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Crosstalk between IGF-1 and estrogen receptor non-genomic signaling pathway in breast cancer

Ali Choucair

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**Crosstalk between IGF-1 and estrogen
receptor non-genomic signaling pathway
in breast cancer**

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Ali,

Lyon 13th of March 2018.

Caractéristiques Générales

Titre

Crosstalk entre la signalisation de l'IGF-1 et du récepteur des oestrogènes dans le cancer du sein

Mots-clés

IGF-1R, ER α , Signalisation, Méthylation, Crosstalk, Cancer de sein .

Résumé

Le cancer du sein est un problème majeur de santé publique qui touche 1 femme sur 5. Quarante-vingt percent des cancers sont hormono-dépendants et sont traités par des hormonothérapies qui ciblent les œstrogènes ou le récepteur des œstrogènes (ER α) et inhibent leurs effets tumorigènes. En parallèle de la voie génomique des œstrogènes, il existe une voie non génomique dans laquelle ER α recrute Src et PI3K à la membrane, et active des cascades de phosphorylation comme Akt, qui aboutit à la survie et la prolifération des cellules cancéreuses. Notre équipe a montré que la méthylation de l'arginine 260 par les œstrogènes, est un prérequis à la formation du complexe non génomique régulant la prolifération cellulaire. En 2012, l'équipe a montré que la signalisation non génomique des œstrogènes est activée dans les tumeurs mammaires agressives, représentant de nouvelles cibles thérapeutiques. Le crosstalk entre les œstrogènes et les facteurs de croissance impliquant des phosphorylations a été largement décrit. C'est pourquoi nous avons cherché si la méthylation d'ER α sur l'arginine 260 pouvait être impliquée dans ce crosstalk. Parmi plusieurs facteurs de croissance, nous avons mis en évidence que IGF-1 était le seul facteur capable d'induire la méthylation d'ER α de façon œstrogéno-indépendante. En effet, comme pour les œstrogènes, IGF-1 induit une méthylation rapide et transitoire par l'arginine méthyltransférase 1 (PRMT1), et la formation du complexe ER α /Src/PI3K. En utilisant plusieurs approches, nous avons obtenu des résultats intéressants, montrant que PRMT1 probablement *via* la méthylation d'ER α , joue un rôle crucial dans la signalisation d'IGF-1. D'autre part, nous avons montré qu'IGF-1 phosphoryle ER α au niveau de son domaine de liaison à l'ADN, modulant son interaction avec IGF-1R. De plus, l'analyse d'une cohorte de 440 tumeurs mammaires a mis en évidence que l'expression d'IGF-1 est corrélée à l'activation de la voie non-génomique des œstrogènes, renforçant les résultats obtenus *in vitro* et ouvrant de nouvelles perspectives thérapeutiques qui cibleraient les 2 voies de signalisation.

General Features

Title

Crosstalk between IGF-1 and estrogen receptor non-genomic signaling pathway in breast cancer

Keywords

IGF-1R, ER α , Signaling, Methylation, Crosstalk, Breast cancer

Abstract

Breast cancer is a major health problem currently affecting 1 out of 5 women. Seventy percent of breast cancers are hormone-dependent, and are treated by hormonal therapies targeting estrogen receptor and consequently the inhibition of its pro-tumorigenic effects. In parallel to the genomic estrogen signaling, non-genomic signaling has been described, where ER α recruits Src kinase and PI3K at the plasma membrane and thus activates downstream phosphorylation cascades like AKT, which in turn leads to survival and proliferation of cancer cells. Our team has found that estrogen-induced methylation of arginine 260 of ER α is a prerequisite for the formation of this non-genomic complex, regulating cell proliferation. In 2012, we have shown that this pathway is activated in aggressive breast tumors representing a new potential target for breast cancer therapy. Crosstalk between estrogen and growth factors signaling involving phosphorylation has been largely described. For this reason, we investigated if ER α R260 methylation could be involved in this crosstalk. Among several growth factors, we found that IGF-1 was the only one able to induce methylation of ER α in an estrogen-independent manner. Similarly to estrogen, IGF-1 induces a rapid and transient methylation of ER α by the Protein Arginine Methyltransferase (PRMT1) concomitant with the formation of ER α /Src/PI3K complex. Using several approaches, we found significant results showing that PRMT1 probably *via* ER α methylation plays a crucial role in IGF-1 signaling. Interestingly, we have recently found also that receptor tyrosine kinase IGF-1R phosphorylates the DNA binding domain (DBD) of ER α that could modulate the latter downstream signaling. In line with these results, we found on TMAs of a cohort of 440 breast tumors that IGF-1 expression is correlated with ER α non-genomic signaling. These results report new insight into estrogen and IGF-1 interference, which open new perspectives of combining therapies targeting the two pathways.

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Scientific Communications

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- **CRCL 3rd International Symposium, Lyon, September 2017** – Poster presentation
- **NVIC conference, Berlin, June 2017** – Short talk
- **Cancéropôle CLARA meeting, Lyon, April 2017** – Poster presentation
- **EDBMIC UCBL1 meeting, Lyon, December 2016** – Short talk
- **CRCL department meeting, Aussois, June 2016** – Short talk
- **Nuclear Receptor Meeting, Paris, May 2016** – Short talk
- **CRCL 4th Scientific Day, Lyon, May 2016** – Poster presentation
- **Cancéropôle CLARA meeting, Lyon, April 2016** – Poster presentation
- **EDBMIC UCBL1 meeting, Lyon, November 2015** – Poster presentation
- **CRCL 2nd International Symposium, Lyon, September 2015** – Poster presentation
- **CRCL department meeting, Aussois, July 2015** – Poster presentation
- **Cancéropôle CLARA meeting, Lyon, April 2015** – Poster presentation

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List of abbreviations

4EBP1:	4E-binding protein 1
a.a:	Amino acid
ALS:	Acid-labile subunit
BCS:	Breast cancer survival
BMI:	Body mass index
BRCA1:	Breast cancer 1
CK:	Casein kinase
CNS:	Central nervous system
CSC:	Cancer stem cell
DBD:	DNA binding domain
DDT:	Dichlorodiphenyltrichloroethane
DFS:	Disease-free survival
DNA:	Deoxyribonucleic acid
EGF:	Epidermal growth factor
EGFR:	Epidermal growth factor receptor
EMT:	Epithelial-mesenchymal transition
ER:	Estrogen receptor
ERE:	Estrogen response element
ERK1/2:	Extracellular signal-regulated kinase
FAK:	Focal adhesion kinase
FBC:	Familial breast cancers

FGF:	Fibroblast growth factor
FOXA1:	Forkhead box protein A1
FSH:	Follicle stimulating hormone
GH:	Growth hormone
GHRH:	Growth hormone-releasing hormone
GPER:	G-protein estrogen receptor
GPR30:	G-protein coupled receptor 30
GRB2:	Growth factor receptor-bound protein 2
HER2:	Human epidermal growth factor receptor
Hsp:	Heat-shock protein
IGF:	Insulin-like growth factor
IGF-1R:	Insulin-like growth factor -1 receptor
IGFBP:	Insulin-like growth factor binding protein
IHC:	Immunohistochemistry
IP:	Immunoprecipitation
IR:	Insulin receptor
IRS:	Insulin receptor substrate
LBD:	Ligand-binding domain
LH:	Luteinizing hormone
M6P:	Mannose-6 phosphate
mAb:	Monoclonal antibody
MAPK:	Mitogen-activated protein kinase ERK1/2
MBC:	Male breast carcinoma

MMP:	Matrix metalloproteinase
MNAR:	Modulator of Non-genomic Activation of ER
mTOR:	Mammalian target of rapamycin
mTORC:	mTOR complex
OS:	Overall survival
PAHs:	Polycyclic aromatic hydrocarbons
PDGF:	Platelet-derived growth factor
PDK:	Phosphatidylinositol-dependent kinase
PDK:	Pyruvate dehydrogenase kinase
PDX:	Patients' derived xenografts
PFOSA:	Perfluorooctane-sulfonamide
PGE:	Prostaglandin
PIP ₂ :	Phosphatidylinositol 4, 5 phosphate
PIP ₃ :	Phosphatidylinositol 3, 4, 5 phosphate
PKB/Akt:	Protein kinase B
PKC:	Protein kinase C
PLA:	Proximity ligation assay
PR:	Progesterone receptor
PRMT:	Protein arginine Methyltransferase
PTHrP	Parathyroid hormone-related protein
PTM:	Post-translational modification
RIP40:	RIP kinase 40
RNA:	Ribonucleic acid

ROS:	Reactive oxygen species
RTK:	Receptor tyrosine kinase
S1P:	Sphingosine-1 phosphate
SBC:	Sporadic breast cancers
SBC:	Sporadic breast cancer
SBR:	Scarff-Bloom-Richardson
SERD:	Selective estrogen receptor degrader
SERM:	Selective estrogen receptor modulator
Shc:	Src homology collagen
SHP:	Src homology region 2 domain-containing phosphatase
siRNA:	Small inhibitory RNA
SK:	Sphingosine kinase
SOS:	Son of sevenless
TGF- β :	Transforming growth factor- β
TKI:	Tyrosine kinase inhibitor
TMA:	Tumor microarrays
TNBC:	Triple-negative breast cancer
TNM:	Tumor-nodes-metastases
VEGF:	Vascular Endothelial Growth Factor
WT1:	Wilms' tumor protein

INTRODUCTION

I. Breast Cancer

A. Prevalence

World Health Organization (WHO) reported in its most recent statistical study in 2012, that breast cancer is the second type of cancer affecting women worldwide and in Europe (globocan, iarc). Around 1.7 million new cases of breast cancer were reported in the world, which means 12% of all new cancer cases, and almost a quarter of all the feminine cancers (**Figure 1**).

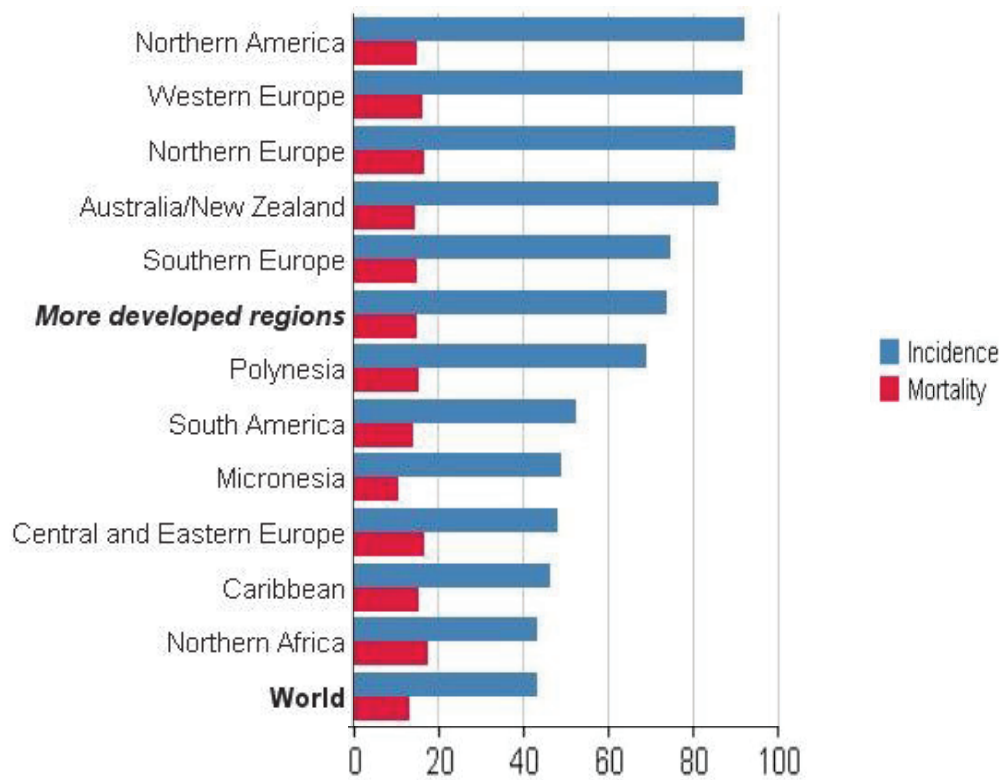


Figure 1: Estimated age-standardised rates per 100,000 of Incidence and mortality of breast cancer in 2012. (source: globocan, iarc)

In France, breast cancer is the leading feminine cancer taking a portion of one-third of all cancers types. Most of the developed European countries showed the same proportion, which makes this cancer the most common neoplasm among women (Dai et al., 2016).

The incidence trend of breast cancer kept on rising since 1975, reaching twice the rate between 2005 and 2010. However, it was shown that the mortality rate was decreased during this period (**figure2**). The European Union scored an overall decrease of 17% in breast cancer mortality among women between 2002 and 2012 (Carioli et al., 2017), and this could be explained by the advancement of diagnostic tools and the integrated efficient treatments of breast cancer.

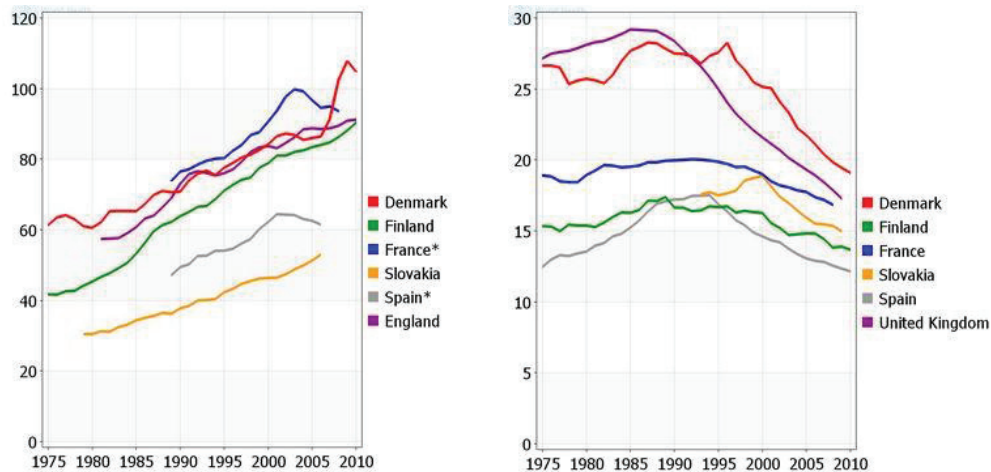


Figure 2: Trends in incidence (A) and mortality (B) of female breast cancer in selected countries: age-standardised rate (W) per 100,000. (source: globocan, iarc)

Despite its dominance in women, breast cancer still affects the men in minor percentages accounting for 0.1% of all masculine cancers (Javidiparsijani et al., 2017). Male breast carcinoma (MBC) mechanism and causes are yet to be better understood, but in general, it is mainly linked to genetic factors and their expression.

B. Mammary Gland

1. Anatomy of the mammary gland

The breast lactation function is carried by the mammary glands; they are highly innervated and vascularized organelles that are composed of two different compartments, epithelial and mesenchymal compartments, each with specific biological characteristics and physiological roles (**figure3**).

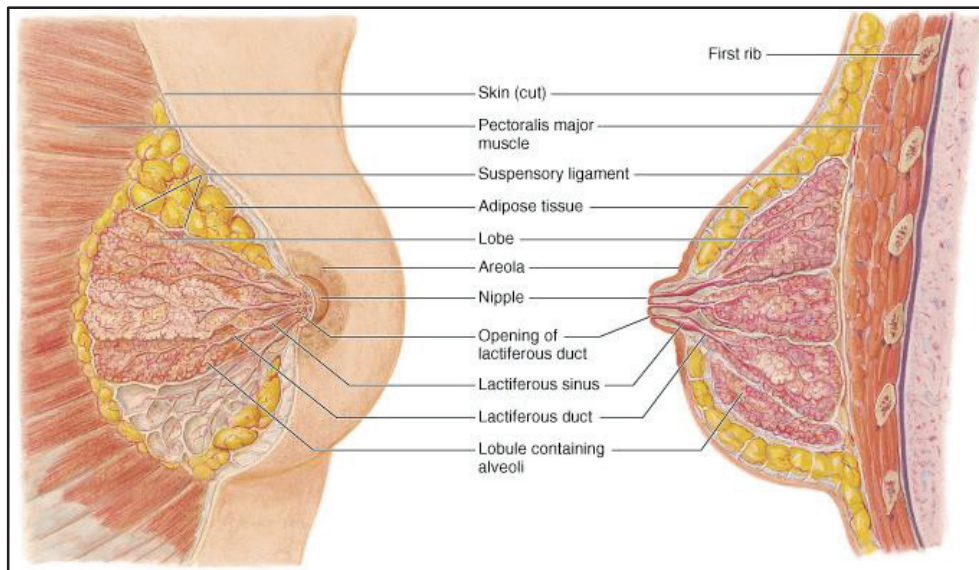


Figure 3: Mammary gland anatomy in cross section (left) and sagittal section (right) of the breast.
 (source: *apsubiology.org*)

a. The Epithelial compartment

This compartment is composed of lobules arranged in acini joined by the galactophorous canals. The lobules role is to transport the milk during breastfeeding to the nipples. Canals and lobules are composed of two cell types: luminal cells that line the lumen of the canals, and the surrounding myoepithelial cells that are in contact with the basal membrane (Hennighausen and Robinson, 2005). GATA-3 is one of the key regulators of mammary gland cells differentiation and a plays a major role in determining the fate of epithelial progenitor and stem cells (Asselin-Labat et al., 2007; Fang et al., 2009).

b. The mesenchymal compartment

In addition to the normal epithelium of the breast mammary gland, there is the mammary gland stroma, which is invaded by the epithelium during mammary gland development. The stroma is composed of adipocytes, fibroblasts, nerves, blood vessels and lymphatic network that is connected to the axillary ganglion (Hennighausen and Robinson, 2005). This plays a major role in the transfer of breast cancer cells through the ganglion causing metastasis (Weigelt et al., 2005).

2. Development of the mammary gland

The mammary gland development occurs mainly during embryogenesis, puberty, pregnancy and remains during all the reproductive life (Watson and Khaled, 2008). This development and growth is paracrinely controlled by the parathyroid hormone-related protein (PTHrP) (Wysolmerski et al., 1998); where it regulates the invasion of the mammary gland mesenchymal layer by the epithelial layer to start the so-called branching process. This branching allows the epithelium to begin sprouting in the fat layer of the mesenchyme, stimulating the formation of mammary-specific mesenchyme that later forms fibrous threads, lymph nodes and blood vessels (Hens and Wysolmerski, 2005).

Several hormones play role in mammary gland development, estrogen and growth hormone (GH) together induce ductal development; where IGF-1 induces GH that upregulates estrogen receptor expression (Kleinberg and Ruan, 2008). In addition to these hormones, several sex hormones and growth factors i.e. epidermal growth factor (EGF), transforming growth factor beta (TGF- β), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) are involved in the mammary gland and breast development (El-Attar and Sheta, 2011; Hynes and Watson, 2010).

C. Etiology of breast cancer

1. Sexual Factors

Breast cancer is considered to be a feminine disease for 99% of the cases. However, it still affects men in minority of the cases due to some genetic factors that should be better understood. Moreover, male breast carcinoma (MBC) was noticed to be increased in the past three decades (Kamińska et al., 2015). In all cases, treatments for males and females are clinically the same.

2. Hereditary Factors

Breast cancer is generally related to the family history, where it shows an increased incidence risk correlated with the hereditary breast cancer. Many germline mutations were linked to breast cancer, but there are two main mutations in DNA repair proteins, breast cancer 1 (BRCA1) and breast cancer 2 (BRCA2), that greatly increase the risk of breast cancer incidence. These mutations were shown to be occurring in 6% of men breast cancers and up to 85% in women ones before the

age of 70, which makes up to 10% of the population (Dorval et al., 2011; Fackenthal and Olopade, 2007; Wolpert et al., 2000).

3. Hormonal factors

Women are subject to fluctuating hormonal levels for a long period, starting from puberty at the age of 12 to their menopause at 50 years. Breast cancer risk rises with longer exposure to sex hormones, like early menses and/or late menopause (Glade, 1999; Colditz, 1998).

In addition to long exposures to hormones, early pregnancies increases the breast cancer risk by 25% and it proportionally increases with the number of births given after. On the other hand, long-term breastfeeding showed to decrease the risk by one-third when comparing women who breastfed their children for long periods with others who did it briefly. This could be due to the increase in prolactin accompanied with declined levels of estrogen during the breastfeeding period; which leads to lesser risk of breast cancer development (Layde et al., 1989; 1994).

4. Environmental factors

Most of the European developed countries showed an increase in the incidence of the breast cancer in the last 25 years. This fact is probably explained by the increased pollution that accompanied this technological advance. Exposure to toxicants like dichlorodiphenyltrichloroethane (DDT), dioxins, perfluorooctane-sulfonamide (PFOSA) during breast development at childhood showed higher risk of carcinogenesis. In addition, air pollutants from fuel combustion and all polycyclic aromatic hydrocarbons (PAHs) were shown to significantly increase breast cancer risk in women with genetic susceptibility i.e. mutations and genetic variations (Rodgers et al., 2018). Last but not the least, ionizing radiation is well known to augment breast cancer risk due to breast sensitivity to this DNA-damaging radiation (Boice, 2001).

5. Physiological factors

a. Age

It is well known that incidence risk of breast cancer increases with age due to longer exposure time of hormones (Colditz, 1998). Also, it is put to evidence that breast cancer attacks preferably postmenopausal women with age more than 55 years; however, one in 300 women are diagnosed with breast cancer before the age of 40, which is probably due

to sedentary life and early usage of contraceptive pills. Early-age diagnosis of breast cancer is a negative predictor of survival and bad prognostic factor, which commonly correlates with recurrence and metastasis (Narod, 2012).

b. Obesity

Body fatness is very well associated with increased risk of breast cancer and especially in postmenopausal women (Hidayat et al., 2017). This is probably due to the fact that the adipose tissue is a site of storage and metabolism of steroid hormones, also it is the site of sprouting of the epithelium in the mammary gland mesenchymal layer (Boice, 2001; Macias and Hinck, 2012).

On the other hand, body mass index (BMI) was inversely correlated with menopause. A study showed that below-average BMI in premenopausal women and above-average BMI in postmenopausal women increased risk of breast cancer diagnosis. This suggests that BMI associates in different manner with age, leading to an increase in breast cancer incidence risk (Brouckaert et al., 2017).

c. Physical Activity

Healthier life style and practicing physical activity on regular basis are showing great impact on breast cancer incidence (Fontein et al., 2013). Although a lot of studies in this field are yet to be completed, most of them generally show high association between physical activity and breast cancer recurrence and death (Lahart et al., 2015). It was also shown that physical activity was correlated with breast cancer risk reduction, mainly in postmenopausal women (Gonçalves et al., 2014), and lead to a 25% decrease in breast cancer incidence risk in physically active women compared to the least active ones (Friedenreich, 2011). Nonetheless, there was a consistent evidence from several studies that physical activity is associated with reduced breast cancer-specific mortality (Ballard-Barbash et al., 2012).

Physical exercises proved to be safe and efficient for patients with cancer and led to reduced cancer-related fatigue and muscular weakness (Brown et al., 2012; van Vulpen et al., 2016), in addition to the positive physical, psychological and social impacts on patients (Zopf et al., 2014).

d. Smoking

Exposure to tobacco is a main cause of lung cancer and a reason for one third of all cancer deaths. Although it is not a main cause of breast cancer, but in the last 25 years, tobacco smoke proved that it has a cumulative role in increasing breast cancer incidence. This is due to all the carcinogenic chemicals and PAHs present in the tobacco smoke (Reynolds, 2013).

Many publications studied the relationship between breast cancer and smoking tobacco or simply living in places with heavy smokers; conclusions were drawn to the fact that smoking is associated with reduction in the overall survival (OS) and breast cancer-specific survival (BCS) (Bérubé et al., 2014). Also, studies have shown that smokers have a 28% increase in breast cancer-associated mortality compared to people who have never smoked (Sollie and Bille, 2017).

e. Alcohol consumption

Alcohol consumption is attributed to ~4% of all cancers worldwide (Cao and Giovannucci, 2016). In many epidemiological and experimental studies, a positive correlation was found between alcohol consumption and risk of breast cancer incidence. Alcohol mechanism in promoting cancer formation can be explained by the increased amount of reactive oxygen species (ROS) produced during alcohol metabolism, and its interference in the bioavailability of some growth factors like IGF-1 and EGF. (Wang et al., 2017).

In breast cancer specifically, alcohol exposure was associated with invasiveness and metastasis. This is due to alcohol's ability in stimulating mobility and invasiveness of breast cancer cells and promoting cellular malignancy by increasing their epithelial-mesenchymal transition (EMT) (Wang et al., 2017). In addition, alcohol impaired endothelial integrity and thus, helped in transition of breast cancer cells and a consequent metastatic breast cancer. This is explained by the fact that alcohol alters the breast cancer cells rendering them cancer stem cells (CSC), and that leads to tumor initiation, promotion and recurrence (Xu and Luo, 2017).

D. Breast Cancer Classification

Breast cancer has different levels of classification depending on the morphological characteristics of the tumor at the time of diagnosis, type of tumor progression and response to treatment. After examination and diagnosis, breast cancers are classified to different stages of progression according to several criteria:

1. Histological Criteria

In the most of cases, breast cancers initiate from the epithelial layer of mammary gland forming the so-called adenocarcinoma. This adenocarcinoma can be either *in-situ* adenocarcinoma or infiltrating adenocarcinoma, where the first is linked to good prognosis and the second is linked to metastasis and poor survival. In the latter case, tumor cells are more likely to infiltrate through blood vessels and lymphatic nodes invading other sites and probably initiating new tumors. Adenocarcinomas can be either ductal or lobular adenocarcinomas, where the first arises from the mammary gland ducts and the second from the mammary lobules (**Figure 4**). Invasive ductal carcinoma constitutes about 70-80% of breast carcinomas (Pourteimoor et al., 2016).

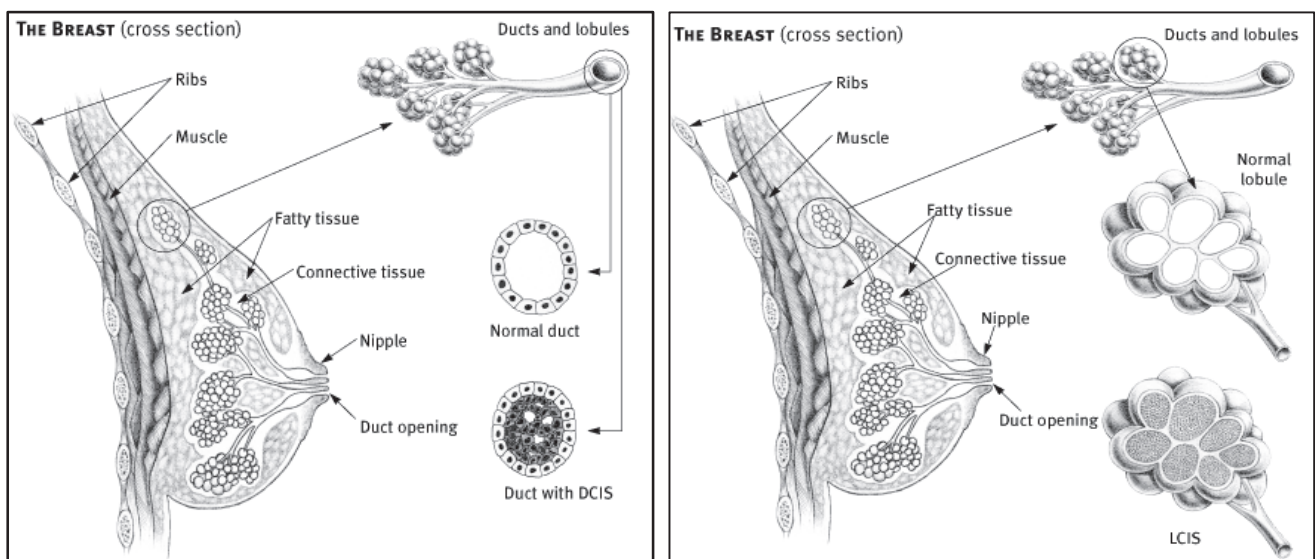


Figure 4: Breast Ductal adenocarcinoma (left) and lobular adenocarcinoma (right) tissue formation.
(source: cancervic.org)

2. Anatomopathological Criteria

Tumors in mammary glands are classified according to stage and prognostic factors. Several aspects are examined to determine the stage of cancer like tumor size, nodular ganglions, tumor infiltrant cells and presence of metastases. Accordingly, breast cancers are divided into tumor stages, TNM classification, and tumor grades, SBR classification.

a. TNM Classification

The International Union against Cancer and the American Joint Committee on Cancer (AJCC) have adapted this classification. It is based on Tumor location, vastness and size (T), involvement of lymph nodes (N) and presence or absence of metastases (M). This classification criteria divides breast cancer into 4 main stages. When they are stages I and II, this means that the tumor is still localized to the region and did not spread outside the tissue. Stage III means the tumor is in larger area of the organ with possibility of tumor cells spreading into the surrounding tissue and lymph nodes.

Table 1: TNM classification of breast tumors. Letter T symbolizes the initial tumor graded from T0 to T4. Letter N symbolizes the lymph node involvement in the tumor and graded from N0 to N3. Letter M symbolizes the metastatic phase of breast tumors.

Tumours	T0/Tis	T1	T2	T3	T4
Tumour Size	T0: No primary tumour. Tis: Tumour only in breast ducts or lobules.	0-2 cm	2-5 cm	>5 cm	Tumor of any size with extension to chest wall/skin or ulceration **inflammatory breast cancer is staged as T4.
Nodes	N0	N1	N1mi	N2	N3
	No lymph node metastases.	Cancer cells present in 1-3 axillary lymph nodes.	Lymph node tumor > 2 mm.	Cancer cells present in 4-9 axillary lymph nodes.	Cancer cells in infra or supraclavicular lymph nodes, or in >10 axillary lymph nodes.
Metastasis	M0	M1			
	No evidence of cancer metastasis.	Cancer found in other areas of body.			

Finally, when it is stage IV, this means that it is a metastatic cancer and with secondary organ infection. Detailed stages of breast cancer classification are summarized in **Table 1**.

b. SBR Classification

This classification is named Scarff, Bloom and Richardson histoprognostic grade (Bloom and Richardson, 1957). It allows assessing the aggressivity of tumors, by studying the nuclear pleomorphism, proliferative activity and tubules formation (Rakha et al., 2010). Some types of cancer have their own grading systems but generally, there are three grades. They are described as:

Grade 1 – The cancer cells look very similar to normal cells and are growing slowly.

Grade 2 – The cells look unlike normal cells and are growing more quickly than normal.

Grade 3 – The cancer cells look very abnormal and are growing quickly.

Clinicians and pathologists analyze microscopically the tumor sections and determine the characteristics of the tumor cells. As the latter are more likely to normal cells and well differentiated, as it is a better prognostic factor. On the other hand, as tumor cells are less differentiated, less localized and proliferating rapidly, this have a bad prognosis **Table 2**.

Table 4: SBR grading of breast tumors. (source: medind.nic.in)

Feature	Score
Tubule formation (%)	
Majority of tumor (>75)	1
Moderate degree (10-75)	2
Little or none (<10)	3
Nuclear pleomorphism	
Small, uniform cells	1
Moderate increase in size/variation	2
Marked variation	3
Mitotic counts (per 10-40×fields)	
0-5 (histo) or 0-1 (cyto)	1
6-10 (histo) or 2-4 (cyto)	2
>11 (histo) or >5 (cyto)	3
Grade 1 (well-differentiated) (sum)	3-5
Grade 2 (moderately differentiated) (sum)	6-7
Grade 3 (poorly differentiated) (sum)	8-9

SBR=Scarff-Bloom-Richardson

Therefore, the tumors with higher SBR grade are tumors with worse prognostic factors; hence, this grading is considered a predictive marker for response to hormonotherapy and chemotherapy.

3. Molecular Criteria

In order to better correlate breast cancer subtypes with prognostic factors and to determine the appropriate treatment for breast cancer patients, several molecular markers are taken into consideration to decide the therapeutic strategy that will be followed.

Breast cancers are classified to several types depending on gene expression status of the following molecular markers: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) (Perou et al., 2000; Sotiriou et al., 2003).

Accordingly, breast cancers are classified to ER-positive and/or PR-positive, HER2-positive and there exists the most invasive triple-negative breast cancer where all the main 3 molecular markers are absent (Kumar and Aggarwal, 2016).

Hormone receptor (HR) positive breast cancers i.e. ER and PR-positive tumors constitute 75-80% of all breast cancers (Sotiriou and Pusztai, 2009).

a. Estrogen Receptor

Although estrogen receptor α (ER α) is expressed in 10% of normal mammary tissue cells, seventy percent of breast cancers are ER α -positive (Nadji et al., 2005). Therefore, these tumors depend on estrogen receptor ligand “Estradiol” for growth; hence, these breast cancers are considered hormone-dependent cancers. For this reason, estrogen receptor is a main molecular and prognostic biomarker of breast cancer, and at the same time, it is a main target of hormone therapy in estrogen receptor positive breast cancers (Johnston, 2015).

b. Progesterone Receptor

Progesterone receptor and estrogen receptor expression are strongly correlated to one another. Progesterone receptor is a functional marker of ER α since the latter induces PR expression; in other words, PR is one of the estrogen-target genes (Allan et al., 2001).

There are different isoforms of progesterone receptor, PR-A and PR-B (Kastner et al., 1990). PR-B is transcriptionally more active than PR-A in certain cell types; however, PR-A acts as a repressor of PR-B and estrogen receptors and other steroid receptors in cell-specific and promoter-specific manner (Kraus et al., 1995; Vegeto et al., 1993). Some studies show that the PR-A/PR-B ratio can alter the response to endocrine therapy for ER/PR positive patients that poorly respond to hormone therapy (Hopp et al., 2004).

Recently a breakthrough study has revealed that PR does not actually have the attributed passive role of ER α activity report (Figure 7). Instead, PR activity has been shown to change ER binding sites to DNA, directly modulating ER function (Baird and Carroll, 2016). When progesterone and estrogen receptor positive tumors are compared with ER-positive, PR-negative tumors, the estrogen receptor DNA binding sites are distinct, with different genes being switched on and off as a result (Mohammed et al., 2015).

c. HER2 Receptor

Human epidermal growth factors (HER) belong to receptor tyrosine kinase family type I. They are one of peptide growth factor receptors that regulate growth and differentiation of both normal and breast cancer cells (Konecny et al., 2003; Slamon et al., 1987).

HER are receptors which can bind to epidermal growth factor (EGF), and hence activate their transmembrane kinase activity to promote downstream cellular signaling leading to cell growth, migration or adhesion (Holbro et al., 2003).

There exist 4 monomers of human epidermal growth factor receptor (HER-1,2,3,4), HER-2 heterodimerizes with other receptors in order to be functionally active (**figure 5**).

Slamon et al. in 1987 were the first to report the amplification of this oncogene (HER2/neu); they showed that it was increased by 2-20 folds in 30% of human primary breast tumors. Moreover, this gene amplification was significantly and inversely correlated with the patient overall survival and time to relapse. HER2/neu gene overexpression was better than the hormone receptors as a prognostic factor in lymph node-positive disease.

Nowadays, HER2 is targeted by blocking agents during treatments in order to stop its downstream signaling in breast cancer. Several studies demonstrated an association

between HER-2/neu overexpression and tamoxifen resistance in primary or advanced HR-positive tumors (Konecny et al., 2003); however, recently it was shown that treating patients with metastatic breast tumors that are HR-negative, HER2-positive with combination of anti-HER2 monoclonal antibodies, has significantly improved the overall survival in patients (Swain et al., 2015).

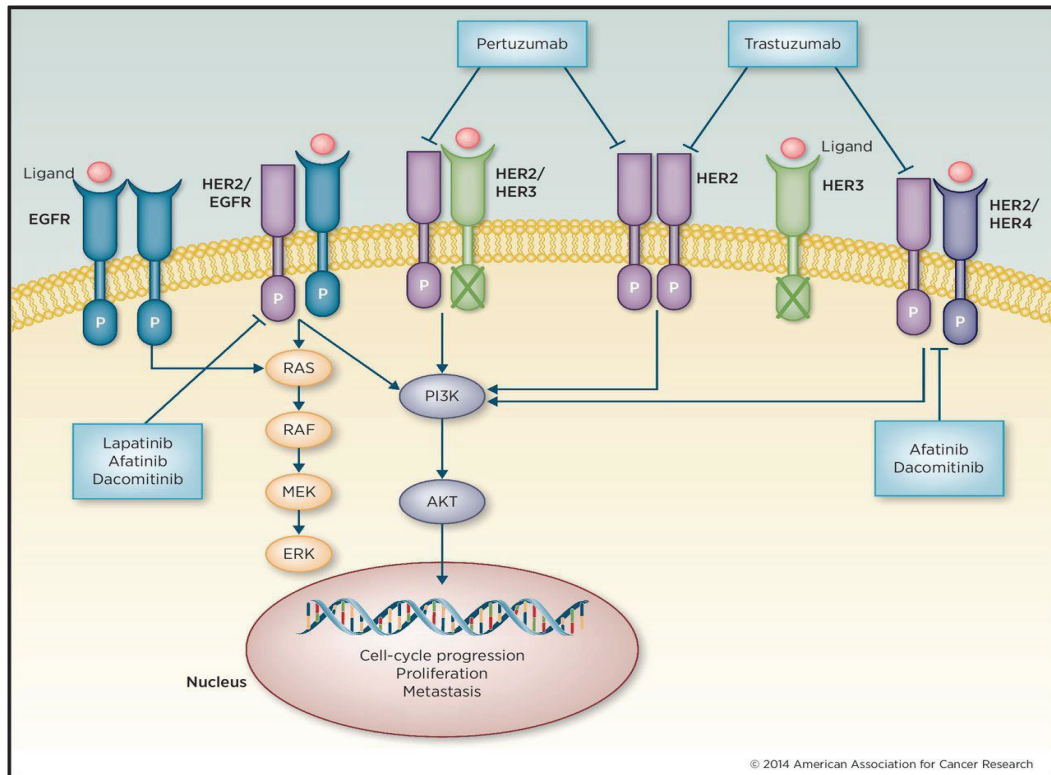


Figure 5: Schematic representation of HER2 receptor hybrids signaling and their blocking drugs. (source: American Association for Cancer Research)

4. Transcriptome Analysis

Advanced technology, high-throughput genomic, transcriptomic and cluster analyses; along with better understanding the molecular aspect of breast cancer, all together have led to detailed discrimination in breast cancer subtypes.

Sporadic breast tumors were initially classified into five main subtypes based on expression profiling patterns and clinical outcomes (Melchor et al., 2008; Sorlie et al., 2003).

In addition to the proposed classification of Perou et al. of breast cancer to 4 main “intrinsic” molecular classes; *luminal A*, *luminal B*, *HER2 positive* and *basal-like triple negative breast cancers*; *normal-like* and *claudin-low* were added to breast cancers subgroups.

- **Luminal-A cancers**, which represents about 50-60% of breast cancer subtypes, are mostly ER/PR-positive, histologically low-grade. Luminal subgroup of breast cancers usually express low molecular weight cytokeratins such as CK8/18 and genes associated with active ER pathway (Parker et al., 2009). Usually luminal A cancers have low SBR grade and good prognostic factor compared to other breast cancer subtypes. In addition, luminal A breast tumors show favorable response to hormonal treatments and a good survival rates.
- **Luminal-B cancers**, which are also mostly ER-positive but may express low levels of hormone receptors and are often histologically high-grade. This subgroup may be HER2 positive or negative, with strong expression of Ki-67 antigen (Dai et al., 2016; Eroles et al., 2012). Luminal B cancers constitute 10-20% of breast cancer subtypes. They are less differentiated, more aggressive, rapidly proliferating tumors, with higher SBR grade and worse prognosis compared to luminal A tumors (Geyer et al., 2009).
- **HER2-positive cancers**, which show amplification and high expression of the *ERBB2* gene and several other genes of the *ERBB2* amplicon. These subgroups correspond reasonably well to clinical characterization on the basis of ER and HER2 status, as well as proliferation markers or histologic grade. HER2-positive cancers constitute 15-20% of all breast cancer subtypes, and generally they are ER/PR-negative cancers with bad prognosis and associated with metastases (Pourteimoor et al., 2016).
- **Basal-like “triple-negative” breast tumors**, which mostly correspond to ER-negative, PR-negative, and HER2-negative tumors. It the most aggressive subtype of breast tumors that represents 10-20% of cancer cases. Concerning their expression profile, basal-like tumors show high expression of “proliferation signature genes” (Thorner et al., 2009). As their name indicates, basal-like breast tumors highly express basal epithelial cell markers and cell cycle regulators such as CK5,6,7,14,17, P-cadherins, CD44, in addition

to a variety of growth factor receptors such as EGFR, IGF-1R, HGFR, c-kit etc... (Sorlie et al., 2003; Sotiriou and Pusztai, 2009; Sotiriou et al., 2003).

Generally, this subgroup of cancer is linked to mutations in onco-suppressor genes such as P53. These “triple-negative tumors” have the highest SBR grade and bad prognosis associated with low overall survival.

- **Normal-like breast cancers**, these constitute a subtype that is not very well understood yet. Some studies linked it to fibroadenoma in which adipose tissue genes are amplified rather than epithelial tissue genes (Peppercorn et al., 2008; Pourteimoor et al., 2016). Although normal-like tumors show absence of ER, PR and HER2, but they share many molecular features with the luminal A subtype, including low proliferation rate; however, the normal-like subgroup exhibits improved prognostic behavior (Guedj et al., 2012; Kinalis et al., 2017).
- **Claudin-low breast tumors**, as their name infers, this type of breast tumors expresses low level of claudin proteins that are involved in intercellular adhesion and junction. Generally, this subgroup of breast cancers is closer to basal than luminal subtypes; where it has low to absent expression of luminal differentiation markers, enriched in mesenchymal markers expression and have high epithelial to mesenchymal transition (EMT) capacity (Sabatier et al., 2014). Claudin-low breast tumors have an intermediate prognosis between basal and luminal breast cancer (Prat et al., 2010).

In addition to the sporadic breast cancers (SBC), there exist the hereditary ones-the familial breast cancers (FBC)-which include tumors from patients carrying mutations in the two known genes for breast cancer susceptibility, *BRCA1* (Miki et al., 1994) and *BRCA2* (Wooster et al., 1995).

BRCA1 expression is important in DNA repair, activation of cell-cycle checkpoints, maintenance of chromosomal stability (Sotiriou and Pusztai, 2009), and germline mutations of *BRCA1* and *BRCA2* are strong predictors of breast cancer development (Fackenthal and Olopade, 2007)

Breast cancer susceptibility gene 1 (*BRCA1*) aberrant function is a distinctive feature of basal-like breast tumors (Turner et al., 2007). Dysfunction of *BRCA1* occurs either through its gene promoter methylation or through its transcriptional inactivation (Hedenfalk et al., 2001; Matros et al., 2005)

Generally, almost all breast cancers that are associated with *BRCA1* mutations, whether sporadic or hereditary cancers, have triple-negative phenotype. In addition, more than half of these cancers have basal-like tendency (Kreike et al., 2007; Rakha et al., 2007; Sotiriou and Pusztai, 2009).

Due to intensive studies of gene signatures of breast cancer cells, there has been recently some new subgroups of breast cancer classified. Based on signaling pathways, copynumber alterations, histopathological and clinical features, including metastatic sites and relapse free survival, Guedj et al. refined the taxonomy by introducing six stable molecular subtypes. They have added the molecular apocrine and luminal C subtypes instead of HER2-positive tumors.

- **Molecular apocrine breast cancers**, are characterized by positive expression of androgen receptor (AR). This subtype of cancers usually differ from luminal subtypes by negative expression of ER and PR (Farmer et al., 2005; Kinalis et al., 2017), but 72% of cancers in this subgroup (AR+/ER-/PR-) were overexpressing *ERBB2/HER2* gene with no co-amplified genes (Guedj et al., 2012).

Due to activation of androgen receptor, *MYC* oncogene, a target of AR signaling, displays a high functional outbreak leading to increased proliferation rate in this molecular subgroup (Pourteimoor et al., 2016).

- **Luminal C breast cancers**, are a subtype that highly expresses vimentin, the protein involved in cellular adhesion, mobility and mesenchymal identity. At the level of hormonal receptor expression, Luminal C was at the boundary between ER+ and ER- , and 40% of these tumors were overexpressing *ERBB2/HER2* gene (Guedj et al., 2012).
- **Interferon cancer subtype**, is characterized by high expression level of interferon-regulated genes such as *STAT1*, and consequently its down-regulated genes like *MYC* and *Pin1*. This leads to increased proliferation rate of this subtype similarly to luminal B cancer subtype (Hu et al., 2006).

E. Breast cancer treatments

Several approaches are used to treat breast cancer, which remains a complex and diverse disease. The major approach to treat breast cancer is the surgical removal of tumor tissue that might be coupled with radiotherapy. Besides these, there are systemic treatments such as chemotherapy or targeted treatments like hormonotherapy and other molecular targeted approaches. These treatments significantly affect breast cancer remission and survival.

1. Surgery

Breast cancer surgery is a local treatment to control the tumor. When the latter is too voluminous, a neo-adjuvant therapy is administered to shrink tumor volume before surgery. Two types of breast surgeries are currently practiced: tumorectomy whereby only the tumor and some surrounding breast tissue, and mastectomy where the whole breast tissue is removed. In both cases, sentinel lymph node is analyzed to evaluate if cancer cells have migrated through the lymphatic system. Following breast surgery, a systemic treatment can be proposed to decrease the risk of tumor reappearance or metastasis.

2. Radiotherapy

Radiotherapy generally follows breast cancer surgery. It helps in preventing local relapse. Radiotherapy can also be applied to axillary lymph nodes if they are present with tumor cells. In some cases, curietherapy can also be given to the patient where radioactive sources are implanted in the tumor core or around the tumor to target it directly.

3. Chemotherapy

Most commonly administered through intravenous way, chemotherapy is a way of destroying proliferating cells. They can be used alone or more frequently in combinations. However, these treatments are not specifically targeted to tumor cells. **Paclitaxel**, which forms part of the taxane family of molecules, inhibit microtubule depolymerization thereby leading to a mitotic catastrophe. **Cyclophosphamide**, which is an alkylating agent. In functions by alkylating DNA thus inhibiting replication. **Doxorubicin**, of the family of anthracyclins, which intercross with DNA and are Topoisomerase I inhibitors.

They also function through inhibition of DNA replication. **Fluoro-uracil**, an anti-metabolite that is incorporated into RNA molecules and leads to transcription failure and cell death. **Methotrexate**, which inhibits dihydrofolate reductase, thereby inhibiting folic acid synthesis and blocks mitotic cells in the S-phase.

4. Hormonotherapy

a. Anti-estrogens

They can be classified into two main categories, SERM and SERD. While selective estrogen receptor modulators (SERM) include **tamoxifen** and its analogs (**raloxifen**, **toremifen** and **arxoxifen**), can act as antagonists or agonists depending on the tissular context, selective estrogen receptor downregulators (SERD), pure anti-estrogens, are devoid of any agonist activity and induce ER α degradation. Majority of breast tumors express ER α and are defined to be hormone-dependant. Since their discovery in 1963, anti-estrogens have not been used clinically to treat breast cancer until 1970 (Lerner and Jordan, 1990), where tamoxifen was discovered. It became the standard treatment for targeted hormone-therapy (Lerner and Jordan, 1990). It reduced breast cancer relapse by 50% and reduces breast cancer related death by almost 35% annually.

SERMs, like tamoxifen for example, binds to ER α and induces a conformational change in the receptor structure which renders it only partially active, and thereby reduces its ability to induce gene expression (Jordan, 1994). Futhermore, tamoxifen bound ER α will recruit co-repressor complexes and histone deacetylases, which leads to transcriptional arrest.

SERDs or pure-antiestrogens have been discovered in 1987, and the molecule **Fulvestrant** or **ICI 182,780** is the most active one of them (Wakeling et al., 1991). It binds to ER α and prevents its nuclear import and directs it to the endopasmic reticulum where ER α is ubiquitinated and undergoes proteosomal degradation (Wakeling, 1991). This molecule has been used to treat metastatic hormone-dependent breast cancer where the use of tamoxifen has failed.

b. Anti-aromatases

To treat breast cancer, recent endocrine therapies have been used targeting the physiological synthesis of estrogens. Estrogen production relies mainly on the aromatization of endogenous androgens. Anti-aromatases such as **Anastrozole** or **Letrozole** target the enzyme responsible for this aromatization and reduce estrogen production in peripheral and adipose tissue (Simpson and Dowsett, 2002). These molecules have been used successfully in post-menopausal women and have a high anti-proliferative activity. Anti-aromatases are also used in anti-estrogen resistant breast tumors. Side effects include skeletal disorders such as osteoporosis.

5. mTOR inhibitors

The mammalian target of rapamycin (mTOR) signaling pathway integrates both intracellular and extracellular signals and serves as a central regulator of cell metabolism, growth, proliferation and survival. Numerous cellular stimuli can lead to mTOR pathway activation such as tyrosine kinase receptors, which constitute the canonical activation pathway, but also other stimuli such as genotoxic stress, inflammation and hypoxia. The mTOR protein serine-threonine kinase that belongs to the phospho-inositide 3-kinase (PI3K)-related kinase family. mTOR nucleates at least two distinct multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).

The different interactions in this signaling pathway give rise to a complex network in the regulation of cell growth. Deregulation of this pathway has been linked to breast cancer progression and is also linked to resistance to endocrine therapies. Inhibitors targeting mTOR pathway are of three generations.

- **The first generation** of inhibitors of the mTOR pathway has focused on the use of rapamycin, along with three rapamycin analogues: **temsirolimus**, **everolimus**, and **deforolimus**. In vitro, Rapamycin and its analogues bind to their intracellular receptor, FKBP12, and this complex binds to mTORC1 at the FKBP12-rapamycin binding domain (FRB domain) that is adjacent to the kinase domain. This leads to a decrease in phosphorylation of the mTORC1 effectors, 4EBP1 and S6K1; and thus, a decrease in cellular growth. Failure to inhibit mTORC2 is related to the fact

that the FRB domain in this complex of mTOR is not accessible to the rapamycin-FKBP12 complex (Zoncu et al., 2011).

- **The second generation of inhibitors** corresponds to Small-molecule inhibitors of mTOR kinase, which inhibit the kinase-dependent function of both mTORC1 and mTORC2. These agents have demonstrated the ability to block mTORC1 and mTORC2 effectors namely, S6K1 and Akt, respectively (Zoncu et al., 2011).
- **The third generation of inhibitors**, commonly named dual-inhibitors, have the property of inhibiting both the mTORC1/2 complexes, as well as PI3K, thus preventing any feedback from the PI3K pathway. **BEZ235**, is an inhibitor under development, is actually in Phase I and II trials, and has demonstrated anticancer properties especially in HER2+ cancers (Zhu et al., 2015).

6. Monoclonal antibodies

a. HER2 anti-bodies

HER2 amplification and overexpression in some breast cancers was clearly shown. Hence, a monoclonal antibody has been generated for the treatment of breast tumors expressing HER2. This antibody, **trastuzumab** or **Herceptin**, targets the extracellular domain of HER2 (Baselga et al., 1998). In fact, the antibody binding to the extracellular part of the receptor will inhibit receptor dimerization (Arnould et al., 2006), leads to its internalization and degradation, and will attract immune cells on the tumor site leading to ADCC. Herceptin has also been shown to inhibit the MAPK and PI3K/Akt pathways leading to cell cycle arrest. Other molecules targeting the ATP binding sites in the intracellular domains of HER2 have been developed, such as **Lapatinib**, which has proven to be efficient at targeting both HER2 and EGFR signaling (Rusnak et al., 2001).

b. VEGF anti-bodies

Vascular Endothelial Growth Factor (VEGF) is the main actor of angiogenesis in physiology and during tumoral neoangiogenesis, which is essential for tumor progression and metastasis (Presta et al., 1997). **Bevacizumab** (commercially known as **Avastin**) is a monoclonal antibody targeted to bind VEGF and prevent the fixation of the latter to its receptor, thereby inhibiting its action. Clinically, treatment with bevacizumab alone does not provide efficient results, but its efficiency increases when used in combination with chemotherapy. Patients with metastatic breast cancer benefited from this combination in terms of survival (Gerber and Ferrara, 2005).

F. Conclusion

Although to treat breast cancer, there are several kinds of treatments, and these depend on the subtype of the breast tumor and its classification. So far in clinics, only ER, PR and HER2 are targeted in breast cancer therapies; and this urges the current cancer research to develop optimal treatment conditions, avoiding any side-effects and blocking any “escape” possibility of hormone therapies that leads to the resistance of tumors and its recurrence with time.

II. The IGF Signaling System

A. Introduction

Insulin was the first discovered member of this signaling system in 1921, and then it was subsequently investigated by Frederic Bantling in its role in glucose metabolism and treating diabetes mellitus (Majumdar, 2001). In addition to insulin, the insulin-like growth factor (IGF) signaling family is comprised of, the two factors similar to insulin named IGF-I and II (Brahmkhatri et al., 2015). The existence of the IGFs was first proposed by Salmon and Daughaday in 1957 when they noticed that there is a hormone-controlled serum factor stimulating cartilage sulfation and replacing the “sulfation factor activity” of the growth hormone (GH) (Salmon and Daughaday, 1957). The terminology “insulin-like” was used because these factors are able to stimulate glucose uptake into fat cells and muscle, and, indeed, both IGF-1 and IGF-2 show approximately 50% homology with insulin (Blundell et al., 1983; Rinderknecht and Humbel, 1978). Insulin-like growth factors are natural growth hormones and play crucial role in normal growth, morphogenesis and development. Moreover, this system is a major player in the pathophysiology of atherosclerosis, cancer, obesity and diabetes (Clemmons, 2007a; Crudden et al., 2015)

All these factors directly regulate cellular functions by interacting with specific cell surface receptors and activating various intracellular signaling cascades. The cellular responses to insulin and to IGFs are mediated primarily by insulin receptor (IR) and IGF-1 receptor (IGF-IR) respectively. The IGF-1 receptor is a member of the family of tyrosine kinase growth factor receptors. In addition to the ligands and their receptors, there are soluble IGF binding proteins (IGFBPs) and IGFBP proteases regulating IGFs actions. The IGFBPs comprise a superfamily of six proteins (IGFBP-1-6) that bind to IGFs with high affinity and specificity and a family of IGFBP-related proteins (IGFBP-rPs), which are structurally similar to the IGFBPs but bind IGFs with much lower affinity (Brahmkhatri et al., 2015). **(Figure 6)**

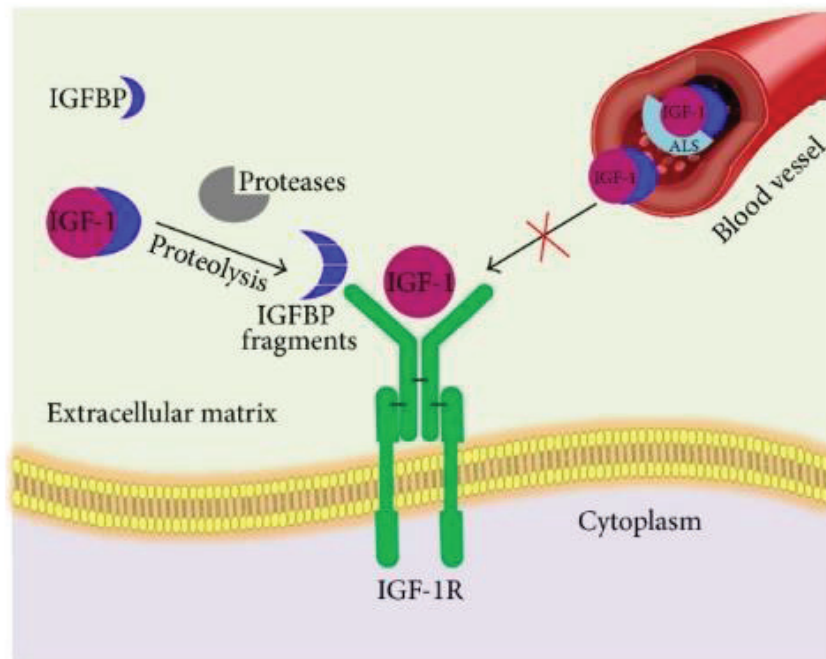


Figure 6: IGF signaling axis. Bound IGF in blood stream exists in a binary complex with IGFBP and/or ternary complex with IGFBP and ALS where IGF action is blocked (left), or free form after proteolytic action of proteases on IGFBP-IGF complex (right) where the free ligand can bind to IGF-IR and promote signaling actions. (*Brahmkhatri et al., 2015*)

B. IGF system components

1. Ligands

All the three ligands belonging to the insulin-like growth factor (IGF) family: Insulin, IGF-1 and IGF-II share structural similarities, although the IGFs are larger than insulin, 7.5kDa and 5.8kDa respectively, and contain two additional domains (Daughaday and Rotwein, 1989; Rinderknecht and Humbel, 1978; Smith and Ciszak, 1994). Mature IGF-I and IGF-II peptides consist of A, B, C, and D domains. The A and B domains of IGFs are homologous to the A and B domains of insulin, and the C domains of IGFs share sequence homology to the C peptide of proinsulin, which is cleaved off in mature insulin during prohormone processing. IGFs contain an additional D domain, which is not found in insulin (Adamo et al., 1993; LeRoith and Roberts, 1993).

IGF-I and IGF-II primarily regulate post-natal and fetal growth respectively, while insulin is a major regulator of metabolism and glucose homeostasis (Gluckman and Pinal, 2003; Saltiel and Kahn, 2001). The synthesis of IGF-I, IGF-II, and Insulin is regulated by distinct mechanisms

(Jones and Clemmons, 1995). Unlike insulin and other peptide hormones, IGFs are not synthesized and stored within specialized cells in a given tissue, but are ubiquitously produced and released by virtually every tissue. Insulin concentration *in vivo* is primarily gauged by blood glucose fluctuation.

IGF-I is mainly produced in the liver in response to growth hormone (GH) and released into circulation in order to regulate post-natal growth (Adamo et al., 1993; Clemmons, 2007a).

In addition to stimulating growth, IGF-I acts as a negative feedback regulator by inhibiting GH secretion at the level of the hypothalamus and pituitary (Giustina and Veldhuis, 1998). Although the majority of IGF-I is produced in the liver as part of the endocrine system, it is also produced in extra-hepatic tissues to function in an autocrine/paracrine fashion (Frystyk et al., 1994; Yakar and Adamo, 2012). IGF's are expressed ubiquitously and act in autocrine/paracrine manner through binding to the IGF-I receptor. The bioavailability of IGF in tissues is determined by both local and systemic factors such as the amount of receptors expressed, various IGFBPs and their respective proteases; these that specifically regulate the circulating levels of IGFs which are shown to be frequently overexpressed in cancer cell lines (**Figure 7**) (Brahmkhatri et al., 2015).

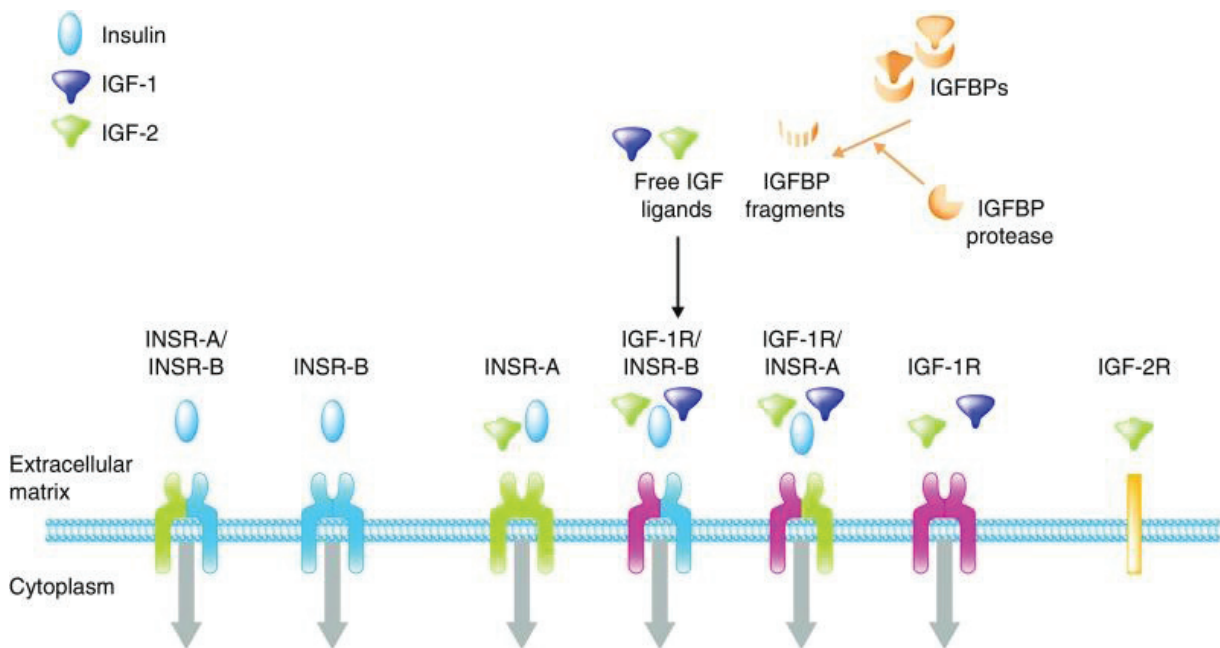


Figure 7: Insulin/IGF signaling system components. It consists of ligands (insulin, IGF-1 and IGF-2), receptors (IR-A, IR-B, IGF-1R, IGF-1R/IR and IGF-2R), IGFBPs 1-6 and IGFBP proteases. In the extracellular matrix, bound IGF-IGFBP complex are subject to proteolytic cleavage by IGFBP protease that renders the ligand free to bind to its receptor. Insulin binds to IR, IGF-1R and IGF-1R/IR hybrids; IGF-1 binds to IGF-1R and IGF-1R/IR hybrids; IGF-2 binds to IR-A, IGF-1R, IGF-1R/IR and IGF-2R. Ligand-receptor binding triggers glucose homeostasis and signaling cascades in the cytoplasm. (Simpson et al., 2017)

IGF-I expression can be also influenced by reproductive hormones, stress hormones (ACTH), as well as diet and nutrition (Dunn et al., 1997; Forbes et al., 1989; Han et al., 1988). Local IGF-I can be produced in response to injury; more specifically it is induced by epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) (Clemmons, 2007b).

Unlike IGF-I, IGF-II expression is not responsive to GH, but is regulated by genomic imprinting (Edén Engström et al., 2006). During fetal development, IGF-II is produced in a wide variety of somatic tissues. IGF-II is not produced in mice after weaning, however, in humans IGF-II production is continued into adulthood and is the prevalent IGF in circulation (Holly and Perks, 2012; Yakar and Adamo, 2012). Despite its lower concentration, IGF-I is still expected to be the primary functional IGF after birth, in part because IGF-I has a three-fold higher binding affinity for the IGF-IR than IGF-II (Denley et al., 2005); and because IGF-I production fluctuates based on hormonal control, surging during puberty, while post-natal IGF-II levels are relatively stable (Yu 2000). Beyond development, the role of IGF-II in human physiology is not well understood. The roles of IGFs in normal physiology are reviewed in more detail by Yakar and Adamo (2012), and Holly and Perks (2012).

At the genetic level, the expression of *IGF-I* and *IGF-II* genes is subjected to complex regulatory mechanism at the levels of transcription and translation, including, but not limited to, multiple promoters, different transcription initiation sites, alternative splicing, and different polyadenylation signals (Hall et al., 1992; Kim et al., 1991). For instance, IGF-II expression in human liver has been shown to be regulated by four promoters with three promoters used in the fetal liver and all four promoter used from the age of 2 months after birth (Li et al., 1996). In addition, *IGF-II* gene is imprinted - only one allele is active, depending on parental origin - and this pattern of expression is maintained epigenetically in almost all tissues (Chao and D'Amore, 2008).

IGFs are critical for normal growth in mammals and other vertebrates. *IGF-I* or *IGFII* knockout mice have 60% the birth weight of normal control mice. *IGF-I* knockout mice also have increased neonatal death rate and reduced postnatal growth rate if they survive. Mice with null mutations in both *IGF-I* and *IGF-II* die invariably after birth (Baker et al., 1993; Liu et al., 1993). Compared with *IGF-I* knockout mice, *GH/IGF-I* double-knockout mice have more dramatically reduced body

growth weighing only approximately 17% of normal mice (Lupu et al., 2001). Therefore, the GH-IGF pathway is the main determinant of the body growth.

IGFs are also important in the development and function of the central nervous system (CNS), skeletal muscle, and reproductive organs. In humans, a homozygous partial deletion of *IGF-I* gene is associated with mental retardation and sensorineural deafness, in addition to prenatal and postnatal growth retardation (Camarero et al., 2002). On the other hand, IGF-I expression in the central nervous system positively was correlated with increased brain growth, promoted neurogenesis, process outgrowth and synaptogenesis, and inhibited neuronal apoptosis (D'Ercole et al., 2002).

Studies have shown that reduced circulating IGF-I levels are associated with Type I diabetes, and IGF-I treatment improves glucose and protein metabolism and attenuates diabetic cardiomyopathy (Carroll et al., 2000; Norby et al., 2002). Moreover, overexpression of IGF-I in mouse pancreatic β cells specifically leads to improvement of type I diabetes (George et al., 2002); also, IGF-I treatment increases insulin sensitivity and improves glycemic control in patients with type 2 diabetes (Moses et al., 1996).

In addition, IGF-I has been shown to have beneficial effects on bone, muscle and neuronal tissues; where overexpression of IGF-I in the osteoblasts of transgenic mice leads to improved bone structure, including increased bone density and mineralization (Zhao et al., 2000). IGF-I overexpression in skeletal muscle tissue caused muscle hypertrophy and sustained regenerative capacity (Barton-Davis et al., 1998; Musarò et al., 2001).

2. Receptors

a. The IGF-I receptor

At the cellular level, IGFs induce a variety of cellular responses, including cell proliferation, differentiation, migration, and survival. IGFs exert these biological actions primarily through binding the receptor tyrosine kinase IGF-I receptor (IGF-IR) (**Fig. 7**). The IGF-IR pro-receptor is cleaved by furin into α - and β -subunits, 135kDa and 95kDa respectively (Czech and Massague, 1982; Ward et al., 2001). The IGF-IR has two α -subunits forming the extracellular ligand binding domain (Garrett et al., 1998), and two β -subunits containing the transmembrane and intracellular tyrosine kinase domains (Pautsch

et al., 2001) linked by disulfide bonds to form a functional receptor (Adams et al. 2000). The α -subunit contains a cysteine-rich IGF binding site and the β -subunit has tyrosine kinase activity, which depends on an ATP binding and tyrosine phosphorylation. Binding to ligand will induce conformational changes of the receptor and facilitate tyrosine autophosphorylation, and eventually leading to receptor activation (Hubbard and Till, 2000). Due to its functional importance, the tyrosine cluster in the β subunit has been subjected to intense study (Cianfarani et al., 2007).

Ligand binding of the IGF-IR α -subunits induces its autophosphorylation at the key tyrosine (Y) triplet residues Y1131, Y1135 and Y1136 in the activation loop of the IGF-1R, leading to transphosphorylation of the opposing β -subunits (Hubbard and Till, 2000). The activated IGF-IR in turn activates multiple signal transduction cascades; including the phosphatidylinositol 3-kinase (PI3K)-Akt cascade and the Raf- mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (MEK)-extracellular signal-regulated kinase (ERK1/2) cascade (Coolican et al., 1997; Duan et al., 2000; Imai and Clemmons, 1999).

After phosphorylation of the key triplet tyrosines in the activation loop, other tyrosine residues are autophosphorylated enhancing IGF-1R activity. For example, phosphorylation of Y950 in the juxtamembrane region generates docking sites for adaptor proteins that recognize specific sequences containing the phosphorylated tyrosines, the P-Y1250/P-Y1251 in the kinase domain serve in anchorage-independent growth, migration and invasion, and P-Y1346 in carboxy-terminus of the beta subunits (Kooijman, 2006; Kurmasheva and Houghton, 2006). In addition to the tyrosine residues, mutations in serine (S) residues 1280-1283 (S1283 being phosphorylated during the signaling process) have been shown to abrogate the transforming ability of the IGF but still maintain mitogenicity (Li et al., 1996).

Steroid hormones and growth factors regulate the expression of IGF-1R (Sepp-Lorenzino, 1998; Stewart and Rotwein, 1996). Since high IGF-1 levels result in a low levels of IGF-1R, IGFs may act as negative feedback signals to suppress expression of IGF-1R (Hernández-Sánchez et al., 1997; Yang et al., 1996). In contradiction of the effect of IGFs, other growth factors, including basic FGF, PDGF and EGF, stimulate IGF-1R expression (Rosenthal et al., 1991; Rubini et al., 1994). Estrogens, glucocorticoids, GH, FSH,

lutinizing hormone, and thyroid hormones also stimulate the expression of IGF-1R (LeRoith et al., 1995). On the other hand, tumour suppressor gene products, such as wild type p53 protein and WT1 (Wilms' tumour protein), inhibit expression of IGF-1R (Prisco et al., 1997; Werner, 1998; Werner et al., 1993). IGF-1R levels are also affected by nutrition (Qu et al., 1997; Zhang et al., 1998).

The IGF-1R is a key component mediating IGF functions *in vivo*. Due to the ubiquitous expression pattern, nearly every tissue is adversely affected by a lack of IGF function, including skeletal muscle, bone, lung, skin, and nervous system (Allan et al., 2001; Bondy et al., 1990; Jones and Clemmons, 1995). For example, mice lacking a functional IGF-1R gene exhibited even more severe growth retardation, weighing only 45% that of their wild type littermates, and these mice died shortly after birth from respiratory failure (Baker et al., 1993; Liu et al., 1993). Conditional knockout is used to further characterize the IGF-1R function in individual tissues and has yielded detailed information with IGF function in specific tissue and development. Selectively disrupting *IGF-1R* gene in mouse osteoblasts caused a striking decrease in bone volume, connectivity, and trabecular number, which is also accompanied by a significant decrease in the rate of mineralization (Zhang et al., 2002).

Deletion of the *IGF-1R* in pancreatic β cells caused defects in glucose-stimulated insulin secretion and impaired glucose tolerance (Kulkarni, 2002). IGF-1R inactivation in the brain impaired remyelination in response to neurotoxicant induced demyelination (Mason et al., 2003). Conditional IGF-1R inactivation in adipose tissue did not affect adipogenesis and instead resulted in increased adipose tissue mass. Insulin-stimulated glucose uptake into adipocytes was unaffected by the deletion of the *IGF-1R*. Surprisingly, *IGF-1R* deletion in adipose tissue led to elevated IGF-I concentration in circulation and had a systemic effect on somatic growth (Klötting et al., 2008). *IGF-1R* conditional knockout in the liver decreased the capacity for regeneration and revealed IGF-1R/IRS-1/ERK signaling cascade as the intracellular pathway controlling the cell cycle progression in the regenerating liver (Desbois-Mouthon et al., 2006). Of note, the *in vivo* function of the IGF-1R is evolutionarily conserved, as studies in zebrafish have generated similar results. Schlueter

et al. showed IGF-IRs in zebrafish are required for embryo viability and proper growth, and especially the development of eye, inner ear, heart, and muscle (Schlueter et al., 2006).

Further study, using antisense morpholino oligonucleotides (MO) or a dominant-negative IGF-IR fusion protein, revealed the cellular actions of this essential pathway during vertebrate embryogenesis. At the cellular level, IGF-1R inhibition increased caspase activity and neuronal apoptosis. Coinjection of antiapoptotic bcl2-like mRNA attenuated the elevated apoptosis. Cell cycle analysis demonstrated cell cycle progression defects in IGF-IR-deficient embryos independent of apoptosis (Schlueter et al., 2007).

b. IGF-IR signal transduction and function

Signaling pathways are common to many growth factors' receptors, including the IR; however, slight differences in the recruitment and activation of intracellular mediators allow for specific effects of each receptor (Boucher et al., 2010; Frasca et al., 2008).

The engagement of the various downstream signaling cascades is mediated through the interaction of the activated IGF-1R with adaptor proteins including Src homology collagen (Shc) proteins (p46/p52/p66) and insulin receptor substrate (IRS) (Dupont and LeRoith, 2001). (**Figure 8**)

Shc is composed of three distinct domains containing an amino-terminal region called the phosphotyrosine binding (PTB) domain, a collagen homology (CH) domain, and a carboxyl-terminal Src Homology 2 (SH2) domain. Shc has been shown to be important primarily in the process of the activation of p21^{ras}-MAPK, which plays a pivotal role in mitogenic signal transduction initiated by receptor tyrosine kinases, including the IGF-1 receptor (Sasaoka et al., 2001). As an example, upon IGF-1 stimulation, phosphorylation of Y950 in the juxtamembrane domain of the IGF-1R forms a “docking” site to recruit and phosphorylate the SH2 domain-containing Shc adaptor protein (Kurmasheva and Houghton, 2006; Tartare-Deckert et al., 1995). This binding leads to the phosphorylation of Tyrosines 239, 240 and 317 in the CH domain of Shc, that leads to its association with the adaptor protein growth factor receptor-bound protein 2 (GRB2), which forms a complex with SOS, a p21^{ras} guanine nucleotide exchange factor (Sasaoka et al., 1996). Shc-Grb2 binding is mediated by the SH2 domain of Grb2 binding to phosphorylated tyrosine residues within the CH domain of Shc (Salcini et al., 1994).

Ishihara et al. stated that the Tyr-317 is most likely to be the main effector tyrosine in the activation of the RAS/RAF/MEK/ERK pathway through Shc- GRB2 recruitment and binding. (Ishihara et al., 1998a).

Activated RAF in turn phosphorylates the MEK dual-specificity serine/threonine and tyrosine kinases, which then phosphorylate and activate the serine/threonine kinases, ERK-1 and ERK. Activation of the ERKs leads to both phosphorylation of cytoplasmic substrates and nuclear translocation and activation of various transcription factors (e.g., c-Myc, Ets factors, CREB, AP1) that control the expression of many genes. Ultimately, this signaling cascade culminates in the generation of pro-proliferative and anti-apoptotic effects in many cell types (Kolch, 2000).

For example, one of the cytoplasmic substrates of ERK includes procaspase-9 - the zymogen form of the pro-apoptotic caspase-9 protein; the phosphorylation of procaspase-9 at threonine 125 by ERK has been demonstrated to prevent the conformational change of the proenzyme to active caspase-9, thus enhancing cellular survival (Allan et al., 2003).

GRB2 can also be recruited by the insulin receptor substrate (IRS)-1, another IGF-1R adaptor protein (White, 1998). In addition to the RAS/RAF/MEK/ERK signaling cascade, activation of the IGF-1R can engage the PI3K/AKT pathway through the adaptor protein IRS-1. It is one of the well-established primary substrates that has been extensively studied (Lavan et al., 1997; Sun et al., 1991). IRS-1 is a large protein (>1200 a.a) that is composed of pleckstrin homology (PH) that acts as a module linking it to the IGF-1R, and phosphotyrosine-binding (PTB) domain that acts as a docking site of IRS-1 to either phosphorylated Y950 or the Y1131, Y1135 and Y1136 triplet motif of the IGF-1R (Hennessy et al., 2005; Kooijman, 2006; White, 1998). IRS-1 is then phosphorylated by the IGF-1R at its long carboxyl-terminal tail region on the tyrosines 612 and 632. Since IRS-1 acts as a multisite “docking” protein by binding to downstream signal-transducing molecules; It recruits to its binding motif (pYXXM) the p85 regulatory subunit of phosphatidylinositol-3 kinase (PI3K), which then activates its p110 catalytic subunit (Coppes and White, 2012). Activated PI3K phosphorylates membrane-associated phosphatidylinositol 4,5 phosphate (PIP₂) to generate phosphatidylinositol 3,4,5 phosphate (PIP₃), in turn resulting in membrane localization of phosphatidylinositol-dependent kinase

(PDK)-1 (Hennessy et al., 2005). PDKs then activate other protein kinases including Akt/Protein Kinase B, p70S6, and protein kinase C (PKC) (Cianfarani et al., 2007). PDK1 which in turn will phosphorylate Akt at Thr308; additional phosphorylation of Akt at Ser473 by mTORC2 fully activates Akt (Mora et al., 2004).

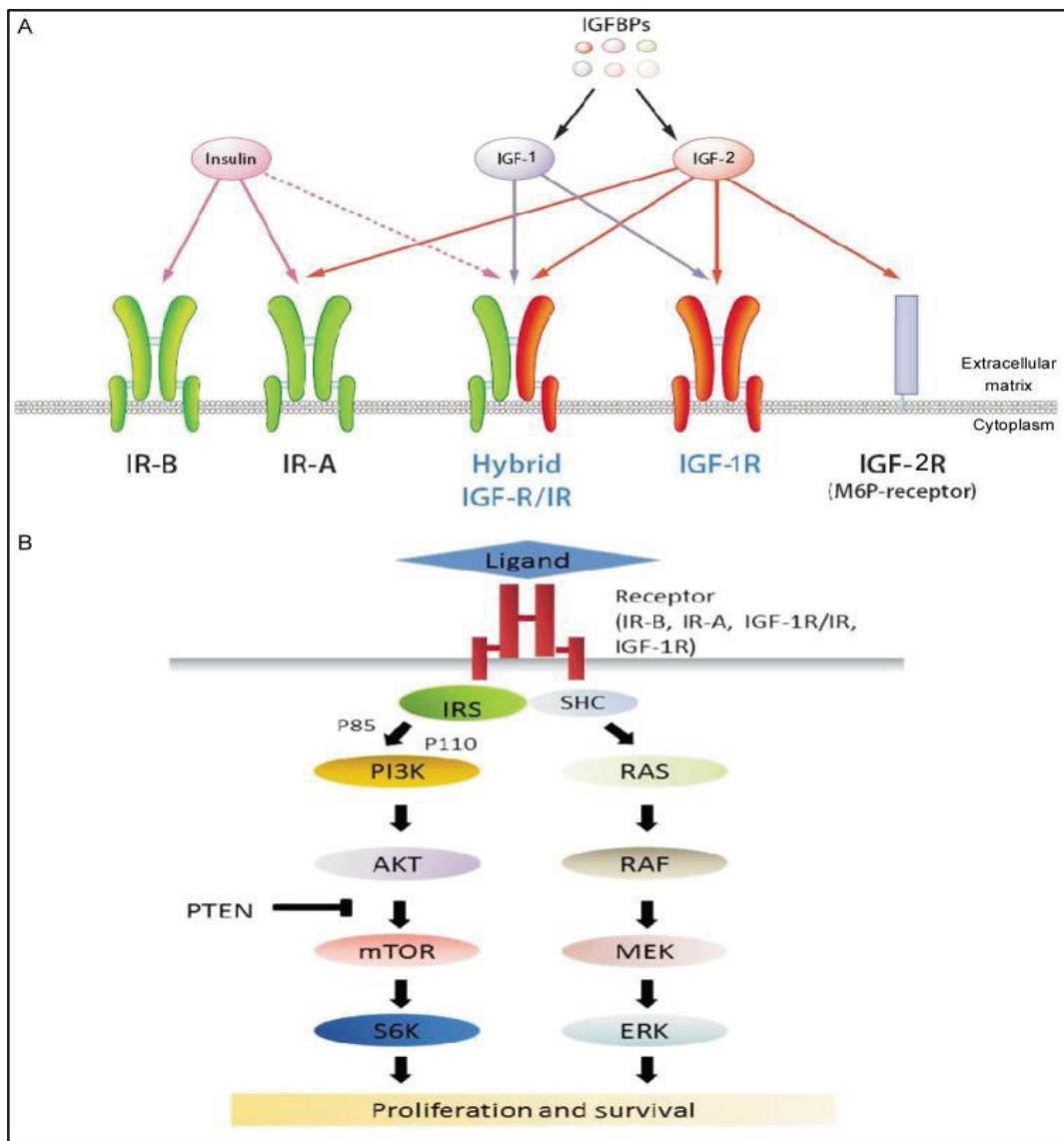


Figure 8: IGF signaling system. **A:** IGF binding proteins control the free form of IGFs, where free ligands bind to their corresponding receptors IGFR, IR and hybrid IGFR/IR. **B:** Binding of ligand to the receptor triggers the signaling of PI3K/Akt/mTOR and Ras/Raf/ERK pathways through adaptor proteins IRS1 and Shc respectively. (Chen and Sharon, 2013)

Another important component of this pathway is the mammalian target of rapamycin (mTOR). Akt phosphorylates TSC2, which in complex with TSC1 relieves its inhibitory effect on the G-protein Rheb allowing activation of mTOR complex 1 (mTORC1). The mTORC1 increases protein synthesis and promotes cell growth and cell cycle progression primarily through the effectors p70S6K and 4E-BP1 (Manning and Cantley, 2007).

IGF-stimulated cell cycle progression from G1 to S phase is promoted by an increase in cyclin D1 expression (Samani et al., 2007). IGF caused an increase in cyclin A and B as well as cyclin dependent kinases, which are involved in the G2 to M phase transition (Furlanetto et al., 1994). IGF-1 induces an Akt-dependent upregulation of MDM2, which can be activated by phosphorylation by Akt and is an ubiquitin ligase responsible for degradation of the tumor suppressor p53 (Du et al., 2013; Mayo and Donner, 2001).

Akt is also involved in cell cycle progression through the modulation of DNA repair and their checkpoints (Trojanek et al., 2003). Also, it is an important mediator of IGF-induced cell survival. Akt directly inhibits pro-apoptotic proteins Bcl-2 family member BAD, the transcription factors forkhead box proteins (FOXO-1,3,4), and caspase 9 (Kulik et al., 1997; Schmidt et al., 2002). Furthermore, Akt signaling increases expression of anti-apoptotic proteins Bcl-2, Bcl-XL (Minshall et al., 1997; Párrizas et al., 1997). Akt increases activation of the pro-survival transcription factors NF- κ B (through IKK phosphorylation) and CREB (Girnita et al., 2014).

In addition to regulating proliferation and survival, Akt regulates cell metabolism. Glycogen synthase kinase-3 (GSK-3) inhibition by Akt leads to increased glycogen synthesis and glycolysis (Manning and Cantley, 2007).

IGFs promote migration and invasion through the above mentioned pathways by increasing the expression of proteins that will influence cell adhesion and the microenvironment, such as matrix metalloproteinases and VEGF (Zhang et al., 2002). The IGF-IR can influence migration beyond the classical signaling through cross-talk with integrins and focal adhesion kinases (FAKs), which can be mediated by IRS-1 or Gab1/Shp2 (Girnita et al., 2014; Goel et al., 2004).

c. Insulin Receptor

Alternative splicing of exon 11 produces two variants of the insulin receptor, IR-A (exon 11-) and IR-B (exon 11+) (Benecke et al., 1992). Exon 11 corresponds to a 12 amino acid sequence at the c-terminus of the α -chain; therefore, these isoforms will exhibit different ligand affinities. The IR-A and IR-B monomers can form homodimers or heterodimers containing both isoforms. While both IR isoforms have a high affinity for insulin, IR-A has a relatively high affinity for IGF-II as well (Denley et al., 2006; Frasca et al., 1999). Both IR isoforms express a low affinity for IGF-I (Frasca et al., 1999; Sciacca et al., 2003). The difference in ligand affinities combined with differential expression of the isoforms indicate varying function, with the mitogenic IR-A predominantly expressed in fetal tissues (Frasca et al., 1999) and metabolic IR-B highly expressed in classically insulin-responsive adult tissues (Benecke et al., 1992; Sesti et al., 1994).

d. IGF-IR/IR hybrids

The IGF-IR and the IR share 60% structural similarity (Benyoucef et al., 2007). More specifically, they share 45-65% structural similarity in the ligand binding domain and 60-85% in the tyrosine kinase domain and substrate recruitment domain (Mynarcik et al., 1997; Ullrich et al., 1986; Whittaker et al., 2001; Yip et al., 1991). There are major differences in the α -subunits which result in differences in ligand binding (Lawrence et al., 2007).

The IGF-IR responds to ligand stimulation by IGF-I and II, and although the IGF-IR can bind insulin, this low affinity interaction typically does not occur at physiologic concentrations (Pandini et al., 1999). Similar structure between IR and IGF-IR renders any IR isoform able to dimerize with an IGF-IR protein monomer to form a functional hybrid receptor (Benyoucef et al., 2007; Pandini et al., 2002).

Ligand binding properties of hybrid receptors were evaluated by radio ligand competition using BRET. Results revealed that IGF-IR/IR hybrids containing either IR isoform had a relatively low affinity for insulin compared to IR homodimers and the presence of hybrid receptors decreased insulin stimulated IR activation. Alternately, the hybrid receptors bound IGF-I and IGF-II with a similar affinity to the IGF-IR irrespective of splice variants (Benyoucef et al., 2007). As expected, these hybrid receptors were activated more strongly

by IGF-I than insulin. Similar results were seen in purified hybrid receptors (Slaaby et al., 2006). In contrast, another study using semi-purified receptors reported differential ligand activation of hybrid receptors containing IR-A or IR-B (Pandini et al., 2002). This could be due to differences in hybrid receptor purification and unintended capture of IR homodimers. Given the significant structural similarity between IGFs and insulin, and their respective receptors, it is not surprising that cases of cross-activation and hybrid receptors have previously been reported, though the functional importance of these interactions remains elusive (Soos et al., 1990; Taguchi and White, 2008).

e. The IGF-II receptor

The IGF-IIR is a membrane-bound truncated receptor that preferentially binds to IGF-II, and it has about 100 times more affinity to IGF-II than IGF-I, with no binding affinity to insulin (Ewton et al., 1987; Lee et al., 1986; Tong et al., 1988). The IGF-IIR binds and targets IGF-II with high affinity for lysosomal degradation without inducing a specific cellular response, as it has no intrinsic tyrosine kinase activity (Kornfeld, 1992). Mouse knockout models of the IGF-IIR suggest that it mainly functions as a sink for IGF-II to prevent overgrowth during fetal development (Wylie et al., 2003), through receptor internalization and subsequent degradation (El-Shewy and Luttrell, 2009; Scott and Firth, 2004).

The extracellular domain of the receptor, which is composed almost exclusively of cysteine-based repeats, disassociates upon proteolytic cleavage from the cell membrane as a soluble fragment, circulates in the blood with the ability to bind to IGF-2 facilitating its degradation (Costello et al., 1999; Kiess et al., 1987; Zaina and Squire, 1998). These receptors, additionally to the IGF-BPs, provide an extra control on the circulating levels of IGF-II.

The IGF-IIR also acts as a mannose-6-phosphate (M6P) receptor and is distinct from the IGF-IR both structurally and functionally. The IGF-IIR will, however, bind proteins with a mannose-6 phosphate (M6P) motif, such as transforming growth factor-beta (TGF- β), and is often referred to as the IGF-II/M6P receptor (El-Shewy and Luttrell, 2009). Loss of the imprinted IGF-II/M6P receptor results in fetal overgrowth and perinatal lethality (Lau et al., 1994). Interestingly, studies showed that IGF-II binding to IGF-II/M6P receptors

stimulates the ERK/MAPK cascade by triggering sphingosine kinase (SK)-dependent transactivation of sphingosine-1 phosphate (S1P) receptors (El-Shewy et al., 2007).

IGF treatment promoted translocation of SK from the cytosol to the plasma membrane and thereby caused a significant increase in S1P concentration, which is essential for IGF-stimulated ERK/MAPK activation. Endogenous IGF-IR and IGF-IIR can independently initiate this signaling pathway. Knockdown of IGF-IR expression by siRNA reduced the IGF-I response largely than the IGF-II induced response. In contrast, IGF-IIR knockdown markedly reduced IGF-II-stimulated ERK phosphorylation, with no effect on the IGF-I-induced response. El-Shewy et al. further demonstrated that PKC mediates IGF-II activated ERK/MAPK phosphorylation via SK activation (Lee et al., 2008).

3. IGF binding proteins

IGF-1 circulates in relatively high concentrations in plasma, approximately 150–400 ng per mL, where it mostly exists as the protein-bound form. The free ligand concentration is very little that is less than 1%. IGFs in circulation are protected from degradation by forming a complex with a family of high affinity IGF binding proteins (Firth and Baxter, 2002). There are six high-affinity insulin-like growth factor binding proteins (IGFBP1-6) that mainly function to regulate IGF bioavailability (Hjortebjerg and Frystyk, 2013; Scagliotti and Novello, 2012). IGFBPs function as carrier proteins for circulating IGFs and regulate IGF turnover, transport and tissue distribution, thus determining physiological concentrations of IGFs (Jones and Clemmons, 1995). Moreover, IGFBPs can bind to ligands in circulation in order to stabilize them and prolong their half-lives while they are transported to tissues for action (Frystyk et al., 1999; Guler et al., 1989).

In addition, compared to insulin, IGF is present in higher concentrations in extracellular body fluid. However, IGF predominantly exists in complex with IGFBP. Thus, the IGF/IGFBP complexes in circulation and tissues help to prevent potential hypoglycemic effect generated by cross-binding of IGFs to the insulin receptor (Rajaram et al., 1997).

IGFBP-3 is the most abundant binding protein in circulation with 70-80% of circulating IGFs bound in complex with IGFBP-3 and the acid-labile subunit (ALS) (Baxter et al., 1989; Yu et al., 1999). At these target sites, IGFBPs can bind to IGFs essentially sequestering them and preventing ligand binding and subsequent receptor activation (Yu and Berkel, 1999). IGFs are released from the IGFBP complex by proteolysis of IGFBP by enzymes such as matrix metalloproteinases

(Nakamura et al., 2005), by IGFBP binding to components of the extracellular matrix (ECM), or by phosphorylation of IGFBP (Firth and Baxter, 2002). IGFbps (1,2,3, and 5) are dual acting and can both potentiate or inhibit IGF action in a tissue and context specific manner (Duan and Xu, 2005). Furthermore, there is evidence supporting IGF-independent activity of IGFbps (1,2,3 and 5) regulating migration, apoptosis, proliferation and differentiation (Ammoun et al., 2012; Yamanaka et al., 1999); therefore IGFbps have important role in cancer progression. (**Table 3**)

Table 5: IGFBP expression sites and their inhibition effect on cancer cases.

IGFBPs	Expression	Results of inhibiting IGFBP activity
IGFBP-1	Liver	It can induce or inhibit the IGF actions in many types of cells. As an example of the inhibiting activity of IGFBP-1, it inhibited IGF-I-induced growth in MCF-7 breast cancer cells.
IGFBP-2	Liver, adipocytes, reproductive system, and central nervous system	IGFBP-2 level changes were associated with the development of different types of cancer including breast and prostate cancer. In prostate cancer, high level of serum IGFBP-2 was associated with low grade prostate cancer.
IGFBP-3	Circulating carrier protein, expressed in many tissues	IGFBP-3 plays important role in different types of human cancers. IGFBP-3 can induce apoptosis by increasing the ratio of proapoptotic to antiapoptotic proteins in breast cancer cells.
IGFBP-4	Liver, bone tissue, and muscles	IGFBP-4 showed a strong inhibitory effect on IGF-1 by preventing the activation of the IGF-1R, when the IGFBP-4 is found in the tissue. Conversely, intravenous administration of IGFBP-4, in the presence of a protease, will promote cellular proliferation.
IGFBP-5	Mammary glands	In breast cancer, IGFBP-5 induced apoptosis and inhibited cellular differentiation in an IGF-dependent manner.
IGFBP-6	Epithelial layer of human bronchial organ	It can inhibit IGF-2 activity mediated through the IGF-1R, including proliferation, differentiation, migration, and survival in different cell lines.

(Brahmkhatri et al., 2015)

IGFBP regulates the endocrine functions of IGF by increasing the halflife as well as facilitating the transportation and tissue distribution of IGF ligand. For example, the ternary complex formed between IGF, IGFBP-3 and ALS is the major existing form of IGF in circulation (Jones and Clemmons, 1995). IGFBP-1 is predominantly expressed in liver. Its expression is subjected to nutrient and hypoxic stress regulation (Kajimura et al., 2005; Seferovic et al., 2009).

In addition to the endocrine function, IGFbps also modulate IGF autocrine and paracrine activity in local tissues. The expression of most IGFbps, including IGFBP-2 to -6, is detected in many peripheral tissues. In culture, most mammalian cells express more than one IGFbps. IGFbps bind to IGFs with high affinity, and are subjected to a variety of post-translational regulations that play important roles in regulating IGFBP actions, including proteolysis, phosphorylation, and

glycosylation. Therefore, they have been postulated as local regulators for paracrine and autocrine IGF actions. The six IGFBPs exhibit diverse actions in modulating IGF actions, including cell proliferation, differentiation, survival, and migration (Firth and Baxter, 2002; Jones and Clemmons, 1995). IGFBP can both inhibit and potentiate IGF actions, depending on specific cellular context and experimental conditions (Firth and Baxter, 2002).

For example, IGFBP-2 and IGFBP-4 both inhibit IGF-I-stimulated DNA synthesis and cell migration. IGFBP-5 has an inhibitory effect on IGF-I-stimulated DNA synthesis, but it strongly potentiates IGF-I induced cell migration (Hsieh et al., 2003). Some IGFBPs have been shown to have intrinsic biological activities that are IGF-independent. It was reported that the binding of IGFBP-1 to integrins via its RGD motif stimulates cell migration (Jones et al., 1993).

Post-translational modification of IGFBPs in turn affects their stability, binding capacity to IGF ligands, and association with cell surface, which therefore is subjected to intense investigation. Three potential N-glycosylation sites (Asn-X-Ser/Thr) have been located in the L-domain of the human IGFBP-3 sequence (Asn89, Asn109, and Asn172). Single and combinational mutants of these sites are generated. Mutations of these glycosylation sites, however, affect neither ligand binding nor ALS binding. It is proposed that glycosylation in IGFBP-3 may regulate the turnover rate and proteolysis of IGFBP-3 (Firth and Baxter, 2002). IGFBP-6 is O-glycosylated. The glycosylation sites of IGFBP-6 have been identified as Thr126, Ser144, Thr145, Thr146, and Ser152. Glycosylated IGFBP-6 exhibited greater resistance to proteolysis by chymotrypsin and trypsin than non-glycosylated IGFBP-6 (Neumann et al., 1998). IGFBP-1 and -3 have been shown to be phosphorylated on serine residues (Coverley and Baxter, 1997; Hoeck and Mukku, 1994). IGFBP-1 phosphorylation has been shown to be physiologically important (Jones et al., 1991).

4. IGFBP Proteases

IGFBP proteases belong to a superfamily of proteases with specificity towards IGFBPs, thereby regulating the action of IGFBPs. These proteases are prime factors in modulating the levels of IGFBPs and ultimately the bioactivity and downstream actions of IGFs (Yamada and Lee, 2009). IGFBP proteases broadly fall into three major super families-serine proteinases (kallikrein-like serine protease), matrix metalloproteinases (MMPs), and cathepsins (Rajah et al., 1995; Romero et al., 2011).

Proteolytic cleavage directly controls IGFBP abundance and releases IGFs from the IGFBP-IGF complexes (Bunn and Fowlkes, 2003). Detection of IGFBP fragments in circulation and other biological fluids, as well as various conditioned media from *in vitro* cultured cells, supports that physiological importance of IGFBP proteolysis as a means of regulating IGFBP availability. Some IGFBPs bind to components of the extracellular matrix (IGFBP-2, -3, and -5) or the cell membrane (IGFBP-1, -2, -3, and -5), thus providing a potential mechanism to concentrate IGF activity within discrete regions (Clemmons, 1998). Most of the proteolytic sites are identified in the L-domain of IGFBPs and the proteolytic fragments have greatly reduced or no affinity for IGFs (Firth and Baxter, 2002). Some of the proteases responsible for IGFBP proteolysis have been identified, including plasmin, thrombin, complement protein 1s (C1s), metalloproteinases (MMPs), pregnancy-associated plasma protein A (PAPP-A), and PAPP-A2 (Bunn and Fowlkes, 2003). Complement components C1r and C1s secreted by human fibroblasts have been purified and identified as IGFBP-5 specific proteases, as no protease cleavage activity is detected against IGFBP-1 (Busby et al., 2000). The functional importance of IGFBP proteolysis has been demonstrated by *in vivo* studies (Nichols et al., 2007; Ning et al., 2008).

IGFBP proteolysis may be directly involved in disease pathogenesis. For example, infusing protease-resistant form of IGFBP-4 significantly inhibited IGF-I actions and cell proliferation (Nichols et al., 2007).

This seems to show that proteases play a significant role in tumor progression and tumor cell survival considering the autocrine-paracrine actions in the IGF axis. Thus, IGFBP proteases have potential clinical implications in cancer research (Brahmkhatri et al., 2015).

C. Therapeutic strategies targeting IGF system in Cancer

Therapeutic strategies targeting various components of the IGF system, with varying degree of success, have been developed for treatment of different types of cancer (Brahmkhatri et al., 2015). IGF system was reported to be involved in progression and development of several cancers such as gastrointestinal, gynecological, lung, prostate, and breast cancers. Different changes in the IGF system, such as overexpression of IGF-I and IGF-II, alterations in receptor expression, and reduction of circulating IGFBP levels, contribute to tumor promotion (Farabaugh et al., 2015). Furthermore, the association of increased serum levels of IGF-I with the risk of development and

mortality of cancer has been observed in several cancer types (Samani et al., 2007; Wu and Yu, 2014). However, IGF signaling in tumor cells is driven by the presence of the ligands rather than receptor aberrations (Riedemann and Macaulay, 2006), and there is evidence that a low circulating IGF-1 concentration can protect against tumorigenesis (Guevara-Aguirre et al., 2011; Steerman et al., 2011).

IGFs were found to be abnormally high in various tumor cells; therefore, they are generally considered as potent survival factors and mitogens. Epidemiological studies have identified increased level of IGF-I level as a risk factor for development of breast, prostate, colon, and lung cancer (Renehan et al., 2004).

Since IGF-2 is maternally imprinted, loss of this imprinting results in biallelic expression, resulting in increased IGF-2 production and a suspected mechanism of cancer development and progression in many conditions (Cui et al., 2003; Ogawa et al., 1993; Vorwerk et al., 2003).

These higher levels of IGF-1 and IGF-2 promote IGF-1R signaling and the consequently activated downstream pathways. Increases in IGF-1R have been shown in different types of cancer, melanoma, and other carcinomas (Almeida et al., 1994; Zhang et al., 1993). **(Figure 9)**

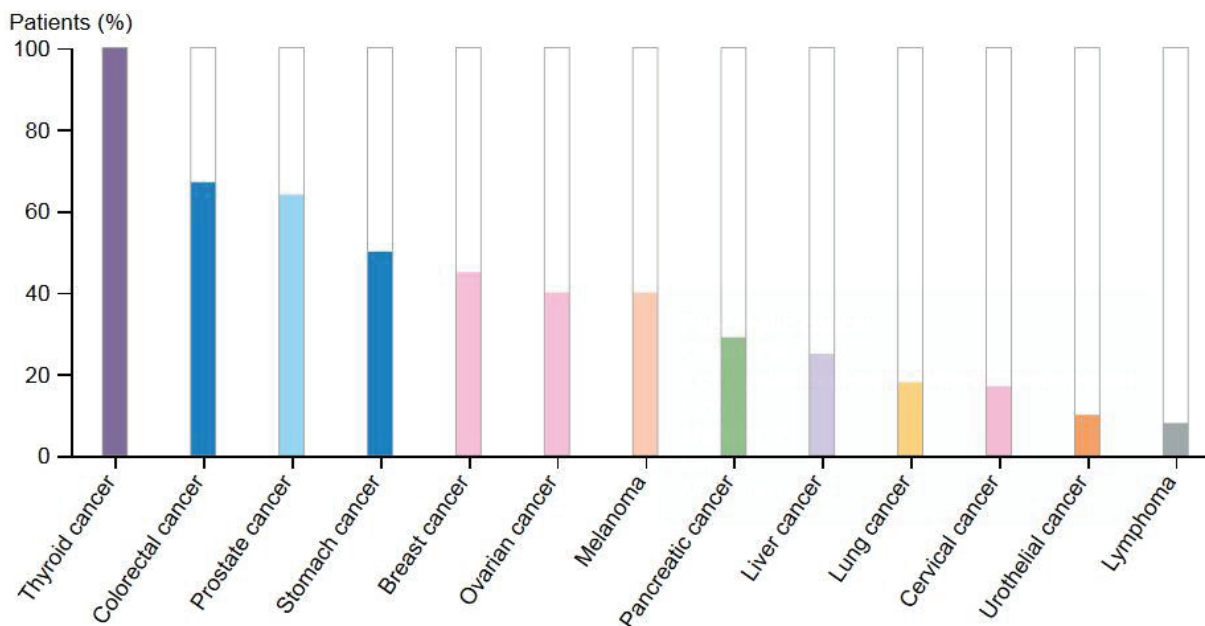


Figure 9: Expression of IGF-1R in different types of cancer. (Source: *proteinatlas.org*)

It was shown that IGF-1R signaling has an important role in response to cancer therapy (Casa et al., 2008; DeAngelis et al., 2011; Goel et al., 2013). However, it seems the role of IGF-1R and its prognostic value in tumor progression is related to the type of cancer (Motallebnezhad et al., 2016;

Wu and Yu, 2014), and the presence of a functional IGF-1R has been shown to be essential for malignant transformation (Sell et al., 1994).

Genetic alterations of IGF-1R leading to varying levels of their expression are found to have a link in cancer (Takeuchi and Ito, 2011). These receptors maybe activated in the tumor cells in an unregulated manner (mutation, chromosomal translocation, abnormal stimulation, and loss of genomic imprinting). Studies have shown that the expression of IGF-IR is significantly increased in breast, colorectal, endometrial and gastric cancers, and this overexpression has been correlated with disease development, aggressive phenotype, poor clinical outcome, and therapy resistance (Christopoulos et al., 2015).

IGF-1R overexpression can result from the loss of tumor suppressor genes, including p53, breast cancer gene-1 (*BRCA1*), von Hippel-Lindau protein and Wilms's tumor suppressor WT1 (Riedemann and Macaulay, 2006; Werner and Roberts, 2003; Werner et al., 1996).

Considering disease prognosis, therapeutic approaches based on targeting IGFRs seem to be promising in cancer research. However, some of these approaches proved to be disappointing due to the role played by the crosstalk between IGF-IR and IR at the receptor level and signaling pathway level (Singh et al., 2014). Since both receptors were proven to be overexpressed in cancer cases, along with the broad binding capacity of both receptors to IGF-I, IGF-II and Insulin, hybrid IGF-IR/IR targeting became one of the cancer therapeutic strategies (Brahmkhatri et al., 2015).

5. IGF-IR in breast cancer

IGF-1 is an essential mitogen for terminal end bud formation and ductul morphogenesis during mammary development (Ruan and Kleinberg, 1999). In addition, IGF-1 has been shown to stimulate proliferation and increased survival of mammary epithelial cells promoting mammary tumorigenesis (Tian et al., 2012). For this reason, role of IGF-IR in breast cancer growth, survival and metastasis had been always under investigation. The correlation of IGF-IR overexpression with breast cancer development and the role of its signaling in the promotion of proliferation of breast cancer cell lines have been indicated in several studies (Christopoulos et al., 2015; Gross and Yee, 2003; Karey and Sirbasku, 1988). Interestingly, data obtained from preclinical tumor xenograft models indicated that IGF system plays an important role in tumor growth stimulation via paracrine or autocrine mechanisms (Karamouzis and Papavassiliou, 2012). In addition,

genomic changes in the IGF system have been observed in 15 % of The Cancer Genome Atlas (TCGA)-documented breast cancers (Cancer Genome Atlas Network, 2012).

Analysis of mRNA levels has shown that there are molecular alterations in the IGF family members in 45.3 % of breast cancers. In 9 % of the tumors, IGF-IR is overexpressed, somatically mutated, or amplified (**figure 10 A**), and 50% of breast tumors express active form of IGF-IR (Farabaugh et al., 2015).

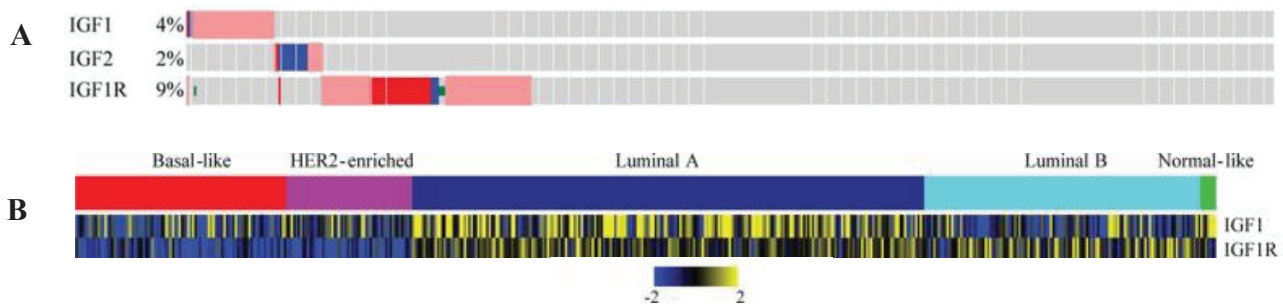


Figure 10: Genomic and transcriptomic variations of IGF-1R expression in breast cancer.
A: Each gray box represents an individual breast tumor indicated with the percentage of expression of IGF-1, IGF-2 and IGF-1R. Red color represents gene amplification and blue color represents gene deletion.
B: Expression levels of IGF-1 and IGF-1R in different breast cancer subtypes ranging from -2 (blue) to +2 (yellow). (Farabaugh et al., 2015)

Many Studies were conducted to estimate the correlation between IGF-IR expression and breast cancer subtypes (**figure 10 B**). As an example, IGF-IR was found to be overexpressed in luminal B subtype patients more than HER2+ patients. This overexpression was correlated with a better prognosis and specific survival, in contrast to the luminal A subtype patients results that showed no specific correlation between the both (Kolacinska et al., 2012; Yerushalmi et al., 2012). On the contrary, IGF-IR overexpression showed poor survival in TNBC where it was overexpressed in 22-46% of those tumors (Farabaugh et al., 2015). In early breast cancer patients, IGF-1R correlates with good prognostic marker and is differentially expressed with variable prognostic impact among breast cancer subtypes (Yerushalmi et al., 2012).

IGF-IR promoter activity is known to be controlled by BRCA-1(Abramovitch et al., 2003); For this reason, increased cell survival and reduced apoptosis were observed in BRCA1-deficient tumors with overexpression of IGF-I and IGF-IR. Also, targeting of IGF-IR activity reduced proliferation of BRCA1 mutant cells. Collecting these results together suggest that IGF system is

involved in the survival and proliferation of BRCA1 mutant breast cancer cells (Motallebnezhad et al., 2016).

Despite hyper-phosphorylation and overexpression of IGF-IR in human breast cancers, IGF-IR expression cannot be used as a prognostic marker because there is no defined cut-off point for IGF-IR overexpression (Karamouzis and Papavassiliou, 2012).

The role of IGF-IR in resistance to apoptosis has been shown in many studies. Treatment of fibroblast and breast cancer cells with IGF-I leads to resistance of these cells to chemotherapeutic drugs and protects them from cell death induced by radiation. In addition, overexpression of IGF-IR is correlated with recurrence of breast cancer after radiation therapy and lumpectomy (Gross and Yee, 2003).

It is noteworthy that the anti-apoptotic effects of IGF-I are mediated through IGF-IR. Multiple anti-apoptotic signaling pathways have been defined for the IGF-IR. One of these pathways is PI3K/AKT, which phosphorylates and inactivates the proapoptotic protein B-cell /lymphoma 2 antagonist of cell death (BAD) (Motallebnezhad et al., 2016; Peruzzi et al., 1999).

On the other hand, metastasis is a main cause of death in women with breast cancer; thus, identification of factors involved in metastasis is important for the development of therapeutic strategies. Previous studies have shown that IGF system is involved in breast cancer metastasis (Karey and Sirbasku, 1988; Samani and Brodt, 2001). It has been revealed that IGF is able to stimulate migration of breast cancer cell lines by IGF-IR activation, in vitro (Gross and Yee, 2003). Additionally, signaling pathways downstream of IGF-IR contributes to the promotion of breast cancer metastasis (Zhu et al., 2011). Sachdev et al. showed that a dominant negative mutant of the IGF-IR suppresses human cancer cell metastasis (Sachdev et al., 2004). Moreover, Saldana et al. demonstrated that inhibition of IGF-IR signaling pathway leads to reduction of breast cancer metastasis to the brain (Saldana et al., 2013).

Long et al. reported that IGF-IR is involved also in extracellular matrix degradation and tumor vascularization (Long et al., 1998). At the same time, Dunn et al. demonstrated that suppression of IGF-IR expression leads to inhibition of metastasis, invasion, and adhesion of breast cancer cells (Dunn et al., 1998).

6. IGF-IR, a potential target of breast cancer therapy

Insulin-like growth factor-1 receptor is a well-described target in breast cancer and multiple clinical trials examining this receptor have been completed (Ekyalongo and Yee, 2017).

Targeting of IGF system, and specifically IGF-IR, and inhibition of their complex signaling pathways are necessary for the inhibition of tumor growth and increase of therapeutic interventions. Targeting this system relies mainly on four therapeutic strategies (**Figure 11**):

IGF-I/-II and IGF-IR monoclonal antibodies (mAb), IGF-IR Tyrosine Kinase Inhibitors (TKI), gene silencing siRNAs and truncated IGF-IR.

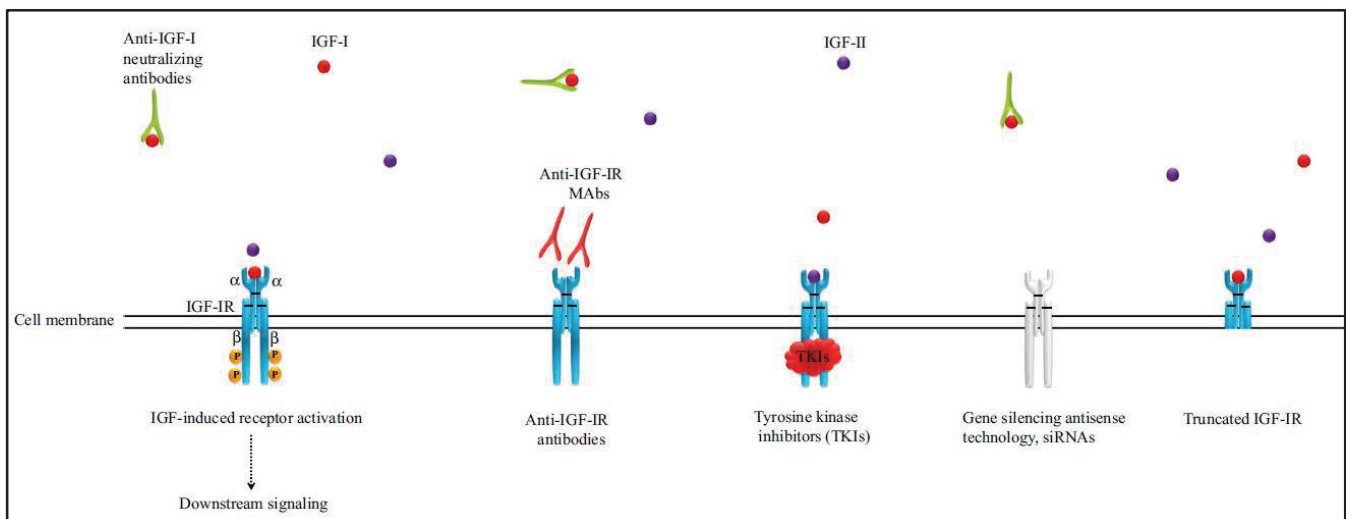


Figure 11: Anti-cancer therapies targeting IGF-IR. Representation of the different therapeutic strategies targeting IGF-IR in cancer; Ligand-neutralizing antibodies, anti-IGF-IR antibodies, inhibitors of tyrosine kinase activity, gene silencing and truncated receptor. (Motallebnezhad et al., 2016)

a. Ligand and Receptor monoclonal antibodies

IGFs binding to the receptor leads to the activation of the latter, which triggers the downstream proliferative signaling cascade, leading to the increase of the tumorigenic identity of the cell. Thus, blocking or reducing the ligand-receptor binding and/or receptor activation may play a beneficial role in cancer therapy. IGF-targeted therapies recorded by clinicaltrial.gov were 625 clinical trials, and since very few therapies depended on the ligand-neutralizing antibody, only two trials used IGF-I/-II mAb.

MEDI-573 is a dual IGF-I/-II neutralizing antibody that inhibits IGF proliferative signaling by inhibition of IGF-I and IGF-II binding to IGF-IR, IGF-IR/IR and IR (Gao et al., 2011). Further studies combined MEDI-573 with other types of signaling inhibitors, such as mTOR inhibitors, showed significant decrease in tumor growth rate (Zhong et al., 2014). Another antibody, named BI836845 or Xentuzumab, is currently in clinical trials to study its effect on human tumor growth. Phase I trial Xentuzumab showed decreased ligand activation of IGF-1R/IR and slower cellular proliferation rate; for this reason, phase II trial was continued by combining Xentuzumab with everolimus (mTOR inhibitor) and exemestane (aromatase inhibitor) in metastatic cancer patients (Ekyalongo and Yee, 2017). In addition to the monoclonal antibodies used to reduce IGF levels, growth hormone-releasing hormone (GHRH) antagonists such as JV-1-38 and growth hormone antagonists such as pegvisomant are also an additional strategy to have the same effect (Motalebnezhad et al., 2016). Monoclonal antibodies targeting IGF-IR, in order to block its activity and induce receptor internalization and subsequent degradation, were produced numerously. Many of them had encouraging preclinical activity i.e. ganitumab, figitumab, dalotuzumab etc (Simpson et al., 2017).

Table 4 summarizes all the current mAbs clinical trials in breast cancer.

Table 4: Potential anti-IGFs and anti-IGF-IR currently used in breast cancer treatment trials

Drug type	Breast cancer indication	IHC-criteria	Phase of trial	Drugs supplements	Estimated date/clinical trial phase
<i>IGF-1 and IGF-2 neutralizing mAbs</i>					
Dusigitumab* (MEDI-573)	Metastatic	HR+/HER2-	n = 188 Phase II	Aromatase inhibitor	06/2011 to 09/2017
Xentuzumab (BI836845)*	Metastatic	HR+/HER2-	n = 174 Phase II	MTOR and Aromatase inhibitor	05/2014 to 04/2018
<i>IGF-IR mAb</i>					
Cixutumumab (IMC-A12)*	Locally advanced Metastatic	HER2/neu+	n = 64 Phase II	Capecitabine	07/2008 to Ongoing
R1507 ^ó	Metastatic		n = 48 Phase II	MTOR inhibitors	10/2008 to Ongoing
	Metastatic		n = 8 Phase II	None	07/2009 to 12/2010
Dalotuzumab* (MK0646)	Metastatic	HR+/HER2- Ki67 \geq 15%	n = 84 Phase II	Aromatase inhibitors	10/2012 to 03/2017
Ganitumab (AMG479)*	Stage II-III	HR+/HER2+, Mamma Print low	n = 1920 Phase II	Anti-hyperglycemic	03/2010 to 05/2018

(Ekyalongo and Yee, 2017)

Although IGF-IR specific mAb lead to downregulation of IGF-IR and IGF-IR/IR hybrids, but still it does not inhibit the IR activation by Insulin or IGF-II ligands binding (Gao et al., 2011). Therefore, the pathophysiological signaling of IR persists, leading to limitation of this therapy. In addition to the crosstalk between both receptors, IGF-IR antibody therapy resulted in hyperglycemia and metabolic syndrome most likely due to disruption of IGF-IR homeostasis and subsequent growth hormone elevation. This elevation induces insulin resistance, hence a subsequent elevation of insulin and a potential for activation of the insulin receptor (Ekyalongo and Yee, 2017). Limitations of using mAb derived necessity to follow other therapeutic strategies such as inhibitors of the IGF-IR tyrosine kinase activity.

b. Tyrosine Kinase Inhibitors (TKI)

Targeting the kinase activity of IGF-IR is one of the strategies to inhibit its downstream signaling. TKIs are small molecules that target the kinase activity by competing with ATP for the respective binding site on the catalytic domain of the receptor; hence, preventing autophosphorylation of the RTK and inhibiting the propagation of the proliferative signal. Several studies have shown that using TKI effectively inhibited cell proliferation, enhanced apoptosis and suppressed tumor growth; therefore, linsitinib (OSI-906) and BMS-754807 were subjected to clinical trials to study their efficiency on humans (Carboni et al., 2009; Mulvihill et al., 2009; Wittman et al., 2009). In 2011, Linsitinib clinical trial combined with letrozole (an aromatase inhibitor) was terminated at phase II due to metabolic toxicities and side effects. Similarly, BMS-754807 with and without letrozole were tested in HR+ patients resistant to non-steroidal aromatase inhibitor, but there was no clear results

assigned (Ochnik and Baxter, 2016). However, inhibitors targeting IGF-IR can also target IR-A and IR-B due to the homology of IGF-IR with both receptors. Thus, receptor tyrosine kinase such as linsitinib, BMS-754807 and KW-2450 are called dual IGF-IR/IR inhibitors (Simpson et al., 2017). Ekyalongo and Yee recently stated that these dual tyrosine kinase inhibitors are not being further developed likely due to their metabolic toxicities and concerns about affecting host glucose uptake (Ekyalongo and Yee, 2017). The following table summarizes the obstacles that faced some therapies.

Table 5: Toxicities associated with anti-IGF-IR therapies

Compound type				
	Estimated enrolled patients	Metabolism and nutrition disorders (grade 3 and 4)	Hyperglycemia (grade 3 and 4)	Clinical trial evolution
<i>IGF-IR mAbs</i>				
Figitumumab (CP-751,871)	115	47.91%	52.08.48%	Terminated at Phase II
Cixutumumab (A12)	19	43.75%	56.24%	Terminated at Phase II
Dalotuzumab (MK0646)	11	(-)	(-)	Terminated at Phase II
AVE1642	18	(-)	(-)	Terminated at Phase II
<i>Non-ATP antagonist TKIs</i>				
Linsitinib (OSI-906)	11	70.58	29.41%	Terminated at Phase II

(Ekyalongo and Yee, 2017)

c. *IGF-IR* gene silencing and truncated IGF-IR

In addition to the proteomic level, IGF-IR could be targeted at the genomic level. Suppression of IGF-IR expression and function by microRNA and siRNA is another approach for targeting the IGF-IR. Studies in which small interfering RNAs (siRNAs) induce potent IGF-1R gene silencing without affecting the IR; and this demonstrate that siRNAs block IGF signaling, thereby enhancing radio and chemosensitivity (effective chemo- and radiotherapy-induced apoptosis) and paving yet another way of therapeutic potential (Macaulay, 2004; Riedemann and Macaulay, 2006).

Recently, Durfort et al. showed that the use of anti-IGF-IR siRNA leads to downregulation of its expression and inhibits tumor growth (Durfort et al., 2012). Targeting of IGF-IR expression by specific microRNA has also been effective in the inhibition of tumor growth, metastasis, and invasion (Shen et al., 2012; Wang et al., 2012; Yang and Yee, 2012).

In addition, dominant negative strategies, like truncated IGF-IR, have also been efficient in IGF-IR targeting. The mechanism of action of these dominant negative mutants involves ligand sequestration and formation of inactive chimeras with the endogenous receptor (Bähr and Groner, 2004; Brahmkhatri et al., 2015).

Clinical efficacy of targeting IGF-IR depends on major factors: the role of IGF-IR itself in the tumors, potential inhibition of siRNAs and antisense therapies in vivo, and compensation of other signaling pathways due to IGFR loss (Macaulay, 2004).

All these data prove the potential genetic blockade studies of IGF-1R, and its efficacy and prognosis in several malignancies including breast, lung, colon, and pancreatic carcinoma (Adachi et al., 2004).

7. IGF-1R combinatorial therapies

Treating IGF-1R alone in breast cancer was insufficient in rising up to expectations, and this may be due to the complex activity played by this receptor in many metabolic processes. For this reason, recently there has been many clinical trials combining the IGF-1R-directed therapies with other chemotherapies and anti-estrogens. The role of IGF-1R in promoting resistance to chemotherapy is well established; therefore, modulating the expression of IGF-1R when using chemotherapy proved to be successive strategy in treating breast cancer. In phase 3 NEOZOTAC trial in 2013, breast cancer HER2-negative patients showed improved clinical outcome when IGF-1R expression was reduced during and after the neo-adjuvant chemotherapy (de Groot et al., 2016).

Moreover, combinatorial therapies for different types of breast cancer patients, although not as expected, but showed some promising results in the stability of the disease and overall survival. In TNBC cell lines, a lot of preclinical studies showed that cotargeting IGF-1R and PI3K axis was able to decrease the viability of certain subsets of these cells (de Lint et al., 2016). Targeting mTOR pathway (ridaforolimus) with anti-IGF-1R also potentiated the anti-tumor activity of these cells.

Here, it is noteworthy to mention the effects of combining therapies against IGF-1R and other growth factors like EGFR, VEGFR, HER2 etc, due to the crosstalk between the RTK and their induced signaling pathway. So the inhibitory effect of targeting IGF-1R will be partially compensated by the activation of the others. For example, HER-targeted therapy like lapatinib and neratinib combined with IGF-1R siRNA or inhibitor (NVP-AEW541) showed reduced resistance and anti-tumor activity of HER+ breast cancer cells (Simpson et al., 2017). Moreover, studies suggested that in addition to inhibiting PI3K/Akt/mTOR and Ras/Raf/ERK/MAPK pathways, co-targeting IGF-1R with CDK4/6 and S6K pathways may be useful to diminish the proliferative IGF-1R signaling effects. **Figure 12** shows all the possible combinatorial therapies that are used or may be used in the future to co-target the IGF-1R complex signaling pathways.

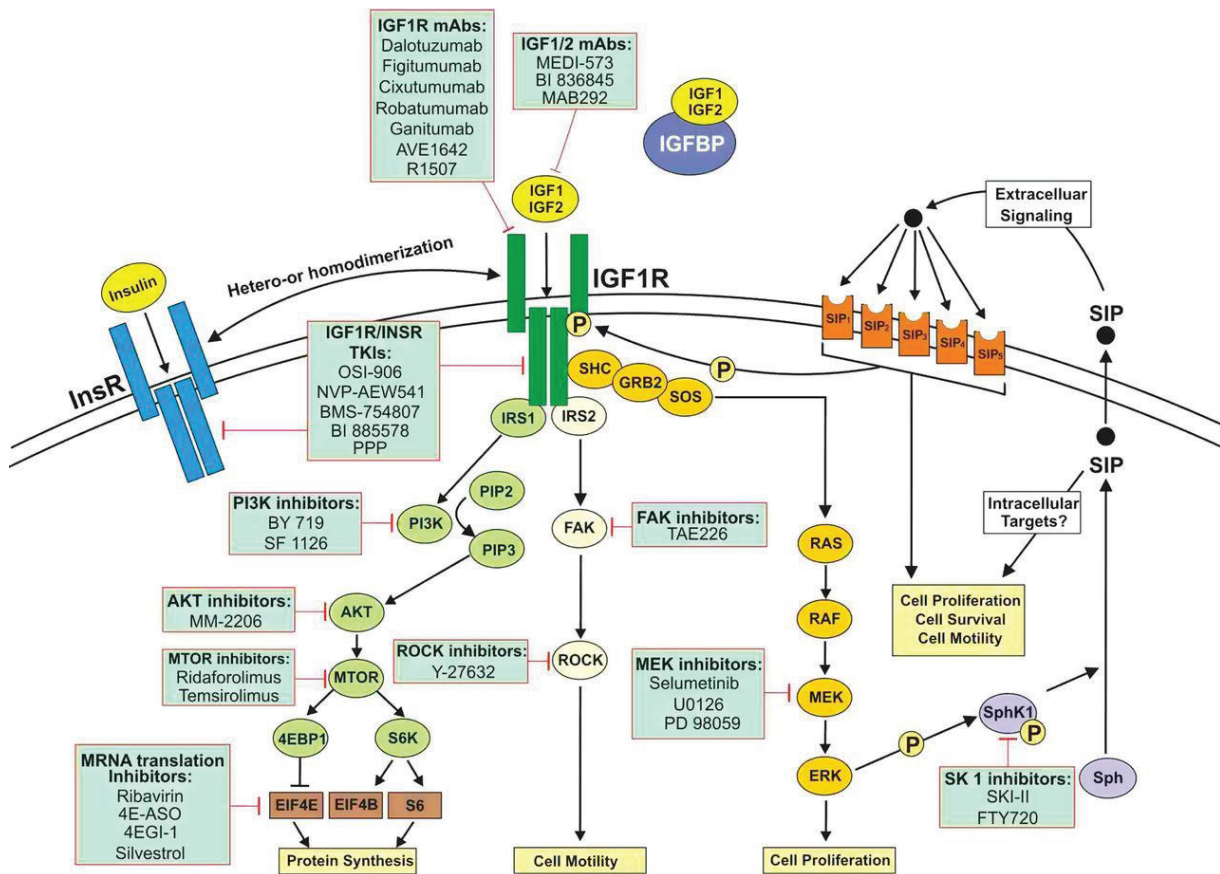


Figure 12: Therapeutic targeting of IGF-1R expression and activity in breast cancer. Different anti-IGF-1R therapies inhibiting IGF-1R activity and its downstream effector cascades, including PI3K/Akt/mTOR/S6K and RAS/RAF/MEK/ERK pathways, that are developing in breast cancer therapeutics. (Ochnik and Baxter 2016)

8. Conclusion

IGF axis targeting is an attractive therapeutic strategy for all cancers generally and for breast cancer specifically, due to its implications in disease progression and resistance to chemotherapy. So targeting IGF-1R has drawn a lot of clinical attention and importance, and IGF-1R therapies were produced and tested rapidly. However, treating IGF-1R alone through mAb and inhibitors showed post-treatment difficulties like metabolic diseases and toxicities, and this lead to the necessity of combining IGF-1R inhibitors with other chemotherapies and anti-estrogens. Unfortunately, defining a proper therapeutic strategy of co-targeting IGF-1R and other signaling components did not work perfectly. This is due to the complexity of its signaling, and the crosstalk with other RTK and their signaling cascades. On the other hand, combining anti-IGF-1R with PI3K inhibitors and other chemotherapy in TNBC, and with anti-estrogens in hormone-sensitive HR+ breast cancer showed promising results. For this reason, we can foresee the great effect of well targeting this receptor in breast cancers that are strongly correlated with its expression. Therefore, there is a need for further clinical trials to define a proper IGF-1R targeting strategy, and better clinical outcome depending on the molecular type of breast cancer.

III. Estrogen Signaling

A. Introduction

Estrogens are well known as important regulators of the female reproductive functions and are perceived mainly as ovarian sex hormones responsible for cellular proliferation and growth of tissues related to reproduction. In addition, they are commonly accepted to have important functions in both female and male physiology and pathology (Vrtačnik et al., 2014). Estrogens are synthesized in both sexes, but are mainly produced in non-menopausal women. In premenopausal women, ovaries represent by far the most important source of circulating estrogens, although during pregnancy, placenta also secretes significant amounts of estrogens into the circulation. In addition to the effects on sexual traits and their participation in controlling the menstrual cycle, estrogens also play a significant role in the regulation of skeletal homeostasis, lipid and carbohydrate metabolism, electrolyte balance, skin physiology, the cardiovascular system and the central nervous system (Nilsson and Gustafsson, 2011). Due to this wide regulatory role, it is not surprising that estrogens also have an important function in male physiology and cannot be viewed solely as female sex hormones. Men and postmenopausal women, due to the decline in their ovary function, are largely dependent on local synthesis of estrogens in extragonadal target tissues. This local production of estrogens extends their signaling from endocrine to paracrine, autocrine and intracrine (Labrie, 2003).

B. Estrogen synthesis and metabolism

Estrogens are derived from cholesterol with a 4-cycle carbon skeleton. Estradiol (E2) is obtained after testosterone processing, and it is the principal form of estrogen secreted by the women. In addition to E2, Estriol (E3) is produced by the placenta during pregnancy and Estrone (E1) produced after menopause by androgen processing. In non-pregnant women, estrogens are mainly synthesized by the ovaries, while during pregnancy, a large amount is synthesized by the placenta. Androgens are secreted by the ovaries in thecal cells then diffuse to the granulosa where they will be processed into estrogen by aromatization and demethylation (**Figure 13**).

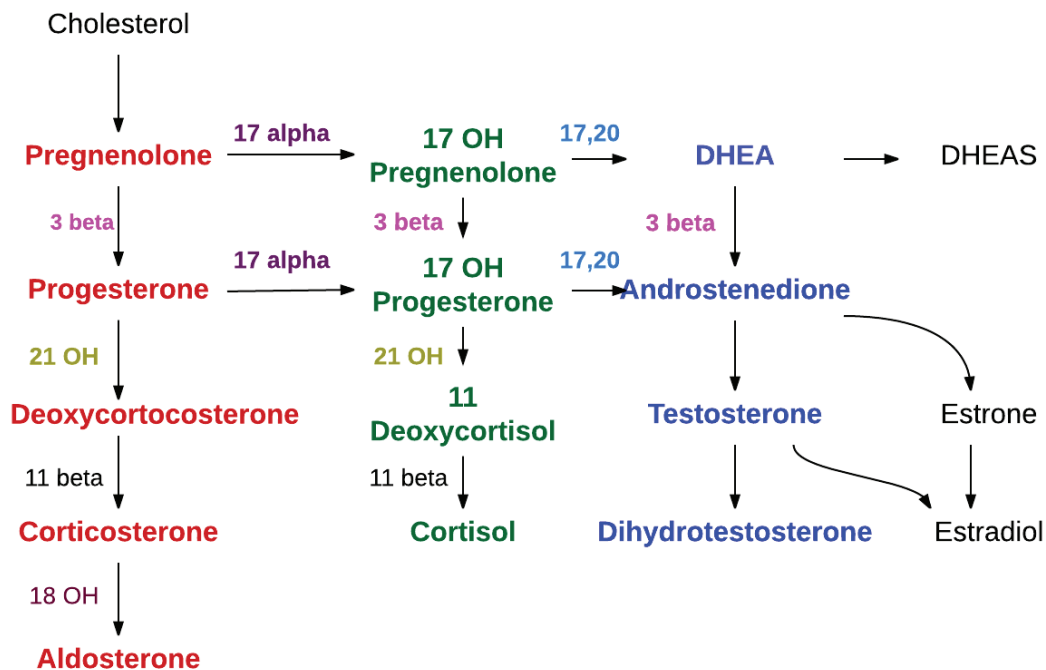


Figure 13: Adrenal steroid hormone synthesis. Estradiol synthesis arises in gonads from cholesterol, leading to the formation of progesterone then androgens. Androgens are then aromatized into estrone and testosterone into estradiol. (Source: [wikimedia.org](https://www.wikimedia.org))

C. The Menstrual Cycle

Estrogen production is under control of the hypothalamus-pituitary axis. Gonadotropin Releasing Hormone (GnRH) secreted by the hypothalamus, acts on the anterior pituitary gland and promote secretion of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH).

During the first days of the cycle, FSH and LH secretion cause the maturation of ovarian follicles, which in turn secrete estrogens, which is responsible for the thickening of the uterine lining to be prepared for an eventual fertilization. During the follicular phase, estrogen concentrations increase induces the secretion of LH by a positive feedback loop. This causes rupture of the ovarian follicle and ovulation takes place. The ruptures follicle, now known as corpus luteum secretes progesterone. If the egg is not fertilized, the corpus luteum is evacuated leading to a drastic progesterone decrease, which results in the shedding of the uterine lining. Another cycle can then take place (**Figure 14**).

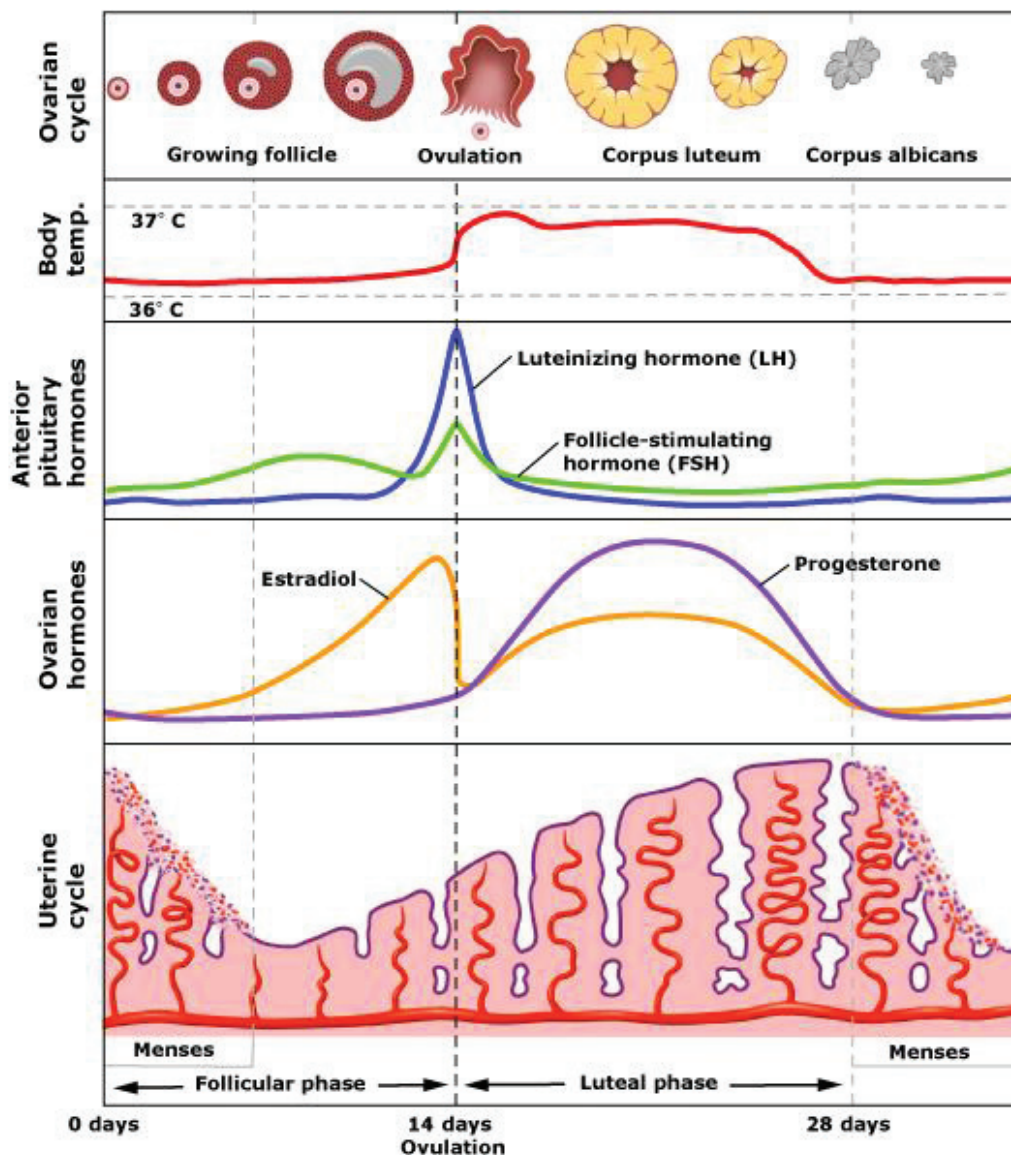


Figure 14: Estradiol changes in menstrual cycle. The cycle is under strict hormonal control by pituitary hormones, estrogen and progesterone. The follicular phase begins on the day of menses and last 14 days, and its end is marked by the ovulation; then begins the luteal phase. (source: *biology.com*)

In addition to their functions in reproduction, they have several roles in the development and the maintenance of the female reproductive organs and in the development of feminine traits (Carpenter and Korach, 2006). Furthermore, they are deeply involved in the development of breast and endometrial cancer.

Estrogens play a natural role in the regulation of blood cholesterol levels by the liver by modulating lipoprotein receptors (Paganini-Hill et al., 1996). In the skeletal system, estrogens help maintain bone density, which explains that post-menopausal women are more susceptible to develop osteoporosis, and are more prone to fractures. In vascular system, estrogens have an anti-apoptotic role in endothelial cell thereby maintaining endothelial integrity (Spyridopoulos et al., 1997). Estrogens have also been described as being a pro-angiogenic factor. In the nervous system, estrogens can play a role in synaptic remodeling and memory (Woolley et al., 1997). Furthermore, studies have shown that estrogens can mediate a neuroprotective effect against cell death. The following figure summarizes most of estrogen physiological functions.

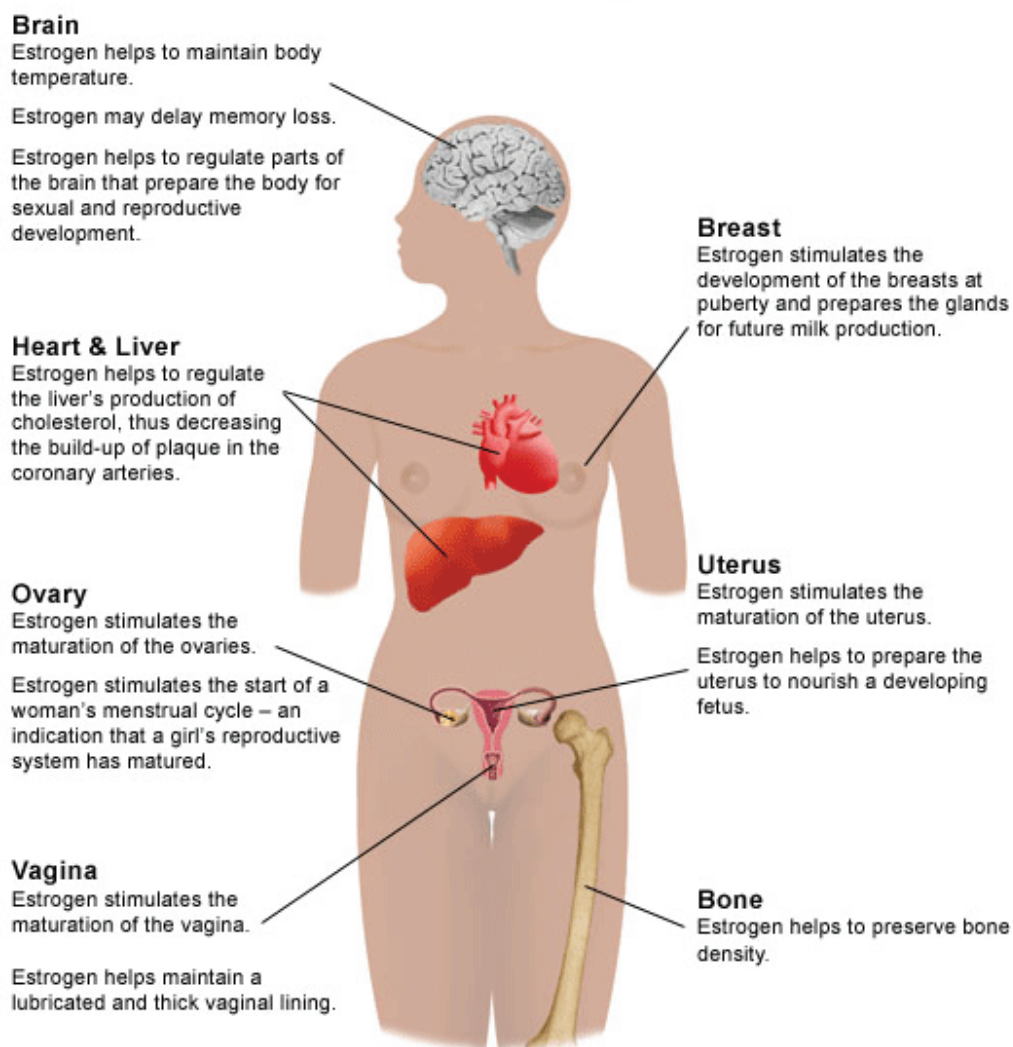


Figure 15: Physiological effects of estrogens (source *hopkinsmedicine.org*)

D. Estrogen Receptors

Estrogen receptors (ER) belong to the nuclear receptor superfamily binding steroid hormones. These receptors function mainly after their activation by the ligands estrogens. ER α was first identified in 1962 and cloned in 1986 (Green et al.; Greene et al., 1986); ten years later, Estrogen Receptor β (ER β) was identified (Kuiper et al., 1998).

1. Structure of Estrogen Receptors

a. Genomic Structure

The ER α gene, named *ESR1* is localized in chromosome 6q25.1 in humans (Menasce et al., 1993). Previously the *ESR1* gene was supposed to contain 8 coding exons, until in 2005, when a ninth exon was discovered downstream exon 8 (Wang et al., 2005). The coding exons 1-8 are highly conserved in between different species, except for exon 9, which is found only in humans and chimpanzees (**Fig. 16 A**). The variable 5' extremity of the *ESR1* gene and the existence of multiple promoters in this region may account for the differential expression of ER α in different tissues and during development (Kos et al., 2001). The ER β gene, named *ESR2* is localized on chromosome 14q23.2 and is composed of 8 coding exons (Enmark et al., 1997). (**Figure 16 B**)

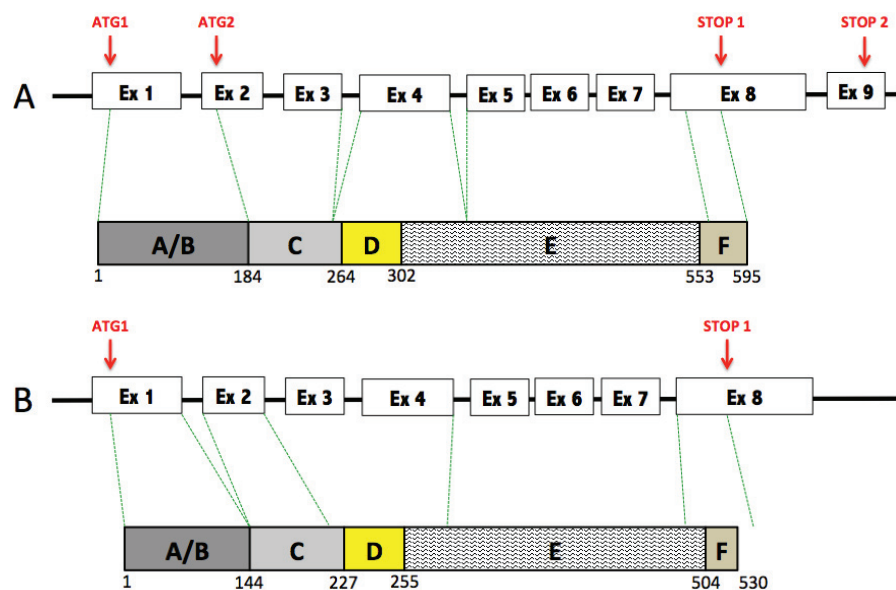


Figure 16: Genomic and protein arrangement of Estrogen Receptors (all intronic sequences between exons are not to scale) A: Structure of *ESR1* and corresponding ER α protein. **B:** Structure of *ESR2* and corresponding ER β protein. Numbers below the proteins represent amino acid numbers from the N-terminus. (Adapted from Le Romancer et al., 2011)

b. Protein Structure

The nuclear receptor superfamily represents a great diversity of receptors and despite that, all these receptors have a relative homology in their organization and function. For most of these factors, the common fact is that they are capable of DNA binding in response to their ligands.

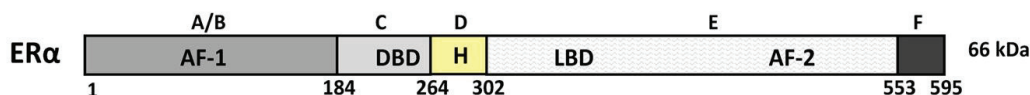


Figure 17: ER α domains structure. (Adapted from Le Romancer et al., 2011)

In accordance to the structure of the nuclear receptor superfamily, ER α is composed of 6 functional domains named A – F (Kumar et al., 1987). (**Figure 17**)

- The A/B domain is composed of the transcription transactivation domain AF1 (activation function 1), and is responsible for ligand independent transcription.
- The C domain, also called the DNA Binding Domain allows for the recognition of estrogen response elements (ERE) on DNA, generally located on the promoters of target genes. It is made up of two zinc-finger structure. A P-Box present in the first zinc finger structure is important for recognition of the ERE while a D-Box in the second zinc finger structure is responsible for receptor dimerization (Ponglikitmongkol et al., 1988).
- The D Domain serves as a Hinge between domains C and E and is responsible for providing flexibility to the DBD for adopting different conformations. This region also carries three Nuclear Localization Sequences, permitting nuclear import of the receptor
- The E domain carries the Ligand Binding Domain (LBD) and has a main function in the dimerization of ER. It also carries the AF2 (activator function 2) transcription transactivation domain, which is ligand dependent. After ligand binding, helix 12 of ER will close on the ligand-binding pocket and this will lead to a stabilization of the dimeric form of the receptor, dissociation from co-repressors and the creation of new interaction sites for interactions with co-activators. Helix 12 also plays a crucial role in the conformation of the receptor when bound to different ligands and will be responsible for the agonist or antagonist actions of the receptor (Ruff et al., 2000).

- The F-Domain, located on the C-Terminal part of the protein is still not fully characterized. It could have a role in modulating ER α activity by modulating protein-protein interactions with co-activators such as SRC1 (Steroid Receptor Co-activator 1) (Koide et al., 2007).

At the functional level, ER α activity is associated with cell proliferation. Also, ER α KO mice present with infertility, uterine atrophy and impaired mammary development. Furthermore these mice are obese and there is no feedback loop on the hypothalamic-pituitary axis in regard to LH secretion (Emmen and Korach, 2003).

2. Estrogen Receptor isoforms

Besides the described ER α and ER β , many variants arising from either alternative splicing or alternative promoters have been characterized. The most described variants of ER α are ER α 46 and ER α 36, named thus due to their respective protein sizes (46kDa and 36 kDa respectively) (Figure 18 A)

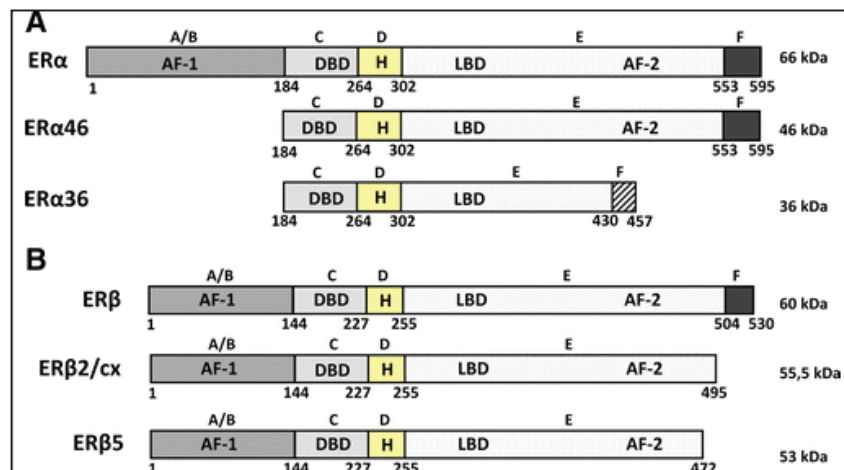


Figure 18: Isoforms of ER. A: ER α 36 and ER α 46 are variants of ER α . B: ER β 2/cx and ER β 5 are isoforms of ER β (Le Romancer et al., 2011).

Isoform ER α 46 was identified in 1996, and is transcribed from an alternative promoter in Exon 2. It has been demonstrated to inhibit the transcriptional activity of ER α via the recruitment of co-repressors (Flouriot et al., 2000; Penot et al., 2005).

Isoform ER α 36 was found by Wang et al. in 2005, and is transcribed from a promoter in the first intron of *ESR1*. Due to its localization in the cytoplasm and the membrane, ER α 36 is believed to be involved in the rapid extranuclear non-genomic signaling (Wang et al., 2005). ER α 36 is involved also in several signaling pathways like PI3K/Akt, Src, ERK1/2, EGFR, PKC, Cyclin D1, C-Myc and Jun pathways.

3. Estrogen receptor signaling pathways

Estrogen receptor is the main mediator of estrogen action by regulating the expression of estrogen dependent genes involved in proliferation, development and differentiation of the mammary gland.

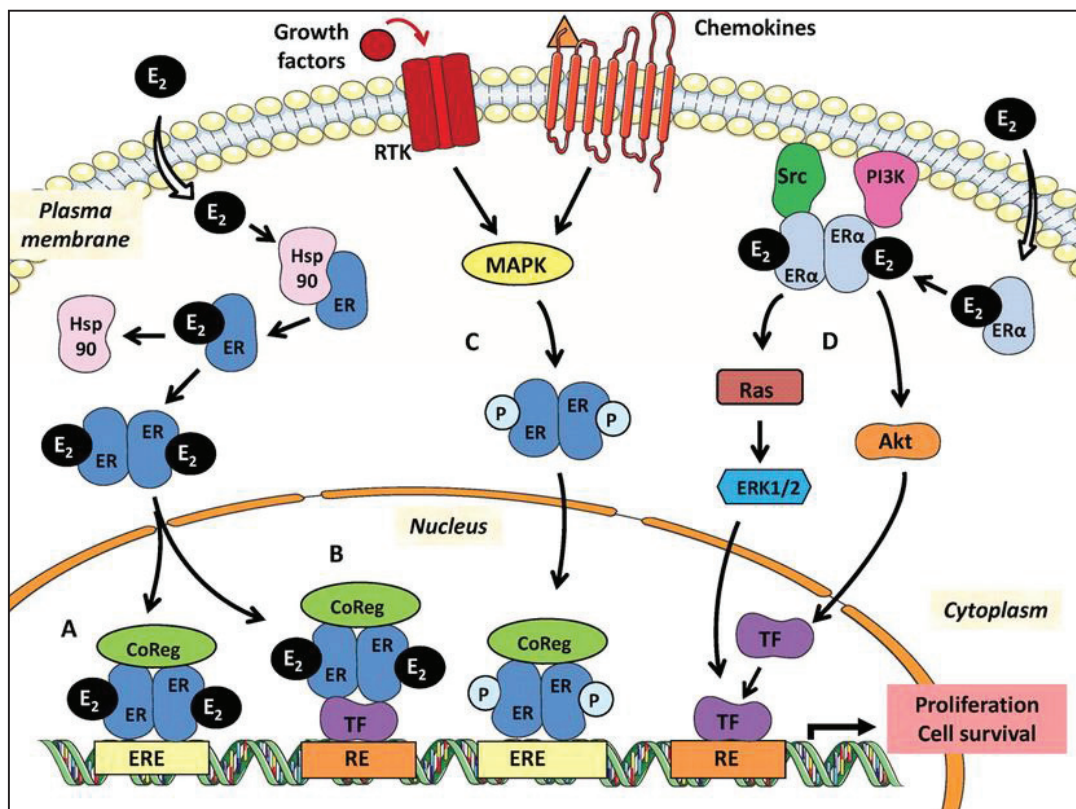


Figure 19: ER Signaling Pathways. ER designates either ER α or ER β . **A,B:** Classical Genomic Pathway, **C:** Non classical genomic pathway, **D:** Non-genomic signaling pathway (Le Romancer et al., 2011)

After ligand binding, the receptor will dimerize and translocate into the nucleus to bind directly onto ERE or indirectly via binding to transcription factors, this is the classical genomic pathway (Figure 19A, B). Otherwise, there is a ligand independent pathway, which relies on the phosphorylation of ER by growth factor receptor activation; this is non-classical genomic pathway (Figure 19C). Finally, there is the non-genomic pathway, which involves a cytoplasmic fraction of ER α and the recruitment of cytoplasmic kinases to initiate the downstream signaling pathways (Figure 19D)

a. ER α inactive form

Steroid receptors have been found to interact with Hsp90 (Heat shock protein) as well as other chaperones, which participate in maintaining the inactive state of these receptors (Sanchez et al., 1987). In eukaryotes, Hsp 90 plays a major role in the folding, localization and degradation of various proteins (Becker and Craig, 1994). Hsp90 is a dimer made up of three distinct domains, an ATP binding N-Terminal Domain, a central domain involved in protein recruitment and binding specificity and a C-terminal domain responsible for dimerization (Prodromou et al., 2000).

Besides Hsp90, many other chaperones have been described as steroid receptor couples such as Hsp40, Hsp70, which are also involved in maintaining the inactive state (Picard, 2006). Hsp90 will bind to the LBD of ER α and the C-terminal ends dimerize after ATP fixation which folds over ER α (Cintrón and Toft, 2006; Johnson et al., 1994).

Alongside these Hsp's, other proteins called immunophilins can form part of the complex to maintain ER α in its inactive state. Several of these immunophilins have been described such as FKBP52 (p59), FKBP51, Cyclophilin 40 (Ratajczak and Carrello, 1996; Renoir et al., 1990). In the absence of ligand, this complex around Hsp90 sequesters the receptor but also allows ER α to acquire a structure having maximal affinity to its ligand. Upon ligand binding, ATP hydrolysis allows the opening of the clamp around ER and ligand fixation.

b. ER α genomic signaling

• The Classical Pathway

Hormone binding to the receptor brings a conformational change, which dissociates it from the complex of chaperone proteins. The receptor dimerizes and translocates in the nucleus (Sabbah et al., 1996). The receptor dimer can then bind to estrogen response elements (ERE), located in the promoter of target genes. The minimal conserved sequence between ERE is a 13bp palindromic sequence divided by 3 random nucleotides (n) 5'-GGTCANNNTGACC-3' (Walker et al., 1984). However a limited number of ER α regulated genes process this sequence and in most cases, the receptor dimer will bind on imperfect ERE or half-palindromic sequences (Ramsey and Klinge, 2001). Depending on the cell type, the type of ERE used and the ligand, the receptor can have either positive or negative trans-activation on target genes.

• ER α transcriptionally active form

As ER α is bound to one of the ERE, it can mediate gene transcription through its AF1 or AF2 transactivation domains and it can also recruit several co-activators.

There are three major co-activator complexes that have been identified for ER α transcriptional activity and play a key role in transcription activation (Rosenfeld et al., 2006).

The first complex is the p160/SRC family of co-activators. SRC-1, SRC-2 and SRC-3 are three members of this family and contain LXXLL motifs that allow binding to ER α hydrophobic pocket on the AF2 domain (Leers et al., 1998). Furthermore, they contain two transcription activation domains AD-1 and AD-2. AD-1 is involved in the recruitment of CBP/p300 and AD-2 in the recruitment of PRMT1 and CARM1 which are involved in histone methylation and chromatin decompaction (Chen et al., 1999). SRC's N-terminal ends also have the capacity to recruit several co-activators including Fli-I which is involved in recruiting the SWI/SWF complex.

The second co-activator complex is CBP/p300. This complex contains an ER α binding site via a consensus LXXLL motif and its role in ER α activation has been well documented. The third is SWI/SNF complex which is recruited to ER α target genes in

the case of estrogenic signaling by BAF's (BRG1 associated factors), for instance BAF57 in the recruitment of ER α . In addition to the fore mentioned complexes, the Mediator Complex is large co-activator complex made up of more than 26 protein sub-units and is also called the TRAP/SMCC/DRIP complex. It has a role in maintaining ER α dependent transcription after the CBP/p300 complex (Kim et al., 2006).

Other proteins have been described to act as ER genomic regulators. For instance, GREB1 has been identified as an ER co-factor that will serve to stabilize the binding of ER to other cofactors and mediate ER transcriptional activity. To sum up, these complexes bring forward enzymatic activities that will allow histone modification and chromatin opening to facilitate transcription of target genes.

- **ER α transcriptionally repressed form**

In addition to binding capacity of co-activators, the AF2 domain of ER α can also recruit various co-repressors. Two main proteins, RIP140 and SHP have co-repressor activities and act in a SRC antagonist manner.

The RIP140 protein, identified as a co-repressor of ER α , includes 9 LXXLL repeats and can therefore be easily recruited onto steroid receptors (Cavaillès et al., 1995). RIP140 has the ability to recruit various transcriptional co-repressors such as HDACs I and II. RIP140 also has the capacity to recruit CtBP proteins, which act as negative transcriptional regulators. Other studies however have described RIP140 to act as a positive ER transcriptional regulator and to serve as a co-activator (Nautiyal et al., 2013). SHP is a nuclear orphan receptor and can interact with ER α through a LXXLL motif. It can repress ER α activity by direct interaction with its AF2 domain (Johansson et al., 2000).

Upon binding to antagonists like Tamoxifen, a different conformation of the receptor is induced which leads to ERE binding but with the induction of binding to co-repressors like N-Cor, SMRT which participate in the recruitment of HDAC for chromatin compaction and transcriptional repression (Shang et al., 2000).

- **The Non-Classical Pathway**

ER is also capable of inducing the transcription of genes devoid of ERE by indirect DNA binding through interactions with other transcription factors. Thus, ER can modulate the activity of transcription factors such as AP1, SP1 or NF- κ B.

Concerning AP-1, this is a transcription factor complex, which includes JUN, and FOS, which binds to AP-1 sites in gene promoters. It was also shown that estrogen activated ER α can bind to the AP-1 complex through the p160 coactivator family (Webb et al., 1999). Regulated genes through ER α and AP-1 interplay include c-fos, Cyclin D1 and IGF, all involved in cell proliferation and motility. In some cells types, this AP-1 interplay with ER α could account for the differential effects of anti-estrogens such as tamoxifen and fulvestran (DeNardo et al., 2005). Regarding SP-1, it was identified as forming part of an ER α /SP-1/DNA complex in the study of Cathepsin D, an estrogen-regulated gene. In this context, the ER α /SP-1 complex has been found responsible for mediating transcription of c-myc, Cyclin D1 and Bcl-2 proteins, thus having a role in cell proliferation and apoptotic resistance (O'Lone et al., 2004).

ER α has been also reported to modulate NF- κ B transcriptional activity by acting as a transcriptional repressor. This pathway has been evidenced to maintain the bone homeostasis to inhibit the NF- κ B induced IL-6 upregulation. ER α is thought to function by blocking NF- κ B's ability to bind DNA (Kalaitzidis and Gilmore, 2005).

- **The Ligand Independent Pathway**

ER can be modulated in the absence of ligand by extracellular signals. EGF and IGF have been reported to phosphorylate ER, thus inducing the transcription of downstream target genes (Le Romancer et al., 2011). The AF-1 ligand independent transactivation domain of ER carries this out. The main described phosphorylation site on ER α is Serine 118 (Bunone et al., 1996). This phosphorylated ER had been found to bind DNA and locate on the promoters of several target genes.

- **ER acting on genome**

While most studies focus on the proximal ERE in the promoters of genes for ER binding, genome wide studies revealed that most ER binding sites are located at

significant distances from TSS. Further investigation demonstrated that the Forkhead factor FoxA1 had a crucial role to play in ER chromatin binding in the sense that ERE which had a Fox A1 binding site in close proximity were much more likely to be bound by ER (Carroll et al., 2005) . Furthermore, ER binding sites throughout the genome can be altered during drug resistance mechanisms. For instance, in an endocrine-resistance setting, ER has been shown to relocate with SRC-1 and the chromatin protein HMGB2 and bind a different subset of non-ER related regulatory elements (Redmond et al., 2015).

c. Estrogen non-genomic signaling

In addition to the fore-mentioned genomic effects, there are rapid effects mediated by estrogens which take place in the minutes following estrogen exposure, meaning they are way too fast to be mediated by transcriptional activation (Pietras and Szego, 1977).

• ER α mediated signaling

Several studies demonstrate the existence of a pool of ER α located at the plasma membrane (Chambliss and Shaul, 2002; Clarke, 2000; Pappas et al., 1995), this localization has been aslo reported in several IHC studies (Norfleet et al., 1999). However, these observations do not provide a mechanism for ER α location at the plasma membrane, keeping in mind that ER α does not process any membrane insertion signal or peptide, no hydrophobic domains nor any glycosylation. The protein shuttles between the cytoplasm and the nucleus through nuclear localization and export sequences.

Acconcia et al, found that the palmitoylation of ER α on Cysteine 447 was shown to contribute to ER α cytoplasmic localization. A mutation of this cysteine residue prevented cytoplasmic ER α localization and abrogated the rapid estrogen induced MAPK activation (Acconcia et al., 2004). Palmitoylation of ER α allows its interaction with Caveolin-1 at the plasma membrane in lipid rafts (Acconcia et al., 2005).

Furthermore, protein association seems to be necessary for ER α localization near the plasma membrane. The adaptor protein Shc which plays a role in IGF-1R signaling and upon auto-phosphorylation of IGF-1R, Shc is recruited and allows

the recruitment of ER α (Ravichandran, 2001). These describe Shc and IGF-1R as key regulators for ER α membrane localization (Song et al., 2004a).

In addition, serine 522 localized in the E-domain of ER α seems to play a role in its membrane localization. Mutants of this serine fail to locate to the membrane and fail to co-localize with Caveolin-1 (Razandi et al., 2003).

- **The ER α /Src/PI3K Complex**

ER α has no intrinsic kinase activity, and could not transduce extracellular signals by itself. Therefore, its association with different kinases is necessary to initiate rapid membrane signaling. Ligand bound ER α dimers can bind to many different proteins for this purpose, mainly the tyrosine kinase Src and p85, the regulatory sub-unit of the PI3K. The Src and PI3K proteins form part of the core of the non-genomic signaling complex (Castoria et al., 2001). This association has been observed firstly in endothelial cells in the induction of eNOS and the activation of the Akt pathway (Simoncini et al., 2000). Then it was observed that estrogen induced the rapid and transient formation of a ER α /Src/PI3K complex (Castoria et al., 2001).

Estrogens thus induce the activation of PI3K increasing the intracellular PIP3 concentration. In parallel, estrogens mediate Src kinase activity and the activation of the Src pathway (Castoria et al., 2001).

Phosphorylation of tyrosine 537 of ER α is essential for its fixation with the SH2 domain of Src (Migliaccio et al., 2000). Pharmacological abrogation of this interaction with a peptide abrogated the ER α /Src interaction and the downstream signaling including the arrest of Cyclin D1 expression (Varricchio et al., 2007).

There seems to be an interplay between the Src and PI3K activities mediated through ER α . In fact, inhibition of Src kinase activity abrogated the estrogen induced PI3K activation, and the formation of the core ER α /Src and PI3K complex (Cabodi et al., 2004). Inversely, the use of a PI3K inhibitor abrogates Src activity (Castoria et al., 2001). In addition to the ER α /Src/PI3K complex, it is noteworthy mentioning the adaptor protein P130^{Cas}. This Crk-associated protein, is a major Src substrate. It is involved in cytoskeleton remodeling during cell migration and transformation. It has been shown to be transiently associated with ER α upon estrogen stimulation and this association depends on Src kinase activity (Cabodi et al., 2004). Moreover, MNAR

(Modulator of Non-genomic Activation of ER) is an adaptor protein that helps making up the ER α /Src/PI3K complex. It functions as a scaffold which favors the ER α /Src interaction and it is thought to be crucial in the recruitment of p85 to the complex (Greger et al., 2007).

- **ER α splice variants signaling**

ER α splice variants could also be involved in the cytoplasmic localization. ER α 46 can be palmitoylated and inhibition of this palmitoylation impairs its membrane localization in endothelial cells (Li et al., 2003).

The ER α 36 isoform has been reported to be involved in the non-genomic signaling by its physical ability to interact with Src, activating the EGFR and ERK1/2 pathways. In addition, it has been shown that ER α 36 mediated the PI3K/Akt signaling in breast and endometrial cells (Deng et al., 2014; Gu et al., 2014)

- **GPR30 mediated signaling**

The G-protein coupled receptor 30 is a 7-transmembrane receptor (Carmeci et al., 1997) which has been shown to mediate estrogen response in ER α negative cells (Filardo et al., 2000). Referred as GPER1 (G-protein coupled estrogen receptor 1), GPR30 has been shown to interact with and mediate estrogen dependent MAPK activation, which leads to the accumulation of downstream c-fos. GPR30 signaling can also activate the PI3K/Akt pathway in a G-Protein independent manner suggesting its potential role as an estrogen receptor (Revankar et al., 2005). The signaling of GPR30 can induce rapid post-translational modifications of several transcription factors such as CREB with downstream accumulation of FOS and JUN and their respective target genes (Prossnitz and Maggiolini, 2009). The role of GPER in physiology is a bit less clear since mice devoid of GPR30 do not present major problems with mammary gland development or tumorigenesis (Marjon et al., 2014).

d. Downstream non-genomic pathway

ER α non-genomic signaling has mainly been linked to two major pathways, the Ras/Raf/ERK/MAPK and PI3K/Akt pathways

- **The MAPK pathway**

Estrogen stimulation induces the ER α /Src interaction, which rapidly leads to Src activation. Src's tyrosine kinase properties will activate downstream RAS. This will in turn lead to the activation of MEK, which will specifically lead to the activation of ERK1/2. Phosphorylated ERK1/2 will migrate to the nucleus where they activate the transcription of proliferative genes such as cyclin D1 (Zassadowski et al., 2012). The activation of the MAPK pathway has been described in numerous cells types such as nervous cells, endothelial cells and mammary cells (Hammes and Levin, 2007).

- **The PI3K/Akt pathway**

Estrogen rapidly leads to the interaction between ER α and the regulatory subunit of the PI3K, p85. This will lead to the activation of the catalytic subunit p100 and leads to an increase in intracellular PIP3. The kinase Akt is then relocalized to the plasma membrane where it is activated and can lead to downstream substrate activation (Castoria et al., 2001).

The PI3K/Akt pathway is linked to an increase in cell proliferation by favoring S-phase entry of cells as well as the induction of Cyclin D1 (Castoria et al., 2001). Akt can also phosphorylate the pro-apoptotic protein BAD which leads to its sequestration. BAD phosphorylation also leads to the release of anti-apoptotic proteins like Bcl-2 and Bcl-XL. Through this mechanism, estrogens protect against apoptotic cell death (Fernando and Wimalasena, 2004).

The two pathways were extensively described in the previous chapter.

4. Post-translational modifications of ER α

The discovery of post-translational modifications (PTM) of proteins has brought forward a huge leap in understanding protein function diversity. In this perspective, the decrypting of ER α post-translational modifications is crucial to understand the global estrogen signaling, be it the genomic or the non-genomic signaling.

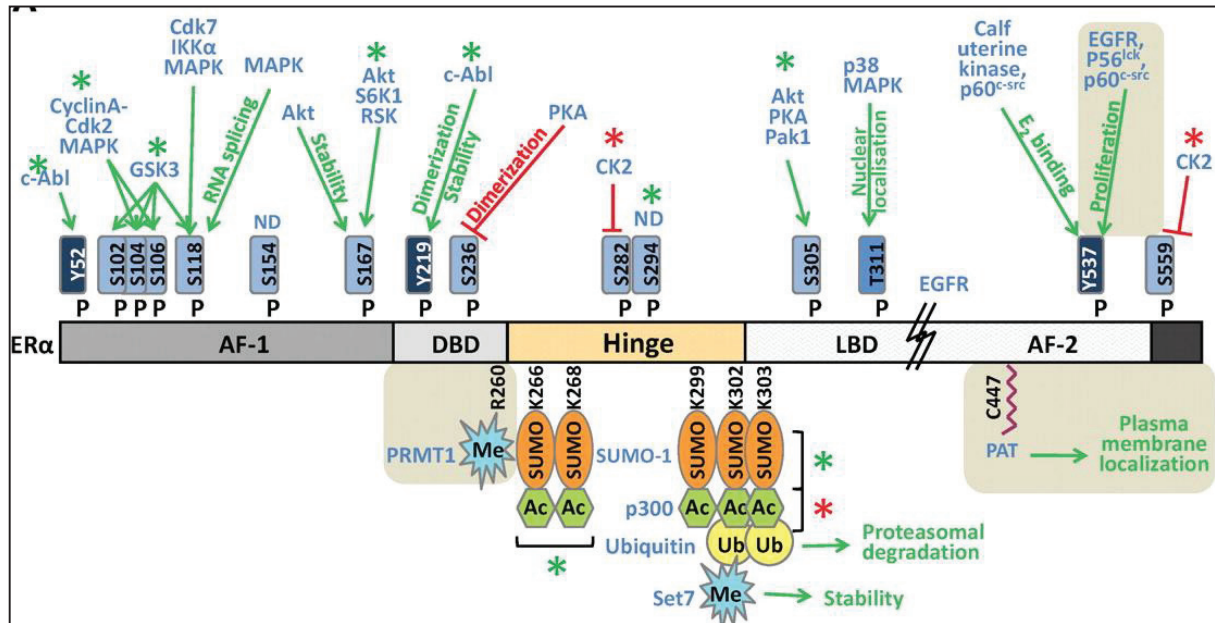


Figure 20: Post-Translational Modifications of ER α . Me: Methylation, P: Phosphorylation, Ub: Ubiquitination, SUMO: Sumoylation. Modification functions are represented in red for inhibitory and in green for activator. (*Le Romancer et al., 2011*)

a. PTM regulating the genomic pathway

• Phosphorylation.

ER α is subject to numerous phosphorylation along all its length and these are mainly involved in the activation of transcriptional activity. The serine 118 for instance can be phosphorylated by MAPK, thereby regulating ER α genomic pathway in an estrogen-independent manner (Bunone et al., 1996). Phosphorylation's on serines 104, 106, 167 and 305 are also involved in transcriptional activation (Le Romancer et al., 2011).

Other properties of ER α are also affected by phosphorylation such as phosphorylation on serine 236 which inhibits receptor dimerization (Sheeler et al., 2003). Phosphorylation of threonine 311 by p38 inhibits ER α nuclear import (He et al., 2010). Furthermore, phosphorylation of tyrosine 537 by the Src family of kinases regulates estradiol fixation on the receptor.

- **Acetylation**

Histone acetyltransferase p300 has been shown to acetylate ER α . When acetylated on lysines 302 and 303, ER α transcriptional activity is repressed (Popov et al., 2007). Estrogen dependent acetylation of lysines 266 and 268 by p300 however stimulate the binding of ER α to DNA and boosts its transcriptional activity (Kim et al., 2006).

- **Ubiquitination**

The proteasome pathway is involved in ER α turnover. The two lysines concerned with ubiquitination are lysines 302 and 303, which can also be acetylated (Berry et al., 2008). This turnover is essential to allow cells to respond quickly to changing hormonal concentrations.

- **Sumoylation**

Our team showed that ER α can be sumoylated in its hinge domain by the E3 ligases PIAS1 and PIAS 3. This PTM affects lysines 266, 268, 299, 302 and 303 and allows a boost of ER α transcriptional activity (Sentis et al., 2005). Sumoylation of ER α is a dynamic and reversible process, which can be a way to regulate the dynamics of ER α transcriptional complexes.

- **Methylation**

ER α is methylated by the SET7 methyltransferase on lysine 302 located in the hinge domain. It is necessary for the recruitment of ER α to promoter regions and leads to transcriptional activation. This process is thought to be very rapid and transient and followed by a rapid deactivation by an unidentified demethylase (Subramanian et al., 2008).

Table 6: Modification sites of ER α and their functions. ND, Not determined; ICI, ICI 172 780; PAK1, p21-activated kinase-1; PMA, phorbol myristate acetate; ROS, reactive oxygen species; Tam, tamoxifen; CK2, casein kinase 2, CXCR4, (CXC motif) receptor 4; DPN,

Amino acid	Modification	Activator	Enzyme involved	Function
Y52	Phosphorylation	Ligand independent	c-Abl	Activates stability, transcription
S102	Phosphorylation	Constitutive, E ₂	GSK3	Activates transcription
S104/106	Phosphorylation	Constitutive, E ₂	GSK3	Activates transcription
		Constitutive, E ₂ , Tam	Cyclin A-Cdk2	Activates transcription
		E ₂ , Tam, ICI, PMA	MAPK	Activates transcription
		E ₂	MAPK	Dimerization
S118	Phosphorylation	E ₂ , Tam, ICI	ND	Activates transcription
		EGF, IGF-I	MAPK	Activates transcription
		E ₂	Cdk7	Activates transcription
		ND	MAPK	Activates RNA splicing
		E ₂	GSK3	Activates transcription
		E ₂	IKK α	Activates transcription
		Prolactin	ND	Activates transcription
		ROS	MAPK	Down-regulation
		E ₂	MAPK	Dimerization
S154	Phosphorylation	Constitutive, E ₂ , EGF	ND	ND
S167	Phosphorylation	Constitutive	Akt	Activates transcription
		ROS	Akt	Down-regulation
		EGF	p90 RSK	Activates transcription
		Insulin, PMA	S6K1	Activates transcription
		ND	Akt	Activates stability
S236	Phosphorylation	Constitutive	PKA	Inhibits dimerization
Y219	Phosphorylation	ND	c-Abl	Activates: dimerization, DNA binding, stability, and transcription
R260	Methylation	E ₂	PRMT1	Nongenomic signalling
K266	Acetylation	E ₂	p300	Activates transcription
	Sumoylation	E ₂ , Tam	SUMO-1	Activates transcription, DNA binding,
K268	Acetylation	E ₂	p300	Activates transcription
	Sumoylation	E ₂ , Tam	SUMO-1	Activates transcription, DNA binding
S282	Phosphorylation	E ₂	CK2	Inhibits transcription
S294	Phosphorylation	E ₂	ND	Activates transcription
K299	Acetylation	Constitutive	p300	Inhibits transcription
	Sumoylation	E ₂ , Tam	SUMO-1	Activates transcription, DNA binding
K302	Acetylation	Constitutive	p300	Inhibits transcription
	Sumoylation	E ₂ , Tam	SUMO-1	Activates transcription, DNA binding
	Ubiquitination	Constitutive, E ₂ , ICI	Ubiquitin	Proteasomal degradation
	Methylation	Constitutive	SET7	Activates stability
K303	Acetylation	Constitutive	p300	Inhibits transcription
	Sumoylation	E ₂ , Tam	SUMO-1	Activates transcription, DNA binding
	Ubiquitination	Constitutive, E ₂ , ICI	ubiquitin	Proteasomal degradation
S305	Phosphorylation	ND	PAK1	Activates transcription
		ND	PKA	Activates transcription
		ND	Akt	Resistance to AI
T311	Phosphorylation	E ₂	p38-MAPK	Nuclear localization
C447	Palmitoylation	Constitutive	PAT	Plasma membrane localization
Y537	Phosphorylation	E ₂	Calf uterine kinase	E ₂ binding
		Constitutive	Src	Dimerization, DNA binding
		Constitutive	Src	E ₂ binding
		EGF	EGFR	Proliferation
S559	Phosphorylation	Constitutive	CK2	Inhibits transcription
S80 (S60)	Glycosylation	ND	ND	Inhibits ER β degradation
	Phosphorylation	ND	ND	Stimulates ER β degradation
S94 (S75)	Phosphorylation	Constitutive	MAPK	Stimulates ER β degradation
S106 (S87)	Phosphorylation	EGF, Ras	MAPK	Enhances the recruitment of SRC-1
		Constitutive	MAPK	Stimulates ER β degradation
		CXCR4/SDF-1	MAPK	Activates transcription
S124 (S105)	Phosphorylation	EGF, Ras	MAPK	Enhances the recruitment of SRC-1
		E ₂ , DPN, genistein	ND	ND
S255 (C399)	Phosphorylation	Erb-B2, Erb-B3	Akt	Inhibits transcription
	Palmitoylation	ND	PAT	Plasma membrane localization & activation of proapoptotic cascades
Y507	Phosphorylation	Constitutive	Src	Activates transcription

b. PTM regulating the non-genomic pathway

Our team clearly demonstrated that arginine methylation is a crucial step in the activation of the non-genomic pathway by estrogens and IGF-1. Following estrogen exposure, ER α is methylated on Arginine 260 by the (PRMT1) (Le Romancer et al., 2008b). This methylated form of ER α is exclusively cytoplasmic and is an essential prerequisite for the formation of the ER α /Src/ PI3K complex. The methylation of ER α is rapid and transient, and also our team has identified the arginine demethylase JMJD6 to be involved in the negative regulation of the methylation process (Poulard et al., 2014, 2015).

Two other modifications are involved in the regulation of the non-genomic pathway. Palmitoylation of ER α on cysteine 447 which allows its anchorage to the plasma membrane and ER α phosphorylation of Tyrosine 537 which favors the interaction between Src and ER α through Src's SH2 domain (Migliaccio et al., 2000).

In the proposed model of ER α non-genomic pathways, steroid deprivation induces palmitoylation of an ER α pool, which localizes to the plasma membrane through association with Caveolin-1. Estrogen binding to the receptor induces a conformational change, which leads to the disruption of the Caveolin-1/ER α complex. ER α dimerizes and can be methylated by PRMT1 and phosphorylated on tyrosine 537 by Src. This induces the recruitment of the Src/PI3K complex to induce downstream Akt signaling and downstream signaling cascades and proliferative physiological responses (Le Romancer et al.).

c. PTM deregulated in breast cancer

Protein modifications leads to changes in the different roles and dynamicity of the proteins; thus, their implications in breast cancer cannot be neglected.

A few post-translational modifications of ER α have been found in breast cancer with respect to their expression levels, we can count serine 118, 167, 305; arginine 260 and lysine 303.

- **Serine 118**

High Serine 118 phosphorylation on ER α has been found in low grade tumors with a good prognosis (Murphy et al., 2004). Furthermore, it has been concluded that this modification is associated with a better response to endocrine therapies such as tamoxifen and anti-aromatases (Generali et al., 2009).

- **Serine 167**

In ER α positive tumors, phosphorylation of serine 167 is associated with good prognosis and an increase in global survival and disease free survival (Jiang et al., 2007).

- **Arginine 260**

Our team demonstrated, using a specific antibody recognizing methylated R260 in IHC, that cytoplasmic methylated ER α expression is increased in 50% of mammary tumors (Le Romancer et al., 2008b). Our team later demonstrated that tumors expressing high levels of methylated ER α had a higher expression of the ER α /Src and ER α /PI3K complexes. This also correlated with an increase in phosphorylated Akt in these tumors. We found that patients with tumors expressing high levels of these complexes had a poorer outcome in terms of disease free survival (Poulard et al., 2012a).

- **Lysine 303**

A study revealed that 505 of breast tumors have a somatic A908G mutation conducting to the lysine being replaced by an arginine. This modification is associated with a poor prognosis and induces a hypersensitivity of cells to estrogen (Herynk et al., 2007). This suggests that this lysine has a very important role to play in ER α regulation and it is subject to many PTM such as acetylation, ubiquitination and simulation.

- **Serine 305**

Phosphorylation of ER α on serine 305 seems to play a role in tamoxifen resistance. It is a target site for PKA and induces a conformational change allowing the fixation of ER α with SRC-1 despite the presence of tamoxifen and therefore leads to an agonist action of tamoxifen on the receptor (Zwart et al., 2007).

These new data suggest that ER α PTM can be considered as new predictive and prognostic markers in breast cancer. In this objective, the decrypting of all modified sites and mechanisms hold a huge potential in developing targeted therapies in breast tumorigenesis.

IV. Crosstalk between IGF-1 and estrogen pathways

A. Introduction

Insulin-like growth factor and estrogen axes are very well known synergistic signaling pathways, in both normal and cancerous tissue. These two proliferative pathways were studied at different transcriptional and translational levels, especially in mammary gland and breast cancer. Studies have shown that the crosstalk between the IGF and estrogen pathways plays an important role in the etiology, maintenance and development of breast tumors. This crosstalk is due to the fact that, estrogen can activate the growth stimulatory properties of the IGF pathway via ER's genomic and non-genomic functions; also that most of the estrogen-responsive breast tumors were additionally stimulated by endogenous IGFs that augmented the proliferative response of breast tumors. **(Figure 21)**

The IGF-1 and -2 are amongst the most potent mitogens for mammary epithelial cells and there is accumulating evidence that they interact with the E₂ axis to regulate mitogenesis, apoptosis, adhesion, migration and differentiation of mammary epithelial cells. Such interactions are bi-directional and E₂ has been shown to regulate the expression and activity of IGF axis genes with the general effect of sensitizing breast epithelial cells to the actions of IGFs and insulin.

Resistance of breast tumors to anti-estrogen therapies, like tamoxifen or any SERM, is believed to be due to the increased activation of other growth factor signaling routes. These may substitute the inhibitory effect of therapies on ER, and affect the way that they are interacting with each other. For example, tamoxifen resistant MCF-7 breast cancer cells or xenografts have demonstrated reduced IGF-1R levels; however, phosphorylation of the receptor is equivalent to those in the tamoxifen sensitive ones (Knowlden et al., 2005). Furthermore, several studies have shown that the blockade of ER function can inhibit IGF-mediated mitogenesis; similarly, blocking of IGF action can inhibit estrogen stimulation of breast cancer cells.

For all these reasons, crosstalk between IGF and estrogen pathways is a corner stone of building the studies of mammary gland tumorigenesis, and is an essential way to better understand the escape mechanisms of anti-estrogen based breast tumor therapies.

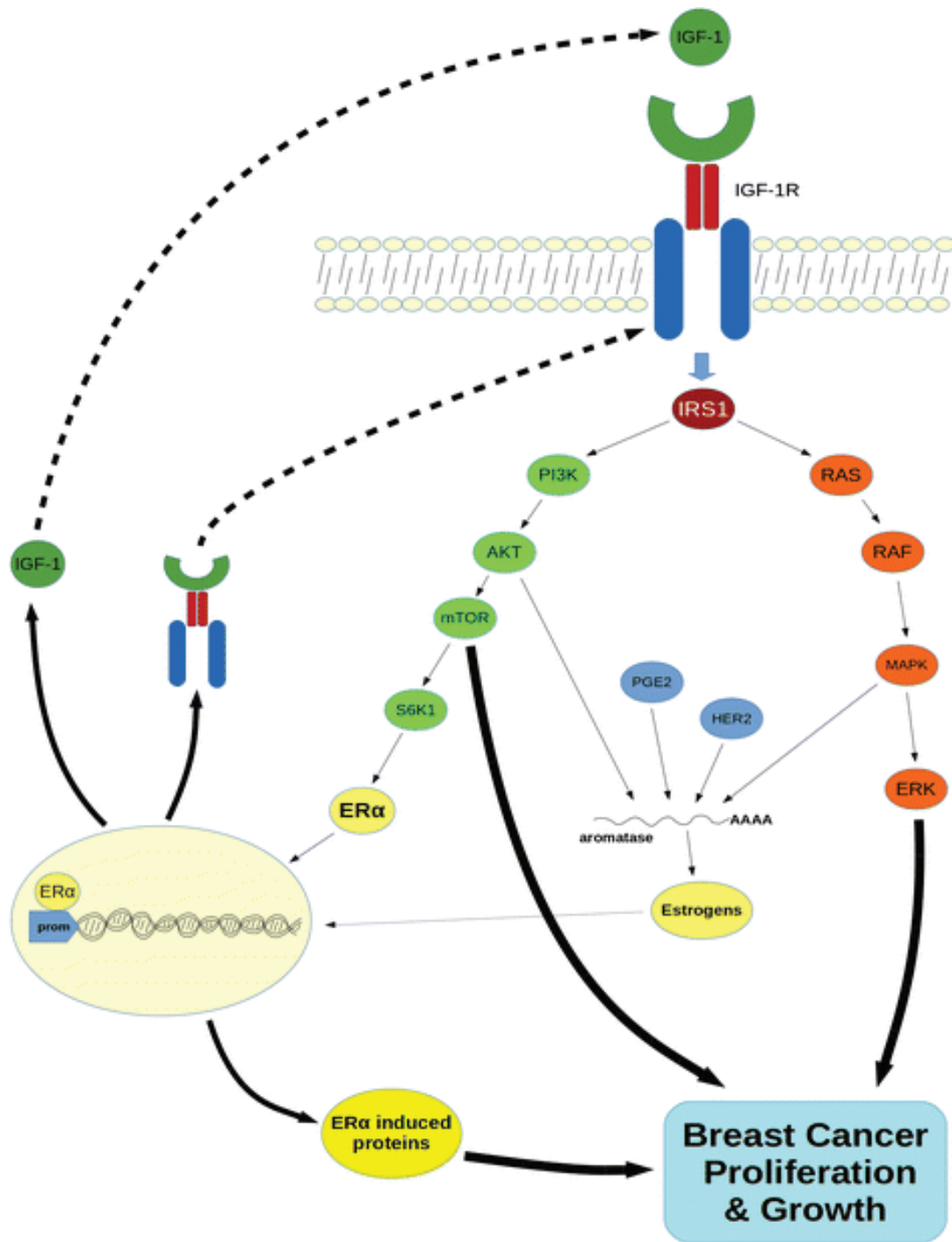


Figure 21: IGF-1 and estrogen crosstalk in breast cancer. Activated IGF-1R leads through its proliferative signaling to increase in the phosphorylation of ER α , which acts as a transcription factor on the promoter sites of the IGF-1, IGF-1R and IRS-1. In addition to Akt and MAPK activation, IGF-1R enhances the activity of Prostaglandin E2 (PGE) and HER2, which induces the aromatase activity leading to increased estrogen production; and thus repeating the cycle of breast cancer proliferation and growth. (Christopoulos et al. 2015)

B. The bi-directional crosstalk

The crosstalk between IGF and E₂ signaling pathways is bi-directional because we cannot truly separate the effects of each pathway on the other. This is exemplified in early studies in uterine cell cultures demonstrating reciprocal phosphorylation of ER α and IGF-1R following treatment of cell cultures with IGF-1 or E₂ respectively (Klotz et al., 2002). The direction of E₂ crosstalk to the IGF axis displays an extra level of complexity derived from the multi-component nature of the IGF axis, and E₂ affects the expression and activity of many of these components like IGFs, IGFRs and IGFBPs. In the mammary gland, although the crosstalk between IGF and E₂ is important to its function, it is worth mentioning that studies have shown that IGF and E₂ independently regulate an overlapping set of genes in mammary tissue, and they co-regulate up to 450 genes in MCF-7 cells (Casa et al., 2012). Interestingly this pool was enriched in genes involved in aspects of DNA replication and metabolism; similarly, co-repressed genes included known or putative tumor suppressors. These findings were highlighted in ER α -positive breast tumor samples where an “activated IGF–E₂ signature” was associated with a shorter time to metastasis in these patients.

In several clinical studies, IGF-1R was found to be up-regulated and activated in tamoxifen-resistant breast cancer patients; similarly, it was shown that diabetic women who are treated with metformin have a lower risk of breast cancer. Previous results from a retrospective cohort study showed that diabetic breast cancer patients receiving metformin had a better response rate with neoadjuvant chemotherapy than patients who received other forms of diabetic medication (Jiralerspong et al., 2009). As a result, trials were conducted examining the effects of 5 years of adjuvant metformin versus placebo in pre- and postmenopausal women, with either ER-positive or ER-negative breast cancer post-surgery and chemotherapy. These trials had promising results of decreased activation of proliferative pathways like PKB/Akt and ERK1/2 pathways, along with beneficial anticancer effects of metformin, for patients that are responsive for metformin (Dowling et al., 2015). Moreover, Engels et al. found that adding metformin to exemestane, an inhibitor of the aromatase activity, improved the clinical outcome in HR-positive BC patients with higher IGF-1R expression (Engels et al., 2016).

C. IGF effects on estrogen signaling

The main route described for IGF cross talk to estrogen signaling mechanisms is through the regulation of ER α activity by IGF-1. In vitro this has usually been reported as an alteration in phosphorylation status of ER α following IGF-1 treatment. There is some evidence that this route of E₂ independent activation of ER α may be partly involved in the development and maintenance of tumorigenesis in breast tissue (Sachdev, 2008). This is the main belief behind the escape of ER α -positive breast tumors from antiestrogen therapeutic regimens (Mawson et al., 2005).

1. IGF-1R activation of ER α activity

It is well-known that IGF-I can increase the transcriptional activity of the ER α . For example, in breast cancer cells, IGF-I can increase the expression of progesterone receptor, a transcriptional target of the ER α (Katzenellenbogen and Norman, 1990). Furthermore, IGF-I can directly increase the transcriptional activity of ER α in the absence of estradiol in MCF-7 cells (Lee et al., 1997). Combined treatment with both ligands enhanced the transcriptional activation of the ER α more than either ligand alone. It has also been shown that IGF-BP-1, an inhibitor of IGF-I action, inhibited IGF-mediated activation of ER α and had a significant inhibitory effect upon estrogen-mediated activation of the ER α (Figueroa et al., 1993). These observations suggest that IGF activation of ER α may be needed for maximal estrogen mediated receptor activation.

2. IGF-1R induces ER α phosphorylation

Although ER α is subject to extensive PTM, the growth factor mediated phosphorylation of serine residues S118 and S167 in the AF-1 domain of human ER α has been the most intensively studied. Activated IGF-1R stimulates serine phosphorylation of ER α at S118 by S6K1 in an Akt dependent manner, and the receptor is subsequently translocated to the cell membrane with resulting activation of the MAPK cascade that in turn phosphorylates S118 (Fagan and Yee, 2008). This suggests that both Akt and MAPK signaling pathways converge at S118. IGF-1 also acts via PI3K/Akt to phosphorylate at S167 within the AF-1 domain of ER α and this leads to subsequent association of ER α with many components of the IGF signaling pathway including IRS proteins, Shc, PI3K and IGF-1R itself (Hawsawi

et al., 2013). In this manner, ER α may regulate the expression, stability and intracellular localization of these signaling intermediates.

In addition to S118 and S167, IGF-1R stimulates the phosphorylation of S305 in the ligand-binding domain ER α by S6K1 kinase through IRS-1/Akt signaling axis. This modification may regulate the nearby acetylation of K303 that plays role in regulating the sensitivity of ER α to estradiol. (**Figure 22**)

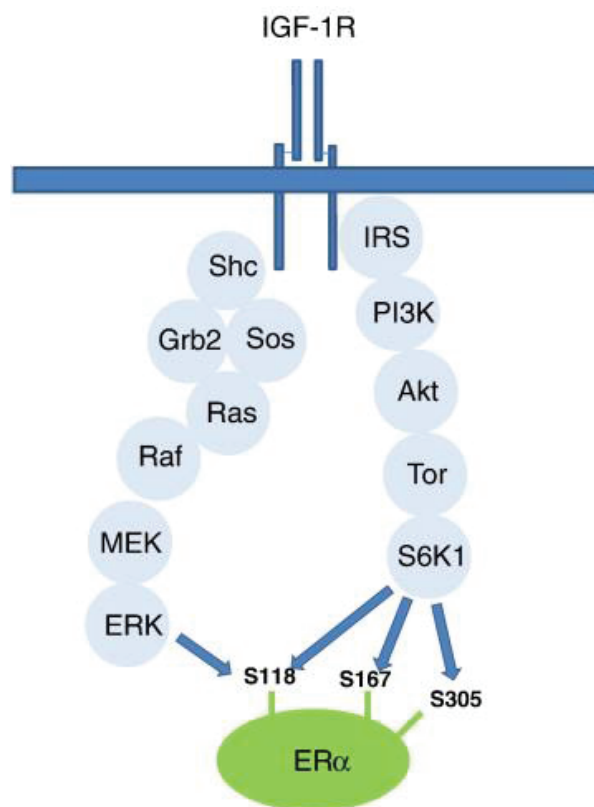


Figure 22: IGF-1R regulated phosphorylation of ER α . IGF-1 induced IGF-1R leads to the phosphorylation of ER α by S6K1 at the S118, 167 and 305 through IRS/Akt pathway. S118 is also phosphorylated by ERK through the Shc/Grb2/MEK pathway. (*Hawsawi et al., 2013*)

Signaling downstream IGF-1R through the PI3K/Akt pathway results in increased protein translation via its downstream effector mTOR. It was once thought that Akt directly activated mTOR via an Akt phosphorylation site on the protein; however, more recent evidence indicates Akt may activate mTOR by relieving it of TSC2-mediated inhibition

(Samani et al., 2007). Once relieved of inhibition, mTOR phosphorylates multiple substrates. Unphosphorylated 4E-BP1 binds eIF4E, preventing the initiation of cap dependent mRNA translation. mTOR phosphorylates 4E-BP1, allowing the release of eIF4E. Liberated eIF4E forms a protein complex (eIF4F) that binds to the 5' mRNA cap, unwinds and scans the RNA. The eIF4F complex promotes the translation of proteins such as IGF-II, cyclin D, c-myc, and VEGF. Thus, IGF-1R activation could act to enhance translation of ER-mediated genes by increasing eIF4E liberation and activation (Fagan and Yee, 2008).

D. Estrogen effects on IGF signaling

Estrogen increases positive growth factor signaling elements and down-regulates the negative signaling elements. For example, it is known that mammary gland stromal cells express IGF-1 and -2, which provide a source of local growth factors (Yee et al., 1991); at the same time, estrogen decreases the expression of IGFBP-3, which can inhibit breast cancer cell growth by binding and sequestering these IGF ligands (Nickerson et al., 1997). Furthermore, estrogen can also downregulate IGF-IIR expression that has high affinity to IGF-II, leading to inhibition of breast cancer cell proliferation (Mathieu et al., 1991).

Several studies have shown that anti-estrogens, like tamoxifen, can inhibit IGF mediated growth (Wakeling et al., 1989) and anti-IGF therapies can inhibit estrogen-mediated ones. These findings have led to the suggestion that IGFs are partially responsible for estrogen-mediated signaling and that estrogen can affect every level of the IGF signaling pathway like ligands, receptors, adaptor proteins and the complex signaling agents.

1. ER α affects IGF-1R signaling pathway

There is compelling data that E₂ can increase the expression of both IGF-1R and IRS-1 in breast cancer cells, resulting in the sensitization of cells to the mitogenic effects of paracrine IGF-1 following E₂ exposure (Thorsen et al., 1992). Up regulation of IGF-1R expression may occur through interaction between liganded ER α and the trans-acting zinc finger transcription factor Sp1 at the IGF-1R promoter (Santen et al., 2009). More recent evidence indicates that in the PC3 prostate cancer cell line E₂ also up regulates IGF-1R expression via a c-Src/ERK1/2 mediated

activation of the cyclic AMP response element binding (CREB) protein which subsequently acts as a trans activating factor within a defined enhancer region in the IGF-1R promoter (Genua et al., 2009).

The long-term effects of estrogen regulation of the IGF pathway may be attributed to genomic (transcriptional) effects; however, more recently reports have indicated that estrogen has more rapid effects on the IGF pathway, which cannot be attributed to nuclear events. Although ER does not contain a membrane localization sequence, it was found to co-immunoprecipitate with IGF-1R in the membrane (Kahlert et al., 2000). Song, et al. (2004) suggested that the interaction involves an intermediate adaptor molecule, the Shc protein. In MCF-7 cells estrogen stimulated tyrosine phosphorylation of IGF-1R, and this is dependent on ER α expression and Shc activity, the IGF-1R adaptor protein. These latter two proteins physically associate through interaction between the AF-1 domain of ER α and the phosphotyrosine binding SH2 domain of Shc, and this interaction was inhibited using the anti-estrogen ICI182780. The necessity of Shc in estradiol-mediated MAPK activation was demonstrated using Shc mutants with mutated tyrosine residues, rendering them unable to be phosphorylated. As Shc is known to associate with IGF-1R, the group hypothesized IGF-1R was also involved in activating MAPK through Shc. Further work by the group has led to a model in which estrogen stimulates cytosolic ER to interact with adaptor proteins, such as Shc or the p85 subunit of PI3K. This complex then translocates to the membrane where it associates with IGF-1R leading to activation of downstream signaling cascades.

Estrogen also influences the expression and activity of other IGF signaling components, like IRS. In MCF-7 xenografts, estrogen was also shown to highly regulate IRS-1 expression and phosphorylation. These same tumors also contained high levels of phosphorylated MAPK, indicating an active signaling cascade through IRS-1. Activation of MAPK may be through the matrix metalloproteinase-2/9 (MMP-2/9) mediated release of heparin bound EGF (HB-EGF) and subsequent activation of EGF receptor signaling (Santen et al., 2009); and in this sense estrogen-mediated IGF-1R and IRS-1 activation can be viewed as lying up stream of EGFR. Such non-genomic mechanisms may provide alternative routes for ER-positive tumor escape from SERM based chemotherapy. Removal of estrogen halted tumor growth and decreased IRS-1 expression and MAPK activity. Thus, estrogen up-regulation of both IGF-1R and IRS-1 enhances IGF-mediated signaling and may explain the observed synergy between the two ligands. Thus, as for

IGF-1R, E₂ can enhance IRS-1 expression via activation of transcription factor Sp1. In addition, ER α is reported to bind and stabilize IRS-1 protein leading to increased signaling through the IGF-1 pathway (Lee et al., 1999). The sum effect of ER α action on IRS-1 is to reinforce the stimulatory action on IGF-1R function and provide a mitogenic drive towards cell division.

Estrogen can also regulate the expression of nuclear key transcription factors that are necessary for IGF signaling, such as c-myc, c-fos, and c-jun. ER α can also regulate the expression of cell cycle components, such as cyclin D1 and p21. IGF and ER may exhibit synergy at the level of cell cycle progression via ER down-regulating p21 (a cdk inhibitor) and IGF subsequently activating cdk complexes.

In this context, it is important to appreciate that the ultimate molecular effect of IGF–E₂ cross talk, is to enhance the transition of cells through the cell cycle process, and involves the regulation of G1-S phase cell cycle progression through key regulators such as cyclinD1 or c-myc. The E₂–IGF control of cell cycle regulation is an important area of research with respect to BC progression, and the interplay between the axes in the control of this process, is an area of active study.

E. The crosstalk therapeutic implications in cancer

With the description of growth factor receptor pathway effects on ER function, it is becoming clearer that co-targeting of these pathways could be of benefit.

The development of resistance to SERM treatment in ER-positive breast tumors presents a major therapeutic challenge in the treatment and management of this cancer. Escaping SERM treatments was subjected to intense studies through gene arrays and proteomic analyses, which were designed to investigate this phenomenon. Predictions of the problem were going towards the “alternative” mitogenic signaling pathways that resulted in increased survival, proliferation and metastasis of tumor cells. Hence, studies of signaling crosstalk between IGF and estrogen may have some clinical relevance.

As previously described, increased IGF-1R expression in tamoxifen-resistant MCF-7 cells enhanced E₂ independent growth of the cells. Nonetheless, there is evidence that in these resistant cells, which have also developed resistance to the EGFR inhibitor AG1478, showed that the subsequent cell growth is largely dependent on activation of the IGF-1R (Lee et al., 1999).

This may provide a mechanism of escape of BC cells from both anti-estrogen and anti-EGFR based therapy. Moreover, ER may function in a non-genomic fashion when tumors are deprived of estrogen therapy; this model mimics resistance to aromatase inhibitors. IGF-1R also results in changes in ER phosphorylation, which could influence the ability of SERMs or pure steroidal anti-estrogens to function. Creighton et al. (2008) have shown that ER-positive breast cancer cells that have an “IGF-I gene transcription program” have poor prognosis. These data support the idea that IGF-1R activation might interact with ER function to adversely affect clinical outcome.

For this reason, clinical therapies considered combining the anti-estrogen with the three different anti-IGF-1R compounds: monoclonal anti-IGF-1R, less commonly monoclonal anti-IGF-1/-2 and the TKI to inhibit the IGF-1R activity and thus signaling.

Unfortunately, data have not been encouraging with the of lack of efficacy of the humanized anti-IGF-1R ganitumab in combination with either exemestane (AI) or fulvestrant (SERD) in increasing progression free survival compared to placebo with anti-estrogen therapy.

Indeed, the first reports of the combination of trastuzumab plus anastrozole suggest a benefit for the combination over anastrozole alone (Mackey et al., 2006). Further, combined treatment with anastrozole and an IGF-1R tyrosine kinase inhibitor (NVPAEW541) was shown to exhibit synergism in inhibiting proliferation and inducing apoptosis in breast cancer cells. For this reason, co-targeting tyrosine kinase growth factor receptors plus ER could have benefit. Hence, a combination of mAb/TKI directed therapy is clearly feasible and may have advantages over treatment with either agent alone. Recently, the development of specific kinase inhibitors has allowed the combination of anti-IGF directed therapies with inhibitors of the mTOR serine/threonine kinase in treatment of solid tumors and has generated some encouraging preliminary data (Ghayyad and Cohen, 2010). In addition, there are animal model systems suggesting that tamoxifen plus an anti-IGF-1R antibody may be effective (Cohen et al., 2005).

Objectives of the thesis study

Upon my arrival to the lab, IGF-1 was demonstrated as the only growth factor able to induce methylation of the estrogen receptor in an estrogen-independent manner. Likely to estrogen, IGF-1 induces a rapid and transient methylation of the estrogen receptor (ER α) concomitant with the formation of ER α /Src/PI3K complex. However, IGF-1 induction is not terminated when using the PI3K inhibitor as estrogen induction is affected.

In consequence, my research project was set to investigate several aspects in breast cancer.

1. Study the molecular mechanisms that regulate IGF-1 induced ER α methylation.
2. Study the role of methylated ER α in IGF-1 signaling.
3. Identify the nature of IGF-1R and ER α interaction.
4. Identify the activation biomarkers of the crosstalk between the ER α and IGF1 signaling.
5. Identify the therapeutic targets capable of inhibiting both pathways.

RESULTS

Introduction to the article

Estrogen signaling plays a major role in breast physiology and development, and thus, is implicated in breast tumor signaling. Estrogen signaling occurs in two different pathways, genomic and non-genomic signaling. Our team has been interested in studying non-genomic signaling pathway, where ER α forms a cytoplasmic complex with Src and PI3K, leading to activation of the proliferative downstream signaling pathways such as Akt and ERK1/2. In 2012, we have shown that ER α is methylated by PRMT1 at the R260, and this an initiative event to the formation of the ER α /Src/PI3K complex. Growth factors' signaling plays a major role in cancer cell tumorigenicity; and targeting these pathways is a main objective in any breast cancer treatment, in addition to the anti-estrogen therapies used. To study the effect of growth factors on methylation of ER α , we found that only IGF-1 induced this methylation in MCF-7 cells, in a similar manner to estrogen.

We decided to use several approaches to elucidate the crosstalk between IGF-1R and estrogen non-genomic signaling pathway; and we found that they interact together at several points, including the arginine methyltransferase PRMT1, and the IGF-1R adaptor protein, Shc.

Our worked allowed us to identify new players in the interaction between IGF-1 and estrogen receptors, and this will help to better understand the crosstalk between those two pathways.

The interplay between IGF-1R and ER α signaling in breast cancer involves the arginine methyltransferase PRMT1

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Keywords: IGF-1, Estrogen receptors α , Breast cancer, Estrogen signaling, arginine methylation

Abbreviated title: metER α regulates IGF-1 signaling

Abstract

Besides nuclear action, oestrogen also mediates its effects through cytoplasmic signalling. Our team has shown that ER α methylation is central to the rapid transduction of oestrogen signalling. Crosstalk between oestrogen and growth factors signalling involving phosphorylation of ER α has been largely described. Here, we investigated whether growth factors can trigger ER α methylation (metER α). Among several growth factors, we found that only IGF-1 treatment of MCF-7 cells induced rapid ER α methylation by the arginine methyltransferase PRMT1, and the binding of metER α to IGF-1R. In addition, in a cohort of breast tumours, we found that IGF-1R expression is correlated with ER α /Src and ER α /PI3K, hallmarks of non-genomic signalling, reinforcing the link between IGF-1R and ER α methylation. By several approaches, we showed that PRMT1 binds constitutively IGF-1R and upon IGF-1 stimulation, PRMT1 became activated. Moreover, we found that silencing PRMT1 or its activity inhibits ER α methylation and IGF-1 signalling. Indeed, it impedes binding of IGF-1R to its partners Shc and IRS1 as well as for the downstream signalling mediated by Akt and ERK. The adaptor protein Shc plays a significant role in this crosstalk as it is involved in the binding of IGF-1R with metER α , whereas the adaptor IRS1 has no effect. In addition, we demonstrated that IGF-1R binds directly and phosphorylates ER α on the Y219 residue, a docking site stabilizing their interaction.

These results report new insights into estrogen and IGF-1 interference, and open new perspectives of combining endocrine therapies with PRMT1 inhibitors in ER α -positive tumors.

Introduction

Breast cancer is the second most common cancer affecting women worldwide after lung cancer (globocan,iarc). Although patients are often diagnosed in the early and curable stages, the treatment of metastatic breast cancer remains a major clinical challenge. So far, estrogen has been largely involved in breast cancer development, as 80% of breast cancers express its receptor, ER α and these patients are treated by hormone therapy, but unfortunately, a problem of acquired hormonal resistance emerged highlighting that novel treatment strategies are required to improve clinical outcomes (Johnston 2010).

To date, only the nuclear ER α is taken in account to orientate the treatments; however, ER α signaling is much more complex and involves many actors. Besides the genomic signaling pathway, there exists a non-genomic pathway where cytoplasmic ER α recruits Src, PI3K and other proteins to form a big complex, that activates downstream proliferative signaling pathways such as MAPK and PI3K/Akt (Castoria et al., 2001; Levin 2005). Few years ago, our team demonstrated that upon estrogen stimulation, ER α is methylated by the arginine methyltransferase PRMT1 at the R260 residue located at the junction between the DNA binding domain and the hinge region. We have shown that this event is a prerequisite for the formation of ER α /Src/PI3K complex and the activation of the downstream signaling (Le Romancer et al., 2008a). Moreover, we also found that this pathway was activated in aggressive human breast tumors and could constitute a new prognosis marker (Poulard et al., 2012).

In addition, ER α activity is regulated by estrogen-independent pathways involving kinases that are activated by phosphorylation involving growth factor receptors like epidermal growth factor

receptor (EGFR) and insulin like growth factor receptor (IGF-1R) (Kato et al. 1995; Song et al. 2007). For example, IGF-1 activates ER α transcriptional activity *via* its phosphorylation on Ser167 residue through Akt/mTOR/S6K1 axis (Becker et al. 2011). We then investigated whether growth factors could trigger ER α methylation and found that only IGF-1 induced ER α methylation. IGF-1R is a tyrosine kinase cell surface receptor which is involved in the regulation of cell growth and metabolism (Dupont and Holzenberger 2003). Previous studies have shown that activation of the IGF-1R signaling pathway promotes proliferation, survival, and metastasis of breast cancer cells (Christopoulos et al. 2015). IGF-1R, when activated by ligand binding, gets auto phosphorylated on tyrosine residues in the kinase domain, thus activating adaptor proteins such as Src homology and collagen domain protein (Shc) and insulin receptor substrate (IRS) (Baserga et al., 1997; Ishihara et al., 1998b; Pollak et al., 2004). Then, IGF-1R activation triggers the proliferative signaling *via* two main pathways; ERK1/2 pathway and PI3K/Akt pathway through Shc and IRS1 respectively (Christopoulos et al., 2015; Simpson et al., 2017).

Numerous papers have highlighted a crosstalk between IGF-1R and ER α in breast tumour cells. Indeed, the dual treatment of cells with oestrogen and IGF-1 results in greater proliferation than exposure to either ligand individually (Stewart et al. 1992; Yee and Lee 2000). Moreover, exposure to the carcinogens (DMBA) in dwarf rats that exhibit reduced circulating level of IGF-1, present a reduced ability to develop ER α -positive breast tumours than in normal rats. Administration of exogenous IGF-1 restores a normal tumour development (Thordarson et al. 2004). The potential synergy between ER α and IGF-1R is underscored by studies showing enhanced tumour efficacy upon combining anti-oestrogen agents with IGF-1R inhibitors (Chakraborty et al. 2010; Lisztwan et al. 2008). At the molecular level, it has been clearly demonstrated that the crosstalk between ER α and IGF-1 is bidirectional as ER α regulates the IGF-1 pathway, and as IGF-1 activates ER α

in a ligand-independent manner. For example, IGF-1 induces ER α expression, phosphorylates it as well as its coactivators, leading to its transcriptional activity (Kato et al. 1995; Lannigan 2003). Conversely, oestrogen influences the IGF-1 pathway by increasing the expression of both IGF-1R and IRS1 in breast cancer cells (Jackson and Yee 1999). Moreover, ER α was also shown to regulate the degradation of the IRS1 in breast cancer cells (Morelli et al. 2003).

The overall aim of our study was to elucidate the molecular mechanisms responsible for IGF-induced ER α methylation and the role of metER α in IGF-1 signalling. Herein, we reveal a novel crosstalk between ER α and IGF-1R, *via* PRMT1-induced ER α methylation. Furthermore, these data provide a rationale to use PRMT1 inhibitors in order to target at the same time the IGF-1 and oestrogen non-genomic pathways.

Materials and Methods

Cell culture and transfections

MCF-7, an ER α -positive human breast adenocarcinoma cell line, was purchased from American Type Culture Collection (ATCC) Organization. MCF-7 cells were maintained in monolayers at 37°C in humidified atmosphere of 5% CO₂, and cultured in DMEM Glutamax medium supplemented with 10% fetal bovine serum, 2% Penicillin/Streptomycin and 1% non-essential amino acids (*Life Technologies*).

Prior to performing treatment with ligands, cells were grown for 48 hrs in phenol red-free medium supplemented with 10% charcoal-stripped serum (*Biowest*), in order to remove steroid hormones (steroid-depletion) or in serum free for IGF-1 treatment. The cells were then treated for different times with E₂ (*Sigma*) 10⁻⁸ M or IGF-1 (4 x 10⁻⁵ μ g/ μ l) from *Peptotech*. When stated, cells were treated with the Src inhibitor PP1, the PI3K inhibitor LY294002, the IGF-1R inhibitor OSI-906 (*Calbiochem*) or the PRMT1 inhibitor MS023 (*Tocris Bioscience*).

For knockdown experiments, MCF-7 cells were seeded in complete medium a day prior to transfection, at a cell density of 1.0 x 10⁶ per petri-dish or 9 x 10⁵ cells in a 12-well plate containing cover slips. A transfection mixture composed of the required IGF-1R, ER α , PRMT1, Shc, IRS1-specific siRNAs or the scramble siRNA (*Eurogentec*) (50 nM) and lipofectamine 2000 reagent (*Invitrogen*) were transfected into MCF-7 cells according to the manufacturer's protocol. For overexpression experiments, pSG5 Flag-tagged vectors were transfected into the cells using XtremeGENE reagent (*Roche*) according to the manufacturer's protocol.

PDX tumors

We used tumors from human breast patient's derived xenograft (PDX) from Dr Marangoni of Curie Institute, Paris., HBCx-17, HBCx-34 have been characterized previously (Marangoni et al. 2007). HBCx-17 does not express ER α and IGF-1R and HBCx-34 expresses both ER α and IGF-1R.

Immunoprecipitation and western blotting

Cells were lysed using RIPA buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 0.25% deoxycholate) supplemented with protease inhibitor tablets (Roche Molecular Biochemicals) and phosphatase inhibitors (1 mM NaF, 1 mM Na₃VO₄ and 1 mM b-glycerophosphate). Protein extracts were incubated with primary antibodies overnight at 4°C by shaking. Protein G or A-Agarose beads were added and the mixture was incubated 2 hrs at 4°C. The immunoprecipitated proteins were separated on SDS-PAGE and visualized by ECL (*Roche Molecular Biochemicals*).

Proximity Ligation Assay (PLA)

This technology was developed by Olink Bioscience (Sweden) to visualize protein/protein interactions *in situ* (≤ 40 nm) (Soderberg et al. 2007).

Fluorescence revelation

90 000 MCF-7 cells were seeded in coverslips in 12-well plates, and serum-starved for 48-72 hrs. Following the kinetic treatment with IGF-1, cells were fixed with cold methanol for 2 min. After saturation, the different couples of primary antibodies (rabbit and mouse) were incubated for 1 hr at 37°C. The PLA probes “mouse minus” and “rabbit plus”, consisting of secondary antibodies

conjugated with complementary oligonucleotides, were incubated 1 hr at 37°C, After ligation of nucleotides, the amplification occurred for 100 min at 37°C. Afterwards, the samples were mounted with the Duolink mounting medium containing Dapi, and then analyzed on fluorescence microscope.

Bright field revelation

Fixed tumor tissues were initially incubated in a hydrogen peroxide solution, for 5 min at room temperature, to avoid peroxidase quenching. The following steps were to those described above. For the detection, the probes were labeled with horseradish peroxidase after two washes in high purity water. A nuclear staining solution was added onto the slides and incubated 2 min at room temperature. After washing the slides 10 min under running tap water, the samples were dehydrated in ethanol and in xylene. Samples were mounted in non-aqueous mounting medium and then analyzed using a bright-field microscope. To detect ER α /Src and ER α /PI3K interactions, we used the antibodies already described (Poulard et al. 2012)

Immunofluorescence

MCF-7 cells (7×10^4) were grown on coverslips in 12-well plates. After treatment, cells were fixed in methanol for 2 min and washed twice in PBS. None specific binding was blocked using 1% gelatin solution for 30 min at room temperature and cells were incubated with primary antibodies for 1 hr at 37°C. After PBS washes, the cells were incubated for another hour at 37°C with the secondary antibody Alexa Fluor 488 (Molecular Probes) in Dako diluent, then washed in PBS and mounted on glass slides in mounting solution (*Dako*). The images were acquired using a fluorescent microscope.

Cloning and vectors

Ligation in pGEMT Easy vector was carried on using the *Promega® Rapid Ligation pGEMT Easy Kit*. IGFR fragments were also ligated in pGEX 4T1 vector that had the Bam H1 & Xho 1 restriction enzymes overhangs. Two μl of the ligation product were added to 25 μl of *DH5 α* bacteria and kept in ice for 30 min, a heat shock is done at 42°C for 20 seconds and the bacteria were kept in ice for 5 min. Six hundred μl of 2YT 1x medium were added to the bacteria and kept on a shaker for 1 hr at 37°C. After incubation, the bacteria were centrifuged for 5 min at 6500 g, 400 μl of the supernatant were removed and the bacteria were resuspended in the 225 μl left and seeded on Ampicillin containing LB Agar, Incubated at 37°C overnight.

Alkaline Lysis Mini Preps were prepared, DNA samples were digested with BamH1 and Xho 1 restriction enzymes. Samples were migrated on 1% agarose gel.

Protein Production

The genes of interest, in this case IGF-1R, cloned into a pGEX-4T1 Plasmids were transformed into *BL21* Protein Producing Bacteria from *Novagen®* and selected on Ampicillin containing LB Agar. One colony of the transformant bacteria was amplified overnight in 25mL 2YT 1x medium containing 0.1% Ampicillin at 37°C under agitation. 10mL of the overnight culture was seeded into 500ml 2YT 1x medium containing 0.1% Ampicillin and the culture was incubated at 37°C under agitation until the bacterial culture reached an optical density of 0.6 on a spectrophotometer. IPTG was added to the culture medium to a final concentration of 0.1mM and induction was carried out for 3h at 28°C. Following induction, the bacterial culture was centrifuged at 4000g for 15min at 4°C and the bacterial pellet was conserved at -80°C overnight.

The bacterial pellet was resuspended in 12ml Lysis Buffer containing Tris 50mM, KCl 100mM, EDTA 1mM, DTT 1mM, Benzonaze 25U/ml and 1 Protease Inhibitor Complex tablet (*Roche*). 200µL Lysozyme was added to the bacterial suspension and incubated at 4°C under agitation. The bacterial lysate suspension was sonicated twice 30 secs, diluted with 12ml Lysis Buffer and sonicated twice 30 secs once more. 2.5mL Triton10% was added to the bacterial lysate and incubated 30 min at 4°C under agitation. The lysate was centrifuged at 10000g for 30 min at 4°C. The supernatant was put into contact with 300µl Glutathione-Sepharose Resin for 2h at 4°C under agitation. The resin was then washed thrice with cold PBS, once with PBS Triton 1% and twice more with cold PBS.

Glutathione transferase (GST) pull-down assay

ER α expression plasmid was transcribed and translated *in vitro* using T7-coupled reticulocyte lysate in the presence of [³⁵S] methionine. 5 µg of purified recombinant GST-fusion proteins were incubated with labelled proteins or 500 µg of protein extracts in 200 µl of binding buffer (Tris 20 mM pH7.4, NaCl 0.1 M, EDTA 1 mM, Glycerol 10%, Igepal 0,25%) with 1 mM DTT and 1% milk) for 2 hrs at room temperature. After washing, bound proteins were separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.

***In vitro* methylation assays**

Immunoprecipitated PRMT1 from MCF-7 cells or GST-PRMT1 fusion protein were incubated with GST-Hinge of ER α as already described (Le Romancer M. et al. 2008) in the presence of S-adenosyl-L [methyl-³H] methionine ([³H] SAM 85 Ci/mmol from a 10.4 mM stock solution in dilute HCl/ethanol 9/1 [pH 2.0–2.5]; *Perkin Elmer*) for 1 hr at 30°C. Methylation reactions were

quenched by the addition of 10 μ L of 2x Laemmli sample buffer, heated at 100°C for 5 min, and separated on SDS-PAGE. Following electrophoresis, gels were soaked in Amplify reagent (*Sigma*) according to the manufacturer's instructions and visualized by autoradiography.

***In vitro* phosphorylation assays**

The assays were performed by incubating the IGF-1R active protein (*Merck*) with GST-fusion proteins of interest according to the manufacturer's protocol, in the presence of adenosine 5'-triphosphate, [γ -³²PATP] (*Perkin Elmer*) for 30 min at 30°C. Phosphorylation reactions were quenched by the addition of 10 μ L of Laemmli buffer, heated at 95°C for 5 min, and separated on SDS-PAGE 10% acrylamide gel. Following electrophoresis, gels were dried for 45 min at 80°C and revealed by autoradiography.

Immunohistochemistry staining

Paraffin embedded tumor tissues fixed in formalin were used for analysis. The pathologist selected representative areas from breast invasive carcinomas. Triplicates from each tumor were inserted in TMA blocks which contained 40 tumors each. Eleven TMA (440 tumors) were analyzed. After deparaffinization and rehydration, tissue sections were boiled in 10 mM citrate buffer pH 8 at 95°C for 40 min. The slides were then incubated in 5% hydrogen peroxide in sterile water to block the activity of endogenous peroxidases. The slides were then incubated at 37°C for 1 hour with the anti-IGF-1R antibody. The slides were subsequently incubated with a biotinylated secondary antibody bound to a streptavidin peroxidase conjugate (Envision Flex kit Ref: K800021-2, Dako). Bound antibodies were revealed by adding the substrate 3, 3-diamino benzidine. Sections were counterstained with haematoxylin.

Image acquisition and analysis

The hybridized fluorescent slides were viewed under a *Nikon Eclipse Ni microscope*. Images were acquired under identical conditions at x60 magnification. Image acquisition was performed by imaging DAPI staining at a fixed Z Position while a Z stack of +/- 5µm at 1 µm intervals was carried out. The final image was stacked to a single level before further quantification. On each sample, at least one hundred cells were counted. Analyses and quantifications of these samples were performed using *Image J* software (free access). PLA dots were quantified on 8-bit images using the 'Analyse Particles' command, while cell numbers were numerated using the cell counter plugin.

IHC images were also acquired using *Nikon Eclipse Ni microscope* at x40 magnification and PLA dots were quantified as described above.

Human breast cancer samples collection

The tumors from 440 CLB patients with invasive breast cancer, whose clinical and biological data were available from the regularly updated institutional database, were analyzed. Written informed consent was obtained from each patient. The institutional ethics committee approved the study protocol. Patient's characteristics are presented in Table S1.

Statistical analysis

Descriptive analysis. Clinical parameter's distributions (cancer subtype, clinical, histological and immunohistochemical data) were presented in the form of numbers and percentages. Correlations of expression with clinical and biomarkers parameters were done using Fisher's exact test.

Survival analysis. Overall Survival (OS) defined as time from diagnosis to death or date of last follow-up and Disease Free Survival (DFS) defined as time from diagnosis to death or relapse or date of last follow-up (for censored patients) were studied. Survival distributions were estimated by Kaplan-Meier method and compared between expression's level groups using the Log-Rank test. Hazard ratios for relapse or death were estimated by cox regression model. Statistical analyses were done using SPSS v20.0 software (IBN, USA). A statistical significant interaction was considered if the alpha error was less than 5%.

Results

IGF-1 treatment induces ER α methylation

To investigate whether growth factors can trigger ER α methylation, we performed treatment of MCF-7 cells with insulin, EGF, IGF-1 or E₂ for different times. Cellular lysates were then analysed for ER α methylation by immunoprecipitation with an antibody that specifically recognized ER α di-methylated on R260 and revealed with ER α antibody as already described (Poulard et al. 2012). Upon the different treatments IGF-1 was the only growth factor triggering ER α methylation (Figure 1A). This time course is reminiscent of what is found for E₂ treatment, as it is rapid and transitory. As we have previously shown that ER α methylation is a prerequisite for the formation of the complex containing ER α /Src/PI3K, we wanted to determine whether it was equivalent upon IGF-1 stimulation. Figure 1B shows that like E₂, IGF-1 triggers the interaction of metER α with Src and p85 of PI3K. Moreover, PRMT1 is also responsible for IGF-1-induced ER α methylation as its knockdown strongly reduced ER α methylation (Figure 1C). As IGF-1R and its adaptors IRS1 and Shc have already shown to form a complex with ER α (Song et al. 2004; Tian et al. 2012), we assessed whether metER α was involved in this complex formation. Figure 1D shows that IGF-1 stimulation triggers the interaction between metER α with IGF-1R. We next studied the regulation of ER α methylation using inhibitors targeting the kinase activities present in the complex. We found that the IGF-1R inhibitor, OSI-906, partially disrupted ER α methylation similarly to Src inhibitor PP1, although PI3K inhibitor LY294002 had a modest effect (Figure 1E). As expected OSI-906 abolished completely IGF-1 signalling (P-Akt and P-ERK), but PP1 had a stronger impact on ERK signalling and LY294002 on Akt signalling. To confirm our results showing that IGF-1 regulates metER α , we studied IGF-1R expression by IHC in a cohort of 440 breast tumours specimen in which we have already studied ER α /Src and ER α /PI3K expression, shown to be strongly correlated

with metER α (Le Romancer M. et al. 2008). Statistical analyses showed that ER α /Src and ER α /PI3K expression was correlated with IGF-1R expression (Figure 1F and 1G). Pictures of a tumour expressing high level of ER α /Src, ER α /PI3K and IGF-1R and one expressing low level of the 3 markers are shown in FigS1.

Altogether, these results show that IGF-1 triggers PRMT1-induced ER α methylation and the recruitment of Src and PI3K; comforting the idea that oestrogen non genomic signalling could interfere with IGF-1 signalling.

IGF-1R interacts with PRMT1 and regulates its activity

We next investigated whether IGF-1 was able to regulate PRMT1 activity. MCF7 cells were treated with IGF-1 for different times, then cell extracts were precipitated with an anti-PRMT1 antibody, and its enzymatic activity was tested by a methylation *in vitro* assay using the hinge fragment of ER α containing R260 residue as an exogenous substrate. We found that the level of methylation was low in the absence of growth factor (Figure 2A); it increased significantly after 5 min of exposure to IGF-1, and then decreased at 15 min. Then, we studied a putative interaction between IGF-1R and PRMT1 in MCF-7 cells by the proximity ligation assay (PLA). To analyse the specificity of the interaction we knock down IGF-1R or PRMT1 (efficacy of the extinction is shown on Figure 2B). Figure 2C shows that IGF-1R interacted with PRMT1 in the cytoplasm of the cells independently of IGF-1 stimulation, as indicated by the presence of red dots (panels a,b,c). The interactions were quantified by counting the number of dots per cell (Figure 2D). The signals strongly decreased in MCF-7 cells in which expression of IGF-1R (panels d,e,f) or PRMT1 (panels g,h,i) was knock down showing the specificity of the signal. We also analysed a putative direct interaction between the 2 proteins by a GST pull down approach and we found that the

intracellular domain of IGF-1R (ICD) interacts specifically with GST-PRMT1 and not with GST (Figure 2E).

We next wanted to evaluate whether the IGF-1R adaptors Shc and IRS1 were involved in PRMT1 binding to IGF-1R. To answer to this question, we silenced Shc and IRS1 in MCF-7 cells (Figure 3A) and studied the IGF-1R/PRMT1 interaction by coimmunoprecipitation and PLA assays. As shown in Figure 3B, the knock down of Shc decreased significantly IGF-1R/PRMT1 interaction whereas IRS1 has no effect. These results were confirmed by PLA (see photos in Figure 3C and counting in Figure 3D).

PRMT1 interferes with IGF-1 signalling

To determine whether PRMT1 was involved in IGF-1 signalling, we knock down PRMT1 in MCF-7 cells and we studied the main events of IGF-1 signalling. As shown in Figure 4A, PRMT1 is required for the interaction of IGF-1R with ER α . This result was confirmed by the PLA approach (Figure S3 A to C). We also found that PRMT1 is involved in IGF-1R interaction with Shc and IRS1. In addition, the downstream events as phosphorylation of Akt and of ERK are also strongly impaired upon PRMT1 silencing. At the contrary, phosphorylation of IGF-1R on tyrosine residue remained unchanged. We next wanted to confirm these results using a specific PRMT1 inhibitor MS023 (Figure 4B) (Eram et al. 2016). We first checked whether it was able to decrease ER α methylation and found that the inhibitor decreased ER α methylation in a dose dependent manner (Figure S3D). Moreover, the downstream signalling of IGF-1 was impaired although upstream IGF-1R phosphorylation remained unchanged (Figure 4B).

Then, we tested whether Shc and IRS1 play a role in IGF-1R/ER α interaction. For this purpose, we silenced Shc and IRS1 in MCF-7 cells, and we analysed IGF-1R/ER α interaction by co-immunoprecipitation after IGF-1 treatment. Shc silencing decreases strongly IGF-1R/ER α interaction whereas IRS1 has no effect (Figure 5A). In addition, Shc knock down also significantly abolished IGF-1-induced ER α methylation. We confirmed these results by the PLA. Figures 5B and 5C show that Shc silencing prevents IGF-1R/ER α interaction although IRS1 silencing has no effect.

IGF-1R interacts with ER α and triggers its phosphorylation

To study more in details IGF-1R/ER α interaction, we first investigated their interaction in breast tumours by PLA where the presence of interaction is revealed by brown dots. As shown in Figure 6A, we can see dots in the cytoplasm of a patient-derived breast tumour (PDX) (panel, c) that expresses strongly IGF-1R (panel a), however the ER α -negative PDX that do not express IGF-1R (panel b) does not show any dots. These results have been quantified on the graph presented in Figure 6B. We next assessed a direct interaction between IGF-1R and ER α by a GST pull down approach. We found that radioactive ER α interacts specifically with the ICD of IGF-1R independently of the presence of E₂ (Figure 6C), and more precisely at the level of the D2 domain that contains the kinase activity of IGF-1R (Figure S4A and S4B). We then investigated whether ER α could be a substrate for IGF-1R. To answer to this question, we performed a phosphorylation *in vitro* assay with the active IGF-1R, in the presence of purified fragments of ER α fused to GST (Figure 6D). We found that the fragment containing the DBD is the only one to be phosphorylated (Figure 6E). When we looked at the DBD sequence, we found that it contains 3 tyrosine residues, Y195, Y197 and Y219 (Figure 7A). The substitution of the tyrosine by phenylalanine residues

showed that only the Y219 substitution had an impact of IGF-1R-induced phosphorylation (Figure 7B). We then studied if ER α phosphorylation on Y219 could play a role in IGF-1R/ER α interaction. To assess this point, we transfected MCF-7 cells with psG5-FlagER α wild type or the mutant Y219F (Figure 7B) and we studied IGF-1R/ER α interaction by PLA using the anti-Flag and the anti-IGF-1R antibodies. The interaction is strongly increased upon IGF-1 activation in cells where WT ER α was transfected; however in cells transfected with the mutant Y219F ER α , the interaction is strongly decreased (Figure 7C and 7D).

Discussion

Approximately 80% of breast cancers express ER α and endocrine therapies have led to significant improvements in patient survival. However, their efficacy is limited by intrinsic and acquired therapeutic resistance. Among the causes of resistance, receptor tyrosine kinase signalling has been shown to play an important role. As an example, increased expression of IGF-1R can elicit tamoxifen resistance (Musgrove and Sutherland 2009). This can be explained by the bidirectional crosstalk between ER α and receptor tyrosine kinase signalling.

In this study, we shed on light new links between ER α and IGF-1R involving PRMT1 enzymatic activity.

Similarly to E₂, IGF-1 is able to trigger ER α methylation in MCF-7 cells. This event is not common to growth factors as insulin and EGF did not induce this post translational modification. The time course is similar to E₂, as ER α methylation is rapidly induced and transitory, suggesting the removal of the methylation mark. We can hypothesize that the arginine demethylase JMJD6 could be involved, as it has already been shown that ER α is a substrate (Poulard et al. 2014). However, the regulation of ER α methylation induced by IGF-1 does not seem to be identical to that of E₂ because Src or PI3K inhibition does not have any effect on metER α , although they are part of the complex formed upon IGF-1 stimulation (Figure 1B). Moreover, the analysis of IGF-1R expression in a cohort of breast tumour specimen exhibits a strong correlation between IGF-1R and ER α /Src and ER α /PI3K expression. In a previous cohort, we have shown that ER α /Src and ER α /PI3K expression is strongly correlated with metER α and downstream Akt activation (Poulard et al. 2012). In conclusion, these results highlight the crosstalk between IGF-1 and metER α *in vivo*.

We then wanted to decipher how IGF-1 triggers ER α methylation. By different approaches, we demonstrated that PRMT1 binds constitutively IGF-1R, and PRMT1 becomes activated upon IGF-

1 treatment. This result could appear startling because PRMT1 is mainly expressed in the nucleus (Bedford and Clarke 2009), where it regulates transcription *via* histone methylation (Strahl et al. 2001). However, several articles related PRMT1 binding to membrane receptors. In 1997, a two hybrid screen identified PRMT1 as a partner of the type I interferon receptor, independently of interferon (Abramovich et al. 1997). PRMT1 also binds and methylates the Ig α subunit of the B cell antigen receptor, to regulate B cell differentiation (Infantino et al. 2010). IGF-1R/PRMT1 interaction is direct and does not require the presence of the adaptors Shc and IRS1, as their knock down does not modify their interaction. Based on our results, we hypothesized that PRMT1 could regulate IGF-1 signalling pathway by methylating IGF-1R, but we were unable to detect any methylation (data not shown). However, we only investigated the putative methylation on the intracellular domain of the receptor, and we cannot exclude that PRMT1 could methylate extracellular domains, as it was demonstrated for EGFR (Liao et al. 2015).

We found that PRMT1 plays a crucial role in IGF-1 signalling and its silencing or pharmacological inhibition impairs downstream signalling, such as Akt and Erk phosphorylation (Figure 4A and B). This effect may be due to the decrease of IGF-1R interaction with its adaptors Shc and IRS1 and with ER α . A recent study showed that treatment of cells with IGF-1 induced a partial relocalisation of ER α in the cytoplasm (Yu et al 2013). This is in agreement with our study that shows that upon IGF-1 treatment, metER α binds to IGF-1R.

In addition, we showed that Shc plays an important role in the IGF-1R/ER α interaction. This is consistent with the work of Santen team that showed that Shc is a central actor in the formation of a complex containing Shc/ER α /IGF-1R, upon E₂ stimulation (Song et al. 2004). Moreover, we confirmed the interaction *in vivo*, as demonstrated by PLA in a breast PDX. Of interest, we demonstrated that ER α binds directly with the intracellular domain of IGF-1R, allowing IGF-1R

to phosphorylate ER α on Y219 residue. The site of phosphorylation is located in the DBD of ER α . Interestingly, ER α Y219F mutant lose its capacity to bind to IGF-1R, suggesting that Y219 phosphorylation could stabilize the interaction. This tyrosine residue has already been shown to be phosphorylated by the kinase c-Abl to regulate ER α transcriptional activity *via* the modulation of its binding to DNA. In addition, a glutamic acid mutant which mimics the phosphorylation, increased cell proliferation and invasion (He et al. 2010). According to our results, we can hypothesize that the observed effects could also be due to activation of the IGF-1 signalling.

Based on our results, we can propose a model of the IGF-1 signalling involving PRMT1 enzymatic activity. In absence of ligand, IGF-1R is bound constitutively with PRMT1, Shc and IRS1. The presence of IGF-1 causes PRMT1 activation, which in turn, methylates ER α , triggering its binding to IGF-1R and its phosphorylation on the Y219, stabilizes the interaction. Then, IGF-1R phosphorylates also IRS1 and Shc on tyrosine residues, which forms docking sites for PI3K and Grb2, activating Akt and ERK pathways, respectively (Figure 7E) (Simpson et al. 2017). PRMT1 plays a crucial role in this pathway as its silencing or inhibition completely abolished the downstream activation of Akt and Erk pathway.

Even, if our results suggest that the PRMT1-induced ER α methylation is involved in IGF-1 signalling, we cannot exclude that it could involve other PRMT1's substrates. To answer to this point, we plan to use genome editing to generate MCF-7 cell lines harbouring R260K *ESR1* mutation to decipher the precise role of metER α in IGF-1 signalling.

Increased expression of IGF-1R and/or IGF-1 has been observed in various cancers, including breast cancer. In breast cancer cells, IGF-1R and ER α are often coexpressed (Happerfield et al. 1997; Heskamp et al. 2015). IGF-1R has also been shown to be upregulated in TAM-resistant breast cancer cells (Massarweh et al. 2008; Simpson et al. 2017) and to participate in anti-estrogen

resistance (Zhang et al. 2011). IGF-1R expression has different prognostic values for patients with breast cancers of different molecular subtypes. Indeed, in hormone-receptor breast cancer, it was correlated with favourable survival, although in triple negative breast cancer, it predicted worse survival (Heskamp et al. 2015; Yan et al. 2015).

So far, the monotherapies targeting IGF-1 signalling have been largely disappointing maybe and success has been limited by the lack of validated predictive biomarker. In addition, due to their lack of specificity, IGF-1R tyrosine kinase inhibitors are associated with hyperglycaemia because of interference with insulin signalling (Simpson et al. 2017).

In this context, our work could provide significant improvement in breast cancer treatment. Indeed, targeting PRMT1 could be a specific way of inhibiting IGF-1 signalling, since insulin does not trigger ER α methylation. Moreover, PRMT1 inhibitors could target at the same time, ER α non-genomic signalling and IGF-1 signalling, two pathways largely involved in breast cancer development.

Legends of figures

Figure 1: IGF-1 triggers ER α methylation.

A) MCF-7 cells grown in serum free medium were treated with E₂ (10⁻⁸M), insulin (100 ng/ml), EGF (100 ng/ml) or IGF-1 (40 ng/ml) for the indicated times. Then, ER α methylation was assessed by performing immunoprecipitation with the anti-metER α antibody followed by western blotting with antibody against ER α . ER α and p-Akt in inputs are shown.

B) MCF-7 cells were treated with E₂ or IGF-1, and then tested for ER α methylation. The immunoprecipitates were blotted with anti-ER α , anti-Src and anti-p85 of PI3K antibodies.

C) Lysates of MCF-7 cells transfected with control siRNA duplexes or siRNAs targeting PRMT1 were tested for IGF-1-induced ER α methylation as in A.

D) MCF-7 cells were treated with IGF-1 for the indicated times. Then, metER α was immunoprecipitated with the specific antibody followed by western blotting with anti-ER α , anti-IGF-1R antibodies.

E) MCF-7 cells were treated or not with OSI-906 (5 μ M), 24 hrs before IGF-1 treatment, or with PP1 (5 μ M) or LY294002 (20 μ M) 20 min before IGF-1 treatment, then incubated with IGF-1 for the indicated times. ER α methylation was evaluated as described above. IGF-1R was immunoprecipitated and revealed for the presence of ER α by western blotting. p-IGF-1R, p-Akt and p-ERK expression in input are shown in the lower panel.

F) Correlation studies between ER α /Src and IGF-1R expression were performed using Fisher's exact test.

G) Correlation studies between ER α /PI3K and IGF-1R expression were performed using Fisher's exact test.

Figure 2: IGF-1R interacts with PRMT1

A) MCF-7 cells were treated with IGF-1 for the indicated times, then cell lysates were immunoprecipitated with anti-PRMT1 antibody and its enzymatic activity was evaluated by performing an *in vitro* methylation assay using GST-Hinge of ER α as a substrate, revealed by western blotting using the anti-metlER α antibody.

B) MCF-7 cells were transfected with siScrambled or siRNA targeting IGF-1R or PRMT1 for 72 hrs, then treated with IGF-1 for different times. The efficacy of protein inhibition was verified by western blotting using the corresponding antibodies.

C) After fixation, PLA experiments were performed to evaluate the interactions between IGF-1R/PRMT1 using IGF-1R and PRMT1 specific antibodies. The detected dimers are represented by red dots. The nuclei were counterstained with mounting medium containing DAPI (blue) (Obj: X60).

D) Quantification of the number of dots per cell was performed by computer-assisted analysis as reported in the method section. The mean \pm s.e.m of three experiments is shown. The *P*-value was determined using the Student's *t*-test. *** indicates a $P < 0,001$.

E) Radioactive GST pull down assay was performed by incubating labelled *in vitro* ^{35}S -labeled intracellular domain of IGF-1R (IGF-1R-ICD*) with GST and GST-PRMT1. The corresponding Coomassie-stained gel is shown in the right panel. * indicates the full length fusion proteins.

Figure 3: The adaptor Shc regulates IGF-1R/PRMT1 interaction

A) MCF-7 cells were transfected with siScrambled or siRNA targeting IRS1 or Shc for 72 hrs, then treated with IGF-1 for different times. The efficacy of protein inhibition was verified by western blotting using the corresponding antibodies. IGF-1R was immunoprecipitated and revealed for the presence of IGF-1R and PRMT1 by western blotting.

B) PLA experiments were performed to evaluate the interactions between IGF-1R/PRMT1 using IGF-1R and PRMT1 specific antibodies. The detected dimers are represented by red dots. The nuclei were counterstained with mounting medium containing DAPI (blue) (Obj: X60).

C) Quantification of the number of dots per cell was performed as described. The mean \pm s.e.m of three experiments is shown. The *P*-value was determined using the Student's t-test. *** indicates a $P < 0,001$.

Figure 4: metER α interferes with IGF-1 signalling

A) MCF-7 cells were transfected with si Scrambled or a pool of siRNAs targeting PRMT1 for 72 hrs, and then treated with IGF-1 for different times. Cell lysates were immunoprecipitated with anti-IGF-1R antibody and revealed by western blotting for the presence of ER α , Src, IRS1, Shc, and IGF-1R, using the corresponding antibodies (left panel). The expression of met-ER α , ER α , p-IGF-1R, p-Akt, p-ERK and PRMT1 were also evaluated by western blotting (right panel).

B) MCF-7 cells were treated or not with the PRMT1 inhibitor MS023 (60 nM), 24 hrs before IGF-1 treatment. Then, IGF-1 signalling was analysed as in A.

Figure 5: The adaptor Shc regulates IGF-1R/ER α interaction

A) MCF-7 cells were transfected with siScrambled or pools of siRNAs targeting IRS1 or Shc for 72 hrs, and then treated with IGF-1 for different times. The efficacy of protein inhibition was verified by western blotting using the corresponding antibodies. ER α and metER α expression was also evaluated. Cell lysates were immunoprecipitated with anti-IGF-1R antibody and revealed by western blotting for the presence of ER α .

B) PLA experiments were performed to evaluate the interactions between IGF-1R and ER α . The nuclei were counterstained with mounting medium containing DAPI (blue) (Obj: X60).

C) Quantification of the number of dots per cell was performed as described. The mean \pm s.e.m of three experiments is shown. The *P*-value was determined using the Student's t-test. *** indicates a *P*<0,001.

Figure 6: IGF-1R interacts with ER α and triggers its phosphorylation.

A) Tumors from PDX models of breast cancer were embedded in paraffin. IGF-1R expression was assessed by IHC staining. (panel a and b). A bright field PLA was performed to study ER α /IGF-1R interaction in the 2 PDX models. The brown dots represent protein-protein interactions (x40 magnification).

B) The interactions were quantified as described in Figure 2b. The *P*-value was determined using the Student's test. *** *P*<0.001.

C) Radioactive GST pull down assay was performed by incubating labelled *in vitro* ³⁵S-labeled ER α or luciferase as a negative control with GST and GST-IGF-1R/ICD in the presence or in

absence of E_2 ($10^{-6}M$). The corresponding Coomassie-stained gel is shown in the right panel. * indicates the fusion proteins.

D) $ER\alpha$ is divided into functional domains ER1 contains the activation Function-1 (AF-1), ER2 contains the DNA binding domain (DBD) and the hinge domain and ER3 contains the ligand-binding domain (LBD) and the activation Function-2 (AF-2).

E) *In vitro* phosphorylation experiment was performed by incubating active IGF-1R with [^{32}P] ATP and GST or $ER\alpha$ fragments fused to GST (ER1, ER2, DBD, Hinge, ER3). The phosphorylated proteins were visualized by autoradiography (left panel). The corresponding Coomassie-stained gel is shown in the right panel. * indicates the full length fusion proteins.

Figure 7: $ER\alpha$ phosphorylation regulates $ER\alpha$ binding to IGF-1R

A) A part of $ER\alpha$ DBD sequence is shown and the 3 tyrosine residues are highlighted in red.

GST, GST-DBD WT or Y/F mutants Y195F, Y197F and Y219F were used as substrates for IGF-1R phosphorylation (left panel). The corresponding Coomassie-stained gel is shown in the right panel. * indicates the full length fusion proteins.

B) MCF-7 cells were transfected with pSG5-Flag $ER\alpha$ WT or the mutants pSG5-Flag $ER\alpha$ Y219F. The expression of the transfected $ER\alpha$ was assessed by western blotting using the anti-Flag antibody.

C) PLA experiments were performed to evaluate the interactions between IGF-1R and transfected $ER\alpha$. The nuclei were counterstained with mounting medium containing DAPI (blue) (Obj: X60).

D) Quantification of the number of dots per cell was performed as described. The mean \pm s.e.m of three experiments is shown. The *P*-value was determined using the Student's *t*-test. *** indicates a $P < 0,001$.

E) Model of IGF-1 signaling

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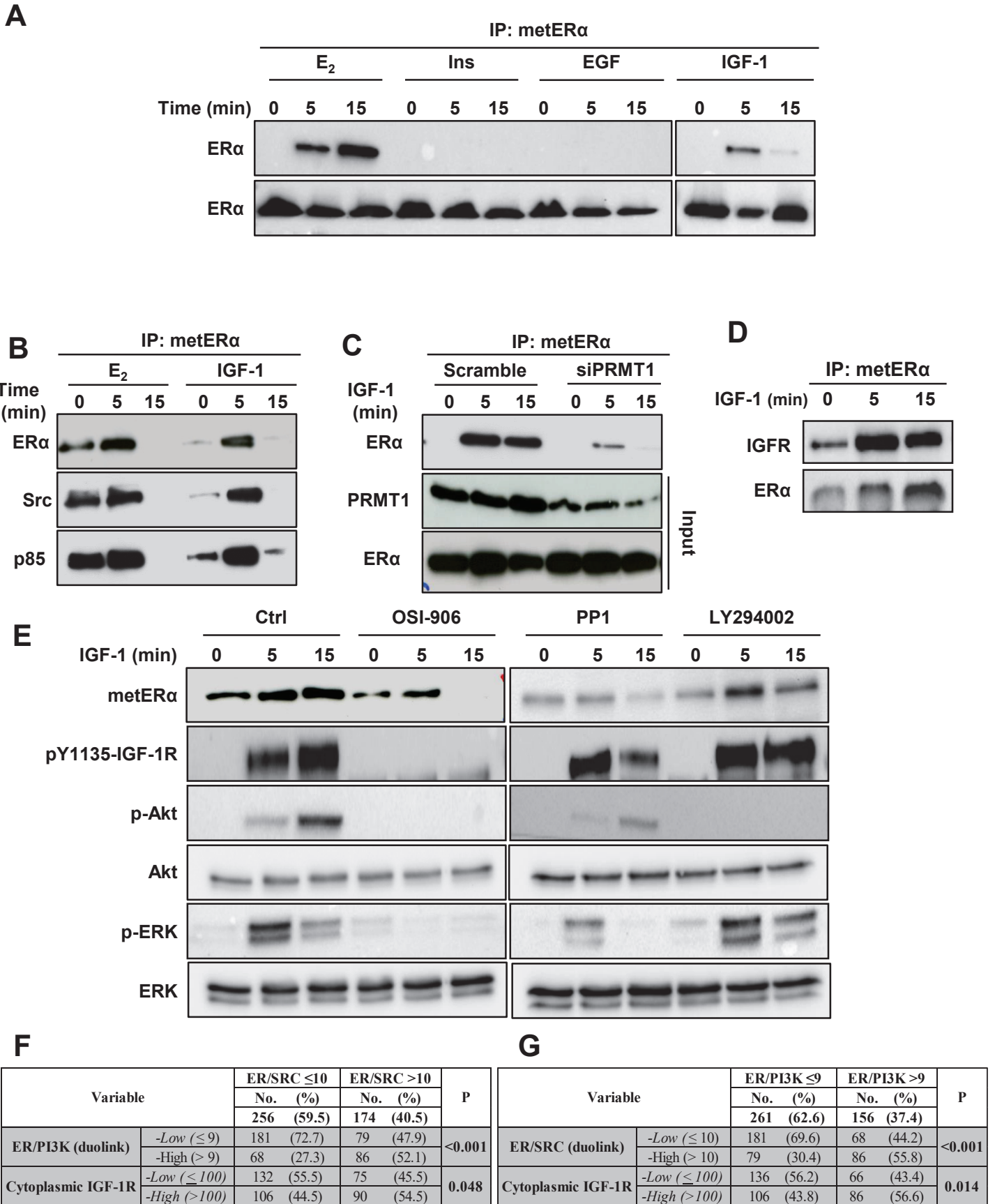


Figure 1

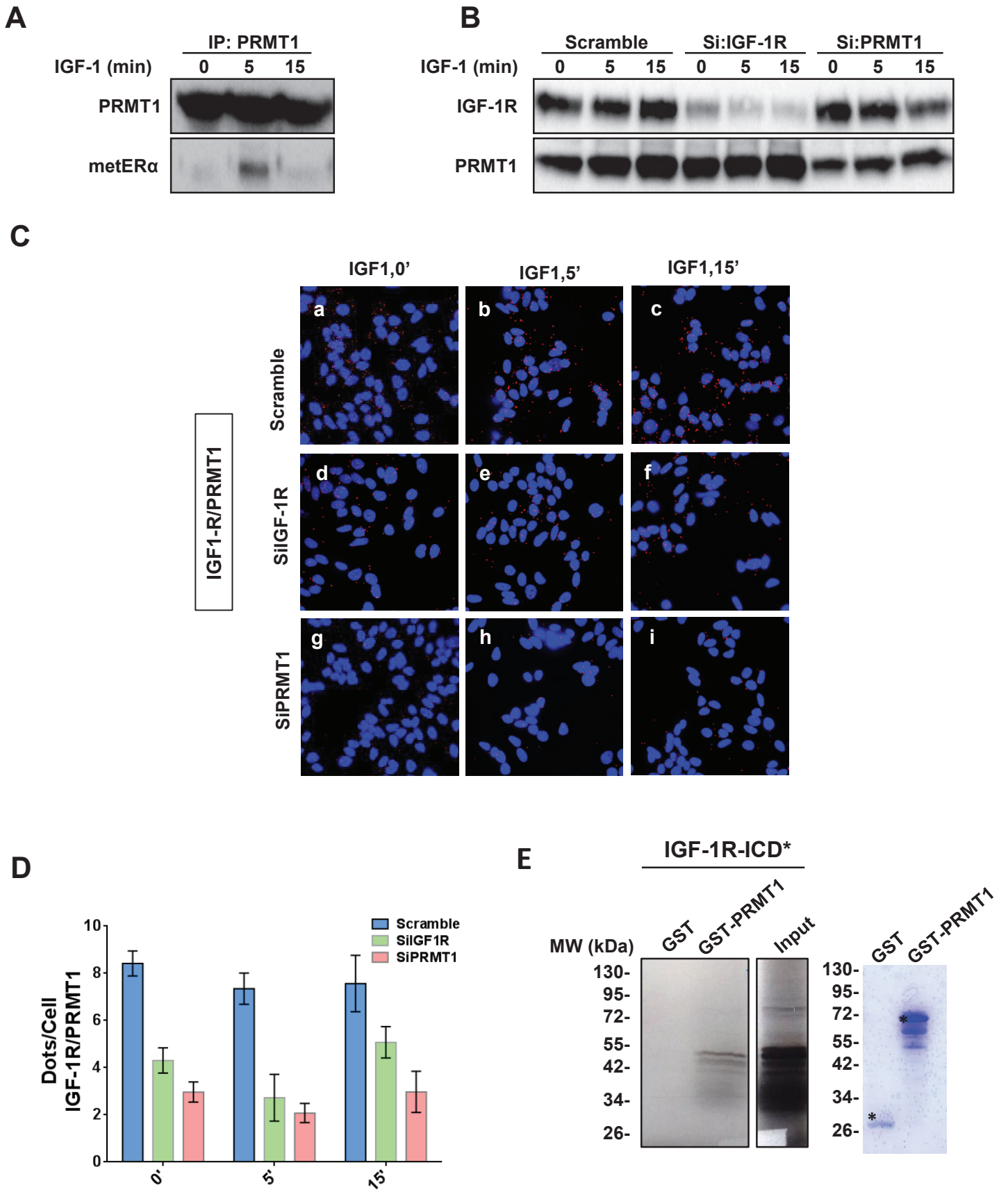


Figure 2

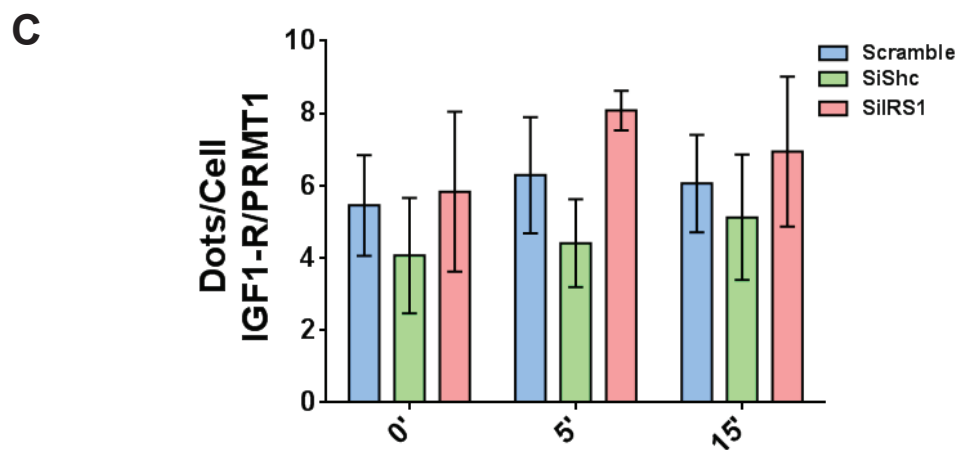
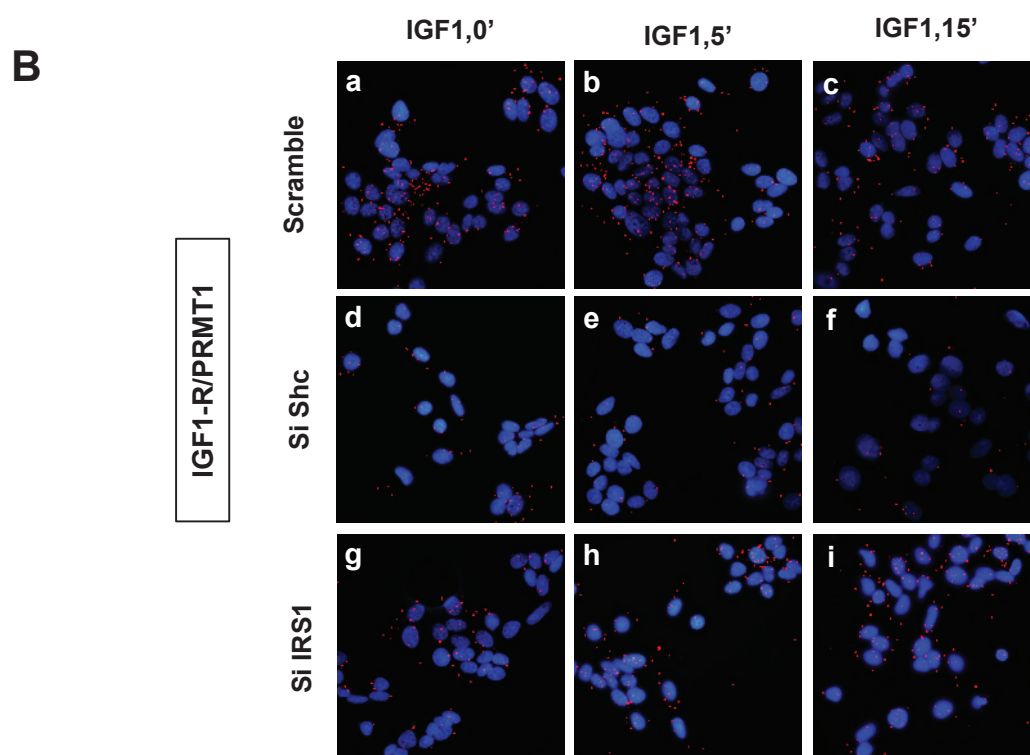
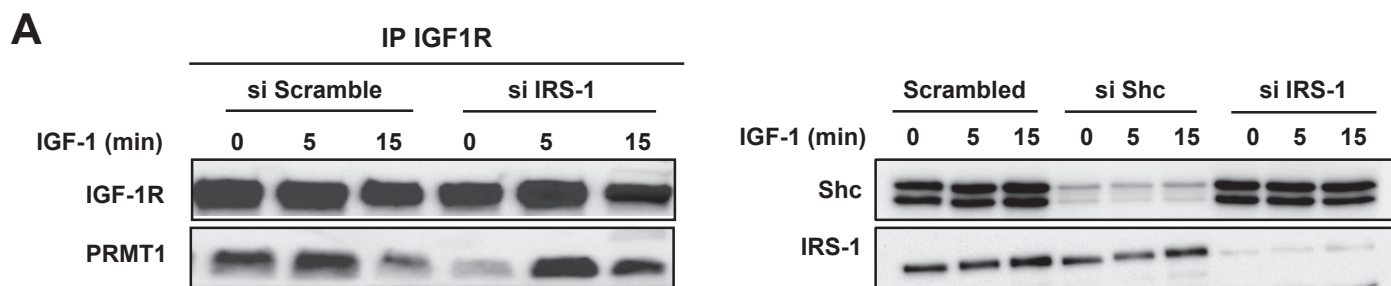


Figure 3

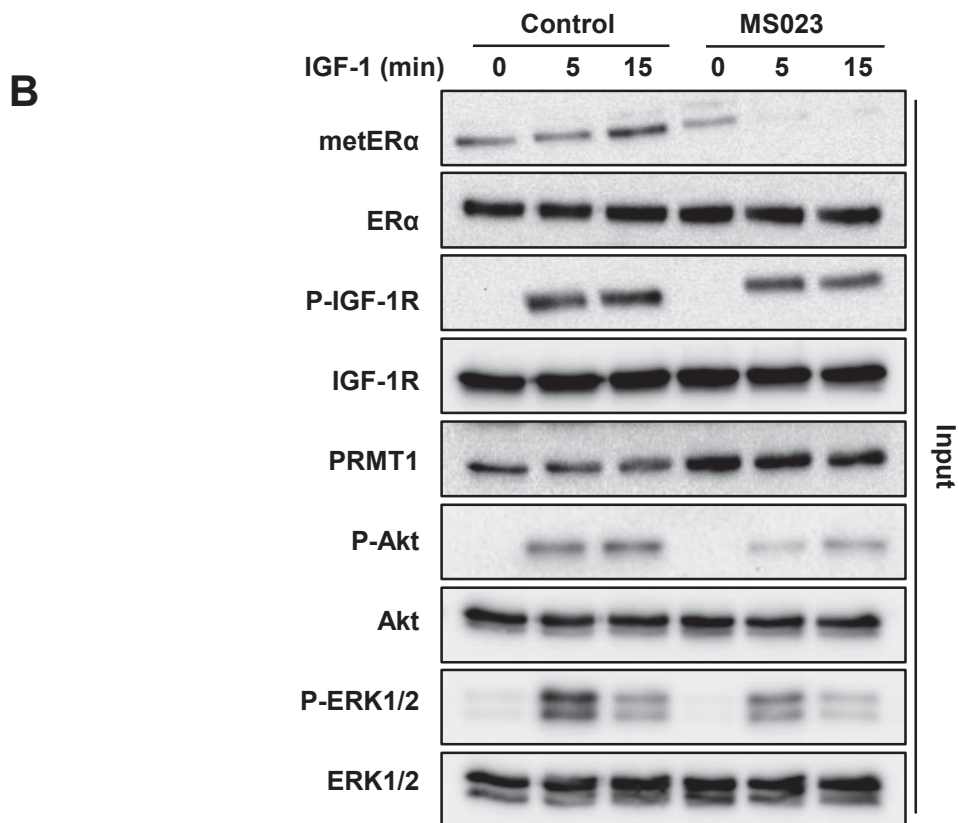
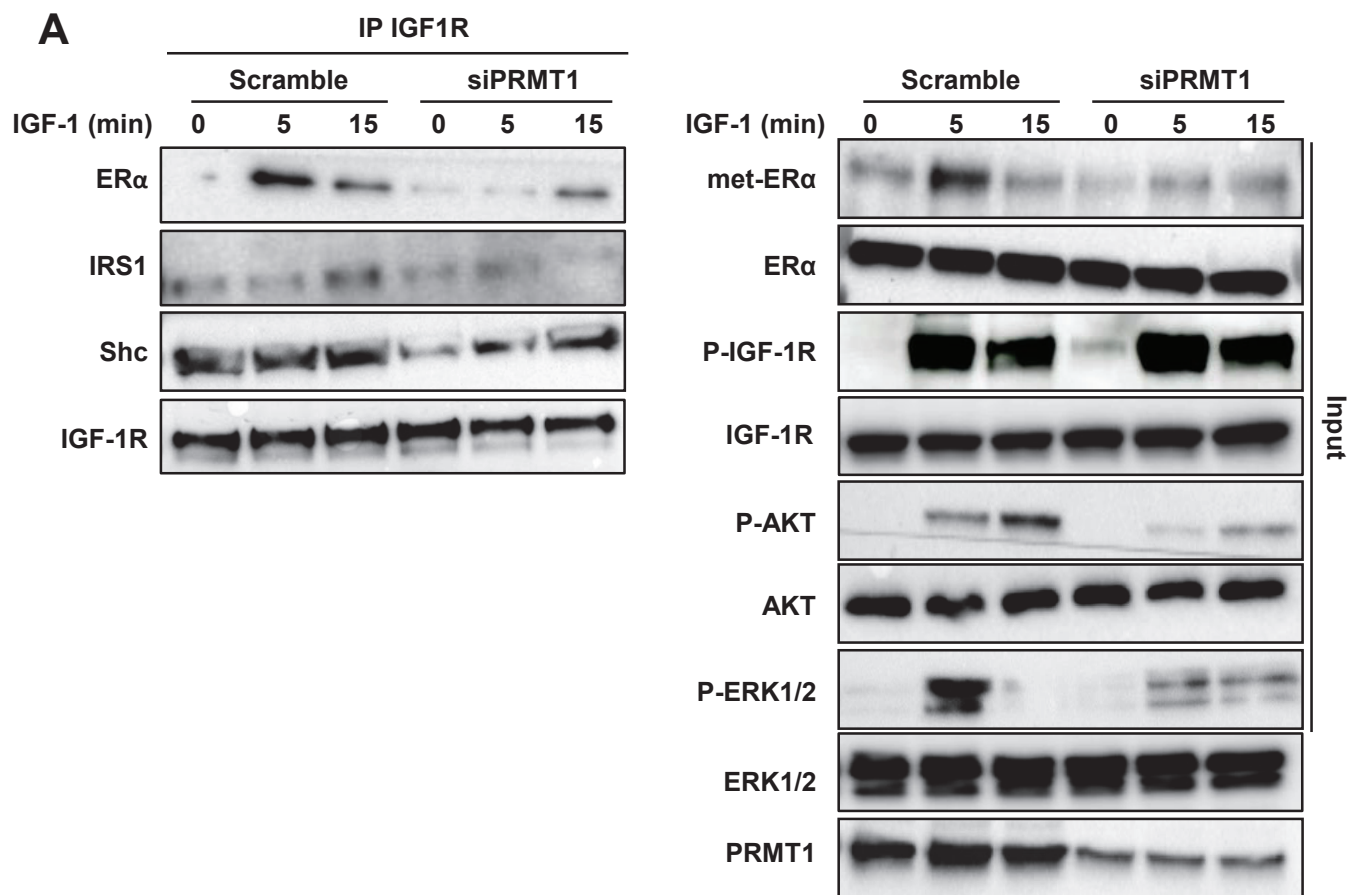


Figure 4

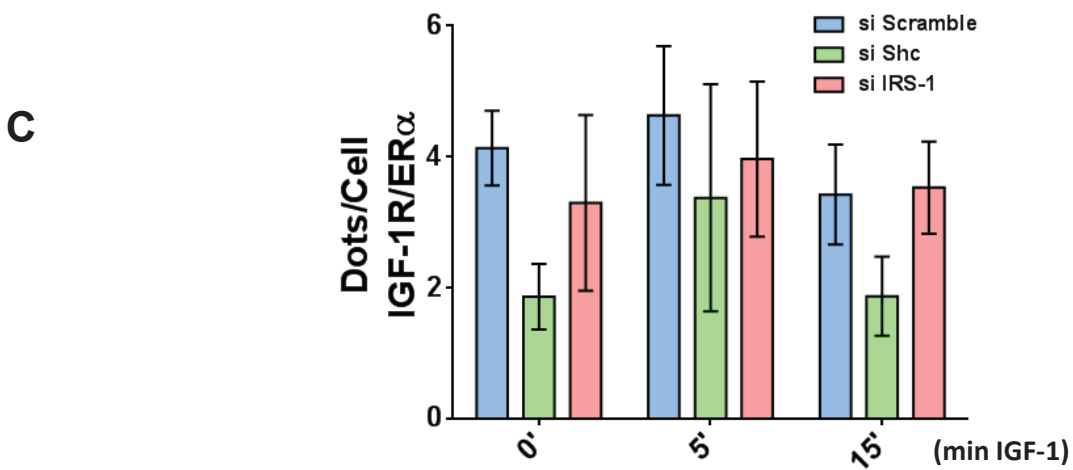
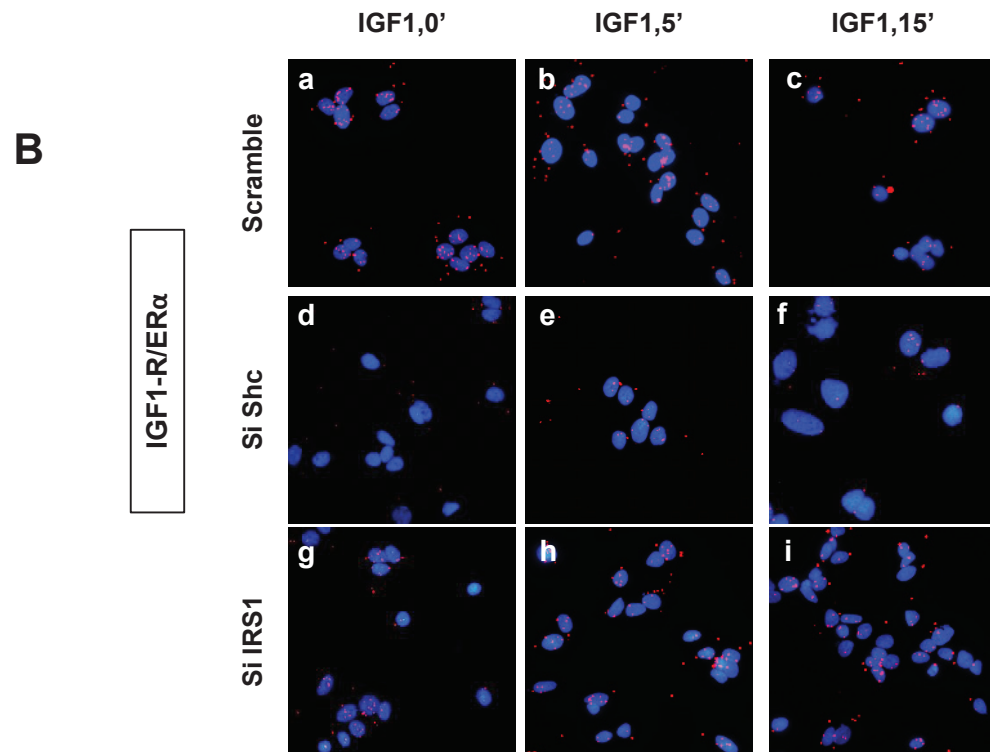
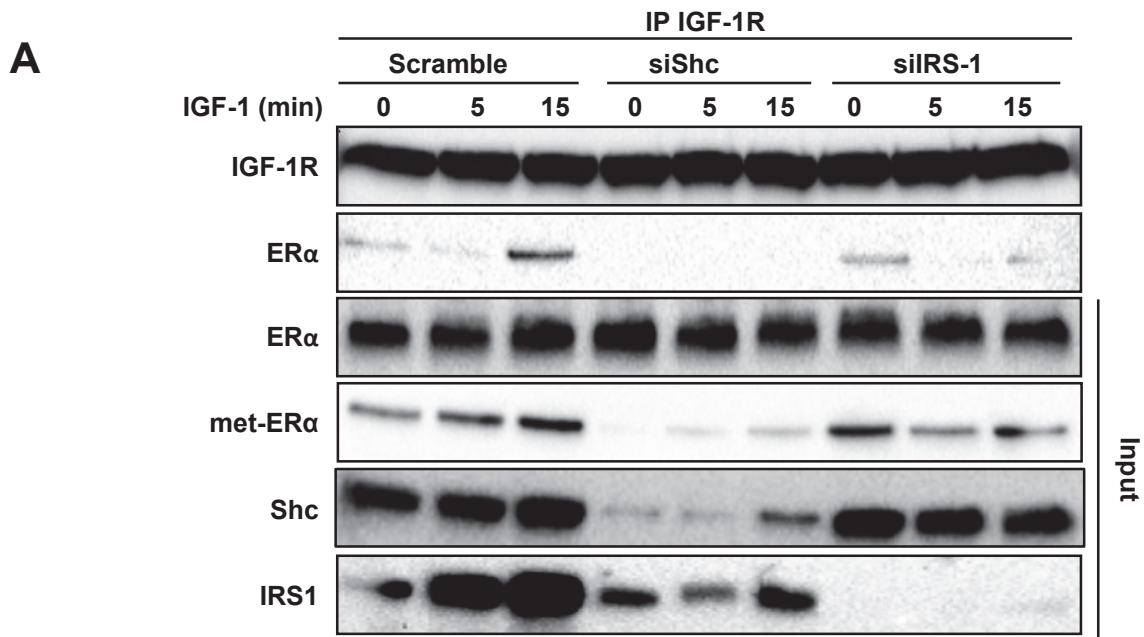


Figure 5

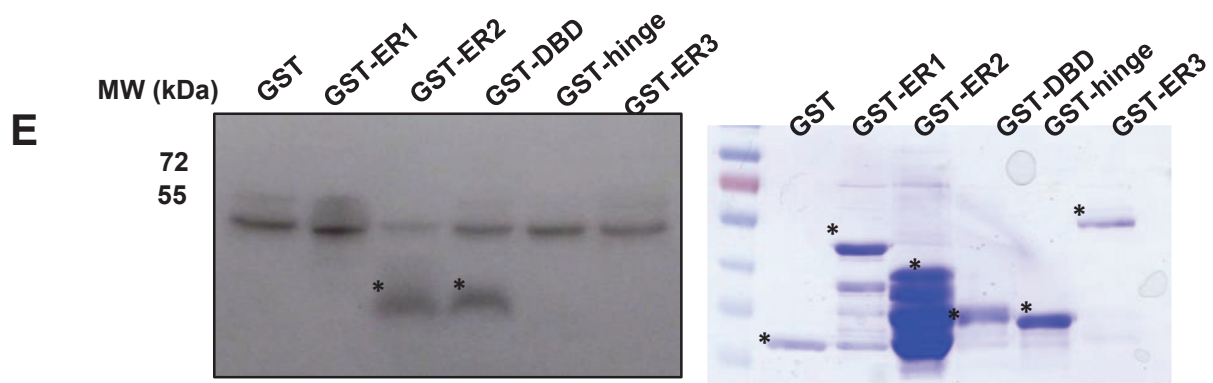
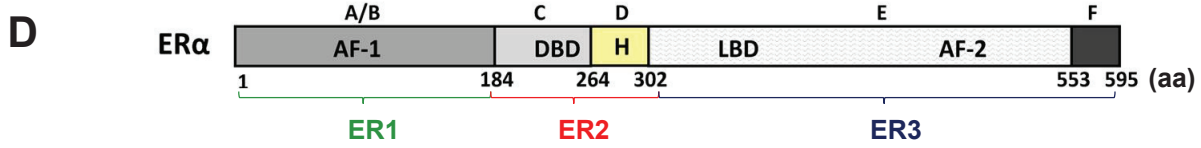
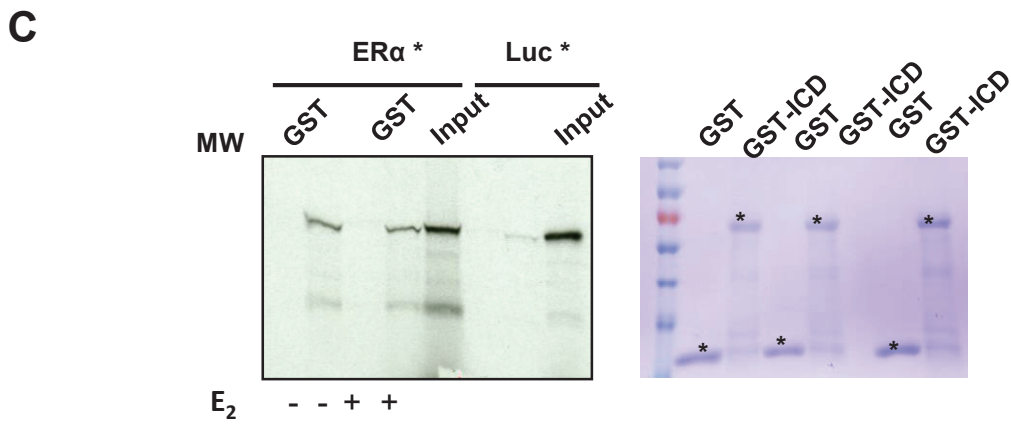
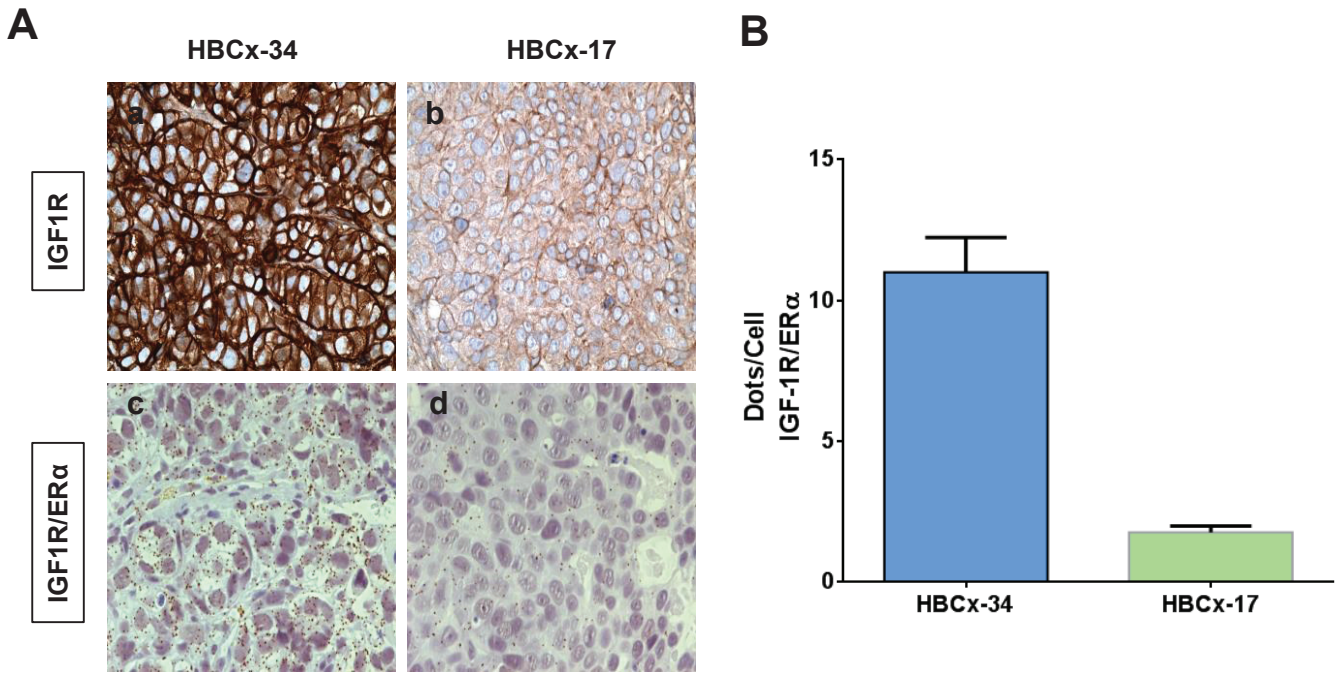
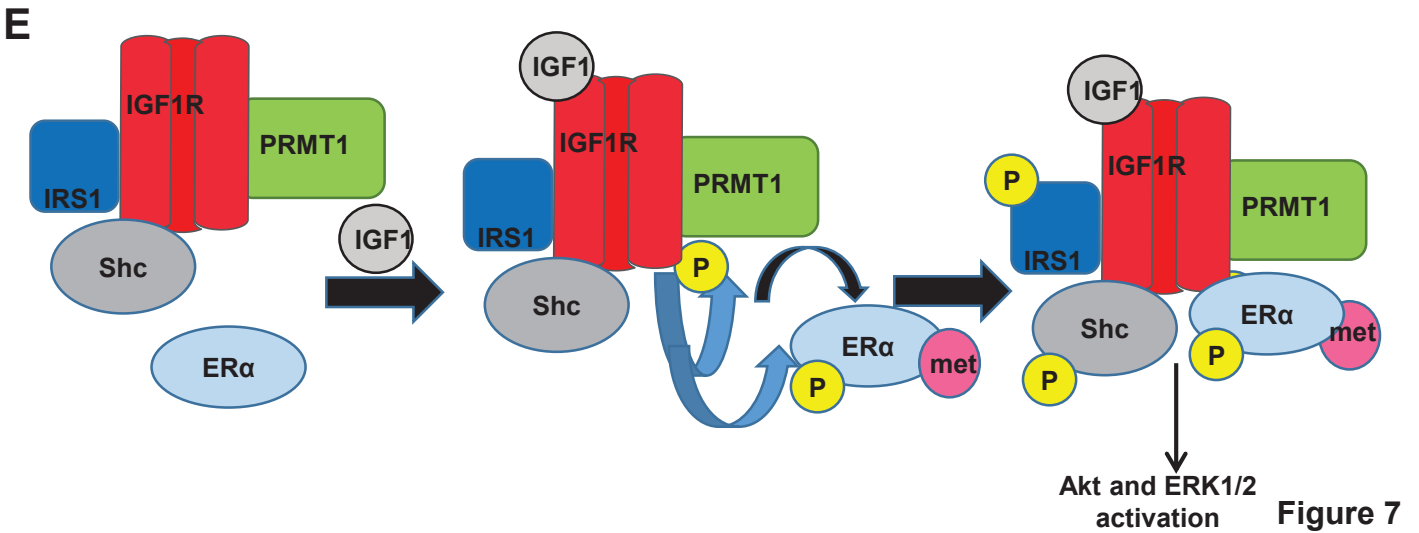
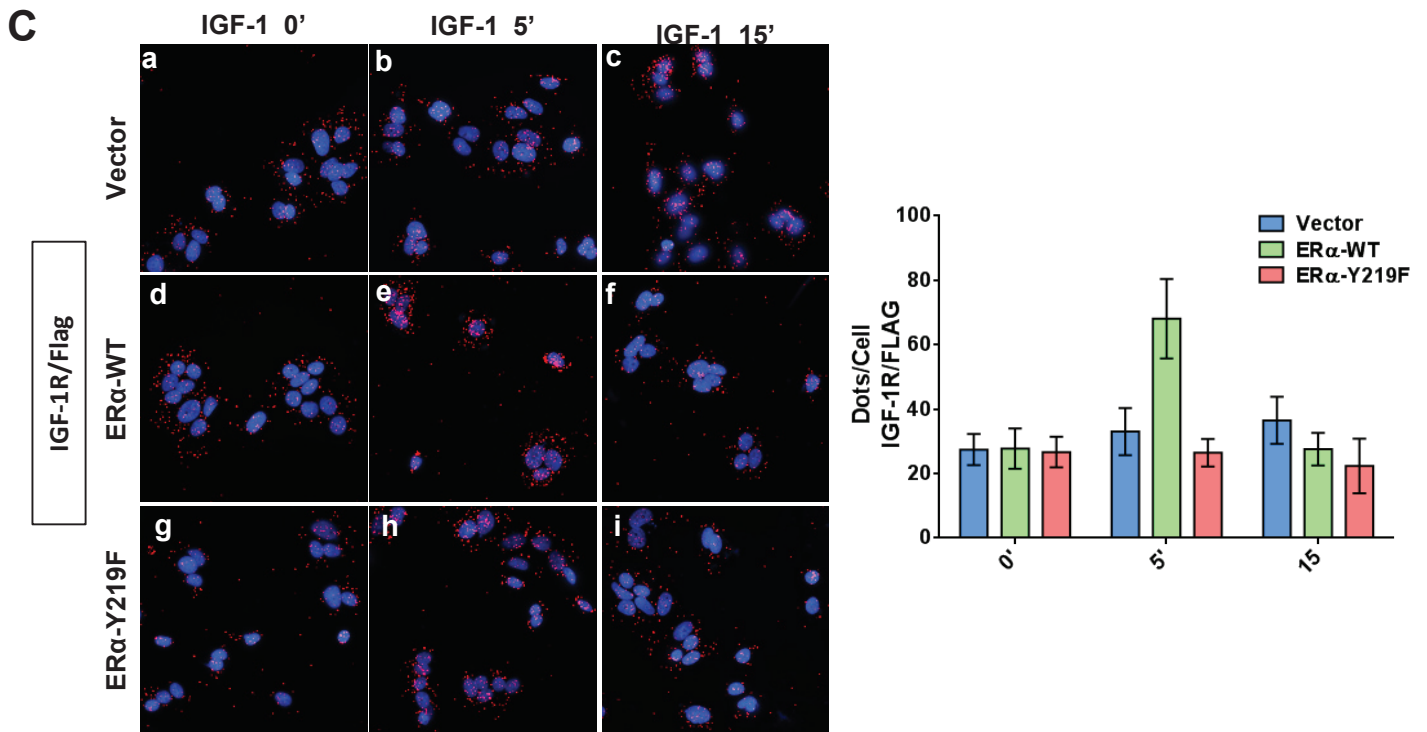
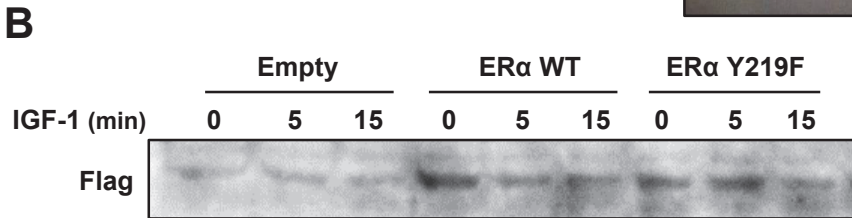
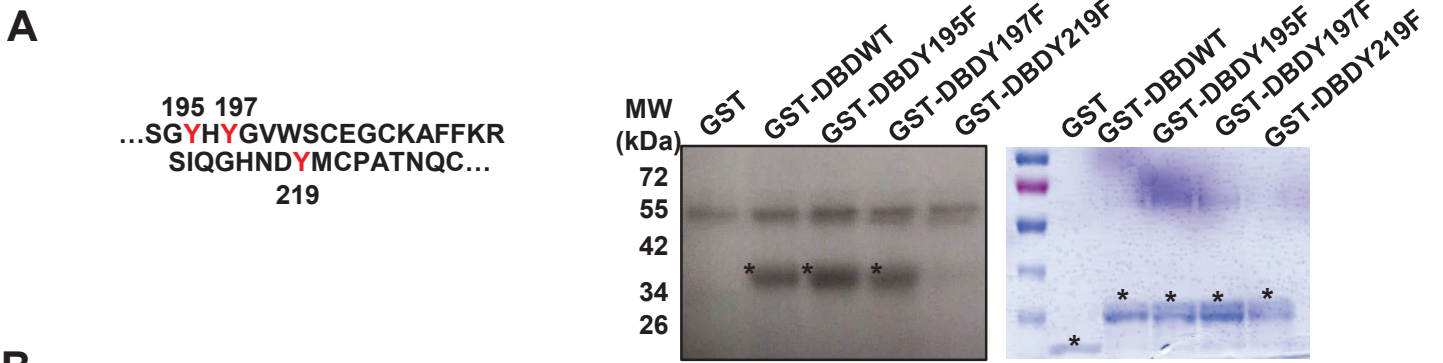


Figure 6

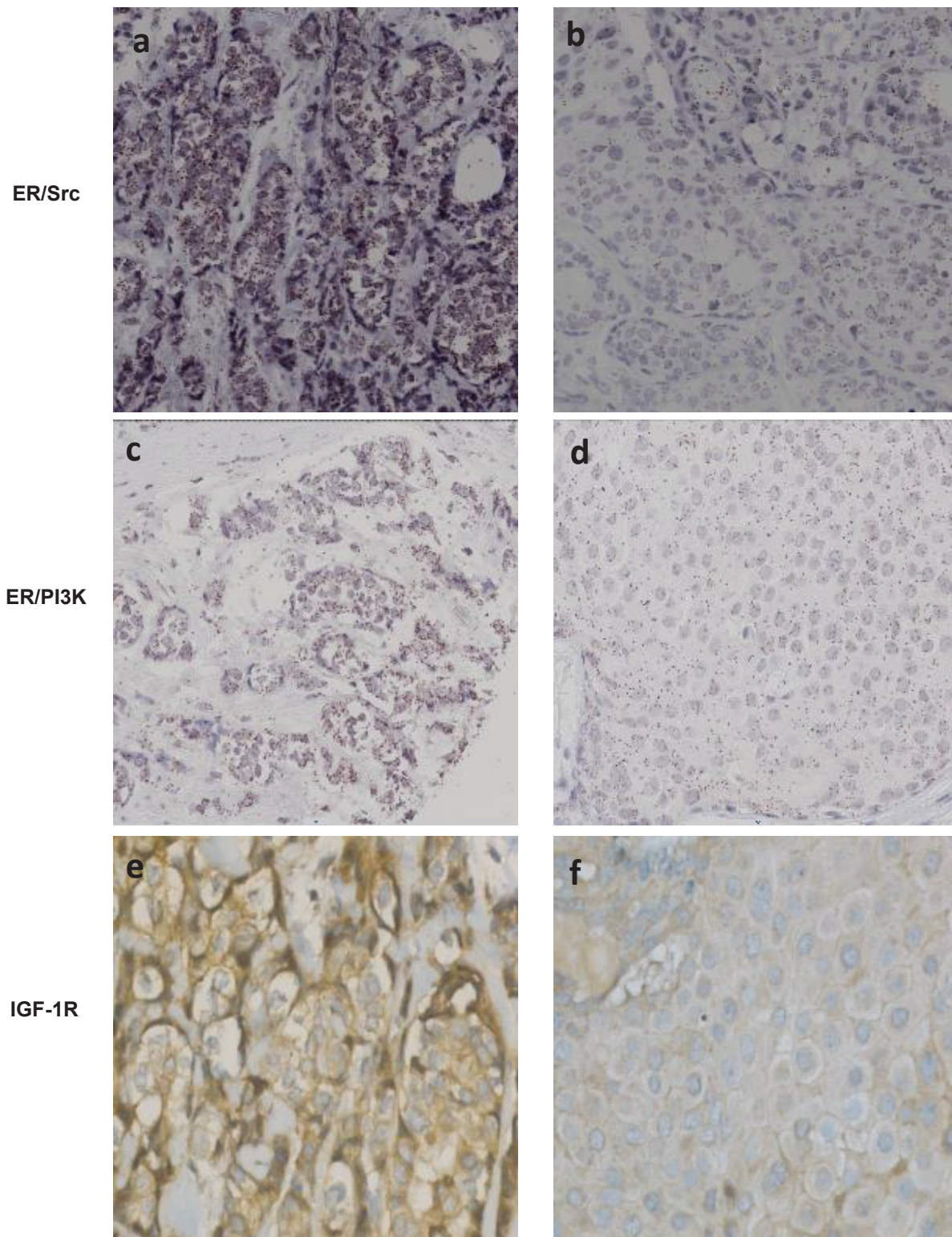


Supplementary Information

The interplay between IGF-1R and ER α in breast cancer involves the arginine methyltransferase PRMT1

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Olivier Trédan⁶, Juliette Rambaud^{1,2,3,7}, Elisabetta Marangoni⁸, Laura Corbo^{1,2,3}, Isabelle
Treilleux^{1,2,3,9} and Muriel Le Romancer^{1,2,3}

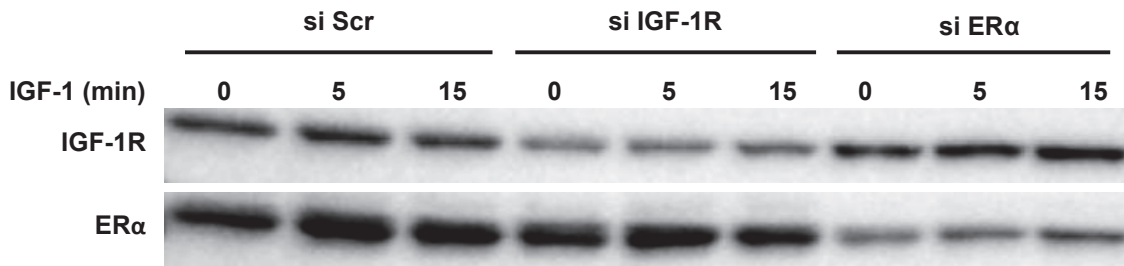
Supplementary Figures



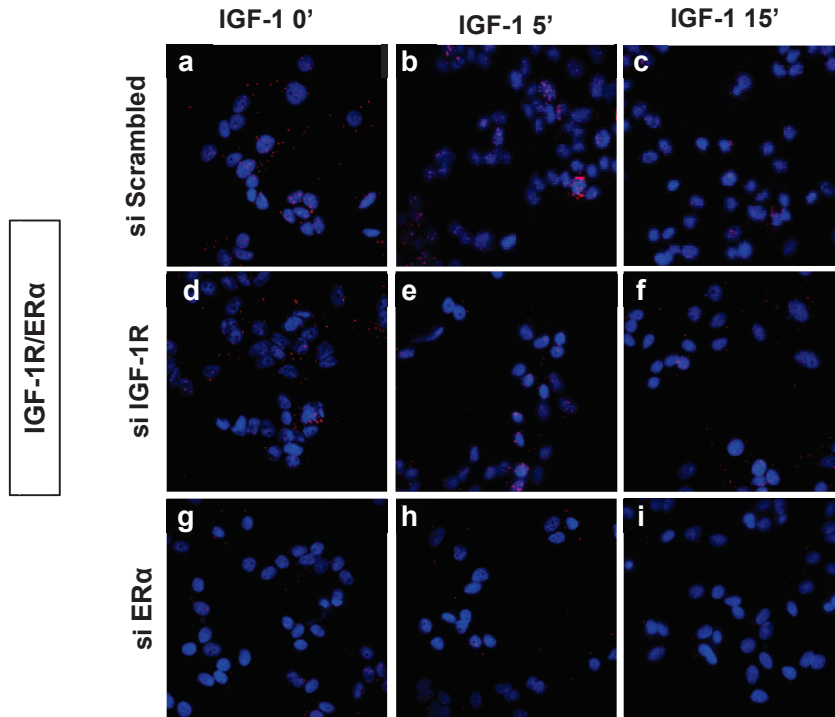
Supplementary Figure 1: Expression of ER α /src, ER α /PI3K and IGF-1R in human breast tumours.

For each tumour, we analyzed by Proximity ligation assay (PLA) the levels of ER α /Src (panels a,b), ER α /PI3K (panels c,d) and IGF-1R expression by immunohistochemistry (panels e,f).

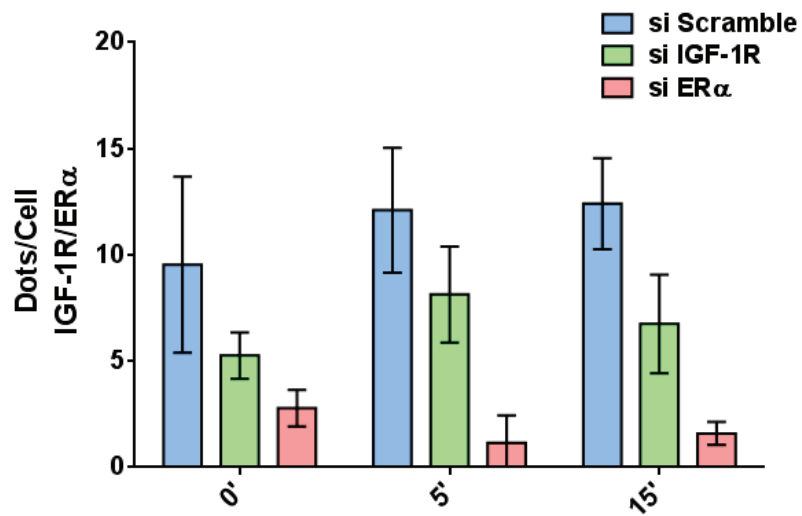
A



B



C

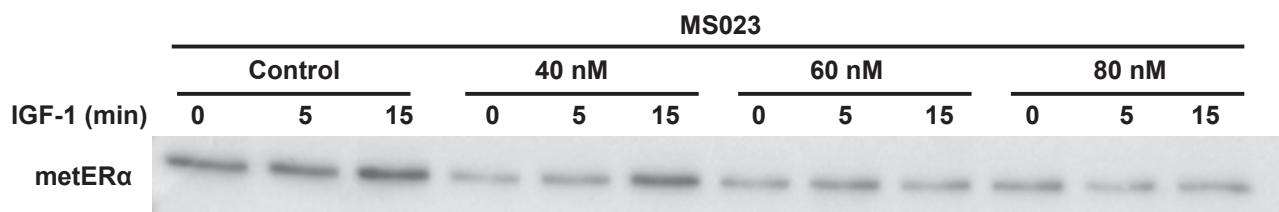


Supplementary Figure 2: IGF-1R/ER α interaction

A) MCF-7 cells were transfected with siScramble or siRNA targeting IGF-1R or ER α for 72 hrs, then treated with IGF-1 for different times. The efficacy of protein inhibition was verified by western blotting using the corresponding antibodies.

B) After fixation, PLA experiments were performed to evaluate the interactions between IGF-1R/ ER α using IGF-1R and ER α specific antibodies. The detected dimers are represented by red dots. The nuclei were counterstained with mounting medium containing DAPI (blue) (Obj: X60).

C) Quantification of the number of dots per cell was performed by computer-assisted analysis as reported in the method section. The mean \pm s.e.m of three experiments is shown. The *P*-value was determined using the Student's t-test. *** indicates a *P*<0,001.

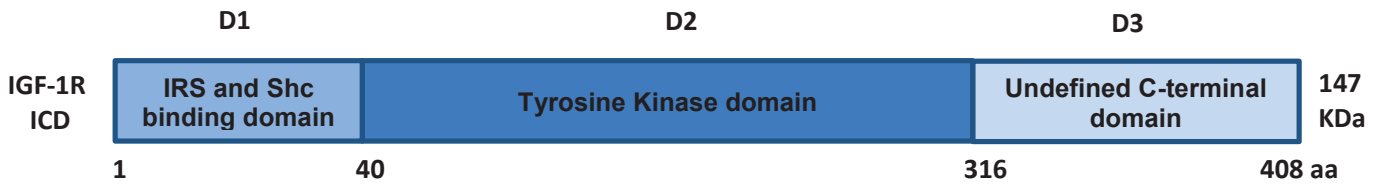


Supplementary Figure 3: Role of PRMT1 inhibition on ER α methylation

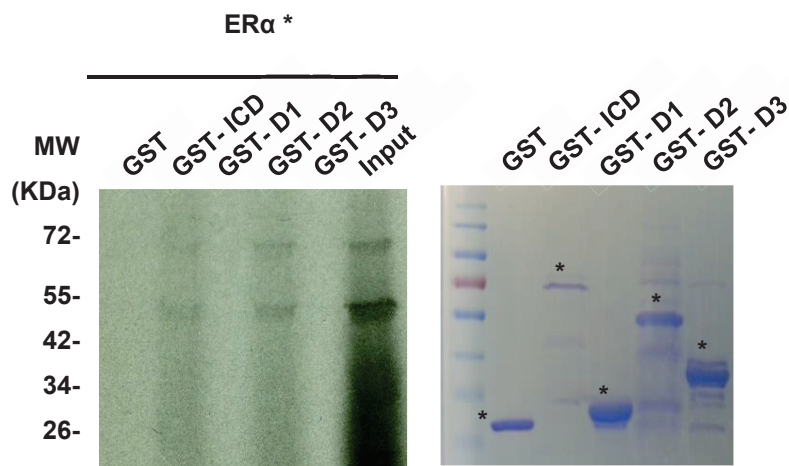
MCF-7 cells were treated or not with the PRMT1 inhibitor MS023 (60 nM), 24 hrs before IGF-1 treatment.

Then, ER methylation was assessed by western blotting.

A



B



Supplementary Figure 4: Study of the interaction between ER α and IGF-1R

A) This figure presents the different structural domains of IGF-1R intra cellular domain. The ICD was divided in 3 domains: the D1 domain contains the IRS1 and Shc binding domain, the D2 domain contains the kinase activity and the D3 domain has no known function.

B) Radioactive GST pull down assay was performed by incubating labelled *in vitro* ^{35}S -labeled ER α with GST, GST-ICD, GST-D1, GST-D2 and GST-D3 of IGF-1R. The corresponding Coomassie-stained gel is shown in the right panel. * indicates the full length fusion proteins.

Table S1: Sample description: Distribution of clinical parameters.

Clinical parameters were analyzed for the 440 patients included in the TMA study.

Characteristic		Number	percent
Age group	<i>≤ 50 years</i>	113	26.1%
	<i>> 50 years</i>	320	73.9%
Menopausal status	<i>Premenopausal</i>	121	28.5%
	<i>Post-menopausal</i>	303	71.5%
	<i>Unknown</i>	9	
BMI	<i>≤ 25 kg/m²</i>	258	61.9%
	<i>> 25 Kg/m²</i>	159	38.1%
		16	
Tumor size	<i>≤ 2cm</i>	252	58.2%
	<i>> 2cm</i>	181	41.8%
Axillary LN metastasis	<i>No</i>	184	42.5%
	<i>Yes</i>	249	57.5%
SBR grade	<i>I</i>	82	18.9%
	<i>II</i>	207	47.8%
	<i>III</i>	144	33.3%
ER status	<i>Negative</i>	56	12.9%
	<i>Positive</i>	377	87.1%
PR status	<i>Negative</i>	109	25.2%
	<i>Positive</i>	324	74.8%
HER2 status	<i>Negative</i>	397	92.8%
	<i>Positive</i>	31	7.2%
	<i>Missing</i>	5	
Breast cancer subtype	<i>Luminal A</i>	243	56.1%
	<i>Luminal B</i>	134	30.9%
	<i>HER2 enriched</i>	11	4.6%
	<i>TNBC</i>	45	10.4%
Adjuvant hormonal regimen	<i>Tamoxifen</i>	173	46.6%
	<i>Tam-AI</i>	198	53.4%
	<i>Missing</i>	62	

Supplementary Experimental Procedures.

Antibody	Company	Ref.	Species	WB	IP	PLA	IHC
Akt	Cell Signaling	9272	mouse	1:1000			
ER α -60C	Millipore	04-820	rabbit		2 μ g	1:500	
ER α -F10	Santa Cruz	sc-8002	mouse	1:1000			
ER α -HC20	Santa Cruz	sc-543	rabbit			1:750	
Flag	Sigma	F7425	rabbit			1:500	
Flag	Euromedex	EL1-B11	mouse	1:1000			
IGF-1R β	Cell Signaling	D23H3	rabbit		2 μ g		
IGF-1R β	Santa Cruz	Sc-81167	mouse	1:1000		1:750	
IRS-1	Cell Signaling	2382	rabbit	1:1000			
IRS-1	Millipore	05-1085	mouse	1:1000			
MAPK	Cell Signaling	4695	rabbit	1:2000			
metER	Homemade	2D10	mouse	1:1000	2 μ g		
P-Akt	Cell Signaling	9271	rabbit	1:1000			
P-IGF-1R	Cell Signaling	3918	rabbit	1:1000			
P-MAPK	Cell Signaling	5726	rabbit	1:2000			
P-Y-4G10	Millipore	05-321	mouse	1:1000			
P85	Millipore	ABS234	rabbit	1:1000			
PRMT1	Upstate	07-404	rabbit	1:1000		1:750	
PRMT1	Sigma	P1620	mouse	1:1000			
Shc	Millipore	06-203	rabbit	1:1000			
Shc	Santa Cruz	sc-967	mouse	1:1000			
Src B12	Santa Cruz	sc-8056	mouse	1:1000			

Supplementary Table 2: List of the antibodies used in the current work.

Discussion to the article

Majority of breast tumors express ER α , and most of the treatments focus on targeting this receptor as a first line of treatment in ER α -positive tumors. Targeting this receptor was limited by the emergence of resistance to therapy like Tamoxifen and Fulvestrant (Johnston, 2015). Several reasons stand behind this resistance, and one of them is the cancer escape through other signaling pathways, specifically the growth factor ones. For this reason, clinics are using combination of hormonal therapies and other signaling inhibitors, such as mTOR pathway inhibitors and IGF-1R signaling inhibitors (Simpson et al., 2017).

IGF-1 system and IGF-1R specifically, was shown to be involved in the ER α -positive resistant tumors. As discussed previously, IGF-1R was activated and up-regulated in tamoxifen-resistant breast tumors (Knowlden et al., 2005), showing the interference of the IGF-1 signaling with estrogen ones in breast cancer. On the other hand, patients that are ER-positive when treated with metformin showed lowered risk of breast cancer incidence (Jiralerspong et al., 2009); this proves that IGF-1R regulation is important in the advancement of estrogen-regulated breast tumors. Hence, these two signaling pathways interfere with each other, and this crosstalk should be better understood for advanced breast cancer treatments.

In order to better study the effect of growth factors on estrogen signaling in breast cancer, our team investigated their effect on ER α methylation on its R260, the key element of the formation of ER α /Src/PI3K complex, that is activated in aggressive breast tumors (Poulard et al., 2012a). Using MCF-7 breast cancer cell line, we treated with EGF, Insulin and IGF-1, and only the latter was able to induce the ER α methylation similarly to E₂. This very specific IGF-1-induced R260 methylation was rapid and transitory in similar timing to estradiol-induced MCF-7, and was able to induce the proliferative downstream signaling pathways, Akt and ERK1/2. So we went further to decipher the effect of IGF-1 stimulation on the ER α /Src/PI3K complex formation; and remarkably, we found that IGF-1 stimulation was not affected by using LY29004, the PI3K inhibitor, as the E₂ stimulation was inhibited. Starting from this point, we wanted to test the effect of IGF-1 on ER α signaling and vice versa.

In the first step, we investigated the PRMT1 activation by IGF-1, and we found that IGF-1R constitutively binds PRMT1 and activates it in a rapid and transitory manner. This interaction was validated *in vivo* using PLA and *in vitro* using co-IP, GST-pull down and methylation *in vitro* assays. However, this interaction and activation was not affected by the inhibition of the IGF-1R adaptor proteins, Shc and IRS1, using siRNA approaches against the latter proteins. In addition, using co-immunoprecipitation assay, the inhibition of ER α by siRNA approach did not affect the interaction between IGF-1R and PRMT1 (data not shown). This could indicate that the interaction between those two receptors is constant and independent of any intermediate partner.

On the contrary, inhibition of PRMT1 abolished the interaction between IGF-1R and ER α ; also, it decreased the IGF-1 induced signaling pathway through Akt and ERK1/2 pathways. These events were downstream IGF-1R, as the auto phosphorylation of the receptor was not affected. This is enforced by the fact that IGF-1R phosphorylation was not changed, although its expression was increased, in tamoxifen-resistant breast tumors compared with the sensitive ones (Knowlden et al., 2005). We did not exclude the fact that PRMT1 could methylate in its turn IGF-1R, as it is known to bind to membrane receptors and methylates arginine residues (Abramovich et al., 1997). Unfortunately, we were not able to detect any methylation of IGF-1R by PRMT1 using radioactive methylation *in vitro* assays. These results were also reproducible using a specific PRMT1 inhibitor, MS023, where we found that the ER α methylation was inhibited, along with the proliferative downstream signaling pathways.

Yu et al. (2013) demonstrated that IGF-1 induced ER α relocalisation to the membrane, and this is also conformant with our results that IGF-1 induced ER α methylation leads to the formation of ER α /Src/PI3K cytoplasmic complex. Moreover, we have shown that the interaction between those receptors was affected by the inhibition of the Shc adaptor protein. This information fits the finding of Shc acting as a transporter of ER α to the membrane, upon estradiol stimulation, to interact with IGF-1R forming a triple complex of Shc/IGF-1R/ ER α (Song et al., 2004b).

To validate our hypothesis *in vivo*, we checked the expression of IGF-1R and ER α in samples of breast PDX, and found that the two receptors interact in a significant manner when expressed in tumors. In addition, we found in a cohort of 440 breast TMAs that IGF-1R expression was strongly correlated with ER α /Src and ER α /PI3K expression; where this cohort showed before

in a previous study a strong correlation between ER α /Src and ER α /PI3K expression, and the methylation of ER α and Akt activation in those tumors (Poulard et al., 2012b).

Going into details of interaction between IGF-1R and ER α , we cloned the IGF-1R intracellular domain into three main parts, IRS1 and Shc binding domain, tyrosine kinase domain and a third undefined one. Using radioactive GST-pull down approaches, we found that ER α binds only to the tyrosine kinase domain; hence, we proposed that hypothesis of ER α direct phosphorylation by IGF-1R. After cloning the different parts of ER α , we performed *in vitro* phosphorylation assays, and we found that IGF-1R phosphorylates the tyrosine residues of the DBD of ER α .

Using site-directed mutagenesis followed by *in vitro* phosphorylation assays, we found that IGF-1R specifically phosphorylates the Y219 of ER α . This tyrosine residue was shown to be phosphorylated by c-Abl, and this phosphorylation stabilizes the binding of ER α to DNA, and renders it transcriptionally active (He et al., 2010). Following these results, we transfected MCF-7 cells with vectors expressing flag-tagged WT ER α or ER α carrying mutant Y219F. Using PLA approaches, we found that cells expressing ER α with Y219F mutant showed significant decrease of IGF-1R and ER α interaction compared with WT ER α .

However, absence of commercial antibody that recognizes this tyrosine residue was limiting our work continuity *in vivo*; where we would have been able to further investigate its effect on the interaction between IGF-1R and ER α different forms, wild type and mutant, to decipher the effect of mutating ER α Y219 at the proteomic level.

Perspectives to our project

Our study proposes a strong indication of targeting PRMT1 as a specific way of inhibiting both IGF-1 and estrogen non-genomic signaling; and this due to the remarkable effect of inhibiting PRMT1 on decreasing IGF-1R and ER α interaction, and the proliferative downstream signaling pathways. However, this approach is indirect, as the effect could be independent of ER α methylation. For this reason, we intended to generate MCF-7 cell line mutant of the R260K of ER α , the site of PRMT1 methylation, using CRISPR/Cas9 genome editing technique. Thanks to our collaborators in Imperial College London, Dr. Simak Ali and Prof. Laki Buluwela, we are now in the last step of cloning the cells to purify the cells carrying the mutation, and to define the number of mutant alleles of the ER α gene. Successful ER α mutant clones will be later studied for their tumorigenic signaling, and their migration and proliferative capability. All these results will lead to a comprehensive understanding of the methylated R260 of ER α role in cancer non-genomic signaling, and probably to better way of defining ER α modifications in breast cancer.

Moreover, as we have noticed the importance of the Y219 of ER α in its interaction with IGF-1R, we also attempted to generate another MCF-7 cell line carrying the mutation Y219F of ER α . Currently we are in the cloning stage to purify the best mutant clone, and we intend to study the effect of this mutation on the ability of ER α to bind to DNA. In addition, we can foresee that the decreased ER α Y219 phosphorylation by IGF-1R will lead to less activated ER α genomic signaling, and hence down regulation of all proliferative ER α target genes such as CyclinD1, c-myc, c-Jun and IGF-1R itself.

On the other hand, as one of the first objectives of our project was to design a peptide interrupting the direct interaction between IGF-1R and ER α , we attempted to investigate the probable sites of interaction between both receptors. For this reason, we have fragmented the tyrosine kinase domain of IGF-1R to five smaller fragments named A to E; we performed radioactive GST-PD of full ER α with those five domains, and we found that it interacted specifically with the domains A and D of IGF-1R ICD. Our next work is to define the exact site(s) of interaction between the receptors; for this, we will work alongside with our collaborating bioinformaticians in IGBMC, Strasbourg.

Since targeting IGF-1R signaling has recently shown some complications such as hyperglycemia and general metabolic toxicities, there is an urging necessity of setting an efficient breast cancer targeting strategy of the “escape” growth factors’ signaling in ER α -positive breast tumors. Hence, our work has shed some light on the mechanistic details of IGF-1R and ER α signaling, with the identification of novel signaling partners, which could be effectively targeted in future breast cancer therapies.

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