



FOXL2: A regulator of endometrial physiology ? First insights from ruminants

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***FOXL2: A regulator of endometrial physiology?
First insights from ruminants***

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« Lorsqu'on quitte un lieu de bivouac, prendre soin de laisser deux choses. Premièrement : rien.

Deuxièmement: ses remerciements. »

As an autist, things cannot changes in the good way. Fortunately for me, four years in this lab show me the exact opposite. Here comes the moment I fear for years, my thanks, mes remerciements.

First, I would like to sincerely thank the members of my jury. It is a great honor for me to defend my PhD in front of Pr Renato Fanchin, Pr Paul Fowler, Dr Richard Lea and Dr Giovanni Levi and Dr Daniel Vaiman,.

Merci beaucoup, Professeur Fanchin d'avoir accepté d'être le président de mon Jury. J'espère que ce travail vous plaira.

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20th Century FOX...L2 present:

Pirate of the endometrium: FOXL2 crusade!

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« Un ami, c'est quelqu'un qui vous connaît bien et qui vous aime quand même » - Hervé Lauwick

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Kamélia, c'est avant tout une chanson qui lui colle à la peau : « You look like a angel, talk like an angel, walk like an angel, but I got wise.... You are the devil in disguise. Oh yes you are! ». Une jeune femme au visage d'ange qui peut sortir les pires blagues ! Merci ma Kamélia d'avoir été là depuis le début, merci pour ton concentré de folie pur, pour ton rire communicatif. Merci mille fois, d'avoir été là et d'être toujours là !

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II. Abbreviation

α -GSU: alpha-glycoprotein subunit
3- β HSD: 3-beta hydroxysteroid deshydrogenase
ACTB: ctin B
AI: artificial insemination
AMH: anti mullerian hormone
ANOVA: analyse of variance
APH: aminoglycoside phosphotransferase
AR: androgen receptor
ATF3: activating transcription factor 3
B2M: beta-2-microglobulin
BCL2A1: B-cell lymphoma 2-related protein A1
BLBC: basal-Like-Breast Cancer
BMP2: bone morphogenetic protein 2
BPES: blepharophimosis ptosis epicanthus inversus syndrome
BSA: bovine serum albumine
CAR: caruncular area
CCL: chemokine (C-C motif) ligand
cDNA: complementary DNA
CH25H: cholesterol 25 hydroxylase
CL: corpus luteum
Coup-TFII: chicken ovalbumin upstream promoter transcription factor II
COX2: cyclooxygenase2
CRESTAR: Oestrus control in both cyclic & non cyclic cattle (Heifers & cows)
CXCL: chemokine (C-X-C motif) ligand
CYP19: cytochrome P450 aromatase
DMSO: dimethyl sulfoxide
DEG: differentially expressed gene
dGE: deep glandular epithelium
DLX5: distal-less homeobox 5
DNA: Deoxyribonucleic acid

dpo: days post-oestrus

E2: estrogens

ECM: extra-cellular matrix

ED: embryonic disk

EET: extra-embryonic tissues

ERE: estrogen receptor response element

ESR1: estrogen receptor 1

ESR2: estrogen receptor 2

FKH: forkhead domain

FOS: FBJ Murine Osteosarcoma Viral Oncogene Homolog

FOX: forkhead box transcription factors

FOXA1, FOXA2, FOXA3: forkhead box transcription factor, subfamily A, member 1, 2 and 3

FOXC1, FOXC2: forkhead box transcription factor, subfamily C member 1 and 2

FOX E3: forkhead box transcription factor, subfamily E member 3

FOX F2: forkhead box transcription factor, subfamily F member 2

FOX G1: forkhead box transcription factor, subfamily G member 1

FOX I1: forkhead box transcription factor, subfamily I member 1

FOX L2: forkhead box transcription factor, subfamily L member 2

FOX M1: forkhead box transcription factor, subfamily M member 1

FOX N3: forkhead box transcription factor, subfamily N member 3

FOX O1, FOX O1A, FOX O3, FOX O3A, FOX O4: forkhead box transcription factor, subfamily O member 1, 1A, 3, 3A and O4

FOX P1, FOX P2, FOX P3: forkhead box transcription factor, subfamily P member 1, 2 and 3

FOX Q1: forkhead box transcription factor, subfamily Q member 1

FOX S1: forkhead box transcription factor, subfamily S member 1

FSH: follicle stimulating hormone

FST: follistatin

GC: granulosa cells

GCT: granulosa cell tumour

GE: glandular epithelium

GFP: green fluorescent protein

GnRH: Gonadotropin Releasing Hormone

HAND2: Heart- and neural crest derivatives-expressed protein 2

HCl: Hydrochloric acid

HNF, HNF3 α : hepatocyte nuclear factor member 3-alpha

HOX, HOXA10: Homeobox protein member A10

ICAR: intercaruncular area

ICM: inner cell mass

IER3: Radiation-inducible immediate-early gene IEX-1

IFN: interferon

IFNT: interferon-tau

IGFBP1: Insulin-like growth factor-binding protein 1

IgG: Immunoglobulin G

IHh: Indian hedgehog

IL: interleukine

ISG: interferon stimulated gene

IVF: *in vitro* fertilization

KGN: steroidogenic human ovarian granulosa-like tumour cell line

LAMINB: lamine B

LATS1: Large Tumour Suppressor, Homolog 1 Serine/threonine-protein kinase

LE: luminal epithelium

LH: luteinizing hormone

MAPK: Mitogen-activated protein kinases

MHC: major histocompatibility complex

MnSOD: manganese superoxide dismutase

mRNA: messenger ribonucleic acid

MUC1: mucine 1

NaCl: sodium chloride

NOBOX: newborn ovary homeobox protein

NR5A2: nuclear receptor subfamily 5, group A, member 2

OVX: ovariectomy/ovariectomized animals

OXTR: oxytocin receptor

P4: progesterone

PBS: phosphate buffered saline

PBS-T: phosphate buffered saline tween 20

PCR: polymerase chain reaction

PFOXIC: promoter FOXL2 inverse complementary

PGC: primordial germ cells

PGF2 α : prostaglandin F2 alpha

PGH2: prostaglandin H2

PGR-A: progesterone receptor form A

PGR-B: progesterone receptor form B

PGRKO: progesterone receptor knock-out

PIAS1: E3 SUMO-protein ligase

PIS: polled intersex syndrome

PISRT1: polled intersex syndrome regulated transcript 1

POF: premature ovarian failure

PolyAla: poly-alanine

PPARGC1A: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PRE: progesterone receptor response element

PRL: prolactin

pSG5: eukaryotic expression vector

PTGS2: Prostaglandin-endoperoxide synthase 2

RGS2: Regulator of G-protein signaling 2

RIPA: Radioimmunoprecipitation assay buffer

RNA: ribonucleic acid

roIFNT: recombinant ovine interferon-tau

RPL19: 60S ribosomal protein L19

RSAD2: radical S-adenosyl methionine domain containing 2

RSPO1: R-spondin-1

RT-PCR: real time (quantitative)-polymerase chain reaction

SCARA5: scavenger receptor class A, member 5

SCNT: somatic-cell nuclear transfer

SDS: Sodium dodecyl sulfate

SEM: standard deviation of the mean

sGE: superficial glandular epithelium

SHH: sonic hedgehog

SIRT1: Sirtuin 1

SOD2: superoxide dismutase 2

SOX9: SRY (sex determining region Y)-box 9

SP1: specificity protein 1

SRY: sex determining region Y

STAT1: signal transducer and activator of transcription 1

StAR: steroidogenic acute regulatory protein

SUMO1: Small ubiquitin-related modifier 1

SYBR: asymmetrical cyanin dye

TESCO: core 1.3 kb testis-specific enhancer of Sox9

TGF- β : Transforming growth factor beta

TLR: toll-like receptor

TNFAIP3: Tumour necrosis factor, alpha-induced protein 3

TSH: Thyroid-stimulating hormone

UBC9: SUMO-conjugating enzyme

WNT: wingless-related protein

III. Introduction

In the last fifty years, human fertility is declining due to mutations, disease and mostly to environmental factors (Casterline 1989; Sadeu et al. 2010). Current estimates state that 10-15% of couples world-wide remain childless due to infertility, with genetic aetiology making up a significant proportion (Matzuk and Lamb 2008). This fertility decline is a consequence of defects of gonad differentiation and function, gametes production and maturation but also endometrial development and physiology. Due to the progress of Medically-Assisted-Procreation (MAP) in human reproduction and global species conservation, pituitary and gonads infertility can be rescued. Nevertheless, endometrial defects cannot be rescued because beyond the blastocyst stage; endometrium is the only tissue able to drive a successful pregnancy until parturition (Sandra et al. 2011). Given that an achieved pregnancy is characterized by the success of numerous checkpoints, implantation appears to be one of the most critical checkpoints for the outcome of pregnancy (Lee and DeMayo 2004; Bazer et al. 2010). This step is characterized by a tightly regulated communication between a healthy conceptus (embryonic disk and extra-embryonic tissues) and a receptive maternal endometrium. Therefore, it is necessary to define precisely the dialogue occurring during the pre-implantation period between the conceptus and the endometrium and more specifically to study the mechanisms involved in endometrial physiology during the early pregnancy in comparison with the menstrual/oestrous cycle in mammals.

A. *Female reproductive physiology in mammals: the particular case of ruminants*

1. Uterine structure

The endometrium belongs to the female reproductive tract including myometrium, two ovaries, two oviducts, cervix, vagina and vulva. This complex and heterogeneous tissue is one of the key entities crucial to obtain a successful pregnancy (Spencer et al. 2012).

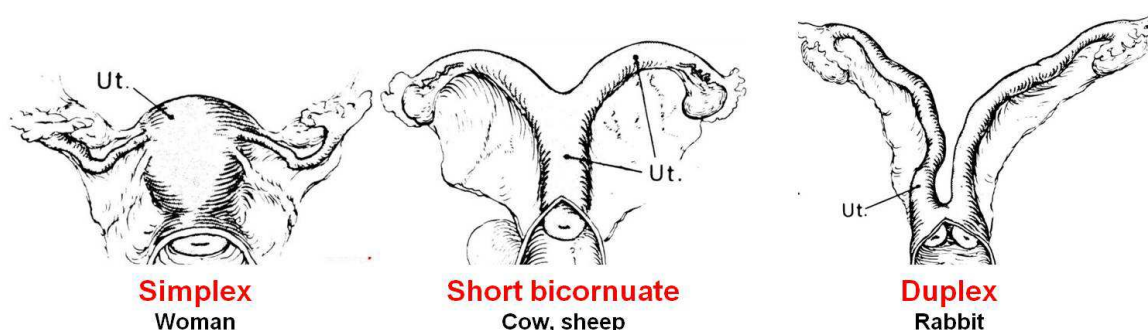


Figure 1: Anatomy of uteri in mammals. Human and non-human primates have a simplex uterus with one cervix and one uterine body. Ruminant have a short bicornuate uterus with two uterine horns, one uterine body and one cervix. Rabbits and rodents have a duplex uterus with two uterine bodies and two cervixes.

All mammal species exhibit different kind of uterus; for instance human have simplex uterus, ruminants have bicornuate uterus, rabbit have duplex uterus (Fig. 1) or carnivore and swine have a long bicornuate uterus. Concerning ruminant, endometrium is not smooth but separated into two different areas, caruncular area or caruncles (CAR, Fig. 2) surrounded by intercaruncular area (ICAR, Fig. 2).

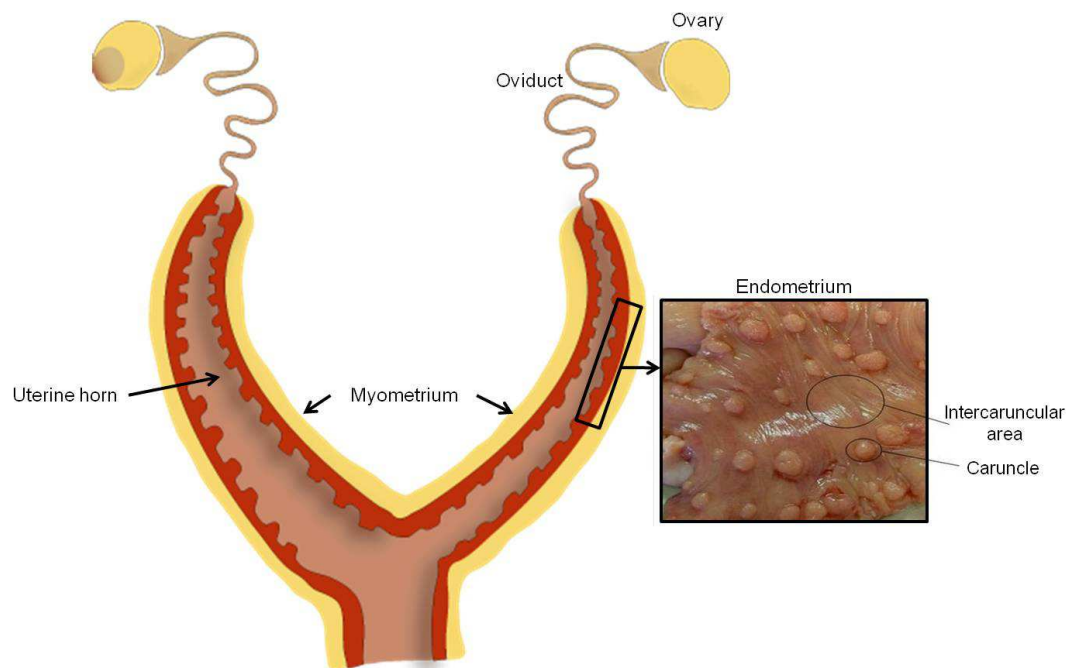


Figure 2: The ruminant female reproductive tract. The bicornuate uterus is composed of myometrium and endometrium which is separated into caruncles and intercaruncular area in both uterine horns.

a) Histology

In mammals, endometrium is constituted with a luminal epithelium overlaying stromal cells, as well as immune and endothelial cells. Endometrial epithelial cells are either luminal or glandular (Cooke et al. 2013). Endometrial glands are localized either close to the lumen namely superficial glandular epithelium (sGE) or in the depth of the endometrium and close to the myometrium namely deep glandular epithelium (dGE). dGE are smaller and more numerous than sGE (Fig. 3). Luminal and glandular epithelia are not a passive barrier to infection but mediate innate immune response through interaction with endometrial dendritic cells, B cells and T cells (Oliveira et al. 2012; Turner et al. 2012; Bauersachs and Wolf 2013). Specifically, ruminant endometrium is separated into CAR and ICAR areas.

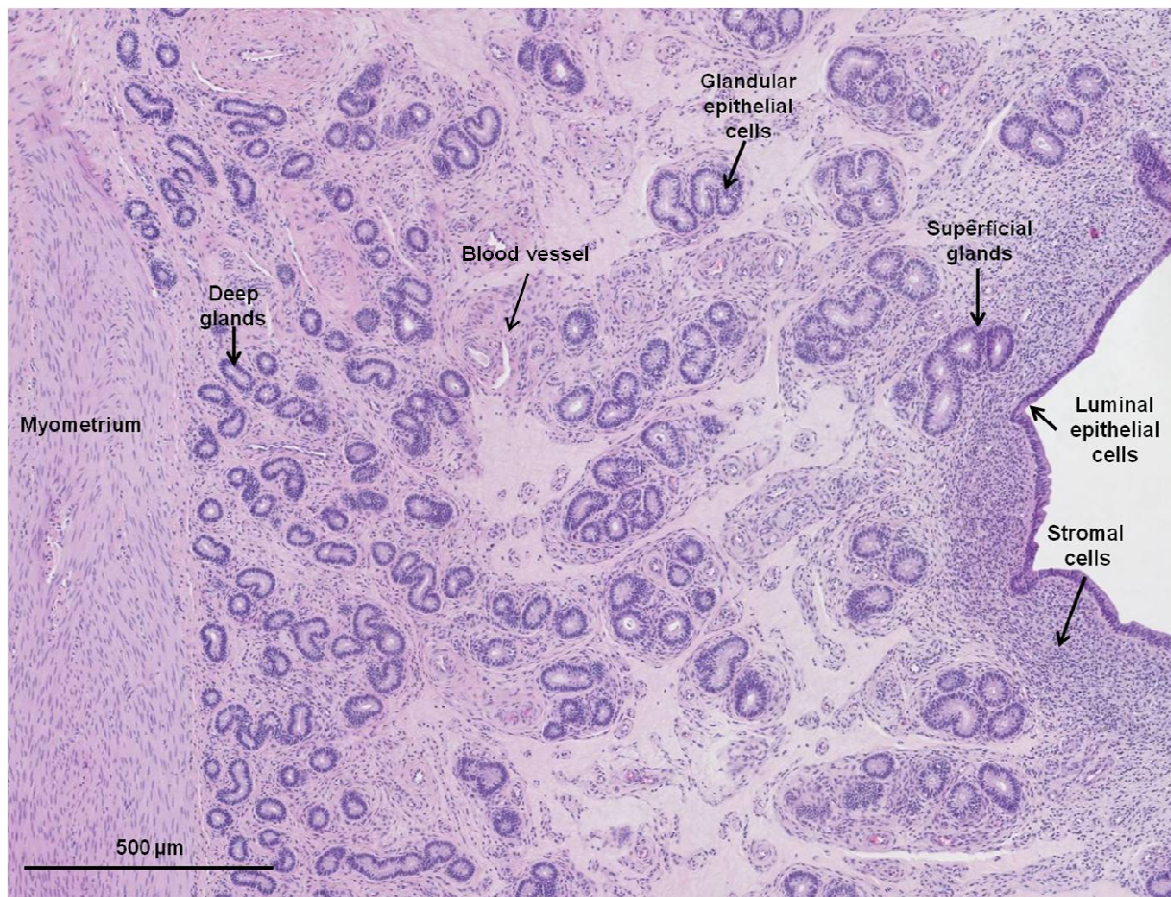


Figure 3: Histology of the bovine uterus. Endometrium is composed by luminal epithelium, stroma, glandular epithelium, immune cells and endothelial cells (blood vessels).

b) Caruncular area

The caruncles are endometrial protuberances, composed almost exclusively of stromal cells, surrounded by a monostratified layer of luminal epithelial cells (Spencer et al. 2012). During pregnancy, the caruncles are the first place of implantation and later, will take part to the placentome units composed of embryonic and maternal villi called cotyledon and caruncle respectively (Nguyen et al. 2012; Touzard et al. 2013). Maternal endothelial and epithelial cells from caruncles are directly apposed to foetal trophoblasts in a variety of crypt-like, villous or folded arrangements.

c) Intercaruncular area

Caruncles are surrounded by the intercaruncular area which is composed of stromal cells (Fig. 3) and a high number of superficial and deep glandular epithelial cells (Spencer et al. 2012). Endometrial glands synthesize and secrete a variety of enzymes, growth factors, cytokines, hormones, transport protein and others molecules collectively termed histotroph (Spencer and Bazer 2002). During pregnancy, maternal histotroph influence the conceptus (embryonic disk and extra-embryonic tissues) development (Bazer et al. 2010).

2. Sexual cycle

In human and ruminant, sexual cycle is characterized by the spontaneous release of the oocyte, at regular intervals. The released gamete takes place at the oestrus, happening even in the absence of the male (Roelofs et al. 2010). In sheep and goats, exposure of seasonally anoestrous females to sexually active males results in synchronized oestrus which is called the “male effect” (Gelez and Fabre-Nys 2004). In mammals, this sexual cycle is dependent of the menstrual/oestrous cycle, the ovarian oestrous cycle and the hormone control of the cycle (Bartlewski et al. 2011).

a) Menstrual cycle

The menstrual cycle lasts approximately 28 days in young healthy women characterizing by an equal duration of follicular and luteal phases (Hawkins and Matzuk 2008; Mihm et al. 2011). Nevertheless, the menstrual cycle length is highly variable between women and correlated with ageing. The variability is mostly dependant of the follicular phase length. At the end of the follicular phase, estrogens rise induces the ovulation of a single ovulatory dominant follicle which is a similar process to cows, mares and women (Ginther et al. 2001).

b) Oestrous cycle

The oestrous cycle is defined by the periodic acceptance of mating just before ovulation occurs. During the oestrus, ruminants exhibit genital (clear vulval discharge) and behavioural (acceptation of mating) events around the oestrus stage (Forde et al. 2011). The oestrous cycle lasts approximately 21 days in cattle and 17 days in sheep (Bartlewski et al. 2011), and can be defined into four stages: proestrus, oestrus, metoestrus and dioestrus (Forde et al. 2011, Fig. 4).

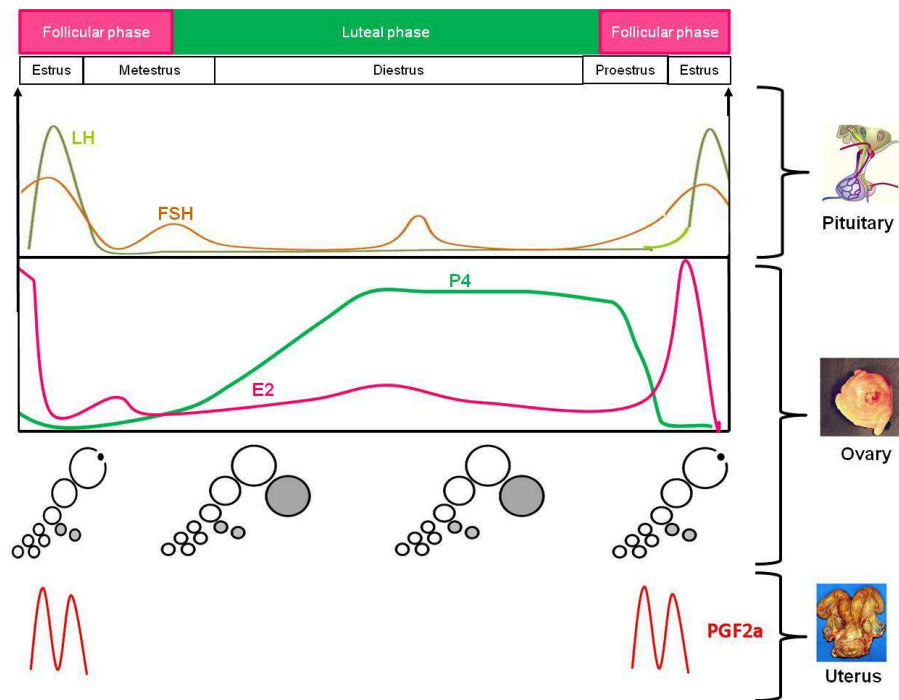


Figure 4: Summary of oestrous cycle events occurring in the pituitary, the ovary and the uterus in ruminants. The pre-ovulatory rise of E2 leads to the LH ovulatory rise then oocytes release. The support ovarian cells differentiate into luteal cells secreting P4. Without fertilization, pulsatile PGF2α release induces the CL regression and the beginning of the next oestrous cycle (Spencer et al. 2007; Forde et al. 2011).

Proestrus takes place at the end of the previous cycle and is characterized by the follicular growth leading to the dominant follicle selection. During the oestrus (from 20 hours to 24 hours in ruminants), the dominant follicle reaches its maximum growth, matures and ovulates (Forde et al. 2011). Metoestrus lasts 2 to 4 days and corresponds to the end of oestrus associated with the Corpus Luteum (CL) formation and the rising of circulating progesterone (P4) levels. Finally, the dioestrus, which is the longest step, is the period of maximum CL size and function leading to high levels of P4 in circulation. At the end of this step, the CL begins to regress which corresponds to the luteolysis phase as proestrus step (Arosh et al. 2002; Bartlewski et al. 2011; Forde et al. 2011).

c) Ovarian oestrous cycle

(1) Follicular phase

The follicular phase takes place during the proestrus and oestrus steps (Forde et al. 2011). This phase is characterized by the follicular growth starting from the primary to the antral follicle also called Graafian follicle. The follicle growth is a continuous phenomenon maintained throughout the pregnancy. During a normal oestrous cycle of 21 days in cattle particularly, three cohorts of follicles begin their follicular growth (Fig. 5, 6 and 7, Ireland et al. 2000). On average, each cohort has an expected lifetime of seven to ten days during which

several follicles will begin their growth but only the dominant follicle will be ovulated. Once selected, the dominant follicle will start the synthesis and the secretion of increasing amount of estrogens (E₂). Remaining follicles will come into follicular atresia (Ireland et al. 2000).

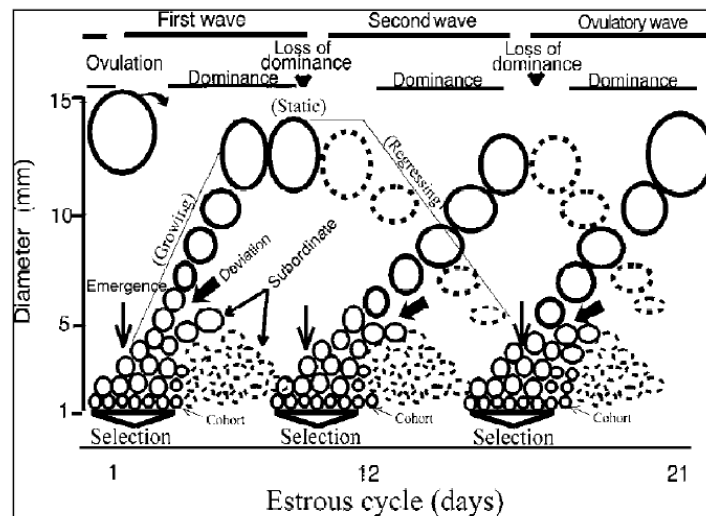


Figure 5: Follicular growth during the bovine oestrous cycle (Ireland et al. 2000). During the oestrous cycle three waves of follicular growth occurred and without fertilization, the third follicular wave leads to the selection of the mature pre-antral follicle to be ovulated. All the unselected follicles degenerate and come into follicular atresia.

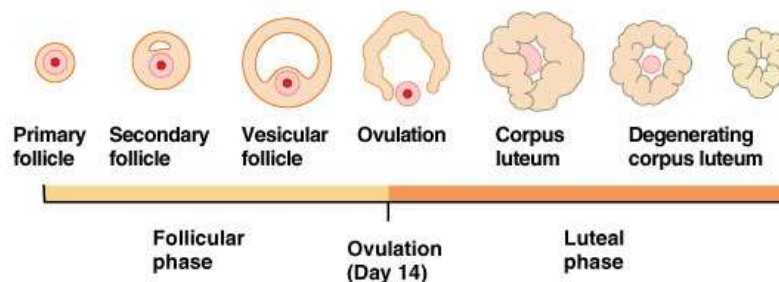


Figure 6: The ovarian cycle in human. Follicular growth leads to the maturation and the selection of ovulatory/ de Graaf follicle. Then, ovulation process leads to a differentiation of ovarian supporting cells into luteal cells secreting P₄ during the luteal phase (B. Cummings, © Pearson Education, 2004).

(2) Luteal phase

After the ovulation, granulosa and interna theca cells of the ovulatory follicle differentiate themselves into large and small steroidogenic luteal cells, respectively. Starting from this step, luteal cells initiate the synthesis and the secretion of P₄ (Fig. 5, 6 and 7; Hawkins and Matzuk 2008; Forde et al. 2011; Mihm et al. 2011). For the differentiation stage, the CL doubles its size and weight until reaching its maximal amount during the active luteal phase (Arosh et al. 2002). Uterus is a major site of prostaglandin synthesis depending on the activity of cyclooxygenase enzymes responsible for the conversion of arachidonic acid to the intermediate precursor PGH₂ then the primary biologically active prostaglandins including prostaglandins F₂ α (PGF₂α, Charpigny et al. 1999). Without fertilization, the CL initiates its regression due to the luteolytic impact of PGF₂α pulses in response to peaks of luteal

oxytocin (Charpigny et al. 1997). Subsequently, P4 synthesis and secretion decrease dramatically (Hawkins and Matzuk 2008).

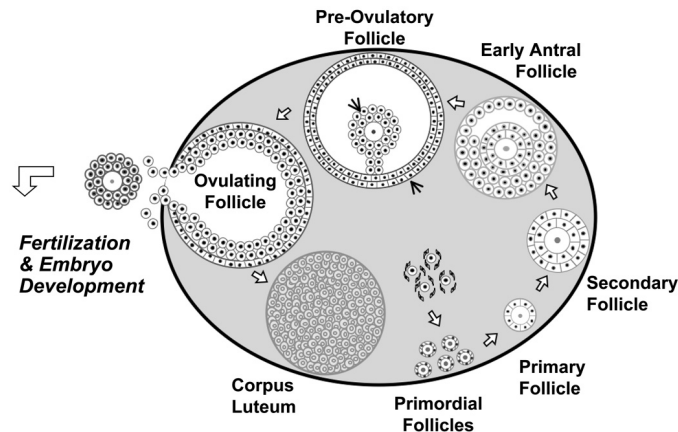


Figure 7: The ovarian follicular growth (Hawkins and Matzuk 2008). Follicular maturation and selection occurred in mammals from primordial to pre-ovulatory follicles then ovulation and CL formation.

d) Hormone control of the oestrous cycle

The oestrous cycle is characterized by the balanced action of ovarian steroid hormone such as E2 synthesized during the follicular phase and P4 during the luteal phase (Miller and Moore 1976; Devroey and Pados 1998; Forde et al. 2011; Mihm et al. 2011). This steroid hormone balance is tightly regulated along the hypothalamo-pituitary-gonadal axis (Fig. 8).

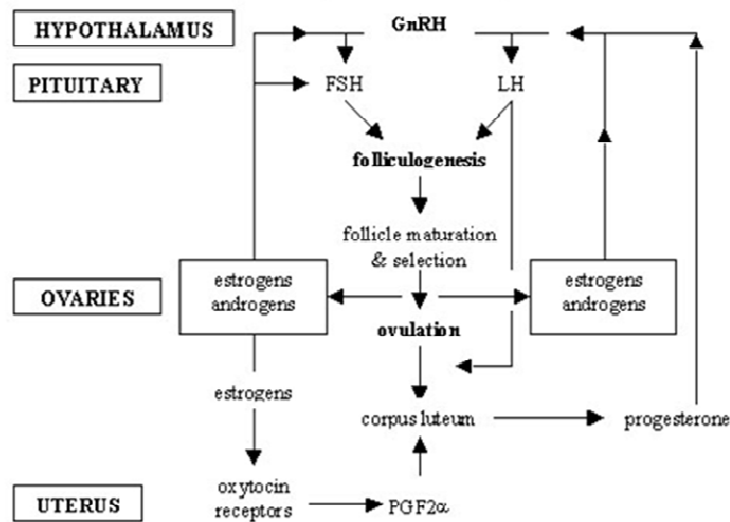


Figure 8: The hypothalamo-pituitary-gonadal axis in mammals (adapted from Whirledge and Cidlowski 2013; Whirledge and Cidlowski 2013). GnRH pulsatile secretion then E2 secretion as well as LH and FSH pulsatile secretions lead to ovarian steroid hormones secretion and ovulation processes. In case of menstrual/oestrous cycle, OXTR then pulsatile secretion of PGF2α induce CL regression.

(1) The hypothalamo-pituitary action

The hypothalamus has to integrate both external (temperature, light or stress) and internal (ovarian E2 and P4) stimuli leading to a pulsatile secretion of a gonadoliberrine hormone,

called GnRH, Gonadotropin Releasing Hormone (Fig. 8). Ovarian steroid hormones can modulate the GnRH secretion and therefore modulate GnRH action on pituitary. The pulsatile secretion of GnRH induces the pulsatile discharge of Follicle Stimulating Hormone (FSH) and the Luteinizing Hormone (LH) by the anterior pituitary. Both pituitary hormones are released at high level leading to the ovulation 24 hours later (Fig. 9; Downey 1980; Ginther et al. 2005; Forde et al. 2011).

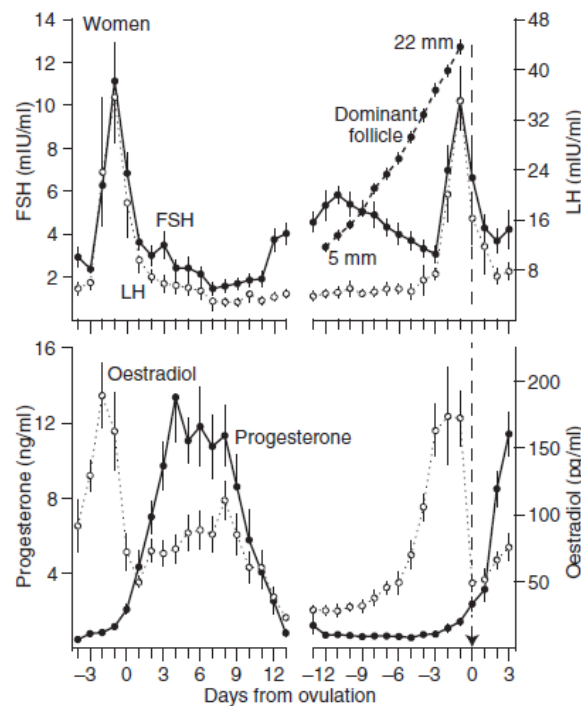


Figure 9: LH, FSH, E2 and P4 concentration during the menstrual cycle. The pre-ovulatory rise of E2 leads to the ovulatory rise of LH then ovulation of the dominant follicle (Ginther et al. 2005).

FSH is responsible for the follicular growth and induces the secretion of E2 by aromatase gene activation. Indeed, aromatase gene, *CYP19*, catalyzes the conversion of androgens into 17 β -estradiol hormone (Pannetier et al. 2006; Forde et al. 2011).

LH is involved in follicular maturation, and, ovulation induced by the pre-ovulatory rise of E2. The ovulatory rise of LH lasts approximately six hours with 50-fold increased level of secretion compared to another step of the oestrous cycle. LH is, then, involved in the differentiation of granulosa and interna theca cells into luteal cells leading to synthesis and secretion of P4 (Messinis et al. 2010).

(2) *Biological action of ovarian steroids*

(a) Estrogens (E2)

During the follicular phase, E2 are synthesized by the granulosa and the interna theca cells in a short time frame with an important discharge 24 hours before the ovulation. E2 stimulate the cyclic activities of hypothalamus and pituitary, emphasizing the secretion of FSH and LH (Downey 1980).

(b) Progesterone (P4)

P4 is mostly secreted by the CL during the luteal phase even if there is a limited production by the ovarian stroma. This luteal secretion rises gradually until P4 secretion reaches a plateau with sufficient concentrations throughout the luteal phase to maintain pregnancy when occurs. P4 is the major pregnancy hormone and avoids the next oestrous cycle with a negative impact on hypothalamus and pituitary preventing behavioural oestrus occurring (Fig. 8; Forde et al. 2011; Whirledge and Cidlowski 2013; Whirledge and Cidlowski 2013). In addition, P4 level during the luteal phase activates the endometrial gland differentiation and secretion occurring during uterine receptivity and promoting conceptus implantation when occurs (Bazer et al. 2010; Forde et al. 2011). In the case of oestrous cycle, oxytocin is secreted by the anterior pituitary and the CL and binds the oxytocin receptor (OXTR) inducing the *COX2/PTGS2* expression (Kim et al. 2003). Uterine pulsatile synthesis of PGF2 α by the rate-limiting enzyme, PTGS2 (Charpigny et al. 1999), leads to CL lyses and then, dramatic decrease of P4 secretion because of the disappearance of the luteal steroidogenic cells (Smith et al. 1994).

(c) Uterine steroid nuclear receptor

Once secreted, E2 and P4 are either transported to the uterus by the ovarian artery or diffuse out of the granulosa and luteal cells respectively, and act in endometrium using mainly nuclear receptor. The steroid hormones bind their own nuclear receptor ER α and β (ESR 1 and 2, respectively) for E2 and PR A and B for P4 (also called PGR form A and B, respectively) which are members of the nuclear receptor super-family of transcription factors capable of activating and repressing transcription of their endometrial target genes (Fig. 10; Franco et al. 2012; Wetendorf and DeMayo 2012).

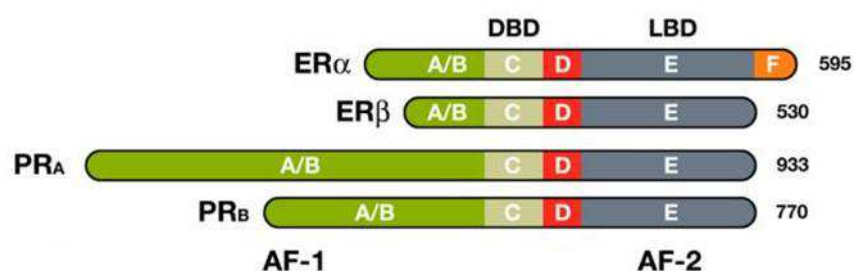


Figure 10: Structure of E2 and P4 nuclear receptor. A/B: N-terminal domain, highly variable. DBD = DNA binding domain. LBD = ligand binding domain. AF = activation function. F: highly variable sequence and unknown function. The steroid hormone receptor abbreviations are ER – estrogen receptor (α, 595 amino acids; β, 530 aa) and PR –progesterone receptor (A, 933 aa; B, 770 aa). Adapted from Aranda and Pascual 2001; Griekspoor et al. 2007.

Many rapid and non-classical actions of P4 are mediated by P4 receptor membrane component 1 and 2 namely *PGRMC1* and *PGRMC2* (Pru and Clark 2013). Endometrial expression of *PGRMC1* in menstrual cycling mammals is most abundant during the proliferative phase of the cycle. *PGRMC2* is highly expressed during the secretory phase in mammals and could be involved in universal non-classical P4 actions in the uterus (Pru and Clark 2013). However, the biological functions of both *PGRMC* remain not clear.

PGR Knock-Out mice are completely infertile due to serious defects on pituitary, ovaries and uterus (Lydon et al. 1995). Mutant mice for both isoforms of *PGR* have a non-receptive uterus which did not undergo decidualisation and then implantation whereas *ESR1* null mice exhibit defects of uterine growth and fail to prepare for blastocyst implantation (Ramathal et al. 2010). Both knock-out mice show uterine receptivity impairment (Fig. 15). *ESR* and *PGR* exhibit a complex expression and localization throughout the oestrous cycle (Clemente et al. 2009; Okumu et al. 2010). Both *ESR* and *PGR* expression are decreased from the follicular to the luteal phase in cattle (Okumu et al. 2010). *ESR* are localized in the stroma cells and either in the superficial glandular epithelium (sGE) or the deep glandular epithelium (dGE) during the follicular phase, specifically during proestrus and oestrus with a maximal level in metoestrus and show a significant decrease of expression during the luteal phase/dioestrus. Similarly, *PGR* localization are increased during oestrus with a maximal level in metestrus (follicular phase) and exhibit a significant decrease in diestrus (luteal phase) and a moderate expression in preestrus (Kimmins and MacLaren 2001).

During the luteal phase, P4 and then *PGR* are responsible of the *ESR1* and *OXTR* silencing leading to a repression of oxytocin action on the endometrium. Interestingly, prolonged action of P4 inhibits the expression of *PGR* leading to oxytocin binding its own receptor and elicits the pulsatile secretion of *PGF2α* (Spencer and Bazer 2002; Bazer 2010) leading to the CL regression.

(3) *Endometrial evolution through the oestrous cycle*

During the oestrous cycle and especially the menstrual cycle (Fig. 11), endometrium undergoes intense cellular reshaping (Maybin and Critchley 2012). The follicular phase exhibits intense endometrial proliferation process under the influence of E2. For instance, E2 induces a massive proliferation of human endometrial cells, increasing the endometrial thickness and angiogenesis prior to the invasive implantation of human embryo when pregnancy occurs (Fig11; Miller and Moore 1976; Devroey and Pados 1998; Mihm et al. 2011). Ruminant endometrium undergoes a moderate proliferative process during the short-follicular phase (Forde et al. 2011). Mitosis occurred in ruminant endometrium in stromal and epithelial cells (Forde et al. 2011). During the luteal phase, P4 inhibits the endometrial proliferation in favor of the endometrial differentiation (Miller and Moore 1976). Human endometrial cells undergo decidualisation process which is a transformation of the endometrium into the decidual tissue, involving the differentiation of endometrial cells and infiltration by large numbers of lymphoid cells (Salamonsen et al. 2003). In ruminant, decidualisation does not occur but endometrium undergoes also a luminal epithelium thickening and morphological changes in endometrial glands that will become angled and curled up associated with a larger diameter (in the ewe; Gray et al. 2001; in the cattle; Bazer et al. 2009). In addition, P4 secreted during the luteal phase stimulates angiogenesis in endometrium and inhibits the myometrial contractions by non-genomic mechanisms (Takamoto et al. 2002; Bazer et al. 2009).

During the luteo-follicular transition, endometrium exhibits a cellular and vascular reshaping with or without bleeding and vascular phenomenon in human and ruminant species, respectively (Forde et al. 2011; Mihm et al. 2011; Gargett et al. 2012). Human endometrium is subjected to cyclical injury and repair throughout a woman's reproductive life. The decline of P4 secretion during the luteo-follicular transition triggers menses leading to the separation of the functional layer of endometrium from the basal layer (Gargett et al. 2012; Maybin and Critchley 2012).

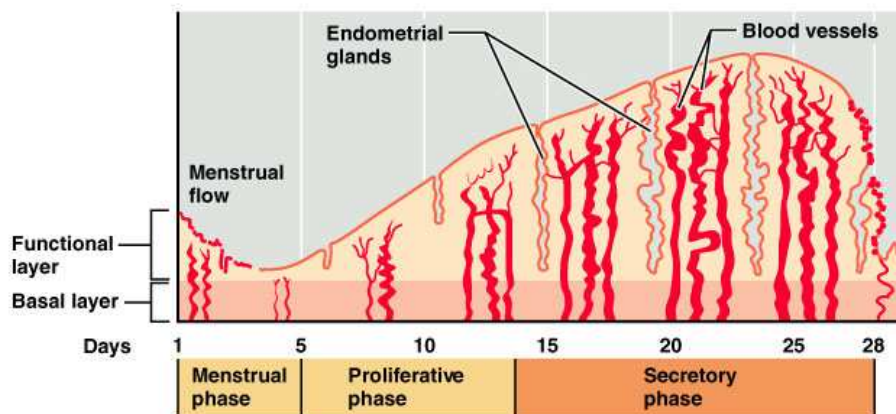


Figure 11: The uterine cycle in human. Human endometrium undergoes intense proliferation then differentiation and decidualisation until menses without fertilization (B. Cummings, © Pearson Education, 2004)

In ruminant, endometrial reshaping is superficial due to the slight proliferation process occurring during the follicular phase.

3. The establishment of pregnancy

a) Early embryo development

After fertilization, the embryo is still localized in the oviduct for few days according to the species and then, come into the uterine cavity (Fig. 12, Thibault © Ellipses, 2001). Early embryo development is characterized by various cellular divisions (blastomeric divisions) and the first differentiations events starting from the morula stage (Guillomot 2001).

Species	Enter in uterus (dpo)	Loss of Zona Pellucida (dpo)	Implantation (dpo)	Duration of pregnancy (dpo)	Placentation
Human	3-4 (morula)	5	6	280	Hemochorial
Mouse	3 (morula)	4	5	20	Hemochorial
Cattle	4 (16-32 cells)	9-10	19-20	280	Syneptihelio-chorial
Sheep	4 (16-32 cells)	8-9	15-16	145	Syneptihelio-chorial

Figure 12: Comparative aspects of pregnancy in four mammal species. Implantation day is highly variable according to the species without any correlation with the duration of pregnancy (adapted from Thibault © Ellipses, 2001).

In mammalian development and more specifically in ruminants, the first cell fate decisions are clearly specified during blastocyst formation, when the trophectoderm (TE) surrounds the inner cell mass (ICM), forming the cavity named the blastocoel (Fig. 13; Young et al. 1998).

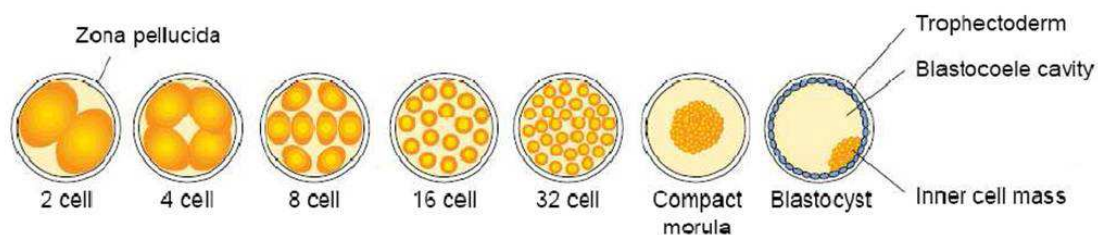


Figure 13: First embryo cellular division from 2-cells stage to blastocyst stage (Young et al. 1998).

The ICM will shape the fate of the embryo and extra-embryonic tissues (EET) that will become the placenta (Hue et al. 2012; Nagatomo et al. 2013). In uterine cavity, bovine blastocyst has a central position because of its future wide expansion whereas human blastocyst has an eccentric position (Wimsatt 1975; Hue et al. 2012). Hatched human and rodent blastocysts undergo implantation process respectively at 6 and 5 dpo. Ruminant hatched blastocysts undergo implantation at 19-20 and 15-16 dpo respectively following an elongation period of the EET (cow: 2 weeks, sheep: 10 days; Bazer et al. 2009) associated with a continuous cross-talk with the uterus (Fig. 14; Degrelle et al. 2005; Hue et al. 2012).

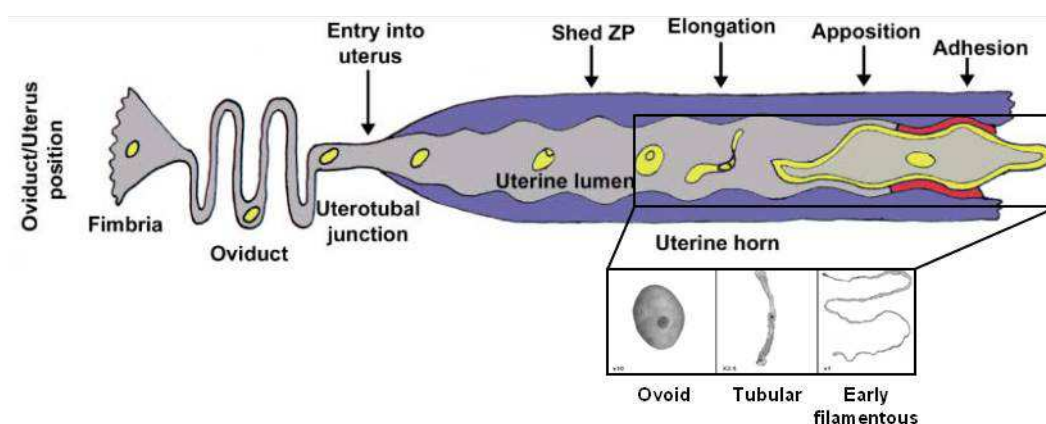


Figure 14: Early pregnancy events in ruminant. Schematic representation of the relative changes in embryo/blastocyst development after fertilization in relation to position in the female reproductive tract from Spencer et al. 2004. Pictures of growth and differentiation of embryonic and extra-embryonic tissues in bovine pre-implanting blastocysts. Ovoid (day 12), tubular and early filamentous blastocysts (day 16-17) increased in size from 150 μ m to 130–150 mm (adapted from Degrelle et al. 2005).

During this elongation period, blastocysts have a free-life period that ends up when the conceptus starts forming loose cellular contacts with the endometrial layer of the uterus (Guillomot 1995; Hue et al. 2012). The major protein product by ovine conceptuses in culture was initially referred to as protein X (Godkin et al. 1982). This protein, secreted by the elongating conceptus trophectoderm, was renamed ovine trophoblast protein-1 then interferon tau (IFNT; Bazer et al. 1979; Martal et al. 1979; Godkin et al. 1984; Godkin et al. 1984). IFNT is a member of type I interferon, sharing high structural homology with other

members including IFN alpha, beta, delta and omega but IFNT is unique to ruminant (Bazer et al. 2010). IFNT which is the major signal of pregnancy maternal recognition is secreted from day 9 to day 18-20 in sheep and from day 12 to day 25 in cattle (Martal et al. 1979; Beal et al. 1981; Knickerbocker et al. 1986; Roberts et al. 2008).

b) Hormonal control of pregnancy maternal recognition

The establishment of pregnancy needs the maintenance of a functional CL. The strong embryonic anti-luteolytic signal, IFNT, abrogates the CL regression and maintains the production of P4 that is permissive to actions of IFNs, growth factors and cytokines responsible for uterine receptivity to implantation (Bazer et al. 2010; Bott et al. 2010).

Specifically, IFNT suppresses the expression of estrogen nuclear receptor 1 (*ESR1*) and estrogen-regulated genes including oxytocin receptor (*OXTR*). In uterine luminal and superficial glandular epithelium, the oxytocin action removal leads to a silencing of the endometrial luteolytic pulses of PGF2 α . The inhibited expression of *ESR1* leads also to the repression of *PGR* and Mucine1 (*MUC1*, anti-adhesive gene) expression in endometrial epithelia which is a prerequisite for uterine receptivity to implantation. *MUC1*, a transmembrane mucin glycoprotein expressed at the apical surface of a variety of reproductive tract epithelia, is a component of the glycocalyx which is an extracellular polymeric material (Spencer et al. 2004). In both human and ruminant species, *MUC1* controls the accessibility of conceptus integrin receptors to their ligands by sterically blocking cell–cell and cell–extracellular matrix (ECM) adhesion (Spencer et al. 2004). Both repression of *PGR* and *MUC1* is a prerequisite for contact with TE and initiation of implantation (Spencer and Bazer 2002; Bazer et al. 2012).

P4 promotes the conceptus semi-allograft in the maternal organism by suppressing the expression of immune recognition like major histocompatibility complex class I molecules (MHC) genes, consisting of an alpha chain, and β 2-microglobulin (B2M) that regulate immune rejection responses (Choi et al. 2003; Bazer 2010). More specifically, in sheep, MHC class I alpha chain and B2M are primarily expressed in LE and sGE from day 10 to 12 of the oestrous cycle and pregnancy but significantly increased in stromal cells and deep glandular epithelium (dGE) during the pregnancy maternal recognition period (day 14 to 20) and not in LE and sGE. Similarly, intrauterine infusion of IFNT leads to an increase of MHC class I and B2M in stromal cells and dGE but not in LE and sGE. Altogether those results show that the MHC class I and B2M molecules local silencing during pregnancy is a prerequisite for maternal tolerance of the allograft conceptus and implantation step (Lea and Sandra 2007; Roberts et al. 2008; Bazer et al. 2010). Immunosuppressive environment is required during pregnancy; P4 is known to induce the expression of Interferon Stimulated

Genes (ISGs) in association with IFNT action leading to the repression of immune response (Bazer et al. 2008). In addition, a combine action of IFNT and P4 induces the expression of Toll-Like Receptors (TLRs), members of the pattern recognition receptors family essential for the endometrial innate immune response (Oliveira et al. 2012). Collectively, endometrial gene expression profile involved in innate immunity exhibits a repression of global immune response prior to the conceptus semi-allograft (Walker et al. 2010).

Normal P4 production and secretion during the oestrous cycle and maintained production during the pregnancy are necessary for endometrial gland differentiation (Gray et al. 2002; Wetendorf and DeMayo 2012). Ovine uterine gland knock-out (UGKO) due to the neonatal exposure of norgestomet; a P4 analogue; exhibit lack of endometrial glands and a markedly reduced surface of luminal epithelium. UGKO ewes cannot support a pregnancy until parturition due to recurrent early pregnancy loss associated with failure of conceptus elongation and survival between 12 and 14 dpo (Gray et al. 2001; Gray et al. 2002). In addition, microarray comparison between cyclic and UGKO ewes show different populations or altered numbers of immune cells in UGKO ewes could be involved in recurrent early pregnancy loss (Gray et al. 2006).

As described above, well-developed endometrial glands are involved in the communication with the conceptus during the peri-implantation period, secreting histotroph. Nutrients, growth factors or enzymes are responsible of the conceptus development; in time and with a normal size; in response of IFNT embryonic secretion (Gray et al. 2006; Spencer et al. 2007; Bazer et al. 2012).

c) Steroid nuclear receptor and early pregnancy

Steroid nuclear receptors control uterine receptivity throughout the oestrous cycle as well as the early pregnancy in association with IFNT action. The biological functions of both members of nuclear receptor transcription factor family are reflected by the regulation of their target genes and were assessed using knock-out mice model or antagonist of hormone receptor (Lubahn et al. 1993; Lydon et al. 1995; Kregge et al. 1998; Couse et al. 2000; Cheon et al. 2002; Fig. 15).

Strategy	α ERKO	β ERKO	$\alpha\beta$ ERKO	PRKO	RU486
Fertility	Infertile	Subfertile, infrequent pregnancies, reduced litter size	Infertile	Infertile	Infertile
Ovary	No ovulation, immature follicle, hemorrhagic cyst	Normal appearance, reduced ovulation	Granulosa differentiation into Sertoli-like cells	No ovulation	ND
Uterus	Hypoplastic uterus, insensitive to E2, no implantation	Normal	Idem α ERKO	Hyperplasia, inflammation	No implantation
Mammary gland	Immature	Normal	Immature	Severely limited development	ND
Publication	Lubahn <i>et al.</i> , 1993	Krege <i>et al.</i> , 1998	Couse <i>et al.</i> , 2000	Lydon <i>et al.</i> , 1995	Cheon <i>et al.</i> , 2002

Figure 15: Comparison of estrogen and progesterone knock-out mice model. Deletion of ER (ESR) and PR (PGR) genes in mice is strongly linked to infertility as abnormal ovarian function and uterine defects.

The deletion of nuclear hormone receptors in mice lead to infertility and altered endometrial gene expression profile binding ERE and PRE of target genes, respectively (Griekspoor et al. 2007). ESR genes can affect cellular proliferation and differentiation using ligand-dependent nuclear transactivation. ESR1-regulated genes are involved in apoptosis, cell cycle, cell adhesion/extracellular matrix and morphogenesis. Classic target genes include TGF β , prolactin, VEGF, angiotensin, cathepsin D, c-Myc and cyclin D1 (O'Lone et al. 2004). In addition, *ESR1* is known to regulate various endometrial genes expression such as *OXTR* and *PGR* genes as previously described above which is a prerequisite to the initiation of uterine receptivity, particularly in ruminants, as previously described above (Bazer et al. 2010).

On the other side, *PGR* is also capable to regulate various endometrial genes such as Indian Hedgehog (*IHh*) which is involved in epithelial cells proliferation and differentiation but also in angiogenesis in mice (Takamoto et al. 2002). *IHh* as an effector of *PGR* is expressed in luminal and glandular epithelia with an increased expression right before the implantation then decreased expression immediately after implantation (Franco et al. 2008). *IHh* is a an early P4-regulated gene inducing the downstream expression of Patched-1 (*Ptc-1*), Hedgehog interacting protein 1 (*Hip1*) and Chicken Ovalbumin upstream Promoter transcription factor II (*CoupTFII*). Bone Morphogenetic protein 2 (*Bmp2*) which is expressed in the endometrial stroma in sites of implantation in mice, is also known to be an effector of *Pgr* inducing the downstream expression of *Wnt4* and *Ptgs2* expression (Franco et al. 2008). The murine transcription factor, Hand2 is involved in the anti-proliferative action of P4 mediated by Pgr which is necessary prior to implantation step (Li et al. 2011). *HOXA10* is a direct target gene of PGR and is involved in embryo development, implantation and conceptus-maternal communications, particularly in pig (Wu et al. 2013). PGR may play a

crucial role in endometrial cancer and endometriosis because of the differential expression of its direct target gene including *HOXA10* and *IHh* genes (Franco et al. 2008).

d) Implantation: the specific case of ruminant

(1) Definition

In mammals, implantation represents a critical checkpoint for the success of pregnancy. This step corresponds to the establishment of permanent cellular interactions between the conceptus (embryo and extra-embryonic tissues) and the endometrium (Lee and DeMayo 2004; Bazer et al. 2010). Compared to the invasive nature of implantation in mice and humans, the process of implantation is superficial in ruminants, leading to a synepitheliochorial placentation. Implantation starts at 20-21 days post-oestrus (dpo) in cattle and 15-16 dpo in sheep (Fig. 12 and Fig. 16; Bowen and Burghardt 2000).

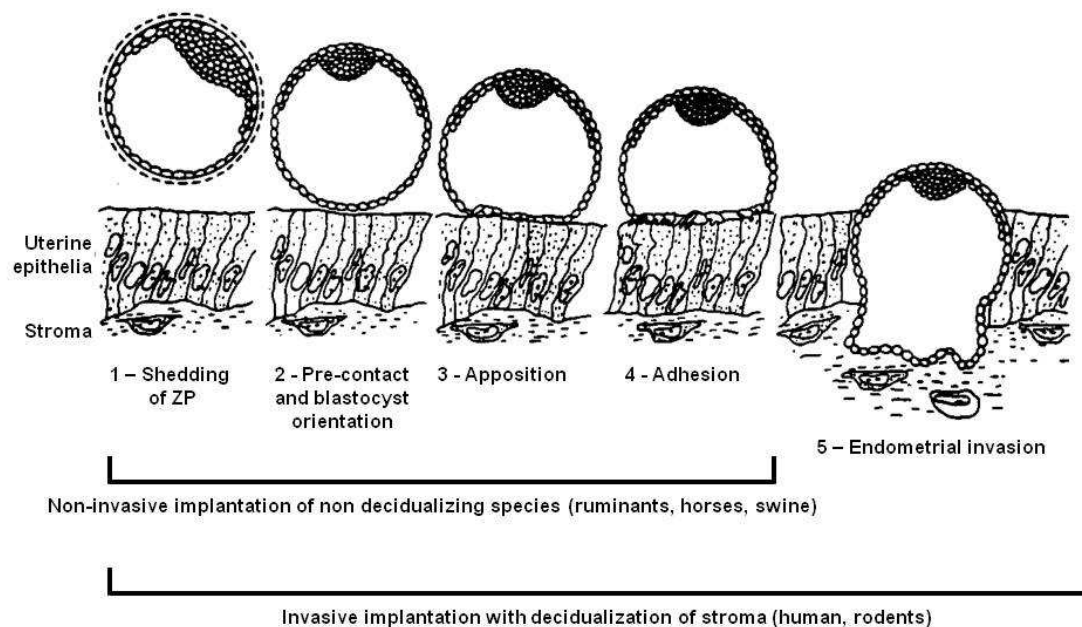


Figure 16: Comparative aspects of implantation (adapted from Thibault © Ellipses, 2001; Bazer et al. 2009). Shedding of ZP, pre-contact, blastocyst orientation, apposition and adhesion are the four common steps of implantation in all mammals. Adhesion represents the ultimate phase of implantation in the species showing the epitheliochorial placental type whereas endometrial invasion lead either to endothelio- or hemochorial placentation.

This critical step of early pregnancy is dependent on the perfect synchronization between the conceptus and the endometrium. Apposition, then adhesion occurs during the window of implantation which is a very short period of time (Fig. 17; Sherwin et al. 2007; Bazer et al. 2009). Prior to implantation, human endometrium undergoes decidual reaction whereas the ruminant conceptus undergoes an important elongation of its EET, a prerequisite for efficient materno-foetal interactions (Hue et al. 2012). At the same time, endometrium undergoes

strong reshaping of stromal and glandular epithelial cells in order to host correctly the conceptus, with an apposition step followed by an adhesion step.

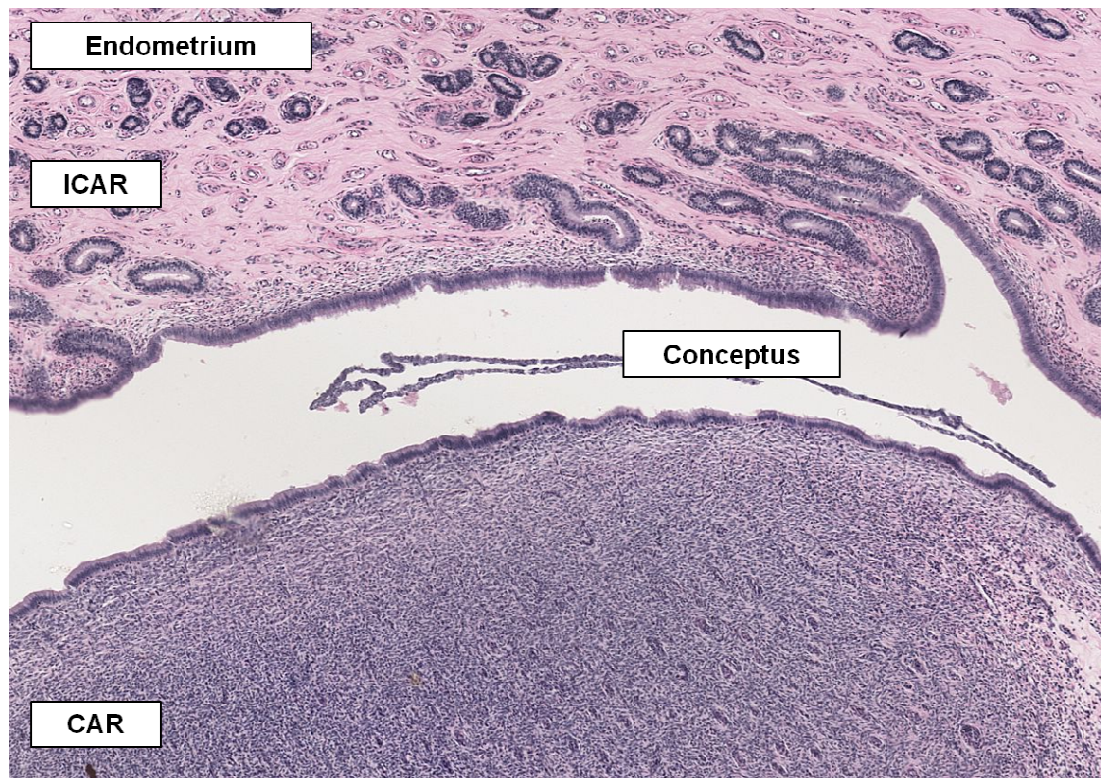


Figure 17: Interaction between ovine elongating conceptus and endometrium at 14 dpo pregnancy (Courtesy of M. Guillomot). Endometrium is separated in caruncular (CAR) and intercaruncular (ICAR) areas. The conceptus (embryonic disk and extra-embryonic tissues) is in the endometrial lumen without cellular contact during the free-life period prior to implantation process.

As described previously above, effective implantation resulted in a tightly regulated communication between both endometrium and conceptus associated with the combine action of IFNT and P4 (Spencer et al. 2007).

(2) *Endometrial reshaping at implantation*

In ruminants, synepitheliochorial implantation is associated with a non-invasion of the endometrium and cotyledonary placentation whereas in human, hemochorial implantation is characterized by a deep invasion of the endometrium and discoidal placentation (Fig. 18).

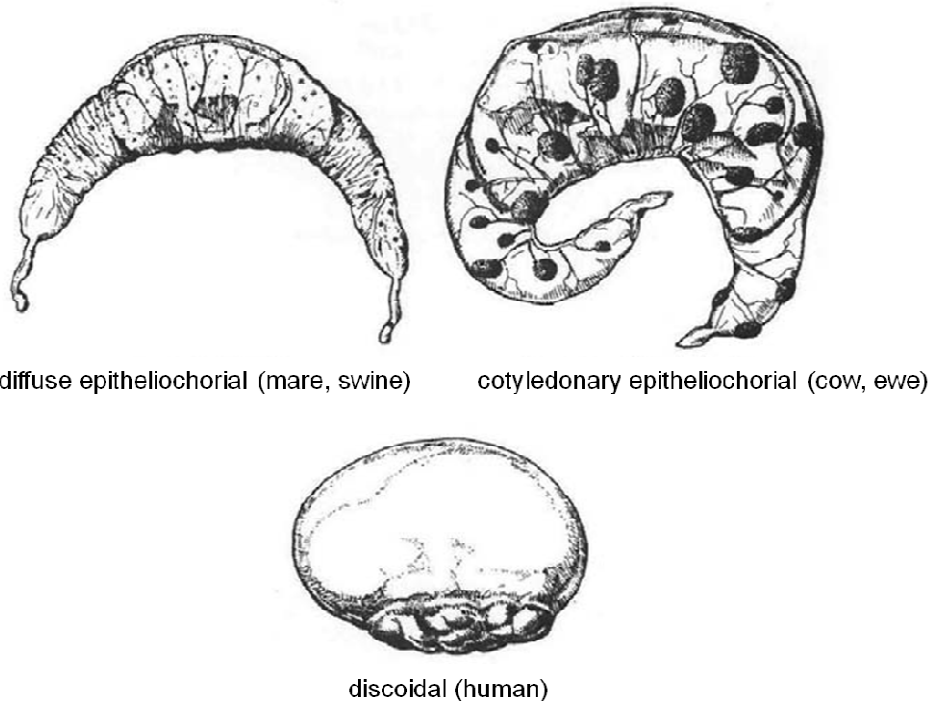


Figure 18: Comparative aspects of placentation from Guillomot 2001. Mare and swine have a diffuse epitheliochorial placentation whereas cow and ewe have cotyledonary epitheliochorial placentation. Human exhibits a discoidal placentation.

The apposition then the adhesion of the conceptus (first, adhesion of embryonic disk (ED) followed immediately by the adhesion of EET) on the endometrium is the first contact between trophoblastic cells and uterine luminal epithelial cells (Guillomot 1995). In sheep and goat, binucleated cells of the trophectoderm which correspond approximately to 20% of trophectodermic cells will migrate and cross the luminal epithelium leading to the syncitium and thus, the synepitheliochorial placentation (Guillomot 1995; Patel et al. 2004). In cows, the situation slightly differs because binucleated cell will not migrate but fuse with one endometrial epithelial cell (Fig. 19; Wooding 1982).

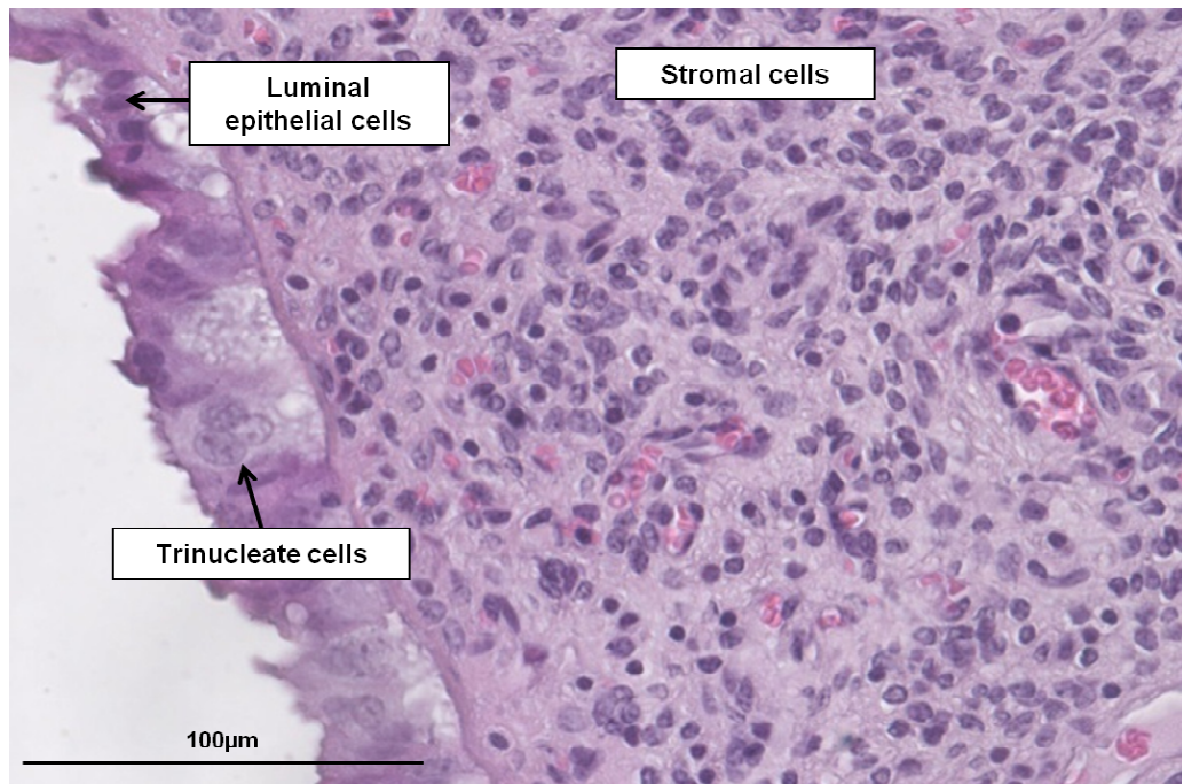


Figure 19: Fusion between binucleated cells of the trophectoderm and luminal epithelial cells at implantation in bovine uterus (C. Eozenou). Hematoxylin/eosin staining of endometrium shows a trinucleate cell specific of cattle.

In human, the hemochorial placenta is discoidal and is characterized by a deep invasion of the endometrium. Thus, chorionic villi are bathed in the maternal blood (Fig. 20). Transplacental exchanges between the conceptus and maternal organism will take place after fusion and invasion respectively in ruminant and in human: placental hormone release in maternal circulation, nutrients necessary for the development of conceptus, etc (Fig. 20; Robbins and Bakardjiev 2012).

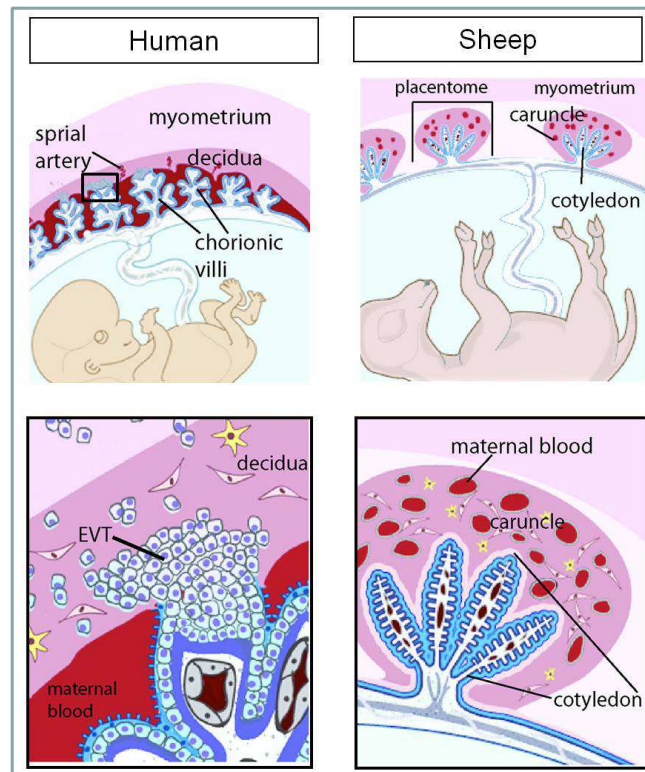


Figure 20: Comparative aspects of placentation between human and sheep (Robbins and Bakardjiev 2012). The hemomonochorial human placenta is a villous structure bathed in maternal blood and anchored deep in the uterine decidua by extravillous trophoblasts (EVT: extravillous trophoblast). The sheep placenta is synepitheliochorial, and composed of multiple placentomes throughout the uterus. Each placentome includes a uterine caruncle partially encapsulating a non-invasive fetal cotyledon.

In ruminant, migration of binucleated cells start at the initiation of implantation and will continue until parturition (Wooding and Wathes 1980; Wooding 1982). Moreover, the apposition followed by adhesion step take place on the entire endometrial surface but syncytia are predominantly made starting from the caruncles leading to the formation of placental units called cotyledons (Fig. 20). Once established, ovine placenta will secrete P4 in place of the CL throughout the pregnancy (Denamur and Martinet 1955) but also others molecules like the bovine Placental Lactogen (bPL) or the Pregnancy Associated Proteins, PAG (Patel et al. 2004; Constant et al. 2011). The bovine placenta only contributes to a minor extent to the peripheral maternal plasma levels of P4 which are rather constant throughout bovine gestation due to CL secretion (Hoffmann and Schuler 2002).

(3) *Embryonic losses and implantation*

A progressive decrease of bovine fertility had been referenced since the 1950's until 2006, particularly in dairy cows. As previously described, implantation is a critical step for the outcome of pregnancy and interestingly, almost 50% of pregnancies abort during the pre-implantation period mostly due to early embryonic death (Diskin and Morris 2008).

(a) Fertilization rate in ruminants

Depending on the ambient temperature, the semen quality, the season (in sheep) and many other environmental factors, the fertilization rate in ruminant is about the average of 90-95%. Moreover, an asynchronicity of fertilization occurred in a very few number of bovine abortion (Diskin and Morris 2008).

(b) Early and late embryonic losses

Since fifty years, fertility in cows and in particular in high-producing dairy cows decreases dramatically due to embryonic losses mostly during the pre-implantation period (up to day 17) but also in post-implantation period (up to day 42, Diskin and Morris 2008). This decrease has multi-factorial causes, like endometrial or mammary infection, female and male gamete genetic, first oestrus delay, maternal ages, energy balance or breeding conditions (Diskin and Morris 2008). The percentage of abortion is more relevant in case of reproductive biotechnologies such as the *In vitro* Fertilization (IVF) or the Somatic Cells Nuclear Transfer (SCNT).

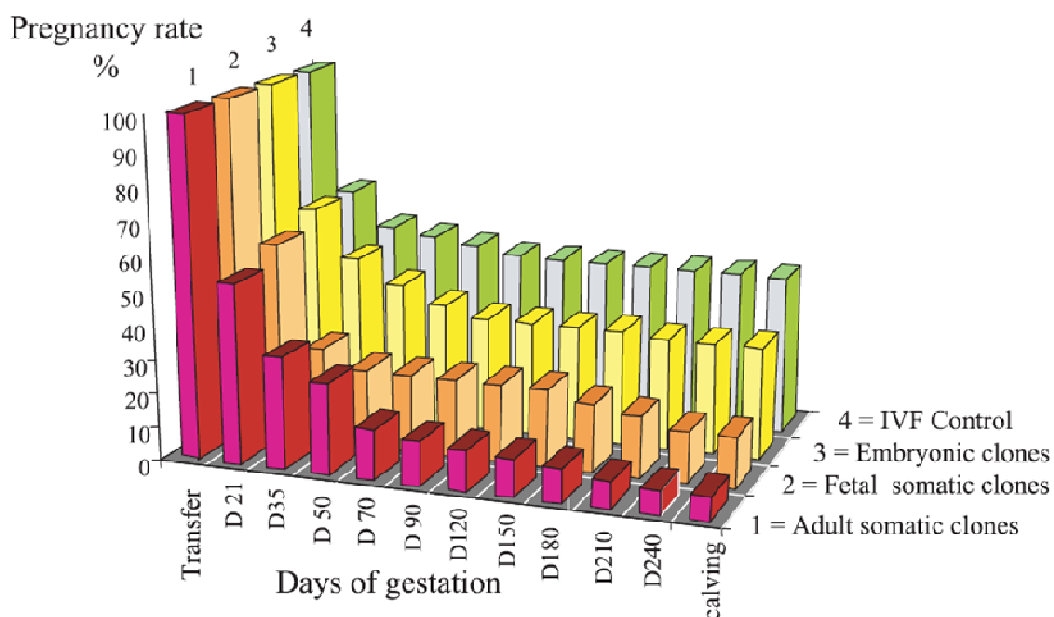


Figure 21: Evolution of pregnancy profiles in bovine recipients after transfer of cloned embryos (from Heyman 2005).

Interestingly, the percentage of fertilization does not change significantly between Artificial Insemination (AI), IVF and SCNT, but the percentage of implantation is lower from the IVF process to the SCNT process (Fig. 21; 70% to 50% of implantation rate; Heyman 2005; Mansouri-Attia et al. 2009). Altogether, those results prompt the need for accurate understanding of the molecular mechanisms involved during the peri-implantation period.

4. Exploratory approach to ruminant endometrial physiology study

Oestrous cycle and early pregnancy are associated to a tremendous alteration of endometrial gene expression. In the last decade, new approaches had been developed to unveil comprehensive genome-wide expression profile throughout the oestrous cycle and the establishment of pregnancy in mammalian species and in ruminant particularly. Various analytical approaches have been developed to profile transcriptomes and the most powerful technologies are microarrays or RNA-sequencing studies (Bauersachs et al. 2008; Bauersachs and Wolf 2012).

In ruminant, high-throughput studies had been carried out using endometrial samples across the oestrous cycle and the early pregnancy.

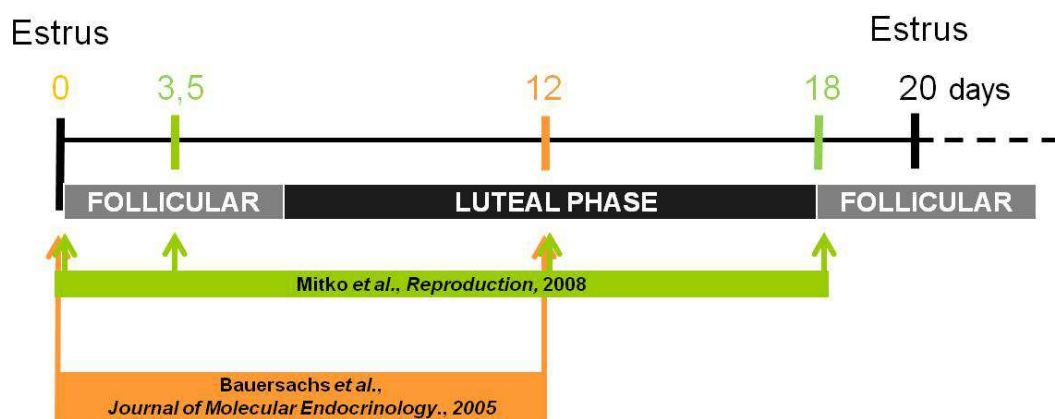


Figure 22: Compilation of microarray studies based in cyclic ruminant endometrium (adapted from O. Sandra).

Bovine endometrial transcriptome had been carried out throughout the oestrous cycle (Fig. 22) at different stage namely oestrus (day 0), metoestrus (day 3.5), dioestrus (day 12 and day 18 as late dioestrus with high circulating level of P4) and proestrus (day 18 associated with a very low circulating level of P4), in German Fleckvieh breed (Bauersachs et al. 2005) and Simmental heifers breed (Mitko et al. 2008). Both high-throughput analyses unveiled the significant differential expression of 133 endometrial genes for Bauersachs *et al*, and 269 endometrial genes for Mitko *et al*, during the oestrous cycle in distinct temporal patterns between all the oestrous stages. Both studies exhibit similar endometrial biological functions expression profile. At oestrus step, differentially expressed genes (DEG) are mostly involved in extracellular matrix remodelling, ions and nutrients transport, cell growth and morphogenesis. Conversely, at dioestrus, DEG are involved in others processes as immune response, angiogenesis, regulation of invasive growth, cell adhesion or embryo feeding (Bauersachs et al. 2005; Mitko et al. 2008).

The impact of the conceptus was also analysed by endometrial transcriptome (Fig. 23). Microarray studies take place at different pregnancy stage including the early conceptus elongation phase, the pregnancy maternal recognition period, and implantation providing the effect of gestational P4, of IFNT, of lactation (Bauersachs et al. 2006; Gray et al. 2006; Klein et al. 2006; Forde et al. 2009; Bauersachs et al. 2011; Forde et al. 2011; Cerri et al. 2012) and others conceptus secretion, on ruminant endometrial gene expressions distributed between CAR and ICAR areas (Mansouri-Attia et al. 2009; Walker et al. 2010).

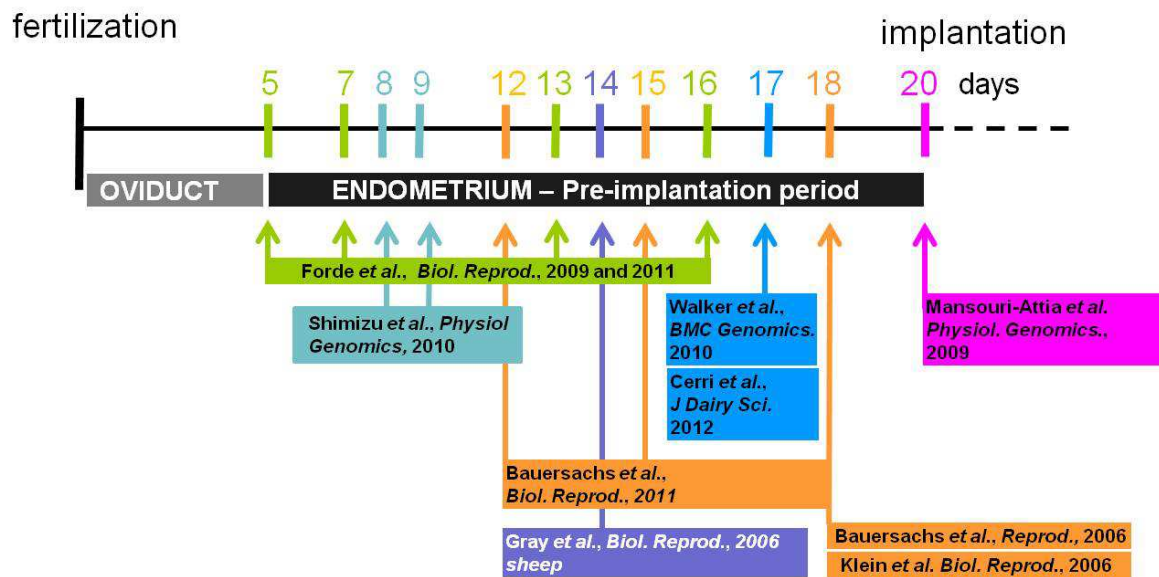


Figure 23: Compilation of nine microarray or RNA-sequencing studies based on bovine endometrium and one microarray study based on ovine endometrium (adapted from O. Sandra).

Each study exhibit a biological function associated to the DEG identified throughout all pregnancy conditions as uterine support of peri-implantation conceptus survival, growth and implantation (Gray et al. 2006; Klein et al. 2006; Mansouri-Attia et al. 2009) associated with the regulation of immune response and promoting the maternal tolerance (Bauersachs et al. 2006; Klein et al. 2006; Walker et al. 2010; Bauersachs et al. 2011; Cerri et al. 2012) but also favouring the transport or triglycerides and glucose as the energy source for the developing embryo (Forde et al. 2009; Forde et al. 2011; Cerri et al. 2012).

Endometrium of ovariectomized cows associated using the physiological supplementation with the ovarian steroid (E2 or P4) had been subjected to transcriptome analysis (Fig. 23; Shimizu et al. 2010). This microarray analysis exhibit several biological functions E2-responsive as cell cycle, morphogenesis and differentiation processes. The profile of P4-responsive genes is associated to a fertility issue such as luteinization, oocyte maturation, and catecholamine metabolism (Shimizu et al. 2010).

These exploratory analyses highlight the expression of numerous gene involved in uterine receptivity, establishment and maintenance of pregnancy and provide new possibilities in understanding the regulation of endometrial genes throughout the oestrous cycle and the early pregnancy.

In particular, compilation and cross-check of all those data exhibit the endometrial differential expression of members of several families of transcription factors as the SP1 family, the NOBOX family, the HOX family, the steroid nuclear receptor and the FOX family.

B. Forkhead Box (FOX) Transcription Factor in female reproductive physiology

Transcription factors have a pivotal role in eukaryotic cells regulating thousands of target genes. Natural mutation or knock-out of transcription factors often lead to early embryonic death due to serious defects in cell division or organogenesis. In addition, conditional deletion of transcription factors induces specific organ defects. In particular, transcription factor constitutes nodes in cellular networks as a molecular integrator of extracellular signals.

The microarray or RNA sequencing analysis unveil a non-exhaustive list of differential expressed FOX factors under several conditions of oestrous cycle and early pregnancy in ruminant endometrium including FOXA1; FOXA2; FOXA3; FOXC1; FOXF2; FOXI1; FOXL2; FOXO1A; FOXO4; FXP1; FOXQ1 and FOXS1 (Bauersachs et al. 2006; Gray et al. 2006; Klein et al. 2006; Mitko et al. 2008; Forde et al. 2009; Shimizu et al. 2010; Walker et al. 2010; Bauersachs et al. 2011; Forde et al. 2011; Cerri et al. 2012).

1. Background of FOX transcription factor

a) Discovery and nomenclature

Forkhead box proteins are transcription factors that belong to basal transcriptional machinery organized around RNA polymerases and also specific transcription factors in response to various biological signals (Carlsson and Mahlapuu 2002; Hannenhalli and Kaestner 2009; Benayoun et al. 2011).

First, FOX factors had been discovered at the end of the 1980's with the genes responsible of the *fork head* phenotype in *Drosophila Melanogaster*. The gene product had a homeotic activity, notably by promoting the development of terminal segments (Weigel et al. 1989).

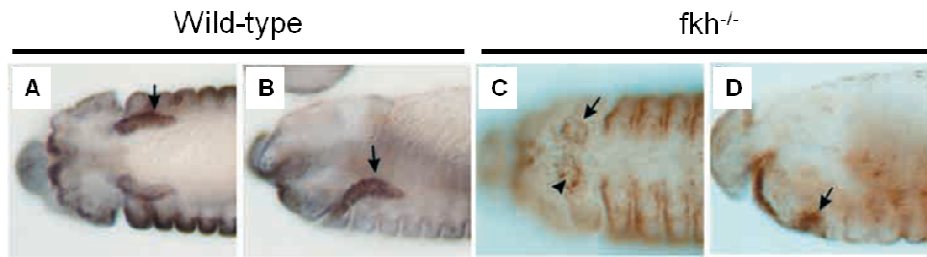


Figure 24: Salivary secretory cells in wild-type and *fkh* mutant fruit fly embryos from Myat and Andrew 2000. (A, B) Wild-type embryos have elongated salivary secretory tubes, which lie along the anterior-posterior axis (arrows). At the same stage, two rings of weakly staining (arrows) are observed near the ventral surface of *fkh* mutant embryos.

In drosophila, *fkh* gene prevents apoptosis supporting secretory cells survival as well as the secretory cell invagination of the salivary glands (Fig. 24; Myat and Andrew 2000).

Later, the cDNA encoding hepatocyte nuclear factor 3 α (*HNF3 α* , *FOXA1*) was cloned in the rat. Both rat and drosophila genes did not present any similarity with the DNA-binding motifs of transcription factors that were known at the time. However, new HNF/FOX factors were cloned whose DNA-binding domains displayed a very high identity with those of *D. Melanogaster* (Kaestner et al. 2000; Lehmann et al. 2003). The DNA-binding site also called Forkhead (FKH) domain is compound of three α -helices, three β -sheets and two “wing” regions that flank the third β -sheet (Fig. 25).

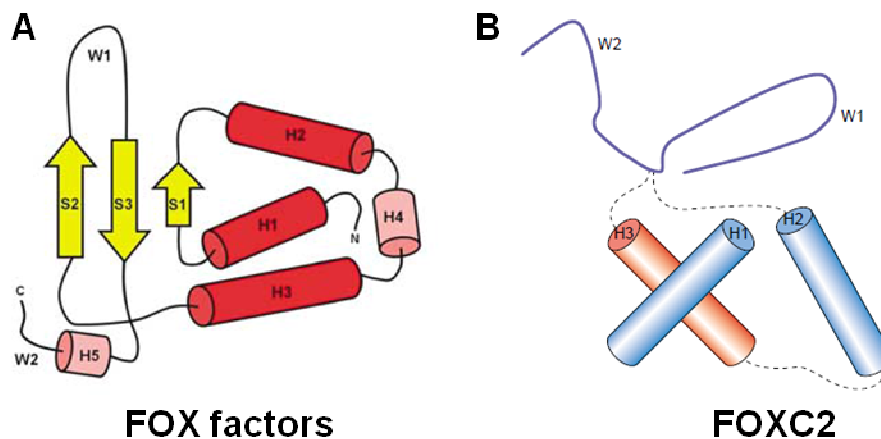


Figure 25: Forkhead domain structure of FOX factors: the example of FOXC2 (adapted from Lehmann et al. 2003; Obsil and Obsilova 2008). (A) Topology of the Forkhead domain. Helices H4 and H5 are present only in certain Forkhead domains. (B) For instance, FOXC2 exhibits only three helices (H1, H2 and H3), three β -sheets (S1, S2 and S3) and two wing-like loops.

Based on the butterfly-like winged structure adopted by the DNA-bound FOX proteins, the FKH domain has also called the winged-helix domain (Degnan et al. 2009; Hannehalli and Kaestner 2009; Benayoun et al. 2011).

Since *fhx* cloning in *D.melanogaster*, hundreds of FOX factors have been discovered in numerous species. At the beginning of 2000's, a FOX classification had been done: based on similarities, FOX proteins were divided into 19 classes from FoxA to FoxS (Hannenhalli and Kaestner 2009).

b) Evolution

The family of FOX proteins encompasses over 2000 members identified in 108 species of fungi and animals sharing not the same number of genes in all species. For instance, *Aspergillus Flavus* fungus has only one gene and *Saccharomyces Cerevisiae* has four. In metazoan, the situation is the same; *Caenorhabditis elegans* genome contains 16 Forkhead genes, 18 in *D.melanogaster*, 49 in the zebra-fish and 50 in humans. The genomic distribution of FOX factor is not random and organized in clusters that reflect the evolutionary history of vertebrate genomes. Recently, two phylogenetically distinct groups of FOX genes have been identified: the first one regrouping the common ancestor of metazoans and Fungi and the second one emerged as a metazoan specific-class (Degnan et al. 2009; Shimeld et al. 2010).

Despite the similarity in their DNA-binding domains, various FOX proteins have evolved distinctly, specifically when duplication events happen (gene duplication can result from unequal crossing over, retroposition or chromosomal (or genome) duplication, Zhang 2003).

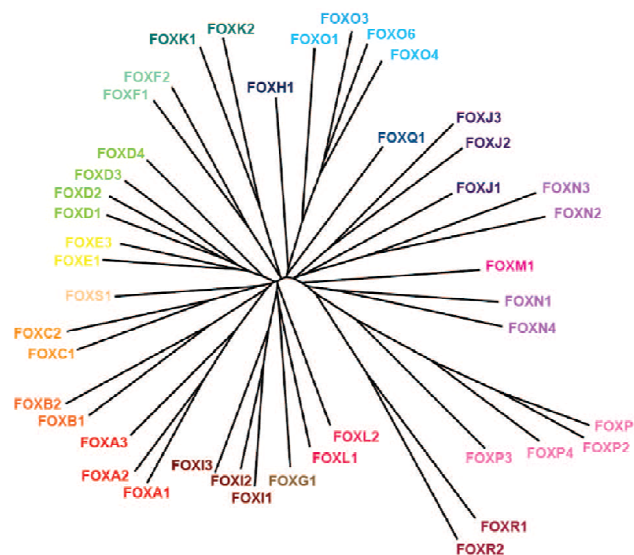


Figure 26: Phylogenetic tree of all known human FOX factors based on similarity of protein sequence (from Uhlenhaut and Treier 2011).

Indeed, newly created members often evolve and acquire distinct functions (Fig. 26). However, the functional diversity among FOX proteins is first dependant on the interaction with partners, i.e. enzymes or co-factors, and secondly dependant on the spatio-temporal expression of FOX genes (Shimeld et al. 2010; Benayoun et al. 2011).

c) Biological functions

Despite the differences between the members of the FOX family, all Forkhead transcription factors are involved in numerous development processes during fetal life but also in the adult organism. More generally, FOX factors act as terminal effectors of many major signal transduction pathways including TGF- β cascade, MAPK pathway, sonic hedgehog (Shh) pathway or Wnt/ β catenine pathway. So, FOX factors act as a molecular integrator of extracellular signals and they might constitute nodes in cellular networks (Hannenhalli and Kaestner 2009; Nef and Vassalli 2009; Horn et al. 2010; Jackson et al. 2010; Benayoun et al. 2011; Feuerborn et al. 2011; Uhlenhaut and Treier 2011; Katoh et al. 2013; Friedman and Kaestner 2006).

Specifically, the FOX factor functions are frequently associated with human genetic diseases (Fig. 27) as mutation of *FOXC1*, *FOXC2*, *FOXE3* and *FOXL2* leading to developmental abnormalities of the ocular region (Benayoun et al. 2011); *FOXP3* and *FOXP1* leading to severe immune defects (Hannenhalli and Kaestner 2009; Katoh et al. 2013); *FOXO3a* and *FOXL2* leading to premature ovarian failure (Uhlenhaut and Treier 2011); and finally mutations of *FOXG1*, *FOXP1* and *FOXP2* lead to mental retardation, autism and speech disorders (Horn et al. 2010).

Human gene	Associated mutation-induced phenotype(s) in humans	OMIM	Orthologous gene knockout/in phenotype in mouse model
<i>FOXC1</i>	Iridogoniodysgenesis type 1 (glaucoma and iris hypoplasia); Axenfeld-Rieger syndrome type 3	601090	Perinatal lethality; numerous developmental anomalies, notably in the eye region
<i>FOXC2</i>	Lymphedema-distichiasis syndrome (lymphoedema of the limbs, double rows of eyelashes and ptosis)	602402	Pre- and perinatal lethality; skeletal and cardiovascular defects; and numerous developmental anomalies, notably in the eye region
<i>FOXE1</i>	Bamforth-Lazarus syndrome (hypothyroidism, spiky hair, cleft palate and choanal atresia); cleft lip and palate	602617	Lethality within 48 hours of birth; cleft palate; and abnormal development of thyroid gland
<i>FOXE3</i>	Primary congenital aphakia; anterior segment mesenchymal dysgenesis (with Peters anomaly)	601094	Viable and fertile; severe cataract and degeneration of lens epithelium
<i>FOXG1</i>	Congenital variant of Rett syndrome; mental retardation, cerebral malformations and microcephaly	164874	Perinatal lethality; severe reduction of the size of cerebral hemispheres and abnormal telencephalon development
<i>FOXL2</i>	BPES and telecanthus; premature ovarian failure	605597	High perinatal lethality; craniofacial abnormalities, with eyelid hypoplasia; anomalies in the development and differentiation of the ovary; females can be either sub- or infertile; partial to total ovary to testis transdifferentiation
<i>FOXN1</i>	T-cell immunodeficiency, congenital alopecia and nail dystrophy	600838	Alopecia and T-cell immunodeficiency, owing to athymia
<i>FOXO3A</i>	Premature ovarian failure	602681	Viable; minor defects in glucose uptake; overproliferation of helper T-cells; age-dependent female infertility owing to premature activation and depletion of ovarian follicles
<i>FOXP1</i>	Mental retardation, with language impairment and autistic features	605515	Early lethality; defects in immune and cardiovascular systems and movement; abnormal development of lungs and oesophagus
<i>FOXP2</i>	Mental retardation, with language impairment and autistic features	605317	Early lethality; motor problems; absence of ultrasonic vocalizations in the young
<i>FOXP3</i>	Immunodysregulation, polyendocrinopathy and enteropathy, x-linked (IPEX)	300292	Overproliferation of CD4 ⁺ CD8 ⁻ T-cells; infiltrations in various organs; increase in levels of various cytokines

Figure 27: Human genetic diseases associated with mutations in FOX genes and corresponding phenotypes of the invalid murine orthologs (Benayoun et al. 2011).

More generally, misregulations (mutations or deregulation) of FOX proteins lead to a process of malignant transformation with stimulation/inhibition of cellular differentiation and proliferation, and apoptosis in adulthood. For instance, overexpressions of *FOXC1* and *FOXC2* detected in metastatic breast cancer confer increased invasive properties and metastatic potential of tumour cells. Similarly, decreased expression of *FOXN3*, by gene

deletion, in mouth, larynx and hepatic carcinoma induce tumour suppressing process (Jackson et al. 2010; Benayoun et al. 2011; Katoh et al. 2013).

FOX factors are associated with ageing process. Indeed, the misregulation of those transcription factors leading to the deregulation of apoptosis process then cancer suggesting the modulation of ageing is tightly balanced by FOX factors. For instance, FoxO factors of *C.elegans* and *D.melanogaster* act as crucial regulators of longevity and stress response. Similarly in mammalian species, Foxo1 and Foxo3a are also involved in longevity regulation (Benayoun et al. 2011; Ghazi 2013). In addition, FoxA was recently shown to have a key role in lifespan expansion in the nematode (Panowski et al. 2007), and in mammals. *FOXM1* deficiencies lead to appearance of early-ageing phenotypes while *Foxm1* overexpression rescues age-associated degeneration of hepatic tissues in older mice (Katoh et al. 2013).

FOX factors are also associated with specific organ ageing as the decreased expression of *FOXO3a* and *FOXL2* in mice ovary leads to ovarian ageing or premature ovarian failure due to an accelerated depletion of their follicular pool (Benayoun et al. 2011; Uhlenhaut and Treier 2011).

The involvement of FOX factors in many signalling pathways and in human disease reflects the complexity and the importance of this family in physiology and pathology. Reproduction as the most critical biological function undergoes intense selective pressure to prevent any modifications which could directly affect the organism's fitness (Lode 2012). Thus, the involvement of FOX factors in reproductive physiology appears crucial to elucidate. FOX factors have been examined for years in female reproductive tract, particularly, and appeared to be strongly linked to fertility issues.

2. FOX factors in female reproduction

Human genetic diseases, natural or conditional mutations in animal model as well as high-throughput analyses allowed a better understanding of the crucial role of FOX factors in female reproductive tract (Benayoun et al. 2011). To date, FOX factors have been investigated mostly in ovary and mammary glands but less in the endometrium. Ovarian Infertility prevents any pregnancies due to POF or POI then endometrium is not studied. Nevertheless, ovaries work in concert with endometrium to ensure a healthy pregnancy suggesting a FOX factor which is involved in ovarian physiology could be involved in endometrial physiology as well.

Ruminant microarray and RNA sequencing studies unveil a list of differentially expressed FOX factors in endometrium. Among this list, many factors had been already described in female reproduction (Bauersachs et al. 2005; Bauersachs et al. 2006; Gray et al. 2006; Klein et al. 2006; Mitko et al. 2008; Forde et al. 2009; Mansouri-Attia et al. 2009; Shimizu et al.

2010; Walker et al. 2010; Bauersachs et al. 2011; Forde et al. 2011; Cerri et al. 2012). For instance, *FOXC1*, *FOXA1* and *FOXP1* have been investigated in mammary gland, *FOXC1* and *FOXO1* in ovary and *FOXA2* as well as *FOXO1A* in endometrium (Fig. 28).

Gene	Mammary gland	Ovary	Endometrium	Mouse Mutant Reproductive Phenotype	Human fertility disorder
<i>FOXA1</i>				ND	Breast Cancer
<i>FOXA2</i>				severely subfertile, disrupted blastocyst implantation	ND
<i>FOXC1</i>			?	infertile, no follicular growth beyond the early antral stage	ND
<i>FOXL2</i>	?			infertile, POF	BPES
<i>FOXO1</i>				ND	ND
<i>FOXO3</i>				Suppression of primordial follicle activation impaired, POF	?
<i>FOXP1</i>				ND	BLBC

Figure 28: Forkhead family members currently known to play a role in female reproductive tract (from Mansouri-Attia et al. 2009; Jeong et al. 2010; Pannetier and Pailhoux 2010; Benayoun et al. 2011; Uhlenhaut and Treier 2011). POF: premature ovarian failure, BPES: Blepharophimosis-Ptosis-Epicanthus-Inversus Syndrome, BLBC: basal-like breast cancer.

a) Mammary gland and breast cancer

Forkhead box transcription factors are often linked in breast cancer with ambivalent actions as oncogene or tumour suppressor (Benayoun et al. 2011).

FOXC1 was originally identified as an important transcription factor that controls development of structures derived from the neural crest and *FOXC1* mutations have long been recognized as a primary cause of Axenfeld-Rieger syndrome, a rare dominant autosomal disorder, which encompasses a range of congenital malformations affecting the anterior segment of the eye (Tumer and Bach-Holm 2009). In addition to its roles in normal function and development of the eye and meninges, *FOXC1* has recently emerged as a possible master regulator of Basal-Like-Breast Cancer (BLBC, Wang et al. 2012).

FOXA1 was first described for its crucial role in controlling pancreatic and renal function required for postnatal survival (Katoh et al. 2013). In addition, *FOXA1* gene is necessary for the normal mammary gland development promoting morphogenesis of ducts and inhibiting the final differentiation of alveoli (Bernardo and Keri 2012). Moreover, *FOXA1* is also involved in breast cancer development associated with good prognosis, regulating the expression of estrogen nuclear receptor (ESR) and androgen nuclear receptor (AR), and could control plasticity between basal and luminal breast cancer repressing basal phenotype and aggressiveness (Bernardo et al. 2013; Katoh et al. 2013; Friedman and Kaestner 2006).

FOXP1 has a pleiotropic expression and has been shown to have a role in cardiac, lung and lymphocyte development. Surprisingly, a loss of *FOXP1* expression in breast cancer is associated with a worse outcome, suggesting *FOXP1* may function as a tumour suppressor in mammary gland (Uhlenhaut and Treier 2011; Katoh et al. 2013).

b) Ovary

In addition to hormones and their receptor as well as signalling molecules, FOX factors as transcription factors have emerged as powerful regulator of ovarian function (Uhlenhaut and Treier 2011). The loss of FOX factors is damaging female fertility in ovary particularly.

Foxc1 null mice exhibit also an impaired migration of the primordial germ cells (PGC) to the gonadal anlage (Mattiske et al. 2006). In the absence of *Foxc1*, the PGCs are trapped in the hindgut and the developing gonad is smaller and disorganize. In addition, when *Foxc1* mutant ovaries were transplanted into wild-type adult mice, the follicles do not develop beyond the pre-antral stage (Mattiske et al. 2006). In addition to its crucial implication in Axenfeld-Rieger syndrome, *FOXC1* may be required for maturation of follicles beyond the early antral stage (Uhlenhaut and Treier 2011). Nevertheless, its potential target genes and regulatory mechanism in ovarian function remained to be investigated.

FOXO factors were first described in *C. elegans* because of their implication in the determination of lifespan as well as the regulation of insulin signalling (Hannenhalli and Kaestner 2009; Hedrick 2009). In addition, FOXOs are also involved in tumour suppression regulating genes linked to the cell cycle inhibition, stress response and pro-apoptotic genes. FOXO family is composed of four members namely FOXO1, FOXO3 and FOXO4 which are ubiquitously expressed whereas FOXO6 is restricted to the brain (Hannenhalli and Kaestner 2009; Hedrick 2009).

Foxo1 deletion leads to an early embryonic death in mice which died at day 10.5 of pregnancy (E10.5) due to severe vascular defects making the study of reproductive phenotype impossible. However, *Foxo1* is known to be regulated by LH and FSH in granulosa cells leading the biosynthesis of lipid and sterol. These data suggest that *Foxo1* may be involved in follicular steroidogenesis (Hosaka et al. 2004; Uhlenhaut and Treier 2011).

In *Foxo3* null mice, primordial follicles are assembled normally but then immediately undergo global activation, resulting in early depletion of the entire primordial follicle pool. Mice could have a normal size litter but quickly reach menopause (Castrillon et al. 2003; Uhlenhaut and Treier 2011). These data suggest FOXO3 is involved in the suppression of follicular growth. On the other side, constitutive activation of FOXO3 in oocytes induces also an ovarian

sterility associated with a constant inhibition of follicular growth preventing the ovulation (Uhlenhaut and Treier 2011).

FOXL2 is a key gene in ovarian differentiation and maintains the ovarian function from foetal life to adulthood (Pannetier and Pailhoux 2010). This part will be discussed in more detail later.

c) Endometrium

As previously describes above, FOX factors are strongly linked to the female reproductive physiology and especially regulates the development and the differentiation of endometrial cells including glandular epithelial cells (Filant et al. 2012).

FOXA2 is a member of the FOXA subfamily such as *FOXA1* and *FOXA3*. This transcription factor is well-known in mice endometrium because of the uniqueness localization in endometrial gland. First, the non-conditional knock-out of *Foxa2* leads to foetal death at E10-11 due to severe defects in node, notochord, neural tube and gut tube formation. The *Foxa2* conditional knout-out in the murine uterus leads to a reduced fertility, disrupting blastocyst implantation associated with severe decreased of endometrial gland number (Bazer 2010; Jeong et al. 2010; Filant et al. 2012; Filant et al. 2013). In addition, ovarian steroid treatments on ovariectomized mice lead to an inhibition of *Foxa2* transcript due to the E2 but not P4 supplementation. In the murine uterus, *Foxa2* is involved in endometrial gland development, crucial step upon the control of ovarian steroids, which is necessary to decidualisation process. Moreover, an alternative approach was setting up to define the FOXA2 cistrome using Chromatin Immuno-Precipitation following by sequencing experiment (ChIP-seq) unveiling more than eight thousands FOXA2 target genes in mice endometrial glands (Filant et al. 2013). In bovine ovariectomized cows supplemented with ovarian steroids (Shimizu et al. 2010), *FOXA2* is differentially expressed in the microarray study mostly due to E2 treatment. This result suggests a similar regulation of endometrial *FOXA2* expression by ovarian steroids between murine and bovine species. Collectively, these data provide strong arguments of the implication of *FOXA2* gene in mammalian endometrial development and physiology (Bazer 2010; Friedman and Kaestner 2006).

FOXO1A is expressed in human and baboon endometrium and up-regulated during the luteal phase of the menstrual cycle and during the early pregnancy, under P4 control (Buzzio et al. 2006). In addition, *Foxo1a* has been shown to act as a master switch in controlling apoptosis and appears to be one of the earliest genes induced in decidualisation process (Buzzio et al. 2006; Takano et al. 2007; Kohan et al. 2010). This induction of *Foxo1a* gene

expression leads to a regulation of numerous downstream genes also involved in decidualisation. For instance, FOXO1A and others specific transcription factor were shown to control promoter activities of decidual prolactin (PRL), IGF-binding protein-1 (IGFBP1), cannabinoid receptor 1 (CNR1), tissue metalloproteinase inhibitor-3 (TIMP3) and decorin (DNC) in a differentiation-dependent manner leading to the initiation of decidualisation (Buzzio et al. 2006).

Collectively, knock-out mice models, human studies or microarrays provide strong arguments that FOX factors are master regulators of female reproductive physiology with a serious lack of information in endometrium (Kohan et al. 2010). Regarding FOXL2, numerous studies show the fundamental role of this transcription factors in pituitary and ovarian physiology with a complete lack of information about its putative implication in endometrial physiology. Nevertheless, FOXL2 appears also expressed for the first time, in endometrium (Mansouri-Attia et al. 2009).

3. FOXL2 and female reproductive physiology

a) Discovery

FOXL2 was first cloned in 2001 as a FOX factor involved in Blepharo-phimosis-Ptois-Epicanthus inversus (BPES) syndrome. Type I BPES is a rare developmental disorder of the eyelid and ovary essentially presenting with an autosomal dominant inheritance. Type II BPES show only eyelid defects but both types map to the chromosome 3q23. Crisponi and colleagues provide the evidence that it is involved in this genetic disease with various known and unknown mutations (Crisponi et al. 2001).

b) Structure

FOXL2 is a single-exon gene of 2.7 kb encoding a highly conserved protein of 376 amino acids (376 in human, 375 in mice and 377 in cattle). As a member of the FOX factors, FOXL2 protein has a Forkhead DNA-binding domain of 110 amino acids (Fig. 29). The sequence and properties of FOXL2 forkhead domain are highly conserved, in eutherian mammals, which is a general characteristic of Forkhead transcription factors (Carlsson and Mahlapuu 2002; Cocquet et al. 2003).

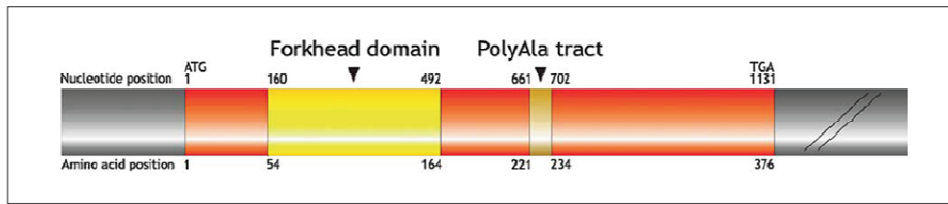


Figure 29: Schematic outline of the human FOXL2 gene and protein. FOXL2 encodes a protein of 376 amino acids (from Verdin and De Baere 2012). The characteristic Forkhead domain and poly-alanine tract are indicated by arrowheads.

Moreover, its protein sequence is made of a PolyAlanine tract (PolyAla) of 14 residues which is strictly conserved in human, goat, cow, mouse, rat and rabbit species and absent in pufferfish, zebrafish and tetraodon species (Fig .29 and Appendix A, Fig. 66). In addition, PolyAla expansions of FOXL2 are pathogenic and represent 30% of all FOXL2 mutations leading to type II BPES inducing a length-dependent loss of response of different target promoters from 14 to 24 alanine (Moumne et al. 2008). A comparison between mammalian and non-mammalian species of protein sequences exhibits FOXL2 amino acids sequence are highly conserved, particularly in C-terminus. Indeed, N-terminus part is less conserved than C-terminus part between human, cow, goat, mouse, tammar wallaby, rabbit, pufferfish, zebrafish and tetraodon species suggesting that N-terminus might be responsible for functional differences among species (Cocquet et al. 2002; Appendix A, Fig. 66).

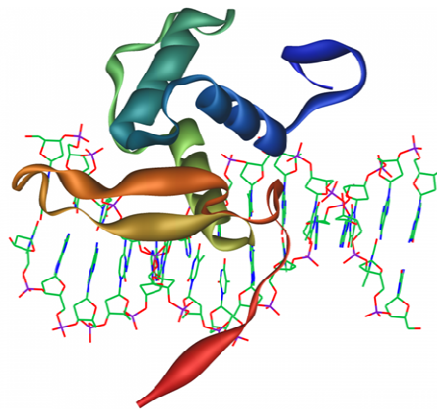


Figure 30: Three dimensional structure of FOXL2 protein (from Benayoun and Veitia 2011). FOXL2 protein exhibit the same classical protein structure of the Forkhead box transcriptions factors namely: three α -helices, three β -sheets and two "wing" regions that flank the third β -sheet.

Foxl2 proteins are more divergent outside of their DNA-binding domain, though a high degree of conservation is still observed even if the three dimensional structure is not completely elucidated (Fig. 30; Benayoun and Veitia 2011).

In mice and rat, two *FOXL2* transcripts had been discovered that would result in alternative polyadenylation without any change in amino-acids sequence length (Cocquet et al. 2003).

c) BPES, Polled Intersex Syndrome and Granulosa Cell Tumour: a FOXL2 story

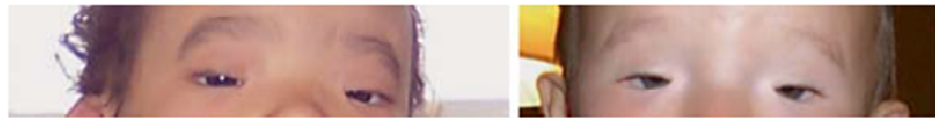
As previously described above, type I and II BPES are part of the same human genetic disease leading to cranio-facial malformations in the eyelid (Type I and II, Fig. 31) associated with a premature ovarian failure (POF, only type II) uniquely caused by *FOXL2* mutations. To date, some genetic defects have been identified including mutations and deletions in the coding sequence, but also deletions located outside the transcription unit (Crisponi et al. 2001; Cocquet et al. 2002; Benayoun et al. 2011).

More specifically, BPES is caused mainly by *FOXL2* intragenic mutations (71% in type I and 11% in type II) and microdeletion upstream or downstream of *FOXL2* promoter (4% in both types, Beysen et al. 2008). Moreover, mutations in the N-terminus side of the protein lead to a truncated protein and a loss of function inducing eyelid malformation and ovarian failure. Extensions by gene duplication are mostly involved in type II BPES. Finally, some genetic rearrangement occurred several kilobases upstream of the gene itself (cis-regulatory elements, Beysen et al. 2008). Similarly, knock-out of *FOXL2* exhibits the same phenotype namely eyelid malformation (Fig. 31) and POF (Uda et al. 2004).

In goat, a natural mutation leads to *FOXL2* invalidation resulting in an absence of horn (polled phenotype, Fig. 31) associated with intersexuality (Pailhoux et al. 2001). The sex-reversal affects only XX gonads in a recessive manner whereas the polled phenotype is dominant in male and female. The heterozygous deletion exhibits the polled phenotype while the homozygous invalidation leads to an association of polled phenotype and an early sex-reversal of the XX gonads presenting an "ovo-testis" gonad (Pailhoux et al. 2001). Altogether, the phenotypes belong to the Polled Intersex Syndrome (PIS) caused by an 11.7 kb DNA deletion involved in tissue-specific regulatory activity. This important deletion leads to the inactivation of 3 transcripts: *PISRT1*, *PFOXIC* and *FOXL2* which is the only transcript encoding for a protein. *PISRT1* and *FOXL2* shared a common transcriptional regulatory region called PIS. *PFOXIC* which is the inverse complementary *FOXL2* promoter is located near *FOXL2* in the opposite orientation. *PISRT1* and *PFOXIC* are two non-coding RNA crucial for *FOXL2* expression and regulation (Pailhoux et al. 2001; Pannetier et al. 2005; Boulanger et al. 2008).

Type I and II BPES

Human



FOXL2 ^{+/+}

FOXL2 ^{-/-}

Mouse



XX PIS ^{+/+}

XX PIS ^{-/-}

Goat



Figure 31: External phenotype of FOXL2 mutation in human, mice and goat (from Pailhoux et al. 2001; Uda et al. 2004; Verdin and De Baere 2012). Both human and mice exhibit the same phenotype caused by FOXL2 mutation/deletion namely POF and eyelid defects whereas goat show a polled phenotype (absence of horn) associated with the XX sex-reversal.

FOXL2 is also involved in Granulosa Cells Tumour (GCT) which represents 5-to-10% of all ovarian cancer. Some mutations of FOXL2 lead to a lack of functional protein leading to an aggressive pattern of progression and may be a factor regulating unregulated granulosa cells proliferation and the apoptosis process (Benayoun et al. 2011; D'Angelo et al. 2011).

Human and caprine mutations of FOXL2 leads to two different phenotypes: a premature ovarian failure and a sex-reversal of the XX gonads respectively. Collectively, those results indicate that *FOXL2* is directly involved in ovarian differentiation and maintenance in species specific manner (Pisarska et al. 2011).

In addition, *FOXL2* has been investigated in rainbow trout and tilapia species confirming *FOXL2* is highly conserved in vertebrates regarding its sequence and its expression in ovary (Baron et al. 2005).



Figure 32: FOXL2 evolutionary event in the classical phylogeny of vertebrates (Geraldo et al. 2013)

Altogether, FOXL2 appears to be expressed in the gonad of a large range of animal species (Fig. 32) and in addition of its expression in ovary, has a cell expression restricted to fetal eyelid and anterior pituitary (Baron et al. 2005; Ellsworth et al. 2006; Uhlenhaut and Treier 2006; Duffin et al. 2009; Geraldo et al. 2013).

d) Fetal eyelid

In all mammals, eyelids start to form as ridges around the margins of the developing eye and undergo a sequence of development, closure and subsequent reopening with a strong Foxl2 expression throughout that period. Expression is high around the mouse cornea, in the protruding ridge from which both eyelids are destined to develop leading to the dysplasia of both eyelids, observed in BPES patients and FOXL2^{-/-} mice (Fig. 33; Uda et al. 2004).

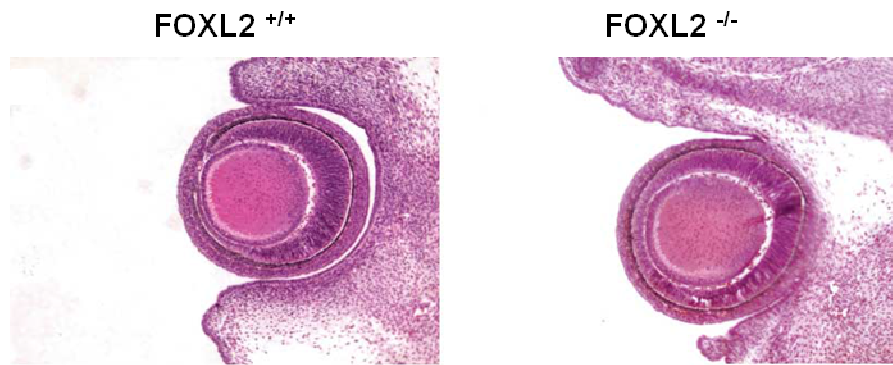


Figure 33: Hematoxylin/eosin staining of eyelid defects at E13.5 in wild-type and *FOXL2*^{-/-} (from Uda et al. 2004). On the right picture, eyelid defects are visible and show an overt eyelid hypoplasia in mice.

Foxl2 is also expressed in the perioptic mesenchyme around the developing mouse eye (Fig. 33). Both male and female suffering from BPES syndrome show typical facies with characteristic eyelid dysplasia, namely small palpebral fissures (blepharophimosis), drooping eyelids (ptosis) and a tiny skin fold running inward and upward from the lower lid (epicanthus inversus; Crisponi et al. 2001; De Baere et al. 2003).

e) Anterior pituitary

Foxl2 is expressed during early pituitary development and is one of the earliest markers of differentiating pituitary cells in mice. *Foxl2* is expressed ventrally in the pituitary with quiescent differentiating cells. Regulating downstream target in pituitary, *Foxl2* can modulate gonadotrope function and reproduction as well as thyrotrope and thyroid function (Ellsworth et al. 2006). FSH and LH are heterodimeric glycoprotein composed of α -GSU and a unique β -subunit. Transcription of this β -subunit is the rate-limiting step for production of mature hormones. Regarding FSH, β -subunit (also called *Fshb*) transcription is regulated by an increased level of activin and P4. Recent study show the evidence that *Foxl2* is involved in the cooperative interaction between activin and P4 signaling pathways in pituitary gonadotrope cells requiring the binding of *Foxl2*, Smad and Pgr to their respective elements on the murine *Fshb* promoter and suggesting physical interaction of these transcription factors (Ghochani et al. 2012). Thus, *FOXL2* may control the regulation of gonadotropin production essential for mammalian fertility.

f) Ovarian differentiation and maintenance

Early ovarian development is not a passive process due to the absence of SRY gene as a default pathway because recent data have shown that two pathways are responsible of the repression of male-specific genes associated with an activation of female-specific genes cascade (Ottolenghi et al. 2007; Nef and Vassalli 2009). The differentiation of the bipotential gonad is possible due to *FOXL2* pathway and β -catenine/WNT/RSPO1 pathway. In addition to its expression in fetal eyelid and in anterior pituitary, *FOXL2* has appeared as a key factor

in ovarian differentiation, development and function (Uda et al. 2004; Nef and Vassalli 2009; Benayoun et al. 2011).

Intragenic and extragenic mutations of *FOXL2* human genes lead to BPES disease which mean eyelid defects as previously described above but especially ovarian defects by premature ovarian failure (POF). POF is defined by the onset of the menopause before the age of 40 years. Various mutations imply various phenotypes which are from primary amenorrhea to irregular menses followed by POF. In addition, mutant ovaries can have different appearance varying from normal ovary to streak gonads (Crisponi et al. 2001; Ottolenghi et al. 2005; Duffin et al. 2009).

Foxl2 homozygous mutant mice are born with open eyes and eyelid hypoplasia but above all with a severe female infertility (Fig. 34).

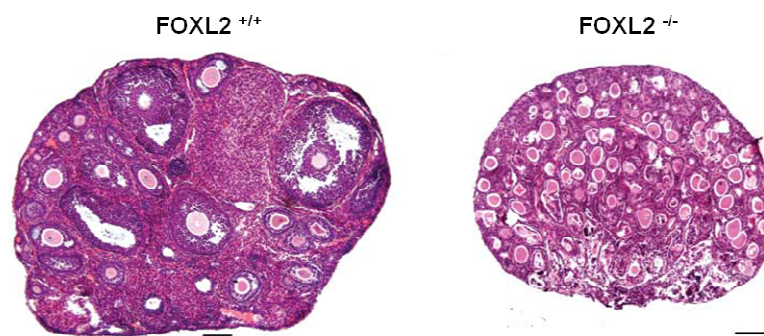


Figure 34: Morphological appearance of wild-type and *FOXL2*^{-/-} ovaries (from Uda et al. 2004). Knock-out mice show slower oocyte growth and somatic cells appear disorganized associated with reduced stroma.

Foxl2 is sexually dimorphic at E12.5 in female gonad but not in male gonad (Uda et al. 2004). Indeed, *Foxl2* is one of the earliest markers of ovarian differentiation in somatic granulosa and theca cells at every follicular stage. Homozygous mutations for *Foxl2* provide the evidence that it is required to granulosa cells differentiation and proliferation. In case of *Foxl2* mutation, granulosa cells do not undergo the squamous-to-cuboidal transition, preventing the follicular development beyond this point. Granulosa cells are supporting follicular development. When mature granulosa cells are absent, oocytes will undergo atresia. The *Foxl2* mutations lead to premature depletion of the primordial follicle pool, then oocytes atresia and finally infertility (Uda et al. 2004; Uhlenhaut and Treier 2006; Pisarska et al. 2011).

Foxl2 is expressed in less differentiated granulosa cells of small and medium follicles (Uhlenhaut and Treier 2006) whereas *Star* gene, Steroidogenic Acute Regulatory which is a direct target of *Foxl2* is expressed in granulosa cells of large pre-ovulatory follicle but not in small and medium immature follicle (Batista et al. 2007; Rosario et al. 2012). *Star* is

responsible of the translocation of cholesterol from the outer to the inner membranes of mitochondria which the rate-limiting step in steroidogenesis. *Foxl2* binds *Star* promoter and suppresses its activity suggesting that transcriptional repression by *FOXL2* prevents *Star* expression in immature follicle. In the same way, *Foxl2* suppresses the expression of *Cyp19* (P450 Aromatase) gene in immature mouse ovary follicle (Pannetier et al. 2006; Pisarska et al. 2011). Sex steroids seem to be important for initiating follicle growth. Collectively, those data suggest that *Foxl2* gene may function as a suppressor of ovarian follicle progression in small and medium follicles by the prevention of premature differentiation and proliferation of granulosa cells preventing the premature depletion of ovarian follicles.

The bipotential gonad is capable to differentiate into an ovary and into a testis depending on the absence and the presence of the Y chromosome respectively governing the sexual phenotype of the organism. The differentiation occurs later with the gender-specific hormone and signalling molecules. Testes and ovaries are composed of three common cell types with a similar function. Firstly, testes and ovaries are made up of germ cells; spermatocytes and oocytes, but also supporting cells; Sertoli cells and granulosa cells and finally steroidogenic cells; Leydig cells and theca cells, respectively (Fig. 35; Nef and Vassalli 2009; Pannetier and Pailhoux 2011).

In the presence of Y chromosome, *Sry* then *Sox9* gene are expressed and will activate the male-specific gene cascades. Conversely, in the absence of Y chromosome, *Foxl2* will suppress the expression of *Sox9* (Fig. 35).

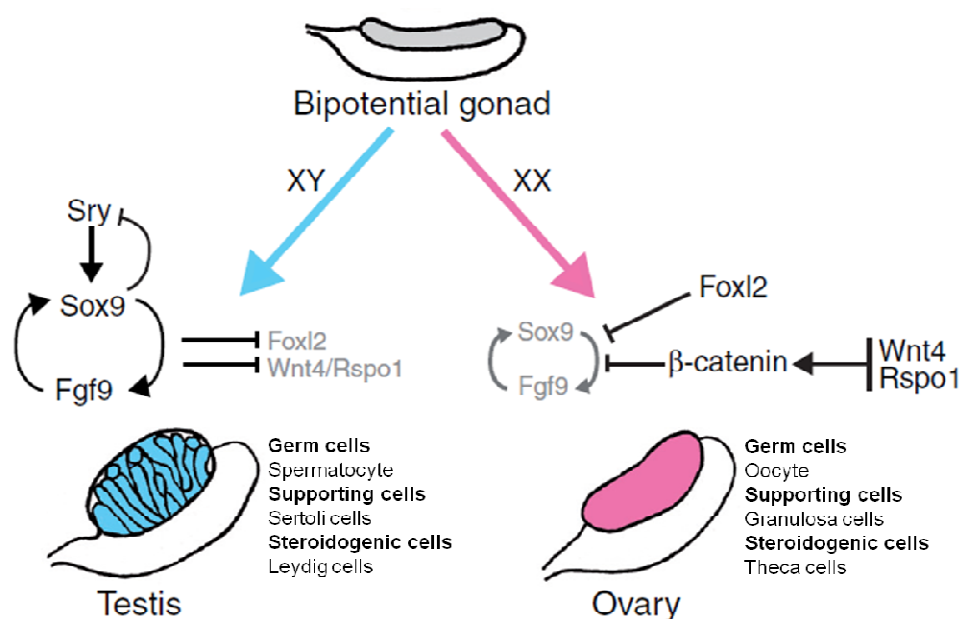


Figure 35: A genetic model for sex differentiation (adapted from Nef and Vassalli 2009). SOX9 or FOXL2 and Wnt/ β -catenin pathways will induce testis or ovary differentiation, respectively.

During the embryonic development, *Sox9* pathway inhibition is due to the *Wnt/β-catenin* pathway and later, in adulthood, the *Foxl2* pathway. Moreover, the invalidation of *Foxl2* in adult mice leads to a de-repression of *Sox9* pathway (Uhlenhaut et al. 2009). Subsequently, granulosa and theca cells trans-differentiate into Sertoli and Leydig cells, respectively, with a global activation of the male-specific genes and an inhibition of the female-specific genes, followed by the loss of oocytes and a production of testosterone (Uhlenhaut et al. 2009). Those results provide the evidence *Foxl2* maintains the ovarian function and differentiation in adulthood, continuously repressing the *Sox9* expression activating the expression of *Cyp19* to catalyze the production of E2. Both actions of *Foxl2* and E2 lead to a repression of *Sox9* expression in the ovary (Pannetier and Pailhoux 2011). In addition, *Foxl2* synergistically interacts with the *Esr1* and 2 to repress directly the gonad specific *cis*-regulatory element of *Sox9* known as a testis specific enhancer of *Sox9*, TESCO (Uhlenhaut et al. 2009). Similarly, the PIS mutation in goat leads to a loss of function of *FOXL2* and a female-to-male sex reversal, because of the de-repression of the male sex determination pathway (Pannetier et al. 2006).

Collectively, these results show the crucial role of *FOXL2* in ovarian physiology, especially during the ovarian differentiation as well as the maintenance of ovarian function in adult organism.

g) Post-translational modifications

Post-translational modifications represent an important mechanism to regulated protein functions. *FOXL2* undergoes several post-translational modifications that have been reported to modulate its activity, its sub-cellular localization and its binding partners (Pisarska et al. 2011).

Foxl2 is known to be sumoylated leading to regulation of its protein stability and intracellular localization. SUMO as Small Ubiquitin-related Modifier is made up of covalent binding of ubiquitin on protein at lysine-residue in a three step conjugation pathway similar to that involved in ubiquitination. *Foxl2* is sumoylated by Sumo1 mediated by Ubiquitin Conjugating Enzyme9 (Ubc9) enhancing *Foxl2* activity in repressing *Star* expression (Kuo et al. 2009; Marongiu et al. 2010; Georges et al. 2011).

Foxl2 is also phosphorylated by the serine/threonine kinase large tumour suppressor 1 (Lats1) enhancing *Foxl2* activity. In addition, the deletion of Last1 in the mouse induces a premature ovarian failure similar to the phenotype obtained by *Foxl2* deletion repressing its phosphorylation then its activity (Georges et al. 2013).

Oxidative stress and heat shock stimulate Foxl2 protein expression associated with a hyperacetylation. Oxidative stress induces an increase of target promoter recruitment as Manganese super-oxide dismutase gene (*Sod2*). *Sod2* has been demonstrated to be a direct target of the acetylated Foxl2 protein. Moreover, *Foxl2* expression is decreased by the NAD-dependent deacetylase sirtuin 1 (*Sirt1*) and *Foxl2* itself induces *Sirt1* transcription demonstrating the importance of acetylation as a regulator of Foxl2 activity and the existence of a feedback loop controlling Foxl2 activity in these cells (Benayoun et al. 2011; Pisarska et al. 2011; Georges et al. 2013).

h) FOXL2 target genes in the ovary

Considering the critical function played by Foxl2 in ovarian differentiation, target genes have been investigated using high-throughput analyses in human and murine models (Fig. 36). In the ovarian context, FOXL2 is known to be involved in the regulation of cholesterol and steroid hormone metabolism genes with *Star* and *Aromatase* genes (to regulate the aromatization of androgens into estrogens), but also, ovarian determination with *Sox9* and *Amh* genes; ovulation with *Cox2/Ptgs2* gene; stress response, ageing or apoptosis with *Sirt1*, *Sod2*, *Tnfaip3* and *Fos* genes. The regulation of the *aromatase* gene induces the production of E2 that act locally in the ovary and act on the hypothalamus regulating the GnRH secretion. Then, Foxl2 regulates the transcription of the α -*Gsu* gene in the pituitary, which encodes the common subunit of all pituitary glycoprotein hormones, namely the Thyroid Stimulating Hormone (TSH), and the gonadotropins LH and FSH, both hormone involved in the ovulation process (Batista et al. 2007; Uhlenhaut et al. 2009).

Depending on the available binding partners, Foxl2 likely had differential effects on a number of genes imperative for ovarian function and female fertility, as the differential regulation of *Ptgs2* ovarian expression (Batista et al. 2007; Moumne et al. 2008; Uhlenhaut et al. 2009; Benayoun et al. 2011). FOXL2 was shown either to stimulate the expression of *PTGS2* in KGN cell line (Batista et al. 2007) or to repress *PTGS2* expression (Kim et al. 2009) in 293FT cells (ER α negative human embryonic kidney) and MDA-MB-231 cells (ER α negative human breast cancer) as a consequence of the availability of its co-factors and binding partners.

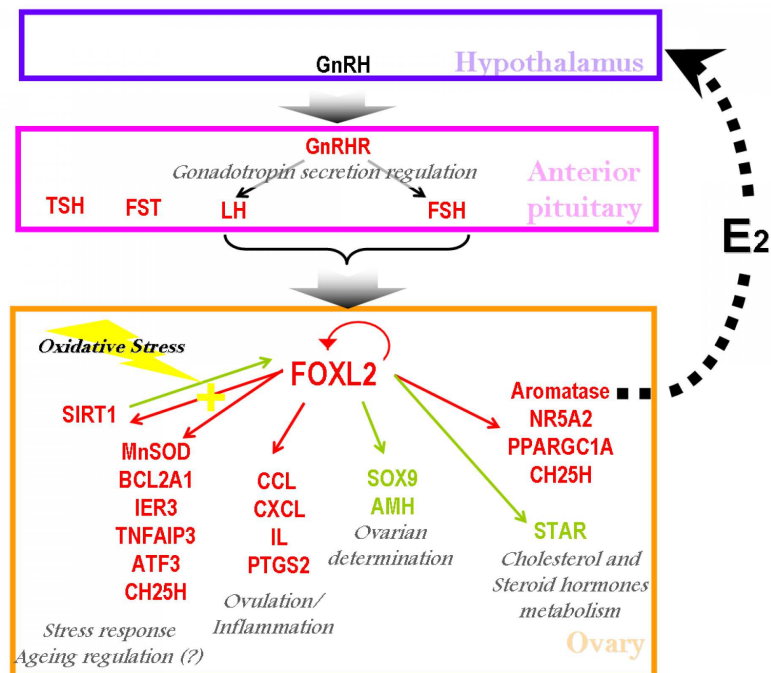


Figure 36: FOXL2 target genes in hypothalamo-pituitary-gonadal axis (from Benayoun and Veitia 2011). Red color represents the stimulation of target genes by FOXL2 whereas green color represents the inhibition.

FOXL2 functions have to be maintained from foetal life to adulthood to stabilize somatic cells fate in both granulosa and theca cells, to maintain “femaleness” and to provide a normal ovarian physiology (Uhlenhaut et al. 2009; Georges et al. 2013). *FOXL2* gene is considered as the gatekeeper of ovarian identity.

IV. Objectives

In the last fifty years, human fertility is declining due to mutations, disease and mostly due to environmental factors (Casterline 1989; Sadeu et al. 2010). This fertility decline is due to defects of gonad differentiation and function, gametes production and maturation but also endometrial development and physiology. Due to the progress of MAP in human reproduction and global species conservation, pituitary and gonads infertility can be rescued whereas endometrial defects cannot be rescued except in case of gestational surrogacy. Given that endometrium is the only tissue able to drive a successful pregnancy until parturition, the study of endometrial physiology appears mandatory (Sandra et al. 2011). Considering an achieved pregnancy is characterized by the success of numerous checkpoints, implantation appears to be one of the most critical checkpoints for the outcome of pregnancy (Bazer et al. 2010). This step is characterized by a tightly regulated communication between a healthy conceptus (embryonic disk and extra-embryonic tissues) and a receptive maternal endometrium. Therefore, it is necessary to dissect precisely the reciprocal interaction occurring during the pre-implantation period between the conceptus and the endometrium.

For ethical reason, human cannot be considered as a model for studying implantation mechanisms and mice represent a difficult model due to the speediness of implantation process. Nevertheless, ruminant species provide a perfect animal model to study implantation. Indeed, cattle and sheep exhibit a long elongation phase of the conceptus prior to the apposition on the endometrium at 20-21 dpo and 15-16 dpo, respectively (Bazer et al. 2009; Sandra et al. 2011). The slow process of implantation in ruminant allows an accurate analyse of conceptus and endometrial molecular mechanisms occurring during this period until the apposition, the common step in all mammals, regardless of the placentation type. Moreover, unlike human and rodents, ruminant implantation is superficial leading to a synepitheliochorial placentation which easily permits to distinguish the maternal to the conceptus tissue (Lee and DeMayo 2004).

In all mammals, the uterine receptivity is ensured by the ovarian secretion of E2 and P4, the major pregnancy hormones (Miller and Moore 1976; Devroey and Pados 1998). E2 is secreted during the follicular phase of the menstrual/oestrous cycle by the developing follicle prior to the ovulation, to enhance the endometrial cells proliferation. P4 is secreted during the luteal phase by the CL, regulating numerous of endometrial genes expression and decidualisation when occurs. Without fertilization, CL regresses, the secretion of P4 stops and the next cycle begins (Forde et al. 2011; Mihm et al. 2011). With fertilization, developing conceptus secretes an embryonic signal which maintains the CL and the P4 secretion. In ruminants, the extra-embryonic tissues of the conceptus secrete interferon-tau (IFNT), which

is the major pregnancy maternal recognition signal in ruminant (Roberts et al. 2008). IFNT is a strong anti-luteolytic signal preventing the regression of CL. Like for the P4, IFNT regulates the endometrial genes expression in order to host correctly the pre-implantatory conceptus (Forde et al. 2011; Bazer et al. 2012).

In the last decade, high-throughput analyses had been developed in mammals and in ruminant particularly to unveil systematically the regulation of endometrial genes expression due to oestrous cycle, early pregnancy as well as the impact of E2, P4 and IFNT (Bauersachs et al. 2005; Bauersachs et al. 2006; Gray et al. 2006; Klein et al. 2006; Mitko et al. 2008; Forde et al. 2009; Mansouri-Attia et al. 2009; Shimizu et al. 2010; Walker et al. 2010; Bauersachs et al. 2011; Forde et al. 2011; Cerri et al. 2012). Recently, a transcriptomic analyse had been done in my team using bovine endometrial samples at 20 dpo of oestrous cycle (late luteal/early follicular phase) compared to pregnancy (implantation) revealing several hundred DEG in CAR and ICAR areas (Mansouri-Attia et al. 2009). Several members of the winged-helix/forkhead domain (FOX) transcription factors family had been differentially expressed in this study including *FOXL2*, *FOXO1A* and *FOXA2*. The FOX family represents more than 40 transcription factors whose biological functions are essentially related to organ development, aging, metabolic and immunoregulatory processes, cell cycling, and cancer. While *FOXO1A* and *Foxa2* are already known to be involved in endometrial gland development, decidualisation and implantation in human and mice respectively (Buzzio et al. 2006; Jeong et al. 2010), microarray study highlights the endometrial expression of *FOXL2*, a key FOX factor for ovarian differentiation and the maintenance of ovarian function (Pannetier and Pailhoux 2011) but unknown in endometrial physiology. Its expression was originally restricted to three cell types: foetal eyelid, anterior pituitary cells and granulosa cells (Pisarska et al. 2011). This ectopic expression of this ovarian master regulator in bovine endometrium questions the implication of *FOXL2* gene in endometrial physiology.

The first goal of my PhD was to evaluate precisely the endometrial expression of *FOXL2* throughout the oestrous cycle and early pregnancy in cattle. The impacts of IFNT as well as altered P4 circulating level were investigated also on the endometrial expression of *FOXL2* using *in vivo* and *in vitro* bovine experimental models.

Then, the impact of ovarian steroid hormones namely E2 and P4 was investigated using ovine and bovine experimental models in order to refine the first analyse.

Finally, the biological functions of *FOXL2* were examined in bovine endometrium. As a transcription factor, its biological functions are associated with its target genes. To date, *FOXL2* target genes were examined using microarray analysis in human immortalized granulosa cells over-expressing *FOXL2* (KGN cell line, Batista et al. 2007) and in case of

conditional deletion of *Foxl2* in adult ovary (Uhlenhaut et al. 2009). *In silico* analyses comparing genes expression of KGN cell line over-expressing FOXL2 with bovine endometrium (comparison between Batista et al. 2007 and Mansouri-Attia et al. 2009), we identified 38 putative endometrial FOXL2 target genes. Based on candidate gene approach, transient transfection of *FOXL2* gene was carried out on bovine primary endometrial stromal and glandular epithelial cells. This strategy gave us first insights about the biological functions of FOXL2 in endometrium. Systematically comparison between target genes from ovary and endometrium had been done to evaluate the FOXL2 biological functions in a tissue-specific manner. The evaluation of endometrial *FOXL2* expression will provide us new insights about its biological relevance in female reproduction.

V. Part I: FOXL2 is regulated during the bovine oestrous cycle and its expression in the endometrium is independent of conceptus-derived interferon tau

A. Introduction

A successful implantation requires a tightly regulated communication between the endometrium and the conceptus. Asynchronous developments of the conceptus lead to implantation failures due to early embryonic death as well as endometrial defects preventing the implantation of a healthy conceptus. On the other hand, only endometrium can drive successfully a pregnancy. But, external events (as infection, nutrition, endocrine disruptors or stress) and intrinsic maternal features altering the biological functions of maternal organs may affect endometrial physiology.

In the last decade, endometrial genes expression had been investigated using high-throughput analysis to evaluate the impact of oestrous cycle, presence and quality of the conceptus (AI, IVF and SCNT) and ovarian steroid hormones. These data have demonstrated the sensor/driver properties of the endometrium as a dynamic and reactive entity.

In particular, these microarrays and RNA-sequencing studies have revealed the expression of several members of the Forkhead box transcription factors family including *FOXA2*, *FOXL2* and *FOXO1A*. Transcription factors and FOX factors particularly, act as a molecular integrator of extracellular signals and they might constitute nodes in cellular networks. FOX factors are widely expressed in various tissues and are involved in many developmental processes, organ ageing, then cancers. In human, rodent and ruminant endometrium, *FOXA2* and *FOXO1A* are known to be both master regulators of endometrial gland development, decidualisation when occurs and implantation. *FOXL2* was shown to be expressed in three cell types: fetal eyelid, anterior pituitary cells and granulosa cells. Indeed, *FOXL2* gene was described originally, as one of the key genes for ovarian differentiation.

To date, *FOXL2* was never studied in the endometrial context but our recent microarray data have revealed the differential expression of this transcription factor between 20 dpo of pregnancy and oestrous cycle in bovine endometrium.

The first part of my PhD was focused on the evaluation of *FOXL2* gene expression during the oestrous cycle and early pregnancy. Using bovine physiological model, we examined the expression of *FOXL2* (mRNA and protein) during the active luteal phase (16 dpo) and the late luteal/follicular phase (20 dpo) of the oestrous cycle compared to the maternal pregnancy recognition period (16 dpo) and the initiation of implantation (20 dpo) during early pregnancy.

IFNT which is the major signal of pregnancy maternal recognition, regulate strongly endometrial genes expression during the early pregnancy. Then, we investigated also the action of IFNT on *FOXL2* endometrial expression using an experimental model. Two groups of cows received an intrauterine infusion of control saline buffer and recombinant ovine IFNT (roIFNT) for 2h at 14 dpo of the oestrous cycle (active luteal phase). This experimental model was designed to study the rapid action of IFNT on endometrial genes expression.

During the luteal phase and the maternal pregnancy recognition period, endometrium undergoes important changes in genes expression mostly due to P4 action. The major hormone of pregnancy is responsible of the uterine receptivity occurring during these periods. We examined the impact of altered P4 circulating level on *FOXL2* endometrial expression using an experimental model derived from collaboration with the team of Patrick Lonergan, Ireland. *FOXL2* expression was studied at 5 dpo (follicular/early luteal phase) and 16 dpo (active luteal phase) during the oestrous cycle and early pregnancy after 2 days-P4 supplementation and 13 days-P4 supplementation respectively.

Finally, cellular and sub-cellular localization of *FOXL2* was investigated during the active luteal phase, the late luteal/follicular phase and at implantation namely 14 and 20 dpo of the oestrous cycle and 20 dpo of pregnancy respectively.

B. Conclusions

This publication showed for the first time an accurate characterization of *FOXL2* endometrial expression in one mammal species.

FOXL2 gene is expressed and regulated during the oestrous cycle and early pregnancy in bovine endometrium. The bovine oestrous cycle is compounded by a short follicular phase and a long luteal phase. In this study, we have demonstrated that *FOXL2* is highly expressed only during the early luteal phase or the follicular phase i.e. 5 and 20 dpo of the oestrous cycle, respectively. In addition, we also showed that *FOXL2* expression is significantly higher during the early pregnancy, at 5 dpo exhibits similar P4 circulating level between the oestrous cycle and early pregnancy. Conversely, *FOXL2* expression is significantly decreased during the luteal phase, the pregnancy maternal recognition period and at implantation i.e. 16 dpo of the oestrous cycle and 16 dpo and 20 dpo of pregnancy, respectively. Moreover, *FOXL2* expression is always significantly lower in the ICAR area compared to the CAR area.

The endometrial expression of *FOXL2* is clearly different between oestrous cycle and early pregnancy at 20 dpo. During the oestrous cycle, 20 dpo is characterized by an absence of

P4 circulating level and a potential rise of E2 while the implantation day is characterized by a maintained high-P4 circulating level associated with a high IFNT secretion by the conceptus and a very low E2 circulating level.

We first examined the action of IFNT on *FOXL2* endometrial expression using *in vivo* and *in vitro* model. *FOXL2* expression was shown to be not impacted by a short (2 hours) and long term (24 hours) treatment of IFNT in both endometrial areas. Our data suggest that *FOXL2* is not an early and late target gene of IFNT in bovine endometrium.

Next, we focused on the P4 action on *FOXL2* expression. Cyclic and pregnant cows were supplemented with exogenous P4 from day 3 to day 5, 2-days-P4 supplementation and to day 16, 13-days-P4 supplementation. *FOXL2* expression is significantly decreased at 5 dpo after the P4 treatment whereas at 16 dpo, P4 supplementation has no impact on *FOXL2* expression. *FOXL2* expression is negatively correlated with P4 circulating level.

Finally, we investigated the endometrial cellular and sub-cellular localization of *FOXL2* during the oestrous cycle and at implantation in cattle. In every conditions, *FOXL2* is completely absent of the luminal epithelial cells. During the luteal phase (14 dpo), *FOXL2* is localized in the nuclei of stromal cells and in both nuclei and cytoplasm of the glandular epithelial cells. During the follicular phase (20 dpo), *FOXL2* is localized in the nuclei of stromal and glandular epithelial cells whereas at implantation (20 dpo) *FOXL2* is only localized in the cytoplasm of stromal and glandular epithelial cells. *FOXL2* nuclear localization is also negatively correlated with the P4 circulating level. Recent work in murine ovary and COS-7 cell line has demonstrated *FOXL2* interacts with PIAS1, a protein of the sumoylation machinery leading to its sumoylation by SUMO1 and UBC9 proteins (Marongiu *et al.*, 2010). This post-translational process induces a change of sub-cellular localization of *FOXL2*. We wonder if *FOXL2* could be sumoylated in bovine endometrium inducing changes in sub-cellular localization observed between follicular and luteal phase as well as at implantation step. Interestingly, the SUMOplot™ Analysis Program software showed seven sumoylation motifs on the bovine protein sequence of *FOXL2*, close to the human and murine motifs including four motifs with a very high probability all localized in the N-terminus side (Fig. 37).

SUMOplot™ Analysis Program

Developed by Abgent, copyright 2003-2013

Protein ID:	gil72534786 ref NP_001026920.1
Defintion:	forkhead box protein L2 [Bos taurus]
Length:	377 aa

1	MMASYPEPEN	ASGALLAPET	GRAAKEFEAP	PPSPGKGGG	GGTGTAPKFP	<div style="display: flex; flex-direction: column; align-items: flex-start;"> <div style="width: 10px; height: 10px; background-color: red; margin-bottom: 2px;"></div> Motifs with high probability </div> <div style="width: 10px; height: 10px; background-color: blue; margin-bottom: 2px;"></div> Motifs with low probability <div style="width: 10px; height: 10px; background-color: cyan; margin-bottom: 2px;"></div> Overlapping Motifs
51	DPAQKFPYSY	VALIAMAIRE	SAEKRLTLTG	IYQYIIAKFP	FYEKNKKGWQ	
101	NSIRHNLSLN	ECFIKVFREG	GGERKGNWT	LDPACEDMFE	KGNYYYYRRR	
151	KRFRPPPAH	FQPGKGLFGA	GGAAGGCGVA	GAGADGYGYL	APPKYLQSGF	
201	LNNSWLPQP	PSPMPYASCQ	MAAAAAAAAA	AAAAAGFGSP	GAAAVVKGLA	
251	GPAASYGPYS	RVQSMALPPG	VVNSYNGLGG	PPAAPPFPPH	PHSHPHAHHL	
301	HAAAAPFPAP	PHHGAAAPFP	QQLSPASPAT	AAPPAPAPT	APGLQFACAR	
351	QPELAMMHCS	YWDHDSKTGA	LHSRLDL			

No.	Pos.	Group	Score	No.	Pos.	Group	Score
1	K115	LNECF IKVP REGGG	0.84	5	K49	TGTAP EKP PAQKP	0.5
2	K151	RRRRR MKRP FRPPP	0.69	6	K37	PPSP GKGG GGGTG	0.5
3	K88	YQYII AKFP FYEKN	0.69	7	K55	KPDPA QKPP YSYVA	0.39
4	K25	ETGRA AKEP EAPPP	0.69				

Figure 37: Sumoylation sites prediction. Bovine sequence of FOXL2 protein showed seven motif of sumoylation in the N-terminus side which is similar to four motifs existing in murine and human sequences (Marongiu et al. 2010).

These *in silico* analyse prompt the need for further investigation about the impact of post-translational modifications on FOXL2 endometrial protein in mammal and especially in cattle.

This publication highlights the first evidence that *FOXL2* is expressed and localized in bovine endometrium and negatively correlated with P4 circulating level. We demonstrated that *FOXL2* expression is not impacted by 2h- or 24h-treatment of IFNT. Nevertheless, the sub-cellular localization of FOXL2 exhibits differences between the luteal phase and implantation day whereas its expression (transcript and total protein) was similar in both phases. Indeed, during the luteal phase, FOXL2 protein was at least detected in the nucleus of the stromal cells whereas at implantation, FOXL2 is absent of the nucleus of glandular epithelial and stromal cells. We hypothesized that the apposition or IFNT independent secretion of the conceptus could induce a cytoplasmic translocation of FOXL2 protein.

In this study, an elevated concentration of P4 leads to a significant decrease of FOXL2 expression in bovine endometrium. P4 acts on the endometrial genes expression using its nuclear receptor; PGR form A and B. P4 binds their receptors, which are translocated to the nucleus regulating genes expression. Therefore, we hypothesized that FOXL2 could be a direct target of P4 and the regulation of its expression could be due to the binding of activated PGR on FOXL2 promoter.

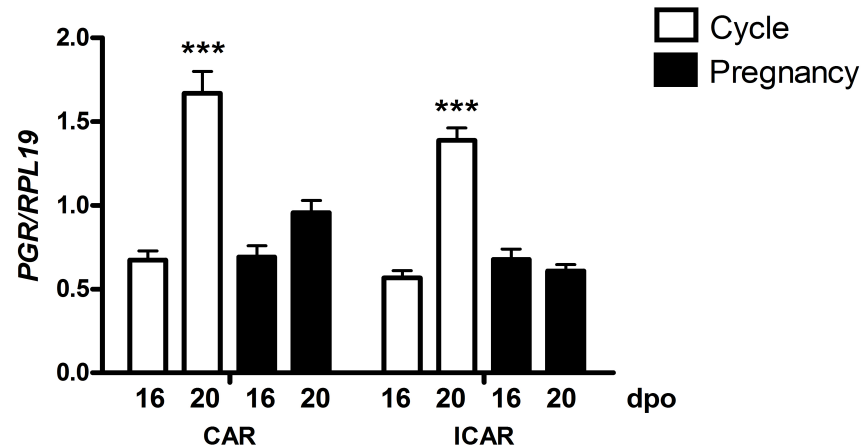


Figure 38: *PGR* expression during the oestrous cycle and the early pregnancy in bovine endometrium. Caruncular (CAR) and intercaruncular (ICAR) endometrial areas were collected from cyclic (n=5 at day 16, n=6 at day 20) and pregnant (preg, n=4 at day 16, n=5 at day 20) cross-bred beef heifers. Quantification of *PGR* mRNA by RT-qPCR. Expression of *PGR* was normalized to *RPL19*. Quantitative data are presented as mean \pm SEM and significant difference is noted, ***: $P < 0.001$.

PGR transcript exhibits a similar expression pattern compared to *FOXL2* expression with a repression during the luteal phase compared to the follicular phase (Fig. 38). As previously published, during the luteal phase, P4 secretion rises and reaches a plateau leading to the inhibition of its own receptors expression prior to the implantation when occurs (Bazer *et al.*, 2010). This result and the literature prompt the need to better define the regulation between P4 circulating level and *FOXL2* endometrial expression. In addition, the potential action of E2 was not investigated in this publication and further analyses will be necessary to dissect the regulation mechanisms of both ovarian steroid hormones on *FOXL2* endometrial expression.

C. Publication

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FOXL2 Is Regulated During the Bovine Estrous Cycle and Its Expression in the Endometrium Is Independent of Conceptus-Derived Interferon Tau¹

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ABSTRACT

FOXL2, a winged-helix/forkhead domain transcription factor, is a key gene involved in the differentiation and biological functions of the ovary. In a recent transcriptomic analysis, we found that *FOXL2* expression in bovine caruncular endometrium was different from that in intercaruncular endometrium. In order to gain new insights into *FOXL2* in this tissue, we determined the expression of this transcription factor during the estrous cycle and the establishment of pregnancy in cattle. The endometrial expression of *FOXL2* did not vary during maternal recognition of pregnancy (Days 16–20). Using an in vivo bovine model and primary cell cultures, we showed that *FOXL2* was not an interferon-tau target gene. Both *FOXL2* transcript and protein were expressed from Day 5 to Day 20 of the estrous cycle, and their levels showed a significant increase during the luteolytic phase. A 2-day progesterone supplementation in heifers led to a clear down-regulation of *FOXL2* protein levels, suggesting the negative impact of progesterone on *FOXL2* expression. Immunohistochemistry data revealed the localization of *FOXL2* in endometrial stromal and glandular cells. *FOXL2* subcellular distribution was shown to be nuclear in endometrial samples collected during the luteolytic period, while it was not detected in nuclei during the luteal phase and at implantation. Collectively, our findings provide the first evidence that *FOXL2* is involved in the regulation of endometrial tissue physiology.

bovine, endometrium, estrous cycle, *FOXL2*, pregnancy

INTRODUCTION

In mammals, implantation represents a critical checkpoint for the success of pregnancy [1]. This step corresponds to the establishment of permanent cellular interactions between the conceptus (embryonic disk and extraembryonic tissues) and the endometrium [2]. Compared to the invasive nature of implantation in mice and humans, the process of implantation

is superficial in ruminants, leading to a synepitheliochorial placentation [3]. In these domestic species, progesterone (P4) and interferon-tau (IFNT) represent two major factors regulating endometrial physiology during the establishment of pregnancy [4]. In sheep and cattle, P4 is indispensable for supporting pregnancy, and alterations in circulating P4 concentration have been reported to significantly affect conceptus elongation as well as endometrial gene expression patterns [5–9]. In these species, IFNT has been shown to be the major signal for maternal recognition of pregnancy [10]. The spatiotemporal secretion of IFNT is restricted to the trophectoderm during the elongation phase of the conceptus [11]. Experimental models and cell lines have been used to show that IFNT inhibits luteolytic mechanisms and induces expression of IFNT-stimulated genes in the endometrium [7, 12–14], circulating immune cells [15], and, more recently, in the corpus luteum during the peri-implantation period [16].

Recent high-throughput data have highlighted the major impact of the conceptus on ovine and bovine endometrial physiology during the peri-implantation period of pregnancy [1, 14]. The endometrial response in small aglandular areas of endometrium (caruncles [CAR]) has also been shown to differ from that in large glandular intercaruncular (ICAR) areas [13, 17, 18]. These transcription profiles have revealed the expression of several families of transcription factors including the forkhead box containing transcription factors (FOX) family [13, 17, 18]. The FOX family represents more than 40 transcription factors whose biological functions are essentially related to organ development, aging, metabolic and immunoregulatory processes, cell cycling, and cancer [19–21]. In the context of the endometrium, murine *Foxa2* and human *FOXO1A* have been shown to be involved in gland development and differentiation of stroma cells and in decidualization [22–24]. In addition to these two FOX factors [13, 18], several other FOX factors including *FOXC1*, *FOXL2*, and *FOXQ1* have been identified in high-throughput analyses of bovine endometrium. Very interestingly, *FOXL2* expression in CAR is different from that in ICAR tissue on Day 20 day of the estrous cycle [13]. Originally, *FOXL2* expression had been restricted to three cell types, namely granulosa cells in the fetal and adult ovary, anterior pituitary cells, and fetal eyelid cells [25, 26]. *FOXL2* was first cloned 10 years ago in humans [27], and it was subsequently isolated in a large variety of animal species ranging from invertebrates (e.g., sea urchins and oysters [28, 29]) to fish, chickens, mice, and goats [30–32]. In vertebrates, including livestock, *FOXL2* has been reported to be a key gene for ovarian differentiation [26, 32–36]. In addition, *FOXL2* has been shown to control GnRH receptor expression [37],

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regulate gonadotropin hormone secretion (follicle-stimulating and luteinizing hormones) in the anterior pituitary [25, 38], and control ovarian function by acting on ovarian differentiation, steroid secretion, ovulation, stress response, and hormone metabolism [34, 38–40]. The present study was conducted to evaluate the expression and regulation of *FOXL2* during the estrous cycle and early pregnancy and the effect of P4 and IFNT on *FOXL2* gene expression and cell localization in bovine endometrium.

MATERIALS AND METHODS

Animals and Cell Cultures

All experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction.

Experiment 1: *FOXL2* expression during the period of pregnancy recognition. Cyclic and pregnant cross-bred beef heifers were synchronized and artificially inseminated as previously described [12]. The day of estrus was considered Day 0. Heifers were slaughtered at Day 16 (cyclic; $n = 5$; pregnant; $n = 4$) and at Day 20 (cyclic; $n = 6$; pregnant; $n = 5$). Uteri were collected, flushed, and, when present, recovered concepti were observed by microscopy to confirm the stage of development [41]. From pregnant and cyclic animals, endometrial CAR and ICAR areas were dissected from the uterine horns ipsilateral to the corpus luteum as previously described [13].

Cyclic and early pregnant Charolais cows were produced using the CRESTAR method and artificial insemination, as previously published [42]. The day of estrus was considered Day 0, and females were slaughtered at Day 20 (cyclic; $n = 5$; pregnant; $n = 5$). Uteri were collected and dissected as described above. In addition, ovaries with the corpus luteum were collected. Some follicles and the corpus luteum were dissected.

Experiment 2: impact of *in vitro* and *in vivo* IFNT infusion on *FOXL2* endometrial expression. The estrous cycles of cyclic cows (Charolais breed) were synchronized as described above. The uterine lumen was infused with recombinant ovine IFNT (roIFNT; 200 μ g/ml; 25 ml/horn) or control solution (saline buffer) at Day 14 postestrus as we previously described [12]. The endometria of 6 females infused with IFNT and 5 control animals were collected and dissected as described in experiment 1.

Culturing of bovine endometrial cells (fibroblasts or glandular epithelial cells) was carried out as we previously reported [12, 13], and samples were treated for 2 h or 24 h with 100 ng/ml roIFNT [12, 13].

Experiment 3: impact of P4 supplementation on *FOXL2* expression. Cyclic heifers and those confirmed to be pregnant (by artificial insemination) received a P4-releasing intravaginal device containing 1.55 g of progesterone (Ceva Animal Health Ltd.) on Day 3 of the estrous cycle or pregnancy to increase circulating concentrations of P4 as previously published [6, 43]. Strips of endometrium (CAR and ICAR) were sampled after 2 days and 13 days of P4 supplementation (Days 5 and 16, respectively) in cyclic and pregnant females displaying normal and high concentrations of P4.

Tissue Collection, Preparation for Immunohistochemistry, and RNA Extraction

Endometrial samples were immediately snap frozen in liquid nitrogen and stored at -80°C prior to analysis [13]. At the same time, sections of the ipsilateral uterine horn were fixed in 4% paraformaldehyde in phosphate buffer and then washed in $1\times$ PBS, followed by a 12-h dehydration treatment in a Shandon Citadel 1000 tissue processor (Thermo-electron Corp.) using increasing concentrations of ethanol. Tissues were embedded in paraffin (Paraplast plus, McCormick Scientific, VWR) and subsequently stored at 4°C . Whole uterus cross-sections were cut (7 μ m) with a rotary microtome (Leica model RM2245) and stored at room temperature until processed. Total RNA from frozen tissue was isolated by homogenization using TRIzol reagent (Invitrogen) as published previously [13]. All RNA samples were purified using Qiagen columns integrating a DNase step (RNeasy mini-kit; Qiagen). Quality and integrity of total extracted and purified RNA were determined using an Agilent 2100 bioanalyzer. Upon addition of RNase inhibitor (RNasin; Promega) total RNA was stored at -80°C .

Real-Time RT-PCR

Purified total RNA samples were used for quantitative RT-PCR (RT-qPCR). One microgram of total RNA was reverse transcribed into cDNA with Superscript II enzyme (Invitrogen) in a 20- μ l volume. RT-PCR reactions were

carried out with Master Mix SYBR Green and Step One Plus system (Applied Biosystems). Primers (Eurogentec) were designed (Primer Express version 2.0 software; Applied Biosystems) to specifically amplified bovine *FOXL2* (forward: CCGGCATCTACAGTACATTATAGC; and reverse: GCACTCGTTGAGGCTGAGGT [NCBI sequence reference, NM_001031750.1]) and *RPL19* (forward: CCCAATGAGACCAAT-GAAATC; and reverse: CAGCCCATCTTTGATCAGCTT) [13]. Amplified *FOXL2* and *RPL19* PCR fragments were sequenced to assess amplification of the correct fragment. According to the relative standard curve method [44], quantification of the amount of *FOXL2* mRNA relative to that of the *RPL19* normalizer gene was calculated.

Western Blot Analysis

Frozen tissues (bovine ovary and endometrium) were ground in liquid nitrogen and then resuspended with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl [pH 8], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, anti-protease [Roche]; 200 μ l of RIPA buffer per 100 μ g of tissue) in a Dounce homogenizer on ice. Samples were centrifuged at 20 000 \times g for 5 min at 4°C , and then the supernatant corresponding to the total protein extract was collected and stored at -80°C . Protein quantification was carried out using the Bradford method with bovine serum albumin (BSA) as the standard (Sigma-Aldrich).

Western blot immunoassays were processed with 25 μ g of total protein extract lysed in SDS buffer and separated using 4%–12% NuPage bis-Tris gel electrophoresis (Novex; Invitrogen). Protein molecular mass markers (Novex Sharp protein standard, 3.5–260 kDa; Invitrogen) were run simultaneously as molecular mass standards. Electrophoretically separated polypeptides were transferred onto a Hybond-P polyvinylidene fluoride membrane (Amersham). Membranes were blocked in $1:1000$ Tween 20-PBS (PBS-T, Euromedex) containing 4% (w/v) non-fat dried milk. Membranes were probed with a rabbit anti-*FOXL2* purified antibody generated against a peptide corresponding to the C-terminal conserved region of mammalian *FOXL2* (WDHDSKTGALHSRLDL; diluted 1:500; Caslo Laboratory) in PBS-T solution containing 4% non-fat dry milk. Then, membranes were incubated with a goat peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) antibody (diluted 1:5000; Santa Cruz Biotechnology). Actin B (ACTB) was assessed as a loading control, using a mouse monoclonal anti-ACTB antibody (diluted 1:2000; Sigma-Aldrich) and goat peroxidase-conjugated anti-mouse IgG antibody (diluted 1:5000; Santa Cruz Biotechnology). Immunoreaction signals were revealed with ECL Plus Western blotting detection reagents (Amersham) and analyzed using an image analysis system (Advanced Image Data Analyzer software; LAS 1000 camera; Fujifilm).

Immunohistochemistry

Sections were rehydrated in xylene (Prolabo; VWR), followed by ethanol baths of decreasing concentration. Sections were incubated in citrate buffer (sodium citrate, 0.01M, pH 6.0) at room temperature for 5 min, followed by incubation at 80°C for an additional 10 min to unmask sections. Endogenous peroxidase activity was quenched by treatment with 0.1% hydrogen peroxide for 30 min. Sections were then incubated with a rabbit anti-*FOXL2* purified antibody (diluted 1:100; Caslo Laboratory) or a goat polyclonal anti-lamin B (Lamin B) antibody (diluted 1:200; Santa Cruz Biotechnology) in phosphate buffer (0.1 M, pH 7.4, with 2% BSA and 1% normal donkey serum) at 4°C overnight. As a negative control, the peptide used to generate the antibody was co-incubated with the rabbit anti-*FOXL2* purified antibody at 37°C for 1 h before incubation on sections. After several washes in phosphate buffer with 2% BSA, anti-rabbit biotinylated antibody (diluted 1:500; Jackson ImmunoResearch) and anti-goat biotinylated antibody (diluted 1:200; Santa Cruz Biotechnology) were applied to slides for 1 h at room temperature. After three washes in phosphate buffer, sections were incubated for 1 h with the ABC Vector Elite kit (Vectastain Elite ABC kit; Vector Labs) in Tris-buffer (Tris 50 mM, NaCl 0.15 M, pH 7.5). Slides were washed three times and incubated with diaminobenzidine substrate and urea (Fast 3.3; Sigma) in Tris-buffer for 2–10 min, until brown staining was detectable. After one wash in water, slides were dehydrated by immersion in increasing concentrations of ethanol baths and then mounted with Eukit mounting medium (Sigma). A section for each physiological condition was stained with hematoxylin-eosin solution to highlight tissue morphology. Images were obtained using a NanoZoomer digital pathology system and then analyzed using NanoZoomer digital pathology virtual slide viewer (NDPView software; Hamamatsu). Each experiment was repeated with four different uterine cross-sections for each animal (three females per biological condition). Quantification of immunohistochemistry staining was processed using ImageJ version 1.45 software (National Institutes of Health, Bethesda, MD; <http://rsbweb.nih.gov/ij/>).

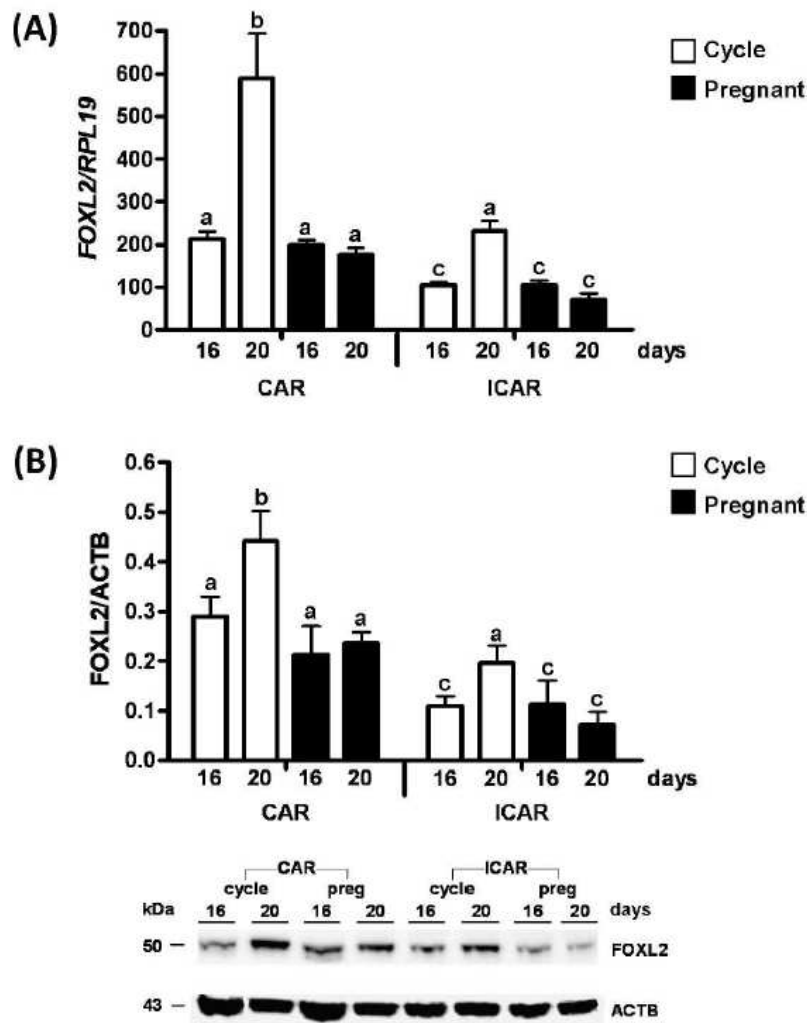


FIG. 1. FOXL2 expression in cyclic and pregnant bovine endometrium on Days 16 and 20. CAR and ICAR endometrial areas were collected from cyclic ($n = 5$ at Day 16; $n = 6$ at Day 20) and pregnant (preg: $n = 4$ at Day 16; $n = 5$ at Day 20) cross-bred beef heifers. A) Quantification of *FOXL2* mRNA by RT-qPCR. Expression of *FOXL2* was normalized to that of *RPL19*. B) Quantification of *FOXL2* protein by Western blotting, normalized to *ACTB* protein level. Quantitative data are means \pm SEM, and bars with different lowercase letters differ significantly ($P < 0.01$ in A; $P < 0.05$ in B).

Statistical Analyses

Statistical analyses were carried out using GraphPad Prism 4 software (GraphPad Software). Quantitative data were subjected to a two-way ANOVA followed by Bonferroni test. For female tissues collected on Days 5, 16, and 20, data were analyzed for the effects of day, pregnancy status (cyclic or pregnant), endometrial areas (CAR and ICAR), and their interactions (day versus status or status versus endometrial areas). Linear regression analysis was used to correlate circulating P4 concentrations with *FOXL2* protein expression.

RESULTS

FOXL2 Is Regulated During the Estrous Cycle in Bovine Endometrium

In cattle, the estrous cycle lasts 20–22 days and is associated with an increase in progesterone secretion (Day 3–4) that

reaches a plateau before decreasing (Day 18) in association with corpus luteum regression. *FOXL2* transcript expression was significantly higher in cyclic heifers on Day 20 when this expression was compared to pregnant heifers on Day 20 and cyclic and pregnant heifers on Day 16 (2.7- to 3.3-fold in CAR area and 2.2- to 3.2-fold in ICAR area; Fig. 1A, $P < 0.01$). However, in CAR and ICAR areas, endometrial expression of *FOXL2* mRNA did not significantly differ between Day 16 and Day 20 in pregnant heifers. At the protein level, a similar pattern of *FOXL2* regulation was reported in CAR and ICAR endometrial areas, with significantly higher *FOXL2* expression in cyclic females on Day 20 (1.5- to 2.2-fold higher) than other conditions ($P < 0.05$) (Fig. 1B). Similar results were obtained with Charolais cows analyzed on Day 20 of the estrous cycle and pregnancy (Supplemental Fig. S1; all Supplemental Data

are available online at www.biolreprod.org). In the ovary (Supplemental Fig. S1B) as well as endometrial CAR and ICAR areas (Fig. 1B), the apparent molecular mass of FOXL2 (~50 kDa) was similar to and consistent with those reported in other animal species [31, 32, 45]. Expression of *FOXL2* mRNA and protein levels were significantly lower in ICAR areas than in CAR areas ($P < 0.05$) (Fig. 1; also see Supplemental Fig. S1).

FOXL2 endometrial expression appeared to be down-regulated during the implantation period. In order to analyze the impact of IFNT (the major signal of maternal recognition of pregnancy in cattle) on *FOXL2* expression, an experimental bovine model was derived using an intrauterine infusion of rolFNT in cyclic cows on Day 14 of the estrous cycle (luteal phase). The amount of endometrial *FOXL2* transcript differed significantly between CAR and ICAR areas, but the short-term IFNT treatment (2 h) did not significantly affect *FOXL2* transcript or protein levels (Fig. 2, A and B).

Bovine endometrial cell cultures (fibroblasts and glandular epithelial cells) were treated with rolFNT in order to investigate the differences between short-term (2-h) and long-term (24-h) treatment with IFNT. Glandular epithelial cells displayed a lower expression of *FOXL2* mRNA than fibroblasts (2 h, 3-fold; 24 h, 5-fold; $P < 0.05$). The short-term (2-h) and long-term treatment (24-h) of IFNT did not significantly alter *FOXL2* expression level (Fig. 2C).

Increased Concentration of Progesterone Affects Endometrial FOXL2 Expression In Vivo

Considering the variation in *FOXL2* gene expression according to the stage of estrous cycle, we addressed the question of *FOXL2* regulation in an experimental bovine model with increased concentrations of circulating P4 induced by exogenous supplementation.

In heifers supplemented with P4 for 48 h (Day 3–5) compared with control animals, a significant 2.55-fold decrease in endometrial *FOXL2* expression was detected in cyclic heifers ($P < 0.05$) (Fig. 3A). The lack of significance in pregnant animals was a consequence of the limited number of samples ($n = 2$). No significant impact of P4 treatment was detected in pregnant or cyclic heifers sampled on Day 16 following 13-day steroid hormone supplementation (Fig. 3A). Nevertheless, a lower *FOXL2* expression was observed in endometrial tissue samples collected from cyclic and pregnant heifers on Day 16 compared with those collected on Day 5 ($P < 0.05$) (Fig. 3A). Interestingly, when circulating concentrations of P4 were plotted against *FOXL2* endometrial expression, the amount of *FOXL2* protein was shown to be inversely correlated with blood concentrations of P4 ($P = 0.0201$) (Fig. 3B).

Cellular Localization of FOXL2 Varies in Bovine Endometrium Collected at Two Different Stages of the Estrous Cycle and at Implantation

The specificity of FOXL2 antibody was confirmed using bovine ovarian tissue and blocking peptide (Supplemental Fig. S2, A and B). In keeping with published data in other vertebrates [46], FOXL2 was clearly localized in nuclei of granulosa cells surrounding the antral follicle (Supplemental Fig. S2A). In the endometrium, FOXL2 cell localization was investigated in cyclic cows on Day 14 (luteal phase) and Day 20 (follicular phase) as well as in pregnant cows at the time of initiation of implantation (Fig. 4, Day 20). The intracellular position of nuclei in the various endometrial cell populations

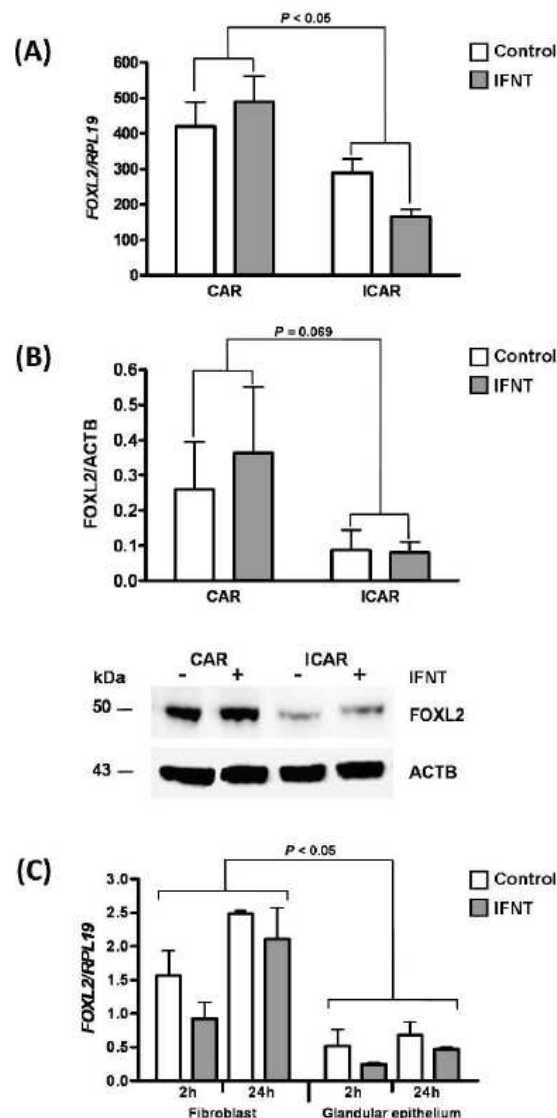


FIG. 2. Regulation of *FOXL2* gene expression by IFNT. A and B) CAR and ICAR endometrial areas were collected from Charolais cows infused with control solution ($n = 5$) or rolFNT (200 μ g/ml; $n = 6$) for 2 h. A) Quantification of *FOXL2* mRNA by RT-qPCR. Expression of *FOXL2* transcript was normalized to that of *RPL19*. B) Quantification of *FOXL2* protein by Western blotting. *FOXL2* expression was normalized to that of *ACTB* protein level. C) Regulation of *FOXL2* expression by IFNT in bovine endometrial primary cell cultures (stroma and glandular epithelium cell culture) was investigated using RT-qPCR. Expression of *FOXL2* was normalized to that of *RPL19*. Quantitative data are means \pm SEM.

was determined using hematoxylin-eosin staining (Fig. 4I) or anti-Lamin B antibody (Supplemental Fig. S2, C and D). On Days 14 and 20 of the estrous cycle, FOXL2 was clearly detected in stroma cells, superficial and deep glandular epithelium, and endothelial cells of blood vessels (Fig. 4, A,

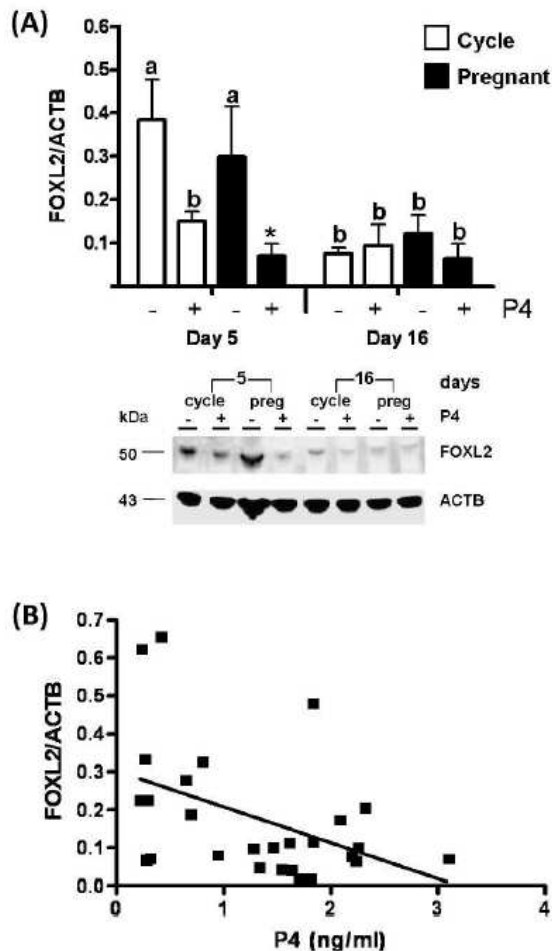


FIG. 3. FOXL2 endometrial expression in cyclic and pregnant heifers supplemented with P4 for 2 or 13 days. **A)** Quantification of FOXL2 protein by Western blotting (normal level of P4 in cyclic heifers, $n = 4$, and in pregnant heifers, $n = 4$, at Day 5; normal level of P4 in cyclic heifers, $n = 3$, and pregnant heifers, $n = 4$, at Day 16; high level of P4 in cyclic plus P4 heifers, $n = 4$, and pregnant plus P4 heifers, $n = 2$, at Day 5; and high level of P4 in cyclic plus P4 heifers, $n = 3$, and pregnant plus P4 heifers, $n = 3$, at Day 16). FOXL2 expression was normalized to that of ACTB. **B)** Correlation between FOXL2 expression and circulating progesterone concentrations ($P = 0.0201$). *Not significant due to a limited number of available samples. Quantitative data are means \pm SEM, and bars with different lowercase letters differ significantly ($P < 0.05$).

B, D, and E). Nevertheless, intracellular FOXL2 localization differed between the two stages of the estrous cycle as FOXL2 nuclear staining was restricted to stroma cells on Day 14 (Fig. 4D) but was visible in stroma and glandular epithelial cells on Day 20 (Fig. 4E). Intrauterine infusion of IFNT did not affect FOXL2 cell localization compared with endometrium collected on Day 14 (Fig. 4G). In pregnant cows at Day 20, endometrial FOXL2 was observed in stroma, glandular, and endothelial cells, but none of these cells displayed a detectable nuclear localization (Fig. 4, C and F). Regardless of physiological condition, staining of luminal epithelium appeared negative for

FOXL2. Quantification of immunohistochemistry staining confirmed that FOXL2 expression displayed significant differences among physiological stages in glandular and stroma cells (Fig. 4J). On Day 20 in cyclic endometrium, FOXL2 staining was significantly higher in stroma and glandular cells than on Day 14 cyclic and Day 20 pregnant tissue. However, at 20 days of pregnancy, stromal FOXL2 expression was significantly lower than in both cyclic stages. Quantification of the FOXL2 intensity signal was in keeping with quantification of the FOXL2 expression on Days 16 and 20 in cyclic and pregnant endometrium (Fig. 1B).

DISCUSSION

FOXL2 has been recognized as a key factor involved in ovarian development and biological functions from fetal life to adulthood [47, 48]. Recently, FOXL2 was listed as a gene whose expression in CAR endometrium differed from that in ICAR endometrium sampled from cyclic females on Day 20 [13]. In the current study, we demonstrated that FOXL2 is expressed in the endometrium and regulated during the estrous cycle and the period of maternal recognition of pregnancy.

Compared with the early preimplantation phase (Day 5), a clear decrease in FOXL2 transcript and protein expression levels was detected during the period of maternal recognition of pregnancy and implantation (Days 16–20). FOXL2 gene expression was not influenced by the conceptus, and short-term infusion of IFNT (the major pregnancy recognition signal in ruminants [10, 14]) affected neither FOXL2 expression nor its cell localization. Similarly, treatment of endometrial cells with IFNT for 2 h or 24 h did not affect FOXL2 mRNA expression. Collectively, our data do not support the hypothesis that the conceptus contributes to the regulation of FOXL2 expression in bovine endometrium. This situation differs from that in humans in which a negative impact of trophoblast tissue on FOXL2 expression has been reported in endometrial stromal cells using a 24-h coculture cell system and gene expression profiling [49]. The down-regulation of FOXL2 expression has also been reported in human endometrial stromal cells co-incubated for 12 h with trophoblast-conditioned medium compared to control conditioned medium [50]. Therefore, experimental data using in vitro human models support the negative effect of trophoblast secretions on FOXL2 expression regardless of P4 action [49, 50]. Type of implantation as well as embryo signals differ between cattle and humans [2], and additional studies across animal species will be necessary to understand the relationships among conceptus secretions, type of implantation, and endometrial regulation of FOXL2 gene expression.

Progesterone is a major hormone of pregnancy, whose biological actions are crucial for the establishment and maintenance of pregnancy in ruminants and in mammals generally [51]. During the estrous cycle, the endometrial expression of FOXL2 decreases between Day 5 and Day 16 (early luteal and late luteal phases, respectively), following which it increases from Day 16 to Day 20 (follicular phase). The endometrial pattern of FOXL2 expression appears to be inversely related to the circulating P4 concentrations determined at similar stages [43, 52]. Furthermore, a two-day P4 supplementation (Days 3–5) led to a significant reduction in FOXL2 expression in both cyclic and pregnant heifers at Day 5. Very interestingly, microarray experiments carried out with human endometrium have also indicated a variable expression of endometrial FOXL2 mRNA across the menstrual cycle in women [53]. During the proliferative phase, FOXL2 expression was high, whereas it dropped during the secretory phase, a period associated with high luteal P4 secretion and a lower

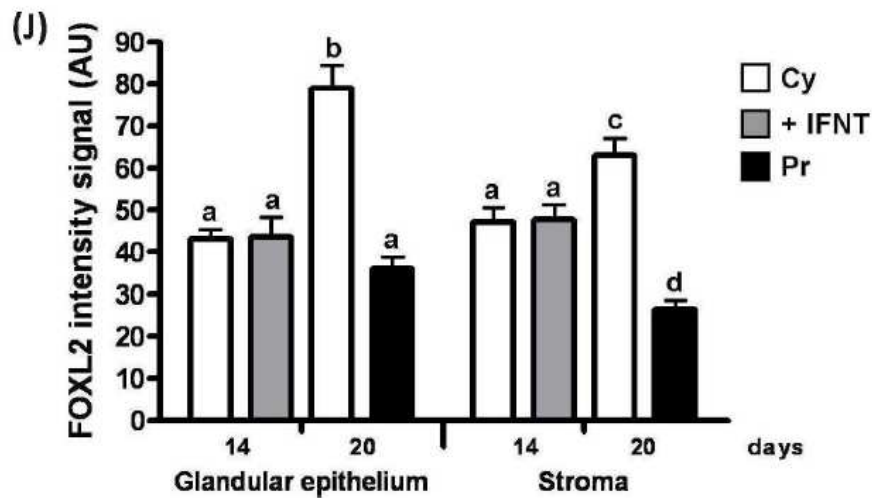
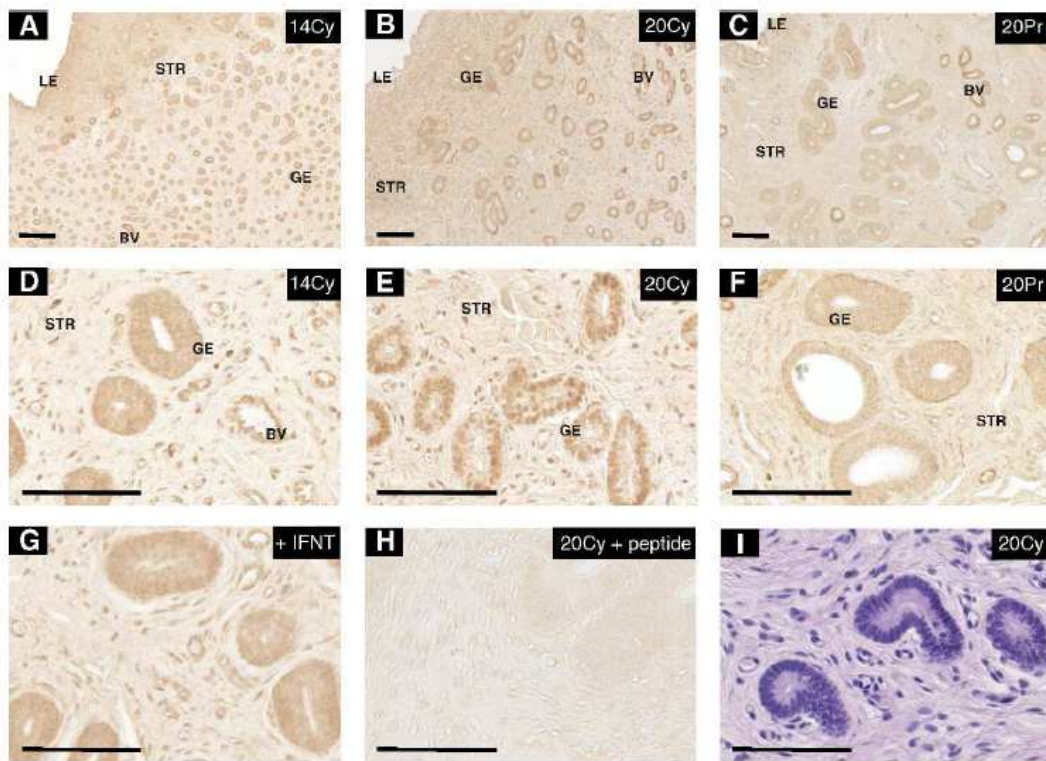


FIG. 4. FOXL2 localization in bovine endometrium during the estrous cycle and at implantation. Immunohistochemistry was performed using sections cut from Charolais endometrium. Females were sampled on Day 14 (14Cy [A and D]), on Day 14 with intrauterine infusion of IFNT (+IFNT [G]), or on Day 20 of the estrous cycle (20Cy [B, E, H, and I]), and on Day 20 of pregnancy (20Pr [C and F]). A–G show tissues incubated with anti-FOXL2 antibody, whereas H shows tissues co-incubated with the anti-FOXL2 antibody and blocking peptide as a negative control. Samples in I were incubated in hematoxylin-eosin solution. Images D–F show higher magnifications of FOXL2 localization shown in A–C, respectively. BV, blood vessel; GE, glandular epithelium; LE, luminal epithelium; STR, stroma. Bars = 100 μ m. K) Quantification of FOXL2 immunohistochemistry intensity signal was processed in glandular epithelium and stroma areas. Quantitative data are means \pm SEM, and bars with different lowercase letters differ significantly ($P < 0.01$).

production of estradiol [53]. Therefore, *FOXL2* appears to be expressed in human endometrium and regulated by ovarian steroid balance. Collectively, these data and our results strongly suggest that *FOXL2* gene expression is negatively regulated by P4 in mammals, and they suggest the need for a detailed regulation of this transcription factor by steroids.

In our study, changes in *FOXL2* expression level were associated with distinct patterns of cell and subcellular localization throughout the estrous cycle and at the initiation of implantation. In endometrium sampled from cyclic cows during the luteal (Day 14) and follicular (Day 20) phases, *FOXL2* was always detectable in the nuclei of stroma cells, whereas nuclear localization in glandular cells was restricted to Day 20. In pregnant endometrium collected when implantation was initiated (Day 20), *FOXL2* was undetectable in the nuclei of stroma and glandular epithelium cells. Interestingly, *FOXL2* has been reported to be the target for post-translational modifications, including sumoylation, acetylation, or phosphorylation, which are associated with changes in *FOXL2* subcellular localization [54, 55]. In transfected cell lines, protein inhibitor of activated STAT-1 (PIAS1) has been shown to interact with *FOXL2*, leading to the sumoylation of *FOXL2* by SUMO1 and UBC9, two proteins of sumoylation machinery [45, 56]. Using several mutated *FOXL2* proteins, Marongiu et al. [45] also demonstrated that sumoylation site-mutated *FOXL2* exhibited neither nuclear localization nor transcriptional activity. In the context of endometrium, sumoylation of proteins has been reported [57] and may represent a key process for the regulation of *FOXL2* intracellular localization along the estrous cycle. Additional analyses of sumoylation machinery regulation (SUMO and PIAS proteins) will be required in order to validate this assumption, as well as to identify *FOXL2* binding partners in mammalian endometrium.

In the context of ovarian development and physiology, microarray analyses and various experimental models have been used to identify *FOXL2*-regulated genes. Studies using human granulosa cell line (KGN) overexpressing *FOXL2* [39] and *Foxl2* knock-out mouse strains [26, 58] led to the identification of potential *FOXL2* target genes including *MNSOD/SOD2*, *RGS2*, and *COX2/PTGS2*, which are known to be involved in apoptosis, stress response, gonadotropin responsiveness in granulosa cells, and ovulation [38, 59, 60]. Interestingly, these three *FOXL2*-regulated genes have also been unveiled in transcriptome analyses carried out with bovine endometrium collected from pregnant and nonpregnant females [13, 18]. *PTGS2* has been well characterized in ruminant endometrium [61, 62], and its expression is induced by pregnancy-associated factors such as IFNT. In ovine and baboon endometrium [63, 64], *SOD2* expression and regulation suggest that stimulation of *SOD2* gene expression may improve pregnancy establishment by enhancing antioxidant defenses [65]. *RGS2* expression has been shown to be restricted to implantation sites in the murine pregnant uterus [60]. In a *FOXL2*-overexpressing KGN cell line, *PTGS2*, *SOD2*, and *RGS2* transcript expression was shown to be up-regulated [39], whereas the ovarian expression of these three genes was down-regulated in *Foxl2* knock-out mouse strain [58]. On the other hand, in the endometrium, the high expression level reported for *PTGS2*, *SOD2*, and *RGS2* is associated with a low level of *FOXL2* during the establishment of pregnancy. The discrepancy between the regulation of these genes and *FOXL2* levels according to the ovarian and endometrial context deserves to be further investigated in order to clarify the interplay between *FOXL2* and its target genes as well as the contribution of this transcription factor in regulating the physiology of the endometrium, a key tissue for pregnancy outcome [66].

Forkhead box genes have evolved to acquire specialized functions in various key biological processes [19], and several members of this family such as *FOXA1*, *FOXA2*, and *FOXO1* have been investigated in the human and murine uterus [19, 67]. A recent high-throughput study by Shimizu et al. [68] reported the expression of *FOXA* transcription factors in bovine endometrium, but, overall, the expression, regulation, and biological actions of FOX transcription factors have been poorly analyzed in the endometrium of ruminants. Using bovine endometrium collected during the estrous cycle and early pregnancy, the present study has demonstrated the expression, regulation, and cell localization of *FOXL2*, a gene first reported as an essential factor for ovarian differentiation [31, 36, 38]. In keeping with preliminary elements available from human endometrial gene profiles [49, 50], our data provide strong arguments for the involvement of *FOXL2* in regulating endometrial physiology and support this transcription factor as a key gene in female reproduction.

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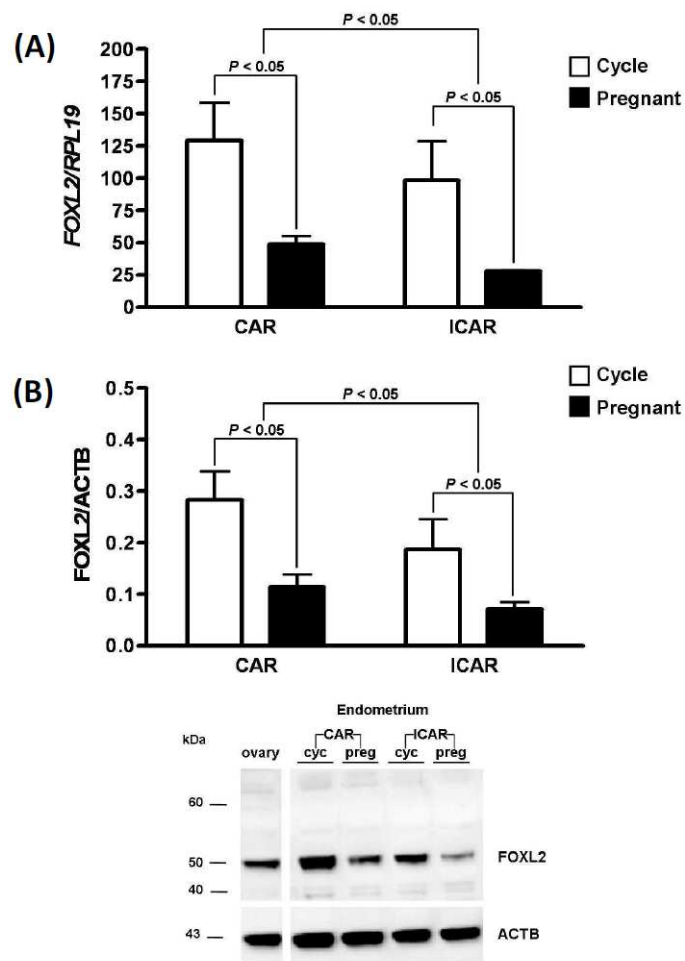
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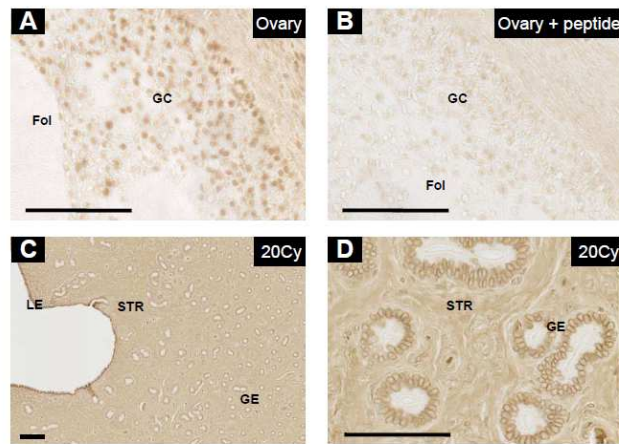
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Supplemental Figure S1. FOXL2 expression in cyclic and pregnant endometrium collected from cows (Charolais breed) at Day 20. Caruncular (CAR) and intercaruncular (ICAR) endometrial areas were collected from cyclic (cyc, n = 5) and pregnant (preg, n = 5) Charolais cows at Day 20. A) Quantification of FOXL2 mRNA by RT-qPCR. Expression of FOXL2 was normalized to RPL19. B) Quantification of FOXL2 protein by Western blotting. FOXL2 expression was normalized to ACTB protein level. Ovary was used as a control tissue. Quantitative data are presented as mean ± SEM.



Supplemental Figure S2. FOXL2 localization in bovine ovary and LAMINB localization in bovine cyclic endometrium at Day 20. Immunohistochemistry was performed using sections cut from Charolais ovary (A and B) and endometrium (C and D). Section A was incubated with the anti-FOXL2 antibody. Section B was co-incubated with the anti-FOXL2 antibody and the blocking peptide as a negative control. Sections C and D were incubated with the anti-LAMINB antibody, with section D representing a higher magnification of LAMINB localization reported in section C. Fol, follicle; GC, granulosa cells;

VI. Part II: *FOXL2* expression is regulated by progesterone in ruminant endometrium

Publication in progress

A. Introduction

In our first publication, the endometrial gene expression of *FOXL2* was precisely evaluated during the oestrous cycle and early pregnancy in cattle. We concluded that *FOXL2* gene expression was not regulated by IFNT but was negatively correlated with P4 blood level.

The uterine receptivity results from the combine action of E2 and P4 during the oestrous cycle. In particular, E2 induces proliferation of endometrial cells whereas P4 regulate maturation and secretory activities of endometrial gland, the expression of various endometrial genes and balance the immune response prior to the apposition of the conceptus on endometrium (and decidualisation when occurs). In this chapter, we aimed to dissect precisely the involvement of ovarian steroids on *FOXL2* endometrial expression.

We used ovine and bovine experimental models as relevant approaches to dissect the steroid hormones regulation of *FOXL2* endometrial expression. Sheep and cattle are closely related ruminant species with similar features in term of oestrous cycle and pregnancy. In sheep, the oestrous cycle lasts 15-16 days with a short follicular phase (2-3 days) and a long luteal phase (14-16 days). During early pregnancy, the ovine conceptus undergoes an elongation phase associated with an IFNT secretion indispensable for maternal recognition of pregnancy prior to implantation at day 15-16.

First, we examined the ovine endometrial expression of *FOXL2* during oestrous cycle and at implantation when occurs. This physiological model provides us a kinetic of *FOXL2* expression during early luteal phase (4 dpo), active luteal phase (8 and 12 dpo) and follicular phase (15 dpo) of the oestrous cycle compared to the implantation day (15 dpo).

Then, we investigated the impact of altered P4 circulating level on *FOXL2* expression in ovine endometrium. Pregnant ewes were daily treated for 11 days with trilostane, an inhibitor of the 3- β HSD activity, preventing therefore the conversion of pregnenolone into P4. This ovine experimental model shows that the lower concentration of P4 did not affect conceptus morphology nor pregnancy rates as determined at 16 dpo but led to changes in endometrial genes expression including *FOXL2* mRNA level.

In the third experiment, we investigated the action of ovarian steroids alone or in combination on *FOXL2* endometrial gene expression. Ovariectomized ewes were supplemented with E2, P4 or both for 12 days at physiological rates in order to mimic the ovarian oestrous cycle and its effect on endometrium. Two control groups were used namely ovariectomized ewes (OVX) and cyclic ewes at 12 dpo. In cattle, a recent high through-put study reported endometrium collected from ovariectomized cows supplemented with E2 and/or P4 (Shimizu

et al. 2010). Among the differentially expressed genes, several FOX factors were listed including *FOXA2*, a major transcription factor involved in uterine gland development (Filant *et al.*, 2013; Shimizu et al. 2010). We established collaboration with Drs Akio Miyamoto and Stefan Bauersachs and we analysed *FOXL2* transcript expression in this bovine model in order to add some comparative aspects between both species of ruminant. Ovariectomized animals brought new insights about the specific action of each steroid on *FOXL2* expression regardless the impact of ovary secretion.

Eventually, we analysed *FOXL2* mRNA expression in endometrial explants incubated with P4 or E2 for 48 hours. This experiment provides us additional results about the steroid regulation of *FOXL2* expression because explants are biopsies of the entire organ preventing the autocrine and paracrine actions of this complex tissue.

B. Conclusion

This second part of my work has brought new insights on the regulation of *FOXL2* gene expression by the ovarian steroid hormones in ruminants.

FOXL2 gene is expressed during the oestrous cycle and at implantation in sheep endometrium. In keeping with our data in the bovine endometrium, *FOXL2* gene expression is higher during the early luteal phase (4 dpo) of the oestrous cycle and the follicular phase (15 dpo) compared to the lowered expression during the luteal phase of the oestrous cycle (8 and 12 dpo) and at implantation (15 dpo). Our data exhibit a similar expression pattern of *FOXL2* between ovine and bovine endometrium. Moreover, ovine and bovine *FOXL2* expression is always significantly lower in the ICAR area compared to the CAR area.

In order to compare our results in bovine endometrium, immunohistochemistry was performed on ovine endometrium slide using the same *FOXL2* antibody described in the first publication (Fig. 39).

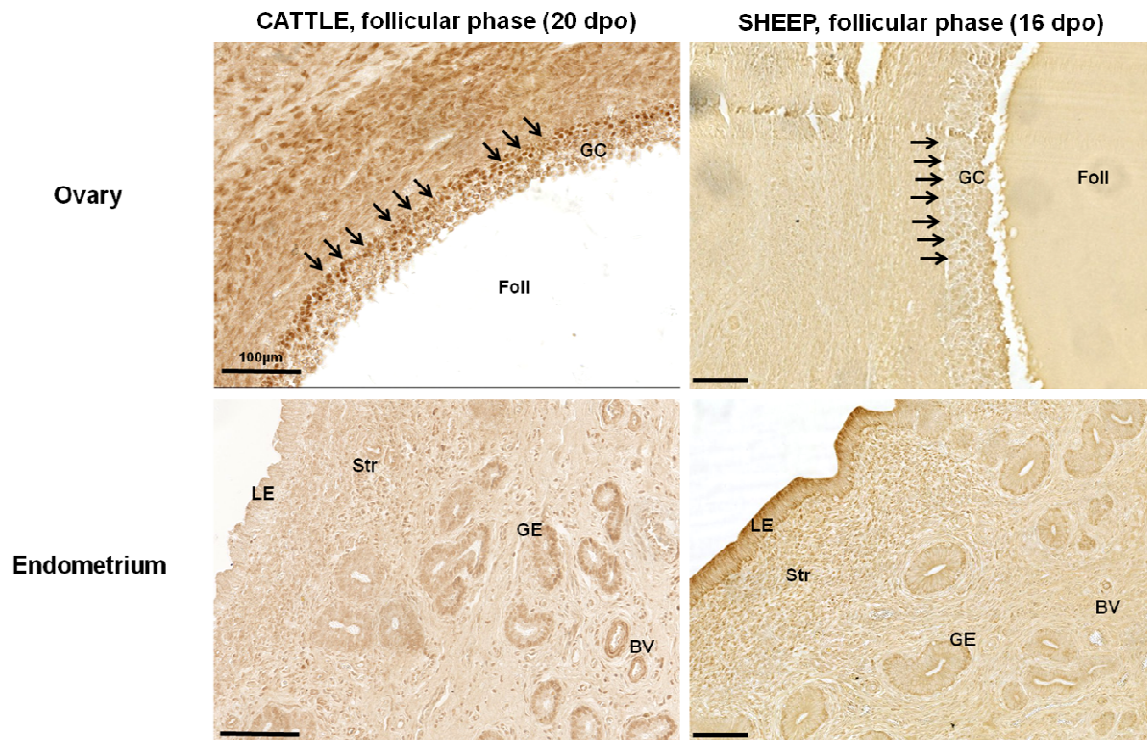


Figure 39: FOXL2 localization in ovary and endometrium of both cattle and sheep during the follicular phase. Foll: follicle; GC: granulosa cells; LE: luminal epithelium; GE: glandular epithelium; Str: stroma and BV: blood vessel. Bovine ovary exhibits a clear nuclear staining of FOXL2 in granulosa cells (black arrowheads), around the follicle whereas ovine ovary does not show FOXL2 localization in the granulosa cells (black arrowheads). Similarly, FOXL2 is detected in the nucleus of glandular epithelium and stroma in bovine endometrium whereas FOXL2 staining appears to be non-specific in ovine endometrium. Black scale bar: 100µm.

Immunohistochemistry was performed on endometrial samples collected during the follicular phase when *FOXL2* expression is higher. *FOXL2* protein was not detected in sheep ovary that was used as a positive control tissue. This result is quite amazing and disappointing since ovine and bovine *FOXL2* primary sequence on the C-terminus side are 100% identical. A 16 amino acids peptide was injected in rabbits to generate the antibody. We can suppose that the three dimensional structure of *FOXL2* protein is slightly different in sheep compared to cattle and our antibody cannot stick to the same epitope than in cow. The identification of intracellular localization of *FOXL2* in ovine endometrium is essential to understand the biological action of this transcription factor. Nuclear localization of *FOXL2* is associated to a functional protein whereas cytoplasmic localization means this transcription factor cannot bind its response element that drives the expression of its target genes. Others antibodies will be tested in order to define the change of *FOXL2* cell localization during the oestrous cycle, early pregnancy and upon ovarian steroid hormones in ovine endometrium.

During early pregnancy, the reduced production of P4 consecutive to the trilostane treatment (Fig. 40A) did affect neither pregnancy rate nor the development of the conceptus up to

implantation (16 dpo) and led to the increase of *FOXL2* mRNA expression in the endometrium. Our data show an increased expression of *FOXL2* transcript associated with a lower P4-circulating level.

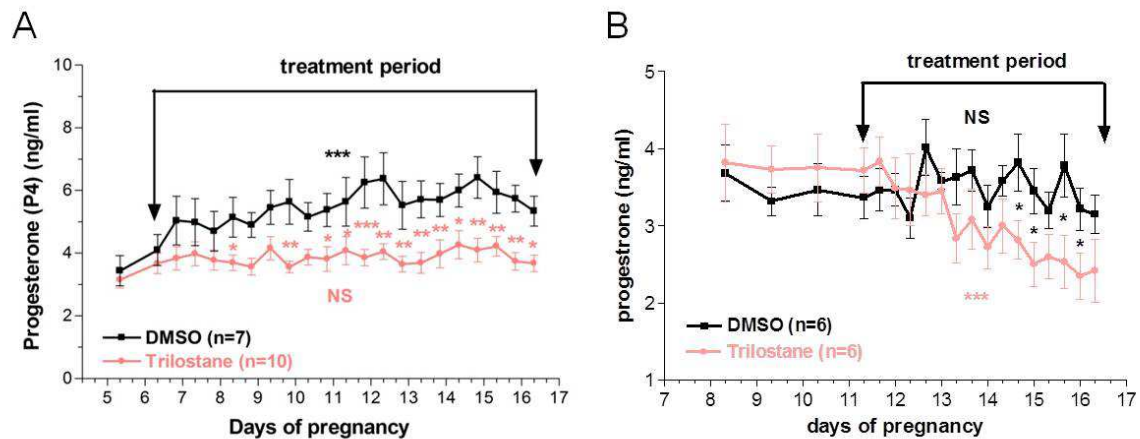


Figure 40: Monitoring of plasmatic concentration of P4 following long and short term treatment of Trilostane (Camous *et al.*, submitted). (A) Trilostane treatment lasts 11 days from 5 dpo to 16 dpo of pregnancy and significantly prevents the rise of P4 circulating level compared to the control group ($p < 0.001$, DMSO). (B) Trilostane treatment lasts 5 days from 11 dpo to 16 dpo of pregnancy and significantly reduces P4 circulating level ($p < 0.001$). (A) and (B) uteri were collected at 16 dpo of pregnancy.

A short term treatment of trilostane was carried out with 5 days of treatment from 11 dpo to 16 dpo of pregnancy (Fig. 40B and Fig. 41). This second experiment aimed to decrease P4 production during the luteal phase whereas the first experiment aimed to prevent the P4 rise during the early luteal phase. The short-term treatment did not prevent also the development of the conceptus up to the implantation day (16 dpo).

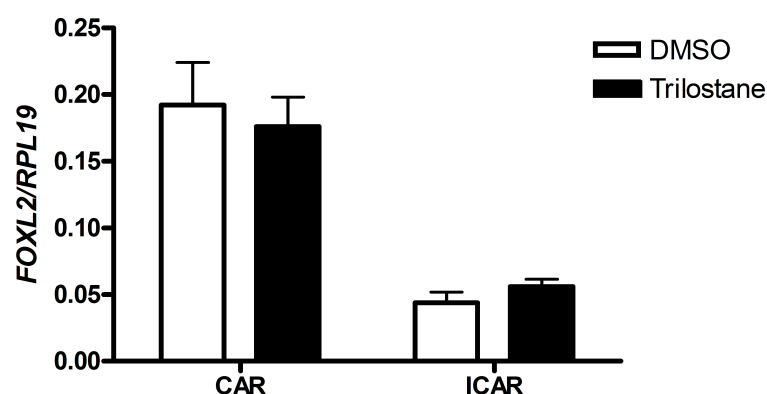


Figure 41: Regulation of *FOXL2* gene expression under the influence of altered P4 circulating level in ovine endometrium. Caruncular (CAR) and intercaruncular (ICAR) endometrial areas were collected from pregnant Pré-alpes du sud ewes treated with DMSO as a control solution (n=7) or trilostane (15 mg/ewe in 1 ml DMSO) for 5 days. Quantification of *FOXL2* mRNA by RT-qPCR. Expression of *FOXL2* was normalized to *RPL19*. Quantitative data are presented as mean \pm SEM.

The short-term treatment of trilostane does not impact the endometrial expression of *FOXL2* at 16 dpo of pregnancy (Fig. 40). However, the differential expression of *FOXL2* is still significant between CAR and ICAR areas ($p < 0.01$).

OVX ewes exhibited a significant increased expression of endometrial *FOXL2* compared to cyclic ewes. E2 supplementation did not impact *FOXL2* gene expression compared to the OVX ewes except in ICAR area where *FOXL2* protein expression is significantly higher suggesting an important E2-responsive function of *FOXL2* in glandular epithelium enriched area. However, P4 treatment inhibits clearly *FOXL2* gene expression compared to the [OVX] and [OVX + E2] groups of ewes. The combine action of E2 and P4 led to an intermediate-level of *FOXL2* gene expression. Similarly, OVX cows exhibited a significant lower expression of endometrial *FOXL2* following [P4] treatment compared to [E2] treatment.

Ex vivo endometrial explants cultivated for 48 hours with control media, E2 or P4 reveal a decreased expression of *FOXL2* transcript under the P4 supplementation.

The regulation of *FOXL2* endometrial expression by E2 remains unclear. In the [OVX+E2] ewes, *FOXL2* expression was similar to the one reported in that OVX ewes suggesting only P4 can alter its expression. P4 has clearly a major impact on *FOXL2* expression but this does not mean E2 has not. We hypothesized the regulation under the E2 treatment could be obscured or compensated by autocrine or paracrine regulation. Further *in vitro* analyses will be necessary to describe precisely the complex mechanisms of the steroid regulation on *FOXL2* endometrial expression.

Collectively, *FOXL2* gene expression in ovine uterus is similar to the one reported in that bovine uterus. In addition, *in vivo* and *ex vivo* data have demonstrated a strong down-regulation of the *FOXL2* expression by P4 in both species of ruminant.

Our first hypothesis was *FOXL2* could be a direct target of P4 meaning that *FOXL2* could be a direct target of *PGR* form A and B genes. In the endometrial explants cultivated with E2, P4 or control media for 48 hours, *PGR* transcript expression was also investigated (Fig. 42).

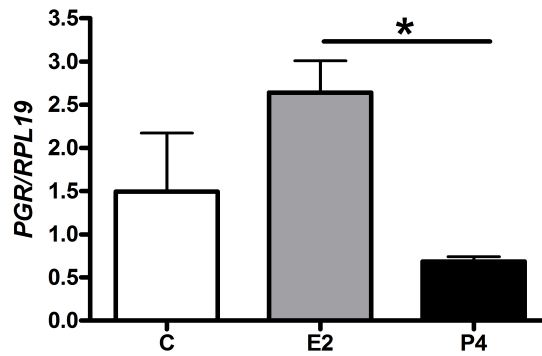


Figure 42: *PGR* expression in endometrial explants cultivated with control media (C), E2 or P4 for 48 hours. Intercaruncular areas were collected from cyclic cows at estrus stage, supplemented with a control solution (OVX, n=2), P4 solution (OVX + P4, n=2) or E2 solution (OVX + E2, n=2). Quantification of *PGR* mRNA by RT-qPCR. Expression of *PGR* was normalized to *RPL19*. Quantitative data are presented as mean \pm SEM and significant differences were noted using * : $P < 0.05$.

PGR transcript expression is significantly higher in the E2 treated explants compared to the P4 treated group (Fig. 42). This result supports former results that E2 stimulates and P4 inhibits the expression of *PGR* in the bovine endometrium (Spencer and Bazer 2002). P4 induces a down-regulation of its own nuclear receptor expression and at the same time, *FOXL2* expression. *PGR* protein was shown to be localized in the nuclei of stromal and either luminal or glandular epithelial cells during follicular phase (Fig. 43; Okumu et al. 2010). The active luteal phase induces changed in cell distribution of this transcription factor. *PGR* protein disappeared during the luteal phase in luminal and glandular epithelia associated with a weak localization in the nuclei of stromal cells (Okumu et al. 2010). Our previous work showed that *FOXL2* protein is detected in the nuclei of stromal and glandular epithelial cells during follicular phase and disappeared from the nuclei of glandular epithelial cells during the luteal phase (Eozenou et al. 2012). In addition, *FOXL2* is localized in the cytoplasm of stromal and glandular epithelial cells at implantation meaning the presence of the conceptus induces a change in sub-cellular localisation of *FOXL2* in stromal cells (Eozenou et al. 2012).

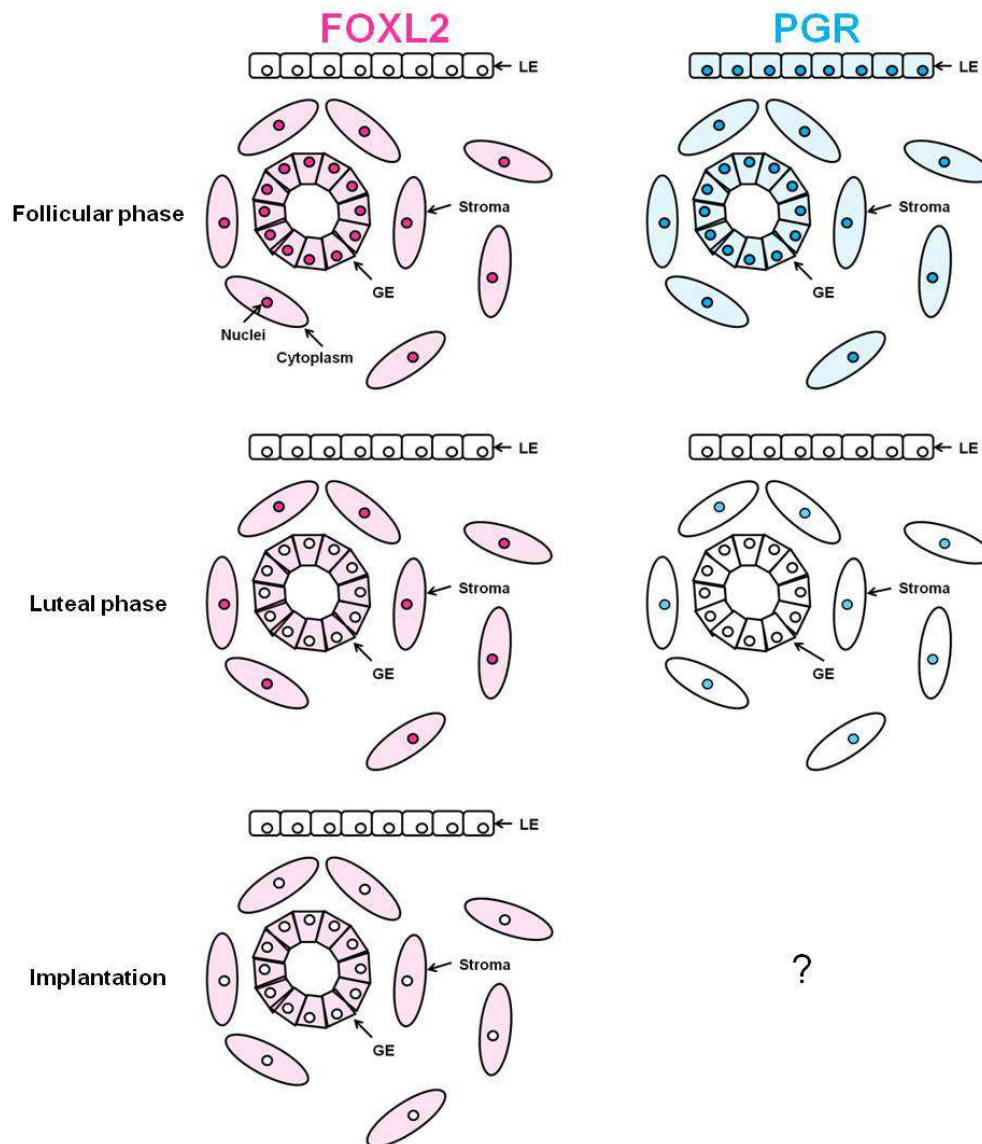


Figure 43: Schematic representation of FOXL2 and PGR proteins distribution in bovine endometrium. FOXL2 and PGR proteins are respectively in pink and blue colors. FOXL2 protein is not detected in the luminal epithelium (LE) whereas the glandular epithelium (GE) and stroma exhibit a nuclear staining (dark pink) during the follicular phase. The luteal phase leads to a nuclear localization of FOXL2 in stroma and cytoplasmic (pale pink) localization in GE. Implantation leads to a cytoplasmic localization of FOXL2 either in stroma or GE (Eozenou et al. 2012). PGR localization is nuclear (dark blue) in LE, GE and stroma during the follicular phase. The luteal phase leads to a cytoplasmic (pale blue) PGR localization in GE and a weak nuclear localization in stroma (Okumu et al. 2010).

In conclusion, PGR and FOXL2 proteins were shown to display the same intracellular localization in stromal and glandular epithelial cells in bovine endometrium. In addition, *FOXL2* promoter sequence from cattle, goat and mouse species exhibited a consensus sequence for Progesterone Response Element (PRE, Appendix C, Fig. 71). Altogether, our data showed strong arguments that P4 could regulate directly *FOXL2* transcriptional activity using its nuclear receptor. In order to confirm or overturn our theory, we designed an *in vitro*

experiment based on transient transfection of *FOXL2* promoter and *PGR* form A and B (courtesy of P. Chambon) genes treated with control media or P4 (Fig. 44).

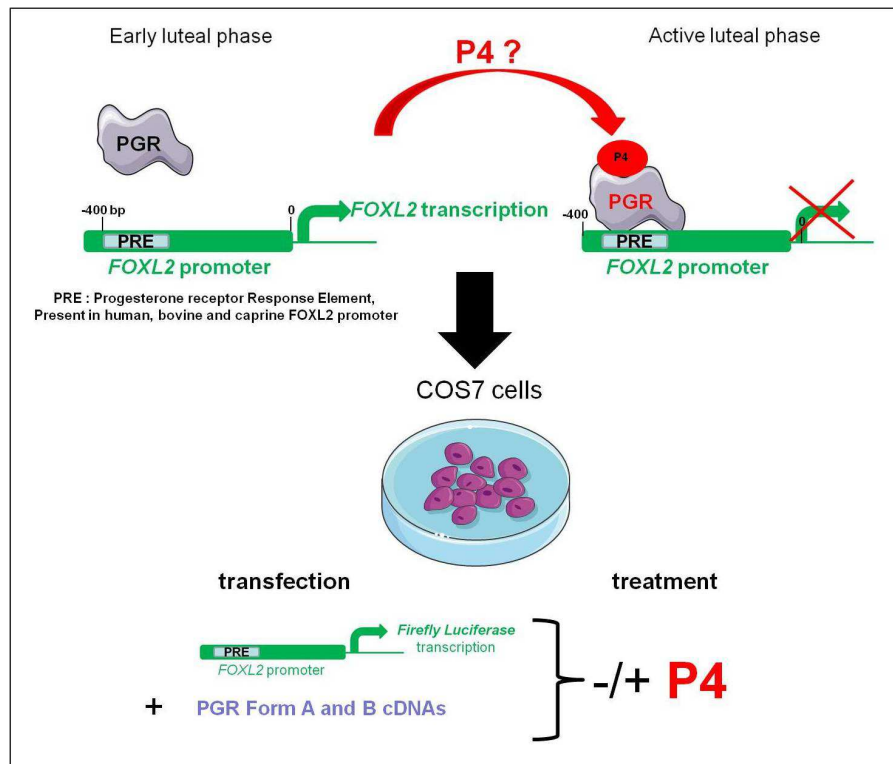


Figure 44: Hypothesis and experimental design of PGR action on FOXL2 promoter in COS7 cell line. COS-7 cell line will be transfected using expressing vector of PGR form A and B associated with FOXL2 caprine promoter coupled to the luciferase gene treated with control media or P4.

Presently, experiments are still in progress.

Collectively, our results in bovine and ovine endometrium provide the evidence that FOXL2 is strongly down-regulated by progesterone circulating level throughout the oestrous cycle and early pregnancy. We suggest that PGR activated by P4 could act directly on FOXL2 promoter to inhibit its expression during the luteal phase and the early pregnancy. Further investigation about the direct action of P4 on FOXL2 transcriptional activity will provide us strong argument about the tightly regulation of FOXL2 endometrial expression in ruminant.

C. *Publication*

FOXL2 gene expression is inhibited by progesterone in ruminants

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Running title: FOXL2, P4 and ruminant endometrium

Summary sentence: FOXL2 is expressed in ovine endometrium; the expression is repressed by P4.

Keywords: FOXL2, endometrium, sheep, cattle, progesterone

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ABSTRACT

FOXL2 -a member of the FOXL sub-class and a key transcription factor for ovarian differentiation- is regulated during early pregnancy in bovine endometrium. Interferon-Tau did not regulate *FOXL2* gene expression but the impact of ovarian steroids was not investigated. In the present study, we analysed the contribution of steroids hormones in the regulation of *FOXL2* gene expression in ruminants using physiological and experimental models derived from cattle and sheep. In sheep, we confirmed that *FOXL2* mRNA and protein are expressed in the ovine endometrium across the oestrous cycle (day 4 to day 15 post-oestrus (dpo)). In keeping with our former report in bovine endometrium, ovine *FOXL2* endometrial expression was low during the luteal phase of oestrous cycle (day 4 to 12 post-oestrus) and at implantation (15 dpo) while mRNA and protein expression significantly increased during the luteolytic phase (day 15 post-oestrus in cycle). In pregnant ewes, inhibition of progesterone production (P4) by trilostane (an inhibitor of 3 β -hydroxysteroid dehydrogenase activity) during the 5-16 dpo period prevented P4 rise and led to a significant increase of *FOXL2* transcript expression in caruncles compared to the control group (1.4-fold, $P < 0.05$). Ovariectomized ewes or cows supplemented with exogenous P4 for 12 days or 6 days respectively exhibited a significant inhibition of endometrial *FOXL2* gene expression compared to control ovariectomized females (sheep, mRNA, 1.8-fold; protein, 2.4-fold; cattle; mRNA, 2.2-fold; $P < 0.05$). Exogenous 17 β -estradiol (E2) treatment for 12 days in sheep or 2 days in cattle did not affect *FOXL2* endometrial expression compared to control ovariectomized females. Moreover, bovine endometrial explants cultivated for 48 hours with exogenous P4 were shown to decrease *FOXL2* expression. In contrast with data reported in mammalian ovary, *FOXL2* gene expression does not appear to be E2-dependant in the endometrium. In ruminants, *FOXL2* expression during the luteal phase of the oestrous cycle and early pregnancy are associated to a low level of P4. Our current findings highlight P4 as a negative regulator of *FOXL2*.

INTRODUCTION

In mammals, successful pregnancy is characterized by an efficient cross-talk between the endometrium and the conceptus (embryonic disk and extra-embryonic tissues) that takes place during the peri-implantation period (Lee and DeMayo 2004; Bazer et al. 2010). By affecting the endometrial function during this period, environmental factors including infections, stress, nutrition or endocrine disruptors can alter these interactions leading to early pregnancy failures or altered progression of pregnancy to term (Sandra et al. 2011). In cattle and more specifically in dairy cows, half of pregnancies abort very prematurely as a consequence of early embryonic death (Diskin and Morris 2008). Therefore, the peri-implantation period is a critical checkpoint for the progression as well as the issue of pregnancy (Bazer et al. 2010; Sandra et al. 2011) and prompts the need for accurately defining the molecular mechanisms involved during this period.

In mammalian oestrous and menstrual cycles, endometrium has to be receptive to host the pre-implantatory conceptus. The receptive status of the endometrium requires the combine action of gonadal steroid hormones, estrogens (E2) and progesterone (P4, Miller and Moore 1976; Devroey and Pados 1998; Al-Gubory et al. 2008; Ozturk and Demir 2010). Estrogens are secreted during the follicular phase that cover a short period of time in ruminants 4 to 5 days (Forde et al. 2011) compared to the long follicular phase described in humans (14 days, Hawkins and Matzuk 2008; Mihm et al. 2011). In ruminants, the short-time secretion of E2 is associated to the absence of endometrial growth (Forde et al. 2011) whereas these steroids regulate the proliferation and the growth of the endometrial cells during the follicular phase to allow the deep invasion of human conceptus when pregnancy occurs (Hawkins and Matzuk 2008; Mihm et al. 2011). The uterine receptivity requires the pleiotropic actions of progesterone, produced by the corpus luteum and required in mammals for the maternal support of conceptus survival and elongation (Spencer et al. 2008; Bazer et al. 2012). Unlike human and rodents species, decidualisation does not occur in ruminant (Lee and DeMayo

2004) leading to a synepitheliochorial implantation associated to a superficial invasion of the conceptus (Lee and DeMayo 2004).

However, P4 controls the endometrial gland differentiation and secretion (histotroph; Lonergan 2011) as well as endometrial angiogenesis (Bazer et al. 2009). P4 has been shown to be permissive to interferon-tau (IFNT) actions, prior to the conceptus implantation (Spencer et al. 2004). IFNT is produced by the trophoctoderm of elongated conceptus and this factor is the major signal of pregnancy maternal recognition in ruminants (Martal et al. 1979; Roberts et al. 2008; Spencer et al. 2008). IFNT acts on the endometrium to prevent development of the luteolytic mechanism and to establish the pregnancy in ruminants (Bott *et al.*, 2010; Bazer et al. 2012). The combine biological action of P4 and IFNT are mandatory for the pregnancy outcome and prompt the need for deciphering their complete actions on endometrial physiology.

In the last decade, microarrays and RNA-sequencing analyses in ruminant endometrium have allowed a genome-wide understanding of endometrial genes involved in endometrial physiology during oestrous cycle and early pregnancy up to implantation (Bauersachs et al. 2008; Satterfield et al. 2009; Bauersachs and Wolf 2012; Ulbrich et al. 2012). Using bovine endometrium at 20 days of oestrous cycle (that corresponds to the follicular phase) and pregnancy (initiation of implantation), the expression of various families of transcription factors was unveiled including several members of the winged-helix/forkhead domain (FOX) transcription factors family (Mansouri-Attia et al. 2009). Based on this analysis, we further characterized the endometrial expression and regulation of *FOXL2* (Eozenou et al. 2012), a key gene involved in ovarian differentiation and maintenance of ovarian function from foetal life to adulthood (Pannetier and Pailhoux 2011; Georges et al. 2013). In our previous study, we demonstrated that IFNT had no effect on *FOXL2* expression whereas high P4 circulating level observed during the luteal phase was negatively correlated with *FOXL2* endometrial expression (Eozenou et al. 2012). The present study aims to evaluate the role of ovarian

steroid hormone balance on *FOXL2* expression in the endometrium of ruminants using physiological situation as well as experimental models.

MATERIALS AND METHODS

Animal and cell cultures

All experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction and approved by the French Ministry of Agriculture according to French regulations for animal experimentation (authorization no. 78-34).

Experiment 1: *FOXL2* expression during the oestrous cycle and at implantation in ovine endometrium.

Cyclic and pregnant ewes of the Préalpes-du-Sud breed are involved in this study and were synchronized and treated as previously published (Al-Gubory et al. 2006, society for endocrinology). Firstly, twelve ewes were randomly separated to three groups (n = 4 ewes per group): day 4, day 8 and day 12 of the oestrous cycle corresponding respectively to the early, mid and active luteal phase. Secondly, eight ewes were randomly separated to two groups (n = 4 ewes per group): day 15 of the oestrous cycle and pregnancy corresponding respectively to the late luteal phase/follicular phase and implantation step. Uteri were collected, flushed and, when present, recovered *concepti* were observed by microscopy to confirm the stage of development (Degrelle et al. 2005). Endometrial caruncular (CAR) and intercaruncular (ICAR) areas were dissected from the uterine horns ipsilateral to the corpus luteum as previously described (Mansouri-Attia et al. 2009).

Experiment 2: Impact of steady progesterone concentration at low level during early pregnancy on ovine *FOXL2* endometrial expression.

The aim of this experiment was to evaluate the effect of a progesterone concentrations reduction during the pre-implantation period on endometrial gene expression at day 16 which

is the initiation of implantation in ewes. Seventeen ewes received either subcutaneous injections of DMSO (n = 7) or Trilostane (n = 10) as previously described (Camous et al. 2013; submitted). Trilostane (15 mg/ewe in 1 ml DMSO) or DMSO were injected every 12 h (08:00 AM and PM) to the ewes from day 6 to day 16 (only at 08:00 AM). Endometrial tissues and concepti were collected as previously described above.

Experiment 3: Impact of steroidal hormone on ruminant FOXL2 endometrial expression.

Ovine ovariectomy

Sixteen ewes of the Préalpes-du-Sud breed were ovariectomized (OVX) as previously described (Al-Gubory et al. 2008). At forty-two days after ovariectomy, all ewes were randomly separated to four groups (n = 4 ewes per group): control ewes (OVX), E2-treated (OVX + E2), P4-treated (OVX + P4), and E2/P4-treated (OVX + E2 + P4) ewes as published (Al-Gubory et al. 2008). This steroid hormone administration protocol has been shown to produce physiological blood concentrations of E2 and P4 (Beard et al. 1994) corresponding to those during the follicular and luteal phases in intact cyclic ewes (Pant et al. 1977). All steroid hormones treatment was administrated in 1 ml of 90% corn oil: 10% ethyl alcohol at intervals of 8 h by intramuscular injection as previously published (Al-Gubory et al. 2008). Blood (monitoring steroid hormone level) and endometrial tissue (CAR and ICAR areas) were collected as described (Al-Gubory et al. 2008).

Bovine ovariectomy

Twelve Holstein cows (3-7 year of age) were ovariectomized (OVX) and after forty-to-fifty days randomly divided into four groups as previously published (Shimizu et al. 2010), the OVX group (n=3, saline solution), OVX + E2 group (n=3, estrogens treatment), OVX + P4 group (n=3, progesterone treatment) and OVX + E2+P4 group (n=3, combination of estrogens and progesterone treatment). Blood (monitoring steroid hormone level) and endometrial tissue (ICAR area) were collected after steroid treatment as described previously

(Shimizu et al. 2010). Endometrial genes expression were analysed in OVX cows treated with estradiol and/or progesterone using microarray analysis (Shimizu et al. 2010).

Bovine endometrial explants

Endometrial explants were dissected in the ICAR area from two cows in the late oestrus stage using a sterile 8-mm punch biopsy and cultured *ex vivo* with control media, E2 media (3pg/ml) and P4 media (5ng/ml) for 48 hours. Explants were then stored at -80°C in TRIZOL reagent prior to RNA extraction.

Tissue and Cell Collection, Preparation for Immunohistochemistry and RNA Extraction

Endometrial samples were immediately snap frozen in liquid nitrogen and stored at -80°C prior to analysis (Eozenou et al. 2012). Cells lamellas were fixed in frozen methanol for 5 min, then washed in PBS 1X and stored at 4°C. At the same time, sections of uterine horn were fixed in 4% paraformaldehyde in phosphate buffer, then washed in PBS 1X and embedded in paraffin as published (Al-Gubory et al. 2008) and subsequently stored at 4°C. Whole uterine cross sections were cut (7 µm) with a rotary microtome Leica RM2245 and stored at room temperature until processed. Total RNA from frozen tissue was isolated by homogenization using Trizol Reagent (Invitrogen, Cergy-Pontoise, France) and purified as published (Eozenou et al. 2012). Total RNA from endometrial cells was extracted with the phenol-based method (Chomczynski and Sacchi 1987).

Real-time RT-PCR

Total RNA samples were used for the quantitative RT-PCR (RT-qPCR). One µg (for the tissue sample) and five hundred ng (for the cell sample) of the total RNA was reverse transcribed into cDNA as previously described (Eozenou et al. 2012). Primers (Eurogentec, Liège, Belgium) were designed (Primer Express Software v2.0, Applied Biosystems) to specifically amplified bovine and ovine *FOXL2* (F – CCGGCATCTACCAGTACATTATAGC; R – GCACTCGTTGAGGCTGAGGT; NCBI sequence reference: NM_001031750.1) and *RPL19*

(F – CCCCAATGAGACCAATGAAATC; R – CAGCCCATCTTTGATCAGCTT, Mansouri-Attia et al. 2009). Amplified *FOXL2* and *RPL19* PCR fragments were sequenced to assess the amplification of the correct fragment. According to the relative standard curve method (Larionov et al. 2005), the relative quantification of mRNA amount of *FOXL2* against the normalizer gene *RPL19* was calculated.

Western Blot Analysis

Total proteins were extracted from frozen tissue as previously published (Eozenou et al. 2012).

Western blot immunoassays were processed with 15 µg of total protein extract as previously described (Eozenou et al. 2012), using a rabbit anti-FOXL2 purified antibody generated against a peptide corresponding to the C-terminal conserved region of mammalian FOXL2 (WDHDSKTGALHSRLDL, CASLO Laboratory, Denmark; diluted 1:500) in PBS-T solution containing 4% nonfat dry milk and then, a goat peroxidase-conjugated anti-rabbit IgG antibody (SantaCruz Biotechnology; Heidelberg, Germany; diluted 1:5 000). Actin B (ACTB) was assessed as a loading control, using a mouse monoclonal anti-ACTB antibody (Sigma-Aldrich, France; diluted 1:2 000) and goat peroxidase-conjugated anti-mouse IgG antibody (SantaCruz Biotechnology; Heidelberg, Germany; diluted 1:5 000). Immunoreaction signals were revealed with Luminata Classico HRP Substrates (Millipore, Guyancourt, France) and analysed using an image analysis system (Advanced Image Data Analyser Software, LAS 1000 camera; Fujifilm, FVST, Courbevoie, France).

Statistical analyses

Statistical analyses were carried out using GraphPad Prism 4 software (GraphPad Software, USA). Quantitative data were subjected to a two-way-ANOVA followed by Bonferroni test. Data were analysed for effects of day, pregnancy status (cyclic or pregnant), treatments (DMSO and Trilostane; E2, P4 and E2+P4) endometrial areas (CAR and ICAR) and their

interactions (day *versus* status or status *versus* endometrial areas). Linear regression analysis was used to correlate circulating P4 concentrations with FOXL2 protein expression.

RESULTS

FOXL2 gene is expressed in the ovine endometrium

In ruminants including sheep and cattle, the oestrous cycle is characterized by a short follicular phase followed by a long luteal phase and associated to the progressive increase of P4 secretion. In the ewe, the oestrous cycle lasts 16-17 days with 2-3 days of follicular phase and 14-16 days of luteal phase. During the active luteal phase, P4 secretion covers day 7 to 12 post-oestrous. Without fertilization, the corpus luteum regresses in association with a dramatic decrease of P4 secretion corresponding to the luteolytic phase and the beginning of the next oestrus.

In the ovine endometrium, *FOXL2* transcript and protein were expressed in endometrial caruncular (CAR) and intercaruncular (ICAR) areas during the oestrous cycle and at the initiation of implantation (Fig. 1). Endometrial *FOXL2* gene expression was significantly higher in the CAR areas than in the ICAR areas (Fig.1 A and B, 3-fold; $P < 0.001$). *FOXL2* expression was significantly higher during the early luteal phase (day 4, 1.5-fold) and the follicular phase (day 15, 2-fold) of the oestrous cycle compared to the mid and active luteal phase of the oestrous cycle and the implantation step especially in the CAR area ($P < 0.05$). In western blot analysis, FOXL2 protein was detectable at 50 kDa as we previously reported in the bovine specie (Eozenou et al. 2012).

Since endometrial *FOXL2* gene expression and P4 circulating level were inversely correlated, two ovine experimental models were derived to further analyse the impact of P4 on *FOXL2* endometrial expression.

Alteration of P4 blood level affects FOXL2 gene expression in the ovine endometrium

Firstly, two groups of pregnant ewes were treated with either a DMSO solution (control group) or a solution of trilostane. Trilostane is an inhibitor of the 3 β -hydroxysteroid-deshydrogenase that catalyzes the conversion of pregnenolone into progesterone. Trilostane treatment was applied from day 6 to day 16 of pregnancy that prevented the P4 rise during the early luteal phase and maintained P4 circulating level at a steady level during early pregnancy (Fig. 2; Camous *et al.*, submitted). Compared to the control group, *FOXL2* mRNA in the trilostane treated ewes was significantly higher in CAR area at 16 dpo in pregnancy (Fig. 2A; 1.4-fold, $P < 0.05$). At the protein level, a trend toward an increase *FOXL2* expression was observed in the CAR areas due to the trilostane injection (Fig. 2B, $P = 0.14$).

In order to mimic the ovarian oestrous cycle, ovariectomized ewes were supplemented for 12 days with control solution (control group) or ovarian steroid solutions: P4, E2 and a combination of both. A group of cyclic ewes at 12 dpo was added to the experimental design in order to determine the effect of the ovariectomy on endometrial genes expression (Al-Gubory *et al.* 2006). *FOXL2* gene was expressed in every experimental condition with a significant decrease in the ICAR area compared to the CAR areas (Fig. 3; transcript and protein, $P < 0.05$). The absence of the ovary led to a significant higher *FOXL2* expression in the OVX ewes compared to the cyclic group. E2 supplementation did not significantly alter *FOXL2* mRNA expression compared to the OVX group (Fig. 3A), but stimulated *FOXL2* protein expression in the ICAR area (Fig. 3B; 2.5-fold, $P < 0.05$). P4 supplementation significantly down-regulated *FOXL2* gene expression (transcript: CAR and ICAR area; and protein: CAR only; $p < 0.05$). On OVX supplemented with [P4 + E2], *FOXL2* gene expression did not significantly differ from the OVX, [E2], [P4] and cyclic groups.

P4 inhibits endometrial FOXL2 gene expression in cattle

Ovariectomized cows were supplemented with E2, P4 or both ovarian steroids as published (Shimizu *et al.* 2010). As in the ovine OVX model, a significant decrease of *FOXL2* endometrial mRNA level was detected in [P4] and [P4+E2] supplemented cows compared to

the OVX group (Fig. 4A, 2-fold, $P < 0.05$). E2 supplementation has no significant impact on endometrial *FOXL2* transcript compared to OVX cows.

In bovine endometrial explants incubated with [E2], [P4] and control media for 48 hours a significant decrease of *FOXL2* mRNA level expression was observed following P4 supplementation compared to the control treatment whereas E2 treatment had no effect (Fig. 4B, 2-fold, $P < 0.05$).

DISCUSSION

Since its cloning and isolation in 2001, *FOXL2* has been recognized to be a key factor in ovarian differentiation and the maintenance of ovarian function from foetal life to adulthood (Crisponi et al. 2001; Uhlenhaut et al. 2009; Pannetier and Pailhoux 2011; Uhlenhaut and Treier 2011). We recently demonstrated that *FOXL2* was expressed and regulated throughout oestrous cycle and early pregnancy in bovine endometrium, independently of the production of the conceptus-derived IFNT, the major signal of pregnancy maternal recognition in ruminants (Eozenou et al. 2012). In this present study, we have demonstrated that (i) *FOXL2* gene expression is regulated throughout oestrous cycle and at implantation in ovine endometrium and (ii) P4 affects endometrial expression of *FOXL2* in cattle and in sheep.

In bovine and ovine endometrium, high and low P4 circulating levels led to a significant decrease and increase of *FOXL2* gene expression, respectively. In addition, ovariectomized animals exhibited a clear drop of *FOXL2* expression under the supplementation of P4 as well as in endometrial explants incubated for 48 hours with [P4] medium. Ovariectomized animals and bovine endometrial explants provide us an accurate demonstration of P4 action on *FOXL2* endometrial expression regardless of the secretion of ovaries as well as the autocrine and paracrine regulations occurring in the endometrium preventing the putative compensatory effects of both ovaries and endometrium. Our results have demonstrated P4

counteracts systematically E2 effect on *FOXL2* endometrial gene expression in ruminants. Collectively, our data suggest strongly that P4 is a master regulator of *FOXL2* endometrial expression. Nevertheless, the data do not still pending the question about P4 direct action on *FOXL2* gene expression. P4 primarily acts through their nuclear receptors (PGR, form A and B) that are involved in uterine receptivity and embryo implantation (Lydon et al. 1995; Franco et al. 2008). Analysis of ovine and bovine *FOXL2* promoter sequence exhibited the presence of Progesterone Receptor response Element (PRE, data not shown) suggesting P4 could regulate *FOXL2* gene expression using its nuclear receptor. PGR are localized in luminal and glandular epithelia as well as stroma in bovine endometrium (Okumu et al. 2010). Its sub-cellular localization is clearly nuclear during follicular/early luteal phase of the oestrous cycle and early pregnancy (5 dpo) and staining intensity of nuclear PGR decreased throughout luteal phase then was completely absent from luminal and glandular epithelia during the active luteal phase (16 dpo; Okumu et al. 2010). *FOXL2* protein which is localized in the nuclei of endometrial stroma and glandular epithelium during the follicular phase (20 dpo) was shown to disappear from the nuclei of glandular epithelium during the active luteal phase as well (14 dpo; Eozenou et al. 2012). Therefore, the literature and our published data reported in bovine endometrium suggest *FOXL2* and PGR proteins displayed the same pattern of cell distribution throughout oestrous cycle. Thus, we hypothesized PGR could stimulate *FOXL2* gene expression in endometrial stroma and glandular epithelium binding its PRE on *FOXL2* promoter. In addition, we suggested also P4 repressed *FOXL2* gene expression as a consequence of *PGR* gene repression. Moreover, many rapid and non-classical action of P4 is mediated by its Progesterone Receptor Membrane component 1 and 2 (PGRMC1 and 2, Pru and Clark 2013). Further *in vitro* investigations will enable us to identify the mechanism of P4 direct action using its receptor on *FOXL2* endometrial gene expression.

In mammals, uterine receptivity is mandatory for the progression of pregnancy and characterized by intensive endometrial cell proliferation mediated by E2 during the follicular

phase and endometrial gland maturation, changes in endometrial genes expression and decidualisation when this step occurs; mediated by P4 during the luteal phase (Miller and Moore 1976; Devroey and Pados 1998; Ozturk and Demir 2010; Spencer and Bazer 2002; Al-Gubory et al. 2008; Spencer et al. 2012). In ruminants, oestrous cycle is characterized by a short follicular phase and a long luteal phase (Forde et al. 2011) where *FOXL2* gene expression was shown to be, respectively, increased and decreased, in both sheep and cattle (Eozenou et al. 2012). Interestingly, in women, where the menstrual cycle is characterized by a long follicular and luteal phase (Hawkins and Matzuk 2008; Mihm et al. 2011), *FOXL2* transcript expression appears to be similar to its expression in bovine and ovine endometrium with a significant higher expression during the follicular phase (Talbi et al. 2005). Our data and the literature suggest that *FOXL2* gene which is expressed during the follicular phase of three mammal species could be a key regulator of the proliferative process of the uterine receptivity. In the ovarian context, *FOXL2* is known to be a master gene in apoptosis regulation (Batista et al. 2007; Caburet et al. 2012). Indeed, the wild-type *FOXL2* gene is considered as a tumour suppressor gene (Benayoun et al. 2011). Conversely, the *FOXL2* (C402G) mutation is involved in adult and juvenile granulosa cell tumours with a poor survival and disease-free survival rate (D'Angelo et al. 2011). Nevertheless, *FOXL2* is involved in the pro-apoptotic process regulating the expression of *BCL2A1* and *ATF3* genes but also the anti-apoptotic process regulating the expression of *TNFAIP3*, *NR5A2* and *FOS* genes in granulosa cells (Batista et al. 2007; Moumne et al. 2008). We can hypothesize that *FOXL2* could play a critical role in the pro- and anti-apoptotic balance during the follicular phase inducing or repressing the proliferation of endometrial cells depending on the species. Further analyses will be necessary to evaluate the expression of pro- and anti-apoptotic genes in ruminant endometrium to confirm the implication of *FOXL2* in the proliferative process of mammal follicular phase in the endometrium. In addition, in case of BPES syndrome, women are infertile caused by premature ovarian failure (Crisponi et al. 2001; Verdin and De Baere 2012). Those women could have access to medically-assisted

procreation to rescue ovarian infertility but in the endometrial context, are they able to be pregnant and able to maintain this pregnancy until parturition? Further analyses will be necessary to better understand the implication of *FOXL2* in mammal endometrial physiology including in BPES syndrome.

Gonadal steroid hormones act as the critical trophic factors necessary for the normal development of many biological systems (Nugent et al. 2012). E2 plays an essential role in female sex determination in non-mammalian vertebrates regardless of the sex determining mechanism (Pask et al. 2010). In mammalian vertebrates, E2 has also a highly conserved role but it is not required for initial ovarian development in mice (Pask 2012). Indeed, alpha and beta estrogen receptors (*Esr1* and *2*) knock-out and *Cyp19*/Aromatase knockout mice have normal early ovarian differentiation (Lubahn et al. 1993; Krege et al. 1998; Britt et al. 2001; Britt and Findlay 2003). However, shortly after birth, granulosa cell fate cannot be maintained and initiate the transdifferentiation to the sertoli cell fate (Lubahn et al. 1993; Krege et al. 1998; Britt et al. 2001; Britt and Findlay 2003). E2 is one of the key modulator of the maintenance of ovarian differentiation throughout the adulthood. In the context of a functional ovary, E2 binds its nuclear receptor, leading to activated ESR and act with *Foxl2* to suppress *Sox9* transcription by directly binding to the *Sox9* enhancer, TESCO element (Uhlenhaut et al. 2009). In the other hand, *FOXL2* is also known to stimulate the *CYP19* gene expression, leading to an increase of E2 production (Pannetier et al. 2006). To conclude, E2 and *FOXL2* are essential for the ovarian biological functions including the ovulation process. Progesterone is the pregnancy hormone in all mammals allowing more particularly, the conceptus implantation (Bazer et al. 2010). In the case of the ovariectomized ewes and cows; and the bovine endometrial explants, only P4 action induces a clear down-regulation of *FOXL2* expression. Conversely, *FOXL2* is up-regulated when P4 production and secretion remains almost constant at a low circulating level. Our data suggest that P4 is one of the most important regulators of *FOXL2* gene in the endometrial context. The

literature and our results exhibit the fact that *FOXL2* is expressed in mammalian ovary and endometrium and is regulated by E2 and P4 respectively in a tissue-specific-manner.

Similarly to the situation we reported in bovine endometrium (Eozenou et al. 2012), *FOXL2* expression in ovine endometrium was inhibited throughout luteal phase (from day 8 to day 12) but increased during the follicular phase (4 dpo and 15 dpo) of the oestrous cycle. Ruminant endometrium is separated in two morphologically distinct areas, the CAR and ICAR areas and exhibits a significant decrease of *FOXL2* expression in the ICAR area. As previously published (Eozenou et al. 2012) and described above, *FOXL2* protein is mostly localized in the nuclei of endometrial stroma and glandular epithelium during the follicular phase and completely absent in the luminal epithelial cells. These data suggest that *FOXL2* gene expression has to be lowered in the ICAR area which is the glandular area, if not, we hypothesized that *FOXL2* would have deleterious effects on endometrial gland differentiation and histotroph secretion which are mandatory processes prior to the establishment of pregnancy and later, implantation (Bazer et al. 2010). Moreover, when the P4 circulating level exceeds a certain threshold, *FOXL2* has also to be lowered in CAR area also, which is the aglandular area. In conclusion, during the luteal phase or the pregnancy maternal recognition period in ruminant endometrium, *FOXL2* expression is low in both endometrial areas suggesting the repression occurring during the luteal phase and the early pregnancy is a prerequisite for uterine receptivity then implantation processes. Nevertheless, the biological functions of endometrial *FOXL2* gene remain unknown. Additional analyses will be necessary to identify the target genes of this transcription factor providing us a better understanding about the implication of *FOXL2* in endometrial physiology. Finally, in mammalian and non-mammalian vertebrates, *FOXL2* appears to be a master gene of the reproductive physiology.

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FIGURE LEGENDS

Figure 1. *FOXL2* expression in cyclic and pregnant ovine endometrium

Caruncular (CAR) and intercaruncular (ICAR) endometrial areas were collected from cyclic (n=4 at day 4, n=4 at day 8, n=4 at day 12) and pregnant (preg, n=4 at day 15) Pré-alpes du sud ewes. (A) Quantification of *FOXL2* mRNA by RT-qPCR. Expression of *FOXL2* was normalized to *RPL19*. (B) Quantification of *FOXL2* protein by western blotting normalized to ACTB protein level. Quantitative data are presented as mean +/- SEM and significant differences were noted using * : $P < 0.05$.

Figure 2. Regulation of *FOXL2* gene expression under the influence of altered P4 circulating level

Caruncular (CAR) and intercaruncular (ICAR) endometrial areas were collected from pregnant Pré-alpes du sud ewes treated with DMSO as a control solution (n=7) or trilostane (15 mg/ewe in 1 ml DMSO) for 11 days. (A) Quantification of *FOXL2* mRNA by RT-qPCR. Expression of *FOXL2* was normalized to *RPL19*. (B) Quantification of *FOXL2* protein by western blotting. *FOXL2* expression was normalized to ACTB protein level. Quantitative data are presented as mean +/- SEM significant differences were noted using * : $P < 0.05$.

Figure 3. *FOXL2* endometrial expression under the influence of ovarian steroid hormones balance in ovariectomized ewes

Caruncular (CAR) and intercaruncular (ICAR) endometrial areas were collected from ovariectomized Pré-alpes du sud ewes supplemented with a control solution (OVX, n=4), [P4] solution (OVX + P4, n=4), [E2] solution (OVX + E2, n=4) or [P4+E2] solution (OVX + E2 + P4, n=4) for 12 days and also from cyclic ewes at 12 days (n=4). (A) Quantification of *FOXL2* mRNA by RT-qPCR. Expression of *FOXL2* was normalized to *RPL19*. (B) Quantification of *FOXL2* protein by western blotting. *FOXL2* expression was normalized to

ACTB protein level. Quantitative data are presented as mean \pm SEM and significant differences were noted using * : $P < 0.05$.

Figure 4. *FOXL2* endometrial expression under the influence of ovarian steroid hormones balance in ovariectomized cows and bovine explants

(A) Strips of both endometrial areas were collected from ovariectomized cows supplemented with a control solution (OVX, n=3), [P4] solution (OVX + P4, n=3), [E2] solution (OVX + E2, n=3) or [P4+E2] solution (OVX + E2 + P4, n=3). (B) Intercaruncular areas were collected from cyclic cows at oestrus stage, supplemented with a control solution (OVX, n=2), P4 solution (OVX + P4, n=2) or E2 solution (OVX + E2, n=2). (A) and (B) Quantification of *FOXL2* mRNA by RT-qPCR. Expression of *FOXL2* was normalized to *RPL19*. Quantitative data are presented as mean \pm SEM and, bars with different superscripts significantly differ ($P < 0.05$) and significant differences were noted using * : $P < 0.05$.

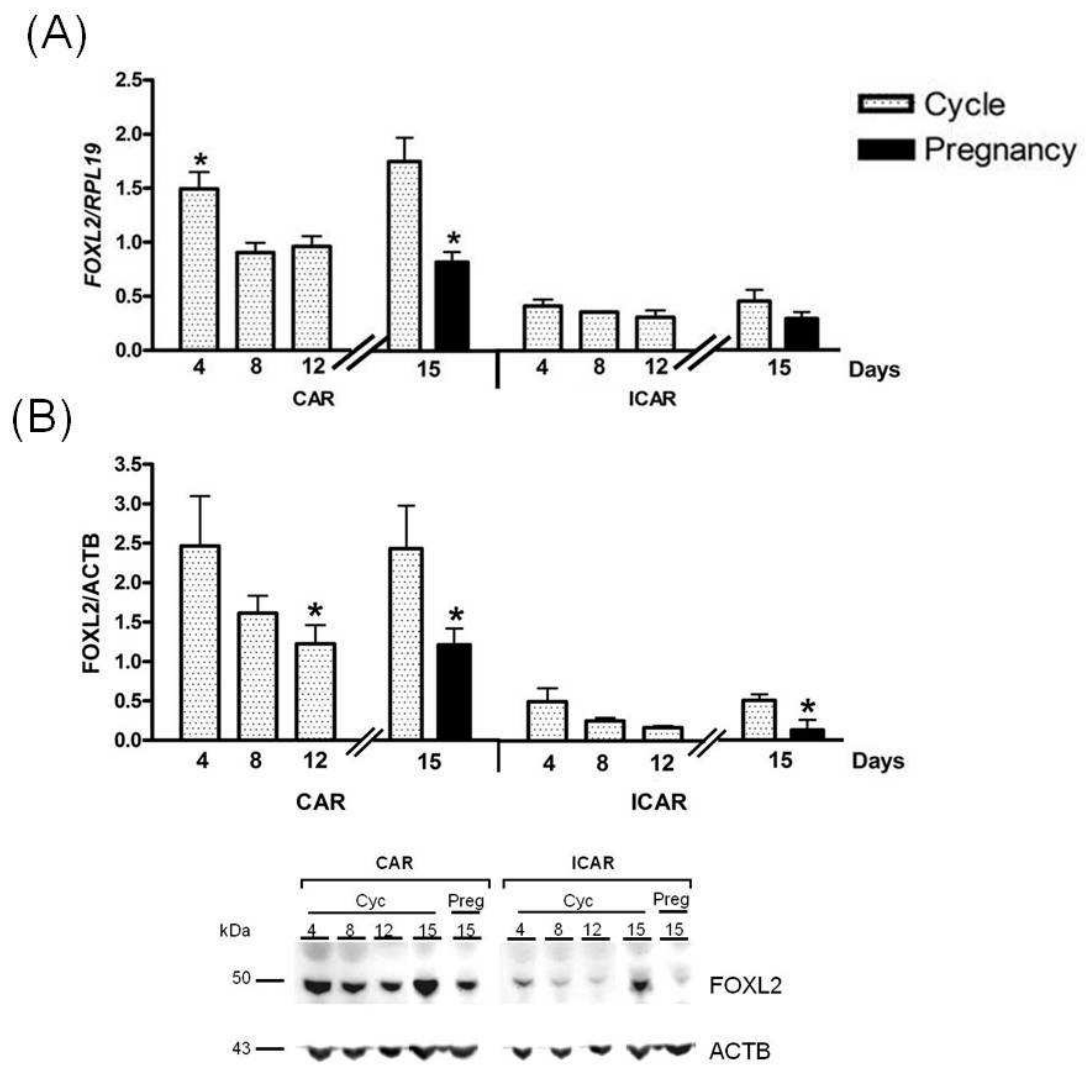


Fig.1

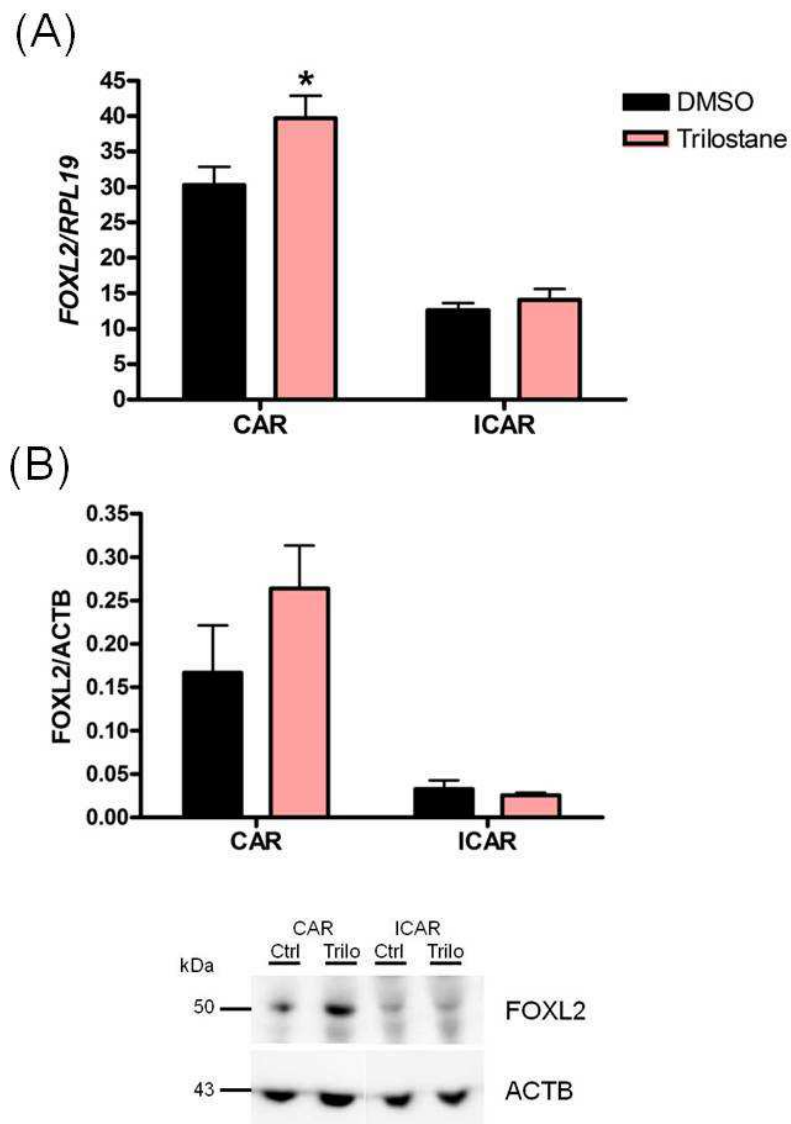


Fig.2

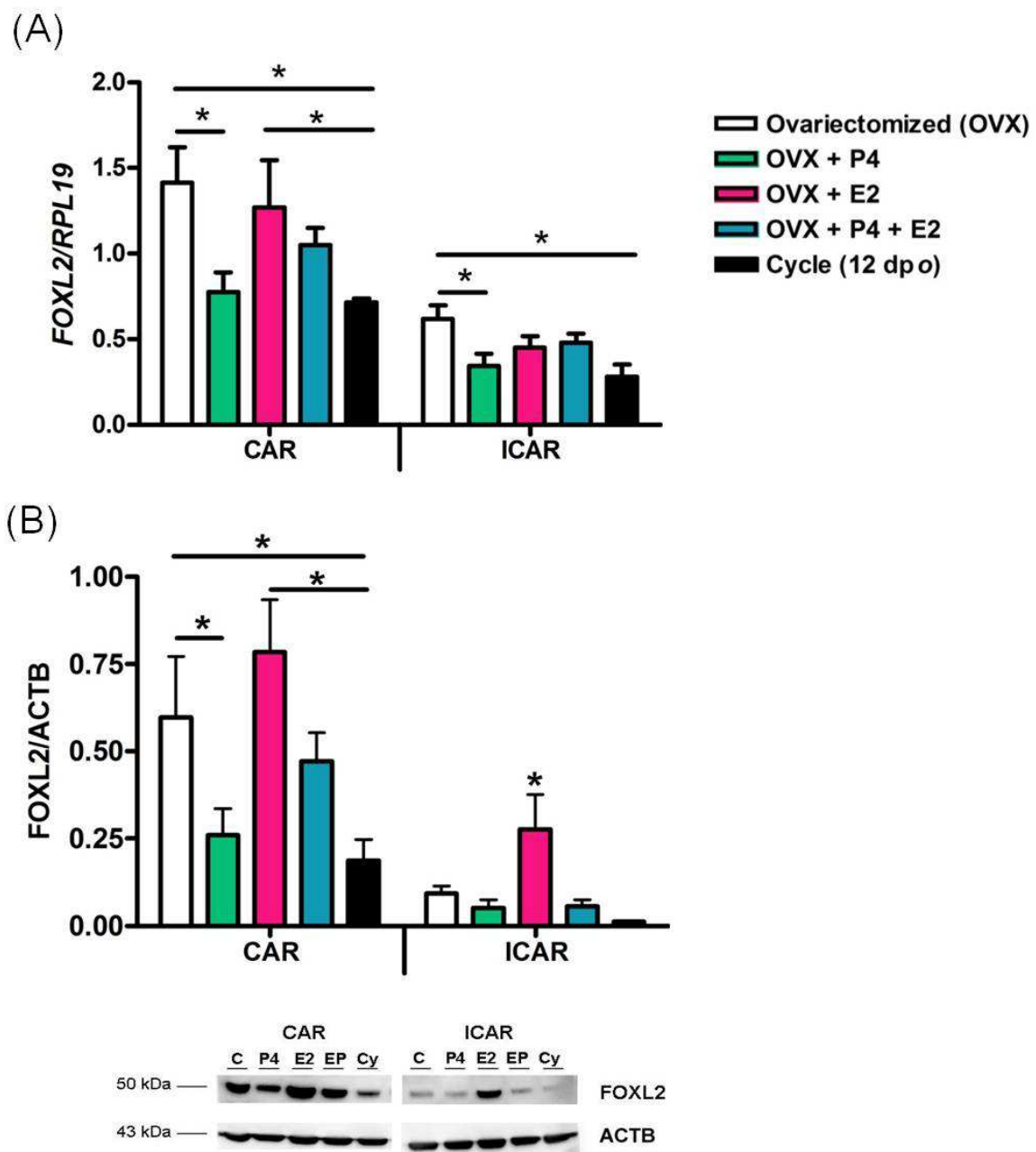


Fig. 3

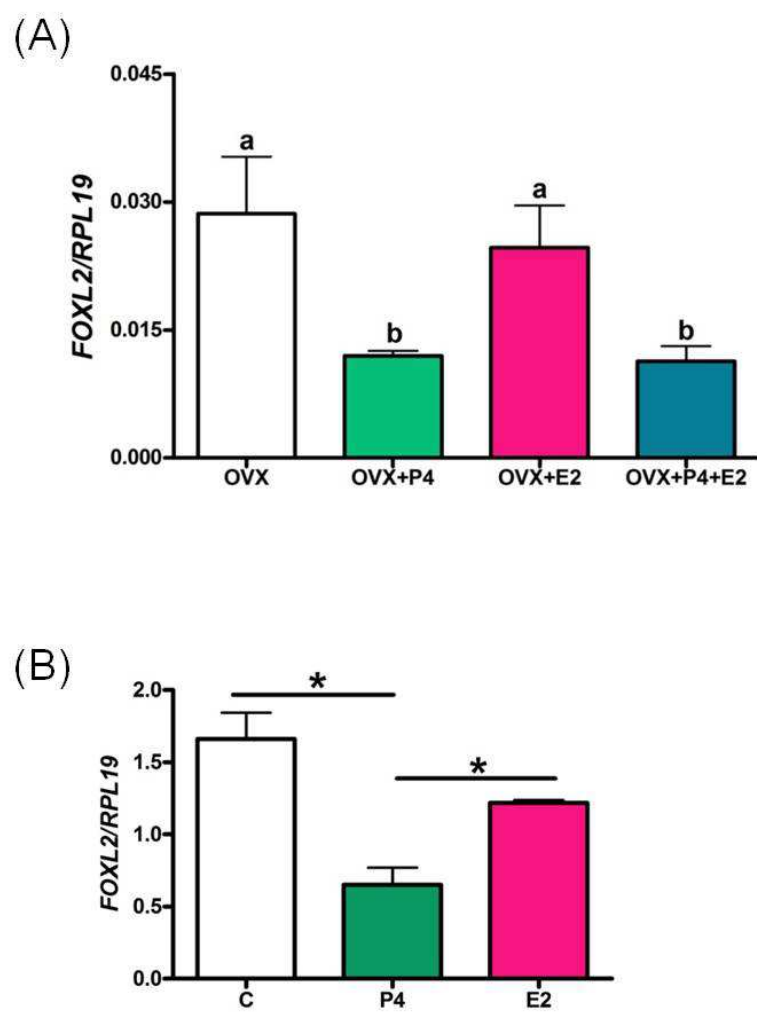


Fig. 4

VII. Part III: First insight about the biological functions of FOXL2 in bovine endometrium

Publication in progress

A. Introduction

The first two parts of my PhD thesis showed an accurate evaluation of *FOXL2* gene expression, confirming the differential expression observed between 20 dpo of pregnancy and oestrous cycle in the microarray study made in our team (Mansouri-Attia et al. 2009). My work has also revealed a higher *FOXL2* gene expression during the follicular phase and early luteal phase compared to the active luteal phase and early pregnancy in endometrium. P4 is a critical regulator of endometrial physiology and regulates endometrial *FOXL2* expression in cattle and in sheep.

Transcription factors constitute nodes in cellular gene networks because of their ability to regulate transcriptional activity of numerous target genes (involved in various biological functions in a tissue-specific manner). The third part of my PhD aims to identify *FOXL2* regulated genes in the endometrium using *FOXL2* transfected cells and candidate genes approach.

FOXL2 has been shown to regulate the expression of several hundred target genes as previously described in the ovarian context (1248 DEG, Batista et al. 2007; Moumne et al. 2008; 922 DEG, Uhlenhaut et al. 2009). *FOXL2* is especially involved in the control of ovarian differentiation suppressing permanently testis differentiation but it is also involved in ovulation process, ovarian ageing and cellular stress response. Interestingly, transcript profiles of bovine endometrium at 20 dpo (pregnancy compared to oestrous cycle) have highlighted the differential expression of *FOXL2* gene but also some genes regulated by *FOXL2* in an ovarian context (Fig. 45). An *In silico* analyse between bovine endometrium and human ovarian granulosa-like tumour cell line (KGN) over-expressing *FOXL2* gene, allows the identification of common orthologs listing 38 genes regulated by *FOXL2* in KGN cell line and expressed in bovine endometrium.

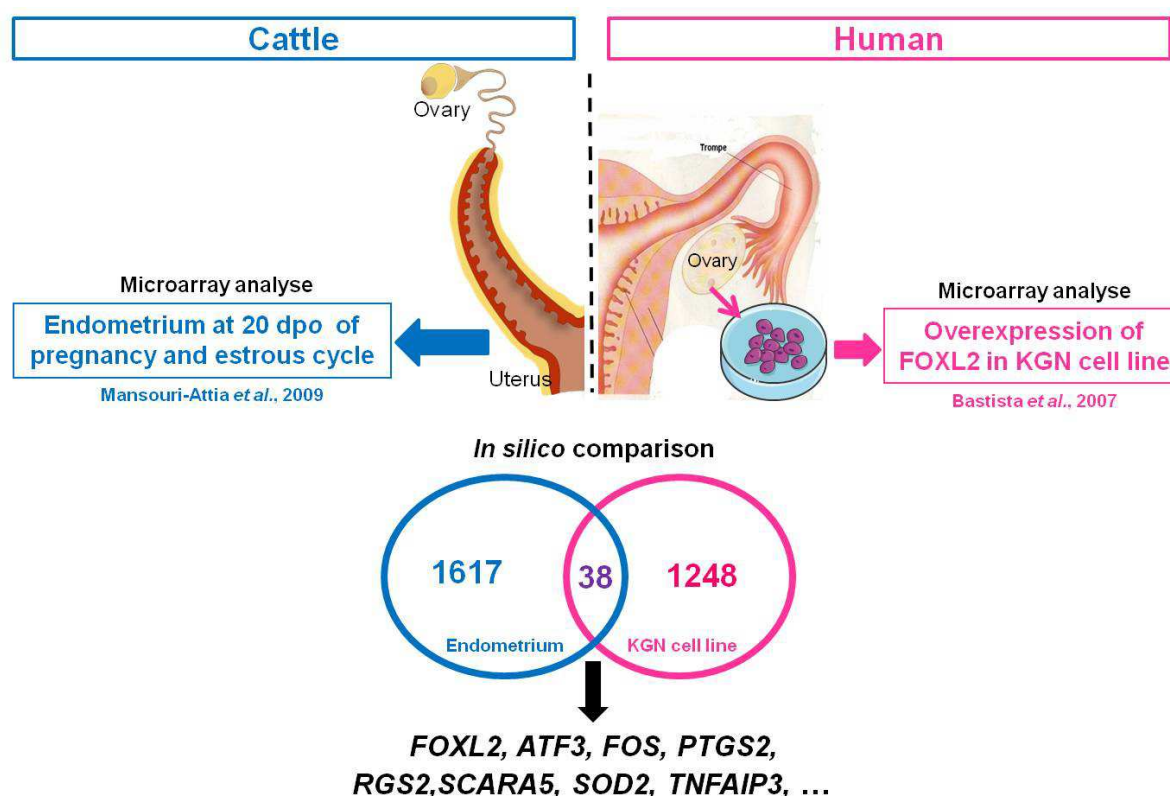


Figure 45: *In silico* comparison of orthologs between bovine endometrium and human ovary based on microarray studies (complete list of common genes in Appendix B, Fig. 67).

Microarray study of *Foxl2* conditional knock-out in adult mice had been add to the previous comparison (Uhlenhaut et al. 2009) showing only one common gene between the three high-through-put analyses, *Scara5*, formerly called *Tesr*, a member of the scavenger receptor involved in the innate immunity (Sarraj et al. 2005; Jiang et al. 2006). Originally, *Scara5* was discovered as gene specifically expressed in epithelial cells and in particular, expressed in the testis (Sarraj et al. 2005; Jiang et al. 2006). Conditional deletion of *Foxl2* in the murine ovary has been reported to induce sex-reversal in adult female, with a transdifferentiation of theca and granulosa cells into leydig-like and sertoli-like cells, respectively (Uhlenhaut et al. 2009). *Scara5* was one of the most up-regulated genes (Uhlenhaut et al. 2009) whereas the over-expression of *FOXL2* in KGN cell line was shown to inhibit the expression of *SCARA5* (Batista et al. 2007; Uhlenhaut et al. 2009). In bovine endometrium, a clear up-regulation of *SCARA5* transcript was reported in our RNA profiles established at 20 dpo of pregnancy compared to the oestrous cycle (Mansouri-Attia et al. 2009). A murine model reported also the stimulation of *Scara5* during the decidualisation process (Duncan et al. 2011). Altogether these data suggest *FOXL2* could repress the expression of *SCARA5*. In addition to *SCARA5*, six other genes were selected: *ATF3*, *FOS*, *PTGS2*, *RGS2*, *SOD2* and *TNFAIP3*. *ATF3*, *FOS*

and *TNFAIP3* were chosen considering their implication in the apoptotic process especially involved in ovarian ageing (Batista et al. 2007). *PTGS2* was also selected because of its essential role during the processes of ovulation in the ovary and uterine receptivity in the endometrium (Charpigny et al. 1999; Georges et al. 2013). In the ovarian context, *PTGS2* formerly called *COX2* is an enzyme involved in ovulation and inflammation processes (Batista et al. 2007). Indeed, *PTGS2* expression is strongly induced in granulosa cells following the gonadotropin rise prior to the ovulation process and appears to be stimulated by *FOXL2* overexpression (Batista et al. 2007; Benayoun et al. 2011). In endometrium, *PTGS2* is the enzyme responsible for the luteolytic surge of $\text{PGF2}\alpha$ (Charpigny et al. 1999). Regarding *RGS2*, this gene was chosen for its role of follicular marker of oocytes developmental competence mediated by *FOXL2* in the ovary (Hamel et al. 2010) as well as for its implication in uterine receptivity process (Huang et al. 2003). Finally, *Sod2/SOD2* was selected for its crucial role in stress response in both ovary (mediated by *Foxl2*) and endometrium (Batista et al. 2007; Al-Gubory et al. 2008).

In order to identify *FOXL2* regulated genes in the endometrium, primary cultures of bovine endometrial cells were transiently transfected with *FOXL2* cDNA. Based on the list of 38 genes (Fig. 45), expressions of *ATF3*, *FOS*, *PTGS2*, *RGS2*, *SCARA5*, *SOD2* and *TNFAIP3* were investigated. In addition, *DLX5*, *HOXA10*, *PGR* and *RSAD2* mRNA expression were analysed. *DLX5* belongs to the homeobox transcription factor family also called Distal-less homeobox member 5 (Robledo et al. 2002). This transcription factor is involved in bone development and fracture healing (Robledo et al. 2002). More specifically, *DLX5* and 6 are involved in craniofacial development with an expression in the distal regions of extending appendages, in differentiating osteoblasts and in Leydig cells (Robledo et al. 2002; Bouhali et al. 2011). Recently, the team of Dr Levi have highlighted the reciprocal regulation occurring between *Foxl2* and *Dlx5* genes in mice (Bouhali et al. 2011). In this species *Foxl2* is strongly expressed in primordial and primary follicles but its expression decreases during the follicle maturation. *Foxl2* ovarian expression activates the transcription of *Dlx5* in granulosa cells. Then, *Dlx5* expression represses *Foxl2* expression in the secondary follicle until mature antral follicle (Bouhali et al. 2011). Nevertheless, *Dlx5* implication has never been reported in the endometrial physiology and prompts the need to clarify it. *HOXA10* and *PGR* gene were selected because these are early P4 target genes in the endometrium and could be related to *FOXL2* expression (Bazer 2010; Zanatta et al. 2010; Mansouri-Attia et al. 2012). Finally, *RSAD2* (Radical S-adenosyl methionine domain containing 2, formerly called viperin) has been shown to be regulated by pregnancy, and to be an IFNT induced genes in ruminant endometrium and a cytoplasmic antiviral protein induced by Type I IFNs (Song et

al. 2007; Mansouri-Attia et al. 2009; Forde et al. 2011). Its expression increased during the maternal pregnancy recognition and implantation periods. In the ovarian context, this gene has not been described yet and is not reported as a *FOXL2* target genes. Nevertheless, *RSAD2* was part of the top list of the up-regulated genes in the endometrium of pregnant ewes or cows (Song et al. 2007; Mansouri-Attia et al. 2009) whereas *FOXL2* is down-regulated at the same time. *RSAD2* were chosen as a negative control gene because it is an IFNT induced gene in ruminant endometrium and should not be regulated by *FOXL2* (Song et al. 2007; Mansouri-Attia et al. 2009; Forde et al. 2011).

B. Experimental procedure

1. Animals

The aim of this experiment was to stimulate actively the expression of *FOXL2* in stromal and glandular epithelial cells in order to regulate the expression of *FOXL2* target genes. In order to over-express significantly *FOXL2* expression, we have sorted animals by physiological stage. Three cows were selected, at the dioestrus stage during the active luteal phase based on CL morphology and weight (Arosh et al. 2002). CL weight and morphology was slightly different between the cows. Here, CL of the second cow (3.5 g equivalent to the days 7-10 of the oestrous cycle) was lighter than the first and the third one (respectively 8.5 g and 6.9 g equivalent to the days 13-15 and 10-12 of the oestrous cycle; Arosh et al. 2002). In addition, the three reproductive tracts were dissected at the slaughterhouse without information about the physiological situation of each cow.

The luteal phase exhibits the lowest level of *FOXL2* expression and represents the ideal stage to transfect *FOXL2* cDNA, and to evaluate changes of *FOXL2* regulated genes expression.

Uteri were dissected, stromal and glandular epithelial cells were separated then cultivated for 5 days. Both cellular types were transfected using either a pSG5 plasmid including caprine *FOXL2* gene or an empty pSG5 plasmid as a control of transfection. The success of transfections has been checked using an immunodetection of *FOXL2* protein then analyses of transcript expression were performed to the eleven candidate genes and when it was possible co-immunofluorescence was performed between *FOXL2* and one target gene.

2. Primary culture of bovine endometrial cells

Filtered digestion buffer was prepared in PBS (Sigma). Considering 100ml of digestion buffer per horn, we used 350 IU/ml of collagenase type IV (SIGMA), 350 IU/ml of hyaluronidase (SIGMA), BSA 2% (SIGMA) and penicillin/streptomycin (10X, SIGMA, PS, P-4333).

The endometrium was carefully dissected from myometrium and cut into strips. Strips were rinsed in Calcium and Magnesium-free PBS for 5min. Strips were chopped in Petri dish. Caruncular tissue has to be chopped very finely in order to go through the cannula. Then minced endometrium was placed in the digestion buffer for 1 to 2 hours at 39°C in water bath. During digestion, minced tissue were aspirated through a cannula (1.6mm of diameter) several times in order to disintegrate the tissue and to facilitate digestion. Subsequently, digested tissue was centrifugated, rinsed in PBS and filtered twice. First filtration used sterile gauze in order to separate cells and cellular aggregates. Using a 31µm cellular nylon sieve filtration (Dutscher, 074013), stromal cells were separated from aggregate of glandular epithelial cells.

Each type of cells was seeded in complete culture DMEM/F12 media containing : "Dulbecco's modified Eagles's Medium: F12" (SIGMA, D6434, 500ml); 10% fetal bovine serum (FBS); 1.25% stabilized glutamine (Glutabio); 0.5% insulin-transferrin-selenium (ITS, SIGMA: I3146); 1% penicillin-streptomycin (PS, SIGMA: P-4333); 0.1% Nystatin (SIGMA: N-3503); 0.1% gentamycin (SIGMA: G1272). The media was sterile-filtered 22µm.

Stromal fraction was incubated for 2 h in 20% CO₂. At the end of this pre-culture step, floating cells were carefully eliminated by washes using PBS and complete culture media. Adherent stromal cells were further cultivated in 5% CO₂ at 39°C (with 2.3×10^7 cells/ 90mm dishes).

Glandular epithelial cell suspensions were seeded into dishes in 5% CO₂ at 39°C (with 8×10^6 cells/ 90mm dishes).

3. Transient transfection on primary culture of bovine endometrial cells

a) Plasmids preparation

Three plasmids were used for cell transfection: GFPmax, expressing the green fluorescent protein as a transfection control (Lonza Amaxa Nucleofector Kit), the pSG5 vector as a negative control and pSG5-FOXL2 expressing the caprine FOXL2 protein (these two vectors were kindly provided by M. Pannetier, BDR, INRA). Bovine and caprine FOXL2 proteins share 99% sequence identity (sequences and alignments are reported in Appendix C, Fig. 70).

b) Transfection kits

Two different transfection protocols were tested: Lonza Amaxa™ kit and Turbofect kit. Due to the lowest efficiency of Turbofect protocol (based on the use of the transfection reagent, Turbofect), Amaxa™ protocol was chosen (the transfection method using Turbofect reagent is described in appendix C). Lonza Amaxa™ protocol is based on the electroporation method.

The Lonza Amaxa™ Basic Nucleofector kits have been applied to primary stromal cells ([optimization guidelines for stromal cells](#)) and primary mammalian epithelial cells ([optimization guideline for epithelial cells](#)). First, various Nucleofector™ programs of transfection were tested using stromal and glandular epithelial cells according to the manufacturer recommendations. The program for each cell type was selected using cell viability and transfection rate but the duration and the intensity of electroporation could not be control and was not noticed in the optimization guideline. Transfection rate for primary stromal cells was higher than transfection rate for primary glandular epithelial cells. We selected the program U-023 for stromal cells which was advised by the manufacturer leading to an average of 40% of transfection rate (Fig. 46).

Program	Basic stromal kit			
	Cells observation in 5 different fields	Cell GFP >0	% survival	% transfection
U-023	448	184	77,31	41,10
Control	579	0	100,00	0

Figure 46: Evaluation of transfection efficiency on transfected stromal cells based on the rate of GFP positive cells. Stromal cells were transfected using Basic stromal kit and its dedicated program (U-023) with an average of 41.1% of transfection rate (from 16% to 68%).

Glandular epithelial cells had undergone the lowest transfection rate due to the numerous tight junctions between cells. Then, seven programs were assessed including the program U-023 dedicated to stromal cells (Fig. 47).

	Program	Cells observation in 5 different fields	Cell GFP >0	% survival	% transfection
Basic stromal kit	U-023	561	54	65,85	9,63
	Control	852	0	100	0
Basic epithelial kit	S-005	901	36	96,6	4
	T-013	274	20	29,4	7,3
	T-020	360	15	38,6	4,17
	W-001	861	26	92,3	3,02
	U-017	429	13	46	3,03
	Y-001	570	69	64,99	12,11
	Control	933	0	100	0

Figure 47: Evaluation of transfection efficiency on transfected glandular epithelial cells based on the rate of GFP positive cells. Glandular epithelial cells were transfected using Basic stromal kit and its dedicated program (U-023) with 9.63% of transfection rate. The Basic epithelial kit was used with six advised program for primary epithelial cells and the program Y-001 was selected with 12.11% of transfection rate.

The program Y-001 was selected for epithelial cells. Transfection rate was always better in the stromal cells compared to glandular epithelial cells.

c) Optimized protocol of bovine primary endometrial cells transfection

For each experiment, four groups were considered: 'Control' (untransfected cells), 'GFP' (positive control of transfection), basic pSG5 ('F-', transfection using an empty pSG5 vector) and pSG5-FOXL2 ('F+', transient transfection of *FOXL2* gene). Transfections were not carried out at the same moment for the three cows.

RNA and protein analyses did need a high density of glandular epithelial cells and even more with the stromal cells. The first group of transfected cells were seeded into two 22mm dishes and did not contain enough endometrial cells for further analyses. For 'control', 'F-' and 'F+' groups, transfected cells were seeded into 60mm dishes (3 per conditions) for RNA analyses, 35mm dishes for protein analyses (3 per conditions) and 12mm dishes (4 per conditions on sterile cover slides) for immunofluorescence experiments representing 8×10^6 cells/groups (total of 2.4×10^7 cells).

For 'GFP' group, transfected cells were seeded into 2 x 22mm dishes for GFP observation 24 hours then 48 hours after transfection representing 8×10^5 cells.

Primary endometrial cells were cultured for 5 days then passaged using trypsinization prior to transient transfection. Culture medium was discarded and endometrial cells monolayers were gently rinsed in Calcium and Magnesium-free PBS then incubated 2 min and 10 min in

Trypsin (Trypselect, Gibco ref 12605) for stromal and glandular epithelial cells respectively. Cell suspension was recovered in classic PBS then centrifugated at 1000 revolutions per minute (rpm) during 5 min. The cell pellet was resuspended in an appropriate volume of culture media.

Transient transfections were made in special certified 'cuvettes'. Four cuvettes were used per condition representing eight cuvettes for 'F-' and 'F+' transfection and one cuvette for the GFP transfection.

One Nucleofection™ sample contains:

- Up to 2×10^6 cells for 'F-' and 'F+' transfections or 8×10^5 cells for 'GFP' condition
- 3µg of plasmids (pSG5, pSG5-FOXL2 or GFPpmax)
- 82µL + 18µL of Basic Nucleofector™ Solution for primary Mammalian stromal/epithelial cells and supplement buffer respectively (final volume 100µL).

Control group of cells were also passaged and seeded into the appropriate dishes for 48 hours (60mm, 35mm and 12mm dishes). Stromal and glandular epithelial cells were electroporated using U-023 and Y-001 programs respectively. Endometrial cells were recovered and pooled for each condition, in an appropriate volume of culture media then immediately seeded in 12mm, 22mm, 35mm or 60mm dishes for 48 hours. After 24 hours, culture medium was discarded and replaced with fresh media for the next 24 hours.

Transient transfections were also carried out for 72 hours but the transfection rate was lower compared to transient transfection for 48 hours.

In the 'GFP' cells group, fluorescence was observed daily after transfection using an inverted microscope to evaluate the transfection rate. All the cells were fixed or harvested for further analyses 48 hours after transfection.

d) RT-qPCR and Western Blot

For protein analysis, cells cultured in 35mm dishes were scratched and recovered in PBS then centrifugated at 10 000 rpm (10 600 g) at 4°C for 10 min. The supernatants were discarded and the dry pellets were stored at -80°C until protein extraction. For RNA analysis, cells cultured in 60mm dishes were directly lysed using a denaturation buffer (Chomczynski and Sacchi 1987; 4M of guanidium thiocyanate, 25mM sodium citrate pH 7, 0.5% sarcosyl, 0.1M 2-mercaptoethanol). Cell lysates were recovered and stored at -80°C until RNA extraction.

RNA and protein extraction were performed then as previously published (Part I, Eozenou et al. 2012; the sequence of the primers are have been listed in Appendix C, Fig. 72). Nevertheless, RNA has not been purified due to the little rate of total RNA recovery. Considering protein extraction, cell membranes were broken using cell disruptor/homogenizer in RIPA buffer.

e) Immunocyto/histochemistry and immunofluorescence

Transfected endometrial cells dishes were cultured on sterile cover slides placed in 12 or 22 mm dishes for 48 hours, fixed 5 min in 100% methanol at -20°C, washed three times in PBS then stored at 4°C in PBS. For immunocytochemistry experiment, cells were permeabilized with 0.5% TritonX-100 for 30 min at room temperature. Cover slides were incubated in citrate buffer (sodium citrate 0.01M, pH 6.0) for 5 min at room temperature followed by incubation at 80°C for a further 10 min to unmask cell monolayer. The endogenous peroxidase activity was quenched by a 0.1% hydrogen peroxide treatment for 30 min. Cells were then incubated with a rabbit anti-FOXL2 purified antibody (see dilution in Fig. 48) in phosphate buffer (0.1 M pH 7.4 with 2% BSA and 1% NDS, Normal Donkey Serum) overnight at 4°C. After several washes in phosphate buffer with 2% BSA, anti-rabbit biotinylated antibody (see dilution in Fig. 48) was applied on slides for 1 h at room temperature. After three washes in phosphate buffer, sections were incubated for 1 h with the ABC Vector Elite kit (Vectastain Elite ABC Kit; Vector Labs, Peterborough, U.K.) in Tris-buffer (Tris 50 mM, NaCl 0.15 M, pH 7.5). Slides were washed three times and were then incubated with diaminobenzidine substrate and urea (Sigma FAST 3.3, SIGMA, France) in Tris-buffer for 90 secondes, until brown staining was detectable. After one wash in water, slides mounted with 40% glycerol mounting medium. Images were collected using a NanoZoomer Digital Pathology System then they were analysed using the NDPView (NanoZoomer Digital Pathology Virtual Slide Viewer, Software, HAMAMATSU, Japan). Experiments were run using primary endometrial cultures derived from three different animals.

Immunohistochemistry experiments were carried out on bovine endometrial slides as previously described (Part I, Eozenou et al. 2012) using a rabbit anti-DLX5 antibody (see dilution in Fig. 48) overnight at 4°C.

For immunofluorescence experiment, non specific binding sites were blocked with 1x PBS containing 2% BSA for 1 hour at room temperature. The incubations (see dilution in Fig. 48) with the rabbit anti-FOXL2 primary antibody, the mouse anti-PTGS2 primary antibody, the mouse monoclonal anti-Vimentin primary antibody and the mouse monoclonal anti-18-Cytokeratin (CK18) primary antibody were performed overnight at 4°C as well as incubation

of rhodamine-labeled phalloidin (a high-affinity F-actin probe conjugated to the red-orange fluorescent dye, tetramethylrhodamine; TRITC) for 1 hour in dark to visualize microfilamentous F-actin. The donkey AffiniPure Anti-Rabbit Cy5-conjugated IgG and the anti-mouse Cy3-conjugated IgG were incubated for 1 hour (See dilution in Fig. 44). DNA was counterstained with DAPI (10 ng/ml; D1306, Invitrogen, France). Preparations were mounted using the fluorescence protector. Labeled endometrial cells were examined using a Zeiss LSM 510 confocal microscope (MIMA2 platform, INRA, Jouy-en-Josas) and analysed with the AxioVision Microscopy Software (Zeiss, Le Pecq, France). Experiments were run using primary endometrial cultures derived from three different animals.

	Name	Supplier	Reference	Dilution	Host	Application
Primary antibody	FOXL2	Caslo Laboratory	Cocquet <i>et al.</i> , 2002	1:1000	rabbit	WB, IHC, ICC, IF
	DLX5	Novus Biologicals	NBP1-19547	1:200	rabbit	IHC
	PTGS2	C. Creminon	Charpigny <i>et al.</i> , 1997	1:200	mouse	IF
	Vimentin	Millipore	Mab3410, clone V9	1:100	mouse	IF
	CK-18	Cliniscience	KS18-174	1:50	mouse	IF
	Rhodamine labeled Phalloidin	Invitrogen		1:200		IF
Secondary antibody	anti-rabbit biotinylated	Abcam	Ab6720	1:200	Goat	IHC
	anti-rabbit HRP	Abcam	Ab6721	1:5000	Goat	WB
	Anti-Rabbit Cy5-conjugated IgG	Jackson Immunoresearch	715-175-152	1:200	Donkey	IF
	Anti-mouse Cy3-conjugated IgG	Jackson Immunoresearch	715-165-152	1:200	Donkey	IF

Figure 48: Antibodies general information (supplier, dilution, host and application). WB: Western Blot; IHC: Immunohistochemistry; ICC: immunocytochemistry; IF: immunofluorescence.

f) Statistical analyses

Transfection results were presented without statistical analyses due to the low number of animals (n=3 for stromal cells and n=2 for glandular epithelial cells). However, classical statistical analyses were carried out using GraphPad Prism 4 software (GraphPad Software, USA) for target genes expression. Quantitative data were subjected to a two-way-ANOVA followed by Bonferroni test. Data were analysed for effects of day, pregnancy status (cyclic or pregnant), treatments (saline buffer or IFNT buffer), endometrial areas (CAR and ICAR) and their interactions (day *versus* status or status *versus* endometrial areas).

C. Results

1. Characterization of bovine primary endometrial cell cultures

Stromal and epithelial cells were observed using light microscopy. Stromal cells appeared elongated and striated whereas glandular epithelial cells grew in insular distribution and showed a cuboid morphology (Fig. 49). Cells confluence reached 80% 6 days after seeding.

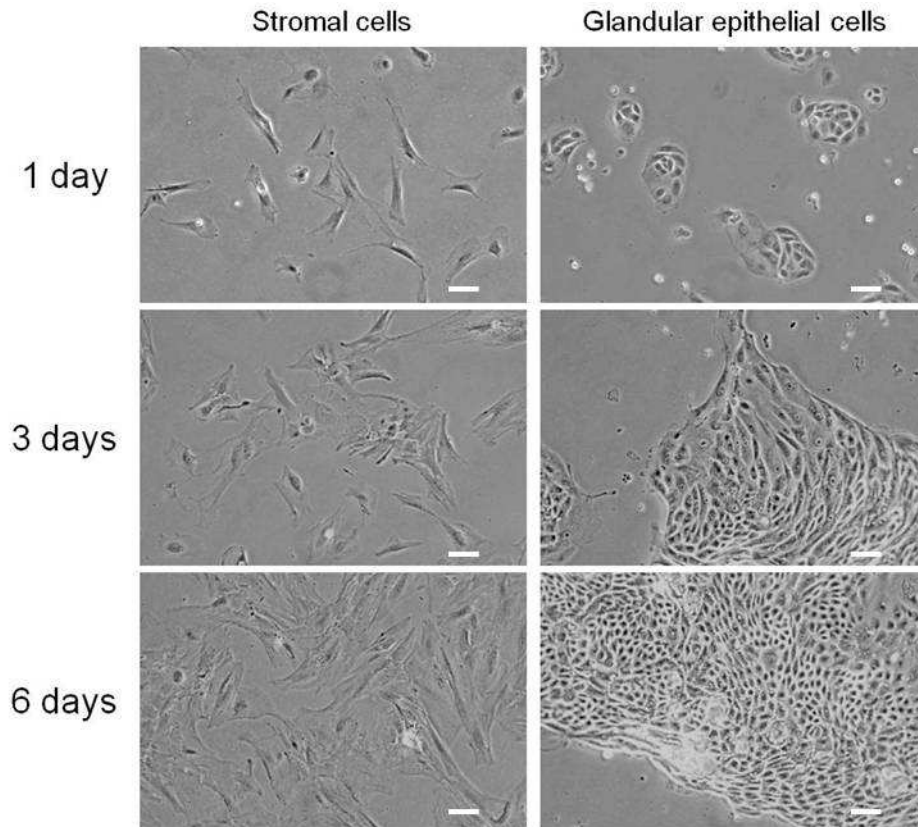


Figure 49: Observation of primary cell cultures using light microscopy; cell cultures here monitored, 1 day, 3 days and 6 days after seeding. Stromal cells are elongated and striated whereas glandular epithelial cells grew in insular distribution and showed a cuboid morphology. White scale bar: 50µm.

Cell purity of either stromal or glandular epithelial cells was checked using immunodetection of vimentine, cytokeratine and phalloidin (Fig. 50).

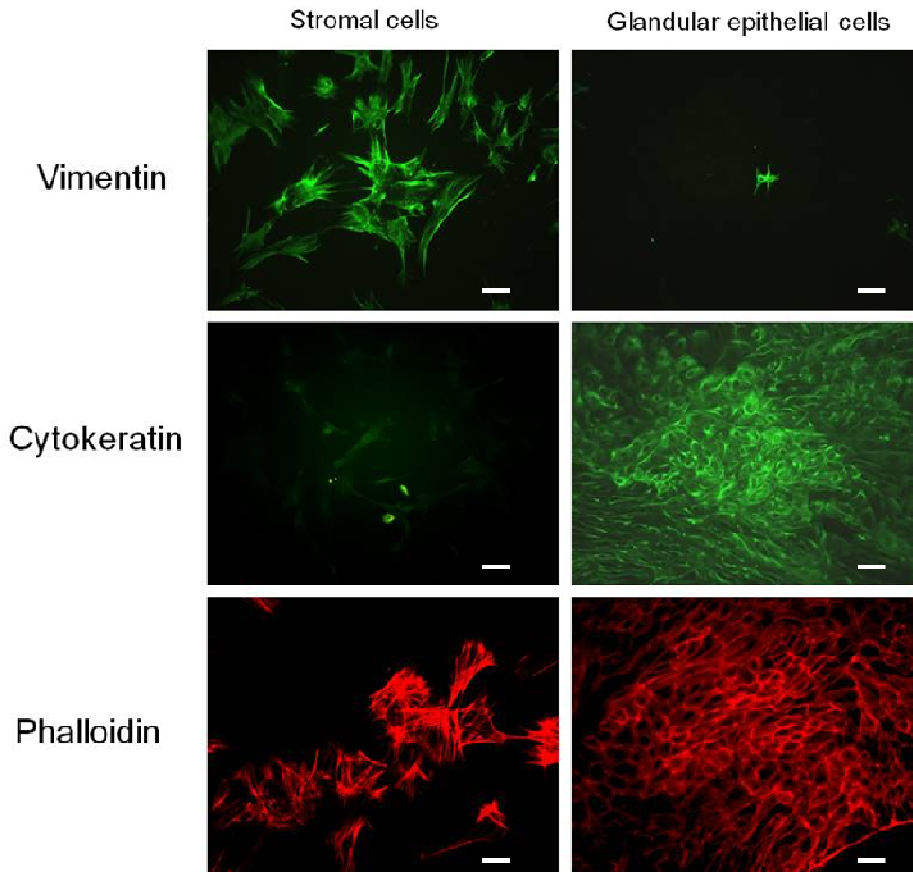


Figure 50: Vimentin, cytokeratin and phalloidin expression by endometrial cells (P. Reinaud). Stromal cells are characterized by the vimentin expression whereas the cytokeratin expression is typical of glandular epithelial cells. Phalloidin allows the identification of microfilamentous F-actin in each cell type. White scale bar 50µm.

Expressions of vimentin, cytokeratin and phalloidin proteins were investigated in endometrial cells for three animals. The presences of cytokeratin proved the epithelial nature of the cells whereas the expression of vimentin confirms the stromal origin of cells and give information on the purity of each cell populations. Phalloidin expression exhibits the mapping of cellular junctions. Analysis of *FOXL2* gene expression was carried out in bovine CAR and ICAR areas as well as in the untransfected primary cells after 5 days of culture in order to evaluate the basal expression of *FOXL2* in those cells prior to the transient transfection (Fig. 51).

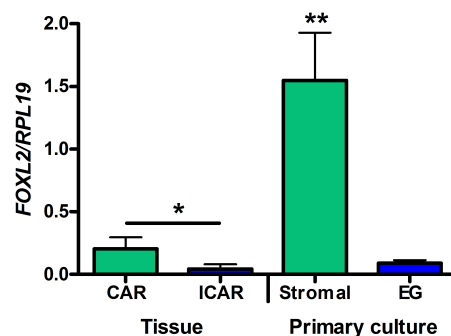


Figure 51: *FOXL2* mRNA level in bovine endometrial CAR and ICAR areas as well as in the stromal and glandular epithelial cells from the selected cows prior to the transient transfection. Caruncular (CAR,

stroma enriched area, green color) and intercaruncular (ICAR, glandular epithelium enriched area) endometrial areas were collected from bovine cyclic luteal uteri (n=3). Stromal and glandular epithelial (GE) cells were collected from the same bovine cyclic luteal uteri and cultured for 5 days. Quantification of *FOXL2* mRNA by RT-qPCR was normalized to *RPL19*. Quantitative data are presented as mean \pm SEM. Significant difference is noted, * : $P < 0.05$; ** : $P < 0.01$.

FOXL2 mRNA level was significantly higher in CAR area compared to the ICAR area (Fig. 51; 2-fold, $P < 0.05$). Surprisingly, *FOXL2* transcript expression was shown to be strongly increased in stromal cells (6-fold, $P < 0.01$) whereas its basal expression in GE cells remained lower. Three cows were selected, at the dioestrus stage during the active luteal phase where P4 circulating level reached its maximal amount. We hypothesized that the cell culture increased mRNA level of endometrial *FOXL2* in stromal cells only. Indeed, the P4 input should be higher in the cyclic luteal uteri than in cell culture leading to a de-repression of *FOXL2* expression. Interestingly, protein analysis has demonstrated that *FOXL2* protein expression is barely detectable in untransfected cells (Fig. 53). Our data suggest cell culture quickly increased the expression of *FOXL2* transcript only associated with increased degradation of the transcripts.

2. Validation of transient transfection of primary cultures of bovine endometrial cells

Transient transfections were carried out with primary endometrial cells of three cows. For each animal, transient transfection efficiency was validated using GFP observations (Fig. 52), *FOXL2* western blot immunoassays (Fig. 53) and immunocytochemistry analyses (Fig. 54).

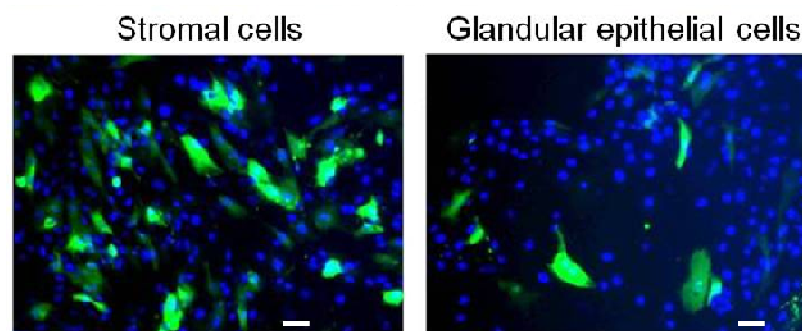


Figure 52: GFP fluorescence observation 48 hours after the transient transfection of GFPmax vector. Nuclei of stromal and glandular epithelial cells are stained in blue using DAPI reagent, and GFP positive cells in green. White scale bar 50 μ m.

GFP fluorescence was checked daily for 48 hours. The GFP signal intensity was not optimal 24 hours after the transfection. Transfection was strongly more effective in stromal cells compared to glandular epithelial cells respectively with an average of 40% and 10% of transfection rate.

FOXL2 transient transfection was also validated using protein detection.

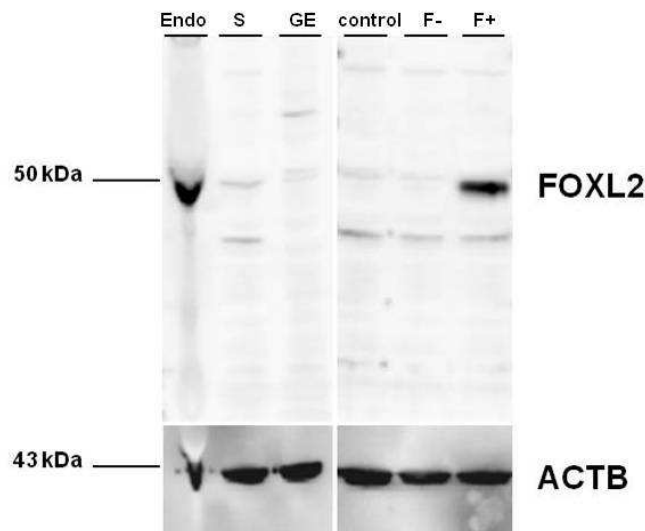


Figure 53: Immunodetection of FOXL2 and ACTB protein in bovine endometrial tissue and cells. Endo: endometrial tissue (CAR area); S: stromal cells; GE: glandular epithelial cells; control: untransfected stromal cells; F-: stromal cells transfected using pSG5 vector; F+: stromal cells transfected using pSG5-FOXL2 vector. FOXL2 is detected at 50kDa and ACTB at 43 kDa.

FOXL2 was strongly detected only in caruncular endometrium and in pSG5-FOXL2 transfected cells (Fig. 53). FOXL2 protein was weakly detectable in 'S', 'control' and 'F-' cells; and barely detectable in GE cells. Nevertheless, the transient transfection led to the over-expression of FOXL2 protein in 'F+' condition.

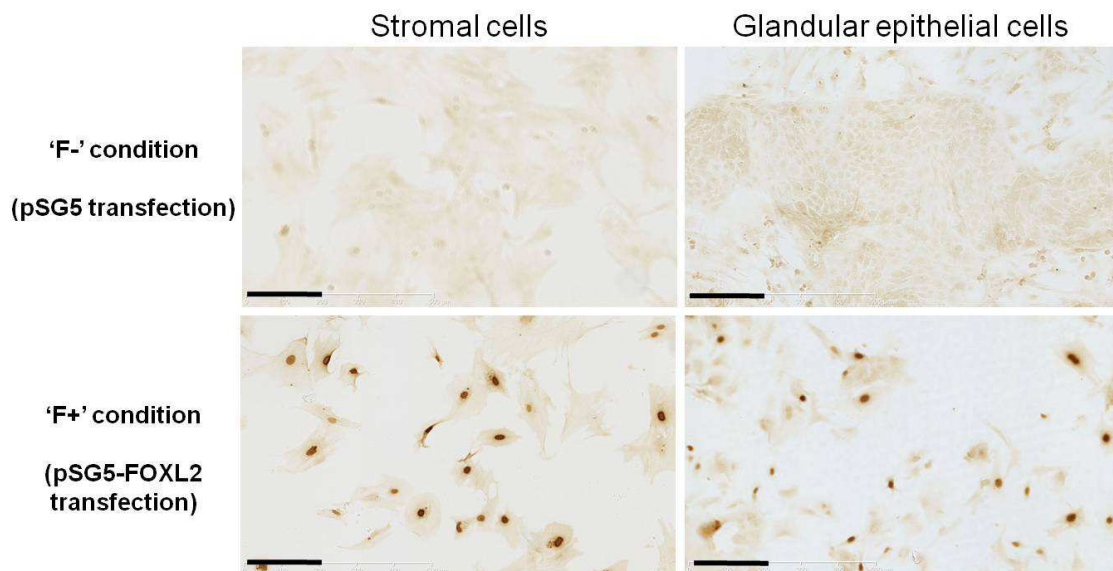


Figure 54: FOXL2 sub-cellular localization in stromal and glandular epithelial cells in mock transfection (pSG5) and FOXL2 transfection (pSG5-FOXL2). Immunocytochemistry was performed on stromal and glandular epithelial cells in 'F-' and 'F+' conditions. FOXL2 protein is barely detectable in stromal and glandular epithelial cells from mock transfection (F- transfection; pSG5). However, FOXL2 is detected in the nuclei of stromal and glandular epithelial cells from FOXL2 transfection (F+; pSG5-FOXL2). Black scale bar: 200µm.

In keeping with the faint signal detected by western blot, FOXL2 localization was barely detectable in stromal and glandular epithelial cells in 'F-' condition considered as a mock transfection (Fig. 54). A similar FOXL2 intensity signal is observed in the nuclei and the cytoplasm of those cells. FOXL2 was strongly localized in the nuclei of stromal and glandular epithelial cells after 'F+' transfection even if the transfection rate is better in stromal cells than in glandular epithelial cells. During the luteal phase, FOXL2 protein was shown to be detected only in the nuclei of stromal cells in bovine endometrial tissue (Part I; Eozenou et al. 2012) whereas stromal cells of 'F-' condition did not show any specific nuclear detection of FOXL2. In addition, FOXL2 protein is clearly detected in caruncular endometrium but almost not detected in stromal and glandular epithelial cells (Fig. 53). These data suggest that the switch from the *in vivo* to the *in vitro* status led to an increase and a decrease of FOXL2 mRNA level and FOXL2 protein expression, respectively.

Altogether, our data show that our protocol allows the effective transfection of primary cultures of bovine endometrial cells. The transfection rate was higher for stromal cells than for the glandular epithelial cells. Nevertheless, in both types of cells, the overexpression of FOXL2 was associated with the nuclear accumulation of the transcription factor a prerequisite for its biological activities.

3. Analyses of putative target genes

Transcript analyzes had been done using RT-qPCR experiment on stromal cells from three animals (Cow n°1, n°2 and n°3) and on glandular epithelial cells from two animals (Cow n°2 and n°3). Epithelial cells from cow n°1 were too much contaminated by fibroblast population and pure epithelial cell culture was not recovered as in Cow n°2 and 3.

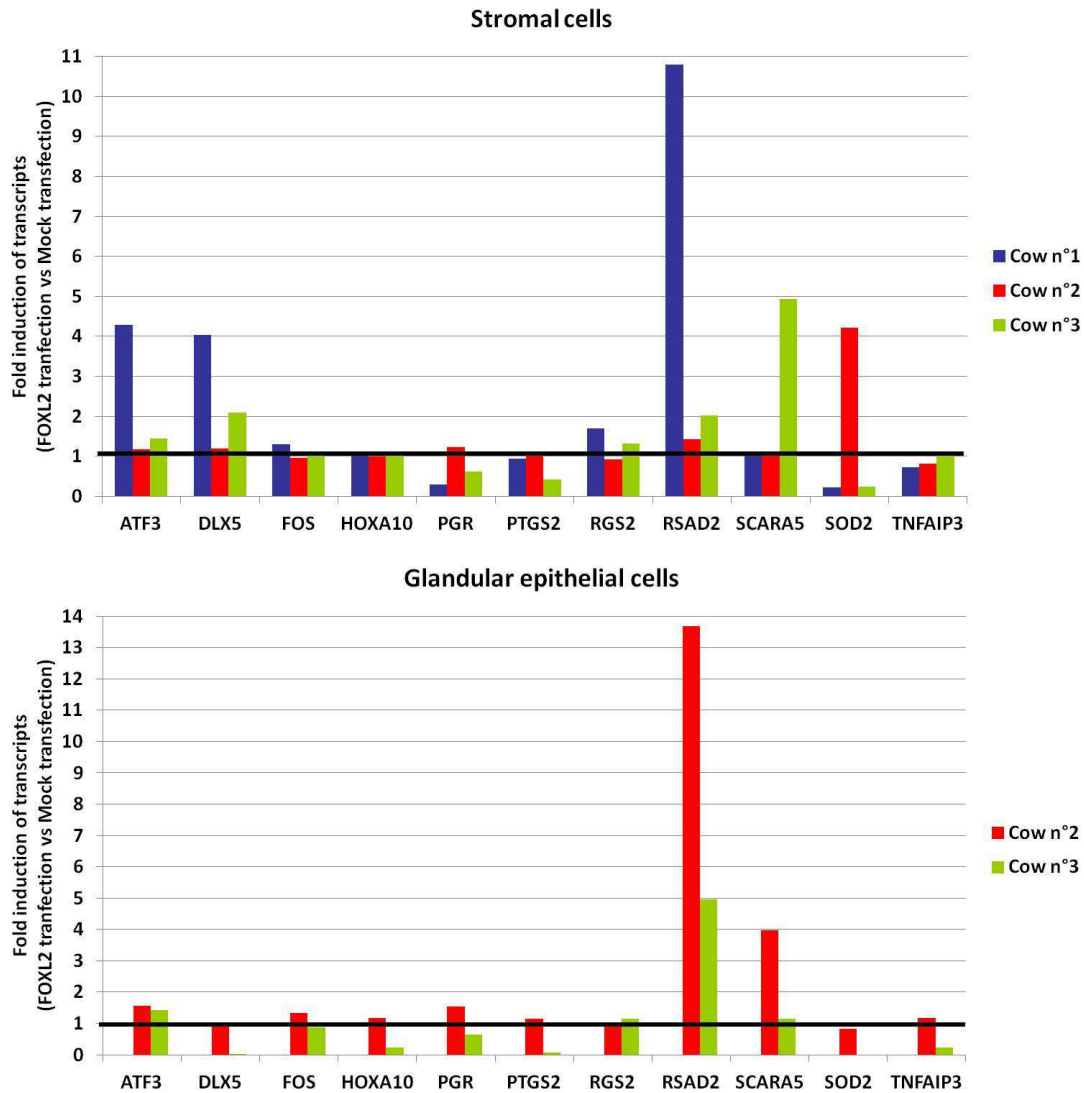


Figure 55: Transcript expression of putative FOXL2 regulated genes in stromal and glandular epithelial cells. Transcript expressions were presented using a ratio of target gene expression in 'F+' condition on 'F-' condition to prevent the impact of only electroporation on stromal and glandular epithelial cells. In stromal cells, *DLX5*, *RSAD2* and *SCARA5* expressions are stimulated at least in cow n°1 and 3. In glandular epithelial cells, *DLX5* and *PTGS2* expression are inhibited whereas *RSAD2* and *SCARA5* expressions were still stimulated.

The transcript analyse show differential regulation between the cows for each candidate gene (Fig. 55). The cows exhibit a complex heterogeneous response to the *FOXL2* over-expression. In stromal cells, the cow n°2 is completely different from the two others in target genes response. For instance, *ATF3*, *DLX5*, *PGR*, *PTGS2*, *RGS2*, *RSAD2* and *SOD2* expressions were completely inverted from cow n°2 to cow n°1 and 3. Nevertheless, analyse of *FOXL2* transfection has revealed the differential expression of some putative target genes despite the heterogeneity of animals.

In stromal cells, *FOXL2* over-expression induced the stimulation of *DLX5* expression (from 2-fold to 4-fold), *RSAD2* expression (from 2-fold to 10-fold) and *SCARA5* (5-fold) and the

inhibition of *PTGS2* expression (from 0.5-fold to 0.9-fold), *PGR* expression (from 0.2-fold to 0.5 fold) and *SOD2* expression (0.2-fold for cows n°1 and 3 and 4-fold for cow n°2).

In glandular epithelial cells, *FOXL2* over-expression led to a stimulation of *RSAD2* (from 5-fold to 13-fold) and *SCARA5* (4-fold) expressions and an inhibition of *DLX5* (0.1-fold), *HOXA10* (0.2-fold), *PGR* (0.7-fold), *PTGS2* (0.1-fold), *SOD2* (0.05-fold) and *TNFAIP3* (0.2-fold) expressions.

RSAD2 and *SCARA5* expression were shown to be the two most regulated transcripts among the eleven candidate genes because of their relevant stimulation either in the stromal or the glandular epithelial cells. *DLX5* expression is differentially regulated from the stromal cells to the glandular epithelial cells.

PTGS2 expression is always inhibited due to the over-expression of *FOXL2* except in the cow n°2 where there was no change of transcript expression.

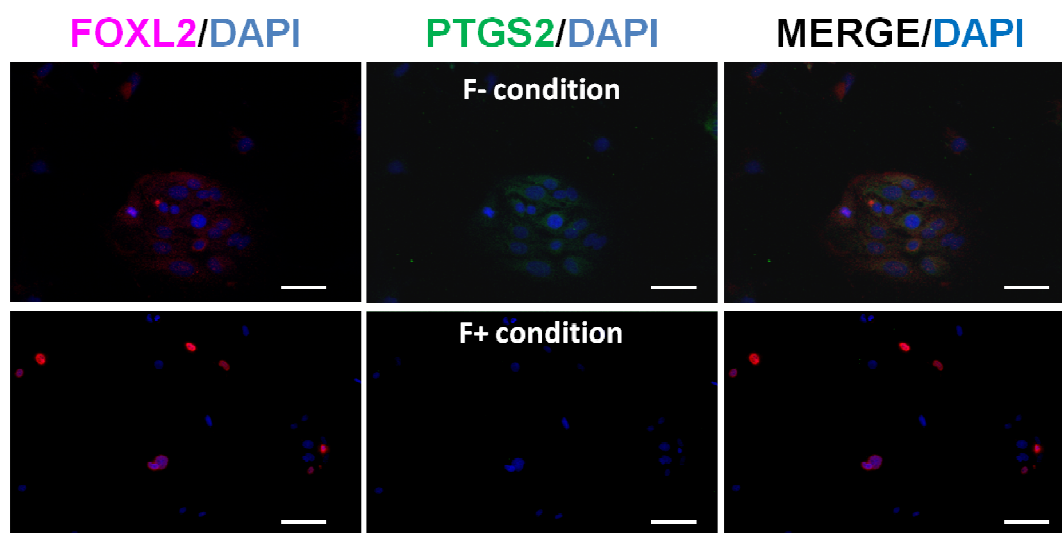


Figure 56: Localization of FOXL2 and PTGS2 proteins in glandular epithelial cells between F- and F+ conditions. FOXL2 protein is stained in red (and pink when co-localization with DAPI occurred), PTGS2 protein is stained in green and nuclei are stained in blue using DAPI staining. White scale bar: 50 μ m.

In 'F-' condition, FOXL2 is barely detected in the glandular epithelial cells and is not detectable in the nuclei (Fig. 56). However, PTGS2 staining is present in the cytoplasm of glandular epithelial cells of 'F-' condition. In 'F+' condition, FOXL2 is clearly detected in the nuclei of glandular epithelial cells whereas PTGS2 is completely absent of these cells. Altogether, our data highlight that FOXL2 over-expression is associated with the down-regulation of PTGS2 protein expression at the cellular level.

4. First insight about the FOXL2 regulated genes in the endometrium

For the first time, transient transfection of a transcription factor was setting up in bovine primary endometrial stromal and glandular epithelial cells. The over-expression of *FOXL2* was validated and the candidate genes approach allows the identification of three *FOXL2* regulated genes: *DLX5*, *RSAD2* and *SCARA5* (Fig. 57).

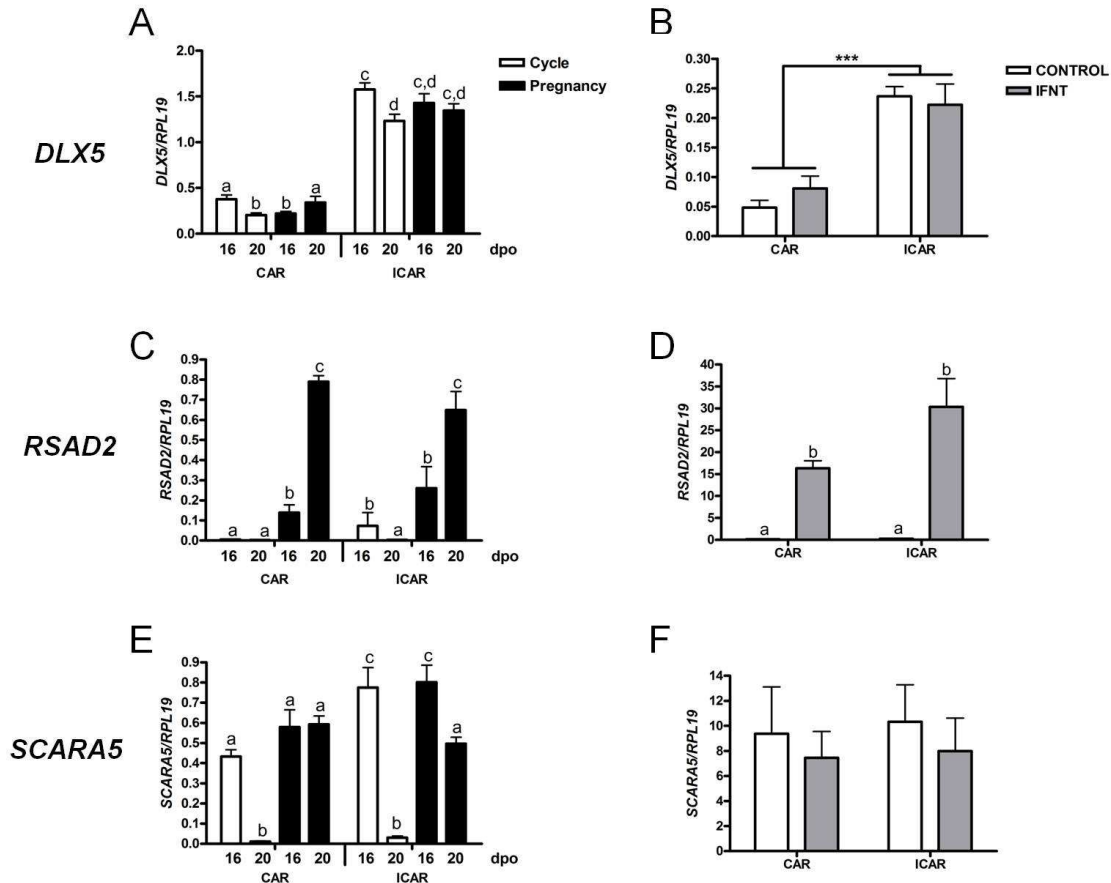


Figure 57: *DLX5*, *RSAD2* and *SCARA5* transcript expressions in bovine endometrium during the oestrous cycle, early pregnancy and under the influence of IFNT. Transcript expression of *DLX5*, *RSAD2* and *SCARA5* were investigated during the oestrous cycle and early pregnancy (respectively A, C and E) and under the influence of IFNT (respectively B, D and F) in bovine endometrium. (A), (C) and (E) Caruncular (CAR) and intercaruncular (ICAR) endometrial areas were collected from cyclic (n=5 at day 16, n=6 at day 20) and pregnant (n=4 at day 16, n=5 at day 20) cross-bred beef heifers. Expressions of transcripts were normalized to *RPL19*. (B), (D) and (F) CAR and ICAR endometrial areas were collected from cyclic (14 dpo) Charolais cows infused with control solution (n=5) or recombinant ovine IFNT (200 µg/ml, n=6) for 2 hours. Expressions of transcripts were normalized to *RPL19*. (D) *RSAD2* expression graph is adapted from Forde *et al.*, 2011. Quantitative data are presented as mean \pm SEM and bars with different superscripts significantly differ (in (A), (C), (D) and (E), $P < 0.01$).

DLX5 mRNA was expressed in both endometrial areas during the oestrous cycle and early pregnancy, was significantly higher in ICAR area (3-fold, $p < 0.01$; Fig. 57A). The oestrous cycle is characterized by a higher *DLX5* expression during the luteal phase (16 dpo; 2-fold, $p < 0.05$) compared to the follicular phase (20 dpo) in both endometrial areas whereas in pregnancy, its expression is lower during the maternal recognition period (16 dpo; 2-fold, $p <$

0.05) compared to the first day of implantation (20 dpo). At 14 dpo of the oestrous cycle corresponding to the luteal phase, *DLX5* expression is also significantly increased in ICAR area (5-fold, $p < 0.001$) after IFNT infusion but not impacted by the short-term treatment (Fig. 57B).

RSAD2 mRNA was expressed during the early pregnancy and significantly higher at implantation (20 dpo; 5.3-fold, $p < 0.001$ in CAR area and 2.3-fold, $p < 0.01$) compared to the maternal recognition period (16 dpo, Fig. 57C). Its expression was almost absent during the oestrous cycle except during the luteal phase (16 dpo) in ICAR area. *RSAD2* as an IFN-induced gene was strongly stimulated by the infusion of IFNT during the luteal phase (Fig. 57D, 14 dpo; 16-fold, $p < 0.0001$ in CAR area and 30-fold, $p < 0.0001$ in ICAR area). These data were adapted from Forde *et al.*, 2011.

SCARA5 mRNA was strongly expressed during the luteal phase of the oestrous cycle, as well as the early pregnancy and its expression was shown to be significantly lower during the follicular phase in both endometrial areas (Fig. 57E, from 43-fold to 58-fold, $p < 0.0001$ in CAR area; from 16-fold to 26-fold, $p < 0.0001$ in ICAR area). In particular, *SCARA5* expression was significantly higher during both oestrous cycle and early pregnancy at 16 dpo compared to the implantation day (20 dpo, 1.6-fold, $p < 0.05$). The short-term treatment of IFNT had no impact on *SCARA5* transcript expression in both endometrial areas (Fig. 57F).

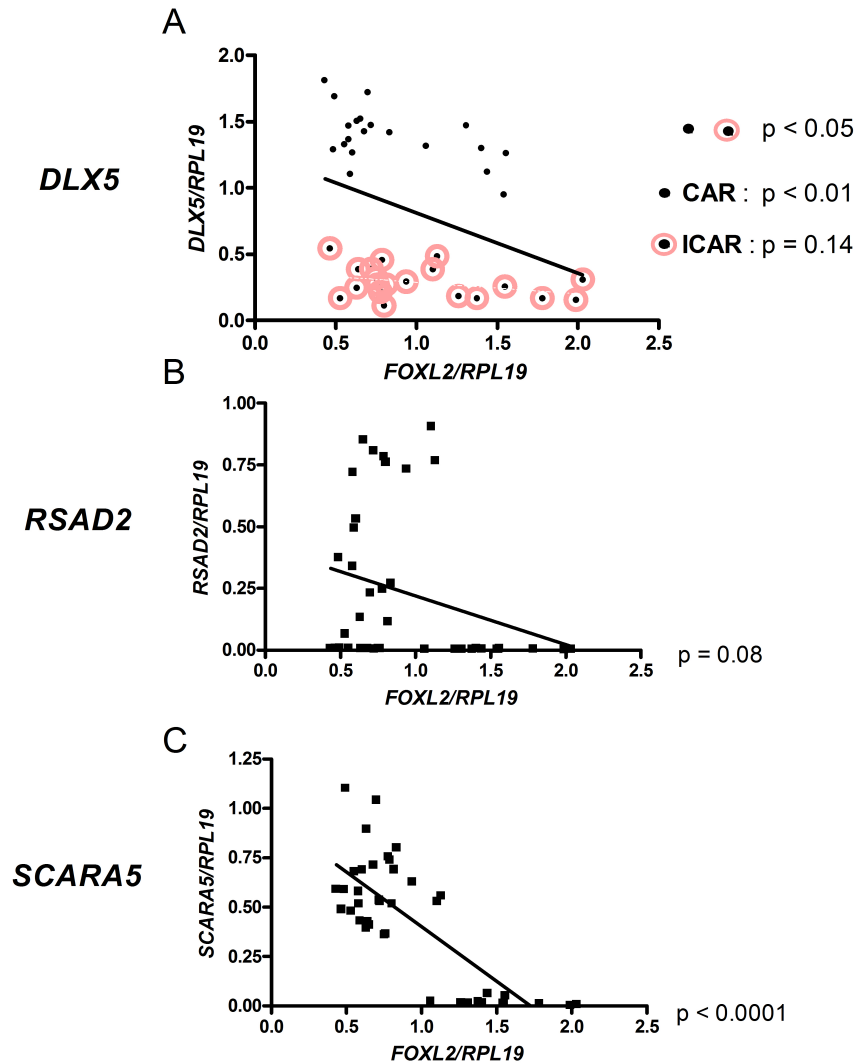


Figure 58: Correlation between the endometrial expression of putative FOXL2 target genes and FOXL2 during the oestrous cycle and the early pregnancy. Correlation was established between *FOXL2* expression and (A) *DLX5* (CAR: black dot, $p < 0.01$; ICAR: pink circled black dot, $p = 0.14$; CAR and ICAR: $p < 0.05$), (B) *RSAD2* and (C) *SCARA5* expressions during the oestrous cycle and the early pregnancy in cattle.

DLX5 and *SCARA5* expressions were negatively correlated with *FOXL2* expression in bovine endometrium (Fig. 58). *DLX5* and *FOXL2* expressions were negatively correlated (Fig. 58A, $p < 0.05$) and two negative slopes were shown corresponding to the inverted expression observed in CAR (black dot, $p < 0.01$) and ICAR (pink circled black dot, $p = 0.14$) areas for each gene. The negative correlation occurring between *FOXL2* and *SCARA5* expressions was highly significant (Fig. 58C; $p < 0.0001$) and separated in two groups: the follicular phase where *FOXL2* is highly expressed and the three remaining group where *FOXL2* expression is lower and steady. The correlation between *FOXL2* and *RSAD2* expressions was not significant (Fig. 58B; $p = 0.08$) but also appeared negative.

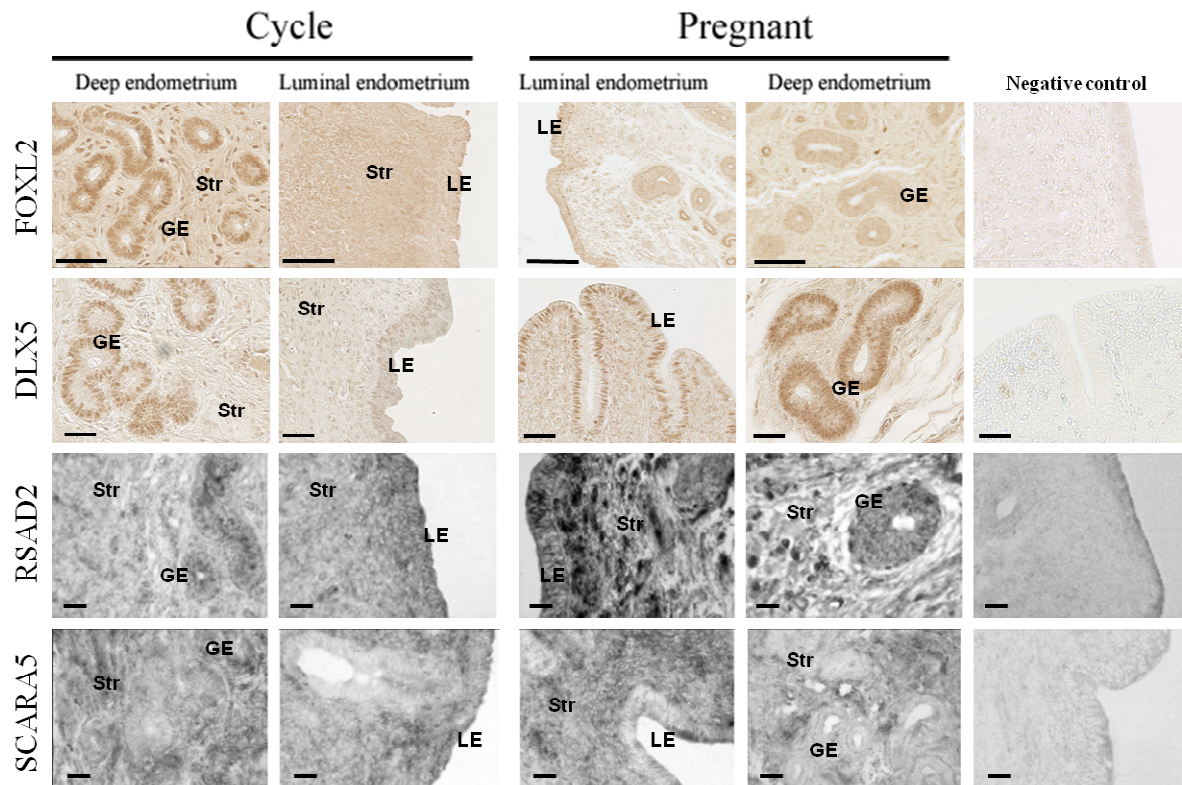


Figure 59: Cell localization of FOXL2 and DLX5 proteins as well as RSAD2 and SCARA5 transcripts in bovine endometrium. FOXL2 and DLX5 proteins were detected using immunohistochemistry (C. Eozenou) whereas RSAD2 and SCARA5 transcripts were detected using *in situ* hybridization (from Mansouri-Attia et al. 2009). Immunohistochemistry and *in situ* hybridization were performed using sections cut from Charolais endometrium. Females were sampled at day 20 of the oestrous cycle as well as day 20 of pregnancy. GE: glandular epithelium; Str: stroma and LE: luminal epithelium. Black scale bar: 50 μ m.

DLX5 protein which is restricted to the nuclei is localized in either luminal or glandular epithelium during the oestrous cycle and show a higher staining intensity during early pregnancy in both types of epithelia (Fig. 59). Interestingly, FOXL2 protein was inversely detected with a higher staining intensity during the oestrous cycle and a strong decrease during early pregnancy, especially in the nuclei of stromal and GE cells. Altogether, our results show that *DLX5* gene is strongly expressed in ICAR area, poorly in CAR area and reversed compared to *FOXL2* gene expression. The expression of *DLX5* transcript and protein varied slightly regarding the complex regulation of *FOXL2* gene expression during the oestrous cycle and early pregnancy.

RSAD2 transcript is localized in the GE and stromal cells as well as in the luminal epithelial cells (Fig. 59). This mRNA is almost absent of the cyclic endometrium compared to the intense staining observed in pregnant endometrium. This localization is completely inverted compared to the localization of FOXL2 protein. FOXL2 is almost absent of the nuclei of endometrial cells during pregnancy whereas during oestrous cycle (20 dpo), FOXL2 protein is strongly detected in the nuclei of stromal and GE cells.

SCARA5 transcript was shown to be localized in the stroma and the glandular epithelium without significant change of intensity between oestrous cycle and early pregnancy (Fig. 59).

D. Conclusions

The aim of this part of my work was to provide first insights on FOXL2 target genes in the bovine endometrium, using functional genomics and various physiological and experimental *in vivo* and *in vitro* models.

In transiently transfected endometrial cells, FOXL2 over-expression led to the alteration of a selection of genes that was selected based on a literature approach. Given the results that were obtained from these preliminary experiments, transfection efficiency and heterogeneous biological response of the transfected cells have to be discussed.

A noticeable problem was the heterogeneity of biological responses in term of FOXL2-regulated genes analysed with the primary endometrial cells that were derived from three different cows. The uteri were taken from a local slaughterhouse and the morphological aspect of the genital tracts and ovaries were recorded. No sign of infection could be seen for either genital tract. For each cow, one single corpus luteum was identified and its physiological status was evaluated according to published criteria (Arosh et al. 2002). The morphology, size and weight allowed the classification of each corpus luteum into three distinct time points of the luteal phase: the first cow was in the active luteal phase (days 13-15 of the oestrous cycle), the second in the early luteal phase and the third one was situated between cow n°1 and 2, in the mid luteal phase. This variability between the three females could account for the heterogeneity of the biological response reported in the primary cultures of endometrial cells before and after transfection. Consequently, further investigations will require deriving primary cultures of endometrial cells from females sampled at an equivalent stage of the luteal phase. If this issue cannot be achieved using biological material collected in local abattoirs, synchronization of experimental females will be necessary that will allow us to recover endometrial cells at a defined day of the oestrous cycle. The cows were sorted in order to over-express *FOXL2* gene expression in endometrial cells. The first part of my PhD revealed that the lowest FOXL2 expression was situated during the active luteal phase (16 dpo) in bovine endometrium. These preliminary data prompt the need to synchronize female and to recover reproductive tract at 16 dpo. Considering the relevant expression of FOXL2 during the follicular phase of the oestrous cycle namely 20 dpo, it could be interesting to inhibit *FOXL2* expression using RNA

interference method on stromal and glandular epithelial cells to complete the identification of FOXL2 regulated genes.

For the gene delivery of *FOXL2* into primary cultures of bovine endometrial cells, we used a method based on the electroporation of non-adherent cells. This method appeared to be the best compromise that yielded a high transfection rate of the stromal cells (on the average of 41%) while preserving their viability (77% of recovered and viable cells upon transfection). Nevertheless, in the case of GE cells, the transfection rate remained low (4-12 %) and could not be improved despite our efforts (change of culture media, FBS, AmaxaTM transfection kit and programs). Since primary cells were used in order to keep the physiological status of the endometrial cells as close as possible to the *in vivo* situation, it seems unrealistic to eliminate the non transfected cells using a selection process based on the use of *Aph* gene combined with a restrictive culture media containing G418 substance (Dutta et al. 2012). Indeed, this process requires several weeks, a time period incompatible with short term cultures of primary cells. Similarly, the stable transfection will not be an option due to the length of cell culture to have a stable transfected culture. Currently one option appears the most relevant to increase the number of transfected GE cells namely the use of cell sorting based on GFP expression. A cell-sorter should be available in the vicinity of the laboratory in a near future.

PTGS2 is an enzyme responsible for the conversion of arachidonic acid into PGH₂, prior to the production of PGF₂ α leading to the CL regression (Smith et al. 1996). This enzyme has been shown to be expressed in bovine and ovine endometrium during the oestrous cycle and early pregnancy (Charpigny et al. 1999; Arosh et al. 2002). *FOXL2* over-expression led to the inhibition of *PTGS2* gene expression in primary cultures of stromal (in cow n°1 and 3) or GE cells (only in cow n°3). Very interestingly, PTGS2 and FOXL2 proteins were not co-expressed as underlined by the absence of co-localization in individual cells. In other words, when FOXL2 was present in the nuclei of endometrial cells, PTGS2 was not detected whereas in untransfected cells, PTGS2 was detected in the cytoplasm when FOXL2 could never been seen in the nucleus nor the cytoplasm of these cells. Collectively, our data show that FOXL2 inhibits the expression of *PTGS2* gene (transcript and protein) in endometrial cells. Immunolocalization of *PTGS2* revealed that endometrial luminal and superficial glandular epithelia are the PTGS2-producing cell types in ovine endometrium whereas in bovine endometrium PTGS2 is mostly localized in luminal epithelium and slightly localized in the glandular epithelium and in stroma (Charpigny et al. 1997; Arosh et al. 2004). FOXL2 could not be detected in luminal epithelium and superficial glandular epithelium whereas it was present in the stromal and outer glandular epithelium (Eozenou et al. 2012). *FOXL2* and

PTGS2 were shown to be up-regulated during the follicular phase but are localized in different endometrial cell types. Endometrial cell localization of *FOXL2* and *PTGS2* is therefore consistent with our *in vitro* results and strongly suggests that *FOXL2* is involved in the control of uterine receptivity through the regulation of *PTGS2* synthesis. *FOXL2* was shown either to stimulate the expression of *PTGS2* in KGN cell line (Batista et al. 2007) or to repress *PTGS2* expression (Kim et al. 2009) in 293FT cells (ER α negative human embryonic kidney) and MDA-MB-231 cells (ER α negative human breast cancer) suggesting *FOXL2* could differentially regulate its target genes and in the latter extent, *PTGS2*, in different cellular context due to the availability of its co-factors and binding partners. Future experiments will examine the binding of *FOXL2* on *PTGS2* promoter in order to confirm this gene as a direct *FOXL2* target gene in the endometrium.

RSAD2 is a class I IFNs induced gene whose expression is strongly increased during early pregnancy in bovine endometrium (Song et al. 2007; Mansouri-Attia et al. 2009; Forde et al. 2011). *RSAD2* has not been described yet in the ovarian context and was not reported to be regulated by *FOXL2*. Nevertheless, *RSAD2* was selected for its essential endometrial role at the maternal/conceptus interface prior to implantation. *FOXL2* is not an IFNT-induced gene *in vivo*, then we hypothesized that *FOXL2* could not regulate the expression of *RSAD2* representing a negative control of gene expression. Interestingly, in our bovine primary endometrial cells, *RSAD2* was the most up-regulated transcript by the over-expression of *FOXL2*. The previous investigations about *RSAD2* biological functions show the evidence its expression could modulate uterine receptivity by induction of antiviral state and modulation of immune cell functions in the pregnant ruminant endometrium (Mansouri-Attia et al. 2009; Forde et al. 2011). *FOXL2* protein and *RSAD2* transcript were shown to be localized in the same endometrial cell types, GE and stromal cells. During the normal oestrous cycle, *RSAD2* mRNA is expressed at a low level in the endometrium whereas *FOXL2* transfection stimulates its expression in bovine endometrial cells from cyclic uteri. We could hypothesized the earlier expression of *RSAD2* during oestrous cycle due to *FOXL2* expression could be deleterious for uterine receptivity and in the latter extent, implantation step. Further investigations will be necessary to evaluate precisely the expression of *RSAD2* during the follicular phase where *FOXL2* is widely expressed but also to determine if *RSAD2* is a direct target gene of *FOXL2* in endometrium.

Scara5, formerly called *Tesr* (Sarraj et al. 2005) appeared as one of the most up-regulated genes resulting from *Foxl2* conditional deletion in the adult murine ovary (Uhlenhaut et al. 2009). This class A scavenger receptor has been shown to be involved in innate immunity

and decidualisation but it was described also as a ferritin receptor mediating iron delivery (Li et al. 2009; Mansouri-Attia et al. 2009; Duncan et al. 2011). *SCARA5* was identified as a differentially expressed gene in endometrial tissues of pregnant cows compared to cyclic females at 20 dpo (Mansouri-Attia et al. 2009). The present work has demonstrated that *SCARA5* is not an IFNT-induced gene *in vivo* in keeping with former data based on primary cultures of IFNT-treated endometrial cells (Mansouri-Attia et al. 2009). Moreover, *SCARA5* transcript and *FOXL2* protein were shown to be localized in the same endometrial cell types namely GE and stromal cells. Then, *SCARA5* and *FOXL2* endometrial expression were negatively correlated during the oestrous cycle and early pregnancy suggesting *FOXL2* could repress *SCARA5* expression in bovine endometrium. Interestingly, *SCARA5* mRNA expression was up-regulated in *FOXL2* transfected stromal and GE cell cultures. These data suggest *SCARA5* is regulated by *FOXL2* in the endometrial context but the *in vitro* situation differs from the *in vivo* situation. Then, we hypothesized that *FOXL2* regulate *SCARA5* expression depending on the cellular context and probably mediated by co-factors or binding partners as the regulation of *PTGS2* expression described above. Complementary analyses are needed to identify *SCARA5* as direct target gene of *FOXL2* as well as binding partners or co-factors of *FOXL2* protein in the endometrium.

DLX5 and *DLX6* transcription factors have been shown to be involved in the control of steroidogenesis in granulosa cells (Bouhali et al. 2011). A reciprocal regulation between the expression of *Dlx5* and *Foxl2* has been described in the murine ovary (Bouhali et al. 2011). In this species *FOXL2* is strongly expressed in primordial and primary follicles but its expression decreases during the follicle maturation. *Foxl2* ovarian expression activates the transcription of *Dlx5* in granulosa cells. Then, *Dlx5* expression represses *Foxl2* expression in the secondary follicle until mature antral follicle (Bouhali et al. 2011). In our cell transfection experiments, *FOXL2* over-expression is associated to a cell-specific regulation of *DLX5* expression. Indeed, the low level of *DLX5* mRNA expression in stromal cells is up-regulated in *FOXL2*-transfected stromal cell cultures whereas *FOXL2* over-expression led to the repression of *DLX5* transcript expression in epithelial cell cultures whose basal level of *DLX5* mRNA was high. Collectively, our results evidence that regulation of *DLX5* gene expression by *FOXL2* differs with the type of endometrial cells. The differential expression of *DLX5* gene between CAR and ICAR areas could also explain the differential regulation of this gene following *FOXL2* over-expression. Indeed, the over-expression of *FOXL2* induces a stimulation of *DLX5* transcript expression in stromal cells whereas its basal expression in CAR area is very low in association with a poor staining intensity of *DLX5* protein in this stroma enriched area. Inversely, *DLX5* expression is down-regulated in epithelial cells after

FOXL2 over-expression whereas its expression is very high in ICAR area associated with a high staining intensity of DLX5 in this endometrial gland area. These data suggest a complex regulation of *DLX5* expression by *FOXL2* dependant on the expression level of *DLX5*.

Finally, *DLX5* and *FOXL2* expressions were shown to be negatively correlated throughout the oestrous cycle and early pregnancy. Our data suggest that the reciprocal regulation between *DLX5* and *FOXL2* originally found in the ovary does exist in the endometrium associated with a cell-specific regulation of *DLX5*. Further experiments will be necessary to decipher the biological meaning of this reciprocal regulation in the context of the endometrial physiology.

Collectively, our results provide the first insights on the regulation of endometrial genes by FOXL2. In our primary cultures of endometrial stromal and GE cells, FOXL2 over-expression led to (i) the inhibition of *PTGS2* and the stimulation of *SCARA5* and *RSAD2* transcript expression in both cell types (ii) the up-regulation and the down-regulation of *DLX5* mRNA level in stromal and GE cells respectively. In cattle, FOXL2 then appears to participate to the regulation of endometrial physiology through the regulation of the prostaglandins metabolism and innate immune function prior to conceptus implantation. Beyond the candidate gene approach we initiated, a large-scale analysis of FOXL2-regulated genes based on the use of transient transfected endometrial cells associated to RNA-sequencing would be relevant in order to determine the contribution of this transcription factor to the regulation of endometrial physiology.

VIII. General discussion

FOXL2 was, originally, described as a key gene for ovarian differentiation, maintenance of ovarian function from foetal life to adulthood with mutations leading to premature ovarian failure, granulosa cell tumour and more generally for ovarian fertility (Crisponi et al. 2001; Pailhoux et al. 2001; Uda et al. 2004; D'Angelo et al. 2011; Verdin and De Baere 2012). Premature ovarian insufficiencies or failures consecutive to *FOXL2* mutations could be partially replaced using the progress of medically assisted procreation (MAP; Devroey and Pados 1998). The question about the impact of those pathologies on endometrial physiology is still pending. Indeed, beyond the blastocyst stage, a successful pregnancy has to be conducted by the uterus and MAP cannot rescue endometrial defects. To date, *FOXL2* was never studied in the endometrium. But now, *FOXL2* transcript has been found differentially expressed in bovine endometrium at 20 dpo between pregnancy and oestrous cycle (Mansouri-Attia et al. 2009). In ruminants and in high-producing dairy cows specifically, half of pregnancies abort during the peri-implantation period as a consequence of early embryonic death and endometrial defects (Diskin and Morris 2008). Therefore, peri-implantation period appears to be a critical checkpoint for the progression and the issue of pregnancy. My work focused on the implication of *FOXL2* gene in ruminant endometrial physiology.

In order to understand the implication of *FOXL2* in endometrial physiology, physiological and experimental models were derived from ruminant. First, *FOXL2* gene was shown to be expressed during the oestrous cycle and the early pregnancy in both cattle and sheep endometrium. Its endometrial expression is under the control of ovarian steroid hormones with a minor positive effect of E2 and a strong inhibition consecutive to P4 biological action. Each model exhibits a decreased expression of *FOXL2* in ICAR area compared to CAR area. Finally, the over-expression of *FOXL2* leads to the regulation of *DLX5*, *PTGS2*, *RSAD2* and *SCARA5* expressions in bovine endometrial stromal and glandular epithelial cells. The ectopic expression of *FOXL2* in endometrial cells from luteal uteri exhibits the altered regulation of genes involved in uterine receptivity suggesting the inhibition of *FOXL2* expression during the luteal phase and the pre-implantation period is a prerequisite for conceptus implantation (Fig. 63, 64 and 65).

The differential expression of *FOXL2* between 20 dpo of pregnancy and oestrous cycle in bovine endometrium was first considered as an IFNT induced regulation. Using *in vivo* and *in vitro* bovine models, we have demonstrated that either short- (2 hours) or long-term (24 hours) treatment of IFNT had no significant impact on *FOXL2* endometrial expression (Fig. 64). This situation differs from that in human since human trophoblast secretions lead to the

down-regulation of *FOXL2* transcript expression as observed in endometrial gene expression profiling (Fig. 60; Hess et al. 2006; Popovici et al. 2006).

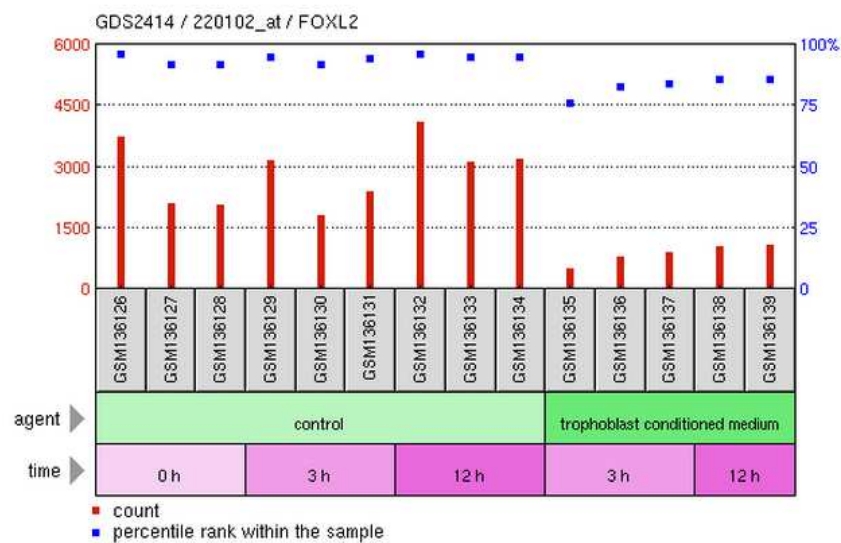


Figure 60: FOXL2 transcript expression in decidual stromal cell response to trophoblast conditioned medium (GEOProfile n°GDS2414 from Hess et al. 2006).

Nevertheless, in cattle, FOXL2 protein localization appears to be regulated by the conceptus apposition. Indeed, during the luteal phase, FOXL2 was localized in the nuclei of stromal cells and in the cytoplasm of glandular epithelial cells whereas at implantation, FOXL2 was detected in the cytoplasm of both stromal and glandular epithelial cells. At implantation, P4 circulating level is similar to the maximal secretion obtained during the luteal phase. But its sub-cellular localization differs between both phases. We hypothesized that conceptus may affect intracellular localization of FOXL2 in stromal cells. The combine action of P4 and conceptus lead to an inhibition of *FOXL2* expression (mRNA levels and cytoplasmic localization). Bovine conceptus secretion could be investigated using a secretome gene expression profile to identify others embryonic signals regulating endometrial gene expression in association with the IFNT action. Interestingly, change of intracellular localization was reported for FOXL2 in the ovarian context (Marongiu et al. 2010; L'Hote et al. 2012). FOXL2 binding partners can either stabilize or destabilize FOXL2 protein. SIRT1, PIAS1, SUMO and SMAD proteins were shown to be binding partners of FOXL2 and could induce an intracellular translocation of FOXL2 (Marongiu et al. 2010; Georges et al. 2011). Our data and the literature suggest the cytoplasmic localization of FOXL2 occurring at implantation in bovine endometrium could be due to the presence or the absence of specific binding partners. Method of Yeast-Two-Hybrid could be considered in endometrial cells in order to identify specific binding partners of endometrial FOXL2 protein followed by the analysis of FOXL2 activity (localization and transactivation).

FOXL2 expression is clearly regulated by E2 and P4 circulating levels in endometrium of both cattle and sheep (Fig. 64 and 65). More specifically, *FOXL2* gene expression is inhibited by P4 circulating level during the luteal phase and the establishment of pregnancy in sheep and cattle. Similar to this situation, *FOXL2* transcript is higher in human endometrial biopsies collected during the follicular phase (proliferative phase) compared to the luteal phase (secretory phase; Fig. 61; Talbi et al. 2005).

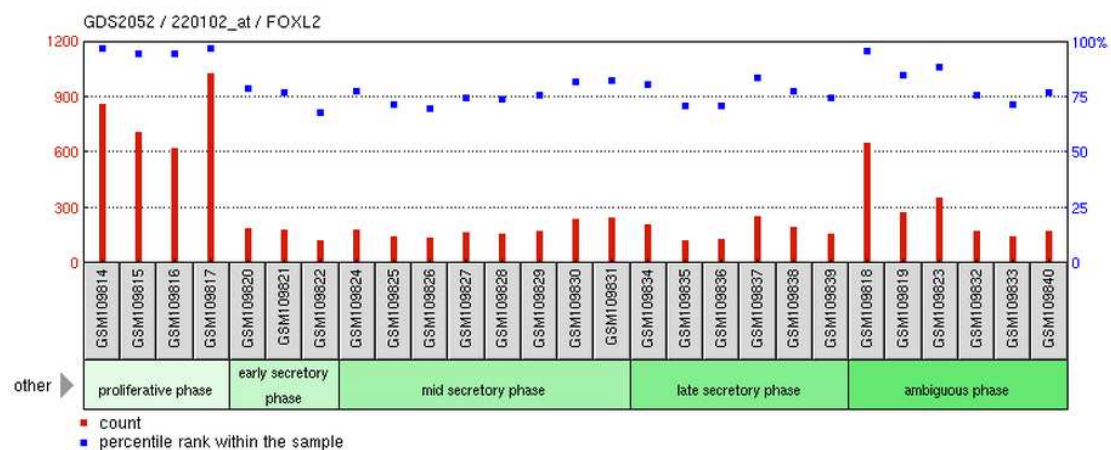


Figure 61: *FOXL2* transcript expression in human endometrium throughout the menstrual cycle (GEOPProfile n°GDS2052 from Talbi et al. 2005).

Interestingly, human proliferative phase controlled by E2 exhibits a higher expression of *FOXL2* transcript than during the human secretory phase controlled by P4 (Talbi et al. 2005). Collectively, our results in cow and sheep as well as in human show *FOXL2* endometrial expression is repressed consecutive to P4 biological action. A pending question is to determine if the action of P4 on *FOXL2* endometrial expression is direct. In the endometrium, nuclear receptors of P4 are responsible of the major direct biological action of P4 (Lydon et al. 1995; Franco et al. 2008; Franco et al. 2012; Wetendorf and DeMayo 2012). During oestrous cycle and early pregnancy, *PGR* gene exhibits the same gene expression profile of *FOXL2* with a high expression during the follicular phase and low expression during the luteal phase and pre-implantation period as well as the same intracellular localization in bovine endometrium (Fig. 43; Okumu et al. 2010; Eozenou et al. 2012). In human endometrium, this situation is the same with a high expression of *PGR* during the proliferative phase and reduced expression during the secretory phase of the menstrual cycle (Talbi et al. 2005).

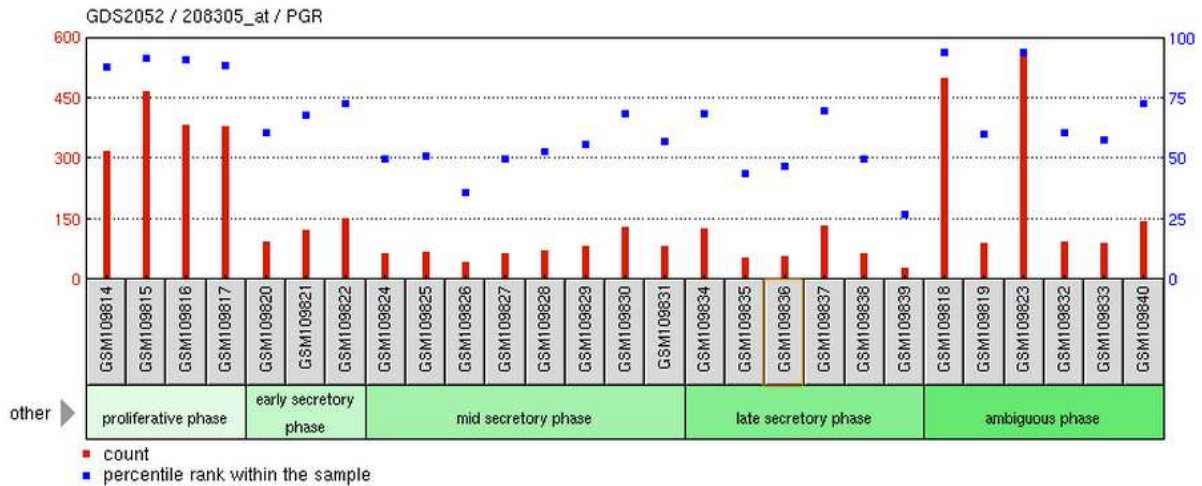


Figure 62: *PGR* transcript expression in human endometrium throughout the menstrual cycle (GEOPProfile n°GDS2052 from Talbi et al. 2005).

In addition, *PGR* expression was up-regulated in endometrial explants incubated for 48 hours with E2 compared to P4 treatment. During uterine receptivity and pre-implantation periods, *PGR* protein is lost in both luminal and glandular epithelium which is a prerequisite for conceptus implantation (Spencer and Bazer 2002). *PGR* play a critical role in endometrial epithelial cells through its regulation of epithelial target genes (namely *HOXA10* and *IHh* genes) and inhibition of E2-induced epithelial proliferation (Franco et al. 2012). Our results and the literature bring evidence about the correlation between *PGR* and *FOXL2* genes expression. In order to identify if *FOXL2* gene is a direct target of P4, *PGR* (form A and B) transient transfection of COS7 cells is carrying out in association with transfection of *FOXL2* promoter reporter plasmid, treated with P4. In addition, experiments of Chromatin-Immuno-Precipitation (ChIP) could be used to decipher the complex regulation occurring between *FOXL2* and *PGR* genes in stromal and glandular epithelial cells. Given that published *PGR* ChIP-sequencing work have revealed direct target genes of *PGR* in the murine uterus (Rubel et al. 2012), it could be interesting to evaluate *FOXL2* in those samples to identify if this FOX factor is a direct target gene of *PGR*. In keeping with *PGR* gene regulation during uterine receptivity, our results suggest the loss of *FOXL2* expression is also a prerequisite for conceptus implantation. As described above, *FOXL2* protein is not detected in GE suggesting that *FOXL2* could be involved in the uterine secretion necessary for conceptus elongation and we could hypothesized that an ectopic expression of *FOXL2* during these periods could be deleterious for conceptus health, elongation and implantation. Given that some types of endometrial cancers and endometriosis are consecutive to a resistance of P4 biological action (Kim and Chapman-Davis 2010; Al-Sabbagh et al. 2012), the implication of endometrial *FOXL2* gene in those pathologies would deserve further investigations in order

to examine if FOXL2 endometrial expression could be over-expressed as our data would suggest it.

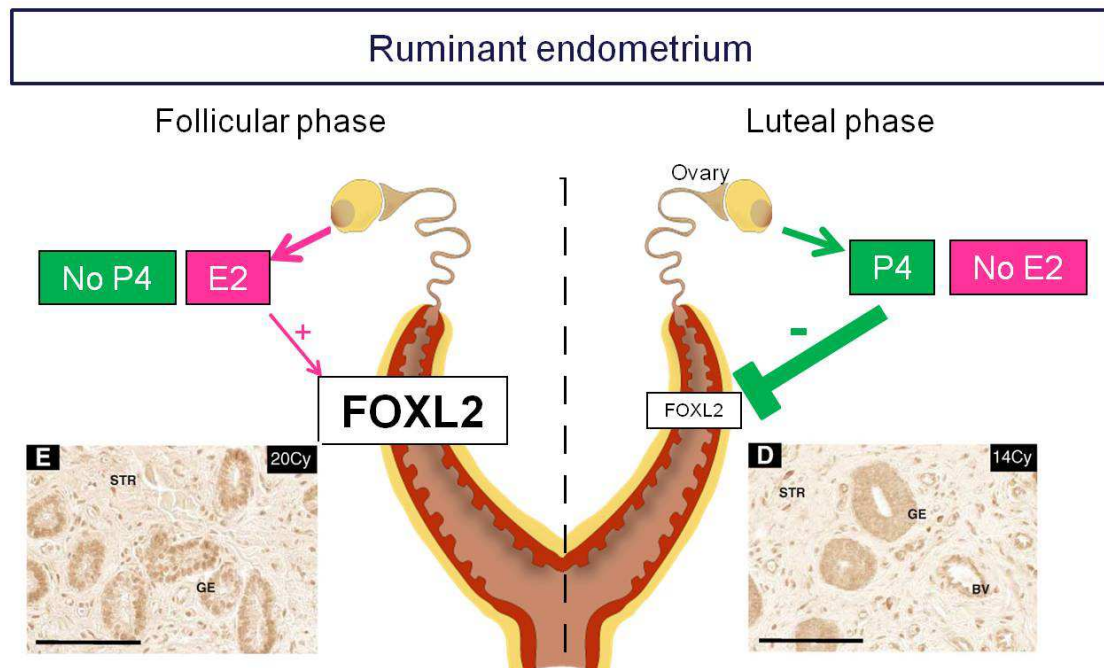


Figure 63: FOXL2 gene expression and localization in ruminant endometrium during the oestrous cycle. FOXL2 was shown to be strongly expressed during the follicular phase under the influence of E2 and to exhibit a clear nuclear localization in ruminant endometrium. During the luteal phase, it has been demonstrated that P4 circulating level inhibited the expression of FOXL2 associated with cytoplasmic localization in the GE cells.

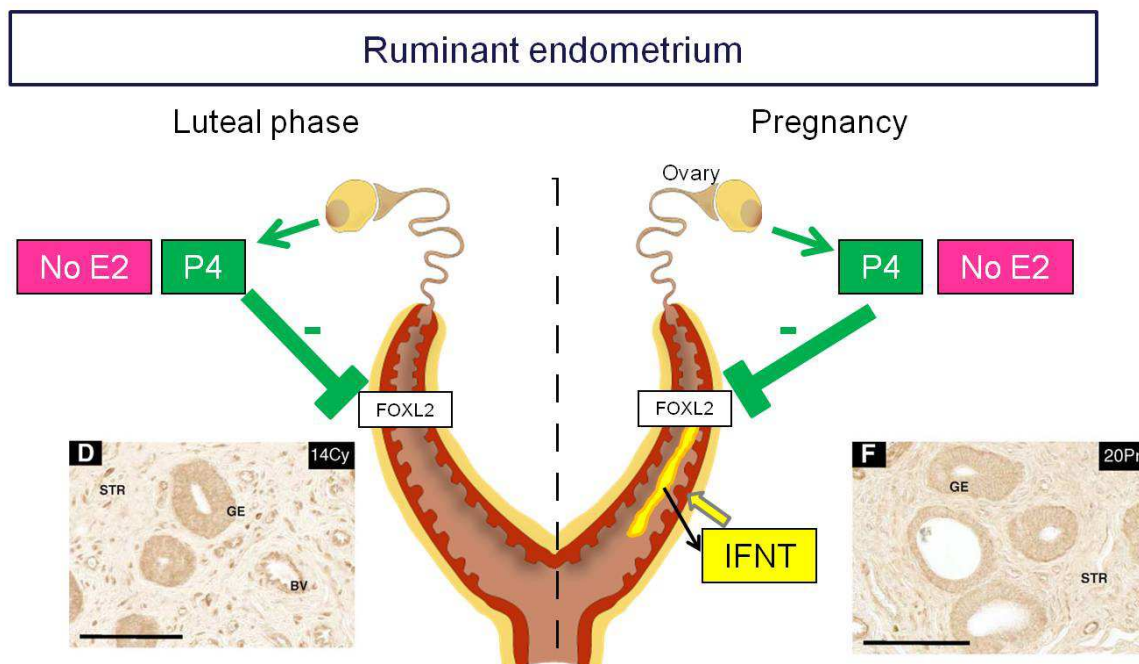


Figure 64: FOXL2 expression and localization in ruminant endometrium between the luteal phase and the early pregnancy. The implantation is a crucial step of the early pregnancy characterized by an elevated P4

circulating level and IFNT conceptus secretion. Only P4 can suppress the expression of *FOXL2*. However, the presence of the conceptus led to the cytoplasmic localization of *FOXL2* of both stromal and GE cells.

Given that uterine receptivity is characterized by intense endometrial cell proliferation during the follicular phase (Miller and Moore 1976; Devroey and Pados 1998; Spencer et al. 2012) and *FOXL2* gene is highly expressed during this phase, we can hypothesized that *FOXL2* could be involved in the regulation of cell proliferation in the endometrium. Interestingly, In the ovary, *FOXL2* gene balances the proliferative process regulating specific pro- (*ATF3*, *IER3*) and anti-apoptotic (*TNFAIP3*, *IER3*, *BCL2A1*) target genes expression (Batista et al. 2007). Its mutations are linked to a worse overall survival and disease free-survival for women suffering from Granulosa Cell Tumour, in association with a higher *FOXL2* protein expression (D'Angelo et al. 2011; Verdin and De Baere 2012). In the endometrial context, ruminant and human species displayed similar pattern of *FOXL2* gene expression during the follicular phase. In ruminant, follicular phase is quite short and associated with a limited proliferation of the endometrial cells whereas human endometrium undergoes a massive proliferation process during the long luteal phase (14 days; Hawkins and Matzuk 2008; Mihm et al. 2011). In addition, ruminant and human concepti exhibit respectively superficial and invasive implantation (Lee and DeMayo 2004). Therefore, we can hypothesize that the length of follicular phase is positively correlated with the proliferation status and the endometrium thickness. In this way, independently of the follicular phase length, *FOXL2* could be a regulator gene of the endometrial proliferation prior to decidualisation (human, mouse) when occurs and mostly prior to implantation stage (human, mouse, cattle and sheep). Regarding BPES syndrome, female patient exhibits eyelid defects and ovarian dysgenesis responsible for a primary amenorrhea due to *FOXL2* mutation (Baron et al. 2005; Verdin and De Baere 2012). Families suffering from BPES syndrome had been studied in order to identify and sequence the differential gene mutations leading to the same phenotype (De Baere et al. 2003). Some women were clinically analysed and exhibited normal ovaries, uterus and regular menses due to exogenous hormonal treatment. Since *FOXL2* gene expression is strongly regulated during both phase of oestrous cycle, the question remains to define the ability of the endometrium to become receptive then to undergo decidualisation but also to drive implantation of conceptus and mostly a successful pregnancy due to an absence of functional *FOXL2* protein. A conditional knock-out of endometrial *FOXL2* could be used to answer this question. The Cre/LoxP method would be considered using floxed *FOXL2* alleles mice (*Foxl2*^{LoxP/LoxP}) crossed with *Pgr*^{Cre} mouse model to invalidate *FOXL2* gene in endometrial epithelial cells (luminal and glandular) and/or crossed with *Amhr2*^{Cre} mouse model to delete *FOXL2* gene in mesenchymal tissue as endometrial stroma or myometrium but also in gonads (Franco et al. 2008). Given that the complex regulation of *FOXL2* in

endometrium, we hypothesized the conditional deletion of endometrial *Foxl2* in mice may affect uterine receptivity, unbalancing proliferation process leading to endometrial hypo- or hyperplasia associated with infertility. Conversely, additive transgenesis using mice and small ruminant model would be considered in order to induce over-expression of *FOXL2* gene during the luteal phase to evaluate endometrial thickness and fertility. In addition, further analyses will be necessary to provide some comparative aspects about the length of follicular phase, the endometrial thickness and *FOXL2* expression in others mammal species including mare, sow or female rat to test our hypothesize. Altogether, our data and the literature suggest the critical role of *FOXL2* endometrial gene expression in the control of cell proliferation during uterine receptivity process.

FOXL2 is a key gene for ovarian differentiation regulating its target genes in the ovary but also in the pituitary and the hypothalamus (Ellsworth et al. 2006; Batista et al. 2007; Moumne et al. 2008; Uhlenhaut et al. 2009; Benayoun et al. 2011; Benayoun and Veitia 2011). Regarding *FOXL2* expression and regulation in the endometrium, we investigated *FOXL2* endometrial target genes using a candidate gene approach based on the comparison of ovarian and endometrial microarrays data. Seven ovarian *FOXL2* target genes expressed in the endometrium were selected and three genes involved in the regulation of endometrial physiology were added to the study. Using bovine primary endometrial stromal and glandular epithelial cells, transient transfection of *FOXL2* cDNA had distinct various impacts on the expression of the eleven candidate genes (Fig. 65). Transcript levels of *RSAD2* and *SCARA5* genes and *PTGS2* gene were up-regulated and down-regulated respectively whereas *DLX5* mRNA level was shown to be up-regulated and down-regulated in stromal and glandular epithelial cells respectively. *ATF3*, *FOS*, *PGR*, *RGS2*, *SOD2* and *TNFAIP3* mRNA expression did not significantly vary. Considering the distinct regulation of *FOXL2* expression in stromal and glandular epithelial cells, investigating *FOXL2* target genes in these two types of endometrial cells appears mandatory. In order to obtain more *FOXL2* transfected glandular epithelial cells, transfection yield will have to be improved in these cell population using alternative methods of transfection associated or not with cell sorting. Nevertheless, in stroma cells, a large scale/exploratory approach of *FOXL2* target genes can be envisaged using RNA-sequencing or microarrays analyses of *FOXL2*-transfected cells compared to control cells. Our laboratory recently validated a protocol for ChIP using frozen endometrial tissue. This method could be applied to *FOXL2* in order to identify *FOXL2* regulated factors in vivo (at 20 dpo of oestrous cycle). Altogether, the complementary in vivo and in vitro methods will bring new insights on the factors/genes which expression is affected by *FOXL2* in bovine endometrium.

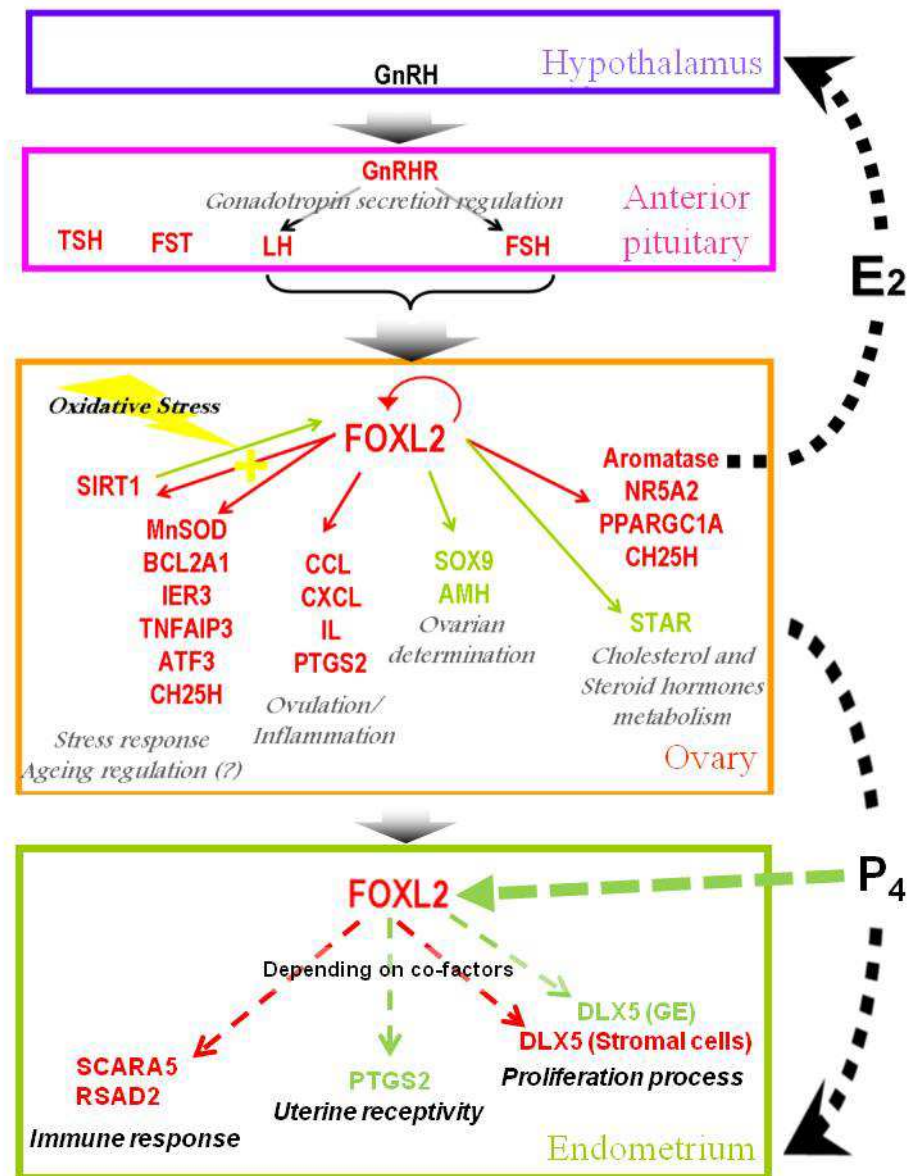


Figure 65: Summary of the biological function of FOXL2 in female reproduction adapted from Benayoun and Veitia 2011. Red arrowheads represent the stimulation of target genes and green arrowheads, the inhibition of target genes by FOXL2.

Reproduction is a critical biological function undergoing intense selective pressure to prevent any modifications which could directly affect the organism's fitness (Lode 2012). Environmental conditions may favor anatomical and physiological adaptations of the mother and embryos for viviparity (Lode 2012). In the last decade, *FOXL2* gene had been considered as the gatekeeper of ovarian identity, controlling reproductive biology of numerous species due to its highly conserved protein sequence including non-vertebrate and vertebrate species (Geraldo *et al.*, 2013). The differences between the species expressing *FOXL2* gene is a consequence of the evolution and the apparition of new tissues. Viviparity has been associated with fertilization and embryogenesis occurring in the female genital tract

including the apparition of placenta of different clades and the apparition of mammary gland in mammals (Lode 2012). GEOprofile and NextProt-Beta software had exhibited the expression of *FOXL2* gene in bovine mammary epithelial cells (GEOProfile n°GDS4081, GDS4009, GDS4437) as well as in murine and human placenta (NextProt: http://www.nextprot.org/db/entry/NX_P58012/expression and GEOProfile n°GDS2990, GDS4037). *FOXL2* gene appears to be listed in microarrays data but has not been described yet in these tissues. The question will deserve to be answered in order to identify *FOXL2* biological function in every reproduction-related tissue: central nervous system, ovary, uterus, oviduct (using NextProt website), placenta and mammary gland.

My PhD thesis provides first and new insights about *FOXL2* biological relevance in the endometrium of mammalian species questioning about its biological functions in female reproductive physiology. With the evolution and the apparition of new tissues, *FOXL2* gene appears to be conserved in female reproductive tract of mammalian species. Further investigations will be required to investigate if *FOXL2* is the gatekeeper of female reproduction in the vertebrate species.

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Book Review:

La Reproduction chez l'Homme et les Mammifères, Charles Thibault and Marie-Claire Levasseur, © Ellipses, 2001

Anatomy and Physiology, Benjamin Cummings, © Pearson Education, 2004

Website:

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<http://pax-db.org/#!/home>

X. Appendices

A. Appendix: Introduction

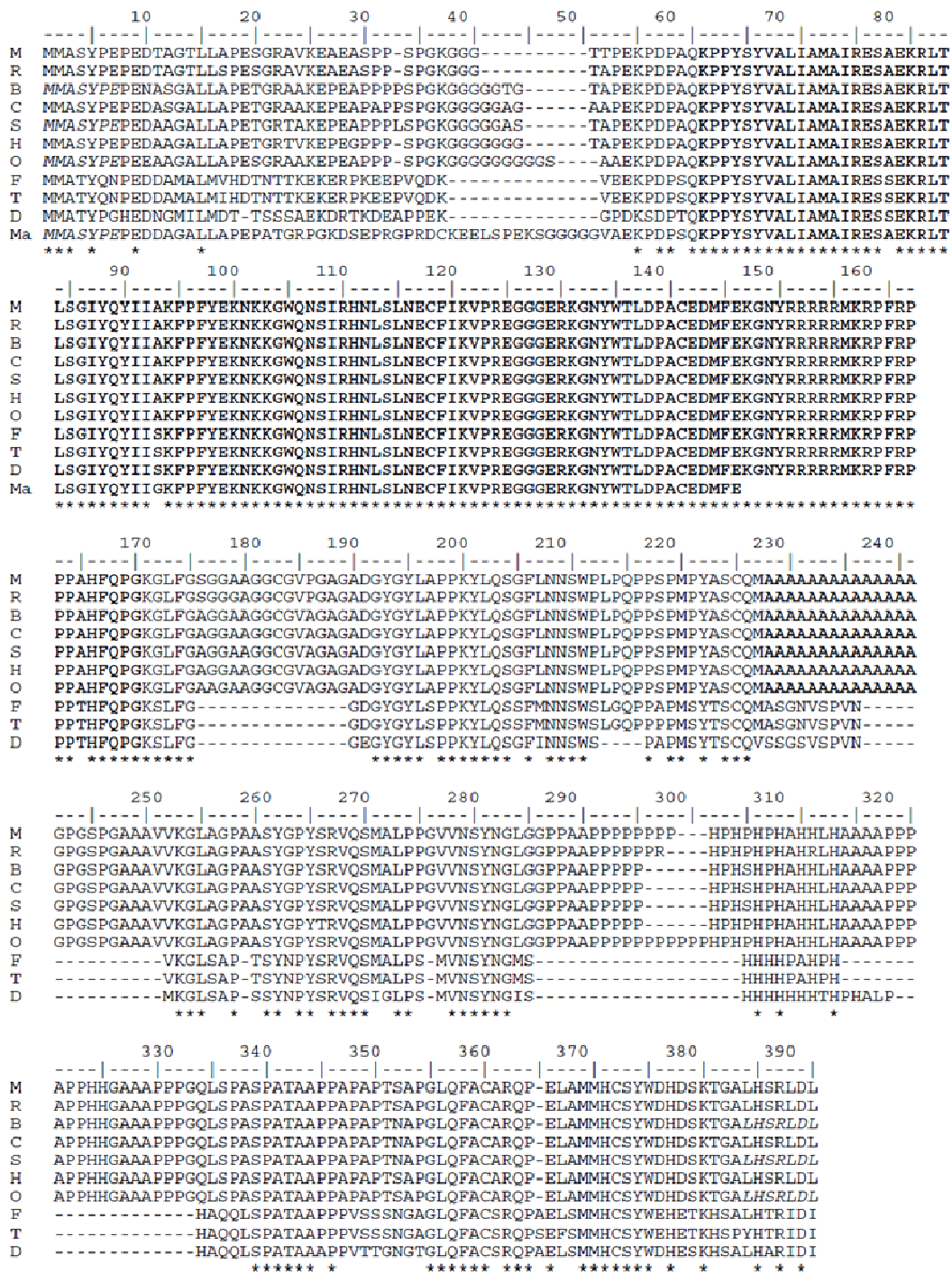


Figure 66: Alignment of FOXL2 protein sequence of ten species (from Cocquet et al., 2003). Forhead domain and poly-alanine tract are indicated in bold. M : *mus musculus* (mouse), R: *Rattus norvegicus* (rat), B: *Bos taurus* (cow), C: *Capra hircus* (goat), S: *Sus scrofa* (pig), H: *Homo sapiens* (human), O: *Oryctolagus Cuniculus* (rabbit), F: *Fugu rubripes* (pufferfish), T: *Tetraodon nigroviridis* (tetraodon), D: *Danio rerio* (zebrafish), FoxL2 partial protein of the *Macropus eugenii* (Ma, tammar wallaby).

B. Appendix: List of putative FOXL2 target genes in endometrium

GENE	GENE INFORMATION
ABLIM2	Actin Binding LIM Protein Family, Member 2
ALS2CL	ALS2 C-Terminal Like
ANKRD12	ankyrin repeat domain 12
ASB2	Ankyrin Repeat And SOCS Box Containing 2
ATF3	activating transcription factor 3
C1orf78	C1orf78
CCND2	Cyclin D2
CHRM2	Cholinergic Receptor, Muscarinic 2
CLDN4	Claudin 4
CXCL10	Chemokine (C-X-C Motif) Ligand 10
CXCL12	Chemokine (C-X-C Motif) Ligand 12
DLX1	Distal-Less Homeobox 1
ENPP1	Ectonucleotide Pyrophosphatase/Phosphodiesterase 1
FOS	FBJ Murine Osteosarcoma Viral Oncogene Homolog
FZD3	Frizzled Family Receptor 3
GDF6	Growth Differentiation Factor 6
GDF8	Growth Differentiation Factor 8
GPRC5B	G Protein-Coupled Receptor, Family C, Group 5, Member B
HES1	Hairy And Enhancer Of Split 1
IL6R	Interleukin 6 Receptor
JUP	Junction Plakoglobin
KCNE3	Potassium Voltage-Gated Channel, Isk-Related Family, Member 3
KLF5	Kruppel-Like Factor 5
LOC387763	Chromosome 11 Open Reading Frame 96
LOC441698	Ras-related protein Rap-2c-like (<i>Homo Sapiens</i>)
MGP	Matrix Gla Protein
PREX1	Phosphatidylinositol-3,4,5-Trisphosphate-Dependent Rac Exchange Factor 1
PTGS2	Prostaglandin-Endoperoxide Synthase 2 (Prostaglandin G/H Synthase And Cyclooxygenase)
RGS2	Regulator Of G-Protein Signaling 2
RRAD	Ras-Related Associated With Diabetes
SCARA5	Scavenger Receptor Class A, Member 5
SELL	Selectin L
SESN2	Sestrin 2
SMPX	Small Muscle Protein, X-Linked
SOD2	Superoxide Dismutase 2
TGFB2	Transforming Growth Factor, Beta 2
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TMEM88	Transmembrane Protein 88

Figure 67: Putative FOXL2 target genes in the endometrium. Gene orthologs were compared between genes profiles in the endometrium (Mansouri-Attia et al. 2009) and genes profiles determined in FOXL2 over-expressing KGN cell line (human, Batista et al. 2007).

C. Appendix: Transient transfection

The pSG5 Vector

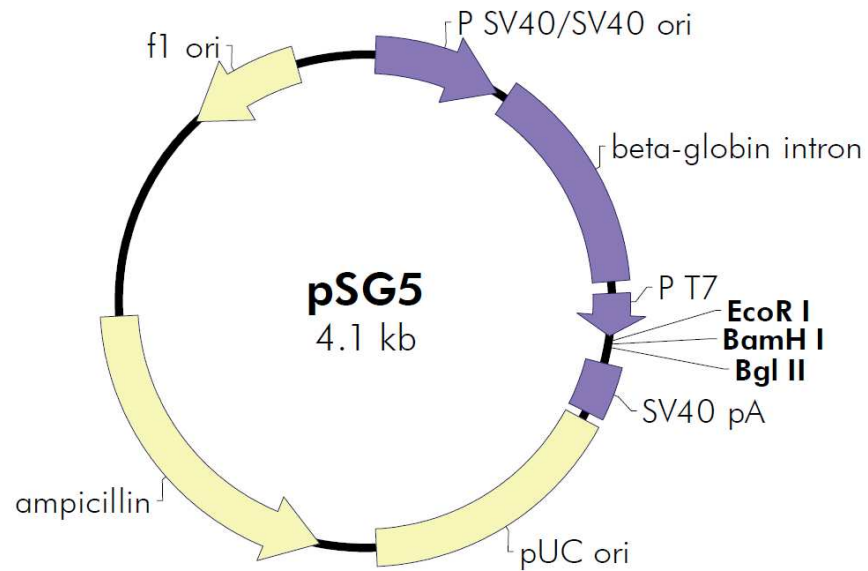


Figure 68: Schematic map of the pSG5 plasmid. Caprine *FOXL2* gene was inserted using BamH1 restriction sites (See Fig. 69).

Green: primers for the amplification of FOXL2 Open Reading Frame from FOXL2-Puc18, for pSG5 cloning

Purple: BamHI site for pSG5 cloning

DBD: DNA binding domain

AAGAGCGCAGAAAAGAAAACCCACCGAGGCGGGGACTGGCCTGGGCGGGGAGGGGCGGCGGAGCTGGA
GCCCCCTCTCTGTTGGGCGGACTCCCCATGGCCAGAGGCTAAGCTCCACTCCCGCCGGCCGCTCCCTTG
GGGAAGGGAAAGAAGAGGAGAGAGGAGCGAGAGGCCGCCAGCAAGAGCGCGGGCGGCATCCGCGAGTC
TGCAGAAGTTTGAGACTCGGCCGTGAACGGACTTGTGCGCCCGGATTCTTTGCGGCGCCAGCGGAAAA
GAGCAGGGACTG**CCCGGCGCGGCGCGCGCGG**TTTGT**CATGATGGCCAGCTACCCCCAGCCCCGAGGAC**
GCCTCGGGGGCCCTGCTGGCCCCGAGACCGGCCGCGCAGCCAAGGAGCCCCGAGGCGCCGGCGCCGCC
CAGCCCCGGGCAAGGGCGGCGGCGGCGGTGCGGGGGCAGCCCCGAGAAG**CCGGACCCGGCGCAGAAGC**
CCCCATACTCTTATGTGGCGCTCATCGCCATGGCGATCCGCGAGAGCGCCGAGAAGAGGCTCACGCTG
TCCGGCATCTACCAGTACATTATAGCCAAGTTCCCGTTCTACGAGAAGAACAAGAAGGGCTGGCAGAA
TAGCATCCGCCACAACCTCAGCCTCAACGAGTGCTTCATCAAGTGCCGCGCGAGGGCGGCGGCGAGC
GCAAGGGCAACTACTGGACGCTGGACCCGGCCTGCGAGGACATGTTTCGAGAAGGGCAACTACCGGCGC
CGCCCGCGCATGAAGCGGCCTTCCGGCCACCGCCCGCGCACTTCCAGCCCCGCAAGGGGCTCTTTGG
GGCAGGAGGCGCTGCGGGTGGCTGCGGCGTGCGGGAGCAGGGGCAGACGGCTACGGCTACCTGGCGC
CCCCAAGTACCTGCAGTCCGGCTTCCTCAACAACCTCGTGCCGCTACCGCAGCCGCTTCGCCCATG
CCCTACGCCTCCTGCCAGATGGCGGCCCGCCGCGCGGCGGCTGCGGCGGCCGCGGCAGCCGCGGGCCC
GGGACGCCCTGGCGCGGCCGCGGTGGTCAAGGGGCTGGCGGGCCCGCCGCTCGTACGGGCCGTACT
CGCGCGTGCAAAGCATGGCGCTGCCTCCCGCGTAGTGAACCTCGTACAACGGTCTGGGAGGCCCTCC
GCCGCGCTCCGCCGCCGCTCACCCCCACTCACACCCGCACGCACACCATCTGCACGCGGCCGCCGC
TCCCCCGCCGGCCCCCTCCGCACCACGGGGCCCGCGCCGCCCCCGGGCCAGCTCAGCCCAGCCAGTC
CGGCCACTGCGCGCCCCCGGCGCCCGCGCCACCAACGCACCCGGCCTGCAGTTCGCCTGCGCCCGG
CAGCCCGAGCTCGCCATGATGCATTGCTCTTACTGGGACCACGACAGCAAGACCGGCGCGCTGCACTC
GCGCCTCGATCT**CTGAGAGTGCAGCGCATGAAGGCTGCGAGGATGCAGGGATCC**GCGACGCCCGCCTT
GGCCGCAGCAGCCGGCCAGA**CCCGCGCGCCCGCTGGGGCCC**CCTGAGCCAGCGCCACCGCCCCCTCTGC
GCCTCCTCGCACTCCTCGGCTCCTCTTGCTCCTCTTTGTCTCCGCTTCTCCCCGTGTTCCGCTCCCTG
CCCTCTTTGCCCTTTTCGCTCCTCTGGCCTGCAGGCCGCTCTCCACGCGTTTTTCCGCAGGCCTAGCCT
TCTCCGGGACCTCAGCGTCGGGGCTAGACACCCGTCGCCAAAATTACGGCGGAGAGGAAGTGCCCCG
GGGCCGAGGTGCAGCCGGGCTTCGGCTGCGCAGACCTCCTGGCCTTTTCTCACAGGTGCGTGCCTCG
CACTCGGCCCTCCGCCGGGCTGCCGCGCCATCCTTGCTAGAAGCGCCGGCATGGGCGGCAACAAGA
CCCGCGCCCGCCGAGAAAGGGACAAACGGATGTTTGCTCCGCGTACCCTTTTGGAGCTATTCAGAAGC
CCCTTCTGGGGCCCACTGGGCAGGGCGGGGATTGGGCTGGATCAGGGAGCCGGGTCTGCCTCGCTCG
GCGGCCTGTACCCGGGTTTACAATAGCATCCCGGCTCGGAGAGAGACTGCGTTTCCTCCGCTCTCCAT
CCCGGCGCTTGGGGCCCCCTCAGGCACGGGCGGCTGGCTCATGTTTTGCTAAAAAGATCACGAGGGCT
ATTTTAGATAATATAAAAAAAATATTTTTTAAAGGAAAAAAATTCACCGGAAAACCGGCGAAGTATT
GTGGCCTTGAGTTTGCTAAAGCAAAACATGAAAATCTTTTGCAGGAGATTGAAGAATTCCTGTGCG
GCCTTCAGAAAGCTATGTTTCAGAGAGGTAGGAGTATATGCCAGTATCAGATGGTTTCCTTTACAAAT
TAGAGCATTTACCCCTCAACCTATCTTTTTTTTAAAAGTCGAAAACAAATTTCCCCCTATCTTCCGA
TGCCAGCCCTGAGCGGGGAGATCACTGTCTGCCTGGCCGAAGCTGCAGGGTCGTCTCATTCCCTCCCT
GATTTTTGTTTTTCAAACGTCTTGCTTCTCCACTTTGGACAAGAGAAATGTGAAACCCGGCAGCAGT
CGGACCAGGCGGGCTTGTGGCTCCGAGCCTACCGGCCACGACTCTAGACCTGGTTGTTTTGTAGTGT
GTCGTTCCGAGGACCAATTTTTTCTAGAAAGAACTAGAGCACTTTTGTTCTTTTGTGTTTGTGTTGTT
GTTGTTGTTTTGTCTTGTCAATTCTCG**AATAAA**

DBD

Figure 69: Caprine FOXL2 sequence (M. Pannetier).

	1	10	20	30	40	50	60	70	80	90	100	
FOXL2_bos_taurus	MNASYPEPENASGILLAPETIGRAAKEPEAPPSPGKGGGGGGTGTAPKPDPAQKPPYSYVILIMAIRESAEKRLILSGIYQYIIAKFPFYEKNKKGMQ											
FOXL2_capra_hircus	MNASYPEPENASGILLAPETIGRAAKEPEAPPSPGKGGGGGGTGTAPKPDPAQKPPYSYVILIMAIRESAEKRLILSGIYQYIIAKFPFYEKNKKGMQ											
Consensus	MNASYPEPENASGILLAPETIGRAAKEPEAPPSPGKGGGGGGTGTAPKPDPAQKPPYSYVILIMAIRESAEKRLILSGIYQYIIAKFPFYEKNKKGMQ											
FOXL2_bos_taurus	101	110	120	130	140	150	160	170	180	190	200	
FOXL2_capra_hircus	NSIRHMLSLNECFIKVPREGGGERKGNVYITLDPACEDMFEKGNYYRRRRRKRPFRPPPAHFQPQKGLFGAGGAAAGGCGVAGAGADGyGYLAPPKYLQSGF											
Consensus	NSIRHMLSLNECFIKVPREGGGERKGNVYITLDPACEDMFEKGNYYRRRRRKRPFRPPPAHFQPQKGLFGAGGAAAGGCGVAGAGADGyGYLAPPKYLQSGF											
FOXL2_bos_taurus	201	210	220	230	240	250	260	270	280	290	300	
FOXL2_capra_hircus	LMSAPLPQPPSPMPYASCGMAAAAAAAAAAAAAAAAAAGPGSPGAAAVYKGLAGPARASYGPRYSRYQSMALPPGVVNSYNGLGGPPAAPPPPPPHSHHPHNNHL											
Consensus	LMSAPLPQPPSPMPYASCGMAAAAAAAAAAAAAAAAAAGPGSPGAAAVYKGLAGPARASYGPRYSRYQSMALPPGVVNSYNGLGGPPAAPPPPPPHSHHPHNNHL											
FOXL2_bos_taurus	301	310	320	330	340	350	360	370	377			
FOXL2_capra_hircus	HRAAAPPAPPHHGAAPPFGQLSPASPATIAAPPAPTNAPGLQFHCARQPELIMHHCSTYMDHDSKTGILHSRLDL											
Consensus	HRAAAPPAPPHHGAAPPFGQLSPASPATIAAPPAPTNAPGLQFHCARQPELIMHHCSTYMDHDSKTGILHSRLDL											

Figure 70: alignment of bovine and caprine FOXL2 protein sequence, both species share 99% sequence identity.

(A)

	2101	2110	2120	2130	2140	2150	2160	2170	2180	2190	2200
Cattle	GGAACTCCGCGGAGCCCATACGAATCAGAACAGAGCGAGGCTCTGGCGCTCTGGAACTCCAGGAGGCGAGCTCCGCCAGAGACGCGGGTCGTGTCTCGG										
Goat	GGAACTCCGCGGAGCCCATACGAATCAGAACAGAGCGAGGCTCTGGCGCTCTGGAACTCCAGGAGGCGAGCTCCGCCAGAGACGCGGGTCGTGTCTCGG										
Mouse	GGAACTCTGCGGAGCCCATACGAATCAGAACAGAGCGAGGCTCTGGCGCTCTGGAACTCCAGGAGGCGAGCTCCGCCAGAGACGCGGGTCGTGTCTCGG										
PRE	AGAACAGAGCGAGGCTCTGGCGCTCTGGAACTCCAGGAGGCGAGCTCCGCCAGAGACGCGGGTCGTGTCTCGG										
Consensus	ggaaactc.gcgagagcccatacgaatcAGAACagagcgaggctcctggcgctctggaaactccaggaggcagctccgccagagacgcgggtcgtgtctcgg										

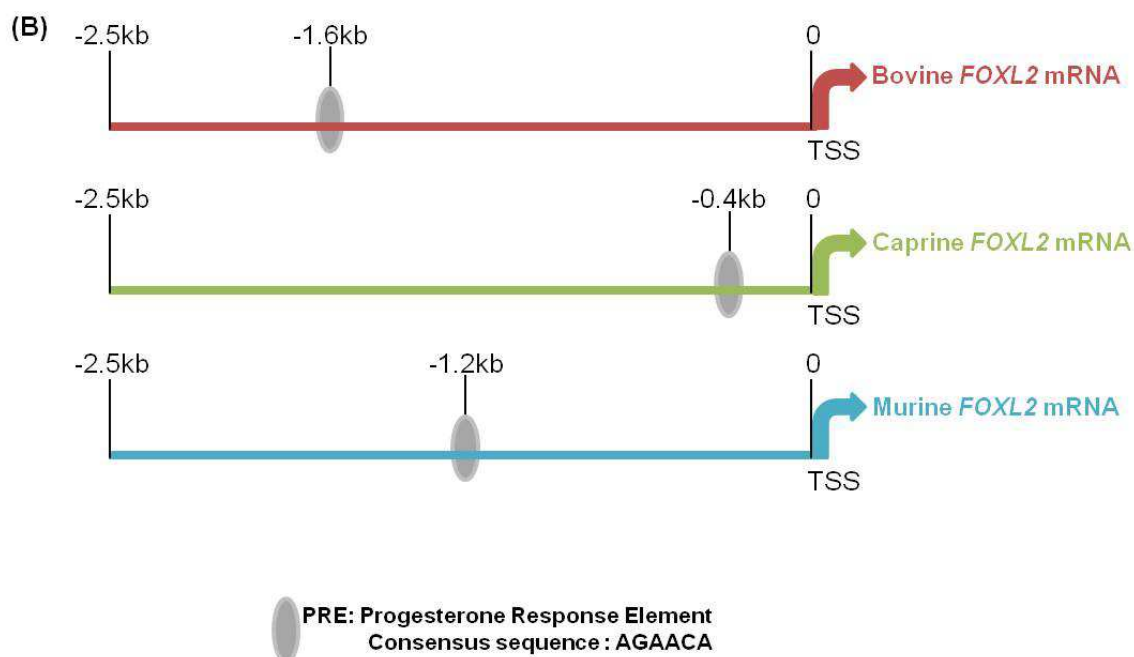


Figure 71: Promoter sequences of *FOXL2* gene in cattle, goat and mouse containing a Progesterone Response Element (PRE). (A) Multalin software (Corpet 1988) was used in order to align promoter sequences of *FOXL2* gene from cattle, goat and mouse species with a consensus site of PRE (namely AGAACA) identified using Transfac Software. (B) Schematic representation of promoter sequences of bovine, caprine and murine *FOXL2* gene. By convention, Transcription Start Site (TSS) were put at the ATG site namely at 0 kb on the sketch.

Transient transfection using turbofect kit protocol

Thermo Scientific, TurboFect Transfection Reagent

Quantities and volumes should be scaled-up according to the number of cells/wells to be transfected.

1. In each well, seed ~5x10⁴ adherent cells or ~5x10⁵ suspension cells in 1 ml of growth medium 24 hours prior to transfection. Note: The recommended confluency for adherent cells on the day of transfection is 70-90% and suspension cells should be in logarithmic growth phase at the time of transfection.
2. Dilute 1 µg of DNA in 100 µl of serum-free DMEM or other serum-free growth medium.
3. Briefly vortex TurboFect reagent and add 2 µl of it to the diluted DNA. Mix immediately by pipetting or vortexing.
4. Incubate 15-20 min at room temperature. Note: Prepare immediately prior to transfection. We recommend starting with 1 µg of DNA and 2 µl of TurboFect per well in a 24-well plate and subsequent optimization may further increase transfection efficiency depending on the cell line and transgene used.
5. Add 100 µl of the TurboFect/DNA mixture dropwise to each well. Do not remove the growth medium from the cells.
6. Gently rock the plate to achieve even distribution of the complexes.
7. Incubate at 37°C in a CO₂ incubator.
8. Analyse transgene expression 24-48 hours later. For stable transfection, cells should be grown in selective medium for 10-15 days.

Gene	primer (5' to 3')		Amplified base pair length
ATF3	F	TGCGGGCCCTCTATTGC	65
	R	GCTGGACTCGTGTTCCTGCTT	
DLX5	F	CTCTCCTACCTCGGCTTCTATG	99
	R	GTAAGCTTTGGCGGGATAGCT	
FOS	F	TGTGGGCCTCAAGGACTTG	66
	R	TCTCCAGAAAGAGGTAAGGACTTGAGT	
FOXL2	F	CCGGCATCTACCAGTACATTATAGC	76
	R	GCACTCGTTGAGGCTGAGGT	
HOXA10	F	TGGAGCTGGAGAAGGAGTT	91
	R	TTGTCTGTCCGTGAGGTG	
PGR	F	CACCATCCCTGCCAATATCTTG	227
	R	CAAGATATTGGCAGGGATGGTG	
PTGS2	F	TCCACCAACTTATAATGTGCAC	81
	R	GGCAGTCATCAGGCACAGGA	
RGS2	F	CTTGAATTCAGCCTGGGTGTTT	110
	R	CTGCCCCAGTACCAGCTTCT	
RSAD2	F	ACTGGACCTGGCTTTTCTGTGA	100
	R	ACGTTGCCCTAGATTACATCCTTATT	
SCARA5	F	CAAGGGTGATGAAGGGAAGC	121
	R	GCCACTGGCATCCACTGTTT	
SOD2	F	TCTGTTGGTGTCGAAGGCTC	350
	R	ACTACAACAGAGCAGCGTAC	
TNFAIP3	F	GGATGTCACCAGGACGTTTAATG	90
	R	GAGCCACTGGAGCTGAGGTT	

Figure 72: Sequences of primers used in Chapter 3 for transient transfection analyse on endometrial stromal and glandular epithelial cells.

D. Appendix: Communication

1. Oral presentation

3rd COST Action FA0702; GEMINI General Conference; Maternal Interactions with Gametes and Embryos; 1st-2nd October 2010; Soustons, France

Characterization of the Forkhead box transcription factor FOXL2 in the bovine endometrium during early pregnancy

Eozenou C.¹, Carvalho A.¹, Gall L.¹, Forde N.², Giraud-Delville C.¹, Pannetier M.¹, Auguste A.¹, Tarrade A.¹, Charpigny G.¹, Roche JF.², Richard C.³, Sandra O.¹

(1) INRA, UMR 1198 Biologie du Développement et Reproduction, F-78350 Jouy en Josas, France

(2) University College Dublin, Veterinary Sciences Center, Belfield, Dublin 4, Ireland.

(3) INRA, UE 1298 Unité Commune d'Expérimentation Animale de Bressonvilliers, Leudeville, France

In mammals, implantation is critical for the outcome of pregnancy and involves a tightly regulated communication between the endometrium and the embryo. Our recent transcriptome analyses using bovine endometrium have revealed the expression of several families of transcription factors, including several Forkhead box (FOX) transcription factors during early pregnancy. Among them, the expression of a member of the FOXL sub-class - FOXL2- has been detected. FOXL2 is one of the most crucial genes involved in ovarian differentiation and until now its expression has appeared to be restricted to pituitary, ovary and foetal eyelid. To determine how FOXL2 contributes to endometrial physiology, we characterized the expression, regulation and cellular localisation of FOXL2 using real-time PCR, western blot and immunohistochemistry analyses under a number of physiological and experimental parameters. The expression of FOXL2 was confirmed at both transcriptional and protein levels in the cyclic and pregnant bovine endometrium. FOXL2 transcript was expressed from day 5 to day 20 post-oestrus and the expression level was independent of systemic progesterone concentrations during the pre-implantation period. Implantation (20 days post oestrus) was associated with a significant decrease of FOXL2 transcript and protein expression in the pregnant endometrium. Interferon-tau, the major signal of pregnancy recognition in ruminants, partially accounted for the reduction in FOXL2 expression. At the cellular level, FOXL2 was found to be localised in the luminal epithelium, the stroma and the glandular cells of the bovine endometrium. Altogether, our results suggest FOXL2 as a major factor associated with the female reproductive axis in mammals. The uterine expression of FOXL2 deserves further investigation to better understand the biological functions of this transcription factor during pregnancy.

Society for Reproduction and Fertility (SRF) conference 2012

9-11 July 2012, Edinburgh

FOXL2 endometrial expression is impacted by P4 and not IFNT in cattle

Caroline Eozenou¹, Anais Vitorino Carvalho¹, Niamh Forde², Maëlle Pannetier¹, Christophe Richard³, Takashi Shimizu⁴, Stefan Bauersachs⁵, Corinne Giraud-Delville¹, Akio Miyamoto⁴, Gilles Charpigny¹, Patrick Lonergan², Eric Pailhoux¹ and Olivier Sandra¹

¹INRA, Jouy-en-Josas, France

²UCD, Dublin, Ireland

³INRA, Leudeville, France

⁴Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan

⁵LMU, Munich, Germany

In mammals, implantation is critical for the outcome of pregnancy and involves a tightly regulated communication between the endometrium and the embryo. Using bovine endometrium, our recent transcriptome analyses listed several families of transcription factors, including several Forkhead box (FOX) transcription factors at day 20 post-oestrus. Among them, a member of the FOXL sub-class -FOXL2- appeared as differentially regulated between the caruncles and the intercaruncular areas. *FOXL2* is a key gene for ovarian differentiation and until now its expression was thought to be restricted to pituitary, ovary and foetal eyelid. In order to gain new insights into FOXL2 biological functions in the endometrium, we characterized the expression and the regulation of this factor during the estrous cycle and establishment of pregnancy in cattle. Both *FOXL2* transcript and protein were expressed from day 5 to day 20 of the estrous cycle, and their levels showed a significant increase (3-fold, $P < 0.01$) during the luteolytic phase. The endometrial expression of *FOXL2* mRNA did not vary during maternal recognition of pregnancy (16 to 20 days post-estrus) and *FOXL2* did not appear to be an interferon-tau target gene. A two-day progesterone supplementation in heifers led to a clear down-regulation of FOXL2 protein levels (2.5-fold, $P < 0.05$). In addition, ovariectomized cows treated with progesterone exhibit a significant inhibition of *FOXL2* expression compared to control ovariectomized cows (2.4-fold, $P < 0.05$). Altogether our results indicate that FOXL2 expression is negatively regulated by progesterone in the endometrium. At the cellular level, FOXL2 was detected in endometrial stromal and glandular cells and its sub-cellular localization was shown to be nuclear in endometrial samples collected during the luteolytic phase while it was not detected in the nuclei during the luteal phase and at implantation. Our findings provide the first evidence that FOXL2 is part of the physiology of endometrial tissue in mammals.

2. Poster

Society for Study of Reproduction's (SSR) 46th Annual Meeting Reproductive Health: Nano to Global

FOXL2 is inhibited by progesterone in ruminant endometrium

Caroline Eozenou¹, Vincent Mauffre¹, Pierrette Reinaud¹, Sylvaine Camous¹, Philippe Bolifraud¹, Jean-Pierre Albert¹, Takashi Shimizu², Akyo Miyamoto², Maëlle Pannetier¹, Fabienne Constant¹, Kaïs H. Al-Gubory¹, Olivier Sandra¹

¹INRA, Jouy-en-Josas, France

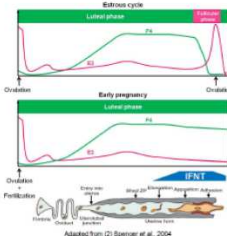
²Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan

In mammals, implantation is critical for the outcome of pregnancy and involves tightly regulated and coordinated interactions between the endometrium and the conceptus (embryo and associated extra-embryonic membranes). The mutual actions of estrogen and progesterone on their uterine receptors are essential for the endometrium receptivity and conceptus implantation. Using bovine endometrium at 20 days post-oestrus (dpo), our microarrays analyses have listed the expression of families of transcription factors, including several members of the winged-helix/forkhead domain (FOX) transcription factors family. We recently demonstrated that *FOXL2* -a member of the FOXL sub-class considered as a key gene for ovarian differentiation- was regulated during early pregnancy in bovine endometrium, independently of the presence of interferon-tau. In the present study, we have aimed to investigate the contribution of steroids hormones in the regulation of *FOXL2* gene expression in ruminants using physiological and experimental models derived in cattle and sheep. First, we have confirmed that *FOXL2* is expressed in the ovine endometrium across the estrous cycle, starting from day 4 to day 15 post-oestrus (dpo) and in pregnancy, at day 15 post-oestrus (day of implantation in ewe). In keeping with our data in the bovine endometrium, *FOXL2* endometrial expression in sheep is low and similar during the luteal phase of estrous cycle (day 4 to 12 post-oestrus) while its expression (RNA and protein) significantly increases during the luteolytic phase (day 15 post-oestrus in cycle). In pregnant ewes, inhibition of progesterone production (P4) by trilostane (an inhibitor of 3 β -hydroxysteroid dehydrogenase activity) during the 5-16 dpo period prevents the P4 rise and leads to a significant increase of *FOXL2* transcript expression compared to the control group (1.4-fold in the caruncular area, $P < 0.05$). Ovariectomized ewes or cows treated with exogenous P4 for 12 days or 6 days respectively have exhibited a significant inhibition of *FOXL2* gene expression compared to control ovariectomized females (in sheep, transcript: 1.8-fold, $P < 0.05$; protein: 2.4-fold, $P < 0.05$; in cattle, transcript: 2.2-fold, $P < 0.05$). Nevertheless, exogenous 17 β -estradiol (E2) treatment for 12 days in sheep or 2 days in cattle did not affect *FOXL2* endometrial expression compared to control ovariectomized females. *In vitro* experiments based on the transient transfection of bovine *FOXL2* promoter are underway in order to confirm the direct impact of P4 and/or E2 treatments on the activity of *FOXL2* promoter. Collectively, our results demonstrate that *FOXL2* gene expression is down-regulated by progesterone in ruminant endometrium. Determining the biological actions of *FOXL2* will be necessary to define the contribution of this transcription factor in the sensor and driver properties of the endometrium.

Research supported by INRA-Phase Department and ANR-08-GENM-037.

FOXL2 expression is inhibited by progesterone in ruminant endometriumC. Eozenou^{1,2}, V. Mauffré^{1,2}, P. Reinaud^{1,2}, S. Camous^{1,2}, P. Bolifraud^{1,2}, J.P. Albert³, T. Shimizu⁴, A. Miyamoto⁴, M. Pannetier^{1,2}, F. Constant^{1,2}, K. H. Al-Gubory^{1,2}, O. Sandra^{1,2}¹INRA, UMR1198 Biologie du Développement et Reproduction, F-78350 Jouy-en-Josas, France, ²ENVA, F-94700 Maisons Alfort, France, INRA, UE1298 Unité commune d'expérimentation animale, F-78350 Jouy-en-Josas, France ; ³Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan**Scientific context**

Implantation is a critical step for the outcome of pregnancy. It is characterized by the first permanent cellular contacts between the conceptus (embryo and extra-embryonic tissues) and the receptive endometrium (1). The mutual actions of estrogen and progesterone on their uterine receptors are essential for the endometrium receptivity and conceptus implantation (2).



In the last decade, exploratory analyses have been developed to unveil genome-wide expression profiles throughout the estrous cycle and the early pregnancy in mammalian species and in cattle particularly (3, 4, 5, 6). Using bovine endometrium at 20 days post-oestrus (dpo), our microarrays analyses have listed the expression of several members of the winged-helix/forkhead domain (FOX) transcription factors family (4). We recently demonstrated that FOXL2, a key gene for ovarian differentiation, was regulated in bovine endometrium throughout the estrous cycle and the early pregnancy, independently of the conceptus-derived Interferon-Tau (IFNT), the major signal of pregnancy maternal recognition in ruminants (7).

In the present study, we have aimed to investigate the contribution of steroids hormones in the regulation of FOXL2 gene expression using physiological and experimental models derived in cattle and sheep.

Experimental design**Physiological models**

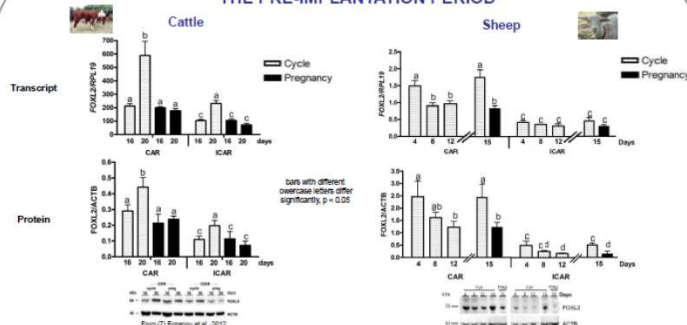
- Cycle (early, active luteal phase & follicular phase)
- Pregnancy (pre-implantation period)

Experimental models

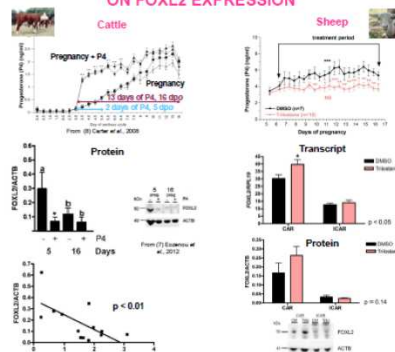
- Pregnant heifers treated with P4 during 2 and 13 days (8)
- Pregnant ewes treated with DMSO or Trilostane (11 days, 30mg/day), 16 dpo (9)
- Ovariectomized ewes supplemented with E2, P4 or both during 12 days (10)
- Ovariectomized Holstein cows supplemented with E2, P4 or both during 8 days (8)

In vitro experiment

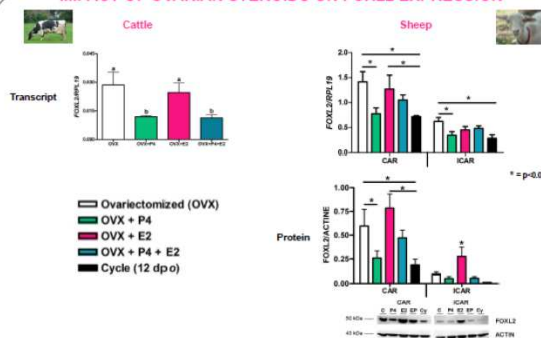
- COS7 cells transfection with FOXL2 promoter-luciferase gene
- +/- Progesterone nuclear receptor cDNAs (PGR, form A and form B)

Results**FOXL2 EXPRESSION DURING THE ESTROUS CYCLE AND THE PRE-IMPLANTATION PERIOD**

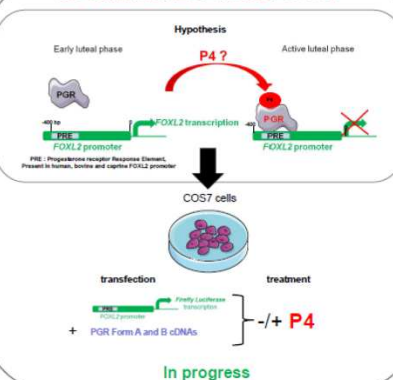
In sheep and cattle, FOXL2 gene expression is :
Higher in the CAR areas vs. ICAR areas
Up-regulated during the follicular phase

IMPACT OF VARIABLE P4 CIRCULATING LEVEL ON FOXL2 EXPRESSION

Altered P4 blood levels
impact FOXL2 gene expression

IMPACT OF OVARIAN STEROIDS ON FOXL2 EXPRESSION

P4 treatment of ovariectomized ewes or cows
down-regulates FOXL2 gene expression

IS FOXL2 A DIRECT TARGET OF P4 ?

In progress

Conclusions & perspectives

- In the endometrium of bovine and sheep origin, FOXL2 gene expression is negatively correlated with P4 blood level. This regulation is similar to the situation in the human endometrium, as determined by microarray datamining based on published study (11)
- In vitro investigations will bring new insights on the regulation of FOXL2 gene expression in mammals
- Determining the biological actions of FOXL2 will be necessary to define the contribution of this transcription factor to the sensor and driver properties of the endometrium

(1) Lee de Mayo, 2004, *Reproduction* 128(6): 166-174
(2) Spencer et al., 2004, *Reproduction* 128(6): 857-868
(3) Mansouri-Attia et al., 2009 *Physiol Genomics* 39(11):14-27
(4) Forde et al., 2009 *Biol. Reprod.* 81(4):784-94.
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(7) Eozenou et al., 2012, *Biol. Reprod.* 87(4):102
(8) Carter et al., 2008, *Reprod Fertil Dev.* 20(3): 368-75
(9) Camous et al., to be submitted
(10) Al-Gubory et al., 2008 *Endocrinology* 149(9):4428-34

(11) Talbi et al., 2006 *Endocrinology* 147(3):1097-1121

3. Publications

- **Analysis of STAT1 expression and biological activity reveals interferon-tau-dependent STAT1-regulated SOCS genes in the bovine endometrium**

Vitorino Carvalho A.^{1,2}, **Eozenou C.**^{1,2}, Healey GD.³, Forde N.⁴, Reinaud P.^{1,2}, Chebrout-Nicolai M.^{1,2}, Gall L.^{1,2}, Rodde N.⁵, Lesage A.^{1,2}, Giraud Delville C.^{1,2}, Leveugle M.⁶, Richard C.^{1,2}, Sheldon IM³, Lonergan P.⁴, Jolivet G.^{1,2}, Sandra O.^{1,2,*}

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Abridged title: *STAT1* regulation in bovine endometrium

Abstract

STAT proteins are a family of transcription factors critical for the regulation of numerous biological processes. In cattle, microarray analyses have identified *STAT1* as a differentially expressed gene in the endometrium during the peri-implantation period. To gain new insights about STAT1 during the oestrous cycle and early pregnancy, we investigated *STAT1* transcript and protein expression, as well as its biological activity in bovine tissue and cells of endometrial origin. The expression of *STAT1* during the oestrous cycle was not affected by progesterone. Pregnancy increased *STAT1* expression on day 16, and protein and phosphorylation levels on day 20. In cyclic and pregnant females, STAT1 was located in endometrial cells but not in the luminal epithelium at day 20 of pregnancy. Interferon- τ (IFNT) stimulated *STAT1* mRNA expression, protein tyrosine phosphorylation, and nuclear translocation, *in vivo* and *in vitro*. Using chromatin immunoprecipitation in IFNT-stimulated endometrial cells, we demonstrated an increase of STAT1 binding on *IRF1*, *CISH*, *SOCS1* and *SOCS3* gene promoters consistent with the induction of their transcripts. Our data provide novel molecular insights into the biological functions of STAT1 in the endometrium in mammals. Future experiments will be necessary to identify factors affecting STAT1 activity as well STAT1 responsive genes in order to better understand the biological functions regulated by this transcription factor in the various cells composing the endometrium during pregnancy maternal recognition and implantation.

Introduction

In mammals, implantation represents a crucial step of pregnancy the success of which relies on the tightly regulated interactions between the growing embryo and the receptive endometrium (Lee & DeMayo 2004). Through its biosensing and biodriving properties, the endometrium plays a critical role in the progression of implantation in various species including ruminants (Spencer *et al.* 2007, Sandra *et al.* 2011). In cattle, large-scale changes in endometrial gene expression at various stages of the oestrus cycle and early pregnancy (before implantation that occurs around 20 day post-oestrous) have been investigated using microarrays and RNA-sequencing analyses (Klein *et al.* 2006, Bauersachs *et al.* 2009, Mansouri-Attia *et al.* 2009a, Mansouri-Attia *et al.* 2009b, Walker *et al.* 2010, Forde *et al.* 2011a, Forde *et al.* 2011b, Bauersachs *et al.* 2012, Forde *et al.* 2012, Mamo *et al.* 2012). The expression of numerous genes has been shown to be altered by the presence of the conceptus as early as day 15 post-oestrus (Forde *et al.* 2011b, Bauersachs *et al.* 2012) but also by interferon-tau (IFNT), a cytokine secreted by trophoctoderm cells and considered as the major signal of maternal recognition of the pregnancy in ruminants (Ealy & Yang 2009). Based on transcriptome profiles, various families of transcription factors have been shown to be expressed in the endometrium, including several members of the winged-helix/forkhead domain (e. g. *FOXL2*; (Eozenou *et al.* 2012) and the Signal Transducer and Activators of Transcription (*STAT*) genes (Klein *et al.* 2006, Bauersachs *et al.* 2009, Mansouri-Attia *et al.* 2009a, Mansouri-Attia *et al.* 2009b, Walker *et al.* 2010, Forde *et al.* 2011a, Forde *et al.* 2011b, Bauersachs *et al.* 2012, Forde *et al.* 2012, Mamo *et al.* 2012).

The STAT proteins represent a family of transcription factors known to be essential for cell proliferation, differentiation and apoptosis (Levy & Darnell 2002, Ivashkiv & Hu 2004). In mammalian cells, the STAT family is composed of seven members transcribed from different genes: *Stat1*, -2, -3, -4, -5A, -5B and -6. Upon phosphorylation by the Janus kinases (JAK), STAT proteins dimerize, translocate to the nucleus where they bind specific motifs of DNA in order to regulate the transcription of their target genes (Stark *et al.* 1998). A wealth of STAT target genes have been identified in various cell models and species, including the Suppressor of Cytokine signalling (SOCS) proteins that are involved in the negative regulation of the JAK-STAT pathway (Rico-Bautista *et al.* 2006, Yoshimura *et al.* 2007).

In the endometrium of humans and mice, *Stat3* and *Stat5* genes have been shown to have significant biological functions during early pregnancy (Maj & Chelmonska-Soyta 2007). Murine *Stat3* has been described in the luminal epithelial cells at implantation (4-5 days post-coitum) and in the decidual cells from 7 to 9 days post-coitum (Teng *et al.* 2004). Phosphorylation of murine STAT3 is induced by embryo implantation (Teng *et al.* 2004) and the inhibition of STAT3 activity is associated to implantation failure due to the absence of stromal cell decidualization (Catalano *et al.* 2005, Nakamura *et al.* 2006). In humans, the level of phosphorylated STAT3 appears to be abnormally low in the endometrium of some patients with unexplained infertility (Dimitriadis *et al.* 2007). In the case of *Stat5*, several studies have highlighted the involvement of prolactin (PRL)-activated STAT5 in the regulation of glandular epithelium development and uterine secretions in humans and mice (Mak *et al.* 2002, Nagashima *et al.* 2008, Bednorz *et al.* 2011, Ji *et al.* 2011). Taken together, these data demonstrate that STAT3 and STAT5 take part in the regulation of uterine physiology in species displaying an invasive implantation.

In ruminants, reports have described a highly specific mechanism involving IFNT and the STAT1 signalling pathways during gestation until implantation. *In vitro* analyses of cultured ovine or bovine epithelial endometrial cells and human STAT1-deficient fibrosarcoma cells have shown that STAT1 phosphorylation represents a major step of the interferon-tau (IFNT) intracellular signalling pathway (Binelli *et al.* 2001, Stewart *et al.* 2001, Stewart *et al.* 2002, Kim *et al.* 2003, Forde *et al.* 2011a). A model has been proposed, describing that STAT1 tyrosine phosphorylation elicited by IFNT leads to

the formation of STAT1 homodimers as well as STAT1-STAT2-Interferon Responsive Factor 9 (IRF9) heterotrimer (known as IFN-stimulated gene factor 3 complex; ISGF3) that translocates to the nucleus and drive the expression of classical IFN-stimulated genes (e. g. *IRF1* and *OAS1*) upon their binding to DNA responsive elements. In the sheep, the expression of *STAT1* gene, that is restricted to the endometrial stromal cells, is up-regulated around implantation (Choi *et al.* 2001). In cattle, the only data available on *STAT1* expression is its identification as a endometrial genes differentially regulated during the maternal recognition period and at implantation (Ivashkiv & Hu 2004, Spencer *et al.* 2007, Sandra *et al.* 2011, Eozenou *et al.* 2012) in caruncular (CAR) and intercaruncular (ICAR) areas (Mansouri-Attia *et al.* 2009a, Walker *et al.* 2010). Thus, no independent investigation has been published on *STAT1* regulation, localization and involvement in the bovine endometrium during the pre-implantation period.

The present study aimed to gain new insights into the contribution of *STAT1* to the regulation of endometrial physiology in ruminants. Using *in vivo* and *in vitro* experimental models, the impact of progesterone (P4), IFNT and pregnancy on the expression and the regulation of *STAT1* transcript and protein as well as its phosphorylation status was analyzed in the bovine endometrium and endometrial cells. In addition, the regulation of *SOCS* genes by *STAT1* was investigated at the transcriptional level using chromatin-immunoprecipitation and primary cultures of stromal cells derived from the bovine endometrium.

Material and Methods

Animals and cell cultures

All the experiments were performed in agreement with European Community Directive 86/609/EC, the Animal Research Ethics Committee of University College Dublin and the French Ministry of Agriculture (authorization B91332). Protocols were registered by the Department of Health and Children (Ireland) or by the Regional Ethical Committee of Animal Experimentation of INRA and AgroParisTech (France, protocol 12-124).

Experiment 1: *STAT1* expression during the maternal pregnancy recognition period

As previously described (Forde *et al.* 2011b, Eozenou *et al.* 2012), synchronized cross-bred beef heifers were artificially inseminated or not inseminated (cyclic cows). Animals were slaughtered at day 16 (cyclic: n=5, pregnant: n=4) or at day 20 post-oestrus (cyclic: n=6, pregnant: n=5). Conceptuses were collected by uterine flushing and their correct stage of development was confirmed by microscopy (Degrelle *et al.* 2005). Endometrial CAR and ICAR tissues were collected separately from the ipsilateral horn to the corpus luteum as previously described (Mansouri-Attia *et al.* 2009b). Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C or fixed in paraformaldehyde 4% (Electron Microscopy Science, Hatfield, USA) in phosphate-buffered saline (PBS, Euromedex, Souffelweyersheim, France) for further analyses.

Experiment 2: Endometrial *STAT1* gene expression at implantation

Charolais cows were synchronized by the Crestar method (Mansouri-Attia *et al.* 2009a) and were artificially inseminated (pregnant n=6; cyclic n=5). On day 20, animals were slaughtered and conceptuses were collected by uterine flushing. Development stage of conceptuses was determined as described in Experiment 1. Endometrium was collected as described in Experiment 1.

Experiment 3: Regulation of endometrial *STAT1* expression by a short-term interferon-tau treatment

As previously described (Forde *et al.* 2011b, Eozenou *et al.* 2012), synchronized cyclic Charolais cows were infused into the uterine lumen with recombinant ovine interferon-tau (roIFNT; 200 µg/ml in saline buffer, 25 ml/horn, 2 h) or a control saline solution at day 14 post-oestrus. Endometrium from 5 IFNT-infused and 5 control cows was collected as described in Experiment 1.

Experiment 4: Impact of P4 supplementation on *STAT1* expression

Synchronized cyclic and pregnant cross bred beef heifers received a P4-releasing intravaginal device containing 1.55 g of P4 (Ceva Animal Health, Libourne, France) on day 3 post-oestrus, as previously published (Carter *et al.* 2008, Forde *et al.* 2011a, Eozenou *et al.* 2012). Females were slaughtered after 2 days (day 5 post-oestrus, normal P4 in cyclic heifers, n=4 and in pregnant heifers, n=4; high P4 in cyclic heifers, n=3, and in pregnant heifers, n=2) and 13 days (day 16 post-oestrus, normal P4 in cyclic heifers, n=3 and in pregnant heifers, n=4; high P4 in cyclic heifers, n=3, and in pregnant heifers, n=5) of P4 supplementation. Endometrium was collected and snap-frozen before being stored at -80°C.

Cell cultures

In order to analyze the regulation of *STAT1*, *IRF1* and *SOCS* genes *in vitro*, epithelial and stromal cell cultures were derived from bovine endometrial tissues sampled during the luteal phase according to ovarian morphology. Primary endometrial epithelial and stromal cells were cultured as previously described (Mansouri-Attia *et al.* 2009a, Cronin *et al.* 2012) and treated with 100 ng/ml or 1000 ng/ml of ovine recombinant IFNT solution for 0.5, 2 and 24 h. Each experiment was performed using stromal and epithelial cells isolated from four independent animals.

RNA extraction

Total RNA was extracted from frozen tissue, using Trizol Reagent (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's recommendations and as previously described (Mansouri-Attia *et al.* 2009b, Eozenou *et al.* 2012). DNase treatment followed by RNA purification was carried out using Qiagen columns according to the manufacturer's protocol (RNeasy Mini Kit, Qiagen, France). Cultured primary cells were washed with 1 ml of PBS, and total RNA was extracted using the RNeasy Mini Kit and the automated system Qiacube (Qiagen, Crawley, UK; (Cronin *et al.* 2012). Quality and integrity of total extracted and purified RNA were determined using an Agilent 2100 bioanalyzer. One µl of RNase inhibitor (RNasin, Promega, France) was added to each sample before storing at -80 °C.

Real time PCR (qPCR)

As previously described (Mansouri-Attia *et al.* 2009b, Eozenou *et al.* 2012), 1 µg of total RNA was reverse transcribed into cDNA using OligodT and SuperScript II in a 20 µl reaction volume (Invitrogen). qPCR assays were performed using SYBR Green Master Mix (Applied Biosystems, Saint Aubin, France) and the Step One Plus system (Applied Biosystems). Primers were designed with Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) or Primer Express Software (Applied Biosystems) and synthesized by Eurogentec (Angers, France). Primers are listed in Table 1. To assess the specific amplification of the expected cDNA fragments, we checked that the sequence of each amplicon was the expected one. Expression of each gene of interest was normalized to that of the most stable reference gene as determined by qBaseplus software (Biogazelle, Gent, Belgium) among six housekeeping genes. Care was taken to consider Ct value within the linear amplification zone. Gene expression was considered as significant when Ct values were lower than 34, and when one single DNA fragment with the expected size and sequence was amplified.

Western Blotting

Frozen tissues were ground in liquid nitrogen and then resuspended (200 µl/100 µg of tissue) in radioimmunoprecipitation assay (RIPA) solution (50 mM Tris HCl [pH 8], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium desoxycholate, containing extemporaneously added protease inhibitor cocktail [Roche, Boulogne-Billancourt, France]). Samples were centrifuged at 20,000 X g for 5 min at 4 °C. The supernatant corresponding to the total protein extract was collected. All samples were stored at -80 °C. Protein quantification was performed using the Bradford method with bovine serum albumin (BSA) as a standard (Sigma-Aldrich, Saint-Quentin Fallavier, France).

Western blot immunoassays were processed as previously reported (Eozenou *et al.* 2012). Membranes were incubated with primary antibody overnight at 4 °C, then incubated with the appropriate peroxidase-conjugated secondary antibody (goat anti-rabbit or goat anti-mouse; dilution 1:5000; Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at room temperature. The optimised primary antibodies were used as follows: rabbit anti-phospho-STAT1 (Tyr701) antibody, (diluted 1:200; #9171, Cell Signaling, Ozyme, Saint Quentin en Yvelines, France) in PBS-Tween 20 (PBS-T, 1:1000) solution containing 5% BSA; rabbit anti-STAT1 antibody (diluted 1:200; sc-346, Santa Cruz Biotechnology) in PBS-T solution containing 4% non-fat dry milk. To control for even loading, blots were hybridized with a mouse anti-actin B (ACTB) antibody (diluted 1:2000; Sigma-Aldrich) in PBS-T solution containing 4% non-fat dry milk. Immunoreactive proteins were detected with Luminata Western HRP chemiluminescent substrates (Millipore, Molsheim, France) and analyzed using an image analysis system (Advanced Image Data Analyzer software; LAS 1000 camera; Fujifilm, Bois d'Arcy, France).

Immunohistochemistry

Sections of endometrium from the ipsilateral horn to the corpus luteum were fixed in 4% paraformaldehyde in PBS overnight at 4 °C. After three washes with PBS, samples were dehydrated with increasing concentrations of ethanol treatment (30%, 50%, 70%, 90%, 100%) in a Shandon Citadel 1000 tissue processor (Thermo Scientific, Courtaboeuf, France) for 12 h. Then, tissues were embedded in paraffin and stored at 4 °C until processing.

Sections (7 µm) were cut with a microtome Leica RM2245 (Leica, Nanterre, France) then immunohistochemistry was performed essentially as described (Eozenou *et al.* 2012). Slides were incubated with the rabbit anti-STAT1 primary antibody (dilution 1:500; sc-346, Santa Cruz Biotechnology) overnight at 4 °C. As a negative control, the primary antibody was incubated for 1 h with gentle rotation at room temperature with the blocking peptide (dilution 1:50; sc-346P, Santa Cruz Biotechnology). The anti-rabbit biotinylated secondary antibody (dilution 1/500; Jackson ImmunoResearch, Suffolk, United Kingdom) was incubated for 1 h at room temperature. Staining was revealed with diaminobenzidine substrate and urea (SIGMAFAST 3,3'-Diaminobenzidine tablets, D4293, Sigma) in Tris-buffer for 1.5 min. Slides were dehydrated then mounted in a Eutkitt mounting medium (Sigma). Images were acquired with a NanoZoomer Digital Pathology System then they were analyzed with the NanoZoomer Digital Virtual Slide Viewer software (Hamamatsu, Japan). Each experiment was repeated for four females per biological condition.

Immunofluorescence

Sub-confluent cultures of bovine stromal cells were incubated with roIFNT solution (recombinant ovine interferon-tau, 100 ng/ml) for 0, 15, 30, 60 and 120 min at 37 °C. Cells were cultured on sterile cover slides and fixed with 90% v/v methanol for 5 min at -20 °C then were permeabilized with 0.5% TritonX-100 for 30 min at room temperature. Non specific binding sites were blocked with 1x PBS containing 2% BSA for 1 h at room temperature. The incubation with the rabbit anti-STAT1 primary antibody (dilution 1:20, 610585, BD Technologies, France) was performed over night at 4 °C. As a negative control, slides were incubated without primary antibody overnight at 4 °C. The donkey

AffiniPure Anti-Mouse Cy5-conjugated IgG (dilution 1:200; 715-175-151, Jackson ImmunoResearch) was incubated for 45 min. DNA was counterstained with DAPI (10 ng/ml; D1306, Invitrogen). Endometrial labeled cells were examined using a Zeiss ApoTome structured illumination system (AxioPlan Imaging, Zeiss, Le Pecq, France; MIMA2 platform, INRA, Jouy-en-Josas) and analyzed with the AxioVision Microscopy Software (Zeiss, Le Pecq, France). Experiments were run using primary endometrial cultures derived from three different animals.

Chromatin Immunoprecipitation

Monolayer cultures of endometrial stromal (fibroblasts) cells (3×10^8) were treated with roIFNT (100 ng/ml) for 30 min. An equal amount of cells were left untreated. Cells were harvested using a silicon scraper, snap-frozen in liquid nitrogen and stored at -80°C until the chromatin immunoprecipitation (ChIP) was processed.

Cells were cross-linked in 1x PBS containing 1% formaldehyde (VWR-Prolabo, Fontenay sous Bois, France) for 10 min at room temperature then quenched with 125 mM glycine (VWR-Prolabo, Fontenay sous Bois, France) for 5 min at room temperature. After centrifugation, cells were washed twice in cold 1x PBS. In order to prepare nuclear-enriched extracts, cells were re-suspended in hypotonic buffer (20 mM Hepes pH 7.9, 10 mM KCl, 1 mM EDTA pH 8, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 0.1 mM sodium orthovanadate and 1x Protease inhibitor cocktail; Roche, Boulogne-Billancourt France) for 15 min and homogenized with a dounce (Dounce B, Dominique Dutscher, Brumath, France; 30 stokes) on ice. The nuclei were collected by centrifugation at $20,000 \times g$ for 3 min at 4°C . Nuclear pellets were incubated in nuclear lysis buffer (10 mM Tris-HCl pH 8, 200 mM NaCl, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 1x Protease inhibitor cocktail) at 4°C and vortexed every 2 min for 10 min. After two washes, nuclei were re-suspended in shearing buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0, 1x Protease inhibitor cocktail). DNA was shorn using a Covaris S2 device (duty cycle : 20%, intensity : 8, cycles per burst : 200, bath temperature : 4°C) for 30 min. Supernatant was recovered by centrifugation at $20,000 \times g$ for 3 min at 4°C then soluble DNA was diluted at least 10 times in dilution buffer (0.01% SDS, 1.1% triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl). Immunoclearing was performed for 1 h at 4°C with protein A-agarose beads coated with salmon sperm DNA (50% of slurry, Millipore, Molsheim, France). 0.1% of pre-cleared DNA was kept as a control sample of DNA fragmentation named input sample. Immunoprecipitation was performed with a rabbit anti-STAT1 antibody (5.6 μg for 50 μg DNA; sc-346, Santa Cruz Biotechnology) or anti-chicken IgG (C2288-1ML, Sigma) overnight at 4°C before adding Dynabeads Protein A (10002D, Invitrogen) for 2 h at 4°C . Immunoprecipitated beads were washed once in washing buffer 1 (0.1%SDS, 1% Triton X100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), once in washing buffer 2 (0.1% SDS, 1% Triton X100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), once in washing buffer 3 (0.25 M LiCl, 1% sodium deoxycolate, 1 mM EDTA, 10 mM Tris-HCl, 10% Igepal) then twice in TE 1X, pH 8.0. Washed beads were incubated in elution buffer (100 mM NaHCO_3 , 1% SDS) for 30 min under agitation. The DNA-containing supernatant was obtained using DynaMag magnet (Invitrogen) and incubated overnight at 65°C to reverse the formaldehyde cross-link. DNA was purified by phenol:chloroform : isoamyl alcohol extraction and precipitation with ethanol. To analyze ChIP experiments, STAT1 binding elements were defined for *IRF1* and each *SOCS* promoter using Genomatix MatInspector Software (Genomatix Software GmbH, Munich, Germany) and primers surrounding these STAT1 binding sites were designed (Primer Express Software; Applied Biosystems). The percentage of immunoprecipitation was calculated as immunoprecipitated DNA/Input sample DNA ratio. ChIP experiments were repeated twice for each promoter.

Statistical analyses

All statistical analyses were performed with the GraphPad Prism 6 software (GraphPad, La Jolla, USA). *STAT1* gene expression (transcript or protein) in tissues was first subjected to a two-way

ANOVA followed by post-hoc Bonferroni tests to analyze effects of day, pregnancy status (cyclic or pregnant), endometrial areas (CAR and ICAR), IFNT and P4 impact and their interactions. IFNT impact on primary cultures of bovine endometrial cells was subjected to a one-way ANOVA analysis followed by Dunnett's multiple comparison tests.

Results

Endometrial STAT1 expression during the oestrous cycle and pre-implantation period

In order to characterize the regulation of STAT1 in the bovine endometrium, transcript and protein expression as well as phosphorylation level were analyzed in the CAR and ICAR endometrial areas on day 16 and day 20 of the oestrous cycle and pregnancy.

No significant variation of *STAT1* expression (transcript or protein) was observed during the oestrous cycle in CAR or ICAR endometrial areas ($P > 0.05$ on days 5, 16, 20; Fig. 1A, 1B). In the pregnant CAR and ICAR areas compared to their cyclic counterparts, *STAT1* transcript level significantly increased at day 16 ($P < 0.05$; Fig. 1A) and day 20 ($P < 0.001$; Fig. 1A). On the other hand, STAT1 protein and phosphorylation were affected by the conceptus in CAR and ICAR endometrial areas only on day 20 of pregnancy ($P < 0.001$, Fig 1B, 1C; supplementary data 1A). In both experiments, no significant difference of *STAT1* gene expression was observed between CAR and ICAR endometrial areas.

On days 16 and 20 of the oestrous cycle (Fig. 2A-C, 2D-F), no significant variation of STAT1 staining was observed: STAT1 protein was visible in the luminal epithelium, glandular epithelium, stromal cells as well as in the endothelial cells of blood vessels. The staining appears to be both cytoplasmic and nuclear. An impact of the conceptus on STAT1 staining was observed on day 20 of pregnancy (Fig. 2J-L) where it increased in the cytoplasm of the stromal, glandular and vascular cells. Moreover, STAT1 nuclear localization was barely detectable in the luminal epithelium at day 20 of pregnancy compared to any other condition (Fig 2K).

Regulation of endometrial *STAT1* expression by two factors involved in bovine pregnancy maintenance

Two major factors have been shown to be necessary for maintaining bovine pregnancy: P4 and IFNT. The first is a steroid hormone secreted by the corpus luteum whereas the second is secreted by the trophoblast of elongated conceptus, during the early pregnancy. In order to investigate the effect of these factors on endometrial *STAT1* expression *in vivo*, two experimental bovine models were developed.

In the endometrium of cyclic or pregnant cross-bred beef heifers, no variation of *STAT1* gene level (transcript and protein) was observed between day 5 and day 16 post-oestrus (Fig. 3). In cyclic and pregnant females, the 2-day treatment of exogenous P4 did not affect STAT1 transcript level but the 13-day treatment led to a significant increase of *STAT1* transcript level ($P < 0.05$; Fig. 3B) in pregnant heifers.

On the second experimental model, the IFNT treatment of cyclic bovine endometrium during a short time (2 h) induced the significant increase of *STAT1* mRNA in CAR and ICAR endometrial areas ($P < 0.05$; Fig. 4A). In accordance, STAT1 mRNA level increased with a 2h and 24h treatment of IFNT in primary cultures of stromal cells while it only increased in epithelial cells with a 24h treatment (Fig. 5). Whereas no significant variation of STAT1 protein level was detected (Fig. 4C), STAT1 phosphorylation status significantly increased in CAR and ICAR areas in cyclic bovine endometrium treated with IFNT ($P < 0.05$; Fig. 4D). Moreover, IFNT treatment induced the translocation of STAT1 to the nuclei in the epithelial, stromal and glandular cells *in vivo* (Fig 4Ev) as well as in primary cultured fibroblastic cells (supplementary data 1B; 15 and 30 min of treatment).

Regulation of *IRF1* and *SOCS* genes by IFNT in primary cultures of bovine endometrial cells

We investigated the regulation of *IRF1* (a transcription factor known to be involved in cytokine signalling pathway) and *SOCS* (a family of negative regulator of cytokine signalling pathway) gene expression in primary cultures of endometrial epithelial and stromal cells treated by rolIFNT (100 ng/ml; 0.5, 2 and 24 h; Fig. 5). No significant difference was observed between treatments with 100 or 1000 ng/ml of IFNT (data not shown). No variation of *SOCS4-7* mRNA levels was observed in epithelial and stromal cells. In IFNT-treated epithelial cells, no significant induction of *CISH* mRNA was apparent, whereas *SOCS3* mRNA expression slightly increased at 0.5 and 2 h ($P < 0.001$). Up-regulation of *IRF1*, *SOCS1* and *SOCS2* mRNA expression was restricted to the 2 h time point ($P < 0.05$). In stromal cells, *IRF1*, *SOCS1*, *SOCS3*, and to a lesser extent *CISH* mRNA induction started as early as 0.5 h after IFNT addition ($P < 0.05$). For these 4 genes, RNA expression peaked at 2 h of IFNT treatment ($P < 0.01$) before declining to a non-significant threshold at 24 h. *SOCS2* expression was increased with a 2 and 24 h IFNT treatment ($P < 0.05$). Thus, stromal cells appeared more sensitive than epithelial cells to IFNT treatment.

STAT1 binding induced by IFNT on *IRF1* and *SOCS* gene promoters.

In order to analyze the putative regulation of *IRF1/SOCS* genes by STAT1, the presence and the position of STAT binding site were investigated in a 1000 bp genomic region upstream of the TSS (Fig. 6) based on literature and MatInspector analysis. *IRF1*, *CISH*, *SOCS1*, *SOCS2*, *SOCS3* and *SOCS7* gene upstream regions presented at least one STAT binding site (Fig. 6) whereas *SOCS4*, *SOCS5* and *SOCS6* did not. Based on the regulation of IFNT early target genes (Fig. 5) and the presence of STAT1 in the nucleus of stromal cells (supplementary data 1B), the binding of STAT1 was analyzed by ChIP on stromal cells with a very short IFNT treatment (30 min, 100 ng/ml; Fig. 7). IFNT induced the increase of STAT1 binding on *IRF1* (4.7-fold), *CISH* (6.3-fold), *SOCS1* (2.3-fold) and *SOCS3* (2.4-fold) promoters. A decrease was observed for *SOCS2* promoter (0.3-fold) and no significant change was observed for *SOCS7* promoter. Thus, a correlation was observed between the recruitment of STAT1 and the transcriptional induction of IFNT early target genes by IFNT.

Discussion

As suspected from pioneer analyses that investigated *STAT1* gene expression and regulation in the ovine endometrium (Choi *et al.* 2001), high-throughput analyses have listed *STAT1* as a differentially expressed gene in the bovine pregnant endometrium compared to the cyclic tissue collected from day 15 to day 20 of the oestrous cycle (Bauersachs *et al.* 2006, Klein *et al.* 2006, Mansouri-Attia *et al.* 2009b, Walker *et al.* 2010, Forde *et al.* 2011b, Bauersachs *et al.* 2012). Based on the use of bovine or ovine cell lines, *in vitro* analyses have also highlighted the transcription factor STAT1 as a major component of the IFNT transduction pathway (Binelli *et al.* 2001, Stewart *et al.* 2001, Kim *et al.* 2003). Collectively, these data prompt the need for characterizing STAT1 in the bovine endometrium. The present study clarifies the spatio-temporal expression pattern of *STAT1* in the bovine endometrium during the oestrous cycle and early pregnancy, including its regulation by P4 and IFNT *in vivo*. Moreover, our analyses provide the first insights into the direct interaction of STAT1 with the promoters of various members of the SOCS gene family in primary cultures of bovine endometrial stromal cells using ChIP.

In the bovine endometrium, our data have shown a sustained *STAT1* transcript level throughout the oestrous cycle (day 5 to day 20) in keeping with the detectable and stable level of STAT1 protein determined in luteal (day 16) and follicular (day 20) stages. This situation resembles that reported for the human uterus whose endometrial glandular and stromal cells expressed STAT1 throughout the menstrual cycle despite a reduced expression at the protein and mRNA level in the active secretory phase (Jabbour *et al.* 1998, Talbi *et al.* 2006). However, our results clearly differ from those on *STAT1* gene regulation reported in the ewe and the sow. In these species, *STAT1* mRNA expression was

shown to decline during the transition from the luteal to the follicular phase (Choi *et al.* 2001, Joyce *et al.* 2007). Moreover, from day 14 to day 20 of the oestrous cycle, the cell distribution investigated in the present study indicated the presence of STAT1 in the endometrial glands, stromal cells as well as the luminal epithelium although in this latter cell population the staining was fainter but still visible at day 20. Again, the sustained expression of STAT1 in the various cell populations of the bovine endometrium contrasts with the barely detectable or undetectable STAT1 protein expression described in the cyclic ovine and porcine endometrium respectively (Choi *et al.* 2001, Joyce *et al.* 2007). Although the biological meaning of this discrepancy between cattle and these two other livestock species is unclear, the regulation of *STAT1* expression by P4 appears consistent between cattle and sheep. Indeed, in the present work, P4 supplemented cyclic heifers did not exhibit any significant impact of the short (2 days) or long-term (13 days) P4 treatment on *STAT1* RNA or protein level. Moreover, *STAT1* gene expression was not correlated with circulating P4 levels (unpublished data). Similarly, in ovariectomized ewes supplemented with physiological level of P4, the injection of a P4 antagonist did not affect the expression of *STAT1* mRNA (Gray *et al.* 2006). Collectively, the results strongly argue for no impact of P4 on the global regulation of *STAT1* gene expression in the endometrium of ruminants.

In the pregnant CAR and ICAR endometrial areas compared to the cyclic tissues, the increase in *STAT1* transcript level initiated during the period of maternal recognition of pregnancy (day 16) and was amplified at implantation (day 20). The time-course of endometrial *STAT1* mRNA expression was consistent with the identification of *STAT1* as a differentially expressed gene in the bovine pregnant endometrium compared to the cyclic endometrium from day 15 of pregnancy onwards (Mansouri-Attia *et al.* 2009b, Walker *et al.* 2010, Forde *et al.* 2011b, Bauersachs *et al.* 2012) and the increase of endometrial *STAT1* transcript level in pregnant ewes and sows (Choi *et al.* 2001, Joyce *et al.* 2007). At day 20 of pregnancy within the present study, the quantification of STAT1 protein revealed a significant increase that could be associated with Tyr701 phosphorylation, a landmark for STAT1 dimerisation, translocation to the nucleus and activation of gene transcription (Levy & Darnell 2002, Mohr *et al.* 2012). This increase in tyrosine phosphorylation was already detectable at day 18 of pregnancy in Holstein heifers (Vitorino Carvalho *et al.* unpublished data) whereas it was barely detectable during the oestrous cycle (present study and unpublished data). Therefore, conceptus secretions and the process of implantation not only stimulate *STAT1* gene expression but they are also associated with STAT1 biological activation in the bovine endometrium.

Among the conceptus secretions that have been studied, IFNT has been identified as the maternal recognition of pregnancy signal in ruminants (Roberts 2007, Ealy & Yang 2009). This type I IFN affects endometrial physiology, as determined by gene expression profiles (Spencer *et al.* 2008, Bauersachs & Wolf 2012). In cyclic ewes, intra-uterine injections of IFNT for 4 days increased endometrial *STAT1* mRNA expression (Choi *et al.* 2001). A similar increase in *STAT1* transcription was reported by Bauersachs *et al.* (2012) using a 2-day intra-uterine release of recombinant IFNA2, another type I interferon. Based on primary cultures of endometrial stromal cells, our *in vitro* analysis showed an acute IFNT effect (15 to 30 min) on the induction of STAT1 nuclear translocation, as previously described in immortalized ovine and bovine endometrial epithelial cells (Binelli *et al.* 2001, Stewart *et al.* 2001). In our primary cultures of endometrial cells, we also determined that the 2 h and 24 h IFNT treatments stimulated bovine *STAT1* gene expression. Investigating the impact of an *in vivo* short-term infusion of IFNT into bovine uteri, we showed a stimulation of *STAT1* transcriptional expression as well as a trigger of tyrosine phosphorylation and nuclear accumulation of STAT1 in endometrial stromal cells, glandular cells and luminal epithelium cells. According to the definition used for IFN-stimulated genes (ISG) in our previous work carried out with bovine endometrium (Mansouri-Attia *et al.* 2009a), the present study highlights STAT1 as an IFNT-induced gene whose phosphorylation and nucleocytoplasmic shuttling are IFNT-regulated.

Although IFNT is recognized as a regulator of STAT1 expression and biological activities, several elements indicate that other IFNs are involved in the modulation of endometrial STAT1 expression and function. In humans, *STAT1* mRNA level was increased in decidual cells co-cultured with trophoblast cells (Popovici *et al.* 2006) or incubated with trophoblast secretions (Hess *et al.* 2007). In the porcine endometrium, *STAT1* transcript expression was stimulated by the conceptus from day 12 of pregnancy onwards (Joyce *et al.* 2007). The *IFNT* gene has not been identified in human and pig genomes, therefore the stimulating actions of the conceptus secretions on endometrial *STAT1* gene expression are thought to be elicited by alternative trophectoderm-produced IFNs including IFNG and IFND or IFNA and IFND in pigs and humans, respectively (Joyce *et al.* 2007, Roberts *et al.* 2008). Consequently, although conceptus signals differ between species, regulation of *STAT1* gene expression and protein activation during early pregnancy appears to be a conserved feature in the mammalian endometrium.

It is worth noting that uterine STAT1 is not exclusively regulated by type I and II IFNs during the establishment of pregnancy. Indeed estrogen produced by conceptuses was shown to stimulate *STAT1* gene expression in the luminal epithelium of the porcine endometrium (Joyce *et al.* 2007). Interestingly, our findings indicated that *STAT1* transcript level peaked at day 20 of pregnancy and remained highly expressed until day 32-34 of pregnancy (Oliveira *et al.* 2010). At day 20 of pregnancy the high level of endometrial *STAT1* mRNA coincides with the sustained protein expression and phosphorylation of STAT1. This high endometrial STAT1 expression questions the nature of the factors that could regulate STAT1 during the peri-implantation period. Ovine placental lactogen, a member of the prolactin/growth hormone (PRL/GH) family has been reported to prolong STAT1 phosphorylation through the heterodimerization of PRL and GH receptors, both expressed in the ruminant endometrium (Postel-Vinay & Kelly 1996, Biener *et al.* 2003). Bovine placental lactogen (PL or CSH) is secreted by the binucleate trophoblast cells from day 18 of pregnancy onwards (Kessler *et al.* 1991, Wooding *et al.* 1992) but, to our knowledge, actions of placental lactogens on the uterine JAK-STAT pathway have not been reported. Consequently, the regulation of endometrial STAT1 by bovine CSH merits investigation.

In sheep and pigs, the up-regulation of *STAT1* gene expression by IFNT, IFNG and/or IFND takes place in the endometrial stromal cells and deep glandular epithelium but not in the luminal epithelium (Choi *et al.* 2001, Joyce *et al.* 2007). In the present work, immunoreactive STAT1 was visible in the cytoplasm and the nucleus of the main populations of endometrial cells including luminal epithelium at day 16 of pregnancy whereas the staining was almost undetectable in the luminal epithelium of day 20 pregnant endometrium. This latter result is consistent with the absence of detectable STAT1 protein in the ovine endometrial luminal epithelium at day 15 of pregnancy (Choi *et al.* 2001). Although the expression of IRF2 has not been investigated in cattle, the inhibition of STAT1 expression in the bovine endometrial luminal epithelium may result from the action of IRF2, known to repress the expression of ISG at the genomic level in the ovine endometrial luminal epithelium (Choi *et al.* 2001).

In keeping with the nuclear localization of the activated phosphorylated STAT1 in IFNT-stimulated endometrial cells (stroma and glandular epithelium) determined *in vivo*, we identified *IRF1* as well as several members of the SOCS family (*SOCS1*, -3 and to a lesser extent *CISH* and *SOCS2*) as IFNT induced genes in primary cultures of endometrial epithelial and stromal cells. Whereas the up-regulation of *IRF1*, *SOCS1*, and *CISH* gene expression was not significant in epithelial cells earlier than 2 h after IFNT treatment, the time course of *IRF1*, *SOCS1*, *SOCS3* and *CISH* gene stimulation started in stromal cells at 30 min then increased at 2 h before dropping at 24 h. When compared to epithelial cells, the reason for the prompter biological response of stromal cells to IFNT is currently unclear but likely reflects a rapid mobilization of STAT1 to the target sites of IFNT target genes. In order to demonstrate that *IRF1*, *CISH*, *SOCS1* and *SOCS3* gene up-regulation was STAT1-dependant, chromatin

immunoprecipitation was performed and it established for the first time the association of STAT1 on identified binding sites of bovine *IRF1*, *SOCS1* and *SOCS3* promoters following a short term IFNT treatment. For these genes as for *SOCS7*, the detection or the absence of STAT1 binding on their promoters was consistent with the regulation of mRNA expression. *SOCS1* and to a lesser extent *SOCS3* gene expression patterns have been reported to fit with the profile of IFNT secretion in the ovine endometrium (Sandra *et al.* 2005). Altogether our findings have identified *SOCS1* and *SOCS3* as two immediate early STAT1 target genes in the endometrium of ruminants. In the study by Song and Shuai (Song & Shuai 1998), *SOCS2* did not block STAT1 activity whereas *SOCS1* was a much stronger inhibitor of STAT1 activation than *SOCS3*. In IFNT-treated endometrial cells, our data strongly suggest a similar hierarchy in the control of the STAT1 signalling pathway.

Collectively our data have demonstrated that STAT1 expression detected in the cyclic endometrium is up-regulated by early pregnancy at the transcript and protein levels including an impact on the phosphorylation level. The present study has identified (i) IFNT as a major regulator of bovine STAT1 expression and biological activities *in vivo*, (ii) phosphorylated STAT1 as a mediator of the rapid effect of IFNT on the transcriptional regulation of ISG in endometrial glandular and stromal cells. We have shown IFNT-induced *SOCS1* and *SOCS3* to be immediate STAT1 target genes that may contribute to the downregulation of STAT1-dependant IFNT signalling pathway in the bovine endometrium. Based on the spatio-temporal regulation of *STAT1* in the endometrium, future experiments will be necessary to identify factors affecting STAT1 activity as well as to characterize STAT1 responsive genes in order to better understand the biological functions regulated by this transcription factor in the various cells composing the endometrium, particularly during pregnancy maternal recognition and the implantation process.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Fig. 1. Quantification of *STAT1* gene expression in cyclic and pregnant bovine endometrium on day 16 and 20 of the oestrous cycle. Caruncular (CAR) and intercaruncular (ICAR) areas were collected from cyclic (day 16: n=5; day 20, n=6) and pregnant (day 16, n=4; day 20, n= 5) cross-bred heifers as well as from cyclic (n=6) and pregnant (n=5) Charolais cows at day 20 of the oestrous cycle. (A) Quantification of *STAT1* mRNA by RT-qPCR in cyclic and pregnant cross-bred beef heifers. Expression of *STAT1* was normalized to that of *RPL19* and *SCL30A6* using qBasePlus. Bars with different lowercase letters differ significantly ($P < 0.05$). (B) Quantification of *STAT1* protein by Western blotting, normalized to *ACTB* protein level. Bars with different lowercase letters differ significantly ($P < 0.05$). (C) The level of *STAT1* mRNA (normalized to *RPL19* and *SCL30A6*) as well as the amount of *STAT1* (normalized to *ACTB*) and *STAT1* phosphorylation level were analyzed by RT-qPCR and Western blotting respectively. Asterisks denote significant effects ($***P < 0.001$).

Fig. 2. Cellular localization of *STAT1* expression in cyclic or pregnant bovine endometrium. Immunohistochemistry was performed using sections cut from cross-bred beef heifers sampled at day 16 (16Cy; A, B, C) and day 20 of the oestrous cycle (20Cy; D, E, F) as well as day 16 (16Pr: G, H, I) and day 20 (20Cy; J, K, L) of pregnancy. A-L sections were incubated with anti-*STAT1* primary antibody. Tissue samples in O were co-incubated with the anti-*STAT1* antibody and blocking peptide as a negative control. Tissue samples in M and N were stained with hematoxylin-eosin solution. BV, blood vessel; GE, glandular epithelium; LE, luminal epithelium; STR, stroma. Bars= 100 μ m.

Fig. 3. *STAT1* endometrial expression in cyclic and pregnant cross-bred beef heifers supplemented with progesterone (P4) for 2 days (day 3 to day 5) or 13 days (day 3 to day 16). *STAT1* mRNA (A) and protein (B) levels was quantified by RT-qPCR and by western blotting respectively (cyclic heifers with normal level of P4, n=4, cyclic heifers supplemented with P4, n=3, pregnant heifers with normal level of P4, n=4, pregnant heifers supplemented with P4, n=2 at day 5; cyclic heifers with normal level of P4, n=3; cyclic heifers supplemented with P4, n=3, pregnant heifers with normal level of P4, n=4, pregnant heifers supplemented with P4, n=5 at day 16). mRNA and protein levels were normalized to *ACTB* and *RPL19* using qBasePlus and to *ACTB*, respectively. Bars (mean \pm s.e.m) represent the different conditions.

Fig. 4. *In vivo* regulation of endometrial *STAT1* expression by interferon-tau (IFNT). CAR and ICAR endometrial areas were collected from Charolais cows infused with control solution (n=5) or recombinant ovine IFNT (roIFNT, 200 μ g/ml; n=6) for 2 h. (A) *STAT1* mRNA level was quantified by RT-qPCR and normalized to *C2ORF29*, *SLC30A6* and *SUZ12* using qBasePlus. (B) Analysis of *STAT1* protein amount and *STAT1* phosphorylation by Western blotting. (C) Quantification of *STAT1* protein amount. (D) Quantification of *STAT1* phosphorylation (P*STAT1*) level. *STAT1* and P*STAT1* expression were normalized to that of *ACTB* and *STAT1* protein level, respectively. Data are the mean \pm S.E.M. $*P < 0.05$, $**P < 0.01$. (E) *STAT1* localization in bovine endometrium at day 14 of the oestrous cycle infused (14Cy+IFNT: ii, v, viii) or not with IFNT (14Cy; i, iv, vii). Tissue samples were incubated with *STAT1* primary antibody. In ix, tissue section was co-incubated with anti-*STAT1* antibody and the blocking peptide as a negative control. Tissue samples in iii, vi and ix were stained with hematoxylin-eosin solution. BV, blood vessel; GE, glandular epithelium; LE, luminal epithelium; STR, stroma. Bars= 100 μ m.

Fig. 5. Regulation of gene expression by interferon-tau (IFNT) in primary cultures of bovine endometrial cells. Stromal (solid line) and epithelial cells (dashed line) isolated from bovine endometrium were treated with recombinant ovine IFNT (roIFNT; 100ng/ml) for 0.5, 2, or 24 hours. *STAT1*, *IRF1*, *CISH* and *SOCS 1-7* mRNA levels were quantified by RT-qPCR. For each gene, mRNA expression was normalized to that of *RPL19* and *ACTB* using qBasePlus. Fold increase was calculated as [IFNT-treated cells/untreated cells] ratio at 0.5, 2, or 24 h. The experiment was repeated with four

independent animals. Significance was evaluated by comparing stimulated versus non stimulated cells. Data are means \pm s.e.m; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 6. Schematic representation of bovine *IRF1*, *CISH* and *SOCS1*, -2, -3, and -7 upstream region (1000 pb) before the Transcription Start Site (TSS). Transcription factor Binding Sites (TFBS) positions for STAT1 were determined using MatInspector (Genomatix) and are indicated with a white circle. No STAT1 TFBS was identified in *SOCS4*, -5 and -6 bovine promoters.

Fig. 7. IFNT-induced recruitment of STAT1 binding to bovine *IRF1*, *CISH*, *SOCS1*, -2, -3 and -7 promoters in primary cultures of bovine endometrial stromal cells. Cells were treated with roIFNT (100ng/ml) for 30 min, harvested then fixed with formaldehyde and sonicated to reduce the DNA length between 500 and 1000 bp. The sonicated chromatin was immunoprecipitated with anti-STAT1 antiserum or IgG (as a control for non-specific immunoprecipitation). After deproteination and reversal of cross-links, the amount of DNA sequence for each promoter was assessed in the immunoprecipitates by real-time PCR. Input indicates samples before immunoprecipitation. Results are presented as the percentage of DNA immunoprecipitated for each gene promoter relatively to input DNA. ND: Not determined.

Supplementary Data

(A) Analysis of STAT1 protein amount by Western blotting. Caruncular (CAR) and intercaruncular (ICAR) endometrial areas were dissected from cyclic and pregnant cross-bred beef heifers collected at day 16 or day 20 of the oestrous cycle. STAT1 was detected using a rabbit anti-STAT1 antibody. Beta actin (ACTB) was used as a control protein to account for variation in loading.

(B) Immunofluorescence analyses of STAT1 protein in primary cultures of bovine endometrial stromal cells. Cells isolated from bovine endometrium were treated with roIFNT (100ng/ml) for 0, 15, 30, 60 or 120 min. Immunoreactive protein was detected using rabbit anti-STAT1 antibody and DNA was counterstained with DAPI. In insert, stromal cells were incubated without primary antibody as a negative control. Results shown are representative of four fields per treatment over three independent experiments. Bars: 20 μ m.

Tables

Table 1. Description of the oligonucleotide primers used for bovine gene quantification by real time RT-PCR.

Gene	Forward	Reverse	Amplicon size (pb)	Accession number
<i>STAT1</i>	GCATAGTCAGGGCCCAATTGTTACAG	GCCCAGATACAGGACAGCTTTGCAC	140	BC151378
<i>IRF1</i>	GCTGGGACATCAACAAGGAT	CTTTCCTCTGGCTCTTGGTG	232	XM_003584726
<i>CISH</i>	AGTCATCCTGGTGCCCGTGA	AGCCTTGTTCTGCACCG	69	NM_001046586.1
<i>SOCS1</i>	CTCGTACCTCCTACCTCTTCATGTT	ACAGCAGAAAAATAAGCCAGAGA	92	XM_864316.2
<i>SOCS2</i>	TGCAAGGATAAGCGGACAGG	AGATGCTGCAGAGGTGGTGCT	101	NM_177523.2
<i>SOCS3</i>	GCCACTCTCCAACATCTCTGT	TCCAGGACCTCCGAATGG	97	NM_174466.2
<i>SOCS4</i>	GCTTTGTTAACCTATGTCTTGGCA	ACAACACACACAGCTTTACCGAAG	100	NM_001076218.2
<i>SOCS5</i>	TACATCCCAGTGGCTGTCGC	GCACAGCAAGCAGAAACATACATT	100	NM_001046182.1
<i>SOCS6</i>	AGAAGGTCAAAAAATGTCACAGGAA	CAATGGTTAGCCTTTTGGCAT	113	NW_003104566.1
<i>SOCS7</i>	CTCCCACTGCCTAAGCCTCTG	GAAATGAGCTGCGCTTCCTT	102	NM_001206013.1
<i>ACTB</i>	CCTGGCACCCAGCACAA	AGCGAGGCCAGGATGGA	90	Y141970
<i>C2ORF29</i>	GCTTTACCACCACAGCCGAG	GGGTCTTTTCCAACCTCTCC	64	XM_002691150.1
<i>GAPDH</i>	CCTTCAAGAGCCCCCTGT	TCATAAGTCCCTCCACGATGC	432	NM_001034034.2
<i>RPL19</i>	CCCCAATGAGACCAATGAAATC	CAGCCCATCTTTGATCAGCTT	72	NM_001040516.1
<i>SLC30A6</i>	TGATGAGGAAACCTAGCCCTGCC	TCGGGCTGCTCCAAAAGCGT	142	NM_001075766.1
<i>SUZ12</i>	CGTTGTGAGCAGTTTTGCCCTGT	ACCACAGTGCTTGGAGTTGGACT	139	NM_001205587.1

Table 2. Description of the oligonucleotide primers used for quantifying immunoprecipitated bovine gene promoters by real time PCR.

Promoter	Forward	Reverse	Amplicon size (pb)	Accession number
<i>IRF1</i>	CTTAGCCGCTAGCTCTACAACAG	CGCGGAGAATCTAAACACTTAGC	138	NW_003104019.1
<i>CISH</i>	CTAATTGGCCCTCCCTGACC	CCTAATCTTTGTCCTCTGTGTCCC	250	NW_003104544.1
<i>SOCS1</i>	GGCCCCACCCGGTTTCCAAG	CCCTAGTCCCGGCGCCTCTA	181	NW_003104571.1
<i>SOCS2</i>	GCAACTGCGCCGGAGTCTCT	GCGCGCGATTTCAGACGTA	175	NW_003103917.1
<i>SOCS3</i>	CGCAGCCCCGAAGCCAAAGA	ACCCGAGAAGCCGAAAG	226	NW_003104499.1
<i>SOCS7</i>	GACTAAAGGCCAGGCGAGAA	CACCGAGCACCACCCC	154	NW_003104495.1

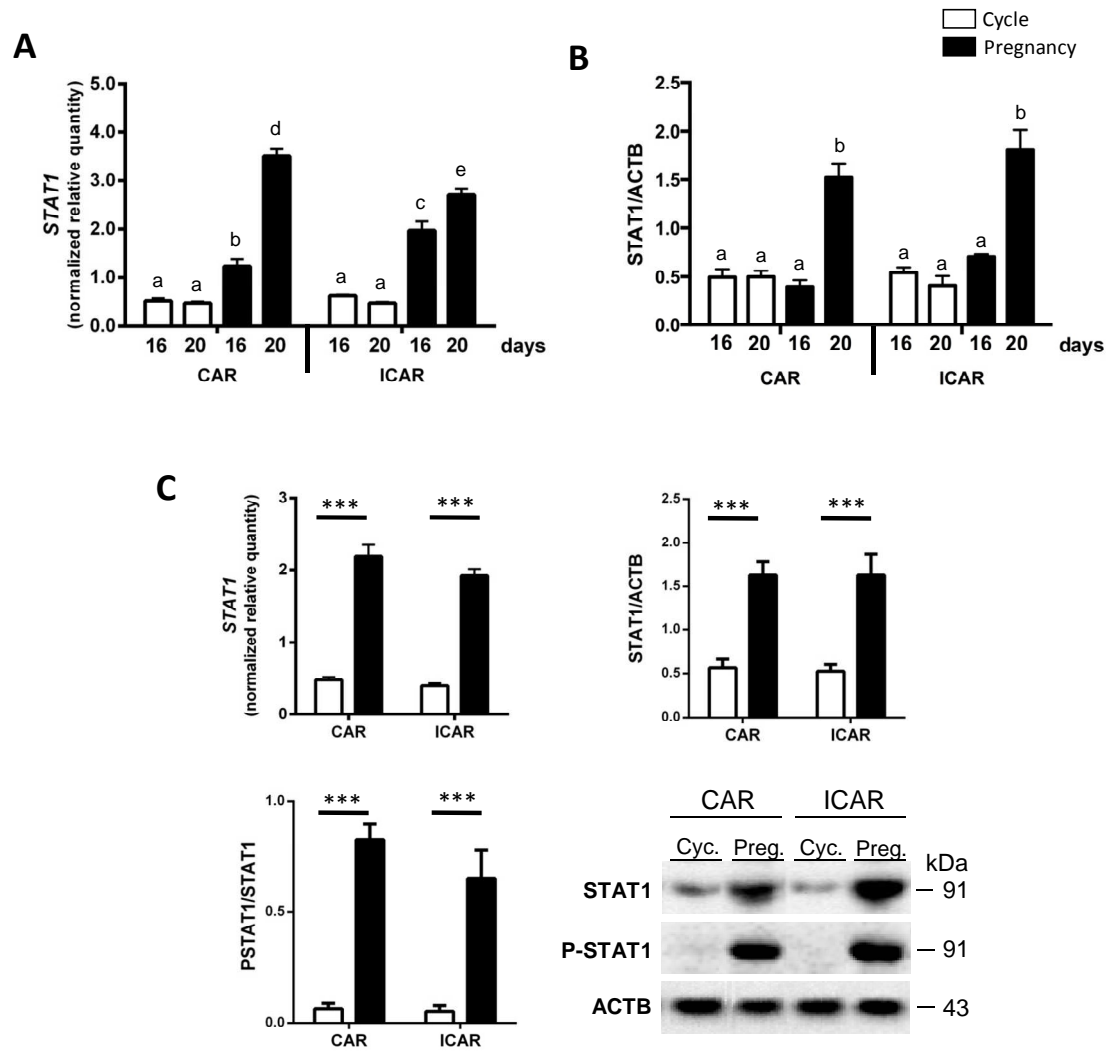


Fig. 1

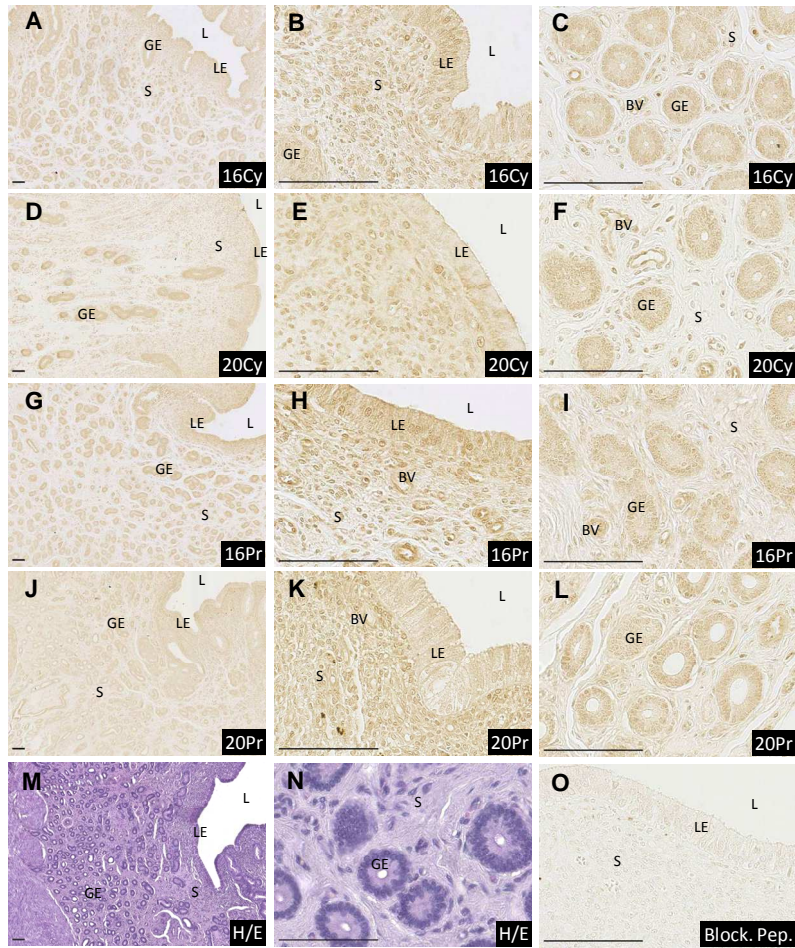


Fig. 2

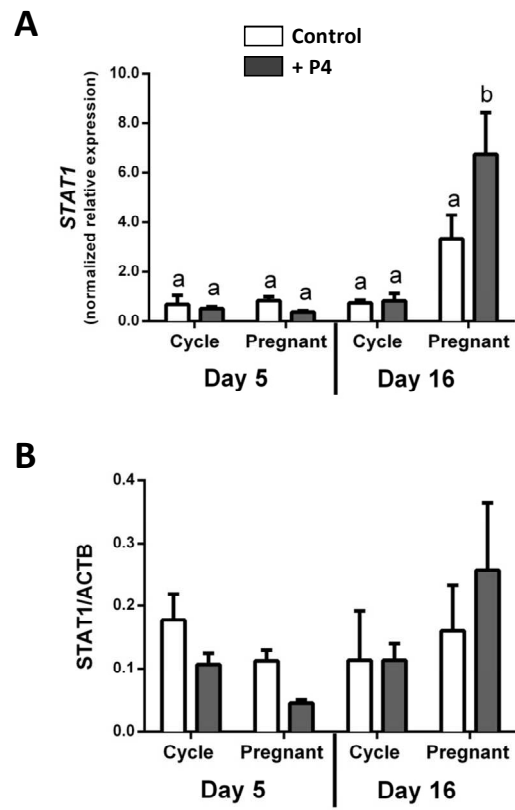


Fig. 3

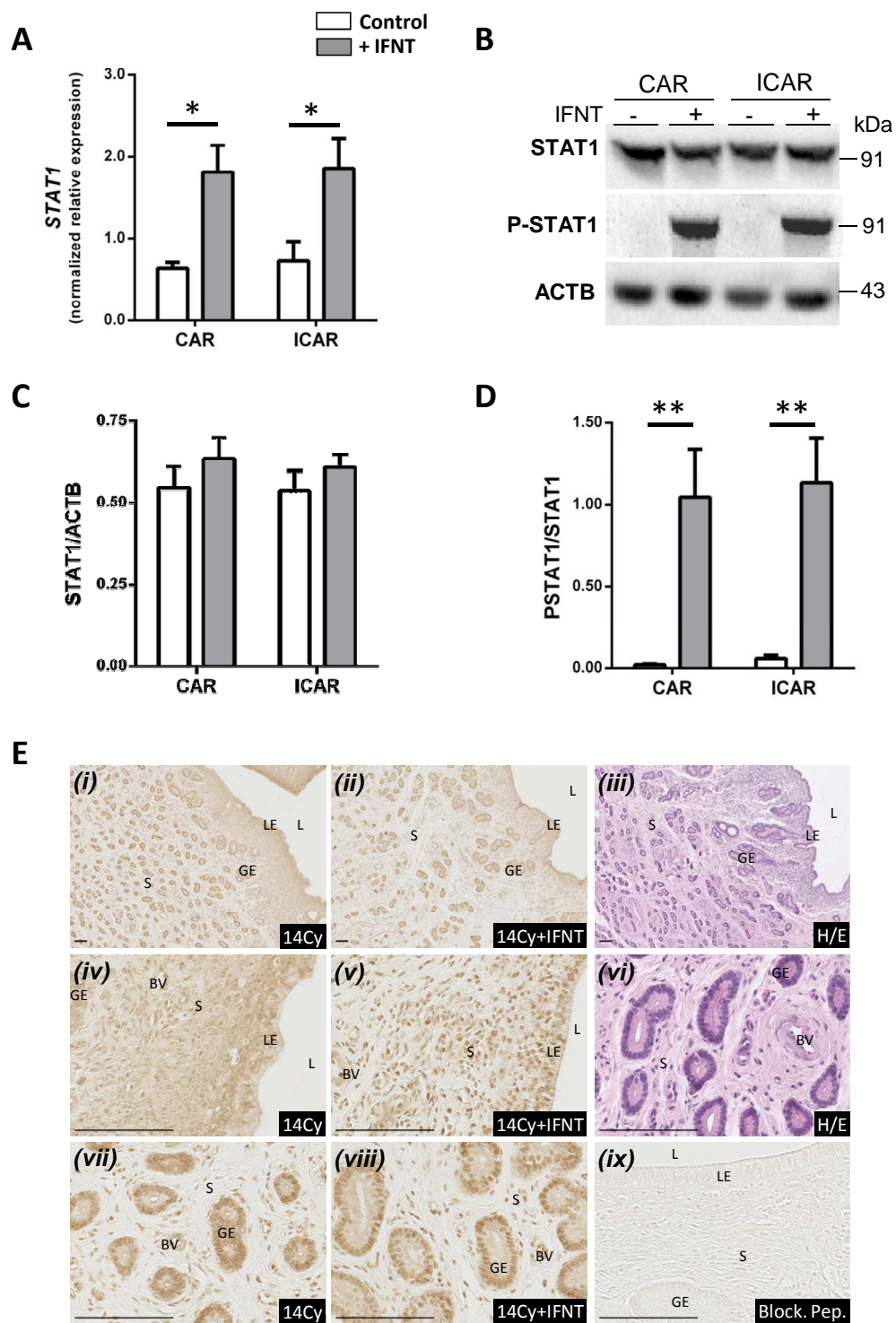


Fig. 4

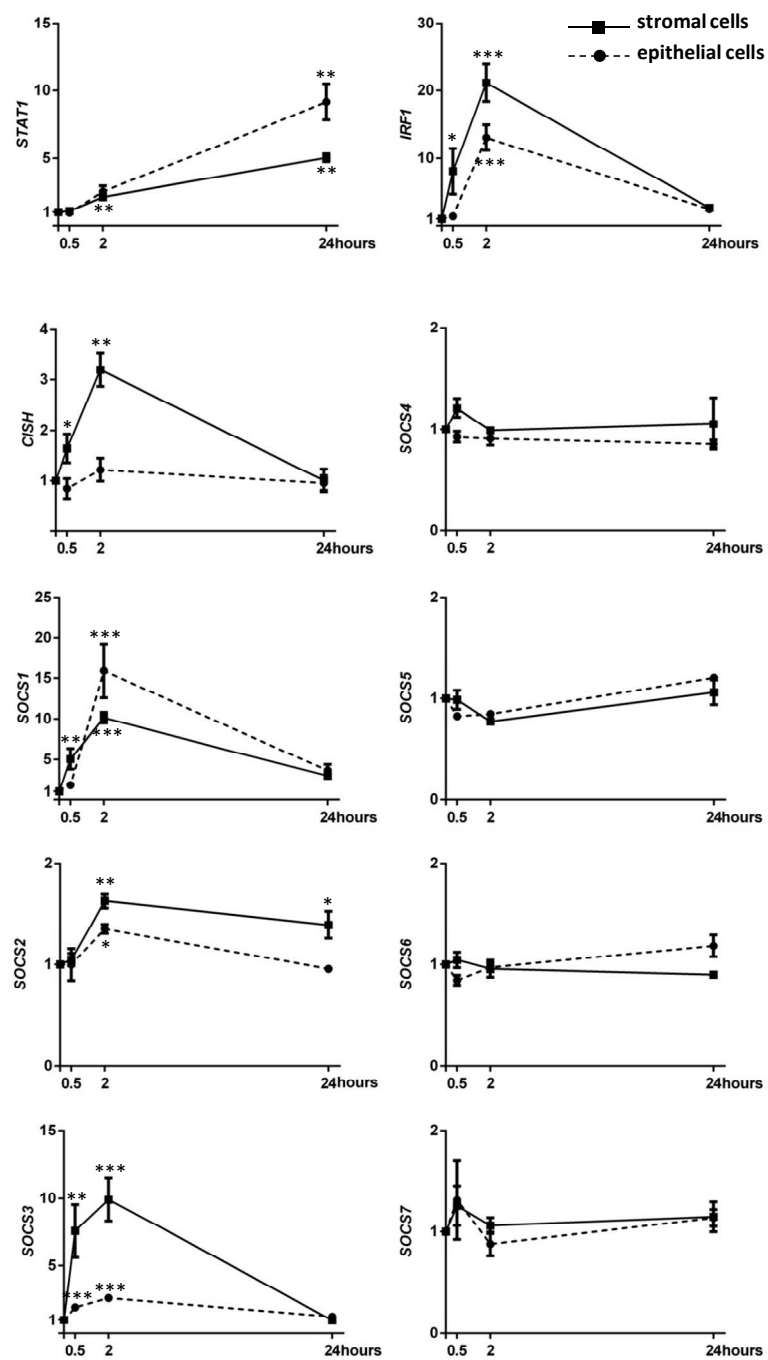


Fig. 5

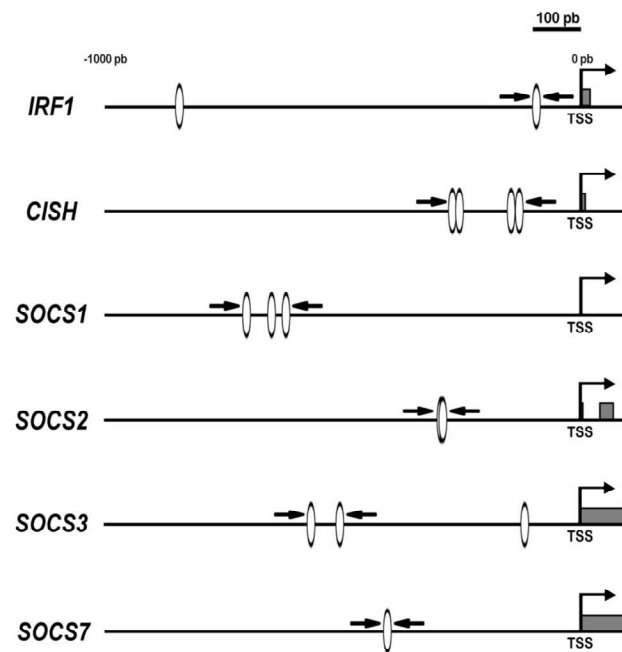


Fig. 6

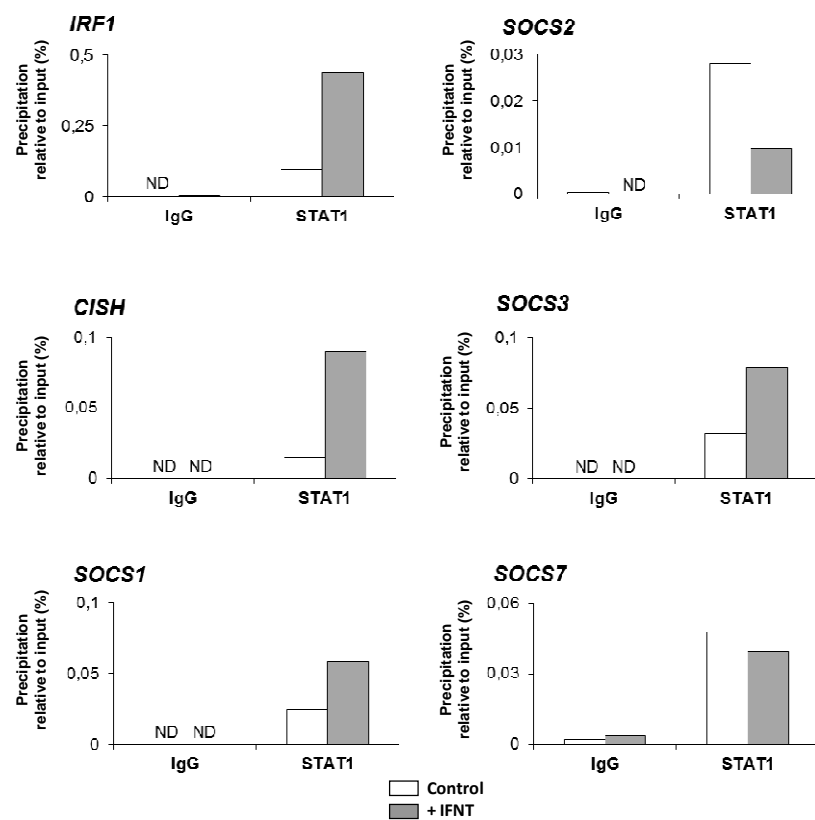
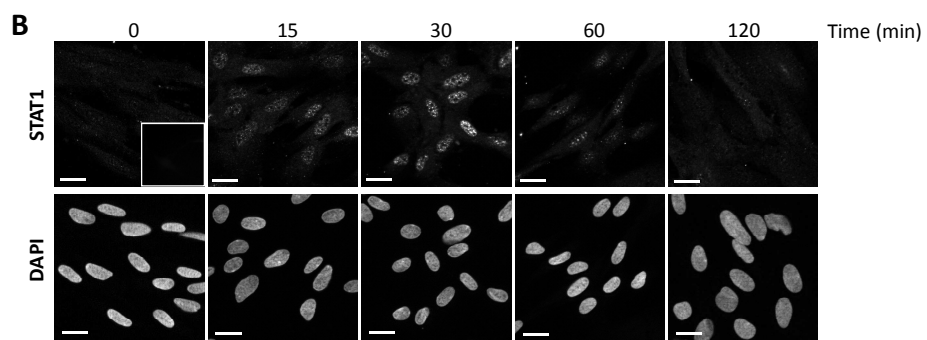
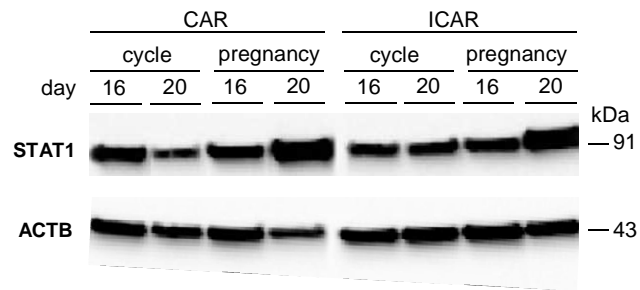


Fig. 7

A



Sup. Data 1

- **Early pregnancy is associated with a marked regulation of SOCS3 and SOCS6 expression in the bovine endometrium**

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ABSTRACT

Suppressor of Cytokine signalling (SOCS) factors control signalling pathways of a wealth of factors whose many contribute to the regulation of uterine physiology in mammals. In order to gain new insight about SOCS biological functions in the endometrium, *CISH* and *SOCS1* to *SOCS7* gene expression was determined during estrous cycle and early pregnancy in cattle. Regulation by interferon-tau (IFNT), progesterone (P4) and concepti displaying distinct potentials of term development (artificial insemination or somatic cell nuclear transfer; SCNT) was also analysed. *SOCS4*, *SOCS5* and *SOCS7* mRNA levels were globally not affected by pregnancy and appeared to be P4-dependent, in keeping with the decline observed during luteolysis. *SOCS1*, *SOCS2* and *SOCS3* mRNA levels presented the highest expression at implantation (Day 20 of pregnancy) suggesting a regulation by factors such as bovine placental lactogen. Experimental bovine models demonstrated that *CISH*, *SOCS1*, -2 and -3 but not *SOCS6* were IFNT-induced genes. Similar *SOCS6* and *SOCS1* expression profiles in pregnant endometrium suggest the contribution of *SOCS6* in the modulation of *SOCS1* expression. *SOCS3* and *SOCS6* cell localisation were also determined. The intense *SOCS3* staining in the glandular epithelium of pregnant endometrium could reflect a control of signalling pathways required for histotroph secretions. Eventually *SOCS3* expression was shown to be affected in SCNT pregnancies in keeping with the altered immune function previously reported in this model of compromised implantation. Collectively, our data suggest that spatio-temporal expression of *SOCS* genes is required to fine-tune endometrial physiology during the acquisition of receptivity, maternal recognition of pregnancy and implantation.

BACKGROUND

The birth of a healthy progeny involves a succession of complex biological steps that have to be passed successfully. Among these steps, a major checkpoint is represented by the implantation process that leads to the apposition (syn- or epitheliochorial implantation) of the embryo or the conceptus (embryonic disk and extra-embryonic tissues) then its anchorage (hemochorial implantation) into the endometrium (Bazer *et al.* 2010). The progression of implantation relies on the coordinated interactions between a developmentally competent embryo and the endometrium, whose receptivity status is controlled by progesterone (P4) in mammals (Wang & Dey 2006, Spencer *et al.* 2007). In the context of early pregnancy, biological functions of the endometrium can be subdivided into abilities (i) to differently respond to embryos displaying various potentials of term development (ii) to affect the development trajectory of the embryo at the epigenetic level, making this tissue an active and dynamic interface combining biosensor and biodriver properties (Sandra *et al.* 2011). Considering the key functions played by this tissue in the control of embryo implantation and pregnancy issue, deciphering the molecular and cellular mechanisms that govern the quality of the endometrium appears therefore mandatory.

In cattle, implantation takes place from 19-20 days post-oestrus onwards (Guillomot 1995). In bovine as well as in other ruminant species the extra-embryonic tissues first elongate before implanting. This elongation process is associated with the secretion of interferon-tau (IFNT), exclusively produced by trophoblastic cells during the peri-attachment period (Roberts 2007, Ealy & Yang 2009, Bazer *et al.* 2010). IFNT has been considered as the major pregnancy recognition signal in ruminants and its biological actions on the endometrium have been abundantly investigated (Roberts 2007, Spencer *et al.* 2007, Spencer *et al.* 2008). Major roles for IFNT have appeared to be related to the inhibition of uterine prostaglandin F2 alpha, therefore preventing luteolysis, and to the modification of the innate immune response (Hansen 2011). Based on experimental models (such as IFNT intrauterine infusions) as well as cultures of endometrial cells incubated with IFNT, target genes for this conceptus cytokine have been identified that cover a wide range of biological processes (Choi *et al.* 2001, Kim *et al.* 2003, Gray *et al.* 2006, Forde *et al.* 2011b, Eozenou *et al.* 2012, Forde *et al.* 2012, Mansouri-Attia *et al.* 2012, Oliveira *et al.* 2012) although the expression of genes strongly affected during early pregnancy has been shown to be IFN-independent (Mansouri-Attia *et al.* 2009a, Eozenou *et al.* 2012). At implantation, endometrial gene expression patterns reflect a combination of several biological processes including the actions of P4, the impact of the embryo-secreted factors and the cellular interactions between the trophoblast and the luminal endometrial epithelium. Consequently, unravelling the regulation of endometrial genes implies to evaluate the respective contribution of these distinct events, taking the morphological and functional heterogeneity of the bovine endometrium (small aglandular caruncles interspaced among large glandular intercaruncular areas) into account (Mansouri-Attia *et al.* 2009a, Walker *et al.* 2010).

Among the signalling cascades taking part to the regulation of the endometrial physiology, analyses have identified the Janus Kinase - Signal Transducer and Activator of Transcription (JAK/STAT) transduction pathway (Choi *et al.* 2001, Catalano *et al.* 2005, Joyce *et al.* 2007, Maj & Chelmonska-Soyta 2007, Spencer *et al.* 2007). To be efficient, the amplitude and the duration of the JAK/STAT signalling cascade require to be tightly controlled by regulatory factors including the Suppressor of Cytokine Signaling (SOCS) proteins (Rico-Bautista *et al.* 2006, Yoshimura *et al.* 2007). The SOCS family includes eight proteins namely the cytokine-inducible SH2-domain containing protein (CIS) and SOCS1-7 that share a central Src Homology 2 (SH2) domain, flanked by a variable N-terminal domain and a C-terminal SOCS box (Yoshimura *et al.* 1995, Endo *et al.* 1997, Naka *et al.* 1997, Starr *et al.* 1997, Hilton *et al.* 1998, Bullock *et al.* 2007). Members of the SOCS family have been identified in numerous vertebrate and some invertebrate species (Delgado-Ortega *et al.* 2013) and they are involved in the control of many biological processes and physiological functions including pregnancy

(Yoshimura *et al.* 2007, Fitzgerald *et al.* 2009). The SOCS proteins have been shown to inhibit the JAK tyrosine kinases activity, by competing with STAT transcription factors for phosphorylated tyrosine residues on cytokine receptors or by targeting bound signalling proteins for proteasomal degradation (Kile *et al.* 2002). The most studied members have been *CIS*, *SOCS1* and *SOCS3* that are expressed at low levels in unstimulated cells and are rapidly and transiently induced by numerous local acting factors and hormones through the JAK/STAT pathway (Fujimoto & Naka 2003). Regarding the endometrium, very few data exist and they have mainly focused on *SOCS1* and *SOCS3* (Fitzgerald *et al.* 2009). These two SOCS factors have been shown to contribute to the decidualization process and the uterine receptivity (Dimitriadis *et al.* 2006, Aghajanova *et al.* 2009). In the ovine endometrium, the expression of *SOCS1* and *SOCS3* as well as *SOCS2* and *CISH* transcripts has been shown to be affected by the presence of the conceptus (Sandra *et al.* 2005). Given the critical functions played by the SOCS factors in cell physiology, the current study aims to gain new insights on the involvement of the *SOCS* genes family in the endometrium physiology. Until now, this aspect has been poorly addressed prompting the need for a comprehensive analysis that we carried out in the bovine endometrium. We investigated (i) the expression of steady state levels of the *SOCS* transcripts in cyclic and pregnant endometrial tissues as well as the cell localization of *SOCS3* and *SOCS6* (ii) the regulation of *SOCS* genes levels by P4 and IFNT using *in vivo* experimental models (iii) the regulation of the *SOCS* transcripts in an experimental model of compromised implantation based on the inappropriate biological response of the endometrium facing somatic cell nuclear transfer (SCNT) bovine concepti (Mansouri-Attia *et al.* 2009b).

MATERIAL AND METHODS

Animals

Animal cares and procedures were completed in accordance with European Community Directive 86/609/EC, the Animal Research Ethics Committee of University College Dublin and the French Ministry of Agriculture (authorization B91332). Protocols were registered by the Department of Health and Children (Ireland) or by the Regional Ethical Committee of Animal Experimentation of INRA and AgroParisTech (France, protocol 12-124). *In vitro* embryo production and embryo transfer protocols were registered by French Veterinary Services (N°FRPB780 and FRTB910).

Experiment 1: Regulation of SOCS expression during maternal pregnancy recognition period

As previously described (Forde *et al.* 2011b, Eozenou *et al.* 2012), synchronised cross-bred beef heifers were artificially inseminated or not (cyclic females) then slaughtered at day 16 (cyclic n=5, pregnant n=4) and at day 20 (cyclic n=6, pregnant n=5). Concepti were recovered by uterine flushing and the correct stage of development was confirmed by microscopy (Degrelle *et al.* 2005). Endometrial caruncular (CAR) and intercaruncular areas (ICAR) were dissected separately from the uterine horn ipsilateral to the corpus luteum (Mansouri-Attia *et al.* 2009a). Samples were immediately frozen in liquid nitrogen and stored at -80°C for further analyses.

Experiment 2: Endometrial STAT1 expression at implantation

Charolais cows were synchronized by the Crestar method (Mansouri-Attia *et al.* 2009b) and were artificially inseminated (pregnant n =6; cyclic n= 5). Concepti were collected by uterine flushing and their development stage was determined as described in the experiment 1. Endometrium was collected as described in the experiment 1. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C or fixed in paraformaldehyde 4% (Electron Microscopy Science, Hatfield, USA) in phosphate buffer saline (PBS, Euromedex, Souffelweyersheim, France) for further analyses.

Experiment 3: Impact of short-term IFNT uterine infusion of endometrial SOCS expression

Cyclic Charolaise cows were synchronised using the CRESTAR method (Mansouri-Attia *et al.* 2009a). At day 14 post-oestrus, ovine recombinant IFNT (200 µg/ml, 25 ml/horn) or control solution (saline buffer) was infused into the uterine lumen, as formerly reported (Forde *et al.* 2011b, Eozenou *et al.* 2012, Forde *et al.* 2012). Cows were slaughtered 2 h after the intra-uterine infusion then the endometrium of 5 IFNT-infused and 5 control cows was collected, dissected and stored as described in experiments 1 and 2.

Experiment 4: Impact of *in vivo* P4 supplementation on the endometrial expression of SOCS transcripts in cyclic heifers

As previously described (Carter *et al.* 2008, Forde *et al.* 2011a, Eozenou *et al.* 2012), cyclic cross breed heifers received a P4-releasing intravaginal device containing 1.55 g of P4 (Ceva Animal Health Ltd.) on Day 3 post-estrus. Females were slaughtered after 2 days (5 days post-estrus, normal P4 level in cyclic heifers, n=5 and high P4 level in cyclic heifers, n=5) and 13 days (16 days post-estrus, normal P4 level in cyclic heifers, n=4 and high P4 level in cyclic heifers, n=5) of P4 supplementation. Strips of endometrium (containing CAR and ICAR areas) were collected, snap-frozen in liquid nitrogen then stored at -80°C for further analyses.

Experiment 5: Impact of bovine SCNT concepti on the endometrial SOCS expression

As previously described (Mansouri-Attia *et al.* 2009b), cyclic Holstein heifers were synchronised using the CRESTAR method. Four females were artificially inseminated whereas 4 other females were transferred with two SCNT embryos produced with adult fibroblast cells line from Holstein heifers (named 5538) (Degrelle *et al.* 2012). Upon slaughtering of the pregnant heifers, the endometrium was collected, dissected and stored as described for experiment 1.

Primary cultures of endometrial cells

Epithelial and stromal (fibroblasts) cells were isolated from cyclic bovine endometrial tissues sampled at day 11-17 of the estrous cycle, based on the ovarian morphology (Cronin *et al.* 2012). Epithelial and stromal endometrial cells were treated with a control solution or with 100 ng/ml or 1000 ng/ml of recombinant ovine IFNT for 2 h (Mansouri-Attia *et al.* 2009a). Each experiment was performed using isolated cells from four independent animals.

Immunohistochemistry

Endometrial samples from the ipsilateral horn to the corpus luteum were fixed in paraformaldehyde 4 % in phosphate buffer saline (PBS), as previously described (Eozenou *et al.* 2012). After three washes with PBS, samples were dehydrated with increasing concentrations of ethanol treatment (30%, 50%, 70%, 90%, 100%) in Shandon Citadel 1000 tissue processor (Thermo Scientific, Courtaboeuf, France) for 12 hours. Then, tissues were embedded in paraffin and stored at 4°C until being processed.

Sections (7µm) were cut with a microtome Leica RM2245 (Leica, Nanterre, France) then immunohistochemistry was performed as previously described (Eozenou *et al.* 2012). Slides were incubated with primary antibodies, anti-SOCS3 (dilution 1:200, sc9323, Santa Cruz Biotechnology, Heidelberg, Germany) and anti-SOCS6 (dilution 1:200, ab53181, Paris, France), in phosphate buffer (0.1 M, pH 7.4, with 2% BSA and 1% normal donkey serum) at 4°C overnight. As negative control, sections were incubated with phosphate buffer without primary antibody. The anti-rabbit secondary antibody (dilution 1:400; sc-346, Santa Cruz Biotechnology, Heidelberg, Germany) was incubated with the slides for 1 h at room temperature. Staining was revealed thanks to one hour incubation at room temperature of ABC Vector Elite Kit and a treatment of 8 min and 6 min for anti-SOCS3 and anti-SOCS6 respectively, with diaminobenzidine substrate and urea (SIGMAFAST 3,3'-Diaminobenzidine tablets, D4293, Sigma, France).

Total RNA extraction

Total RNA was extracted from frozen tissue by homogenisation using Trizol Reagent (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's recommendations and as previously published (Mansouri-Attia *et al.* 2009a, Eozenou *et al.* 2012). Total RNA samples were purified on Qiagen columns according to manufacturer's protocol (RNeasy Mini Kit, Qiagen, Courtaboeuf, France). The cultured primary cells were washed with 1 ml of PBS, then total RNA was extracted using the RNeasy Mini Kit and the automated system Qiacube (Qiagen, Crawley, UK; Cronin *et al.* 2012). Quality and integrity of total extracted and purified RNA were determined using an Agilent 2100 bioanalyzer. One μ l of RNase inhibitor (RNasin, Promega, France) was added to each sample before storing at -80°C .

Quantative real time PCR (qPCR)

As previously described (Mansouri-Attia *et al.* 2009a, Eozenou *et al.* 2012), 1 μ g of total RNA was retro-transcribed into cDNA using OligodT and SuperScript II (Invitrogen, Cergy-Pontoise, France). Quantitative real-time PCR (qPCR) was carried out using Master Mix SYBR Green (Applied Biosystems, Saint Aubin, France) and Step One Plus system (Applied Biosystems, Saint Aubin, France). Primers were designed using Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) or Primer Express Software (Applied Biosystems) then synthesized by Eurogentec (Angers, France). The oligonucleotide primers used for gene quantification are listed in table 1. To assess the amplification of the correct cDNA fragments, every amplicon was sequenced and blasted on NCBI RNA bovine collection. For each gene of interest, relative expression was normalized to the expression of the most stable reference genes as determined by qBaseplus software from the quantification of six housekeeping genes (Biogazelle, Gent, Belgium).

Statistical analyses

All statistical analyses were performed with GraphPad Prism 6 software (La Jolla, CA, USA). *SOCS* genes expression in tissues was first subjected to a two-way ANOVA followed by t-test tests to analyze effects of day, pregnancy status (cyclic or pregnant), endometrial areas (CAR and ICAR), and their interactions (day versus status or status versus endometrial areas). *SOCS* expression in primary cell cultures treated by IFNT was subjected to a two-way ANOVA analysis followed by t-test tests to study the potential differential *SOCS* expression relative to cell lines and to IFNT treatment.

RESULTS

Endometrial SOCS expression during estrous cycle and pre-implantation period

Using bovine endometrium collected from cross bred beef heifers, *SOCS* mRNA levels were analyzed in the CAR and ICAR endometrial areas at Day 16 and Day 20 of the estrous cycle and pregnancy. The threshold for statistical significance was fixed at $P < 0.05$.

In the cyclic endometrium (Fig. 1), no significant difference of *CISH*, *SOCS4* and *SOCS7* mRNA levels was observed between day 16 and day 20 whereas *SOCS1* and *SOCS2* gene expression increased in ICAR areas only (1.7-fold and 2.2-fold respectively). At Day 20 of estrous cycle, *SOCS3* mRNA level increased (1.8-fold in CAR and 2.9-fold in ICAR) whereas *SOCS5* and *SOCS6* gene expression decreased in CAR (0.3-fold and 0.4-fold respectively) and ICAR endometrial areas (0.4-fold and 0.3-fold respectively).

Compared to the estrous cycle no significant impact of the conceptus on *CISH*, *SOCS2*, *SOCS5* mRNA level was noticed whereas a significant regulation of transcript expression was observed for (i) *SOCS1*

in the ICAR areas at Day 16 and Day 20 of pregnancy (1.4-fold and 1.2-fold respectively) (ii) *SOCS3* and *SOCS4* in the CAR areas at Day 20 (2.2-fold and 1.3-fold respectively) (iii) *SOCS6* in the CAR and ICAR areas at Day 16 (1.3-fold and 1.3-fold respectively) that was amplified in each endometrial area at Day 20 (CAR: 3.4-fold and ICAR 2.8-fold). Between the stage of maternal pregnancy recognition (Day 16) and implantation (Day 20), no significant variation of *CISH*, *SOCS2*, *SOCS5* and *SOCS7* mRNA expression was seen in either endometrial area whereas a significant increase was detected for *SOCS1*, *SOCS3*, *SOCS4* and *SOCS6* transcripts in CAR areas (1.6-fold, 2.8-fold, 1.3-fold and 1.6-fold respectively) and for *SOCS1*, *SOCS3* and *SOCS6* transcripts in the ICAR areas (1.3-fold, 2.3-fold and 1.4-fold respectively).

Regulation of endometrial SOCS expression by IFNT

In order to determine the short-term impact of IFNT on SOCS genes expression, an *in vivo* experimental model (Charolais cows at Day 14 of the estrous cycle infused with rolFN for 2 h) and primary cultures of epithelial and stromal cells (incubated with IFNT for 2 h) were used (Fig. 2). No effect of IFNT on *SOCS4*, -5, -6 and -7 transcript levels was seen *in vitro* or *in vivo* (supplementary data 1). Expression of *SOCS1* (4.4-fold, $P < 0.05$ in CAR; 6.8-fold, $P < 0.05$ in ICAR), -2 (1.8-fold, $P < 0.05$ in CAR; 2.8-fold, $P < 0.05$ in ICAR), -3 (4-fold, $P < 0.05$ in CAR) and to a lesser extent *CISH* mRNA (2.2-fold in CAR and 1.7-fold in ICAR, $P < 0.05$) was up-regulated by IFNT treatment in CAR and ICAR endometrial areas (Fig 2. *in vivo*). No significant difference of transcript expression was observed between CAR and ICAR areas.

In primary cultures of epithelial cells (Fig. 2, *in vitro*), *SOCS1* and *SOCS3* mRNA levels were significantly induced by a 2h treatment of IFNT (10-fold, $P < 0.01$ and 2.3-fold, $P < 0.0001$ respectively). In primary cultures of stromal cells, *CISH* and *SOCS1* mRNA levels were significantly up-regulated by IFNT (3-fold, $P < 0.05$ and 8.3-fold, $P < 0.05$ respectively) whereas a trend towards an increase of *SOCS3* gene expression was observed. The variability between the four animals used for the *in vivo* experiment was responsible for the lack of statistical significance. No significant regulation of *SOCS2* transcript expression by IFNT could be seen in either type of primary cultured cells. Interestingly a differential expression of *CISH*, *SOCS1*, *SOCS2* (Fig 2) and *SOCS4* (supplementary data 1) mRNA was observed between the primary cultures of stromal cells compared to the epithelial cells (0.8-fold, $P < 0.001$, 4.6-fold, $P < 0.05$, 75-fold, $P < 0.001$ and 0.6-fold, $P < 0.001$ respectively).

Regulation of endometrial SOCS expression by exogenous progesterone (P4)

In order to investigate the impact of P4 on SOCS mRNA expression, cross-bred beef heifers were supplemented with P4 from Day 3 of the estrous cycle onwards then endometrium was sampled at Day 5 or Day 16 (Fig. 3). Only *SOCS5* gene expression was significantly altered by the 13-days P4 treatment (2.8-fold, $P > 0.01$). When the group of P4-supplemented heifers (Day 5 and Day 16) was compared to the group of control heifers, a significant increase could be seen for *SOCS6* and *SOCS7* mRNA levels in the P4-treated group ($P < 0.05$, $P < 0.01$, respectively). Moreover, a trend toward a significant increase could be observed for *SOCS3* expression in the P4-treated group ($P < 0.1$). No significant impact of P4 was observed for *CISH*, *SOCS1* and *SOCS2* transcripts. Interestingly, for *CISH* only, a significant difference in endometrial mRNA expression was noted between Day 5 and Day 16 of the estrous cycle (Fig. 3; $P < 0.05$). Eventually, no significant correlation between P4 blood concentration and any of the SOCS transcript expression was observed in the endometrium (data not shown).

Immunolocalization of SOCS3 and SOCS6 in bovine endometrium

SOCS3 and *SOCS6* were localized in endometrial tissues sampled from Charolais cows (Fig. 4). During the estrous cycle, *SOCS3* and *SOCS6* were expressed in luminal and glandular cells as well as stromal cells. Whereas *SOCS3* staining was present in the nucleus and cytoplasm of endometrial cells, *SOCS6*

staining appeared to be more specific to the nucleus at day 14 of cycle. The comparison of day 14 and day 20 of estrous showed that SOCS3 staining was not impacted whereas nuclear SOCS6 staining decreased between the mid-luteal phase and late luteal phase.

In this breed, regulation of SOCS gene expression between Day 20 of the estrous cycle and pregnancy was similar to the regulation reported in cross-bred beef heifers (data not shown). In the luminal epithelium, stroma and glandular epithelium at day 20 in cycle (Fig. 4), the staining appeared to be specific to the nucleus. At day 20, SOCS3 and SOCS6 staining increased in the cytoplasm of the luminal epithelium and epithelial stroma. Moreover, their staining was more important in the nucleus of the luminal epithelium, the stroma and the glandular epithelium at day 20 of pregnancy.

Regulation of endometrial SOCS genes by SCNT concepti

The endometrium has been shown to distinguish concepti displaying distinct abilities of development to term pregnancy (Mansouri-Attia et al. 2009b). Compared to control pregnancies, we investigated the expression of endometrial SOCS genes in heifers carrying conceptus produced by SCNT. *SOCS3* mRNA level was perturbed in the endometrium facing SCNT concepti at day 20 of pregnancy (Fig., 5; $P < 0.05$) whereas the other SOCS genes did not present any significant variation in mRNA level (supplementary data 2).

DISCUSSION

The family of the SOCS proteins has been shown to play essential functions in the negative regulation of cell signalling pathways activated by cytokine and hormone receptors, most of them being expressed at the embryo-maternal interface in mammals (Krebs & Hilton 2001, Fitzgerald *et al.* 2009). The endometrium and its physiological status are critical for supporting implantation depending on the development potency of the embryo and its quality (Salamonsen *et al.* 2009, Sandra *et al.* 2011, Weimar *et al.* 2012). Whereas the contribution of various endometrial factors (ligands, receptors and effectors) to implantation control has been unveiled based on descriptive and functional studies, scarce publications have focused on the regulation of SOCS genes and their biological functions in the uterus (Sandra *et al.* 2005, Oliveira *et al.* 2012). In order to provide new insights on the involvement of SOCS in the endometrial physiology, expression of the known SOCS (*CISH*, *SOCS1* to *-7*) genes has been investigated in bovine endometrium in order to (i) establish expression patterns as well as SOCS3 and SOCS6 cell localization during estrous cycle and early pregnancy (ii) define the contribution of interferon-tau and of progesterone (P4) to SOCS genes regulation (iii) determine if SOCS mRNA levels are altered in a model of compromised pregnancy using bovine SCNT concepti.

In the bovine endometrium, our data have shown that every SOCS gene was expressed in the bovine endometrium along the estrous cycle (Day 5 to Day 20). When compared to endometrium in luteal phase (Day 16 of the estrous cycle), analyses of expression patterns unveiled an increase for *SOCS1*, *SOCS2* and *SOCS3* but a decrease for *SOCS5* and *SOCS6* gene expression associated to luteolysis (Day 20 of the estrous cycle). A noteworthy rise of *CISH* gene transcription could also be seen between the early and late stages of the luteal phase (Day 5 *versus* Day 16 of the estrous cycle) that was not followed by a decline at the luteolytic stage (Day 20). During the estrous cycle in the ovine endometrium, results failed to report a significant alteration in *CISH*, *SOCS1*, *-2* and *-3* mRNA levels analysed by dot-blotting (Sandra *et al.* 2005). This robust quantification method is less sensible than the real-time PCR analyses undertaken in the present work and likely accounts for the discrepancy of results. Interestingly, searching for SOCS expression patterns in a microarray analysis of the human endometrium (Talbi *et al.* 2006) revealed variations in *CISH*, *SOCS1*, *SOCS2*, *SOCS3* and *SOCS6* gene levels in the secretory phase compared to the proliferative phase. Altogether these findings obtained in human and cattle indicate an impact of the ovarian steroids on the expression of *SOCS1*, *-2*, *-3*

and -6 genes in the endometrium. Modulation of *SOCS* gene expression by estrogen or progesterone has already been documented in other tissues and cultured cells (Leong *et al.* 2004, Matthews *et al.* 2005, Steyn *et al.* 2008). Progesterone-regulated *SOCS* factors could be involved in the cross regulation of signalling pathways implicated with endometrium remodelling that takes place at the end of the estrous cycle.

Using an *in-vivo* experimental model of P4-supplemented heifers, we showed that P4 treatment (i) led to the significant up-regulation of *SOCS5*, *SOCS6* and *SOCS7* transcript expression consistent with the decline observed at Day 20 of the estrous cycle when P4 blood level drops (ii) did not affect *CISH*, *SOCS1*, *SOCS2* and *SOCS3* transcript expression whereas their mRNA levels rose at Day 20 of the estrous cycle compared to Day 16. Consequently, when regression of the corpus luteum occurs, the increase of endometrial *SOCS1*, -2 and -3 transcript levels implies the participation of regulating factors others than P4. In the endometrium, during luteolysis, various parameters could account for the increased expression of those *SOCS* genes including modifications of the cytokines/hormones balance (Rahman *et al.* 2004) and variations of type and/or number of immune cell populations such as macrophages and dendritic cells that take place in the endometrium (Cobb & Watson 1995, Mansouri-Attia *et al.* 2012, Oliveira *et al.* 2012). In conclusion, whereas increase in *CISH* expression appears to be related to the acquisition of endometrial receptivity, changes in the expression of other *SOCS* genes during the estrous cycle coincide with luteolysis and reflect a direct or an indirect consequence of declining P4 blood level, the identity of intermediary factors remaining to be uncovered.

In the pregnant CAR or ICAR endometrial areas compared to the cyclic tissues, distinct patterns of *SOCS* transcripts regulation were observed. Indeed, for *SOCS6*, mRNA level first rose during the maternal phase of pregnancy recognition (Day 16 of pregnancy) and was amplified at implantation (Day 20 of pregnancy) whereas the highest level of *SOCS1*, *SOCS2* and *SOCS3* transcripts coincided with implantation. Regulation of endometrial *SOCS* mRNA expression by the conceptus is consistent with microarray studies that listed several *SOCS* genes as differentially expressed genes in the bovine pregnant endometrium compared to the cyclic tissue at Day 17 (*SOCS1*, Walker *et al.* 2010), Day 18 (*SOCS1*, *SOCS3*, *SOCS4*, *SOCS6*; Baeursachs *et al.* 2011) and Day 20 (*SOCS6*; Mansouri-Attia *et al.* 2009a) post-estrus. Interestingly, the expression patterns of *CISH*, *SOCS1*, -2 and -3 mRNA reported in the present study differ from the changes we observed in the ovine endometrium (Sandra *et al.* 2005). Compared to day 12 of estrous cycle (luteal phase), *CISH*, *SOCS1*, -2 and -3 mRNA levels did not vary in ovine CAR endometrial areas during the period of maternal recognition (Day 12 of pregnancy) but clearly increased at Day 16 of pregnancy when implantation initiates. In the bovine CAR areas at Day 16 of pregnancy (maternal pregnancy recognition) compared to Day 16 of the estrous cycle (luteal phase), *CISH*, *SOCS1*, -2 and -3 mRNA levels were not up-regulated but they were significantly increased when the apposition of the extra-embryonic tissues on the endometrium initiates. Altogether, the global transcriptional profiles of endometrial *CISH*, *SOCS1*, -2 and -3 genes do not suggest critical functions for these factors during the maternal period of pregnancy recognition but they provide first arguments that essential biological roles are played by these *SOCS* genes for driving a successful implantation in the endometrium of ruminants.

In the bovine species, the present study has shown that *SOCS4*, *SOCS5*, *SOCS6* and *SOCS7* genes expression was not induced by IFNT. In keeping with our *in vivo* analyses in the ovine endometrium (Sandra *et al.* 2005), *SOCS1*, *SOCS3* and to lesser extent *CISH* and *SOCS2* were identified as IFNT-induced genes whose expression is stimulated by a 2h treatment of IFNT *in vivo* and *in vitro*. Interestingly, whereas *SOCS1* mRNA expression was coordinated with the peak of IFNT secretion in the ovine endometrium (Sandra *et al.* 2005), overlapping was not seen in the bovine endometrium. The peak of IFNT secretion occurs at Day 15-17 of pregnancy in cattle, concomitant with the phase of pregnancy maternal recognition (Robinson *et al.* 2006). At Day 16 of pregnancy compared to estrous

cycle, *SOCS1* transcript levels did not significantly vary in the CAR areas and slightly increased in the ICAR areas. On the other hand the increase of *SOCS1* mRNA expression from Day 16 to Day 20 of pregnancy coincides with the raise in STAT protein level recently reported in the bovine endometrium (Vitorino Carvalho *et al.* submitted). These two studies demonstrate that *SOCS1* is an IFNT-induced gene and an immediate early STAT1 target gene, consistent with the ability of this protein to control STAT1-dependant IFN signal duration in various cell models (Alexander & Hilton 2004). Altogether our findings strongly support *SOCS1* as a major down-regulator of STAT1 signalling pathway during the establishment of pregnancy in the endometrium of ruminants.

Although the regulation of several *SOCS* genes depends upon IFNT at Day 16 and 20 of pregnancy, mRNA expression profiles as well cell localization indicate that other factors participate to the regulation of *SOCS* genes in the endometrium. *SOCS3* protein was detected in the cytoplasm and the nucleus of major cell populations that compose the endometrium including luminal epithelium, stroma and glandular epithelium. At implantation, whereas IFNT declines, the nuclear staining for *SOCS3* was more intense in the glandular cells, a localisation previously reported when *SOCS3* is expressed at a high level in cells (Lee *et al.* 2008). Glandular cells secrete histotroph whose production is essential for the development and growth of the conceptus (Gray *et al.* 2001). In ruminants, regulation of endometrial glands activities involves P4, IFNT as well as various peptidic hormones and factors including placental lactogens and the growth hormone/insulin growth factor (GH/IGF) family (Wathes *et al.* 1998, Spencer *et al.* 2004). During the pre-implantation period, IGF-1 and -2 levels have been shown to increase in ovine and bovine endometrium (Geisert *et al.* 1991, Kirby *et al.* 1996, Keller *et al.* 1998, McCarthy *et al.* 2012) and IGF components are principally expressed in luminal and glandular epithelia of the bovine endometrium (McCarthy *et al.* 2012). *SOCS* proteins have been shown to modulate the JAK-STAT pathway activated by IGF and GH (Krebs & Hilton 2001, Fujimoto & Naka 2003). Bovine placental lactogen, a member of the prolactin/growth hormone (PRL/GH) family Bovine placental lactogen (bPL) is secreted by the binucleate trophoblast cells from Day 18 of pregnancy onwards (Kessler *et al.* 1991, Wooding *et al.* 1992) and, as for ovine PL, its biological actions require PRL and GH receptors whose biological actions are mediated by JAK-STAT-*SOCS* pathway (Biener *et al.* 2003, Spencer *et al.* 2004). Thus from Day 20 of pregnancy onwards, *SOCS3* increase in the endometrial glands could reflect the contribution of this *SOCS* factor in the negative control of signalling pathways stimulated by bPL and IGF ligands.

A major finding of this study deals with the first report of *SOCS6* expression in the endometrium that undergoes a clear regulation during the maternal recognition phase and implantation. Our data demonstrate that *SOCS6* is not an IFNT-induced nor regulated gene *in vivo*, confirmed by the inability of IFNT to affect *SOCS6* mRNA expression at 2 h (this study) or 24 h *in vitro* (Vitorino Carvalho *et al.*, unpublished observations). Interestingly, as for *SOCS3*, *SOCS6* protein was detected in the cytoplasm and the nucleus of luminal epithelium, stroma and glandular epithelium of the endometrium. The nuclear staining of *SOCS6* is consistent with the publication reporting that high level of *SOCS* expression leads to its nuclear localization in cells (Hwang *et al.* 2007). *SOCS6* regulators and biological functions have been less investigated than for other *SOCS*, therefore the meaning of *SOCS6* presence in the endometrium is currently unknown. Nevertheless, two relevant hints are provided by reports showing that *SOCS6* can (i) potentiate signaling of IFN type I receptor by targeting *SOCS1* to proteosomal turn-over (Piessevaux *et al.* 2006) (ii) downregulate STAT3 protein levels in HEK293T cells (Hwang *et al.* 2007). *STAT3* is expressed in the endometrium of ruminants (Song *et al.* 2009) and *SOCS6* gene expression is similar to *SOCS1* mRNA profile in pregnant females suggesting the contribution of *SOCS6* in the regulation of STAT3 transcription factor and *SOCS1* availability in the bovine endometrium. Additional studies based on alteration of *SOCS* gene expression in primary cultures of endometrial cells could definitely help in deciphering *SOCS6* biological roles in the endometrium.

Biosensing of the embryo quality is an endometrial property that has been established in cattle first (Mansouri-Attia *et al.* 2009b) then in human (Teklenburg *et al.* 2010). As previously reported by microarrays analyses (Mansouri-Attia *et al.* 2009b), SCNT conceptus elicit an endometrial response that, in turn, affects the progress of implantation associated to pregnancy failures. The present study indicates an increased expression of *SOCS3* mRNA levels in the endometrium of SCNT concepti carrying heifers. In transgenic mice, *SOCS3* overexpression leads to an embryonic lethality (as a consequence of anemia) and to increased Th2 responses (reviewed in Fitzgerald *et al.* 2009). This later phenotype is very interesting and suggests a link between *SOCS3* and the deregulated immune function we reported in the endometrium of SCNT pregnancies (Mansouri-Attia *et al.* 2009b). This increase in *SOCS3* we have reported in the endometrium likely reflects an alteration in the quality or the type of embryonic signals emanating from SCNT concepti. Using day 18 SCNT concepti derived with the 5538 somatic cell line (the same as the one used in our work), the recent transcriptomic analysis has not focused on ligands or secreted factors that differ with AI or *in vitro* produced (IVP) concepti (Degrelle *et al.* 2012). Nevertheless IFNT as well as bPL gene expression are identical between AI concepti and SCNT concepti derived from the 5538 cell line (Degrelle *et al.* 2012; Mansouri-Attia *et al.* 2009b; Vitorino Carvalho A, unpublished observations). Based on recent data reporting the biological functions of embryo signals other than IFNT, glucocorticoids and prostaglandins (PG) produced by the trophoctoderm and the endometrium are part of the orchestra that regulates the expression of endometrial genes during implantation in ruminants (Dorniak *et al.* 2011, Majewska *et al.* 2012, Ulbrich *et al.* 2012). Compared to pregnancies obtained with IVP embryos, uterine flushings from bovine SCNT pregnancies were reported to contain lower levels of PGI2 and PGE2, consecutively to a reduced amount of embryo- and/or endometrium produced PG (reviewed by Ulbrich *et al.* 2012). In rat corpus luteum and hypothalamus, PG have been shown to regulate *SOCS1* and *SOCS3* expression (Curlewis *et al.* 2002, Bonafede *et al.* 2011). Altogether these data suggest that the higher level of endometrial *SOCS3* in SCNT pregnancy could reflect an altered production and/or metabolism of PG. In this model of compromised implantation with long-term effects, the better understanding of the events taking place in the endometrium facing cloned embryos will require a thorough characterization of the extra-cellular signals that differ between cloned and control embryos.

Given the fact that *SOCS* act in a negative feedback loop to inhibit signal transduction but are also cross-talking factors regulating other signaling pathways, disentangling the contribution of the *SOCS* in the regulation of the endometrial function represents an actual challenge. Nevertheless rapid modifications in *SOCS3* and *SOCS6* as well as *SOCS1* and *CISH* gene levels have been shown by our data in the endometrium in various physiological and experimental situations. Consequently, appropriate spatio-temporal expression of *SOCS* genes is likely necessary to fine-tune endometrial physiology during the acquisition of receptivity, maternal recognition of pregnancy and implantation. Alterations of *SOCS* expression may be detrimental for the progression of any of these steps therefore affecting global reproductive capacity of the female. Intensive efforts are currently produced to identify biomarkers of the endometrial quality as well as targets for therapeutic approaches aiming to restore an altered endometrial ability to support term pregnancy or to prevent embryo implantation (contraception). In this context, expression of *SOCS* genes would deserve to be investigated in the mammalian endometrium presenting a suboptimal or a compromised functional capacity as a consequence of environmental insults such as nutrition, stress or infections.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Figure legends

Figure 1: . Quantification of *SOCS* genes expression in cyclic and pregnant bovine endometrium on Day 16 and 20 of the estrous cycle. Caruncular (CAR) and intercaruncular (ICAR) areas were collected from cyclic (Day 16: n=5; Day 20, n=6) and pregnant (Day 16, n=4; Day 20, n= 5) cross-bred heifers. Expression of *SOCS* was normalized to that of *RPL19* and *SCL30A6* using qBasePlus. Bars with different lowercase letters differ significantly ($P < 0.05$).

Figure 2: Regulation of endometrial *CISH*, *SOCS1-3* expression by interferon-tau (IFNT) *in vivo* and *in vitro*. *In vivo*, CAR and ICAR endometrial areas were collected from Charolais cows infused with control solution (n = 5) or recombinant ovine IFNT (roIFNT, 200 µg/ml; n = 5) for 2 h at Day 14 of estrous cycle. *SOCS* mRNA level was quantified by RT-qPCR and normalized to *C2ORF29*, *SLC30A6* and *SUZ12* using qBasePlus. *In vitro*, cells isolated from bovine endometrium were treated with recombinant ovine IFNT (roIFNT; 100ng/ml) for 0, 0.5, 2, or 24 h. For each gene, mRNA expression was normalized to that of *RPL19* and *ACTB* using qBasePlus. Data are the mean ± S.E.M. a, b and c letters indicate a significant difference between means ($p < 0.05$) whereas x and y letters indicate significant difference between control and IFNT samples ($p < 0.05$).

Figure 3: *SOCS* endometrial expression in cyclic and pregnant cross-bred beef heifers supplemented with progesterone (P4) for 2 days (Day3 to Day 5) or 13 days. Females were slaughtered after 2 days (5 days post-estrus, normal P4 level in cyclic heifers, n=5 and high P4 level in cyclic heifers, n=5) and 13 days (16 days post-estrus, normal P4 level in cyclic heifers, n=4 and high P4 level in cyclic heifers, n=5) of P4 supplementation. *SOCS* mRNA level was quantified by RT-qPCR and normalized to *ACTB* and *RPL19* using qBasePlus. Bars (mean ± s.e.m) represent the different conditions. Data are the mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$. x and y letters indicate significant difference between Day 5 and Day 16 samples ($p < 0.05$)

Figure 4: Cellular localization of *SOCS3* and *SOCS6* expression in cyclic or pregnant bovine endometrium. Immunohistochemistry was performed using sections cut from Charolais cows sampled at Day 15 (14Cy: A, D, G, J, M, P) and Day 20 of the estrous cycle (20Cy; B, E, H, K, N, Q) as well as Day 20 (20Pr; C, F, I, L, O, R) of pregnancy. A-I and J-R sections were respectively incubated with anti-*SOCS3* and anti-*SOCS6* primary antibody. Tissue samples in S were incubated without primary antibody as a negative control. Tissue samples in T-U were stained with hematoxylin-eosin solution. BV, blood vessel; GE, glandular epithelium; LE, luminal epithelium; STR, stroma. Bars= 100 µm.

Figure 5: *SOCS3* endometrial expression in Holstein cows carrying embryos produced by artificial insemination (n=4) or with transfer of SCNT produced embryos (n=4) at day 20 of pregnancy. *SOCS* mRNA levels were quantified by RT-qPCR and normalized thanks to qBasePlus on *C2ORF29*, *RPL19* and *SLC30A6*. Bars are representative of the mean value ± S.E.M. x and y letters indicate significant difference between control and SCNT pregnancy ($p < 0.05$).

Supp Data 1: Regulation of endometrial *SOCS4-7* expression by interferon-tau (IFNT) *in vivo* and *in vitro*. *In vivo*, CAR and ICAR endometrial areas were collected from Charolais cows infused with control solution (n = 5) or recombinant ovine IFNT (roIFNT, 200 µg/ml; n = 5) for 2 h at Day 14 of estrous cycle. *SOCS* mRNA level was quantified by RT-qPCR and normalized to *C2ORF29*, *SLC30A6* and *SUZ12* using qBasePlus. *In vitro*, cells isolated from bovine endometrium were treated with recombinant ovine IFNT (roIFNT; 100ng/ml) for 0, 0.5, 2, or 24 h. For each gene, mRNA expression was normalized to that of *RPL19* and *ACTB* using qBasePlus. Data are the mean ± S.E.M. a, b and c letters indicate a significant difference between means ($p < 0.05$).

Supp Data 2: *SOCS* (except *SOCS3*) endometrial expression in Holstein cows carrying embryos produced by artificial insemination (n=4) or with transfer of SCNT produced embryos (n=4) at day 20 of pregnancy. *SOCS* mRNA levels were quantified by RT-qPCR and normalized thanks to qBasePlus on *C2ORF29*, *RPL19* and *SLC30A6*. Bars are representative of the mean value \pm S.E.M.

Table

Table 1: Description of the oligonucleotide primers used for gene quantification by real time RT-PCR.

Gene	Forward	Reverse	Amplicon size	Accession number
<i>CISH</i>	AGTCATCCTGGTGCCCGTGA	AGCCTTGTTCTGCACCG	69	NM_001046586.1
<i>SOCS1</i>	CTCGTACCTCCTACCTCTTCATGTT	ACAGCAGAAAAATAAAGCCAGAGA	92	XM_864316.2
<i>SOCS2</i>	TGCAAGGATAAGCGGACAGG	AGATGCTGCAGAGGTGGTGCT	101	NM_177523.2
<i>SOCS3</i>	GCCACTCTCCAACATCTCTGT	TCCAGGACCTCCCGAATGG	97	NM_174466.2
<i>SOCS4</i>	GCTTTGTAAACCTATGTCATTGGCA	ACAACACACACAGCTTTACCGAAG	100	NM_001076218.2
<i>SOCS5</i>	TACATCCAGTGGCTGTCGC	GCACAGCAAGCAGAAACATACATT	100	NM_001046182.1
<i>SOCS6</i>	AGAAGGTCAAAAAATGTCACAGGAA	CAATGGTTTAGCCTTTTGGCAT	113	NW_003104566.1
<i>SOCS7</i>	CTCCACTGCCTAAGCCTCTG	GAAATGAGCTGCGCTTCCTT	102	NM_001206013.1
<i>SUZ12</i>	CGTTGTGAGCAGTTTGGCCCTGT	ACCACAGTGCTTGGAGTTGGACT	139	NM_001205587.1
<i>C2ORF29</i>	GCTTTACCACCACAGCCGAG	GGGTCCTTTTCCAACCTCTCC	64	XM_002691150.1
<i>SLC30A6</i>	TGATGAGGAAACCTAGCCCTGCC	TCGGGCTGCTCCAAAAAGCGT	142	NM_001075766.1
<i>RPL19</i>	CCCCAATGAGACCAATGAAATC	CAGCCCATCTTTGATCAGCTT	72	NM_001040516.1
<i>GAPDH</i>	CCTTCAAGAGCCCCCTGT	TCATAAGTCCCTCCACGATGC	432	NM_001034034.2

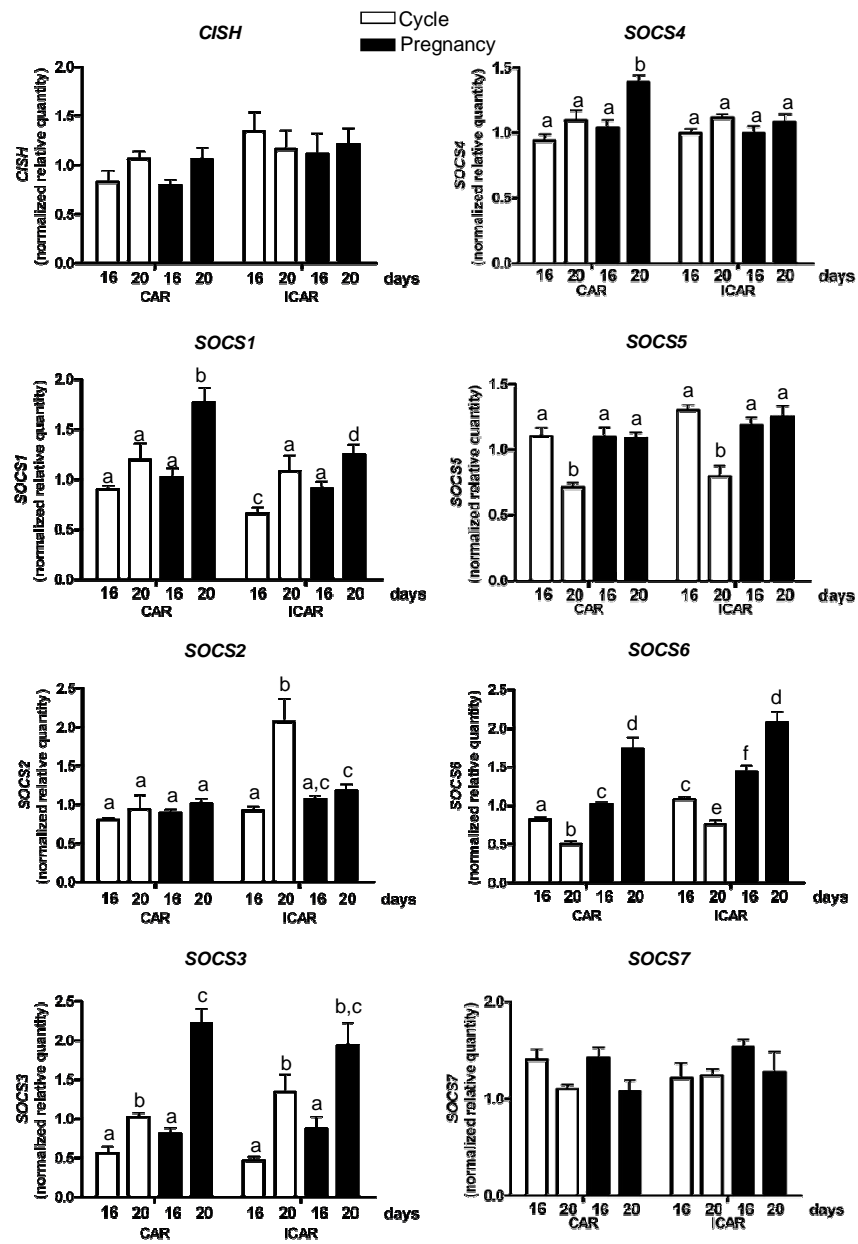


Fig. 1

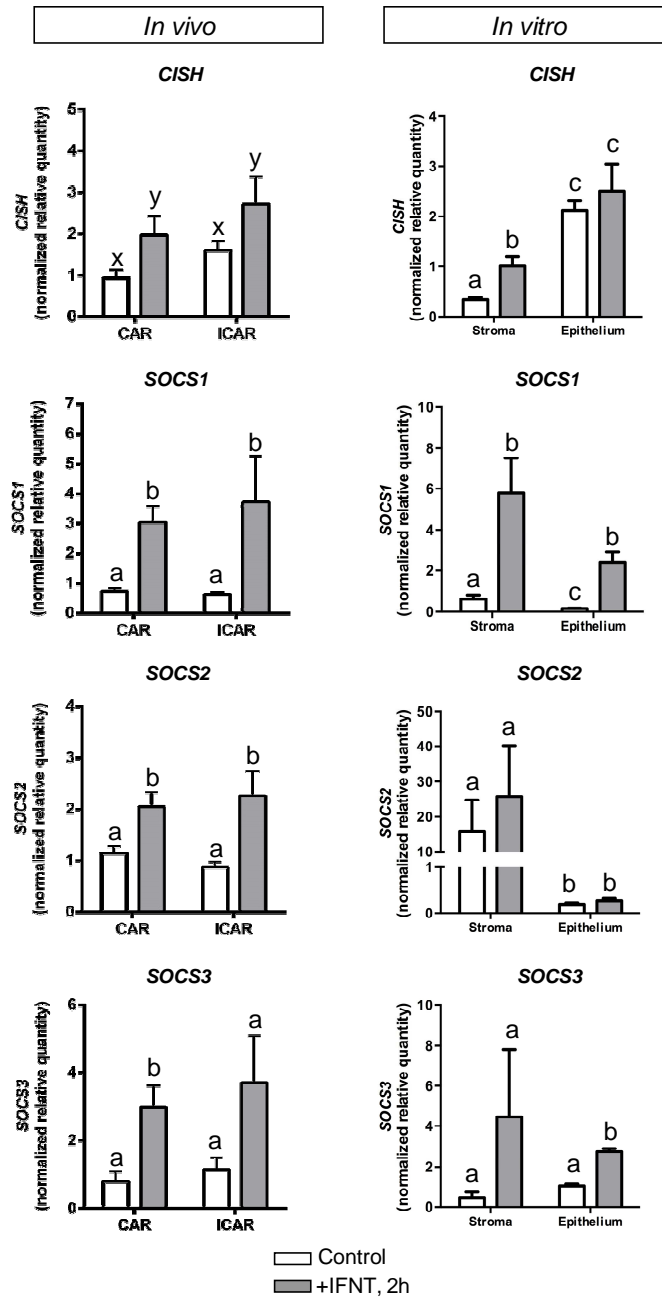


Fig. 2

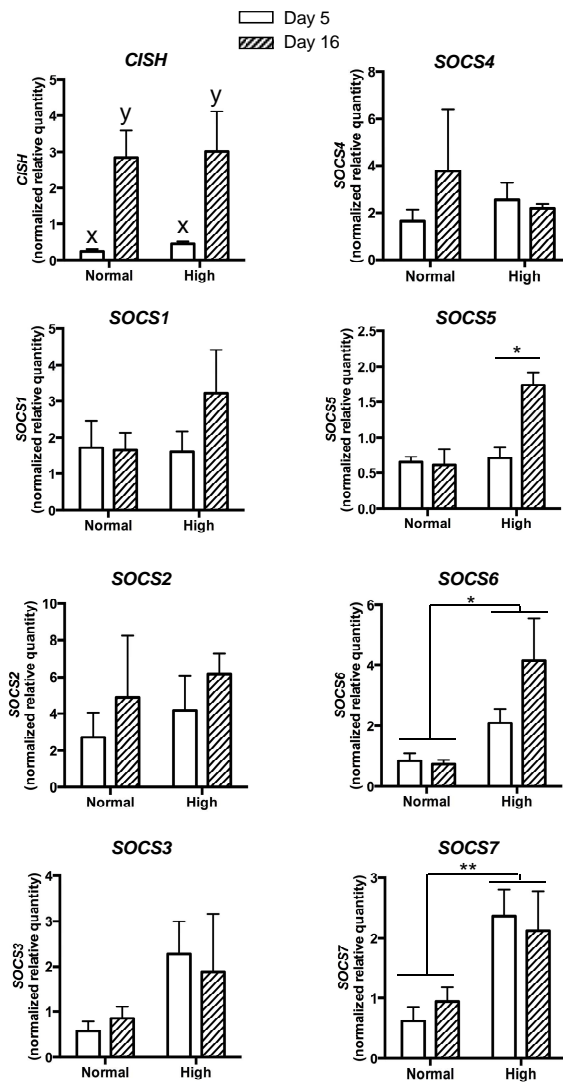
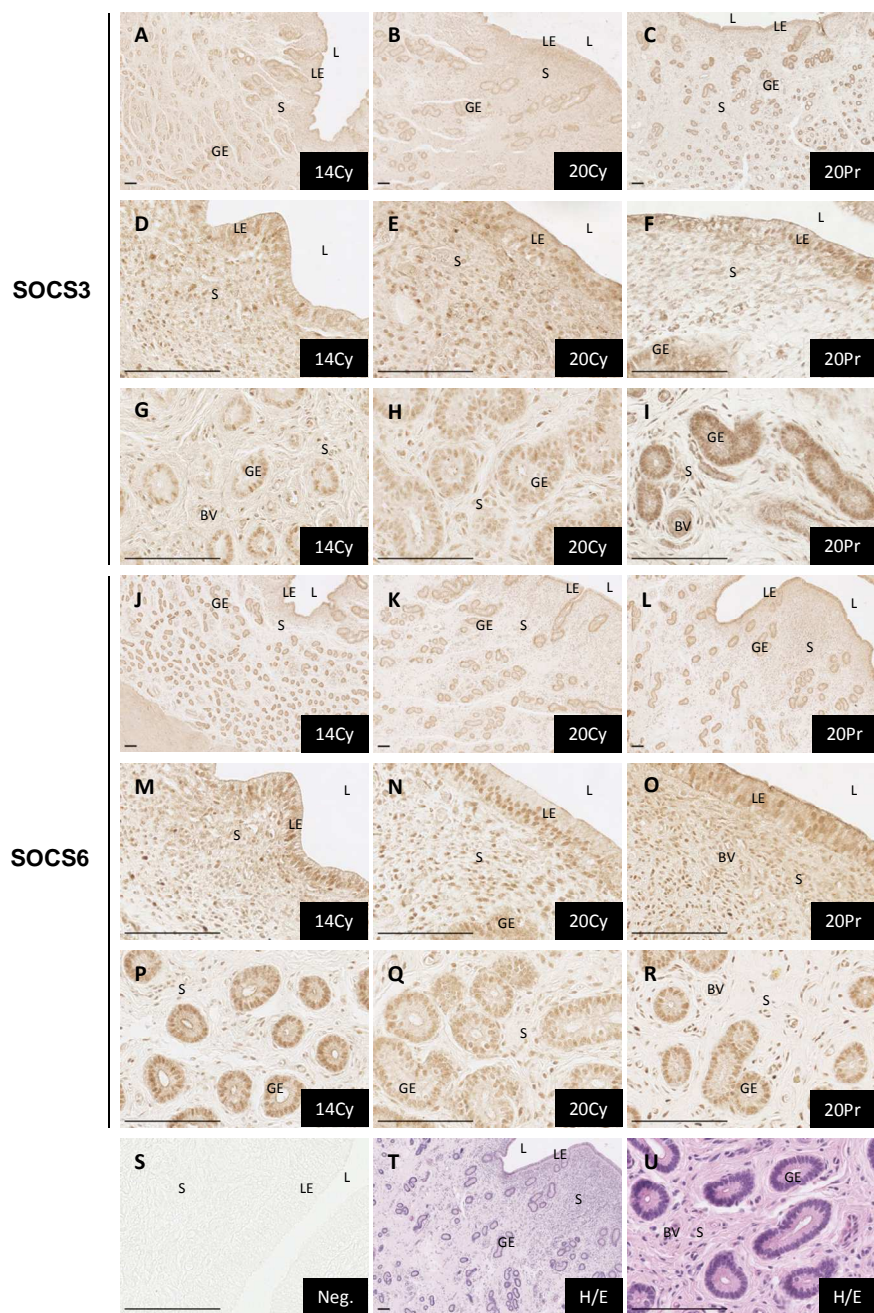


Fig. 3



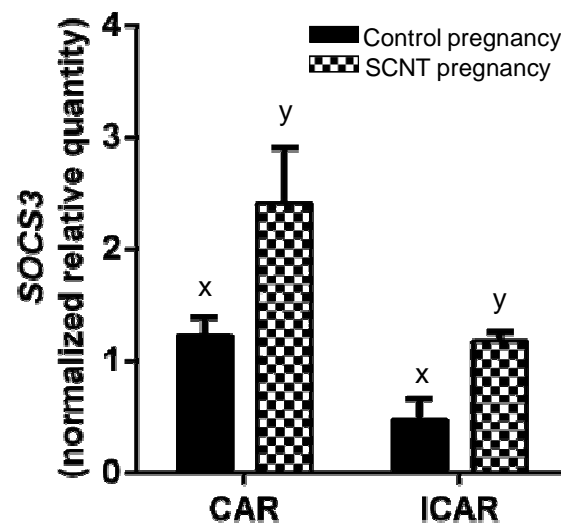
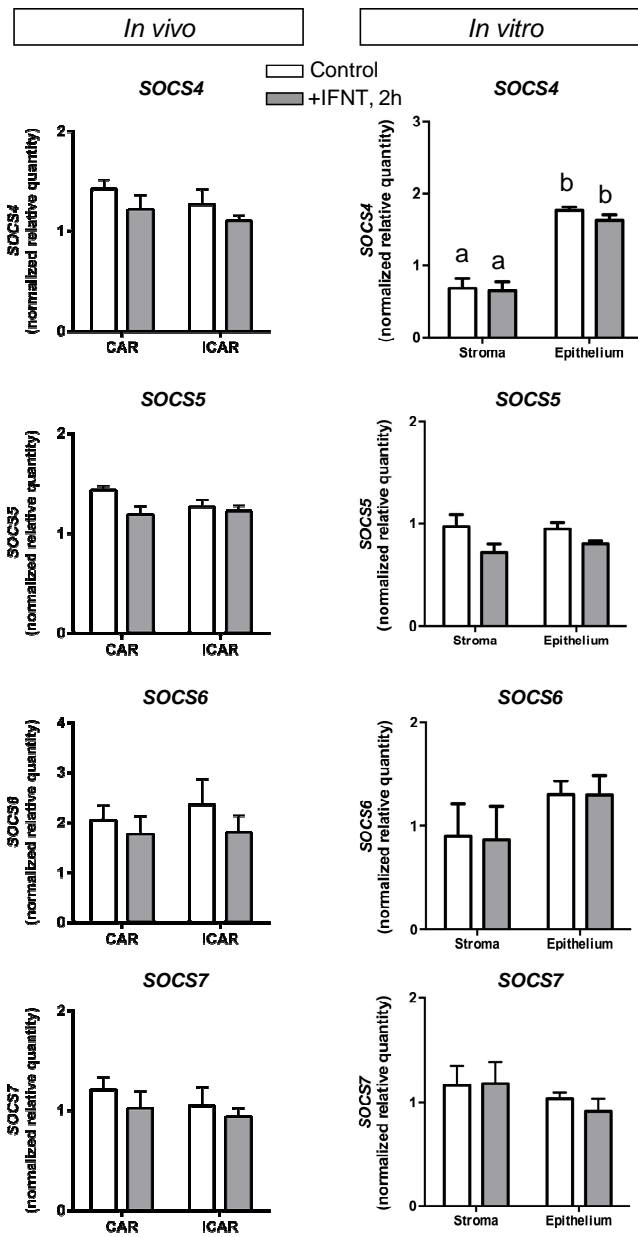
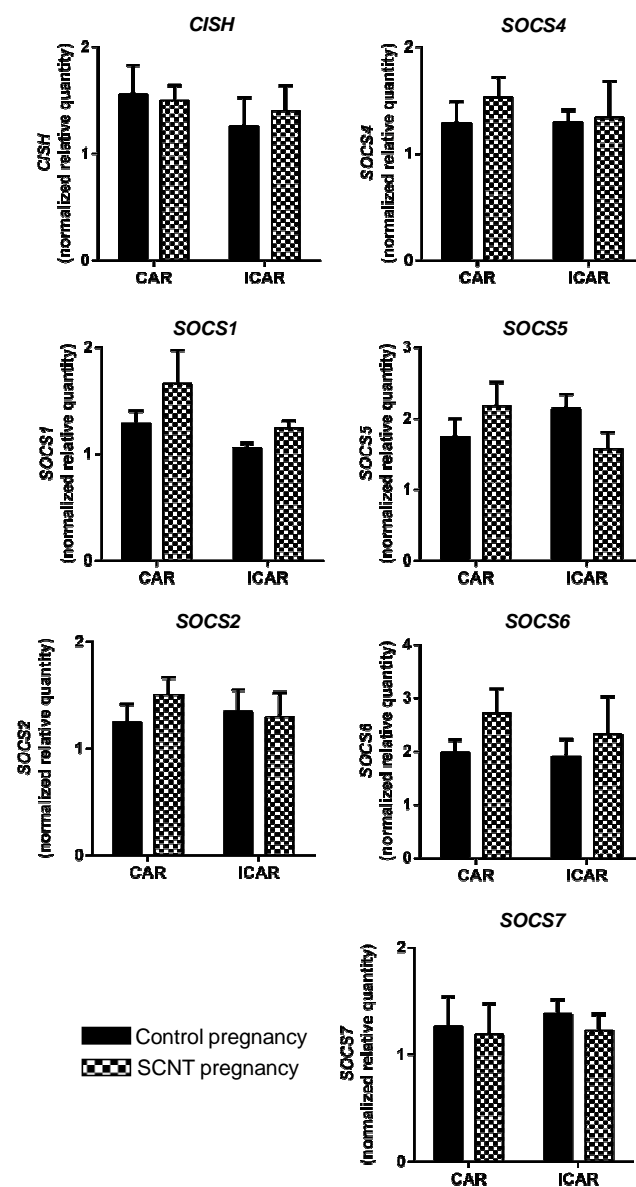


Fig. 5



Supplemental Data 1



Supplemental Data 2