All progestins are not created equal. Steroids 68:879-890
5. **Tsai MJ, O'Malley BW** 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451-486
6. **Georgiakaki M, Chabbert-Buffet N, Dasen B, Meduri G, Wenk S, Rajhi L, Amazit L, Chauchereau A, Burger CW, Blok LJ, Milgrom E, Lombes M, Guiochon-Mantel A, Loosfelt H** 2006 Ligand-controlled Interaction of HBO1 with the N-terminal Transactivating Domain of Progesterone Receptor Induces SRC-1-dependent Co-activation of Transcription. Mol Endocrinol 20:2122-2140
7. **Lu NZ, Wardell SE, Burnstein KL, Defranco D, Fuller PJ, Giguere V, Hochberg RB, McKay L, Renoir JM, Weigel NL, Wilson EM, McDonnell DP, Cidlowski JA** 2006 International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol Rev 58:782-797
8. **Madauss KP, Grygielko ET, Deng SJ, Sulpizio AC, Stanley TB, Wu C, Short SA, Thompson SK, Stewart EL, Laping NJ, Williams SP, Bray JD** 2007 A structural and in vitro characterization of asoprisnil: a selective progesterone receptor modulator. Mol Endocrinol 21:1066-1081
9. **Spitz IM** 2003 Progesterone antagonists and progesterone receptor modulators: an overview. Steroids 68:981-993
10. **Chwalisz K, Perez MC, Demanno D, Winkel C, Schubert G, Elger W** 2005 Selective progesterone receptor modulator development and use in the treatment of leiomyomata and endometriosis. Endocr Rev 26:423-438
11. **Chabbert-Buffet N, Meduri G, Bouchard P, Spitz IM** 2005 Selective progesterone receptor modulators and progesterone antagonists: mechanisms of action and clinical applications. Hum Reprod Update 11:293-307
12. **Wardell SE, Edwards DP** 2005 Mechanisms controlling agonist and antagonist potential of selective progesterone receptor modulators (SPRMs). Semin Reprod Med 23:9-21
13. **Liu Z, Auboeuf D, Wong J, Chen JD, Tsai SY, Tsai MJ, O'Malley BW** 2002 Coactivator/corepressor ratios modulate PR-mediated transcription by the selective receptor modulator RU486. Proc Natl Acad Sci U S A 99:7940-7944.
14. **Shiau AK, Barstad D, Radek JT, Meyers MJ, Nettles KW, Katzenellenbogen BS, Katzenellenbogen JA, Agard DA, Greene GL** 2002 Structural characterization of a subtype-selective ligand reveals a novel mode of estrogen receptor antagonism. Nat Struct Biol 9:359-364
15. **Soderholm AA, Lehtovuori PT, Nyronen TH** 2005 Three-dimensional structure-activity relationships of nonsteroidal ligands in complex

with androgen receptor ligand-binding domain. J Med Chem 48:917-925

16. **Fagart J, Wurtz JM, Souque A, Hellal-Levy C, Moras D, Rafestin-Oblin ME** 1998 Antagonism in the human mineralocorticoid receptor. Embo J 17:3317-3325
17. **Fagart J, Hillisch A, Huyet J, Barfacker L, Fay M, Pleiss U, Pook E, Schafer S, Rafestin-Oblin ME, Kolkhof P** 2010 A new mode of mineralocorticoid receptor antagonism by a potent and selective nonsteroidal molecule. J Biol Chem 285:29932-29940
18. **Williams SP, Sigler PB** 1998 Atomic structure of progesterone complexed with its receptor. Nature 393:392-396
19. **Kettel LM, Murphy AA, Mortola JF, Liu JH, Ulmann A, Yen SS** 1991 Endocrine responses to long-term administration of the antiprogesterone RU486 in patients with pelvic endometriosis. Fertil Steril 56:402-407
20. **Petit-Topin I, Turque N, Fagart J, Fay M, Ulmann A, Gainer E, Rafestin-Oblin ME** 2009 Met909 plays a key role in the activation of the progesterone receptor and also in the high potency of 13-ethyl progestins. Mol Pharmacol 75:1317-1324
21. **Shen T, Horwitz KB, Lange CA** 2001 Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294. Mol Cell Biol 21:6122-6131
22. **Khan JA, Amazit L, Bellance C, Guiochon-Mantel A, Lombes M, Loosfelt H** 2011 p38 and p42/44 MAPKs Differentially Regulate Progesterone Receptor A and B Isoform Stabilization. Mol Endocrinol doi:10.1210/me.2011-1042
23. **Zhang Y, Beck CA, Poletti A, Edwards DP, Weigel NL** 1995 Identification of a group of Ser-Pro motif hormone-inducible phosphorylation sites in the human progesterone receptor. Mol Endocrinol 9:1029-1040
24. **Rosenfeld MG, Glass CK** 2001 Coregulator codes of transcriptional regulation by nuclear receptors. J Biol Chem 276:36865-36868
25. **Amazit L, Alj Y, Tyagi RK, Chauchereau A, Loosfelt H, Pichon C, Pantel J, Foulon-Guinchard E, Leclerc P, Milgrom E, Guiochon-Mantel A** 2003 Subcellular localization and mechanisms of nucleocytoplasmic trafficking of steroid receptor coactivator-1. J Biol Chem 278:32195-32203
26. **Wang D, Wang Q, Awasthi S, Simons SS, Jr.** 2007 Amino-terminal domain of TIF2 is involved in competing for corepressor binding to glucocorticoid and progesterone receptors. Biochemistry 46:8036-8049
27. **Schulz M, Eggert M, Baniahmad A, Dostert A, Heinzel T, Renkawitz R** 2002 RU486-induced glucocorticoid receptor agonism is controlled by the receptor N terminus and by corepressor binding. J Biol Chem 277:26238-26243
28. **Nagy L, Schwabe JW** 2004 Mechanism of the nuclear receptor molecular switch. Trends Biochem Sci 29:317-324
29. **Madauss KP, Deng SJ, Austin RJ, Lambert MH, McLay I, Pritchard J, Short SA, Stewart EL, Uings IJ, Williams SP** 2004 Progesterone receptor ligand binding pocket flexibility: crystal structures of the norethindrone and mometasone furoate complexes. J Med Chem 47:3381-3387
30. **Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, Otto N, Joschko S, Scholz P, Wegg A, Basler S, Schafer M, Egner U, Carrondo MA** 2000 Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J Biol Chem 275:26164-26171
31. **Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, Lambert MH, Moore JT, Pearce KH, Xu HE** 2002 Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. Cell 110:93-105
32. **Bledsoe RK, Madauss KP, Holt JA, Apolito CJ, Lambert MH, Pearce KH, Stanley TB, Stewart EL, Trump RP, Willson TM, Williams SP** 2005 A ligand-mediated hydrogen bond network required for the activation of the mineralocorticoid receptor. J Biol Chem 280:31283-31293
33. **Fagart J, Huyet J, Pinon GM, Rochel M, Mayer C, Rafestin-Oblin ME** 2005 Crystal structure of a mutant mineralocorticoid receptor responsible for hypertension. Nat Struct Mol Biol 12:554-555
34. **Cullingford TE, Pollard JW** 1988 RU 486 completely inhibits the action of progesterone on cell proliferation in the mouse uterus. J Reprod Fertil 83:909-914
35. **Kleywegt GJ, Jones TA** 1994 Detection, delineation, measurement and display of cavities in macromolecular structures. Acta Crystallogr D Biol Crystallogr 50:178-185

FIGURE LEGENDS

Fig. 1. APR6 and APR19 display distinct agonist and antagonist properties. *(A)* MDA-MB-231 cells stably expressing PRB (MDA-PRB) were transiently transfected with GRE^2-luc vector as described in *SI Materials and Methods*. Cells were treated during 24 h with vehicle or progesterone (P4, 10^{-9} M) alone or in combination with increasing concentrations (10^{-8} to 10^{-5} M) of APR6 or APR19. For determining agonist effects, cells were treated with APR6 or APR19 alone (10^{-6} or 10^{-5} M). Luciferase activity was determined and data (means ± SEM) from six independent cell cultures are presented. Agonist or antagonist efficacy was determined as percentage of maximum transactivation (100 %) obtained following P4 treatment as compared to vehicle treated cells. *(B)* MDA-PRB cells were treated with vehicle or P4 (10^{-9} M) or APR19 (10^{-6} to 10^{-5} M) alone or in combination during 6 h and qRT-PCR analysis was performed for indicated gene transcripts. Data are presented as above.

Fig. 2. APR19 inhibits in vivo anti-estrogenic effects of progesterone. Six immature B6D2 female mice were injected IP with indicated compounds, uteri were recovered and weighed as described in *Materials and Methods*. Increase in uterine weight following E2 injection alone is presented as 100 % of estrogenic stimulation. Data (means ± SEM) were obtained from six mice. Statistical significance was determined by non parametric Mann Whitney test and is indicated at $p \leq 0.01$.

Fig. 3. APR19 binding inhibits PR functional interaction with transcriptional co-regulators. *(A)* MDA-PRB cells were transiently transfected with control or pSG5SRC1 vector coding full length co-activator SRC1 along with GRE^2-luc vector encoding luciferase reporter gene under the control of PR responsive element containing promoter. Six independent cell cultures were treated with vehicle or P4 (10^{-9} M) or P4 (10^{-9} M) plus APR19 (10^{-6} or 10^{-5} M) or P4 (10^{-9} M) plus RU486 (10^{-5} M) during 24 h. Data (means ± SEM) representing luciferase activity normalized by total protein content of the corresponding sample is presented. *(B)* MDA-PRB cells were transiently transfected as in *(A)* and cells were treated with vehicle or APR19 (10^{-5} M) or RU486 (10^{-5} M) during 24 h. Data (means ± SEM) from six independent cell cultures are presented. *(C)* HEK293T cells were transiently transfected with vectors; pVPPR coding full length hPR fused to VP16 activation domain, pGALSRC2-NterRID vector coding for co-activator SRC2 1-867 amino acids (corresponding to the N-ter and RID regions) fused to GAL4-DBD, pSG5luc coding for luciferase reporter gene under the control of GAL4-responsive element containing promoter and control vector pcβgal for normalizing transfection efficiency. Cells were treated with increasing concentrations of P4 (10^{-10} to 10^{-8} M) or APR19 (10^{-7} to 10^{-5} M) or P4 (10^{-8} M) plus APR19 (10^{-7} to 10^{-5} M). Data (means ± SEM) from three independent experiments were cumulated and are presented as fold induction of vehicle treated cells. *(D)* HEK293T cells were transiently transfected with vectors as in *(C)* except that pGALSRC2-NterRID vector was replaced by pGALNCoR coding for co-repressor NCoR RID region linked to GAL4-DBD. Cells were incubated with vehicle or increasing concentrations of RU486 (10^{-11} to 10^{-9} M) or APR19 (10^{-7} to 10^{-5} M). Data are presented as in *(C)*. *(E)* HEK293T cells were transiently transfected and treated with ligands as in *(C)* except that pGALSRC2-NterRID vector was replaced by pGALSMRT vector coding for co-repressor SMRT RID region fused to GAL4DBD.

Fig. 4. Accommodation mode of APR6 and APR19 within PR LBD. *(A)* progesterone, *(B)* APR6, (C) APR19, accommodation mode within the PR LBD (PDB ID: 1a28) are shown. The α-helices and β-strands of the PR LBD are depicted as blue ribbons and arrows, respectively. The red chicken wire surface delimits the cavity volume which can be occupied by the ligands. Some residues of the binding pocket are showed with their carbon, oxygen and nitrogen atoms coloured in grey, red and blue, respectively. Ligands are illustrated with their oxygen and fluorine atoms in red and green, respectively. The carbon atoms and the ligand's van der Waals volumes are coloured in grey, light green and yellow for progesterone, APR6 and APR19, respectively. Hydrogen bonds and short van der Waals contacts between the ligands and the residues are depicted as red and green dots, respectively. The figures panels were generated using the Dino package (DINO: Visualizing Structural Biology (2002) (http://www.dino3d.org).

Figure 1

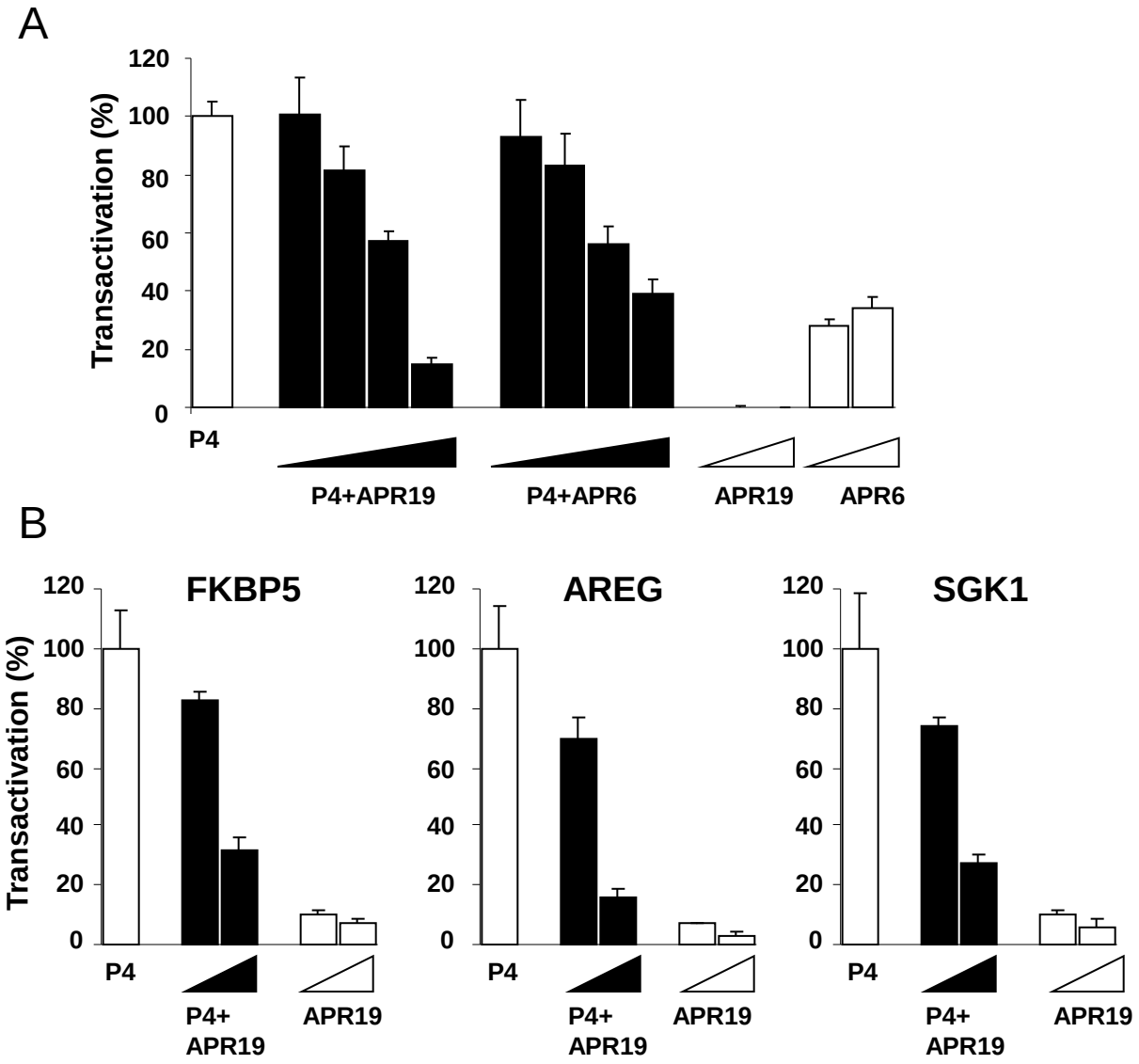

A
Transactivation (%)
120
100
80
60
40
20
0
P4
P4+APR19
P4+APR6
APR19
APR6
B
Transactivation (%)
FKBP5
AREG
SGK1
120
100
80
60
40
20
0
P4
P4+ APR19
APR19

Figure 2

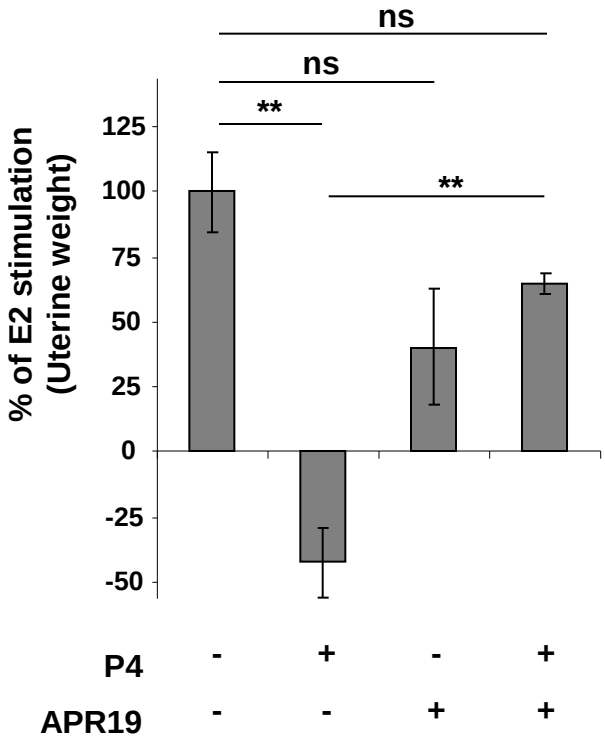

ns
ns
**
**
% of E2 stimulation
(Uterine weight)
125
100
75
50
25
0
-25
-50
P4
-
+
-
+
APR19
-
-
+
+

Figure 3

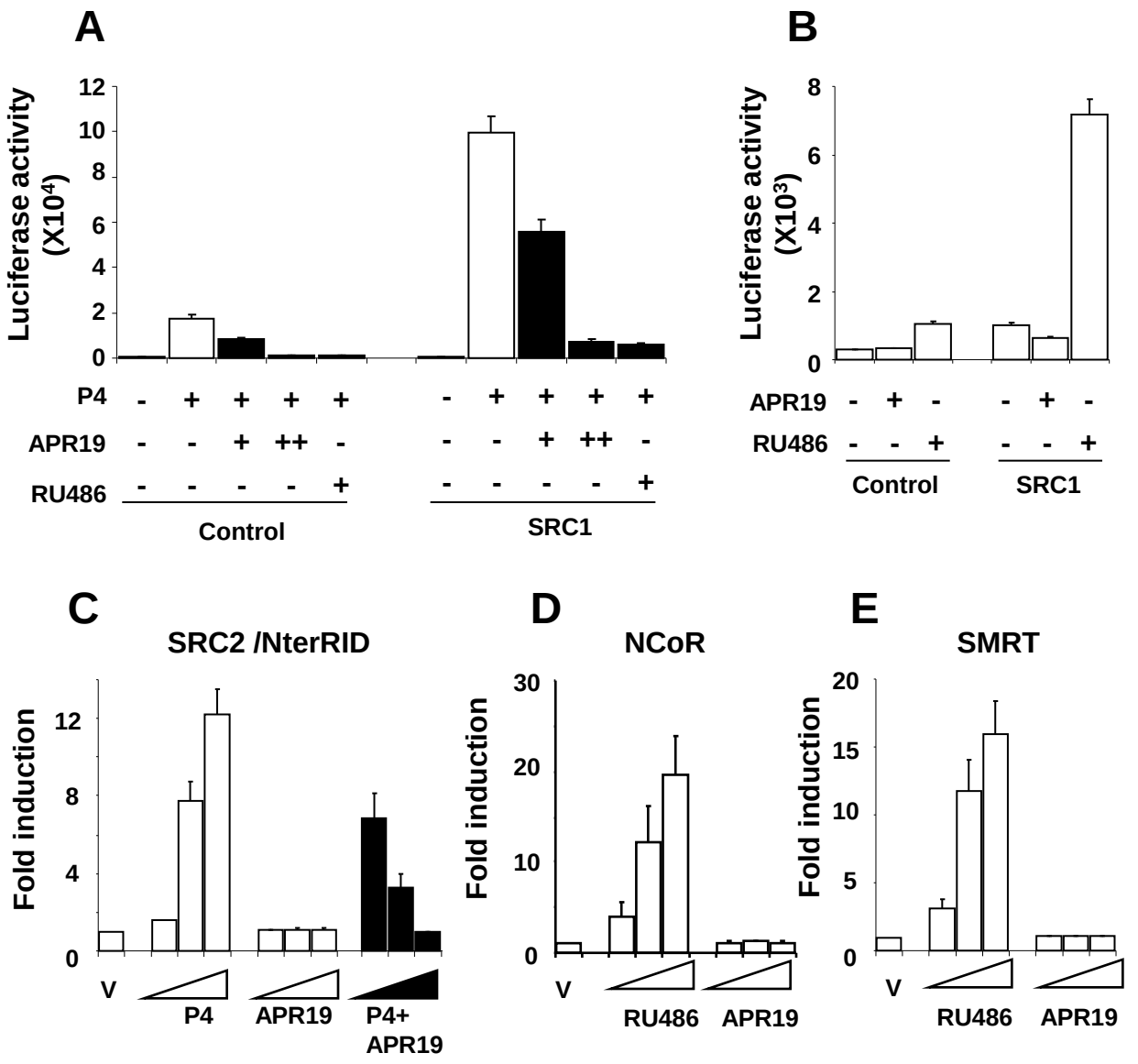
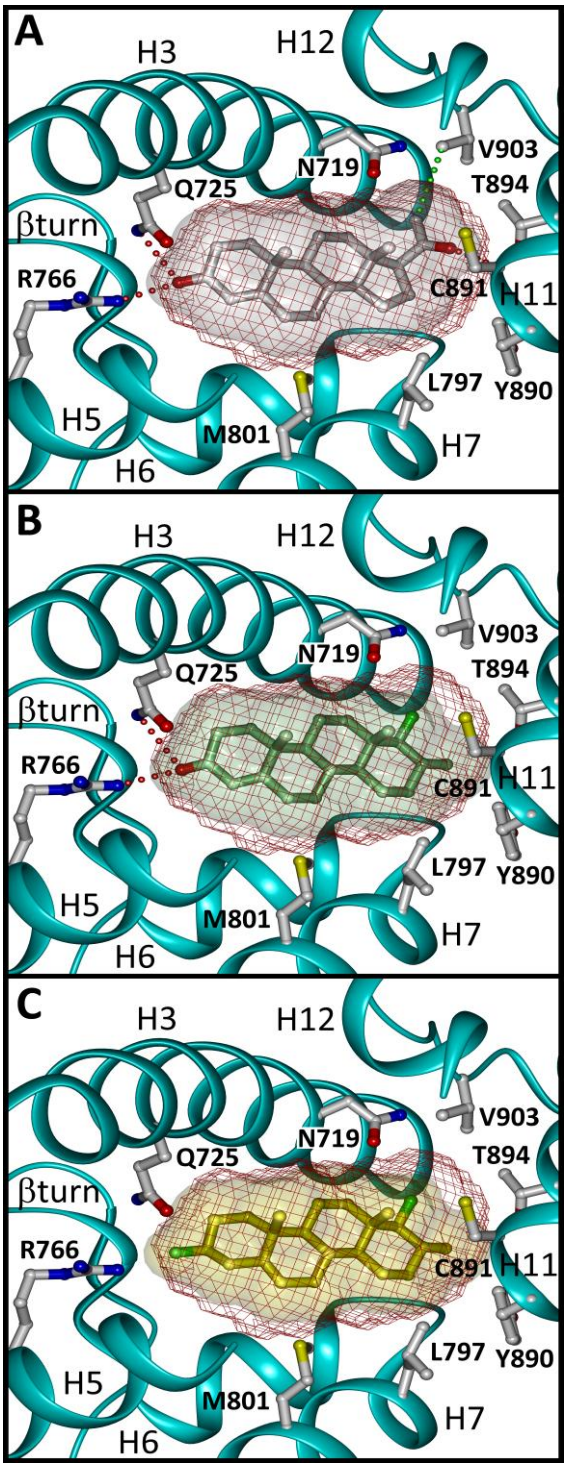

A
Luciferase activity (X10⁴)
12
10
8
6
4
2
0
P4 - + + + + - + + + +
APR19 - - + ++ - - - + ++ -
RU486 - - - - + - - - - +
Control
SRC1
B
Luciferase activity (X10³)
8
6
4
2
0
APR19 - + - - + -
RU486 - - + - - +
Control
SRC1
C
SRC2 /NterRID
Fold induction
12
8
4
0
V
P4
APR19
P4+ APR19
D
NCoR
Fold induction
30
20
10
0
V
RU486
APR19
E
SMRT
Fold induction
20
15
10
5
0
V
RU486
APR19

Figure 4


A
H3
H12
V903
N719
T894
Q725
βturn
R766
C891
H11
L797
Y890
H5
M801
H7
H6
B
H3
H12
V903
N719
T894
Q725
βturn
R766
C891
H11
L797
Y890
H5
M801
H7
H6
C
H3
H12
V903
N719
T894
Q725
βturn
R766
C891
H11
L797
Y890
H5
M801
H7
H6

SUPPLEMENTAT FIG LEGENDS

S1. Synthesis of APR6 and APR19. Synthesis of fluorinated APR6 and APR19. (a) $NaBH_4$, MeOH/THF (6/4), 20 °C. (b) DAST, CH_2Cl_2, -78 to 20 °C; (c) Amberlyst 15, CH_2Cl_2, 20 °C.

S2. Half-maximal inhibitory concentrations (IC50) of APR6 and APR19. Cytosols prepared from MDA-PRB cells were incubated for 4 h at 4°C with [^{3}H]-P4 (10^{-9} M) alone or with increasing concentrations of P4 (10^{-9} to 10^{-6} M) or APR6 (10^{-8} to 10^{-5} M) or APR19 (10^{-8} to 10^{-5} M). Bound and unbound steroids were separated by the dextran-charcoal technique. Results are expressed as the percentage of binding measured with [^{3}H]-P4 alone.

S3. Comparison of agonist/antagonist properties of APR6 and APR19 in HEK293T cells. HEK293T cells were transiently transfected with hPRB and GRE^2-luc vectors. Cells were treated during 24 h with vehicle or P4 (10^{-9} M) alone or in combination with increasing concentrations (10^{-7} to 10^{-5} M) of APR6 or APR19. For agonist effects, cells were treated with APR6 or APR19 alone (10^{-6} M). Luciferase activity was measured and Data (means ± SEM) of three independent experiments are presented as in Fig. 1A.

S4. Agonist/antagonist properties of APR19 for 3 oxo-steroid receptors (GR, AR, MR). *(A)* HEK293T cells were transiently transfected with vectors coding for hGR, reporter GRE2-luc and internal transfection control pcβgal. Cells were treated with dexamethasone (Dex, 10^{-9} M) or APR19 (10^{-6} M) alone or Dex (10^{-9} M) plus increasing concentrations of RU486 (10^{-10} to 10^{-7} M) or Dex (10^{-9} M) plus increasing concentrations of APR19 (10^{-7} to 10^{-5} M). Luciferase activity was measured and Data (means ± SEM) of three independent experiments are presented as percentage of maximal transactivation (100 %) obtained following Dex (10^{-9} M) treatment as compared to vehicle treated cells. *(B)* HEK293T cells were transiently transfected with vectors coding for hAR, reporter GRE2-luc and internal transfection control pcβgal. Cells were treated with dihydrotestosterone (DHT, 10^{-9} M) or APR19 (10^{-6} M) alone or DHT (10^{-9} M) plus increasing concentrations of RU486 (10^{-8} to 10^{-5} M) or DHT (10^{-9} M) plus increasing concentrations of APR19 (10^{-7} to 10^{-5} M). Luciferase activity was measured and Data (means ± SEM) of three independent experiments are presented as percentage of maximal transactivation (100 %) obtained following DHT (10^{-9} M) treatment as compared to vehicle treated cells. *(C)* HEK293T cells were transiently transfected with vectors coding for hMR, reporter GRE2-luc and internal transfection control pcβgal. Cells were treated with aldosterone (Aldo, 10^{-9} M) or APR19 (10^{-6} M) alone or Aldo (10^{-9} M) plus increasing concentrations of spiranolactone (Spiro, 10^{-8} to 10^{-5} M) or Aldo (10^{-9} M) plus increasing concentrations of APR19 (10^{-7} to 10^{-5} M). Luciferase activity was measured and Data (means ± SEM) of three independent experiments are presented as percentage of maximal transactivation (100 %) obtained following Aldo (10^{-9} M) treatment as compared to vehicle treated cells.

S5. APR19 inhibits P4-dependent PR turnover and does not induce PR S294 phosphorylation. *(A)* MDA-PRB cells were incubated with vehicle or P4 (10^{-8} M) alone or in combination with APR19 (10^{-6} or 10^{-5} M) during 24 h and western blot analysis was performed using specific antibody against PR or loading control tubulin. *(B)* MDA-PRB cells were treated with vehicle or APR19 (10^{-5} M) or RU486 (10^{-5} M) during 24 h and western blot analysis was performed as above. PR serine 294 phosphorylated species were detected using specific antibody.

S6. APR19 inhibits P4-dependent PR binding to PRE. *(A)* Electrophoretic Mobility Shift assay performed on MDA-PRB cells following incubation with vehicle or P4 (10-8 M) or APR19 (10-5 M) during 1 h. After 15 min incubation of nuclear extract proteins, with double strand [^{32}P]-labeled oligonucleotide, protein-DNA complexes were separated on a nondenaturing 4.5% polyacrylamide gel and detected by autoradiography. Specific complexes were identified by competition experiments in which to 100 fold unlabeled probe competitor was added. *(B)* Western blot analysis was performed as in *(S5)* for PR detection in nuclear extracts.

S7. APR19 inhibits P4-induced PR-dependent nuclear translocation of SRC1ΔNLS mutant. MDA-PRB cells were seeded on cover slips in 6-well plates and transiently transfected with PRB and SRC1ΔNLS expression vectors during 24 h and treated with vehicle or P4 (10^{-9} M) or APR19 (10^{-6}M) or both during 4 h. Cells were fixed with 4 % paraformaldehyde, incubated overnight at 4 °C with primary antibodies against PR and SRC1 and then with fluorophore-coupled anti-mouse and anti-rabbit antibodies for detecting PRB and SRC1ΔNLS, respectively.

S8. APR19 inhibits P4-dependent PR functional interaction with transcriptional co-activator SRC2 N-ter domain. HEK293T cells were transiently transfected with vectors; pVPPR coding full length hPR fused to VP16 activation domain, pGALSRC2-Nter coding for GAL4 DBD linked to SRC2 1-623 sequence comprising N-ter region, pG5luc coding for luciferase reporter gene under the control of GAL4-responsive element containing promoter and control vector pcβgal for normalizing transfection efficiency. Cells were incubated with vehicle or increasing concentrations of P4 (10^{-10} to 10^{-8} M) or APR19 (10^{-7} to 10^{-5} M) or P4 (10^{-8}M) plus APR19 (10^{-7} to 10^{-5} M). Data (means ± SEM) from three independent experiments were cumulated and are presented as fold induction of vehicle treated cells.

Figure S1

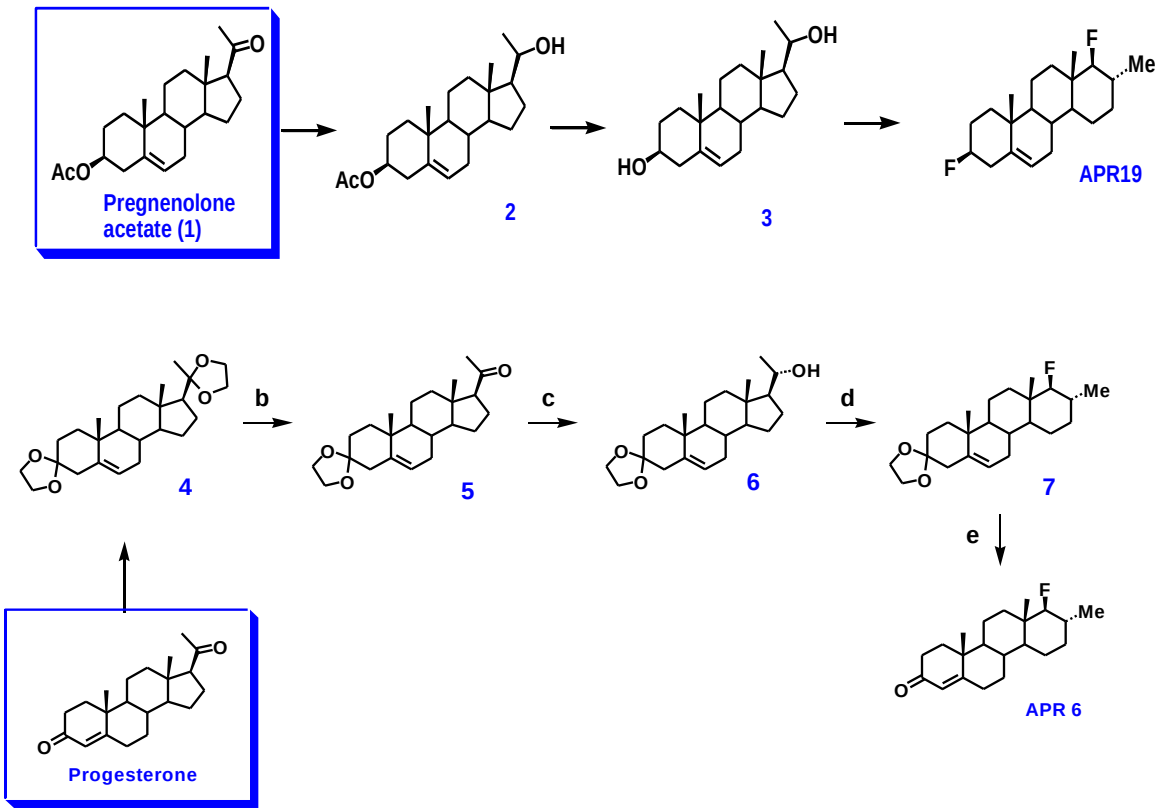

AcO
O
Pregnenolone acetate (1)
AcO
OH
2
HO
OH
3
F
Me
F
APR19
O
O
O
O
4
b
O
O
5
c
OH
O
O
6
d
F
Me
O
O
7
e
F
Me
O
APR 6
O
O
Progesterone

Figure S2

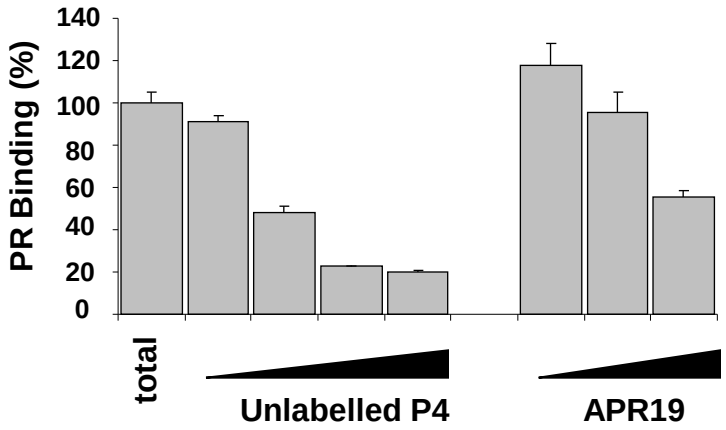


Figure S3

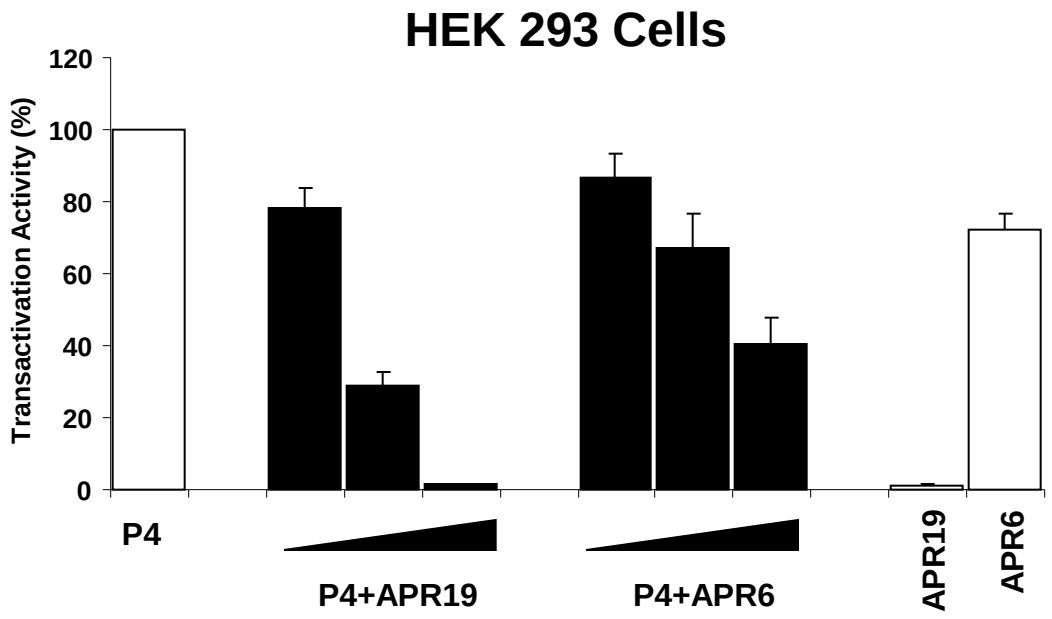

HEK 293 Cells
Transactivation Activity (%)
120
100
80
60
40
20
0
P4
P4+APR19
P4+APR6
APR19
APR6

Figure S4

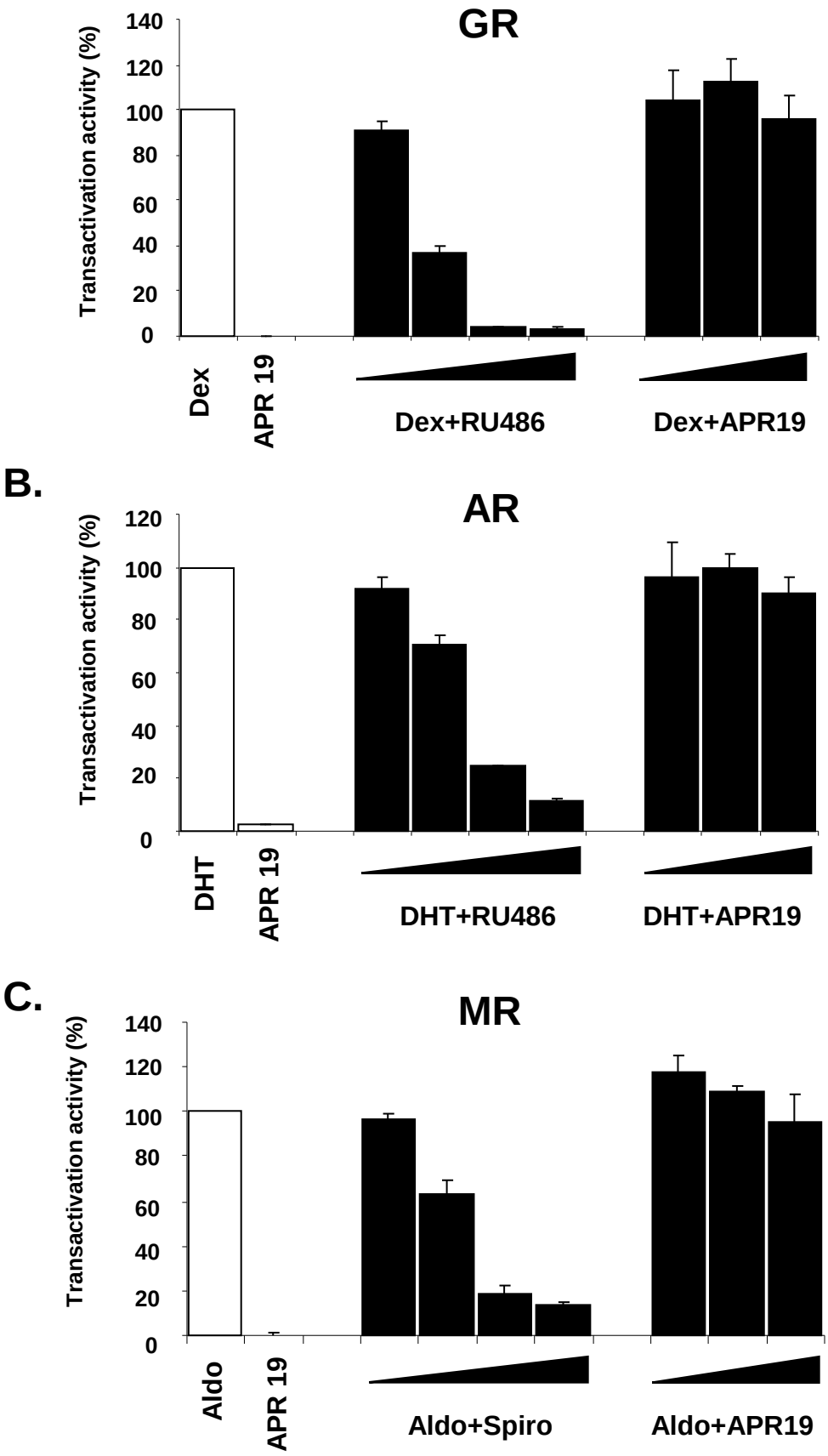
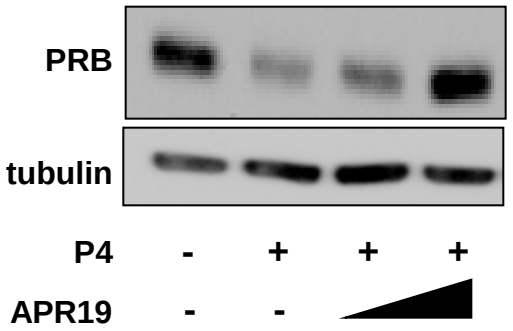

A.
GR
Transactivation activity (%)
140
120
100
80
60
40
20
0
Dex
APR 19
Dex+RU486
Dex+APR19
B.
AR
Transactivation activity (%)
120
100
80
60
40
20
0
DHT
APR 19
DHT+RU486
DHT+APR19
C.
MR
Transactivation activity (%)
140
120
100
80
60
40
20
0
Aldo
APR 19
Aldo+Spiro
Aldo+APR19

Figure S5

A.



B.

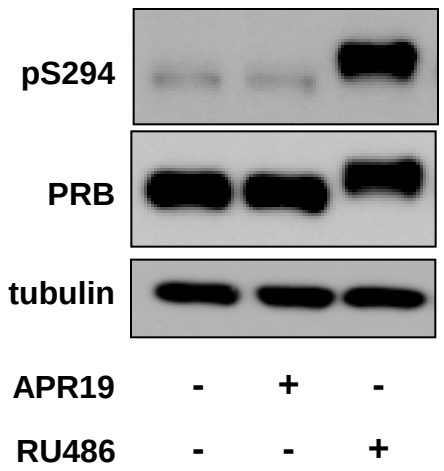
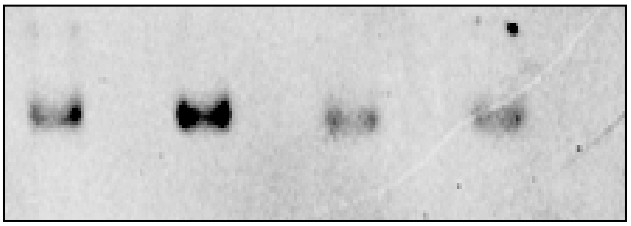


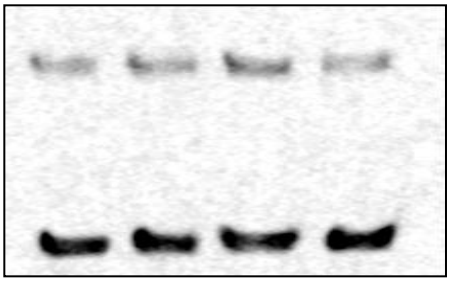
Figure S6

A.



P4	-	-	+	+	-	-	+	+
APR19	-	-	-	-	+	+	+	+
PRE in excess	-	+	-	+	-	+	-	+

B.



P4	-	+	-	+
APR19	-	-	+	+

Figure S7

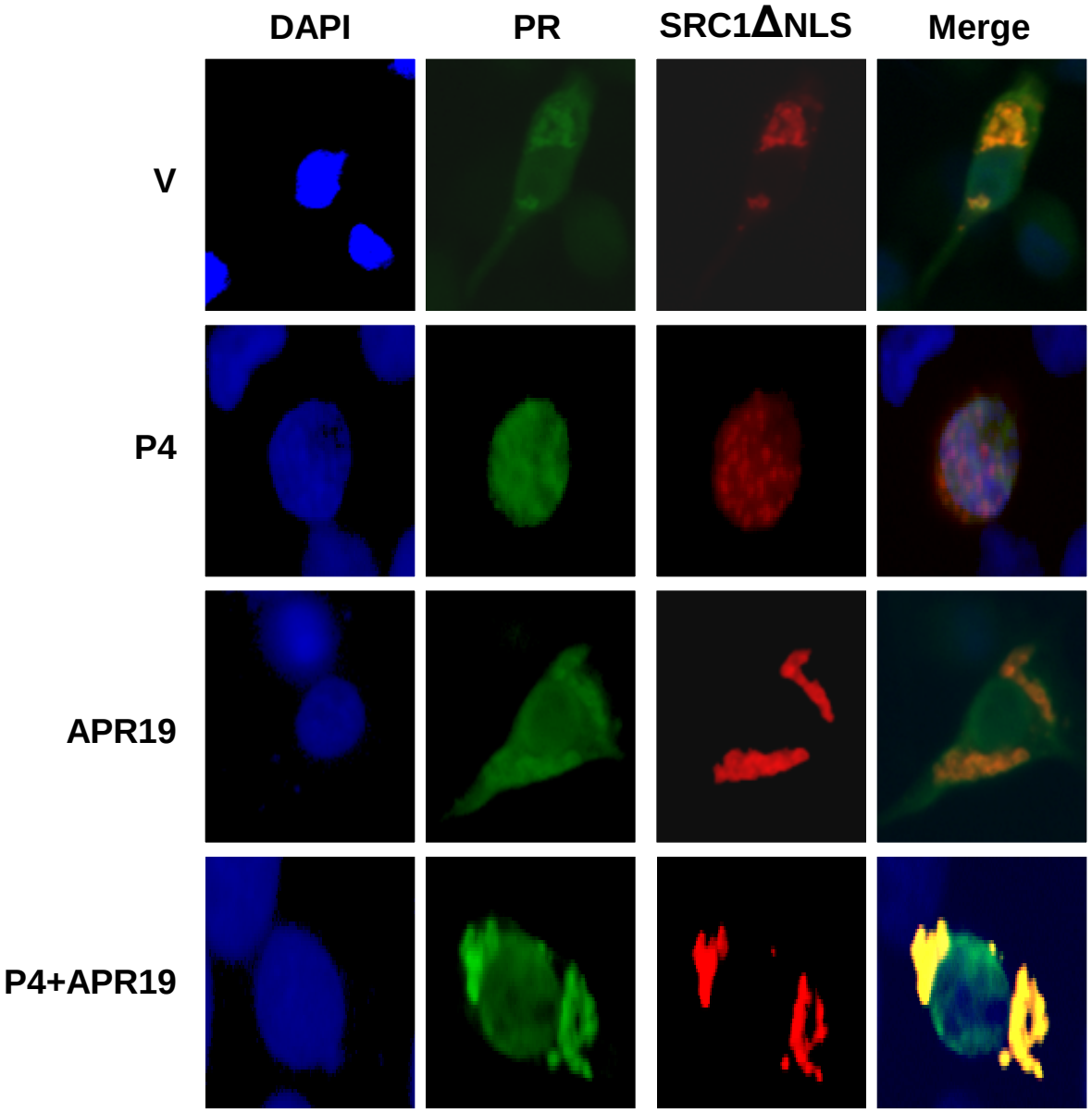
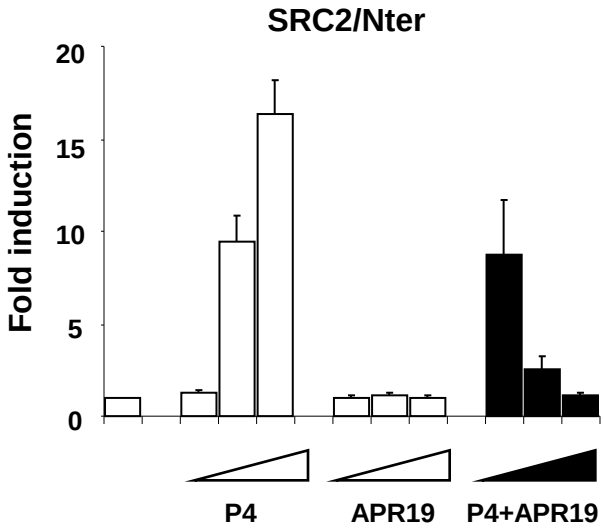
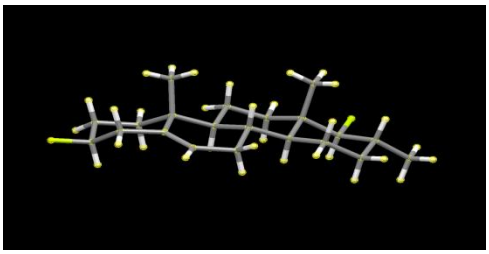


Figure S8

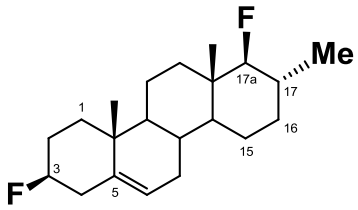


SUPPLEMENTAL MATERIALS AND METHODS

3β,17aβ-Difluoro-17α-methyl-D-homopregn-5-ene (APR19). To a mixture of pregnenolone (2.0 g, 6.32 mmol) in MeOH (60 mL) and THF (40 mL) was added slowly $NaBH_4$ (423 mg, 11.2 mmol) at room temperature and stirring was continued for an additional period of 1 h. The reaction mixture was hydrolyzed with brine; ethyl acetate was added and the organic layer was successively washed with aqueous 1N HCl, aqueous saturated Na_2CO_3 solution, brine, dried over Na_2SO_4, and concentrated under vacuum. Filtration through silica gel (cyclohexane/EtOAc, 60/40) gave pregn-5-en-3β,20-diol, which was dissolved in dry dichloromethane (160 mL), and under an argon atmosphere, DAST (2.3 mL, 18.96 mmol) was added, at -78 °C, and stirring was continued at room temperature for an additional period of 60 h. The reaction mixture was poured into ice water; the organic layer was washed thoroughly with an aqueous saturated Na_2CO_3 solution, brine, dried over Na_2SO_4, and concentrated under vacuum. Purification of the residue by silica gel column chromatography (petroleum ether/EtOAc, 99/1) gave **APR19** (1.16 g, 57% yield, white solid) as a single diastereoisomere. R_f = 0.54 (petroleum ether/EtOAc, 95/5). Mp 131-132 °C (pentane). IR (ν cm^{-1}): 800, 950, 1003, 1382, 1442, 2918. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (s, 3H), 0.98 (d, 3H, J = 6.1 Hz), 1.01 (s, 3H), 0.68-2.17 (m, 18H), 2.45 (m, 2H), 3.60 (dd, 1H, $^2J_{HF}$ = 49.2, $^3J_{HH}$ = 10.2 Hz), 4.38 (m, 1H), 5.38 (m, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): 11.9 (d, $^3J_{CF}$ = 3.1 Hz), 18.6 (d, $^3J_{CF}$ = 2.1 Hz), 19.4, 19.8, 23.5, 28.9 (d, $^2J_{CF}$ = 17.7 Hz), 31.2, 32.1, 32.3 (d, $^2J_{CF}$ = 18.0 Hz), 32.9 (d, $^3J_{CF}$ = 9.8 Hz), 36.2 (d, $^3J_{CF}$ = 10.8 Hz), 36.8, 36.9 (d, $^2J_{CF}$ = 18.0 Hz), 38.5 (d, $^2J_{CF}$ = 17.0 Hz), 39.3 (d, $^2J_{CF}$ = 19.3 Hz), 49.5, 49.9 (d, $^3J_{CF}$ = 5.0 Hz), 92.9 (d, $^1J_{CF}$ = 174.0 Hz), 106.1 (d, $^1J_{CF}$ = 181.1 Hz), 122.8, 139.3 (d, $^3J_{CF}$ = 12.5 Hz). ^{19}F NMR : δ -168.5 (dm, 1F, J = 50.6 Hz), -194.9 (dt, 1F, J = 41.6 and 7.6 Hz). MS (APCI+) m/z 303.0 $[M-(HF)+H]^+$, 283.0 $[M-(2HF)+H]^+$. Anal. Calcd for $C_{21}H_{32}F_2$ (322.25) C 78.22, H 10.00; found C 78.26, H 10.04



X-ray crystal structure of **APR19**



Structure of **APR19**

17aβ-Fluoro-17α-methyl-D-homo-3ethylenedioxo-5-androstene (3). Under an argon atmosphere, DAST (160 µL, 1.2 mmol) was added, at -78 °C, to a solution of steroid **2** (288 mg, 0.8 mmol) in dry dichloromethane (10 mL), and stirring was continued at room temperature for an additional period of 16 h. The reaction mixture was poured into ice water; the organic layer was washed thoroughly with an aqueous saturated Na_2CO_3 solution, brine, dried over Na_2SO_4, and concentrated under vacuum. Purification of the residue by silica gel column chromatography (petroleum ether/EtOAc, 95/5) gave steroid **3** as a single diastereoisomere (200.1 mg, 69% yield). R_f = 0.70 (Cyclohexane/EtOAc : 70/30); Mp 146-147 °C. IR (ν cm^{-1}): 2943, 1455, 1367, 1264, 1142, 1099, 1084, 999, 980, 954, 907, 874, 819. RMN ^{1}H (300 MHz) δ ppm: 0.79 (s, 3H, CH_3), 0.90 (d, 3H, CH_3, ^3J = 6.3 Hz), 0.91 (s, 3H, CH_3), 0.50-2.60 (m, 20H), 3.55 (dd, 1H, $^3J_{H-F}$ = 10.3 Hz, $^2J_{H-F}$ = 49.2 Hz), 3.87 (m, 4H), 5.30 (s, 1H). RMN ^{13}C (75 MHz) δ ppm: 139.9, 121.9, 109.4, 106.0, (CH, d, J_{C-F} = 180 Hz), 64.5, 64.3, 49.7 (CH, d, ^3J = 5.3 Hz), 49.1, 38.4 (Cq, d, $^2J_{C-F}$ = 16.5 Hz), 36.7, 36.0, 32.8, 32.7 (CH, d, ^2J = 18.0 Hz), 32.3, 32.1 (CH, d, ^2J = 18.0 Hz), 31.9, 31.0, 27.0, 23.4, 19.6, 18.8, 18.6, 11.8. RMN ^{19}F δ ppm: -194.9 (dt, 1F, J = 47.0 Hz, J = 8.0 Hz). MS (APCI) : m/z = 363.3 $[M+H]^+$. Anal. Calcd for $C_{23}H_{35}FO_2$ (362.52) C 76.20, H 9.73; found C 76.15, H 9.68.

17a□-Fluoro-17□-methyl-D-homo-pregn-4-en-3-one (APR6). To a solution of **3** (230 mg, 0.63 mmol) in CH_2Cl_2 (5 mL) was added at room temperature Amberlyst (1.0 g) and stirring was continued for 2 h.

The solid phase was separated by filtration and the solid was washed several times with CH_2Cl_2. The organic layer was washed with an aqueous saturated Na_2CO_3 solution, brine, and dried over Na_2SO_4. The extracts were then concentrated and the residue was purified on a silica gel column chromatography (Cyclohexane/EtOAc : 3/7) to yield APR6 (145 mg, 72%). R_f = 0.60 (Cyclohexane/EtOAc : 3/7) ; Mp 170-171 °C. IR (ν cm^{-1}) = 2943, 1677, 1454, 1367, 1264, 1143, 1085, 999, 980, 954, 873, 819, 925. RMN ^1H (300 MHz) δ ppm : 0,90 (s, 3H, CH_3), 0.97 (d, 3H, CH_3, ^3J = 6.0 Hz), 1.17 (s, 3H, CH_3), 0.90-2.50 (m, 20H), 3.60 (dd, 1H, ^3J = 10.0 Hz, ^2J = 49.2 Hz), 5.72 (s, 1H). RMN ^{13}C (75 MHz) δ ppm : 199.5, 171.1, 123.5, 105.8 (d, C-F, $J_{C\text{-}F}$ = 180 Hz), 53.3, 48.9 (CH, d, J = 5.3 Hz), 38.6, 36.8, 35.5, 34.9; 34.0, 32.6, 32.0 (d, CH, ^3J = 18 Hz), 31.4, 29.7, 26.9, 23.3, 19.8, 18.5, 17.6, 11.8. RMN ^{19}F δ ppm : -192.3 (dt, J = 49.0 Hz; J = 7.5 Hz). MS(APCI) : m/z = 319.2 $[M+H]^+$. Anal. Calcd for $C_{21}H_{31}FO$ (318.47) C 79.20, H 9.81; found C 79.12, H 9.75.

Cell Culture and Reagents. MDA-PRB and HEK 293T cells were routinely cultured in a high-glucose DMEM medium (Invitrogen, Cergy Pontoise, France), 20 mM HEPES, 2 mM glutamine, 1X non essential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin supplemented with 10 % fetal calf serum (FCS). Mifepristone (RU486) was from HRA Pharma (Paris, France). Progesterone, dexamethasone, dihydrotestosterone, aldosterone and spironolactone were from Sigma-Aldrich (S^t Louis, MO, USA).

Expression Vectors. The expression vectors pchGR, pchMR and pchPR coding human GR, MR and PRB respectively have been previously described (1-3). The human AR expression vector pcDNA-hAR was kindly provided by G.A. Coetzee (University of Southern California, Los Angeles). The plasmid pcβgal, which contains the β-galactosidase sequence, was used to standardize transfection experiments (4). Synthetic promoter driving luciferase reporter gene expression vector GRE2Luc was the gift of A. Biola-Vidammente and M. Pallardy (Univ Paris Sud, Châtenay-Malabry, France). The expression vector pVPPR encodes full length hPR fused to VP16 activation domain of herpes simplex virus. The pGALSMRT and pGALNCoR vectors coding for chimeric proteins GAL4 DNA-binding domain (DBD) fused to Receptor Interacting Domain (RID) of corepressors SMRT and NCoR respectively, were kindly provided by P. Balaguer (Montpellier, France). The pGALSRC2-RID and pGALSRC2-NterRID vectors, kindly provided by G Pinon (Univ Paris 7-Denis Diderot, France), code for; GAL4 DBD fused to SRC2 624-868 sequence comprising RID and GAL4 DBD fused to SRC2 1-868 sequence corresponding to the N-ter and RID. The pG5luc (kindly provided by P. Fuller) contains the luciferase gene driven by a GAL4-responsive promoter.

Competition binding experiments. MDA-PRB cells were grown in steroid depleted medium during 24 h, rinsed twice with cold PBS before pelleting and freezing in liquid nitrogen. One volume of cells was homogenized in two volumes of TEWG buffer (20 mM Tris-HCl, 1 mM EDTA, 20 mM sodium tungstate, 10% (vol/vol glycerol, pH 7.4, at 20°C) in a Teflon-glass Potter-Elvehjem apparatus. Cytosols obtained after centrifugation at 4°C at 15,000 g for 20 min were incubated for 4 h at 4°C with [^{3}H]-progesterone (10^{-9} M) alone or with increasing concentrations of P4 (10^{-9} to 10^{-6} M) or APR6 (10^{-8} to 10^{-5} M) or APR19 (10^{-8} to 10^{-5} M). Bound and unbound steroids were separated by the dextran-charcoal technique. Results are expressed as the percentage of binding measured with [^{3}H]-progesterone alone. Half-maximal inhibitory concentrations (IC_{50}) were thereafter calculated.

Transactivation assays. The MDA-PRB cells stably expressing PRB were transfected with indicated vectors (100 ng pSG5SRC1, 100 ng GRE2-Luc, 20 ng β-galactosidase) during 24 h, using Lipofectamine 2000 (Invitrogen) in 96-well plates in steroid depleted medium. Cells were treated during 24 h with vehicle or progesterone or indicated antagonist ligand alone or in combination to determine PRB-mediated agonist or antagonist activity. Whole cell extracts were collected using lysis buffer (Promega). Luciferase and β-galactosidase activity or total protein contents (BCA assay) were determined using a luminometer (Victor 378, Perkin Elmer). Luciferase activity was normalized by β-galactosidase activity or total protein contents. For transactivation assays performed in HEK293T cells, 24 h before transfection, the cells were

seeded at $3x10^6$ cells / 80 mm diameter culture Petri dish and cultured overnight in the same medium. Six hours before transfection, the FCS supplemented medium was replaced by the same medium supplemented with 10 % dextran-charcoal treated FCS. Transfections were carried out using the calcium phosphate precipitation method. The calcium phosphate precipitate was prepared with one of the steroid receptor expression vectors (0.5 µg pchPRB plus 1.5 µg pcDNA3, 2 µg pchAR, pchGR or pchMR) together with 7 µg of the reporter vector GRE2-Luc and 1µg of pcβgal for an internal transfection control, in 1 ml 140 mM NaCl, 0,75 mM Na2HPO4, 25 mM HEPES, 125 mM $CaCl_2$ pH 7.05 and added to the cells 30 minutes later. After 16 h incubation, the transfected cells were washed with PBS containing 2.5 mM EDTA, trypsinized and pooled. The transfected cells were replated in 24-well plates (10^5 transfected cells/well). Four hours later, ligands were added to transfected cells and the incubation maintained for 24 h at 37°C. Cells were lysed in 300 µl PBS, 25 mM glycine, 4 mM EDTA, 15 % glycerol, 1% triton X-100, 15 mM $MgSO_4$, pH 7.8 supplemented with 2 mM β-mercaptoethanol. The luciferase activity was quantified using a Mithras LB940 reader microplates (Berthold).

Quantitative RT-PCR. MDA-PRB cells were cultured in 6-well plates and treated during 6 h with vehicle or P4 or APR19 alone or in combination. Total RNA was extracted using TRIZOL reagent (Invitrogen). One µg of total RNA was treated with DNase I Amplification Grade (Invitrogen) and reverse transcribed using cDNA RT kit from Applied Biosystems (Courtaboeuf, France). Following a ten-fold dilution, the cDNA samples were amplified in duplicate by real-time PCR in ABI 7300 apparatus (Applied Biosystems), using the Power SYBR Green PCR Master Mix (Applied Biosystems) in the presence of specific forward and reverse primers (300 nM). A dissociation curve was obtained at the end of the reaction to verify primers specificity. Standard curve for PCR calibration of each gene transcript was obtained with the corresponding amplicon subcloned in pGEMT-easy (Promega) and verified by sequencing analysis. The expression level of each gene transcript was normalized to 18S RNA level, and results are expressed as means ± SEM.

Animals. Immature female B6D2 mice of 4 weeks age (10-12 g) were purchased from Societé Janvier, France and housed with free access to food and water and maintained on a light-dark cycle at 22-24 °C. All animal experiments were conducted in accordance with the National Institute of Health guide for the Care and Use of Laboratory Animals and French legislation on animal welfare.

Model of *in vivo* antiprogesterone efficacy of PR antagonists. A previously established model of screening PR antagonists for their *in vivo* antiprogesterone efficacy (5) was modified. Briefly, after 3 days of acclimatization, all mice were injected intraperitoneally (IP) with 25 ng estradiol (E2) for priming on 1^{st} day of experiment. Mice were separated into five groups (n = 6). On day 3 to 6, mice were injected IP daily with progesterone (50 µg) or APR19 (750 µg) or both. The last injection was supplemented with 50 ng of E2 to induce endometrial proliferation except in control group injected IP with vehicle solvent. Fifteen hours later, visceral organs were examined for gross lesions if any and uteri were recovered and weighed following cervical dislocation of mice.

Mammalian two hybrid assays. Cell transfection was performed in HEK 293T cells as above with 2 µg pVPPR, 5 µg pG5-luc and 1 µg pcβgal together with 1 µg pGALSMRT or pGALNcoR or 2 µg pGALSRC2-RID or pGALSRC2-NterRID. After 16 h incubation, the transfected cells were washed with PBS containing 2.5 mM EDTA, trypsinized and replated in 24-well plates. Four hours later, cells were incubated with APR19 (10^{-8} to 10^{-5} M), RU486 (10^{-11} to 10^{-8} M) or progesterone (10^{-10} to 10^{-8} M). Additional experiments were performed with progesterone (10^{-9} M) in the presence of APR19 (10^{-8} to 10^{-5} M). After 16 h, luciferase and β-galactosidase activities from whole cells extracts was determined using a Mithras reader microplates (Berthold).

Immunoblotting. Western blot analysis was performed as previously described (6). Antibodies used were, PR serine 294 phospho-specific antibody (Affinity BioReagent), anti-PR (NCL-L-PGR-312/2,

Novocastra Laboratories), anti-α-tubulin antibody (Sigma), horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA). Target proteins were detected using ECL Plus reagent (GE Healthcare) and visualized by chemiluminescence.

Electromobility Shift Assay. MDA-PRB cells were cultured in steroid-free medium during 24 h and then treated with indicated ligand. Nuclear protein extraction and gel mobility shift assays using [^{32}P]dCTP-labeled double strand PRE as described previously (7). Oligonucleotide sequences were as follows: sense, 5'-AGCTGCTCAGCTAGAACACTCTGTTCTCTACT-3', antisense, 5'-AGCTAGTAGAGAACAGAGTGTTCTAGCTGAGC-3'.

References

1. **Hellal-Levy C, Couette B, Fagart J, Souque A, Gomez-Sanchez C, Rafestin-Oblin M** 1999 Specific hydroxylations determine selective corticosteroid recognition by human glucocorticoid and mineralocorticoid receptors. FEBS Lett 464:9-13
2. **Fagart J, Wurtz JM, Souque A, Hellal-Levy C, Moras D, Rafestin-Oblin ME** 1998 Antagonism in the human mineralocorticoid receptor. Embo J 17:3317-3325
3. **Petit-Topin I, Turque N, Fagart J, Fay M, Ulmann A, Gainer E, Rafestin-Oblin ME** 2009 Met909 plays a key role in the activation of the progesterone receptor and also in the high potency of 13-ethyl progestins. Mol Pharmacol 75:1317-1324
4. **Fagart J, Huyet J, Pinon GM, Rochel M, Mayer C, Rafestin-Oblin ME** 2005 Crystal structure of a mutant mineralocorticoid receptor responsible for hypertension. Nat Struct Mol Biol 12:554-555
5. **Cullingford TE, Pollard JW** 1988 RU 486 completely inhibits the action of progesterone on cell proliferation in the mouse uterus. J Reprod Fertil 83:909-914
6. **Khan JA, Amazit L, Bellance C, Guiochon-Mantel A, Lombes M, Loosfelt H** 2011 p38 and p42/44 MAPKs Differentially Regulate Progesterone Receptor A and B Isoform Stabilization. Mol Endocrinol
7. **Lombes M, Binart N, Oblin ME, Joulin V, Baulieu EE** 1993 Characterization of the interaction of the human mineralocorticosteroid receptor with hormone response elements. Biochem J 292 (Pt 2):577-583

GENERAL DISCUSSION

Progesterone receptor (PR), a steroid-activated transcription factor, is an important pharmacological target for contraception, female reproductive disorders as well as for hormone-dependent breast and uterine cancers. Alternative transcription of PR gene results in equal expression of two major isoforms PRA and PRB (Kastner et al., 1990; Mote et al., 2002). PRA lacks the 164 N-terminal amino acids, also called the B-upstream segment (BUS) present in PRB (Sartorius et al., 1994). Despite the high sequence similarity, accumulating evidence indicates that PRB and PRA are functionally distinct transcriptional factors (Giangrande and McDonnell, 1999) and exhibit differential physiological responses in target tissues (Mani et al., 2006; Mulac-Jericevic et al., 2003; Mulac-Jericevic et al., 2000). Biochemical and biophysical studies suggest that unique conformation of PRA and PRB (Bain et al., 2001) allows interaction with distinct coregulators (Tetel et al., 1999). Under physiological conditions, the majority of PR positive mammary epithelial cells express both PR isoforms at equimolar levels (Mote et al., 2002; Mote et al., 2007). However, altered PRA/PRB ratio is often associated with breast carcinogenesis (Graham et al., 1995b; McGowan et al., 2004; Mote et al., 2002). This could be explained by three possibilities i) the variations in PRA/PRB ratio is the outcome of the diverse changes in cell signaling pathways associated with pathogenesis ii) the altered PRA/PRB ratio plays a contributing role towards such processes iii) a combination of both. Although, the mechanisms behind alteration in PRA/PRB ratio are largely unknown, each isoform having distinct genomic targets (Richer et al., 2002) and exerting tissue-specific effects (Conneely et al., 2001), PRA/PRB expression ratio should be considered a key biological determinant selecting tissue responsiveness to hormone and growth factors stimuli. Therefore, in this thesis we investigated the mechanisms controlling PR isoforms degradation/stability in response to agonist/antagonist ligands. We identified that distinct MAPK pathways are involved in controlling PR isoforms stability at post-translational level. PRA is stabilized by p38 MAPK whereas p42/44 MAPK selectively control PRB stability leading to opposite variation in PRA/PRB ratio. Extracellular stimuli such as epidermal growth factors or pro-inflammatory cytokines that preferentially activate p42/44 or p38 MAPK respectively may lead to opposite variations in PRA/PRB expression ratio in cancer cells that may lead to dramatic shift in selection of PR target gene subsets thus switching cellular responses to hormonal/growth factor stimuli.

To explore the contribution of individual PR isoform and particularly the role of varied PRA/PRB ratio in cell signalling, we developed a new strategy in the form of bi-inducible promoter system to control PRA and PRB expression in the same cell. This cell based system allowed us to demonstrate that several aspects of PR signalling such as target gene

selection/transcriptional regulation, antiproliferative efficacy of antiprogestin and cross talk with growth factors are highly influenced by variation in PRA/PRB ratio. Our results highlight the importance of understanding the contribution of PR isoforms in several pathophysiological scenarios. A possible therapeutic approach in PR-dependent pathological conditions employs PR antagonists. However, most of the currently available antiprogestins present partial agonist activity and are not selective to PR since they also antagonize other steroid receptors such as GR and AR. Therefore, in a collaborative project we have developed several new class PR antagonists which lack partial agonist character and are selective to PR. Interestingly, these molecules inactivate PR through unusually passive mechanism of action i.e. do not allow recruitment of transcriptional coregulators upon binding to PR. Such PR specific antagonists devoid of partial agonist character might provide important therapeutic perspectives for various reproductive tract abnormalities and certain hormone-dependent uterine and breast cancers.

Distinct MAPK-dependent mechanisms control PRA/PRB ratio at post-translational levels

In PR, at least 14 phosphorylation sites are targeted by multiple kinases, mostly within Serine-Proline motifs in N-terminal domain (Beck et al., 1996a; Dressing et al., 2009; Pierson-Mullany and Lange, 2004; Shen et al., 2001; Weigel and Moore, 2007). Among these phosphorylation sites, PRB serine 294 phosphorylation (pS294-PRB) has been shown to act as a major signal for PR turnover since substitution of serine 294 by an alanine (S294A) decreased PRB turnover (Lange et al., 2000). However, in stably transfected T47D cells, PRB-S294A mutant underwent ligand induced turnover, though to lesser extent as compared to wild type PRB (Skildum et al., 2005) suggesting that PR stability/turnover might also be governed by pS294-independent mechanisms. Knowing that S294-PRB or S130-PRA (S294 equivalent residue in PRA) residue is common in PRB and PRA, the question arises whether the increased PRB degradation is exclusively due to its increased S294 phosphorylation? In other words, whether S294 phosphorylation is the only signal for PRB degradation? We thus investigated the mechanism(s) differentially controlling PR isoforms degradation/stability in the context of agonist and antagonist ligands. RU486 (Mifepristone), a widely used PR antagonist, blocks PR transcriptional activity by favoring corepressors recruitment and has been proposed for hormone-dependent breast cancer treatment (Klijn et al., 2000). Therefore we studied the impact of RU486 in regulating PR stability/turnover. Like progesterone, RU486 stimulates similar early cascade of events including chaperone dissociation, dimerization and post-translational modifications such as sumoylation (Daniel et al., 2007a) and phosphorylation (Beck et al., 1996b; Clemm et al., 2000). We used two cellular models, a breast cancer cell model (MDA-MB-231) and an endometrial cancer cell model (Ishikawa) stably expressing PRA and/or PRB. Both these cell lines have been

previously characterized and widely used to study PR signaling (Petit et al., 2009; Smid-Koopman et al., 2003).

We found that RU486 promotes PRB or PRA protein stabilization despite inducing pS294 or pS130 respectively, indicating that RU486 interferes in downstream events of pS294- or pS130-signalled PR isoforms down-regulation. We found that p42/44 MAPK activity inhibited proteasome-mediated degradation of PRB but not PRA in both cell lines in a ligand-sensitive manner. Although, identical sites are phosphorylated by R5020 and RU486 binding (Beck et al., 1996b), recently, it was demonstrated that RU486-bound PRB conformation, in conjunction with PR coregulatory protein Jun dimerization protein-2, exposes protein interaction surfaces that are distinct from those presented by agonist ligand (Wardell et al., 2010). In agreement with these studies, our results indicate that unique conformation of RU486-bound PRB might strongly facilitate stabilizing effects of p42/44-dependent phosphorylation (on a residue other than S294) which impedes interaction with coregulatory proteins implicated in PRB turnover. Nevertheless, we could not exclude the possibility that a p42/p44-dependent phosphorylation of a partner protein which preferentially recognizes conformation of the PR-RU486 complex and indirectly inhibit its ubiquitination leading to PRB stabilization.

Furthermore, ligand-induced PRB turnover required neosynthesis of a mandatory down-regulating partner whose interaction/function is negatively controlled by p42/44 MAPK. Such regulation strongly influenced expression of various endogenous PRB target genes in a selective manner, supporting functional relevance of the mechanism. Our data does not correlate with previous reports using T47D-YB cells (stably expressing PRB) or in HeLa cells transiently transfected with PRB expression vector showing that p42/44 MAPK accelerate PRB degradation (Lange et al., 2000; Qiu and Lange, 2003). Furthermore, it was reported that EGF, despite inducing pS294, increased PRB stability in T47D-YB cells (Shen et al., 2001), consistent with our observations. In support of our results, it has been recently described that degradation of androgen receptor (AR) is enhanced following p42/p44 inhibition by U0126 in prostate cancer LNCaP cells (Agoulnik et al., 2008). It thus seems very likely that such p42/44 MAPK-dependent stabilizing effect might be conserved for this nuclear receptor subfamily.

Interestingly, in contrast to PRB, PRA stability was specifically increased by MEKK1-induced p38 MAPK activation. Selective inhibition of p42/p44 or p38 activity resulted in opposite variations of PRA/PRB expression ratio. Recent biochemical and biophysical studies suggest that unique conformation of PRA and PRB (Bain et al., 2001) allows interaction with distinct molecular partners (Tetel et al., 1999). Lack of BUS domain in PRA structure might allow p38-dependent phosphorylation that might be inaccessible in PRB due to conformational differences in PR isoforms.

In this study, we employed active kinase strategy along with selective MAPK inhibitors in order to rapidly reverse the observed PR stabilization/turnover. Although the first part of article mainly focused on p42/44 MAPK, in the second part, we challenged parallel kinase signaling pathways by studying p38 as well as pJNK along with p42/44 MAPKs. To minimize toxic effects of chemical inhibitors used in this study, we used minimum recommended concentrations of these inhibitors and did not notice toxic effects on cell morphology by immunocytochemistry. Under similar experimental conditions, same inhibitor concentrations and similar cellular models, the fact that only PRB but not PRA stability is inhibited by selective p42/44 inhibitor (U0126) and only PRA but not PRB stability is inhibited by selective p38 inhibitor (PD169316), also minimizes the chances of artifactual observations resulting from general side effects of such inhibitors. Although the use of chemical inhibitors strongly suggested the differential MAPK-dependent control of PRA and PRB stabilization, the strategy of selective knock-down of p38 or p42/44 MAPK strengthened our conclusions for PR isoform specific stabilization mechanisms.

Given that, both PR isoforms are expressed under estrogen stimulation at equimolar levels under physiological conditions, investigation of PR isoforms stability at post-translational level required the use of synthetic promoters for driving PR isoforms expression to minimize the contributions that may come from transcriptional modulation from their endogenous promoters. To exclude the possibility that such ligand sensitive MAPK-dependent stabilizing mechanisms might be due to artifactual over-expression of PR isoforms, we confirmed that expression levels of PRA or PRB in both of our cellular models is similar to endogenous PR levels observed in wild-type T47D cells expressing both PRA and PRB. Nevertheless, one limitation of our study is that additional mechanisms might also affect PR expression at transcriptional and even post transcriptional levels. The combination of which might also influence PRA/PRB ratio. Also we did not identify MAPK-dependent PR isoforms phosphorylation sites which contribute towards PR stabilization. We and several other laboratories have shown that ligand-induced PR turnover is undetectable in transient expression systems, due probably to excessive production of receptor proteins. Since efficient protein turnover requires functioning of various components of ubiquitin-proteasome pathway, the latter(s) might be the limiting factor in such over-expression systems. Analysis of the role of such phosphorylations in PR stability/turnover under more physiological conditions requires not only their identification but also establishment of stable cell lines using as many mutants as putative sites present in PR sequence. This difficult task was far beyond the scope of this study.

Establishment and characterization of new cellular model conditionally expressing PRA and/or PRB

To establish bi-inducible breast cancer cellular model conditionally expressing either or both PRA and PRB in the same cells, we selected MDA-MB-231 cells (PR-) since they do not express ER and hence enable investigation of PR specific events independently of estrogen. We used RheoSwitch and TRex inducible systems for conditional expression of PRA and PRB respectively. RheoSwitch system is known for minimal background in the absence of inducer ligand and maximum inducible expression of target protein. TRex system was chosen instead of alternate Tet-on/Tet-off systems in order to minimize the background expression in the absence of inducer. Both RheoSwitch and TRex systems require non-steroidal inducer ligands RheoSwitch Ligand (RSL1) and doxycycline (Dox) in contrast to steroid ligand ponasterone used by other laboratories for establishing inducible PR cell lines (Jacobsen et al., 2002).

Since regulatory proteins of both inducible systems are of non-mammalian origin and inducers are non-steroidal ligands, transcriptional drawbacks in human cells are highly limited. Indeed, we did not observe any impact of RSL1 or Dox in regulating transcription of PRE^2-driven reporter gene or endogenous PR target genes that we studied. This newly established bi-inducible breast cancer cellular model is of broad interest since the contribution of PRA and PRB homo- and hetero-dimers in progesterone signaling could be investigated with more confidence given that such cells serve as their own controls when cultured in the absence of inducer(s). Moreover, the role of varied PRA/PRB expression ratio in breast cancer development and metastasis could also be investigated. For instance, mice models xenografted with iPRAB cells and exposed to varying amounts of doxycycline and/or RSL1 to control PRA/PRB ratio might be of particular pharmacological interest. Lastly, since both PR isoforms are selectively expressed within the same cells, this cell-based system is also highly suitable for screening and characterization of new PR antagonists for their PR isoform selective inhibitory properties.

PRA/PRB ratio a critical determinant of target gene selection

The presence of PR and more importantly the PRA/PRB ratio directly influences breast cancer development and metastasis independently of the progestational status (Hopp et al., 2004; Jacobsen et al., 2003; Punglia et al., 2006) and breast cancer patients with PRA dominant tumors have poorer disease-free survival rates (Hopp et al., 2004). The mechanisms by which PR contributes to such tumorigenic processes are not fully known in part due to lack of appropriate cellular models. To validate our strategy of bi-inducible PR isoforms expression, we studied several aspects of PR signaling to investigate the critical role of PRA/PRB ratio in target gene selection, transcriptional regulation and cross-talk with growth factors stimuli.

Progesterone exerts antiproliferative effects in PR expressing cells (Beleut et al., 2010) and few proliferating mammary epithelial cells (MEC) express PR (Clarke et al., 1997; Russo et al., 1999). Recently, the role of progesterone signaling in mammary carcinogenesis via paracrine mechanism gained strength since various independent studies have demonstrated that progesterone drives proliferation of PR(-) MEC leading to carcinogenesis via receptor activator of NF-κB ligand (RANKL), a tumor necrosis factor family member, and its receptor (Beleut et al., 2010; Gonzalez-Suarez et al., 2010; Schramek et al., 2010). Consistent with previous studies (Lin et al., 1999), we found that both progesterone and antiprogestin RU486 exerted antiproliferative effects when either or both PR isoforms were expressed. Interestingly RU486 but not progesterone exhibited stronger antiproliferative effects in PRA- as compared to PRB-expressing cells suggesting that PRA/PRB expression ratio influences the antiproliferative efficacy of antiprogestins.

Cross talk of PR with EGF-initiated pathways has been extensively studied by other laboratories (Faivre et al., 2005). It has been shown that PR action is linked to EGF stimulations at various levels such as subcellular PRB localization, DNA binding and target gene regulation (Daniel et al., 2007a). Paracrine EGF-like growth factors HBEGF and AREG are highly implicated in mammary gland carcinogenesis and metastasis (Bos et al., 2009; Desruisseau et al., 2004) and proposed to be the therapeutic targets of various human cancers including breast cancer (Yotsumoto et al., 2008). The differential role of PR isoforms in regulating HBEGF and AREG expression under progesterone and EGF stimulation is not known. Therefore, using iPRAB cells, we explored the transcriptional regulation of HBEGF and AREG by PR isoforms in the context of progesterone and EGF stimulations. We show that PR isoforms distinctly impact the transcription of HBEGF and AREG genes not only in the absence of hormone but also under progesterone and EGF stimulations independently of contributions from ER. Studies on PRB transactivation from exogenous promoters have shown that EGF strongly enhances progesterone-induced transcriptional response (Daniel et al., 2007b). Whether EGF also potentiates progesterone-induced PRA-mediated transactivation from similar exogenous promoters has not been described. Our results on AREG transcription revealed that EGF strongly potentiates progesterone-induced AREG expression uniquely via PRB similar to synergetic effect of progesterone and EGF on PRB-mediated transactivation from exogenous promoters. Such PR isoforms-dependent synergetic response of EGF and progesterone on AREG transcription are supported by the finding that co-expression of PRA compromised such potentialization effect of EGF. Interestingly, progesterone and EGF exerted opposing effects on HBEGF transcription, progesterone decreased while EGF increased the HBEGF expression. These opposing effects of progesterone and EGF involve PR since such effects are not observed in uninduced (PR -) cells.

Using this original model, we were able to show that epidermal growth factor (EGF) can enhance or repress transcription of progesterone responsive genes depending not only on promoter context but more importantly on PRA/PRB ratio. We identify that PR isoforms play distinct role in transcriptional regulation of heparin-binding growth factor (HBEGF) and amphiregulin (AREG), paracrine growth factors implicated in carcinogenesis. Finally, our results demonstrate that unliganded PRA continuously increases AREG transcription that can be inhibited by antiprogestin RU486. Increased AREG expression by elevated PRA/PRB ratio may provide paracrine growth stimulus for the proliferation of nearby PR negative cells. This newly established cellular model provides a power tool to discriminate the differential impact of PR isoforms in cancer progression.

The iPRAB cells allowed us to show that antiproliferative efficacy of antiprogestins is highly influenced by PRA/PRB ratio that thus should be taken into consideration during therapeutic applications involving antiprogestins. We demonstrate that elevated PRA/PRB ratio is associated with increased AREG expression that was inhibited by antiprogestin RU486. Given the involvement of AREG in cancer development/metastasis as a paracrine growth stimulus, we propose that aggressive phenotype of PRA rich tumors might at least in part be attributed to elevated AREG levels which may enhance the proliferation of nearby PR negative cells.

However, further studies are needed to show AREG expression at protein level. For this western blot analysis of whole cell extract and/or enzyme linked immunosorbent assay (ELISA) should be performed to determine AREG secretion in cell culture media. Remains to be proven is the role of AREG in promoting proliferation of adjacent PR (-) cells. For this, PRA expressing cells should be cultured with PR (-) cells in the absence of hormone and the proliferation should be determined after few days of co-culture compared with conditions where all cells express PRA or not. If the proliferation is higher in co-culture condition, the same experiment needs to be repeated in the presence of small interfering RNA against AREG or with AREG antibody to neutralize the proliferating effect of AREG secreted by PRA expressing cells. Furthermore, histological screening should be conducted to see whether PRA-rich breast tumors express elevated AREG protein as compared to PRB rich tumors or tumors which do not express PR at all. The mechanism of AREG expression by unliganded PRA remains to be further explored. For example, chromatin immunoprecipitation experiments should be performed to explore the promoter region(s) involved in PRA-dependent AREG transcription.

New class of PR antagonists with passive mechanism of action

PR agonists and antagonists elicit profound physiological effects, notably by modulating multiple gene expression profiles in target tissues. There is strong preclinical and clinical evidence for the value of progestin antagonists in treatment of endometriosis, uterine fibroids,

dysfunctional uterine bleeding and breast cancer (Cadepond et al., 1997; Kettel et al., 1998; Murphy et al., 1995; Spitz, 2003). However, currently available antiprogestins also function as effective antiglucocorticoids (Teutsch et al., 1991; Teutsch et al., 1995). Thus, there is need to develop compounds with no or reduced antiglucocorticoid activity, notably for long term administration of antiprogestins required for some diseases (Kettel et al., 1991). However, limitation in identifying compounds which selectively bind PR may relate to the fact that so far most of the principle PR antagonists are steroidal molecules which are characterized by the C11 bulky substituent and therefore may function by similar mechanisms (Teutsch et al., 1995).

Our concept of novel class of PR antagonists was based on crystallographic studies of PR LBD complexed with either progesterone (Williams and Sigler, 1998) or two testosterone-derived progestins norethindrone (Madauss et al., 2004) or levonorgestrel (Petit-Topin et al., 2009). These studies revealed that the network of stabilizing contacts with PR LBD involved two key regions in these ligands. The ketone of steroidal A ring is involved in stabilizing contacts with Gln725 and Arg766 residues of PR ligand binding cavity (Williams and Sigler, 1998). The 13-methyl or ethyl group in the D ring establishes stabilizing contacts with Met909 of the H12 helix either directly or indirectly (Petit-Topin et al., 2009). Additional contacts between the ligand-binding cavity and the C17 hydroxyl group of D ring have also been identified for the testosterone-derived progestins (Petit-Topin et al., 2009). Based on these observations, we propose a novel strategy to inactivate PR by molecules lacking bulky 11-substituent. We report that the replacement of the classical 5-carbon D ring by 6-carbon ring having no polar substituent but keeping C3-keton function confers antagonist character with partial agonist feature (APR6). Interestingly, a second modification within the A ring by replacing the 3-ketone by fluorine (APR19) completely abolished the residual agonist activity resulting in a full PR antagonist. Using transactivation assays in human breast cancer cells stably expressing PRB, we demonstrated that C-3 substitution plays critical role in determining agonist character of homosteroid molecules since 3-ketone in APR6 accounts for its partial agonist feature while APR19 with its 3-fluorine behaves as a full antagonist *in vitro* and exhibited high *in vivo* anti-progesterone activity in a mouse model. Thus, a molecule lacking anchoring via its A and D rings remains capable of binding to PR and displays antagonist feature. Interestingly, the steroidal ligand P1 lacking any substituent at C3 position of the A ring was shown to inactivate MR carrying the mutation S810L (MR_{S810L}), while it was unable to bind to the wild type receptor (Pinon et al., 2004). The antagonist feature of this molecule was related to its inability to contact Gln776 (corresponding to Gln725 in PR). This residue and Leu810 together with the steroidal 19-methyl are involved in a network of contacts critical for the MR_{S810L} activation (Fagart et al., 2005).

Importantly, APR19, in contrast to RU486, is highly selective to PR since it did not inhibit transcriptional activity of other 3oxo-steroid receptors such as GR, AR and MR. All

steroid receptors, except ER present high sequence homology in their LBD. Their cognate natural steroid ligands harbor C3 ketone function in A ring which is strongly anchored to the highly conserved residues in ligand binding cavity of oxo-steroid receptors. This might explain in part the lack of receptor specificity of most of C-11 aryl substituted ligands which all harbor C3 ketone. The presence of fluorine at the C3 position in APR19 preserved its binding to PR but not to the other steroid receptors (GR, AR and MR). This suggests that C3 ketone function of APR19 is dispensable for PR binding but mandatory for their binding to other steroid receptors. It is likely that multiple hydrophobic contacts involved in APR19 binding to PR compensate the lack of A ring anchoring.

A noteworthy feature of the homosteroid APR19 is that PR, upon APR19 binding, is unable to recruit transcriptional coregulators (corepressors and coactivators). This is in contrast to recruitment of transcriptional coregulators by antagonist-bound receptors as it has been demonstrated for RU486 and asoprisnil. Co-crystal structural studies have been performed using peptide of SMRT corepressor along with PR LBD in the presence of asoprisnil (Madauss et al., 2007). The bulky 11beta-benzaldoxime substituent of asoprisnil collided with the side chain of the H12 residue Met909 pushing the latter away and preventing the H12 from adopting the agonist-bound conformation. This steric interference makes room for interaction with corepressor and displaces Glu911 (Madauss et al., 2007), a residue critical for coactivator binding (Bledsoe et al., 2002). Furthermore, high potency of such bulky antagonist might be explained by the formation of direct stabilizing contacts between C11 polar substituent and coregulators since asoprisnil C11 substituent directly interacted with Leu917 of NCoR or the corresponding Leu2263 of SMRT peptide (Madauss et al., 2007). Although, asoprisnil-bound PR LBD preferentially recruited corepressors, interaction with coactivators SRC1, SRC2 and SRC3 was also observed (Madauss et al., 2007) leading to partial agonist character of such antagonists. Indeed it has been shown that the transcriptional outcome following bulky antagonists binding is highly influenced by coactivator/corepressor expression ratio (Liu et al., 2002). Lack of C11 aryl substitution together with C3 ketone function in APR19 allows full PR inactivation due to absence of stable H12 conformation and lack of stabilizing contacts at the A ring. Thus APR19-bound PR is unable to interact with corepressors as well as coactivators and therefore, it could be classified as a prototype molecule belonging to a new class of passive PR antagonists. Such passive PR antagonists present the advantage of avoiding deleterious side effects associated with long term use of currently available antiprogestins.

Numerous steroid receptor ligands, lacking any bulky substituent, act as antagonists even though they are smaller than endogenous ligands. This is namely the case for spironolactone which is smaller than natural MR ligand aldosterone and display high potency to inhibit the aldosterone-induced MR activity (Fagart et al., 2005). Due to their inability to establish the stabilizing contact with Asn770, these molecules form unstable complexes which

are unable to recruit transcriptional coregulators (coactivator or corepressor) (Fagart et al., 2005). The AR antagonist flutamide with a size comparable to testosterone is another example of passive antagonist. Finally, APR19 is the first example of non-steroidal, full PR antagonist inactivating PR by passive mechanism. This lead molecule may open new pharmacological strategies for long-term utilization of PR antagonists, circumventing deleterious side effects of current antiprogestins. This may be particularly promising for treatment or prevention of hormone-dependent breast cancers. The study mainly focused on PRB, whether this molecule exhibits similar antagonistic effects via PRA isoform remains to be studied. Also further *in vivo* studies are required to verify anti-tumoral properties of APR19 in mouse models xenografted with human breast tumors or breast cancer cells.

CONCLUSION

Progesterone receptor (PR) is an important pharmacological target for contraception, female reproductive disorders as well as for hormone-dependent breast and uterine cancers. Human PR is expressed as two major isoforms PRA and PRB which exhibit distinct transcriptional properties, have distinct target tissue specific functions and capable of interacting with distinct molecular partners. Although, under physiological conditions, PR isoforms are expressed at equimolar levels, an altered PRA *vs* PRB expression is often observed under pathological conditions through unknown mechanisms. Any change upshift or downshift in PRA/PRB expression ratio should be considered a key biological determinant selecting tissue responsiveness to hormone and growth factors stimuli. Therefore, in this thesis using breast and endometrial cancer cell models stably expressing PRA and/or PRB, we found that down-regulations of PRB and PRA proteins are negatively controlled by key phosphorylation events involving distinct MAP kinases, resulting in selective PR isoform stabilization. PRA is selectively stabilized by p38 MAPK whereas p42/44 MAPK selectively control PRB stability leading to opposite variation in PRA/PRB ratio. Furthermore, these phosphorylation events are differentially controlled by agonist and antagonist ligands and inhibit PRB degradation via proteasome. In cancer cells, elevated extracellular stimuli such as epidermal growth factors or pro-inflammatory cytokines that preferentially activate p42/44 or p38 MAPK respectively may result in opposite variations in PRA/PRB expression ratio that may lead to dramatic shift in selection of PR target gene subsets thus switching cellular responses to hormonal/growth factor stimuli. These results are of potential relevance in explaining the alteration in PRA/PRB ratios frequently observed in breast cancer and that may contribute to the role of progesterone signaling in breast pathogenesis.

To get a mechanistic understanding of how target cells behave to different stimuli under varied PRA/PRB ratio, we generated an original bi-inducible PR-isoform system allowing selective, reversible and dose-dependent expression of PRA and/or PRB, thus allowing fine-tune adjustment of PRA/PRB ratio to desired extent. This cell-based model provides a biological tool for investigating the functional consequences of PRA *vs* PRB activation in cultured cells as well as in xenografted mouse models. Using this model, we investigated transcriptional regulation by unliganded and liganded PR isoforms and found that several aspects of PR signaling such as target gene selection/transcriptional regulation and antiproliferative efficacy of antiprogestin are highly influenced by variation in PRA/PRB ratio. Moreover, we found that synergetic or antagonistic effect of epidermal growth factor and progesterone stimulations on PR transactivation properties depends not only on target genes but more importantly on PRA/PRB ratio. Interestingly, unliganded PRA increases amphiregulin (AREG) expression independently of estrogen receptor that can be inhibited by

antiprogestins. AREG is known for its important role in breast cancer tumorigenesis, and aggressive phenotype of PRA-dominant tumors might at least in part be attributed to continuous paracrine growth stimulation by AREG. Our results provide mechanistic support for the aggressive phenotype of breast cancers with high PRA/PRB ratio.

A possible therapeutic approach in PR-dependent pathological conditions could be the use of PR antagonists. Most of the currently available antiprogestins such as mifepristone present partial agonist activity and are not selective to PR leading to undesirable side effects. Based on the knowledge of key contacts involved in PR-progestin interaction as revealed by recent crystallographic studies, in a collaborative project we have developed several new class PR antagonists named as APRn. Structure-activity relationship studies allowed identification of the key substitutions in steroidal skeleton responsible for agonist/antagonist character of these molecules. The selected APRn lack partial agonist effect, are selective to PR and inhibit progesterone-induced PR transcriptional properties. Interestingly, these molecules inactivate PR through unusually passive mechanism of action i.e. do not allow recruitment of transcriptional coregulators (both coactivators and corepressors) upon binding to PR. *In silico* docking experiments showed that in contrast to progesterone, APR19 only partially occupies the ligand-binding cavity, preventing PR-APR19 complex to acquire a stable conformation a pre-requisite of PR interaction with coregulators. This might explain the passive mechanism of action of these compounds. Such PR selective antagonists devoid of partial agonist character might provide important therapeutic perspectives for various reproductive tract abnormalities and certain hormone-dependent uterine and breast cancers.

BIBLIOGRAPHY

Abdel-Hafiz, H., Dudevoir, M. L., and Horwitz, K. B. (2009). Mechanisms underlying the control of progesterone receptor transcriptional activity by SUMOylation. J Biol Chem *284*, 9099-9108.

Abdel-Hafiz, H., Takimoto, G. S., Tung, L., and Horwitz, K. B. (2002). The inhibitory function in human progesterone receptor N termini binds SUMO-1 protein to regulate autoinhibition and transrepression. J Biol Chem *277*, 33950-33956.

Abedin, S. A., Thorne, J. L., Battaglia, S., Maguire, O., Hornung, L. B., Doherty, A. P., Mills, I. G., and Campbell, M. J. (2009). Elevated NCOR1 disrupts a network of dietary-sensing nuclear receptors in bladder cancer cells. Carcinogenesis *30*, 449-456.

Africander, D., Verhoog, N., and Hapgood, J. P. (2011). Molecular mechanisms of steroid receptor-mediated actions by synthetic progestins used in HRT and contraception. Steroids *76*, 636-652.

Agoulnik, I. U., Bingman, W. E., 3rd, Nakka, M., Li, W., Wang, Q., Liu, X. S., Brown, M., and Weigel, N. L. (2008). Target gene-specific regulation of androgen receptor activity by p42/p44 mitogen-activated protein kinase. Mol Endocrinol *22*, 2420-2432.

Allen, E., and Doisy, E. A. (1923). An ovarian hormone. Preliminary report on its localization, extraction and partial purification, and action in test animals. J Am Med Assoc *81*, 819-821.

Allen, W. M. (1941). The chemical and physiological properties, and clinical uses of the corpus luteum hormone, progesterone. Annual Meeting of The New York Academy of Medicine. The Bulletin, 508-518.

Amazit, L., Roseau, A., Khan, J. A., Chauchereau, A., Tyagi, R. K., Loosfelt, H., Leclerc, P., Lombes, M., and Guiochon-Mantel, A. (2011). Ligand-dependent degradation of SRC-1 is pivotal for progesterone receptor transcriptional activity. Mol Endocrinol *25*, 394-408.

Anderson, G. L., Limacher, M., Assaf, A. R., Bassford, T., Beresford, S. A., Black, H., Bonds, D., Brunner, R., Brzyski, R., Caan, B.*, et al.* (2004). Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. Jama *291*, 1701-1712.

Antal, M. C., Krust, A., Chambon, P., and Mark, M. (2008). Sterility and absence of histopathological defects in nonreproductive organs of a mouse ERbeta-null mutant. Proc Natl Acad Sci U S A *105*, 2433-2438.

Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science *277*, 965-968.

Arnett-Mansfield, R. L., DeFazio, A., Mote, P. A., and Clarke, C. L. (2004). Subnuclear distribution of progesterone receptors A and B in normal and malignant endometrium. J Clin Endocrinol Metab *89*, 1429-1442.

Arnett-Mansfield, R. L., deFazio, A., Wain, G. V., Jaworski, R. C., Byth, K., Mote, P. A., and Clarke, C. L. (2001). Relative expression of progesterone receptors A and B in endometrioid cancers of the endometrium. Cancer Res *61*, 4576-4582.

Asselin-Labat, M. L., Vaillant, F., Sheridan, J. M., Pal, B., Wu, D., Simpson, E. R., Yasuda, H., Smyth, G. K., Martin, T. J., Lindeman, G. J., and Visvader, J. E. (2010). Control of mammary stem cell function by steroid hormone signalling. Nature *465*, 798-802.

Augereau, P., Badia, E., Balaguer, P., Carascossa, S., Castet, A., Jalaguier, S., and Cavailles, V. (2006). Negative regulation of hormone signaling by RIP140. J Steroid Biochem Mol Biol *102*, 51-59.

Bain, D. L., Franden, M. A., McManaman, J. L., Takimoto, G. S., and Horwitz, K. B. (2001). The N-terminal region of human progesterone B-receptors: biophysical and biochemical comparison to A-receptors. J Biol Chem *276*, 23825-23831.

Bakken, K., Fournier, A., Lund, E., Waaseth, M., Dumeaux, V., Clavel-Chapelon, F., Fabre, A., Hemon, B., Rinaldi, S., Chajes, V.*, et al.* (2010). Menopausal hormone therapy and breast cancer risk: Impact of different treatments. The European prospective investigation into cancer and nutrition (EPIC). Int J Cancer.

Bamberger, A. M., Bamberger, C. M., Gellersen, B., and Schulte, H. M. (1996). Modulation of AP-1 activity by the human progesterone receptor in endometrial adenocarcinoma cells. Proc Natl Acad Sci U S A *93*, 6169-6174.

Banwell, C. M., MacCartney, D. P., Guy, M., Miles, A. E., Uskokovic, M. R., Mansi, J., Stewart, P. M., O'Neill, L. P., Turner, B. M., Colston, K. W., and Campbell, M. J. (2006). Altered nuclear receptor corepressor expression attenuates vitamin D receptor signaling in breast cancer cells. Clin Cancer Res *12*, 2004-2013.

Battaglia, S., Maguire, O., and Campbell, M. J. (2010). Transcription factor co-repressors in cancer biology: roles and targeting. Int J Cancer *126*, 2511-2519.

Baumann, C. T., Lim, C. S., and Hager, G. L. (1999). Intracellular localization and trafficking of steroid receptors. Cell Biochem Biophys *31*, 119-127.
Beato, M., Chalepakis, G., Schauer, M., and Slater, E. P. (1989). DNA regulatory elements for steroid hormones. J Steroid Biochem *32*, 737-747.
Beck, C. A., Weigel, N. L., Moyer, M. L., Nordeen, S. K., and Edwards, D. P. (1993). The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signaling pathways. Proc Natl Acad Sci U S A *90*, 4441-4445.
Beck, C. A., Zhang, Y., Altmann, M., Weigel, N. L., and Edwards, D. P. (1996a). Stoichiometry and site-specific phosphorylation of human progesterone receptor in native target cells and in the baculovirus expression system. J Biol Chem *271*, 19546-19555.
Beck, C. A., Zhang, Y., Weigel, N. L., and Edwards, D. P. (1996b). Two types of anti-progestins have distinct effects on site-specific phosphorylation of human progesterone receptor. J Biol Chem *271*, 1209-1217.
Beleut, M., Rajaram, R. D., Caikovski, M., Ayyanan, A., Germano, D., Choi, Y., Schneider, P., and Brisken, C. (2010). Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland. Proc Natl Acad Sci U S A *107*, 2989-2994.
Benecke, A., Chambon, P., and Gronemeyer, H. (2000). Synergy between estrogen receptor alpha activation functions AF1 and AF2 mediated by transcription intermediary factor TIF2. EMBO Rep *1*, 151-157.
Bern, H. A., Mills, K. T., and Jones, L. A. (1983). Critical period for neonatal estrogen exposure in occurrence of mammary gland abnormalities in adult mice. Proc Soc Exp Biol Med *172*, 239-242.
Bernstein, L. (2002). Epidemiology of endocrine-related risk factors for breast cancer. J Mammary Gland Biol Neoplasia *7*, 3-15.
Bhaumik, S. R., Smith, E., and Shilatifard, A. (2007). Covalent modifications of histones during development and disease pathogenesis. Nat Struct Mol Biol *14*, 1008-1016.
Bledsoe, R. K., Montana, V. G., Stanley, T. B., Delves, C. J., Apolito, C. J., McKee, D. D., Consler, T. G., Parks, D. J., Stewart, E. L., Willson, T. M*., et al.* (2002). Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. Cell *110*, 93-105.
Boonyaratanakornkit, V., McGowan, E., Sherman, L., Mancini, M. A., Cheskis, B. J., and Edwards, D. P. (2007). The role of extranuclear signaling actions of progesterone receptor in mediating progesterone regulation of gene expression and the cell cycle. Mol Endocrinol *21*, 359-375.
Boonyaratanakornkit, V., Scott, M. P., Ribon, V., Sherman, L., Anderson, S. M., Maller, J. L., Miller, W. T., and Edwards, D. P. (2001). Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol Cell *8*, 269-280.
Bos, P. D., Zhang, X. H., Nadal, C., Shu, W., Gomis, R. R., Nguyen, D. X., Minn, A. J., van de Vijver, M. J., Gerald, W. L., Foekens, J. A., and Massague, J. (2009). Genes that mediate breast cancer metastasis to the brain. Nature *459*, 1005-1009.
Brayman, M. J., Julian, J., Mulac-Jericevic, B., Conneely, O. M., Edwards, D. P., and Carson, D. D. (2006). Progesterone receptor isoforms A and B differentially regulate MUC1 expression in uterine epithelial cells. Mol Endocrinol *20*, 2278-2291.
Brisken, C., and O'Malley, B. (2010). Hormone action in the mammary gland. Cold Spring Harb Perspect Biol *2*, a003178.
Brisken, C., Park, S., Vass, T., Lydon, J. P., O'Malley, B. W., and Weinberg, R. A. (1998). A paracrine role for the epithelial progesterone receptor in mammary gland development. Proc Natl Acad Sci U S A *95*, 5076-5081.
Bungay, P. J., Tweedy, S., Howe, D. C., Gibson, K. R., Jones, H. M., and Mount, N. M. (2011). Preclinical and Clinical Pharmacokinetics of PF-02413873, a Nonsteroidal Progesterone Receptor Antagonist. Drug Metab Dispos *39*, 1396-1405.
Burrows, H., and Hoch-Ligeti, C. (1946). Effect of progesterone on the development of mammary cancer in C3H mice. Cancer Res *6*, 608.
Busia, L., Faus, H., Hoffmann, J., and Haendler, B. (2011). The antiprogestin Lonaprisan inhibits breast cancer cell proliferation by inducing p21 expression. Mol Cell Endocrinol *333*, 37-46.
Cadepond, F., Ulmann, A., and Baulieu, E. E. (1997). RU486 (mifepristone): mechanisms of action and clinical uses. Annu Rev Med *48*, 129-156.
Carnevale, R. P., Proietti, C. J., Salatino, M., Urtreger, A., Peluffo, G., Edwards, D. P., Boonyaratanakornkit, V., Charreau, E. H., Bal de Kier Joffe, E., Schillaci, R., and Elizalde, P. V. (2007). Progestin effects on breast cancer cell proliferation, proteases activation, and in vivo development of metastatic phenotype all depend on progesterone receptor capacity to activate cytoplasmic signaling pathways. Mol Endocrinol *21*, 1335-1358.
Catelli, M. G., Binart, N., Feramisco, J. R., and Helfman, D. M. (1985). Cloning of the chick hsp 90 cDNA in expression vector. Nucleic Acids Res *13*, 6035-6047.

Chabbert-Buffet, N., Meduri, G., Bouchard, P., and Spitz, I. M. (2005). Selective progesterone receptor modulators and progesterone antagonists: mechanisms of action and clinical applications. Hum Reprod Update *11*, 293-307.

Chang, A. C., Jellinek, D. A., and Reddel, R. R. (2003). Mammalian stanniocalcins and cancer. Endocr Relat Cancer *10*, 359-373.

Chauchereau, A., Amazit, L., Quesne, M., Guiochon-Mantel, A., and Milgrom, E. (2003). Sumoylation of the progesterone receptor and of the steroid receptor coactivator SRC-1. J Biol Chem *278*, 12335-12343.

Check, J. H., Dix, E., Cohen, R., Check, D., and Wilson, C. (2010a). Efficacy of the progesterone receptor antagonist mifepristone for palliative therapy of patients with a variety of advanced cancer types. Anticancer Res *30*, 623-628.

Check, J. H., Dix, E., Wilson, C., and Check, D. (2010b). Progesterone receptor antagonist therapy has therapeutic potential even in cancer restricted to males as evidenced from murine testicular and prostate cancer studies. Anticancer Res *30*, 4921-4923.

Check, J. H., Sansoucie, L., Chern, J., and Dix, E. (2010c). Mifepristone treatment improves length and quality of survival of mice with spontaneous lung cancer. Anticancer Res *30*, 119-122.

Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S. M., Schurter, B. T., Aswad, D. W., and Stallcup, M. R. (1999a). Regulation of transcription by a protein methyltransferase. Science *284*, 2174-2177.

Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997). Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell *90*, 569-580.

Chen, H., Lin, R. J., Xie, W., Wilpitz, D., and Evans, R. M. (1999b). Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. Cell *98*, 675-686.

Chen, J. D., and Evans, R. M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors [see comments]. Nature *377*, 454-457.

Chen, Y. H., Kim, J. H., and Stallcup, M. R. (2005). GAC63, a GRIP1-dependent nuclear receptor coactivator. Mol Cell Biol *25*, 5965-5972.

Clarke, R. B., Howell, A., Potten, C. S., and Anderson, E. (1997). Dissociation between steroid receptor expression and cell proliferation in the human breast. Cancer Res *57*, 4987-4991.

Clemm, D. L., Sherman, L., Boonyaratanakornkit, V., Schrader, W. T., Weigel, N. L., and Edwards, D. P. (2000). Differential hormone-dependent phosphorylation of progesterone receptor A and B forms revealed by a phosphoserine site-specific monoclonal antibody. Mol Endocrinol *14*, 52-65.

Condon, J. C., Hardy, D. B., Kovaric, K., and Mendelson, C. R. (2006). Up-regulation of the progesterone receptor (PR)-C isoform in laboring myometrium by activation of nuclear factor-kappaB may contribute to the onset of labor through inhibition of PR function. Mol Endocrinol *20*, 764-775.

Connaghan-Jones, K. D., Heneghan, A. F., Miura, M. T., and Bain, D. L. (2008). Thermodynamic dissection of progesterone receptor interactions at the mouse mammary tumor virus promoter: monomer binding and strong cooperativity dominate the assembly reaction. J Mol Biol *377*, 1144-1160.

Conneely, O. M., Jericevic, B. M., and Lydon, J. P. (2003). Progesterone receptors in mammary gland development and tumorigenesis. J Mammary Gland Biol Neoplasia *8*, 205-214.

Conneely, O. M., Mulac-Jericevic, B., Lydon, J. P., and De Mayo, F. J. (2001). Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. Mol Cell Endocrinol *179*, 97-103.

Corner, G. W., and Allen, W. M. (1929). Physiology of the corpus luteum. II. Production of a special uterine reaction (progestational proliferation) by extracts of the corpus luteum. Am J Physiol *88*, 326-339.

Crawford, P. A., Polish, J. A., Ganpule, G., and Sadovsky, Y. (1997). The activation function-2 hexamer of steroidogenic factor-1 is required, but not sufficient for potentiation by SRC-1. Mol Endocrinol *11*, 1626-1635.

Dace, A., Zhao, L., Park, K. S., Furuno, T., Takamura, N., Nakanishi, M., West, B. L., Hanover, J. A., and Cheng, S. (2000). Hormone binding induces rapid proteasome-mediated degradation of thyroid hormone receptors. Proc Natl Acad Sci U S A *97*, 8985-8990.

Daniel, A. R., Faivre, E. J., and Lange, C. A. (2007a). Phosphorylation-dependent antagonism of sumoylation derepresses progesterone receptor action in breast cancer cells. Mol Endocrinol *21*, 2890-2906.

Daniel, A. R., Gaviglio, A. L., Czaplicki, L. M., Hillard, C. J., Housa, D., and Lange, C. A. (2010). The progesterone receptor hinge region regulates the kinetics of transcriptional responses through acetylation, phosphorylation, and nuclear retention. Mol Endocrinol *24*, 2126-2138.

Daniel, A. R., and Lange, C. A. (2009). Protein kinases mediate ligand-independent derepression of sumoylated progesterone receptors in breast cancer cells. Proc Natl Acad Sci U S A *106*, 14287-14292.

Daniel, A. R., Qiu, M., Faivre, E. J., Ostrander, J. H., Skildum, A., and Lange, C. A. (2007b). Linkage of progestin and epidermal growth factor signaling: phosphorylation of progesterone receptors mediates transcriptional hypersensitivity and increased ligand-independent breast cancer cell growth. Steroids *72*, 188-201.

Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998). Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev *12*, 3343-3356.

Dauvois, S., White, R., and Parker, M. G. (1993). The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. J Cell Sci *106 (Pt 4)*, 1377-1388.

DeFranco, D. B., Qi, M., Borror, K. C., Garabedian, M. J., and Brautigan, D. L. (1991). Protein phosphatase types 1 and/or 2A regulate nucleocytoplasmic shuttling of glucocorticoid receptors. Mol Endocrinol *5*, 1215-1228.

Dennis, A. P., Lonard, D. M., Nawaz, Z., and O'Malley, B. W. (2005). Inhibition of the 26S proteasome blocks progesterone receptor-dependent transcription through failed recruitment of RNA polymerase II. J Steroid Biochem Mol Biol *94*, 337-346.

Deroo, B. J., Rentsch, C., Sampath, S., Young, J., DeFranco, D. B., and Archer, T. K. (2002). Proteasomal inhibition enhances glucocorticoid receptor transactivation and alters its subnuclear trafficking. Mol Cell Biol *22*, 4113-4123.

Desruisseau, S., Palmari, J., Giusti, C., Romain, S., Martin, P. M., and Berthois, Y. (2004). Clinical relevance of amphiregulin and VEGF in primary breast cancers. Int J Cancer *111*, 733-740.

Dong, X., Challis, J. R., and Lye, S. J. (2004). Intramolecular interactions between the AF3 domain and the C-terminus of the human progesterone receptor are mediated through two LXXLL motifs. J Mol Endocrinol *32*, 843-857.

Dressing, G. E., Hagan, C. R., Knutson, T. P., Daniel, A. R., and Lange, C. A. (2009). Progesterone receptors act as sensors for mitogenic protein kinases in breast cancer models. Endocr Relat Cancer *16*, 351-361.

Duffy, D. M., Wells, T. R., Haluska, G. J., and Stouffer, R. L. (1997). The ratio of progesterone receptor isoforms changes in the monkey corpus luteum during the luteal phase of the menstrual cycle. Biol Reprod *57*, 693-699.

Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., and Mark, M. (2000). Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. Development *127*, 4277-4291.

Epping, M. T., Wang, L., Edel, M. J., Carlee, L., Hernandez, M., and Bernards, R. (2005). The human tumor antigen PRAME is a dominant repressor of retinoic acid receptor signaling. Cell *122*, 835-847.

Fagart, J., Hillisch, A., Huyet, J., Barfacker, L., Fay, M., Pleiss, U., Pook, E., Schafer, S., Rafestin-Oblin, M. E., and Kolkhof, P. (2010). A new mode of mineralocorticoid receptor antagonism by a potent and selective nonsteroidal molecule. J Biol Chem *285*, 29932-29940.

Fagart, J., Huyet, J., Pinon, G. M., Rochel, M., Mayer, C., and Rafestin-Oblin, M. E. (2005). Crystal structure of a mutant mineralocorticoid receptor responsible for hypertension. Nat Struct Mol Biol *12*, 554-555.

Fagart, J., Wurtz, J. M., Souque, A., Hellal-Levy, C., Moras, D., and Rafestin-Oblin, M. E. (1998). Antagonism in the human mineralocorticoid receptor. Embo J *17*, 3317-3325.

Faivre, E., Skildum, A., Pierson-Mullany, L., and Lange, C. A. (2005). Integration of progesterone receptor mediated rapid signaling and nuclear actions in breast cancer cell models: role of mitogen-activated protein kinases and cell cycle regulators. Steroids *70*, 418-426.

Faivre, E. J., Daniel, A. R., Hillard, C. J., and Lange, C. A. (2008). Progesterone receptor rapid signaling mediates serine 345 phosphorylation and tethering to specificity protein 1 transcription factors. Mol Endocrinol *22*, 823-837.

Faivre, E. J., and Lange, C. A. (2007). Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. Mol Cell Biol *27*, 466-480.

Faus, H., and Haendler, B. (2006). Post-translational modifications of steroid receptors. Biomed Pharmacother *60*, 520-528.

Fels, E., and Slotta, K. H. (1931). U¨ ber das Hormon des Corpus luteum. In: Greenwood AW, editor. Proceedings of the Second International Congress for Sex Research. Edinburgh, London: Oliver and Boyd., 361-366.

Fernandes, I., Bastien, Y., Wai, T., Nygard, K., Lin, R., Cormier, O., Lee, H. S., Eng, F., Bertos, N. R., Pelletier, N.*, et al.* (2003). Ligand-dependent nuclear receptor corepressor LCoR functions by histone deacetylase-dependent and -independent mechanisms. Mol Cell *11*, 139-150.

Fraenkel, L. (1903). Die Function des Corpus luteum. Arch f Gyndk, 438.

Fu, M., Rao, M., Wang, C., Sakamaki, T., Wang, J., Di Vizio, D., Zhang, X., Albanese, C., Balk, S., Chang, C.*, et al.* (2003). Acetylation of androgen receptor enhances coactivator binding and promotes prostate cancer cell growth. Mol Cell Biol *23*, 8563-8575.

Garcia, T., Benhamou, B., Gofflo, D., Vergezac, A., Philibert, D., Chambon, P., and Gronemeyer, H. (1992). Switching agonistic, antagonistic, and mixed transcriptional responses to 11 beta-substituted progestins by mutation of the progesterone receptor. Mol Endocrinol *6*, 2071-2078.

Georgiakaki, M., Chabbert-Buffet, N., Dasen, B., Meduri, G., Wenk, S., Rajhi, L., Amazit, L., Chauchereau, A., Burger, C. W., Blok, L. J.*, et al.* (2006). Ligand-controlled Interaction of HBO1 with the N-terminal Transactivating Domain of Progesterone Receptor Induces SRC-1-dependent Co-activation of Transcription. Mol Endocrinol *20*, 2122-2140.

Giangrande, P. H., Kimbrel, E. A., Edwards, D. P., and McDonnell, D. P. (2000). The opposing transcriptional activities of the two isoforms of the human progesterone receptor are due to differential cofactor binding. Mol Cell Biol *20*, 3102-3115.

Giangrande, P. H., and McDonnell, D. P. (1999). The A and B isoforms of the human progesterone receptor: two functionally different transcription factors encoded by a single gene. Recent Prog Horm Res *54*, 291-313; discussion 313-294.

Gianni, M., Parrella, E., Raska, I., Jr., Gaillard, E., Nigro, E. A., Gaudon, C., Garattini, E., and Rochette-Egly, C. (2006). P38MAPK-dependent phosphorylation and degradation of SRC-3/AIB1 and RARalpha-mediated transcription. Embo J *25*, 739-751.

Girault, I., Lerebours, F., Amarir, S., Tozlu, S., Tubiana-Hulin, M., Lidereau, R., and Bieche, I. (2003). Expression analysis of estrogen receptor alpha coregulators in breast carcinoma: evidence that NCOR1 expression is predictive of the response to tamoxifen. Clin Cancer Res *9*, 1259-1266.

Glass, C. K., and Rosenfeld, M. G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev *14*, 121-141.

Goldman, S., and Shalev, E. (2006). Difference in progesterone-receptor isoforms ratio between early and late first-trimester human trophoblast is associated with differential cell invasion and matrix metalloproteinase 2 expression. Biol Reprod *74*, 13-22.

Gonzalez-Suarez, E., Jacob, A. P., Jones, J., Miller, R., Roudier-Meyer, M. P., Erwert, R., Pinkas, J., Branstetter, D., and Dougall, W. C. (2010). RANK ligand mediates progestin-induced mammary epithelial proliferation and carcinogenesis. Nature *468*, 103-107.

Graham, J. D., and Clarke, C. L. (1997). Physiological action of progesterone in target tissues. Endocr Rev *18*, 502-519.

Graham, J. D., Roman, S. D., McGowan, E., Sutherland, R. L., and Clarke, C. L. (1995a). Preferential stimulation of human progesterone receptor B expression by estrogen in T-47D human breast cancer cells. J Biol Chem *270*, 30693-30700.

Graham, J. D., Yager, M. L., Hill, H. D., Byth, K., O'Neill, G. M., and Clarke, C. L. (2005). Altered progesterone receptor isoform expression remodels progestin responsiveness of breast cancer cells. Mol Endocrinol *19*, 2713-2735.

Graham, J. D., Yeates, C., Balleine, R. L., Harvey, S. S., Milliken, J. S., Bilous, A. M., and Clarke, C. L. (1995b). Characterization of progesterone receptor A and B expression in human breast cancer. Cancer Res *55*, 5063-5068.

Grimm, S. L., Seagroves, T. N., Kabotyanski, E. B., Hovey, R. C., Vonderhaar, B. K., Lydon, J. P., Miyoshi, K., Hennighausen, L., Ormandy, C. J., Lee, A. V.*, et al.* (2002). Disruption of steroid and prolactin receptor patterning in the mammary gland correlates with a block in lobuloalveolar development. Mol Endocrinol *16*, 2675-2691.

Guiochon-Mantel, A., Delabre, K., Lescop, P., Perrot-Applanat, M., and Milgrom, E. (1994). Cytoplasmic-nuclear trafficking of progesterone receptor. In vivo study of the mechanism of action of antiprogestins. Biochem Pharmacol *47*, 21-24.

Guiochon-Mantel, A., Lescop, P., Christin-Maitre, S., Loosfelt, H., Perrot-Applanat, M., and Milgrom, E. (1991). Nucleocytoplasmic shuttling of the progesterone receptor. Embo J *10*, 3851-3859.

Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M., and Milgrom, E. (1989). Mechanisms of nuclear localization of the progesterone receptor: evidence for interaction between monomers. Cell *57*, 1147-1154.

Hager, G. L. (1988). MMTV as a model for gene expression in mammary tissue. Cancer Treat Res *40*, 267-281.

Halachmi, S., Marden, E., Martin, G., MacKay, H., Abbondanza, C., and Brown, M. (1994). Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. Science *264*, 1455-1458.

Han, S. J., DeMayo, F. J., Xu, J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (2006). Steroid receptor coactivator (SRC)-1 and SRC-3 differentially modulate tissue-specific activation functions of the progesterone receptor. Mol Endocrinol *20*, 45-55.

Han, S. J., Jeong, J., Demayo, F. J., Xu, J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (2005). Dynamic cell type specificity of SRC-1 coactivator in modulating uterine progesterone receptor function in mice. Mol Cell Biol *25*, 8150-8165.

Han, S. J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (2007). Distinct temporal and spatial activities of RU486 on progesterone receptor function in reproductive organs of ovariectomized mice. Endocrinology *148*, 2471-2486.

Hard, T., Dahlman, K., Carlstedt-Duke, J., Gustafsson, J. A., and Rigler, R. (1990). Cooperativity and specificity in the interactions between DNA and the glucocorticoid receptor DNA-binding domain. Biochemistry *29*, 5358-5364.

Haslam, S. Z. (1989). The ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones. Endocrinology *125*, 2766-2772.

Haslam, S. Z., and Shyamala, G. (1981). Relative distribution of estrogen and progesterone receptors among the epithelial, adipose, and connective tissue components of the normal mammary gland. Endocrinology *108*, 825-830.

Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature *387*, 733-736.

Heneghan, A. F., Connaghan-Jones, K. D., Miura, M. T., and Bain, D. L. (2007). Coactivator assembly at the promoter: efficient recruitment of SRC2 is coupled to cooperative DNA binding by the progesterone receptor. Biochemistry *46*, 11023-11032.

Hermanson, O., Glass, C. K., and Rosenfeld, M. G. (2002). Nuclear receptor coregulators: multiple modes of modification. Trends Endocrinol Metab *13*, 55-60.

Herrmann, W., Wyss, R., Riondel, A., Philibert, D., Teutsch, G., Sakiz, E., and Baulieu, E. E. (1982). [The effects of an antiprogesterone steroid in women: interruption of the menstrual cycle and of early pregnancy]. C R Seances Acad Sci III *294*, 933-938.

Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G., and Evans, R. M. (1985). Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature *318*, 635-641.

Holmstrom, S. R., Chupreta, S., So, A. Y., and Iniguez-Lluhi, J. A. (2008). SUMO-mediated inhibition of glucocorticoid receptor synergistic activity depends on stable assembly at the promoter but not on DAXX. Mol Endocrinol *22*, 2061-2075.

Hong, H., Kohli, K., Garabedian, M. J., and Stallcup, M. R. (1997). GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. Mol Cell Biol *17*, 2735-2744.

Hong, H., Kohli, K., Trivedi, A., Johnson, D. L., and Stallcup, M. R. (1996). GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. Proc Natl Acad Sci U S A *93*, 4948-4952.

Hopp, T. A., Weiss, H. L., Hilsenbeck, S. G., Cui, Y., Allred, D. C., Horwitz, K. B., and Fuqua, S. A. (2004). Breast cancer patients with progesterone receptor PR-A-rich tumors have poorer disease-free survival rates. Clin Cancer Res *10*, 2751-2760.

Hoppe, T. (2005). Multiubiquitylation by E4 enzymes: 'one size' doesn't fit all. Trends Biochem Sci *30*, 183-187.

Horie-Inoue, K., Takayama, K., Bono, H. U., Ouchi, Y., Okazaki, Y., and Inoue, S. (2006). Identification of novel steroid target genes through the combination of bioinformatics and functional analysis of hormone response elements. Biochem Biophys Res Commun *339*, 99-106.

Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and et al. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature *377*, 397-404.

Horwitz, K. B., Dye, W. W., Harrell, J. C., Kabos, P., and Sartorius, C. A. (2008). Rare steroid receptor-negative basal-like tumorigenic cells in luminal subtype human breast cancer xenografts. Proc Natl Acad Sci U S A *105*, 5774-5779.

Horwitz, K. B., and Sartorius, C. A. (2008). Progestins in hormone replacement therapies reactivate cancer stem cells in women with preexisting breast cancers: a hypothesis. J Clin Endocrinol Metab *93*, 3295-3298.

Hovey, R. C., Trott, J. F., Ginsburg, E., Goldhar, A., Sasaki, M. M., Fountain, S. J., Sundararajan, K., and Vonderhaar, B. K. (2001). Transcriptional and spatiotemporal regulation of prolactin receptor mRNA and cooperativity with progesterone receptor function during ductal branch growth in the mammary gland. Dev Dyn *222*, 192-205.

Hovland, A. R., Powell, R. L., Takimoto, G. S., Tung, L., and Horwitz, K. B. (1998). An N-terminal inhibitory function, IF, suppresses transcription by the A-isoform but not the B-isoform of human progesterone receptors. J Biol Chem *273*, 5455-5460.

Howell, A., and Evans, G. D. (2011). Hormone replacement therapy and breast cancer. Recent Results Cancer Res *188*, 115-124.

Hu, X., and Lazar, M. A. (1999). The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature *402*, 93-96.
Ikeda, M., Kawaguchi, A., Takeshita, A., Chin, W. W., Endo, T., and Onaya, T. (1999). CBP-dependent and independent enhancing activity of steroid receptor coactivator-1 in thyroid hormone receptor-mediated transactivation. Mol Cell Endocrinol *147*, 103-112.
Ikonen, T., Palvimo, J. J., and Janne, O. A. (1997). Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. J Biol Chem *272*, 29821-29828.
Imhof, M. O., and McDonnell, D. P. (1996). Yeast RSP5 and its human homolog hRPF1 potentiate hormone-dependent activation of transcription by human progesterone and glucocorticoid receptors. Mol Cell Biol *16*, 2594-2605.
Jacobsen, B. M., Jambal, P., Schittone, S. A., and Horwitz, K. B. (2009). ALU repeats in promoters are position-dependent co-response elements (coRE) that enhance or repress transcription by dimeric and monomeric progesterone receptors. Mol Endocrinol *23*, 989-1000.
Jacobsen, B. M., Richer, J. K., Sartorius, C. A., and Horwitz, K. B. (2003). Expression profiling of human breast cancers and gene regulation by progesterone receptors. J Mammary Gland Biol Neoplasia *8*, 257-268.
Jacobsen, B. M., Richer, J. K., Schittone, S. A., and Horwitz, K. B. (2002). New human breast cancer cells to study progesterone receptor isoform ratio effects and ligand-independent gene regulation. J Biol Chem *277*, 27793-27800.
Jacobsen, B. M., Schittone, S. A., Richer, J. K., and Horwitz, K. B. (2005). Progesterone-independent effects of human progesterone receptors (PRs) in estrogen receptor-positive breast cancer: PR isoform-specific gene regulation and tumor biology. Mol Endocrinol *19*, 574-587.
Jantzen, H. M., Strahle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R., and Schutz, G. (1987). Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell *49*, 29-38.
Jensen, E. V., and Jacobsen, H. I. (1962). Basic guides to the mechanism of estrogen action. Recent Prog Horm Res *18*, 387-414.
Jenster, G., Spencer, T. E., Burcin, M. M., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997). Steroid receptor induction of gene transcription: a two-step model. Proc Natl Acad Sci U S A *94*, 7879-7884.
Joshi, P. A., Jackson, H. W., Beristain, A. G., Di Grappa, M. A., Mote, P., Clarke, C., Stingl, J., Waterhouse, P. D., and Khokha, R. (2010). Progesterone induces adult mammary stem cell expansion. Nature, 1-10.
Kahmann, S., Vassen, L., and Klein-Hitpass, L. (1998). Synergistic enhancement of PRB-mediated RU486 and R5020 agonist activities through cyclic adenosine 3',5'-monophosphate represents a delayed primary response. Mol Endocrinol *12*, 278-289.
Kalkhoven, E., Wissink, S., van der Saag, P. T., and van der Burg, B. (1996). Negative interaction between the RelA(p65) subunit of NF-kappaB and the progesterone receptor. J Biol Chem *271*, 6217-6224.
Kang, Z., Pirskanen, A., Janne, O. A., and Palvimo, J. J. (2002). Involvement of proteasome in the dynamic assembly of the androgen receptor transcription complex. J Biol Chem *277*, 48366-48371.
Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., and Chambon, P. (1990). Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. Embo J *9*, 1603-1614.
Kemppainen, J. A., Lane, M. V., Sar, M., and Wilson, E. M. (1992). Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones. J Biol Chem *267*, 968-974.
Kettel, L. M., Murphy, A. A., Morales, A. J., and Yen, S. S. (1998). Preliminary report on the treatment of endometriosis with low-dose mifepristone (RU 486). Am J Obstet Gynecol *178*, 1151-1156.
Kettel, L. M., Murphy, A. A., Mortola, J. F., Liu, J. H., Ulmann, A., and Yen, S. S. (1991). Endocrine responses to long-term administration of the antiprogesterone RU486 in patients with pelvic endometriosis. Fertil Steril *56*, 402-407.
Khan, J. A., Amazit, L., Bellance, C., Guiochon-Mantel, A., Lombes, M., and Loosfelt, H. (2011). p38 and p42/44 MAPKs Differentially Regulate Progesterone Receptor A and B Isoform Stabilization. Mol Endocrinol *doi:10.1210/me.2011-1042*.
Khanim, F. L., Gommersall, L. M., Wood, V. H., Smith, K. L., Montalvo, L., O'Neill, L. P., Xu, Y., Peehl, D. M., Stewart, P. M., Turner, B. M., and Campbell, M. J. (2004). Altered SMRT levels disrupt vitamin D3 receptor signalling in prostate cancer cells. Oncogene *23*, 6712-6725.
Kim, H. J., Kim, J. H., and Lee, J. W. (1998). Steroid receptor coactivator-1 interacts with serum response factor and coactivates serum response element-mediated transactivations. J Biol Chem *273*, 28564-28567.

Kim, J. H., Li, H., and Stallcup, M. R. (2003). CoCoA, a nuclear receptor coactivator which acts through an N-terminal activation domain of p160 coactivators. Mol Cell *12*, 1537-1549.
Kim, J. Y., Son, Y. L., and Lee, Y. C. (2009). Involvement of SMRT corepressor in transcriptional repression by the vitamin D receptor. Mol Endocrinol *23*, 251-264.
Kim, M. Y., Woo, E. M., Chong, Y. T., Homenko, D. R., and Kraus, W. L. (2006). Acetylation of estrogen receptor alpha by p300 at lysines 266 and 268 enhances the deoxyribonucleic acid binding and transactivation activities of the receptor. Mol Endocrinol *20*, 1479-1493.
Kino, T., and Chrousos, G. P. (2011). Acetylation-mediated epigenetic regulation of glucocorticoid receptor activity: circadian rhythm-associated alterations of glucocorticoid actions in target tissues. Mol Cell Endocrinol *336*, 23-30.
Klein-Hitpass, L., Tsai, S. Y., Weigel, N. L., Allan, G. F., Riley, D., Rodriguez, R., Schrader, W. T., Tsai, M. J., and O'Malley, B. W. (1990). The progesterone receptor stimulates cell-free transcription by enhancing the formation of a stable preinitiation complex. Cell *60*, 247-257.
Klijn, J. G., Setyono-Han, B., and Foekens, J. A. (2000). Progesterone antagonists and progesterone receptor modulators in the treatment of breast cancer. Steroids *65*, 825-830.
Knotts, T. A., Orkiszewski, R. S., Cook, R. G., Edwards, D. P., and Weigel, N. L. (2001). Identification of a phosphorylation site in the hinge region of the human progesterone receptor and additional amino-terminal phosphorylation sites. J Biol Chem *276*, 8475-8483.
Kouzarides, T. (2007). Chromatin modifications and their function. Cell *128*, 693-705.
Lanari, C., Lamb, C. A., Fabris, V. T., Helguero, L. A., Soldati, R., Bottino, M. C., Giulianelli, S., Cerliani, J. P., Wargon, V., and Molinolo, A. (2009). The MPA mouse breast cancer model: evidence for a role of progesterone receptors in breast cancer. Endocr Relat Cancer *16*, 333-350.
Lange, C. A. (2004). Making sense of cross-talk between steroid hormone receptors and intracellular signaling pathways: who will have the last word? Mol Endocrinol *18*, 269-278.
Lange, C. A., Shen, T., and Horwitz, K. B. (2000). Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci U S A *97*, 1032-1037.
Lanz, R. B., McKenna, N. J., Onate, S. A., Albrecht, U., Wong, J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1999). A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex. Cell *97*, 17-27.
Le Romancer, M., Poulard, C., Cohen, P., Sentis, S., Renoir, J. M., and Corbo, L. (2011). Cracking the Estrogen Receptor's Posttranslational Code in Breast Tumors. Endocr Rev.
Lee, S. K., Kim, H. J., Kim, J. W., and Lee, J. W. (1999). Steroid receptor coactivator-1 and its family members differentially regulate transactivation by the tumor suppressor protein p53. Mol Endocrinol *13*, 1924-1933.
Lee, S. K., Kim, H. J., Na, S. Y., Kim, T. S., Choi, H. S., Im, S. Y., and Lee, J. W. (1998). Steroid receptor coactivator-1 coactivates activating protein-1-mediated transactivations through interaction with the c-Jun and c-Fos subunits. J Biol Chem *273*, 16651-16654.
Lee, Y. H., Campbell, H. D., and Stallcup, M. R. (2004). Developmentally essential protein flightless I is a nuclear receptor coactivator with actin binding activity. Mol Cell Biol *24*, 2103-2117.
Lefebvre, P., Berard, D. S., Cordingley, M. G., and Hager, G. L. (1991). Two regions of the mouse mammary tumor virus long terminal repeat regulate the activity of its promoter in mammary cell lines. Mol Cell Biol *11*, 2529-2537.
Leo, J. C., Wang, S. M., Guo, C. H., Aw, S. E., Zhao, Y., Li, J. M., Hui, K. M., and Lin, V. C. (2005). Gene regulation profile reveals consistent anticancer properties of progesterone in hormone-independent breast cancer cells transfected with progesterone receptor. Int J Cancer *117*, 561-568.
Li, H., Gomes, P. J., and Chen, J. D. (1997). RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. Proc Natl Acad Sci U S A *94*, 8479-8484.
Li, Q., Kannan, A., DeMayo, F. J., Lydon, J. P., Cooke, P. S., Yamagishi, H., Srivastava, D., Bagchi, M. K., and Bagchi, I. C. (2011). The antiproliferative action of progesterone in uterine epithelium is mediated by Hand2. Science *331*, 912-916.
Li, X., Lonard, D. M., and O'Malley, B. W. (2004). A contemporary understanding of progesterone receptor function. Mech Ageing Dev *125*, 669-678.
Liang, Y., Besch-Williford, C., Brekken, R. A., and Hyder, S. M. (2007). Progestin-dependent progression of human breast tumor xenografts: a novel model for evaluating antitumor therapeutics. Cancer Res *67*, 9929-9936.
Lin, V. C., Ng, E. H., Aw, S. E., Tan, M. G., Ng, E. H., Chan, V. S., and Ho, G. H. (1999). Progestins inhibit the growth of MDA-MB-231 cells transfected with progesterone receptor complementary DNA. Clin Cancer Res *5*, 395-403.
Lipford, J. R., and Deshaies, R. J. (2003). Diverse roles for ubiquitin-dependent proteolysis in transcriptional activation. Nat Cell Biol *5*, 845-850.

Liu, Z., Auboeuf, D., Wong, J., Chen, J. D., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (2002). Coactivator/corepressor ratios modulate PR-mediated transcription by the selective receptor modulator RU486. Proc Natl Acad Sci U S A *99*, 7940-7944.

Lonard, D. M., Nawaz, Z., Smith, C. L., and O'Malley, B. W. (2000). The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. Mol Cell *5*, 939-948.

Loosfelt, H., Atger, M., Misrahi, M., Guiochon-Mantel, A., Meriel, C., Logeat, F., Benarous, R., and Milgrom, E. (1986). Cloning and sequence analysis of rabbit progesterone-receptor complementary DNA. Proc Natl Acad Sci U S A *83*, 9045-9049.

Lu, N. Z., Wardell, S. E., Burnstein, K. L., Defranco, D., Fuller, P. J., Giguere, V., Hochberg, R. B., McKay, L., Renoir, J. M., Weigel, N. L.*, et al.* (2006). International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol Rev *58*, 782-797.

Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R., and Sigler, P. B. (1991). Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature *352*, 497-505.

Luo, X., Yin, P., Coon, V. J., Cheng, Y. H., Wiehle, R. D., and Bulun, S. E. (2010). The selective progesterone receptor modulator CDB4124 inhibits proliferation and induces apoptosis in uterine leiomyoma cells. Fertil Steril *93*, 2668-2673.

Lydon, J. P., DeMayo, F. J., Funk, C. R., Mani, S. K., Hughes, A. R., Montgomery, C. A., Jr., Shyamala, G., Conneely, O. M., and O'Malley, B. W. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev *9*, 2266-2278.

Madauss, K. P., Deng, S. J., Austin, R. J., Lambert, M. H., McLay, I., Pritchard, J., Short, S. A., Stewart, E. L., Uings, I. J., and Williams, S. P. (2004). Progesterone receptor ligand binding pocket flexibility: crystal structures of the norethindrone and mometasone furoate complexes. J Med Chem *47*, 3381-3387.

Madauss, K. P., Grygielko, E. T., Deng, S. J., Sulpizio, A. C., Stanley, T. B., Wu, C., Short, S. A., Thompson, S. K., Stewart, E. L., Laping, N. J.*, et al.* (2007). A structural and in vitro characterization of asoprisnil: a selective progesterone receptor modulator. Mol Endocrinol *21*, 1066-1081.

Mahajan, D. K., and London, S. N. (1997). Mifepristone (RU486): a review. Fertil Steril *68*, 967-976.

Mangal, R. K., Wiehle, R. D., Poindexter, A. N., 3rd, and Weigel, N. L. (1997). Differential expression of uterine progesterone receptor forms A and B during the menstrual cycle. J Steroid Biochem Mol Biol *63*, 195-202.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995). The nuclear receptor superfamily: the second decade. Cell *83*, 835-839.

Mani, S. K., Reyna, A. M., Chen, J. Z., Mulac-Jericevic, B., and Conneely, O. M. (2006). Differential response of progesterone receptor isoforms in hormone-dependent and -independent facilitation of female sexual receptivity. Mol Endocrinol *20*, 1322-1332.

Masuyama, H., and MacDonald, P. N. (1998). Proteasome-mediated degradation of the vitamin D receptor (VDR) and a putative role for SUG1 interaction with the AF-2 domain of VDR. J Cell Biochem *71*, 429-440.

McEwan, I. J. (2009). Nuclear receptors: one big family. Methods Mol Biol *505*, 3-18.

McGowan, E. M., Saad, S., Bendall, L. J., Bradstock, K. F., and Clarke, C. L. (2004). Effect of progesterone receptor a predominance on breast cancer cell migration into bone marrow fibroblasts. Breast Cancer Res Treat *83*, 211-220.

McInerney, E. M., Tsai, M. J., O'Malley, B. W., and Katzenellenbogen, B. S. (1996). Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator. Proc Natl Acad Sci U S A *93*, 10069-10073.

McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999). Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev *20*, 321-344.

McKenna, N. J., and O'Malley, B. W. (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. Cell *108*, 465-474.

Meyer, M. E., Gronemeyer, H., Turcotte, B., Bocquel, M. T., Tasset, D., and Chambon, P. (1989). Steroid hormone receptors compete for factors that mediate their enhancer function. Cell *57*, 433-442.

Meyer, M. E., Pornon, A., Ji, J. W., Bocquel, M. T., Chambon, P., and Gronemeyer, H. (1990). Agonistic and antagonistic activities of RU486 on the functions of the human progesterone receptor. Embo J *9*, 3923-3932.

Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., and Auricchio, F. (1998). Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. Embo J *17*, 2008-2018.

Milgrom, E., Atger, M., and Baulieu, E. E. (1970). Progesterone in uterus and plasma. IV. Progesterone receptor(s) in guinea pig uterus cytosol. Steroids *16*, 741-754.
Molenda-Figueira, H. A., Murphy, S. D., Shea, K. L., Siegal, N. K., Zhao, Y., Chadwick, J. G., Jr., Denner, L. A., and Tetel, M. J. (2008). Steroid receptor coactivator-1 from brain physically interacts differentially with steroid receptor subtypes. Endocrinology *149*, 5272-5279.
Montano, M. M., Ekena, K., Delage-Mourroux, R., Chang, W., Martini, P., and Katzenellenbogen, B. S. (1999). An estrogen receptor-selective coregulator that potentiates the effectiveness of antiestrogens and represses the activity of estrogens. Proc Natl Acad Sci U S A *96*, 6947-6952.
Mote, P. A., Balleine, R. L., McGowan, E. M., and Clarke, C. L. (1999). Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle. J Clin Endocrinol Metab *84*, 2963-2971.
Mote, P. A., Balleine, R. L., McGowan, E. M., and Clarke, C. L. (2000). Heterogeneity of progesterone receptors A and B expression in human endometrial glands and stroma. Hum Reprod *15 Suppl 3*, 48-56.
Mote, P. A., Bartow, S., Tran, N., and Clarke, C. L. (2002). Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis. Breast Cancer Res Treat *72*, 163-172.
Mote, P. A., Graham, J. D., and Clarke, C. L. (2007). Progesterone receptor isoforms in normal and malignant breast. Ernst Schering Found Symp Proc, 77-107.
Mote, P. A., Leary, J. A., Avery, K. A., Sandelin, K., Chenevix-Trench, G., Kirk, J. A., and Clarke, C. L. (2004). Germ-line mutations in BRCA1 or BRCA2 in the normal breast are associated with altered expression of estrogen-responsive proteins and the predominance of progesterone receptor A. Genes Chromosomes Cancer *39*, 236-248.
Mulac-Jericevic, B., Lydon, J. P., DeMayo, F. J., and Conneely, O. M. (2003). Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. Proc Natl Acad Sci U S A *100*, 9744-9749.
Mulac-Jericevic, B., Mullinax, R. A., DeMayo, F. J., Lydon, J. P., and Conneely, O. M. (2000). Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. Science *289*, 1751-1754.
Murphy, A. A., Morales, A. J., Kettel, L. M., and Yen, S. S. (1995). Regression of uterine leiomyomata to the antiprogesterone RU486: dose-response effect. Fertil Steril *64*, 187-190.
Mutter, G. L., Bergeron, C., Deligdisch, L., Ferenczy, A., Glant, M., Merino, M., Williams, A. R., and Blithe, D. L. (2008). The spectrum of endometrial pathology induced by progesterone receptor modulators. Mod Pathol *21*, 591-598.
Na, S. Y., Lee, S. K., Han, S. J., Choi, H. S., Im, S. Y., and Lee, J. W. (1998). Steroid receptor coactivator-1 interacts with the p50 subunit and coactivates nuclear factor kappaB-mediated transactivations. J Biol Chem *273*, 10831-10834.
Nawaz, Z., Lonard, D. M., Dennis, A. P., Smith, C. L., and O'Malley, B. W. (1999a). Proteasome-dependent degradation of the human estrogen receptor. Proc Natl Acad Sci U S A *96*, 1858-1862.
Nawaz, Z., Lonard, D. M., Smith, C. L., Lev-Lehman, E., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1999b). The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. Mol Cell Biol *19*, 1182-1189.
Nawaz, Z., and O'Malley, B. W. (2004). Urban renewal in the nucleus: is protein turnover by proteasomes absolutely required for nuclear receptor-regulated transcription? Mol Endocrinol *18*, 493-499.
Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005). A genomic and functional inventory of deubiquitinating enzymes. Cell *123*, 773-786.
O'Malley, B. W. (2007). Coregulators: from whence came these "master genes". Mol Endocrinol *21*, 1009-1013.
O'Malley, B. W., and Means, A. R. (1974). Female steroid hormones and target cell nuclei. Science *183*, 610-620.
O'Malley, B. W., Sherman, M. R., and Toft, D. O. (1970). Progesterone "receptors" in the cytoplasm and nucleus of chick oviduct target tissue. Proc Natl Acad Sci U S A *67*, 501-508.
Onate, S. A., Boonyaratanakornkit, V., Spencer, T. E., Tsai, S. Y., Tsai, M. J., Edwards, D. P., and O'Malley, B. W. (1998). The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. J Biol Chem *273*, 12101-12108.
Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science *270*, 1354-1357.

Ormandy, C. J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Edery, M., Brousse, N., Babinet, C., Binart, N., and Kelly, P. A. (1997). Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev *11*, 167-178.

Owen, G. I., Richer, J. K., Tung, L., Takimoto, G., and Horwitz, K. B. (1998). Progesterone regulates transcription of the p21(WAF1) cyclin- dependent kinase inhibitor gene through Sp1 and CBP/p300. J Biol Chem *273*, 10696-10701.

Palijan, A., Fernandes, I., Bastien, Y., Tang, L., Verway, M., Kourelis, M., Tavera-Mendoza, L. E., Li, Z., Bourdeau, V., Mader, S.*, et al.* (2009a). Function of histone deacetylase 6 as a cofactor of nuclear receptor coregulator LCoR. J Biol Chem *284*, 30264-30274.

Palijan, A., Fernandes, I., Verway, M., Kourelis, M., Bastien, Y., Tavera-Mendoza, L. E., Sacheli, A., Bourdeau, V., Mader, S., and White, J. H. (2009b). Ligand-dependent corepressor LCoR is an attenuator of progesterone-regulated gene expression. J Biol Chem *284*, 30275-30287.

Perissi, V., Staszewski, L. M., McInerney, E. M., Kurokawa, R., Krones, A., Rose, D. W., Lambert, M. H., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1999). Molecular determinants of nuclear receptor-corepressor interaction. Genes Dev *13*, 3198-3208.

Petit-Topin, I., Turque, N., Fagart, J., Fay, M., Ulmann, A., Gainer, E., and Rafestin-Oblin, M. E. (2009). Met909 plays a key role in the activation of the progesterone receptor and also in the high potency of 13-ethyl progestins. Mol Pharmacol *75*, 1317-1324.

Petit, E., Courtin, A., Kloosterboer, H. J., Rostene, W., Forgez, P., and Gompel, A. (2009). Progestins induce catalase activities in breast cancer cells through PRB isoform: correlation with cell growth inhibition. J Steroid Biochem Mol Biol *115*, 153-160.

Picard, D. (2006). Chaperoning steroid hormone action. Trends Endocrinol Metab *17*, 229-235.

Picard, D., Kumar, V., Chambon, P., and Yamamoto, K. R. (1990). Signal transduction by steroid hormones: nuclear localization is differentially regulated in estrogen and glucocorticoid receptors. Cell Regul *1*, 291-299.

Picard, D., and Yamamoto, K. R. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. Embo J *6*, 3333-3340.

Pickart, C. M. (2001). Mechanisms underlying ubiquitination. Annu Rev Biochem *70*, 503-533.

Pierson-Mullany, L. K., and Lange, C. A. (2004). Phosphorylation of progesterone receptor serine 400 mediates ligand-independent transcriptional activity in response to activation of cyclin-dependent protein kinase 2. Mol Cell Biol *24*, 10542-10557.

Pinon, G. M., Fagart, J., Souque, A., Auzou, G., Vandewalle, A., and Rafestin-Oblin, M. E. (2004). Identification of steroid ligands able to inactivate the mineralocorticoid receptor harboring the S810L mutation responsible for a severe form of hypertension. Mol Cell Endocrinol *217*, 181-188.

Poole, A. J., Li, Y., Kim, Y., Lin, S. C., Lee, W. H., and Lee, E. Y. (2006). Prevention of Brca1-mediated mammary tumorigenesis in mice by a progesterone antagonist. Science *314*, 1467-1470.

Pratt, W. B., Galigniana, M. D., Harrell, J. M., and DeFranco, D. B. (2004). Role of hsp90 and the hsp90-binding immunophilins in signalling protein movement. Cell Signal *16*, 857-872.

Proietti, C., Salatino, M., Rosemblit, C., Carnevale, R., Pecci, A., Kornblihtt, A. R., Molinolo, A. A., Frahm, I., Charreau, E. H., Schillaci, R., and Elizalde, P. V. (2005). Progestins induce transcriptional activation of signal transducer and activator of transcription 3 (Stat3) via a Jak- and Src-dependent mechanism in breast cancer cells. Mol Cell Biol *25*, 4826-4840.

Punglia, R. S., Kuntz, K. M., Winer, E. P., Weeks, J. C., and Burstein, H. J. (2006). The impact of tumor progesterone receptor status on optimal adjuvant endocrine therapy for postmenopausal patients with early-stage breast cancer: a decision analysis. Cancer *106*, 2576-2582.

Qiu, M., and Lange, C. A. (2003). MAP kinases couple multiple functions of human progesterone receptors: degradation, transcriptional synergy, and nuclear association. J Steroid Biochem Mol Biol *85*, 147-157.

Qiu, M., Olsen, A., Faivre, E., Horwitz, K. B., and Lange, C. A. (2003). Mitogen-activated protein kinase regulates nuclear association of human progesterone receptors. Mol Endocrinol *17*, 628-642.

Ramamoorthy, S., Dhananjayan, S. C., Demayo, F. J., and Nawaz, Z. (2010). Isoform-Specific Degradation of PR-B by E6-AP Is Critical for Normal Mammary Gland Development. Mol Endocrinol.

Reid, G., Hubner, M. R., Metivier, R., Brand, H., Denger, S., Manu, D., Beaudouin, J., Ellenberg, J., and Gannon, F. (2003). Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. Mol Cell *11*, 695-707.

Richer, J. K., Jacobsen, B. M., Manning, N. G., Abel, M. G., Wolf, D. M., and Horwitz, K. B. (2002). Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J Biol Chem *277*, 5209-5218.

Richer, J. K., Lange, C. A., Manning, N. G., Owen, G., Powell, R., and Horwitz, K. B. (1998). Convergence of progesterone with growth factor and cytokine signaling in breast cancer. Progesterone receptors regulate signal transducers and activators of transcription expression and activity. J Biol Chem *273*, 31317-31326.

Rocha, A., and Soares, R. (2009). Unraveling progesterone-induced molecular mechanisms in physiological and pathological conditions. Curr Clin Pharmacol *4*, 148-153.
Rossouw, J. E., Anderson, G. L., Prentice, R. L., LaCroix, A. Z., Kooperberg, C., Stefanick, M. L., Jackson, R. D., Beresford, S. A., Howard, B. V., Johnson, K. C.*, et al.* (2002). Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. Jama *288*, 321-333.
Rowan, B. G., and O'Malley, B. W. (2000). Progesterone receptor coactivators. Steroids *65*, 545-549.
Russo, J., Ao, X., Grill, C., and Russo, I. H. (1999). Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. Breast Cancer Res Treat *53*, 217-227.
Salama, S. A., Jamaluddin, M., Kumar, R., Hassan, M. H., and Al-Hendy, A. (2007). Progesterone regulates catechol-O-methyl transferase gene expression in breast cancer cells: distinct effect of progesterone receptor isoforms. J Steroid Biochem Mol Biol *107*, 253-261.
Samalecos, A., and Gellersen, B. (2008). Systematic expression analysis and antibody screening do not support the existence of naturally occurring progesterone receptor (PR)-C, PR-M, or other truncated PR isoforms. Endocrinology *149*, 5872-5887.
Sartorius, C. A., Melville, M. Y., Hovland, A. R., Tung, L., Takimoto, G. S., and Horwitz, K. B. (1994). A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. Mol Endocrinol *8*, 1347-1360.
Sartorius, C. A., Shen, T., and Horwitz, K. B. (2003). Progesterone receptors A and B differentially affect the growth of estrogen-dependent human breast tumor xenografts. Breast Cancer Res Treat *79*, 287-299.
Savouret, J. F., Bailly, A., Misrahi, M., Rauch, C., Redeuilh, G., Chauchereau, A., and Milgrom, E. (1991). Characterization of the hormone responsive element involved in the regulation of the progesterone receptor gene. Embo J *10*, 1875-1883.
Scarpin, K. M., Graham, J. D., Mote, P. A., and Clarke, C. L. (2009). Progesterone action in human tissues: regulation by progesterone receptor (PR) isoform expression, nuclear positioning and coregulator expression. Nucl Recept Signal *7*, e009.
Schramek, D., Leibbrandt, A., Sigl, V., Kenner, L., Pospisilik, J. A., Lee, H. J., Hanada, R., Joshi, P. A., Aliprantis, A., Glimcher, L.*, et al.* (2010). Osteoclast differentiation factor RANKL controls development of progestin-driven mammary cancer. Nature *468*, 98-102.
Schumacher, M., Sitruk-Ware, R., and De Nicola, A. F. (2008). Progesterone and progestins: neuroprotection and myelin repair. Curr Opin Pharmacol *8*, 740-746.
Seeler, J. S., and Dejean, A. (2003). Nuclear and unclear functions of SUMO. Nat Rev Mol Cell Biol *4*, 690-699.
Sharma, M., Zarnegar, M., Li, X., Lim, B., and Sun, Z. (2000). Androgen receptor interacts with a novel MYST protein, HBO1. J Biol Chem *275*, 35200-35208.
Shatnawi, A., Tran, T., and Ratnam, M. (2007). R5020 and RU486 act as progesterone receptor agonists to enhance Sp1/Sp4-dependent gene transcription by an indirect mechanism. Mol Endocrinol *21*, 635-650.
Shen, T., Horwitz, K. B., and Lange, C. A. (2001). Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294. Mol Cell Biol *21*, 6122-6131.
Shiau, A. K., Barstad, D., Radek, J. T., Meyers, M. J., Nettles, K. W., Katzenellenbogen, B. S., Katzenellenbogen, J. A., Agard, D. A., and Greene, G. L. (2002). Structural characterization of a subtype-selective ligand reveals a novel mode of estrogen receptor antagonism. Nat Struct Biol *9*, 359-364.
Shyamala, G., Schneider, W., and Schott, D. (1990). Developmental regulation of murine mammary progesterone receptor gene expression. Endocrinology *126*, 2882-2889.
Shyamala, G., Yang, X., Cardiff, R. D., and Dale, E. (2000). Impact of progesterone receptor on cell-fate decisions during mammary gland development. Proc Natl Acad Sci U S A *97*, 3044-3049.
Shyamala, G., Yang, X., Silberstein, G., Barcellos-Hoff, M. H., and Dale, E. (1998). Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor exhibit developmental abnormalities in mammary glands. Proc Natl Acad Sci U S A *95*, 696-701.
Skildum, A., Faivre, E., and Lange, C. A. (2005). Progesterone receptors induce cell cycle progression via activation of mitogen-activated protein kinases. Mol Endocrinol *19*, 327-339.
Smid-Koopman, E., Blok, L. J., Kuhne, L. C., Burger, C. W., Helmerhorst, T. J., Brinkmann, A. O., and Huikeshoven, F. J. (2003). Distinct functional differences of human progesterone receptors A and B on gene expression and growth regulation in two endometrial carcinoma cell lines. J Soc Gynecol Investig *10*, 49-57.
Smith, C. L., and O'Malley, B. W. (2004). Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev *25*, 45-71.

Smith, D. F. (1993). Dynamics of heat shock protein 90-progesterone receptor binding and the disactivation loop model for steroid receptor complexes. Mol Endocrinol *7*, 1418-1429.
Soderholm, A. A., Lehtovuori, P. T., and Nyronen, T. H. (2005). Three-dimensional structure-activity relationships of nonsteroidal ligands in complex with androgen receptor ligand-binding domain. J Med Chem *48*, 917-925.
Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997). Steroid receptor coactivator-1 is a histone acetyltransferase. Nature *389*, 194-198.
Spitz, I. M. (2003). Progesterone antagonists and progesterone receptor modulators: an overview. Steroids *68*, 981-993.
Steinauer, J., Pritts, E. A., Jackson, R., and Jacoby, A. F. (2004). Systematic review of mifepristone for the treatment of uterine leiomyomata. Obstet Gynecol *103*, 1331-1336.
Stumpf, W. E., Narbaitz, R., and Sar, M. (1980). Estrogen receptors in the fetal mouse. J Steroid Biochem *12*, 55-64.
Suen, C. S., Berrodin, T. J., Mastroeni, R., Cheskis, B. J., Lyttle, C. R., and Frail, D. E. (1998). A transcriptional coactivator, steroid receptor coactivator-3, selectively augments steroid receptor transcriptional activity. J Biol Chem *273*, 27645-27653.
Suganuma, T., and Workman, J. L. (2008). Crosstalk among Histone Modifications. Cell *135*, 604-607.
Takeshita, A., Cardona, G. R., Koibuchi, N., Suen, C. S., and Chin, W. W. (1997). TRAM-1, A novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1. J Biol Chem *272*, 27629-27634.
Takeshita, A., Yen, P. M., Misiti, S., Cardona, G. R., Liu, Y., and Chin, W. W. (1996). Molecular cloning and properties of a full-length putative thyroid hormone receptor coactivator. Endocrinology *137*, 3594-3597.
Takimoto, G. S., Hovland, A. R., Tasset, D. M., Melville, M. Y., Tung, L., and Horwitz, K. B. (1996). Role of phosphorylation on DNA binding and transcriptional functions of human progesterone receptors. J Biol Chem *271*, 13308-13316.
Tallec, L. P., Kirsh, O., Lecomte, M. C., Viengchareun, S., Zennaro, M. C., Dejean, A., and Lombes, M. (2003). Protein inhibitor of activated signal transducer and activator of transcription 1 interacts with the N-terminal domain of mineralocorticoid receptor and represses its transcriptional activity: implication of small ubiquitin-related modifier 1 modification. Mol Endocrinol *17*, 2529-2542.
Tanaka, T., Rodriguez de la Concepcion, M. L., and De Luca, L. M. (2001). Involvement of all-trans-retinoic acid in the breakdown of retinoic acid receptors alpha and gamma through proteasomes in MCF-7 human breast cancer cells. Biochem Pharmacol *61*, 1347-1355.
Tetel, M. J., Giangrande, P. H., Leonhardt, S. A., McDonnell, D. P., and Edwards, D. P. (1999). Hormone-dependent interaction between the amino- and carboxyl-terminal domains of progesterone receptor in vitro and in vivo. Mol Endocrinol *13*, 910-924.
Teutsch, G., Gaillard-Moguilewsky, M., Lemoine, G., Nique, F., and Philibert, D. (1991). Design of ligands for the glucocorticoid and progestin receptors. Biochem Soc Trans *19*, 901-908.
Teutsch, G., Nique, F., Lemoine, G., Bouchoux, F., Cerede, E., Gofflo, D., and Philibert, D. (1995). General structure-activity correlations of antihormones. Ann N Y Acad Sci *761*, 5-28.
Tiefenbach, J., Novac, N., Ducasse, M., Eck, M., Melchior, F., and Heinzel, T. (2006). SUMOylation of the corepressor N-CoR modulates its capacity to repress transcription. Mol Biol Cell *17*, 1643-1651.
Tirard, M., Almeida, O. F., Hutzler, P., Melchior, F., and Michaelidis, T. M. (2007). Sumoylation and proteasomal activity determine the transactivation properties of the mineralocorticoid receptor. Mol Cell Endocrinol *268*, 20-29.
Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997). The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function [see comments]. Nature *387*, 677-684.
Tseng, L., Tang, M., Wang, Z., and Mazella, J. (2003). Progesterone receptor (hPR) upregulates the fibronectin promoter activity in human decidual fibroblasts. DNA Cell Biol *22*, 633-640.
Tung, L., Abdel-Hafiz, H., Shen, T., Harvell, D. M., Nitao, L. K., Richer, J. K., Sartorius, C. A., Takimoto, G. S., and Horwitz, K. B. (2006). Progesterone receptors (PR)-B and -A regulate transcription by different mechanisms: AF-3 exerts regulatory control over coactivator binding to PR-B. Mol Endocrinol *20*, 2656-2670.
Vanzulli, S. I., Soldati, R., Meiss, R., Colombo, L., Molinolo, A. A., and Lanari, C. (2005). Estrogen or antiprogestin treatment induces complete regression of pulmonary and axillary metastases in an experimental model of breast cancer progression. Carcinogenesis *26*, 1055-1063.
Vegeto, E., Shahbaz, M. M., Wen, D. X., Goldman, M. E., O'Malley, B. W., and McDonnell, D. P. (1993). Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. Mol Endocrinol *7*, 1244-1255.

Verma, S., Ismail, A., Gao, X., Fu, G., Li, X., O'Malley, B. W., and Nawaz, Z. (2004). The ubiquitin-conjugating enzyme UBCH7 acts as a coactivator for steroid hormone receptors. Mol Cell Biol *24*, 8716-8726.

Voegel, J. J., Heine, M. J., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. (1998). The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways. Embo J *17*, 507-519.

Voegel, J. J., Heine, M. J., Zechel, C., Chambon, P., and Gronemeyer, H. (1996). TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. Embo J *15*, 3667-3675.

Voges, D., Zwickl, P., and Baumeister, W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu Rev Biochem *68*, 1015-1068.

Wallace, A. D., and Cidlowski, J. A. (2001). Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. J Biol Chem *276*, 42714-42721.

Wang, C., Fu, M., Angeletti, R. H., Siconolfi-Baez, L., Reutens, A. T., Albanese, C., Lisanti, M. P., Katzenellenbogen, B. S., Kato, S., Hopp, T.*, et al.* (2001). Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. J Biol Chem *276*, 18375-18383.

Wang, C., Powell, M., Tian, L., and Pestell, R. G. (2011). Analysis of nuclear receptor acetylation. Methods Mol Biol *776*, 169-181.

Wang, J. C., Stafford, J. M., and Granner, D. K. (1998). SRC-1 and GRIP1 coactivate transcription with hepatocyte nuclear factor 4. J Biol Chem *273*, 30847-30850.

Ward, R. D., and Weigel, N. L. (2009). Steroid receptor phosphorylation: Assigning function to site-specific phosphorylation. Biofactors *35*, 528-536.

Wardell, S. E., Boonyaratanakornkit, V., Adelman, J. S., Aronheim, A., and Edwards, D. P. (2002). Jun dimerization protein 2 functions as a progesterone receptor N- terminal domain coactivator. Mol Cell Biol *22*, 5451-5466.

Wardell, S. E., Narayanan, R., Weigel, N. L., and Edwards, D. P. (2010). Partial agonist activity of the progesterone receptor antagonist RU486 mediated by an amino-terminal domain coactivator and phosphorylation of serine400. Mol Endocrinol *24*, 335-345.

Wargon, V., Fernandez, S. V., Goin, M., Giulianelli, S., Russo, J., and Lanari, C. (2011). Hypermethylation of the progesterone receptor A in constitutive antiprogestin-resistant mouse mammary carcinomas. Breast Cancer Res Treat *126*, 319-332.

Wargon, V., Helguero, L. A., Bolado, J., Rojas, P., Novaro, V., Molinolo, A., and Lanari, C. (2009). Reversal of antiprogestin resistance and progesterone receptor isoform ratio in acquired resistant mammary carcinomas. Breast Cancer Res Treat *116*, 449-460.

Wei, L. L., Gonzalez-Aller, C., Wood, W. M., Miller, L. A., and Horwitz, K. B. (1990). 5'-Heterogeneity in human progesterone receptor transcripts predicts a new amino-terminal truncated "C"-receptor and unique A-receptor messages. Mol Endocrinol *4*, 1833-1840.

Wei, L. L., Hawkins, P., Baker, C., Norris, B., Sheridan, P. L., and Quinn, P. G. (1996). An amino-terminal truncated progesterone receptor isoform, PRc, enhances progestin-induced transcriptional activity. Mol Endocrinol *10*, 1379-1387.

Wei, L. L., Norris, B. M., and Baker, C. J. (1997). An N-terminally truncated third progesterone receptor protein, PR(C), forms heterodimers with PR(B) but interferes in PR(B)-DNA binding. J Steroid Biochem Mol Biol *62*, 287-297.

Wei, L. N., Hu, X., Chandra, D., Seto, E., and Farooqui, M. (2000). Receptor-interacting protein 140 directly recruits histone deacetylases for gene silencing. J Biol Chem *275*, 40782-40787.

Weigel, N. L. (1996). Steroid hormone receptors and their regulation by phosphorylation. Biochem J *319 (Pt 3)*, 657-667.

Weigel, N. L., and Moore, N. L. (2007). Steroid receptor phosphorylation: a key modulator of multiple receptor functions. Mol Endocrinol *21*, 2311-2319.

White, M. M., Sheffer, I., Teeter, J., and Apostolakis, E. M. (2007). Hypothalamic progesterone receptor-A mediates gonadotropin surges, self priming and receptivity in estrogen-primed female mice. J Mol Endocrinol *38*, 35-50.

Wiehle, R., Lantvit, D., Yamada, T., and Christov, K. (2011). CDB-4124, a progesterone receptor modulator, inhibits mammary carcinogenesis by suppressing cell proliferation and inducing apoptosis. Cancer Prev Res (Phila) *4*, 414-424.

Wiehle, R. D., Christov, K., and Mehta, R. (2007). Anti-progestins suppress the growth of established tumors induced by 7,12-dimethylbenz(a)anthracene: comparison between RU486 and a new 21-substituted-19-nor-progestin. Oncol Rep *18*, 167-174.

Wijayaratne, A. L., and McDonnell, D. P. (2001). The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. J Biol Chem *276*, 35684-35692.

Wilkens, J., Williams, A. R., Chwalisz, K., Han, C., Cameron, I. T., and Critchley, H. O. (2009). Effect of asoprisnil on uterine proliferation markers and endometrial expression of the tumour suppressor gene, PTEN. Hum Reprod *24*, 1036-1044.

Williams, A. R., Critchley, H. O., Osei, J., Ingamells, S., Cameron, I. T., Han, C., and Chwalisz, K. (2007). The effects of the selective progesterone receptor modulator asoprisnil on the morphology of uterine tissues after 3 months treatment in patients with symptomatic uterine leiomyomata. Hum Reprod *22*, 1696-1704.

Williams, S. P., and Sigler, P. B. (1998). Atomic structure of progesterone complexed with its receptor. Nature *393*, 392-396.

Xu, J., Liao, L., Ning, G., Yoshida-Komiya, H., Deng, C., and O'Malley, B. W. (2000). The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. Proc Natl Acad Sci U S A *97*, 6379-6384.

Xu, J., Qiu, Y., DeMayo, F. J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1998). Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science *279*, 1922-1925.

Xu, J., Wu, R. C., and O'Malley, B. W. (2009). Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer *9*, 615-630.

Yang, S., Thiel, K. W., and Leslie, K. K. (2011a). Progesterone: the ultimate endometrial tumor suppressor. Trends Endocrinol Metab *22*, 145-152.

Yang, Y., Tse, A. K., Li, P., Ma, Q., Xiang, S., Nicosia, S. V., Seto, E., Zhang, X., and Bai, W. (2011b). Inhibition of androgen receptor activity by histone deacetylase 4 through receptor SUMOylation. Oncogene *30*, 2207-2218.

Yokota, K., Shibata, H., Kobayashi, S., Suda, N., Murai, A., Kurihara, I., Saito, I., and Saruta, T. (2004). Proteasome-mediated mineralocorticoid receptor degradation attenuates transcriptional response to aldosterone. Endocr Res *30*, 611-616.

Yotsumoto, F., Yagi, H., Suzuki, S. O., Oki, E., Tsujioka, H., Hachisuga, T., Sonoda, K., Kawarabayashi, T., Mekada, E., and Miyamoto, S. (2008). Validation of HB-EGF and amphiregulin as targets for human cancer therapy. Biochem Biophys Res Commun *365*, 555-561.

Zhang, P. J., Zhao, J., Li, H. Y., Man, J. H., He, K., Zhou, T., Pan, X., Li, A. L., Gong, W. L., Jin, B. F*., et al.* (2007). CUE domain containing 2 regulates degradation of progesterone receptor by ubiquitin-proteasome. Embo J *26*, 1831-1842.

Zhang, Y., Beck, C. A., Poletti, A., Clement, J. P. t., Prendergast, P., Yip, T. T., Hutchens, T. W., Edwards, D. P., and Weigel, N. L. (1997). Phosphorylation of human progesterone receptor by cyclin-dependent kinase 2 on three sites that are authentic basal phosphorylation sites in vivo. Mol Endocrinol *11*, 823-832.

Zhang, Y., Beck, C. A., Poletti, A., Edwards, D. P., and Weigel, N. L. (1994). Identification of phosphorylation sites unique to the B form of human progesterone receptor. In vitro phosphorylation by casein kinase II. J Biol Chem *269*, 31034-31040.

Zhang, Y., Beck, C. A., Poletti, A., Edwards, D. P., and Weigel, N. L. (1995). Identification of a group of Ser-Pro motif hormone-inducible phosphorylation sites in the human progesterone receptor. Mol Endocrinol *9*, 1029-1040.

Zhang, Z., Yamashita, H., Toyama, T., Sugiura, H., Ando, Y., Mita, K., Hamaguchi, M., Hara, Y., Kobayashi, S., and Iwase, H. (2006). NCOR1 mRNA is an independent prognostic factor for breast cancer. Cancer Lett *237*, 123-129.

Zhu, J., Gianni, M., Kopf, E., Honore, N., Chelbi-Alix, M., Koken, M., Quignon, F., Rochette-Egly, C., and de The, H. (1999). Retinoic acid induces proteasome-dependent degradation of retinoic acid receptor alpha (RARalpha) and oncogenic RARalpha fusion proteins. Proc Natl Acad Sci U S A *96*, 14807-14812.

Zhu, Y., Qi, C., Calandra, C., Rao, M. S., and Reddy, J. K. (1996). Cloning and identification of mouse steroid receptor coactivator-1 (mSRC-1), as a coactivator of peroxisome proliferator-activated receptor gamma. Gene Expr *6*, 185-195.