Université de Montréal

EXPRESSION OF STEROIDOGENIC PROTEINS AND GENES IN BOVINE PLACENTA FROM CONVENTIONAL AND SOMATIC CELL NUCLEAR TRANSFER (SCNT) GESTATIONS

par

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RÉSUMÉ

Pendant la grossesse, les hormones stéroïdes jouent un rôle indispensable dans la régulation des principales manifestations physiologiques telles que la reconnaissance maternelle de la gestation, la réceptivité de l'endomètre, le début du développement embryonnaire ainsi que le maintien de la gestation. Cependant, on sait très peu sur la production de ces hormones et les principaux facteurs des voies intracellulaires impliqués dans le processus de stéroïdogenèse dans le placenta bovin pendant les stades initiaux et plus avancés de la gestation. Par ailleurs, certaines anomalies du placenta chez les bovins suite à une mauvaise production de stéroïdes n'ont pas encore été démontrées. Les objectifs de cette thèse étaient donc de : 1) déterminer la présence et la localisation des principales protéines stéroïdiennes dans le placenta de bovins provenant de gestations de 50 à 120 jours, 2) comparer l'expression placentaire d'une série de gènes et de protéines stéroïdiennes entre une gestation impliquant un transfert de noyaux de cellules somatiques (SCNT) et une gestation non-clonale; 3) étudier l'impact des hormones trophiques et des seconds messagers sur la stéroïdogenèse dans le placenta bovin à 140 ±10 jours de gestation.

L'utilisation de techniques d'immunohistochimie, d'immunobuvardage et de PCR quantitatif nous a permis d'évaluer la présence d'un large éventail de gènes stéroïdiens (*STAR, CYP11A1, HSD3B1, CYP17A1 et SCARB1*) qui participent au transport du cholestérol et dans la production de différents types de stéroïdes. Dans cette thèse, nous avons démontré la capacité du placenta bovin d'initier la stéroïdogenèse au début de la gestation et nous avons également déterminé les principales cellules impliquées dans ce processus. Nous avons constaté que les tissus maternels expriment les principaux marqueurs de stéroïdogenèse suggérant une plus grande capacité stéroïdogénique que les tissus fœtaux. En outre, un modèle d'expression des protéines complémentaires stéroïdogéniques entre la caroncule et le cotylédon a été observé, indiquant que la stéroïdogenèse placentaire exige une communication cellule à cellule entre les cellules de la mère et du fœtus.

Après avoir démontré les principales cellules impliquées dans la synthèse des hormones stéroïdiennes dans le placenta bovin en début de gestation, nous avons ensuite étudié les modifications possibles de la stéroïdogenèse dans les tissus SCNT cotylédonaires à 40 jours de gestation. Nous avons identifié d'importantes modifications dans l'expression

des gènes *STAR, CYP11A1, HSD3B1, CYP17A1, et SULT1E1*. Conséquemment, nous postulons que l'expression réduite des gènes stéroïdiens peut provoquer une insuffisance de la biosynthèse des hormones stéroïdiennes, ce qui pourrait contribuer à un développement anormal du placenta et du fœtus dans les gestations SCNT à court ou long terme.

Finalement, nous avons développé un modèle efficace de culture d'explants de placentome qui nous a permis d'explorer les mécanismes sous-jacents spécifiques à la stéroïdogenèse placentaire. Nous avons exploré l'effet stimulant des hormones trophiques et différents messagers secondaires sur l'expression de différentes protéines stéroïdogéniques ainsi que le taux de progestérone (P4) dans les explants de placentome. En utilisant les techniques de RIA et de PCR quantitatif, nous avons constaté que même si les analogues de l'hormone lutéinisante (hCG) ont un effet stimulant sur plusieurs gènes stéroïdiens, le calcium ionophore est le principal modulateur dans la synthèse de la P4. Ces résultats suggèrent que dans le placenta bovin, la synthèse de la P4 est modulée principalement par l'afflux de calcium intracellulaire, et apparemment les nucléotides cycliques ne semblent pas contrôler ce processus.

En conclusion, cette étude contribue de manière significative à une meilleure compréhension des mécanismes d'entraînement de la synthèse des stéroïdes placentaires au début de la gestation et permet aussi d'apporter de nouveaux éclairages sur l'importance des stéroïdes placentaires dans la régulation du développement du placenta et du fœtus.

Mots-clefs : placenta bovin, stéroïdes placentaires, stéroïdogenèse, gestation SCNT.

ABSTRACT

During pregnancy, steroid hormones have essential roles in regulating key physiological events such as maternal recognition, endometrial receptivity, early embryonic development, and maintenance of pregnancy. However, very little is known about the production of these hormones nor about the principal factors and intracellular pathways implicated in the steroidogenic process in bovine placenta, during early and advanced pregnancy. In addition, placental abnormalities in cattle following an improper steroid production in bovine placenta have not been yet demonstrated. The aims of this thesis were to: 1) determine the occurrence and localization of the principal steroidogenic proteins in bovine placenta from day 50 to day 120 of pregnancy; 2) compare the placental expression of a series of steroidogenic genes and proteins between somatic cell nuclear transfer (SCNT) pregnancies and non-SCNT gestations; 3) investigate the impact of trophic hormone, and second messengers on steroidogenesis in bovine placenta at 140 $\pm$10 days of gestation.

Using immunohistochemistry, western blot and qPCR techniques, we evaluated the presence of a wide range of steroidogenic genes (*STAR*, *CYP11A1*, *HSD3B1*, *CYP17A1* and *SCARB1*), that participate in the cholesterol transport and in the production of different types of steroids. In this thesis, we demonstrated the capability of the early bovine placenta to initiate steroidogenesis, and we also determined the principal cells implicated in this process. We found that maternal tissue expresses the principal steroidogenic markers suggesting it has a greater steroidogenic capacity compared to fetal tissue. Moreover, a complementary pattern of steroidogenic protein expression between the caruncle and the cotyledon were found, indicating that placental steroidogenesis requires cell to cell communication between the maternal and fetal cells.

Having shown the principal cells involved in the synthesis of steroid hormones in bovine placenta during early pregnancies, we then studied possible

alterations in steroidogenesis in cotyledonary tissue in SCNT at 40 days of pregnancy. We identified significant alterations in the expression of *STAR*, *CYP11A1*, *HSD3B1*, *CYP17A1* and *SULT1E1* transcripts. Therefore, we postulate that reduced expression of steroidogenic genes may cause an insufficient local biosynthesis of steroid hormones, which might contribute to the abnormal placental and fetal development in SCNT gestations at short or long term.

Finally, we developed an efficient placentome explants culture model that allowed us to explore the specific mechanisms underlying placental steroidogenesis. We explored the stimulatory effect of trophic hormones and different second messengers on the expression of various steroidogenic proteins and the progesterone levels in placentome explants. By RIA and qPCR techniques, we found that although LH-like hormones (hCG), had a stimulatory effect on multiple steroidogenic genes, the calcium ionophore was the principal modulator in the synthesis of progesterone. These results suggest that in bovine placenta, the synthesis of progesterone is modulated principally by intracellular calcium influx, and cyclic nucleotides do not seem to be controlling this process.

In conclusion, these studies significantly contribute to a better understanding of the driving mechanisms of placental steroid synthesis in early gestations and also provide new insights into the importance of placental steroids in the regulation of placental and fetal development.

Key words: Bovine placenta, placental steroids, steroidogenesis, SCNT gestation.

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25-OHD_3	25-hydroxyvitamin D3
A2387	Calcium ionophore
ACTB	Actin beta
ACTH	Adrenocorticotropic hormone
AI	Artificial insemination
ANGPT1	Angiopoietin 1
BTGC or BNGC	Binucleate trophoblast giant cells
Ca^{2+}BPs	Binding proteins known as the calbindins
Ca^{2+}	Calcium
Ca^{2+}BP9k	Calbindin-D9k
calcitriol	1,25-dihydroxyvitamin D_3
cAMP	Cyclic-AMP
CEC	Caruncular epithelial cells
CL	Corpus luteum
CP	Chorionic plate
CRL	Crown-rump length
CSC	Caruncular stromal cells
CSH	Chorionic somatomammotropin hormones
CSH1	Chorionic somatomammotropin hormone 1

	(placental lactogen)
CST3	Cystatin C
CTSL	Cathepsin L, cysteine proteinase
CV	Chorionic villi
Cy3	Cyanine 3
CYP11A1	Cytochrome P450, family 11 subfamily, A polypeptide 1
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1
CYP19A1	Cytochrome P450, family 19 subfamily, A polypeptide 1
DAPI	4',6-diamidino-2-phenylindole
DEDTC	Diethyldithiocarbama
DHEA	Dehydroepiandrosterone
DHEA-S	DHEA sulfate
DHT	Dihydrotestosterone
DKK1	Dickkopf homolog 1 (Xenopus laevis)
DOC	Deoxycorticosterone
E1	Estrone
E1S	Estrone sulfate
E2	Estradiol
E2R	E2 receptor

EGF	Epidermal growth factor
ERK	Extracellular-signal-regulated kinases
ESR1	Estrogen receptor 1
FACS	Fluorescence activated cell sorting method
FGF	Fibroblast growth factor
FGF2	Fibroblast growth factor 2 (basic)
FGFR1	Fibroblast growth factor receptor 1
FITC	Fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GE	Glandular epithelium
GH	Growth hormone
GnRH	Gonadotrophin-releasing hormone
GNRH1	Gonadotrophin-releasing hormone 1
HAND1	Heart and neural crest derivatives expressed 1
hCG	Human chorionic gonadotrophin
HCGR	Human chorionic gonadotrophin
HDL	High density lipoproteins
HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HOXA10	Homeobox A10
HOXA11	Homeobox A11

HSD17B	Hydroxysteroid (17-beta) dehydrogenase
HSD17B1	Hydroxysteroid (17-beta) dehydrogenase 1
HSD17B3	Hydroxysteroid (17-beta) dehydrogenase 3
HSD17B7	Hydroxysteroid (17-beta) dehydrogenase 7
HSD3B	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerases
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
HSD3B2	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2
HSL	Hormone-sensitive lipase
IFIH1	Interferon induced with helicase C domain 1
IFNT	Interferon-tau
IGF1	Insulin-like growth factor 1 (somatomedin C)
IGF2	Insulin-like growth factor 2 (somatomedin A)
IGF2R	Insulin-like growth factor 2 receptor
IGFBP1	Insulin-like growth factor binding protein 1
IGFBP2	Insulin-like growth factor binding protein 2
IgG	Immunoglobulin G
IL-1	Interleukin-1
IMH	Immunohistochemistry
IP	Immunoperoxidase

ISG15	IFN-stimulated gene 15 or ISG15 ubiquitin-like modifier
KDR or VEGFR-2	Kinase insert domain receptor (a type III receptor tyrosine kinase)
LDL	Low density lipoproteins
LDLr	LDL family receptors
LE	Uterine luminal epithelium
LGALS15	Galectin 15; lectin
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LIF	Leukemia inhibitory factor (cholinergic differentiation factor)
MAPK	Mitogen activated protein kinase
Mash 2	Achaete-scute complex-like protein 2
MDF	Macrophage-derived factor
MLN64 or STARD3	StAR-related lipid transfer (START) domain containing 3
MMP	Matrix-metalloproteinases
MMP10	Matrix metallopeptidase 10 (stromelysin 2)
MMP26	Matrix metallopeptidase 26
MMP8	Matrix metallopeptidase 8 (neutrophil collagenase)
mRNA	Messenger RNA

MS	Maternal septum
MTGC	Mononucleate trophoblast giant cells
MUC4	Mucin 4, cell surface associated
MUC5B	Mucin 5B, oligomeric mucus/gel-forming
NG	Non-clone gestations
NPC	Niemann-Pick type C proteins
OXTR	Oxytocin receptor (OXTR)
P13K	Phosphoinositide 3-kinases
P4	Progesterone
PAF	Paraformaldehyde
PAG	Pregnancy-associated glycoproteins
PAP7 or ACBD3	acyl-CoA binding domain containing
PGE (1)	Prostaglandin E(1)
PGE (2)	Prostaglandin E(2)
PGF	Placental growth factor
PGF(2α)	Prostaglandin F2α
PGR	Progesterone receptor
PHLDA2	Pleckstrin homology-like domain, family A, member 2
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate

PRL	Prolactin
PRLR	Prolactin receptor
qPCR	Quantitative real time PCR
RSAD2	Radical S-adenosyl methionine domain containing 2
SAP	Steroidogenic activator polypeptide
SC	Stromal cells
SCARB1 or SRB1	Scavenger receptor class B member I
SCNT	Somatic cell nuclear transfer
SCP2	Sterol carrier protein 2
sGE	Superficial glandular epithelum
SNRPN	Small nuclear ribonucleoprotein polypeptide N
SSP1	Secreted phosphoprotein 1
STAR	Steroidogenic acute regulatory protein
STARD4	StAR-related lipid transfer (START) domain containing 4
STARD5	StAR-related lipid transfer (START) domain containing 5
STARD6	StAR-related lipid transfer (START) domain containing 6
STS	Steroid sulfatase

SULT1E1	Sulfotransferase family 1E, estrogen-preferring member 1
TBS	Tris Buffered Saline
TGC	Trophoblast giant cells
TGFB1	Transforming growth factor beta 1
TNF	Tumor necrosis factor
TSPO	Translocator protein (18 kDa)
TTGC	Trinucleate trophoblast giant cells
UTC	Uninucleate trophoblast cells
VDCA	Voltage-dependent anion channel
VEGFA	Vascular endothelial growth factor A
VEGFR-2 or KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)
WB	Western blotting
WNT7A	Wingless-type MMTV integration site family, member 7A
ZF	Zona fasciculata
ZG	Zona glomerulus
ZP	Zona pellucida
ZR	Zona reticularis

"Sueño con que mi México despierte del profundo letargo en el que se encuentra sumido; y sobre todo sueño con un México que vuelva a vivir sus años de esplendor como la gran Tenochtitlán que fue algún día"

Dedicated to my country

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CHAPTER 1. INTRODUCTION

In the last decade, the manipulation of gametes and embryos in farm animals has improved significantly. New reproductive biotechnologies have had a great impact in the development of the productive and reproductive fields, and are powerful tools that may provide a solution to the coming world food demands. SCNT technology has many potential applications in basic research, medicine and agriculture. Some examples of the potential benefits and research applications of this technology, including conservation of animal genetic resources, study of genetic and epigenetic mechanisms underlying developmental biology, aging and carcinogenesis and preservation of endangered species (Tong*, et al.*, 2002; Mastromonaco & King, 2007; Murphey*, et al.*, 2009; Rodriguez-Osorio*, et al.*, 2009; Liu*, et al.*, 2010). Although bovine cloning by SCNT has improved significantly in the last decade, the cloning process remains largely inefficient because of losses during gestation. In cattle, most SCNT pregnancies are lost in the first trimester and resulting from abnormalities of the embryo or its placenta or alterations in maternal uterine environment or fetomaternal interactions (Wilmut*, et al.*, 1986; Wilmut*, et al.*, 1997; Cibelli*, et al.*, 1998; Wells*, et al.*, 1999; Koo*, et al.*, 2002; Pace*, et al.*, 2002). A variety of placental dysfunctions have been reported for the SCNT pregnancies, including fewer placentomes, rudimentary and reduced number of cotyledons, increased caruncular weight, failures in development of the allantois, and abnormalities of placental vasculature (Hill*, et al.*, 2000; Hashizume*, et al.*, 2002; Lee*, et al.*, 2004; Farin*, et al.*, 2006). Faulty gene expression during implantation and placentogenesis has been suggested to contribute to the pathologies observed in cloned offspring.

Variations in the pattern of expression found in some genes, proteins and factors which control placental growth (IGF1, IGF2, IGF2R, IGFBP1, IGFBP2, VEGFA, VEGFR-2, DKK1 and PHLDA2), have been associated with placental dysfunction, restraining placental growth, delayed parturition and a generalized hypoxic condition in early placental tissues in SCNT pregnancies (Ravelich*, et al.*, 2004) (Ledgard*, et al.*, 2009; Perecin*, et al.*, 2009; Campos*, et al.*, 2010).

Among the key functions of the placenta is the production of steroid hormones, such as progesterone, estrogens and sulfoconjugated estrogens. Placental steroid hormones are required for pregnancy recognition, maternal support of conceptus, regulation of proliferation and/or differentiation of glandular epithelium, conceptus-uterine interactions, placental vascular development and process of parturition (Pepe & Albrecht, 1995; Spencer*, et al.*, 2004; Mesiano & Welsh, 2007; Johnson*, et al.*, 2010). In addition, excess of estrogen sulfoconjugation is the possible cause for a poor sign of parturition in SCNT pregnancies (Hirayama*, et al.*, 2008). Nonetheless, in SCNT gestations, little is known about sub-optimal steroid synthesis, and its possible impact in the placental and fetal development.

Previous research in bovine gestation has demonstrated that ruminant placenta produces estrogens and progesterone, and, as mentioned before, these hormones are important factors controlling caruncular growth, differentiation and function, and also inducing histotrophic and hematotrophic support (Hoffmann & Schuler, 2002; Dunlap*, et al.*, 2008; Schuler*, et al.*, 2008). Furthermore, estrogen and progesterone receptors are present in bovine placenta, suggesting that placental

steroids act as local regulators of placental growth (Hoffmann & Schuler, 2002). Nevertheless, further research is needed to identify the underlying processes of steroidogenesis in bovine placenta on a molecular level.

Given the importance of steroid hormones in placental and fetal development, the objectives of this PhD project were to understand the mechanisms involved in the regulation of steroid synthesis by bovine placenta, and to explore if abnormalities in bovine cloned pregnancies may originate from disturbed placental steroidogenesis.

CHAPTER 2. LITERATURE REVIEW

2.1. PREGNANCY

2.1.1. Definition and general aspects of pregnancy

Pregnancy is defined as a condition where there is carrying of an embryo or fetus inside a female viviparous animal. Establishment and maintenance of pregnancy is an intricate and complex interaction between the conceptus and the mother that comprises pregnancy recognition, endometrial receptivity, implantation, placental and fetal development and parturition. In most species, a complex dialogue between the embryo and the uterus is initiated immediately after fertilization with remarkable and visible micro-remodeling of the endometrium, followed somewhat later by embryo implantation (Hashizume, 2007; Lefevre*, et al.*, 2007).

2.1.2. Pregnancy recognition

In ruminants, pregnancy recognition is the physiological process whereby the conceptus signals its presence to the maternal system and prolongs lifespan of the corpus luteum (CL), allowing it to continue to secrete progesterone (Spencer*, et al.*, 2004). Progesterone is the hormone of pregnancy and unequivocally required in all mammals to stimulate and maintain the uterine functions that will facilitate the early embryonic development, implantation, placentation, and successful fetal and placental development to term (Spencer & Bazer, 2002; Spencer & Bazer, 2004). The role of progesterone and other steroid hormones during gestation will be discussed in more detail in later sections of this review. As mentioned before,

recognition of ruminant pregnancy requires conceptus signals, which alter endometrial physiology, resulting in establishment of the pregnancy. Precisely timed embryo recognition in the genital tract is essential for establishment of an optimal maternal environment for embryo implantation (Spencer*, et al.*, 2007). A deficient signal leads to luteolysis, progesterone level decline and finally, pregnancy loss. The pregnancy recognition factors, critical days of pregnancy recognition and time of conceptus attachment in different mammal species are shown in the following table:

Table 1. Pregnancy recognition factors.

Species	Pregnancy Recognition Factors	Critical Period for Recognition (days after ovulation)
Bitch	None needed	___
Cow	Elongation of the blastocyst and synthesis of interferon-tau (INFT). INFT prevents development of the endometrial luteolytic mechanism	15-16
Ewe		13-14
Mare	Embryo produces substantial amounts of estrogens and prostaglandins. The aforementioned factors apparently facilitate the continuously migration of equine conceptus throughout the uterine lumen, avoiding the luteolysis.	9-16
Sow	Estradiol (E2) produced by conceptuses induces a transient release of calcium into the lumen, changing the direction of endometrial secretion of prostaglandins from an endocrine to an exocrine orientation.	10-12
Mouse	Prolactin release caused by stimulation of the pelvic nerve and activation of a neural reflex arc. Zona pellucida (ZP) derivatives can act as an intrinsic signal from the developing embryo for maternal recognition by the immune system.	Before 4 days
Woman	Embryo produces human chorionic gonadotrophin (hCG), which interacts with luteinizing hormone LH/hCG receptor and promotes the maintenance of the CL.	7-12

(Bazer & Roberts, 1983; Gandolfi*, et al.*, 1992; Roberts*, et al.*, 1996; Duc-Goiran*, et al.*, 1999; Spencer*, et al.*, 2004; Spencer*, et al.*, 2004; Senger, 2005; Bazer*, et al.*, 2008; Fujiwara*, et al.*, 2009; Walker*, et al.*, 2009; Farin*, et al.*, 2010; Klein*, et al.*, 2010).

2.1.3. Animal models of implantation

The embryo implantation is a complex process where local paracrine signaling between embryo and the endometrium occurs in order to obtain a strong attachment between them. In species with invasive placenta, the free floating blastocyst attaches to the maternal endometrium, invades into the stroma and establishes the placenta. This requires a synchronization of a viable blastocyst to implant and an endometrium able to respond to embryonic signals. There is diversity in types and time of implantation among species (see _Table. 2).

Table 2. Different types of placenta, implantation and time of attachment.

Species	Time of attachment (time after ovulation)	Implantation type	Placenta type
Cow	18-22 days	Caruncular areas Centric	Syndesmochorial Cotyledonary
Ewe	15-18 days	Caruncular areas Centric	Syndesmochorial Epitheliochorial Cotyledonary
Sow	14-18 days	Superficial and centric	Epitheliochorial Diffuse
Mare	36-38 days	Endometrial surface	Epitheliochorial Diffuse
Mice and rats	Day 4 (mouse) Day 5 (rat)	Eccentric	Discoid Memoendothelial
Woman	9 days	Interstitial	Discoid Hemochorial

(Dey*, et al.*; Lee & DeMayo, 2004; Senger, 2005)

In general, the first step in implantation after the preattachement is when cells of the trophectoderm from the blastocyst migrate between the epithelial cells, displacing them and penetrating as far as the basement membrane. The second step is when the trophoblast cells invade through the basal membrane coming in contact with the maternal stroma. The final step in this process is the decidualization, which consists of transformation of the endometrial stromal cells in highly secretory cells to prepare the endometrium for implantation (Sharkey & Smith, 2003) (see figure. 1).

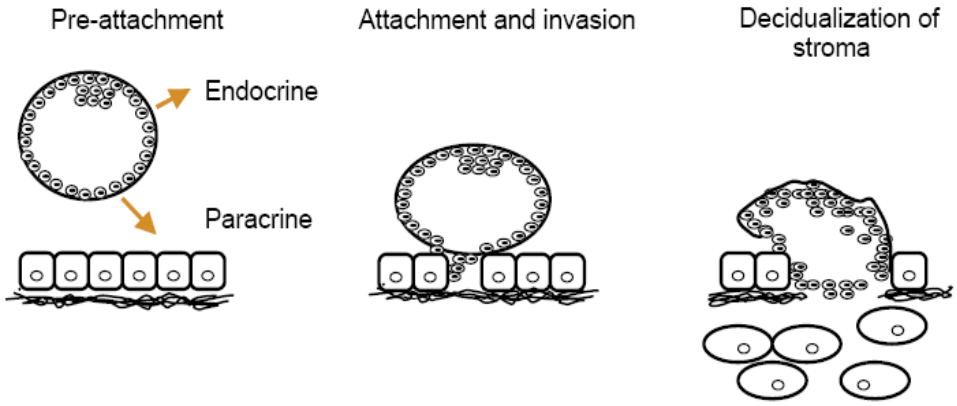

Figure 1. Cartoon representing the initial phases of murine implantation. During the pre-attachment phase, the embryo uses endocrine and paracrine mediators to signal its presence to the mother. Following apposition and attachment, the embryo invades and promotes decidualization of the maternal stroma. Taken from Sharkey and Smith, 2003.

After implantation, the conceptus undergoes massive growth, due largely to the development of extraembryonic membranes. The formation of these membranes is the vital importance in the ability of the embryo to attach in some species. In fact the chorioallantoic membrane is the foetal contribution to the placenta and will provide the surface for attachments to the endometrium (Senger, 2005). Regardless of the placental type, the purpose of attachment is to bring embryonic tissue and conceptus blood vessels into contact with the maternal blood supply to allow nutrient uptake, waste elimination, and gas exchange between the mother and the conceptus (Lee & DeMayo, 2004).

2.2. Bovine pregnancy

2.2.1. Implantation

In ruminants implantation takes place when the spherical blastocysts elongates to a tubular form, after which the embryo and associated membranes begin to adhere strongly to the maternal endometrium (Spencer*, et al.*, 2004; Song*, et al.*, 2010)

2.2.2. Type of placentation

The bovine placenta is classified as synepitheliochorial (Wooding, 1992). In contrast to haemochorial placenta, placentation in the ruminants is characterized by multiple discrete areas of attachment, called placentomes, and formed by interactions of patches of the chorioallantois with the endometrium (Igwebuike, 2006).

2.2.3. Morphometry and histology of bovine placenta

The placentome consists of a fetal portion (the cotyledon) contributed by the chorion, while the maternal contact sites are the caruncles, originated from uterus (Figure 2).

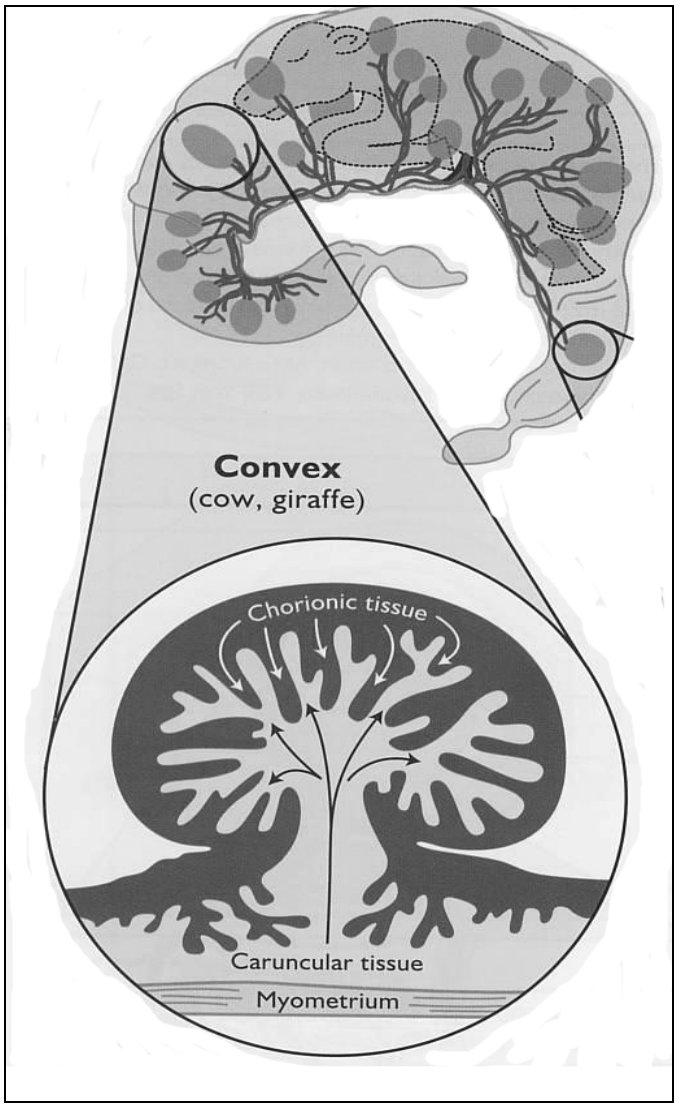

Figure 2. Diagrammatic representation of the bovine placentome. The cotyledonary tissue is characterized by numerous "button-like" structures distributed across the surface of the chorion. The cotyledons (black) fuse with the maternal caruncles (gray), and they form a placentome. Source: (Senger, 2005).

The formation of placentomes is a ongoing process that begins immediately after pregnancy recognition, and the chorion becomes strongly attached to the caruncles of the uterus approximately at day 40 of gestation (Leiser & Kaufmann, 1994; Senger, 2005). In cows there are between 70 to 120 cotyledons distributed across the surface of the chorion, and apparently there is no change in the total number of placentomes during gestation (Laven & Peters, 2001). Moreover, the cotyledons display a villous interdigitation of fetal villi with maternal crypts of the complementary caruncles (Leiser*, et al.*, 1997). This adhesion of the trophectoderm and the endometrial luminal epithelium occurs in caruncular and intercaruncular areas of the endometrium. In the trophectoderm, two morphological and functional cell population have been identified, these are the mononucletae trophoblast cells and the binucleate trophoblast cells (Igwebuike, 2006). Mononucleate trophoblast cells are cuboidal to columnar cells that constitute four-fifths of the trophoblast population, and are primarily involved in nutrient exchange (Igwebuike, 2006) . On the other hand, mature binucleate cells or trophoblast giant cells (TGC) are large cells that contain two nuclei, but the presence of mononuclear and trinuclear forms have been also reported (Klisch*, et al.*, 1999)(See Figure 3).

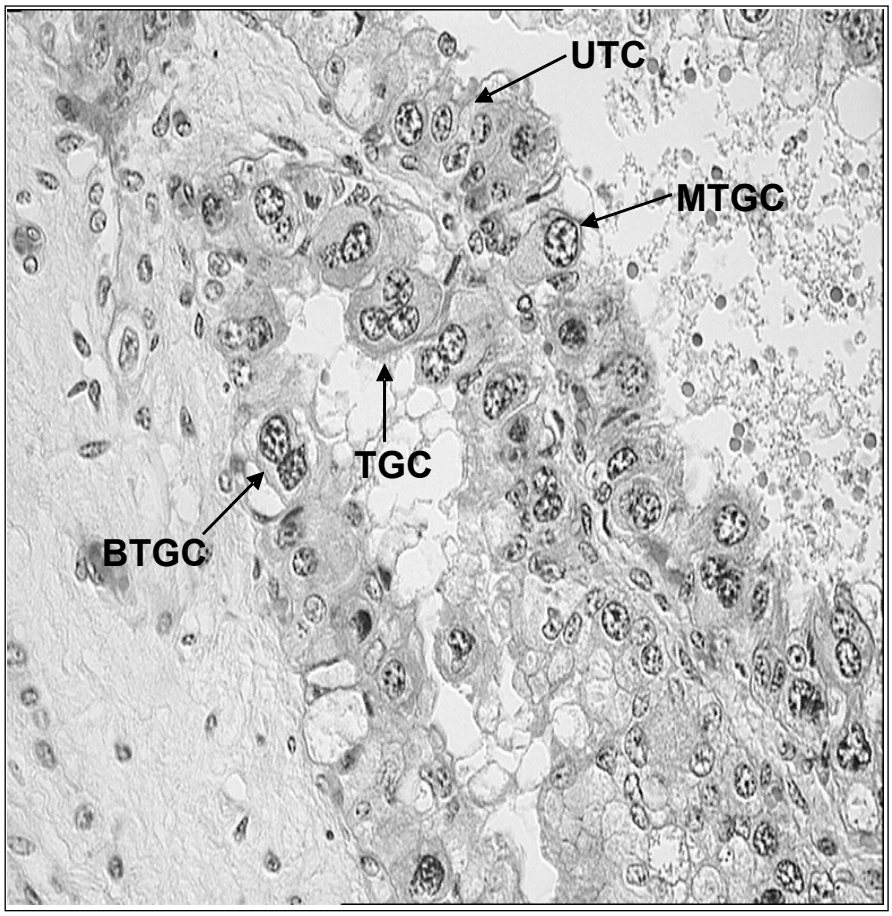

Figure 3. Cross section of a bovine cotyledon at approximately 90 days of gestation. The image shows the different types of trophoblast cells lying in the chorionic villi. UTC, uninucleate trophoblast cells, MTGC, mononucleate trophoblast giant cells, BTGC, binucleate trophoblast giant cells, TTGC, trinucleate trophoblast giant cells (A. Verduzco, unpublished).

2.2.3.1. Bovine Trophoblast Giant Cells

The trophoblast giant cells represent approximately 20% of the trophectoderm and it has been demonstrated that they originate from mononucleate trophoblast cells by acytokinetic mitoses (mitoses uncoupled from cell division) (Igwebuike, 2006). Moreover the occurrence of trinuclear cells is the result of the fusion of binuclear trophoblast cells with uterine epithelial cells (Klisch*, et al.*, 1999). The TGCs are believed to be endocrine cells that produce factors, which are necessary to mediate gas and micronutrient exchange through the placenta and specially to guarantee fetal survival (Wooding, 1992). These cells are the only placental cells that have the capacity to migrate across the feto-maternal interface to fuse with columnar cells of the maternal uterine epithelium. The TGCs have at least two functions: 1. to form a hybrid feto-maternal syncytium essential for successful implantation and subsequent placentomal growth; 2. to synthesize and secrete protein and steroid hormones as placental lactogen (CSH1) and progesterone (P4) (Spencer et al, 2004). Migration and fusion of TGCs with maternal endometrial cells are imperative for the delivery of the aforementioned factors into the maternal compartment and subsequently to the maternal circulation (Senger, 2005; Igwebuike, 2006)(Figure 4).

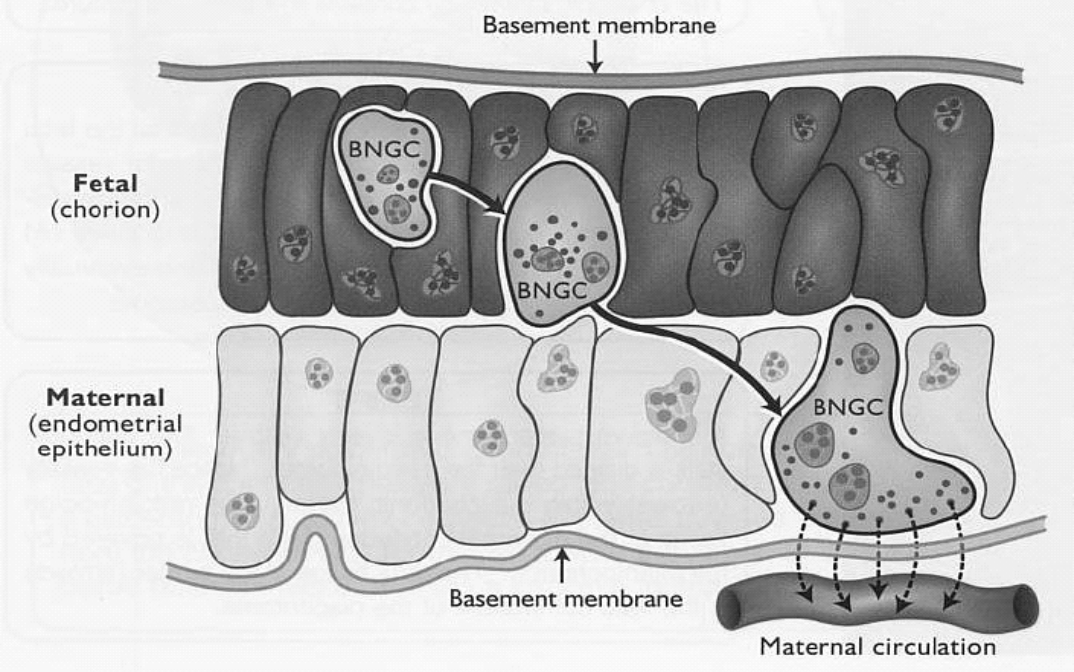

Figure 4. Schematic representation of how binucleate giant cells (BNGC) or Trophobast giant cells (TGCs) migrate from the chorion to the endometrial epithelium in ruminants. These cells are thought to secrete placental lactogens, pregnancy specific proteins and steroid hormones. Source (Senger, 2005).

2.2.4. Pregnancy recognition in cattle

In ruminants the columnar trophoblast cells synthesize and secrete IFNT, the pregnancy recognition signal from Days 8 to 24 of gestation. The effect of the IFNT is the maintenance of a functional CL, abrogation of the endometrial luteolytic mechanism, and also establishment of uterine receptivity to implantation (Kubisch*, et al.*, 2001; Spencer & Bazer, 2004; Bazer*, et al.*, 2009; Farin*, et al.*, 2010). IFNT acts directly on the uterine endometrium blocking the expression of estrogen receptor 1 (ESR1), and therefore indirectly silences oxytocin receptor (OXTR) expression in the endometrial epithelium, preventing endometrial prostaglandin F2α (PGF(2α) synthesis and release (Walker*, et al.*, 2009; Banu*, et al.*, 2010; Farin*, et al.*, 2010).

2.2.4.1. Ruminant endometrial receptivity

Endometrial receptivity is a self-limited period in which the endometrium acquires the ability to adhere the developing blastocyst (Dominguez*, et al.*, 2003). In bovine, this process involves numerous physiological, molecular and cellular interactions between the conceptus, the receptive endometrium, and the corpus luteum; which play a pivotal role in establishment of the pregnancy and development of the placenta (Hashizume, 2007; Farin*, et al.*, 2010).

Steroid hormones, cytokines, chemokines, growth factors, IFNT, pregnancy-associated glycoproteins (PAGs) and chorionic somatomammotropin hormones (CSH) secreted by the ovary, endometrium and developing embryo, have been

shown to be involved in the coordination of the endometrial receptivity (Adamson, 1993; Sharkey, 1998; Hashizume, 2007).

In ruminants the coordinate expression and sequential exposure of the pregnant endometrium to P4, IFNT, CSH1, prolactin (PRL) and growth hormone (GH), are necessary to activate and maintain endometrial remodeling, secretory function, and uterine growth during gestation (Spencer*, et al.*, 1999). In this context, IFNT and P4 are essential factors for endometrial receptivity in early bovine gestation. Exposure of endometrium to steroid hormones, especially P4 and estrogens, prepare the endometrium for implantation and then maintain the gestation by promoting uterine growth, modulating the maternal immune system and suppressing myometrial contractions (Graham & Clarke, 1997; Sharkey & Smith, 2003; Goff, 2004).

The primary action of IFNT is to abrogate the pulsatile secretion of PGF(2α), blocking the luteolytic mechanism (Bazer*, et al.*, 2009). Moreover recent studies in ovine and bovine, indicate that conceptus IFNT also stimulated genes such as radical S-adenosyl methionine domain containing 2 (RSAD2), interferon induced with helicase C domain 1 (IFIH1), IFN-stimulated gene 15 (ISG15); which are important genes in the establishment of uterine receptivity to implantation, conceptus development and modulation of local immune cells in the endometrium (Song*, et al.*, 2007; Yang*, et al.*, 2010) .Figure 5 presents the principal pleiotropic (producing more than one effect) effects of P4 and IFNT in mammal's pregnancy.

In addition to the molecules listed above, other factors and genes such as metalloproteinases (MMP8,MMP10,MMP26), integrins, mucins (MUC4,MUC5B),

secreted phosphoprotein 1 (SSP1), calcitonin, leukaemia inhibitory factor (LIF), homeobox genes (HOXA10 and HOXA11), have been reported essential for uterine receptivity in the human and mouse (Lim*, et al.*, 1999; Sharkey & Smith, 2003; Altmae*, et al.*, 2010; Casals*, et al.*, 2010; Sendag*, et al.*, 2010). Some of the aforementioned molecules have yet proven useful as biomarkers of endometrial receptivity (Casals*, et al.*, 2010; Fogle*, et al.*, 2010; Rackow*, et al.*, 2011).

Although the implantation process is different in bovine compared to human and mice; a recent study points to the potential use of endometrial and embryo gene expression patterns as predictors of pregnancy success in cattle (Salilew-Wondim*, et al.*, 2010). In this study cell division, kinesin, integrins, chemokines, insulin growth factors, solute carriers, WD repeats, adapter protein, leucine reach repeats, claudins, coiled-coil domain, inositol, ATPases, Zink finger, S100 calcium binding protein, collagen families, RNA binding motif, Rho GTPase activating protein, CD molecules, keratins, protein kinases and phosphatase gene clusters were differentially expressed in embryo biopsies resulting in calf delivery.

In addition, transcript levels of many new candidate genes involved in the regulation of transcription, cell adhesion, modulation of the maternal immune system and endometrial remodeling were found to be increased during the implantation window (Bauersachs*, et al.*, 2006).However, the precise mechanism or mechanisms involved in the ruminant endometrial receptivity have not been fully investigated.

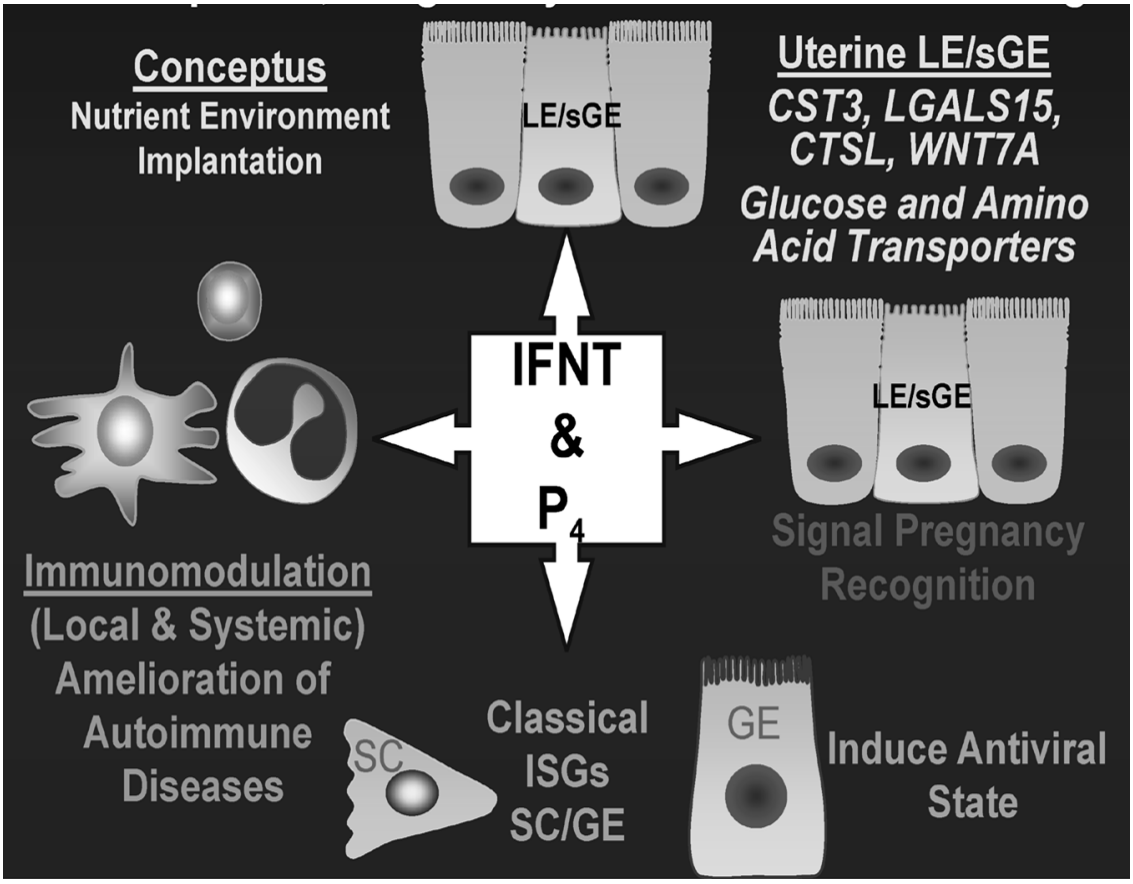

Figure 5. General pleiotropic effects of progesterone (P4) and interferon-tau (IFNT)on conceptus development, pregnancy and maternal well-being in mammal's pregnancy. Uterine luminal epithelium (LE), stromal cells (SC), glandular epithelium (GE), superficial glandular epithelum (sGE), cystatin C (CST3), galectin 15; lectin (LGALS15), cathepsin L, cysteine proteinase (CTSL) and wingless-type MMTV integration site family, member 7A (WNT7A). Source: (Bazer*, et al.*, 2008).

2.3. Steroid Biosynthesis

2.3.1. General aspects

The synthesis of steroids is a vital process for essential physiologic functions such as blood salt balance, carbohydrate metabolism and reproduction. Steroid hormones are synthesized predominantly in the adrenal cortex, testis, ovary, placenta and central nervous system (Simpson & Waterman, 1983; Hornsby, 1987; Payne & Youngblood, 1995; Arensburg*, et al.*, 1999; Pasqualini, 2005; Rekawiecki*, et al.*, 2008; Rone*, et al.*, 2009). The steroids occur in a large diversity of biologically active forms; this variety is due partially by steroid-synthesizing tissues, depending upon the set of enzymes present in it. Steroids include principally: testosterone (androgens), estradiol (estrogen), progesterone (progestin), cortisol/corticosterone (glucocorticoid), aldosterone (mineralocorticoids), neurosteroids and a wide variety of intermediate metabolites (Bogan & Hennebold, 2010). In general, P4 and estradiol are classified as sex-steroids, and are produced primarily in ovary and placenta; whereas glucocorticoids and mineralocorticoids are produced in adrenal glands, androgens in testes, and neurosteroids in brain (Enyeart, 2005; Pasqualini, 2005; Ghayee & Auchus, 2007; Mellon, 2007; Rekawiecki*, et al.*, 2008; Lavoie & King, 2009) (Figure 6) . Although steroid hormones are synthesized with different physiological actions in each tissue, their synthesis depends on a common precursor, namely cholesterol (Ghayee & Auchus, 2007).

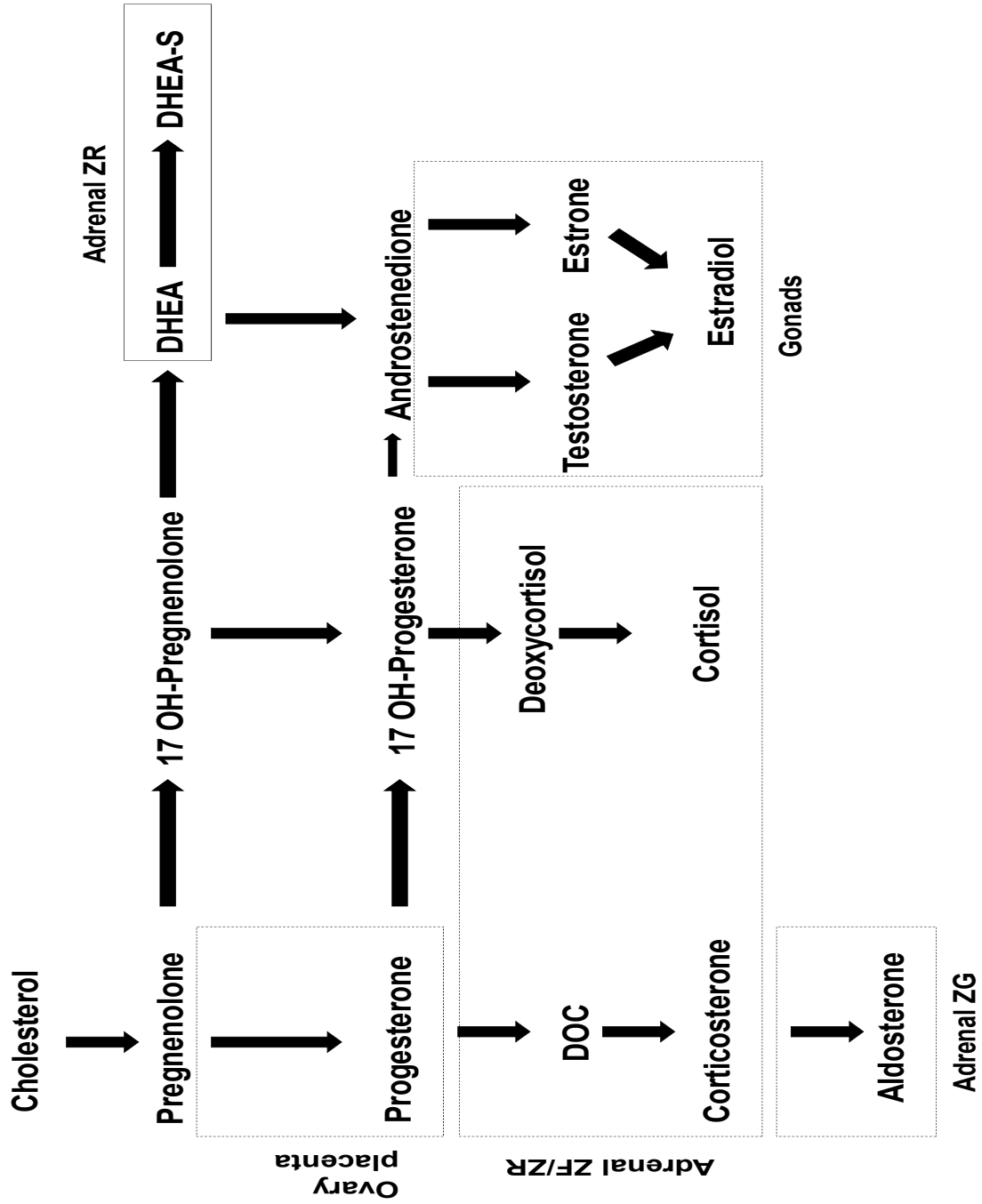
Cholesterol
Pregnenolone
17 OH-Pregnenolone
DHEA
DHEA-S
Adrenal ZR
Progesterone
17 OH-Progesterone
Androstenedione
Testosterone
Estrone
Estradiol
Gonads
Ovary
placenta
Deoxycortisol
Cortisol
DOC
Corticosterone
Adrenal ZF/ZR
Aldosterone
Adrenal ZG

Figure 6. Overview of steroidogenesis and the principal steroid hormones. Boxes indicate the major tissues for each reaction (adrenal glands, gonads and placenta). Abbreviations include DOC (deoxycorticosterone), DHEA (dehydroepiandrosterone), DHEA-S (DHEA sulfate), ZF (zona fasciculata), ZR (zona reticularis) and ZG (zona glomerulus). From Lavoie and King, 2009.

2.3.2. Cholesterol transport

In all mammals, cholesterol is an important sterol found in the cell membranes, which can be derived from the diet or be synthesized *de novo* (Bloch, 1952). It is well known that cholesterol is the starting point for biosynthesis of steroids, oxysterols and bile acids in mitochondria (Russell, 2003; Steck & Lange, 2010). Cholesterol is transported in the blood plasma to the target tissue as lipoproteins (Plosch*, et al.*, 2007). High density lipoproteins (HDL) and low density lipoproteins (LDL) are the principal carriers responsible for cholesterol transport. The relative contribution of HDL or LDL in cholesterol uptake varies among species and tissues. There are two known pathways for cholesterol absorption and import into the cell: a non-selective endocytic pathway and a selective absorption pathway. In the non-selective pathway, LDL are specifically bound and internalized via the LDL family receptors (LDLr); and the receptor fuses with the endosomal pathway for distribution of the lipoproteins (Murphy & Silavin, 1989; Argov*, et al.*, 2004). On the other hand, the selective pathway uses a specific receptor, the scavenger receptor class B member I (SCARB1) situated at the plasma membrane to bind both LDL and HDL (Hoekstra*, et al.*, 2009; Kolmakova*, et al.*, 2010). The selective pathway takes the cholesterol present in the lipoproteins, and it is transferred directly to the cell membrane without absorption of the lipoprotein particles (Argov*, et al.*, 2004; Steck & Lange, 2010). The mRNA expression of the respective receptors for LDLr and scavenger receptor class B member I (SCARB1)

was determined in bovine ovarian cells at different stages of follicular development (Argov*, et al.*, 2004).

Once inside the cell the cholesterol esters and triglycerides in the cell can be stored in lipid droplets, which are bound by a phospholipid membrane (Rone*, et al.*, 2009). The stored cholesterol esters present in the lipid droplets are converted to free cholesterol. In steroidogenic cells, upon hormonal stimulation there is increased cholesterol absorption through the plasma membrane; and cholesterol esters are hydrolyzed by lipases, principally hormone-sensitive lipase (HSL)(Kraemer & Shen, 2002). HSL interacts with various cholesterol-binding proteins to direct the cholesterol to the outer mitochondrial membrane, where the first step in the steroidogenic process takes place (Lavoie & King, 2009).

2.3.2.1. Importing cholesterol into the mitochondria

In the acute steroidogenic response, cells must deliver large amounts of cholesterol: from the cytoplasm to the outer mitochondrial membrane, and from there to the inner mitochondrial membrane. It is believed that two strategic pathways could be the responsible for the movement of cholesterol across aqueous cytoplasmic spaces (Soccio & Breslow, 2004; Rone*, et al.*, 2009). In the first "the vesicular pathway", the cholesterol is incorporated into vesicular membranes (e.g. lysosomes, endosomes, peroxisomes) which subsequently fuse with other membranes, delivering cholesterol into the inner mitochondrial membrane. However this route appears to be a minor pathway. On the other hand, in the

second route “the non-vesicular pathway”, cholesterol can be solubilized and transferred by cholesterol-binding proteins, through the cytosol to the mitochondria (Soccio & Breslow, 2004; Rone*, et al.*, 2009).

Several candidate proteins, including SNARE complexes, vimentin-intermediate filaments, nonspecific transporters like sterol carrier protein 2 (SCP2), voltage-dependent anion channel (VDCA) and a subfamily of lipid binding proteins referred to as STARD4, STARD5 and STARD6 have been proposed for the cytoplasmic cholesterol transfer to the outer membrane (Bogan & Hennebold, 2010). The transfer of cholesterol from the outer to the inner membrane is considered as a rate-limiting step in steroid formation; and until now the prototypical steroidogenic acute regulatory protein (STAR), is the principal molecule implicated in this process. Mutations in STAR protein cause a specific phenotype in intracellular cholesterol distribution (lipoid congenital adrenal hyperplasia)(Lin*, et al.*, 1995; Sugawara*, et al.*, 1995). Nevertheless studies suggest that STAR proteins presumably work in concert with the translocator protein (18 kDa, TSPO) and endozepine, which is the natural ligand for TSPO (Niswender, 2002; Papadopoulos*, et al.*, 2006; Rone*, et al.*, 2009). Figure 7 illustrates the trafficking of cholesterol to the mitochondria for steroidogenesis more precisely.

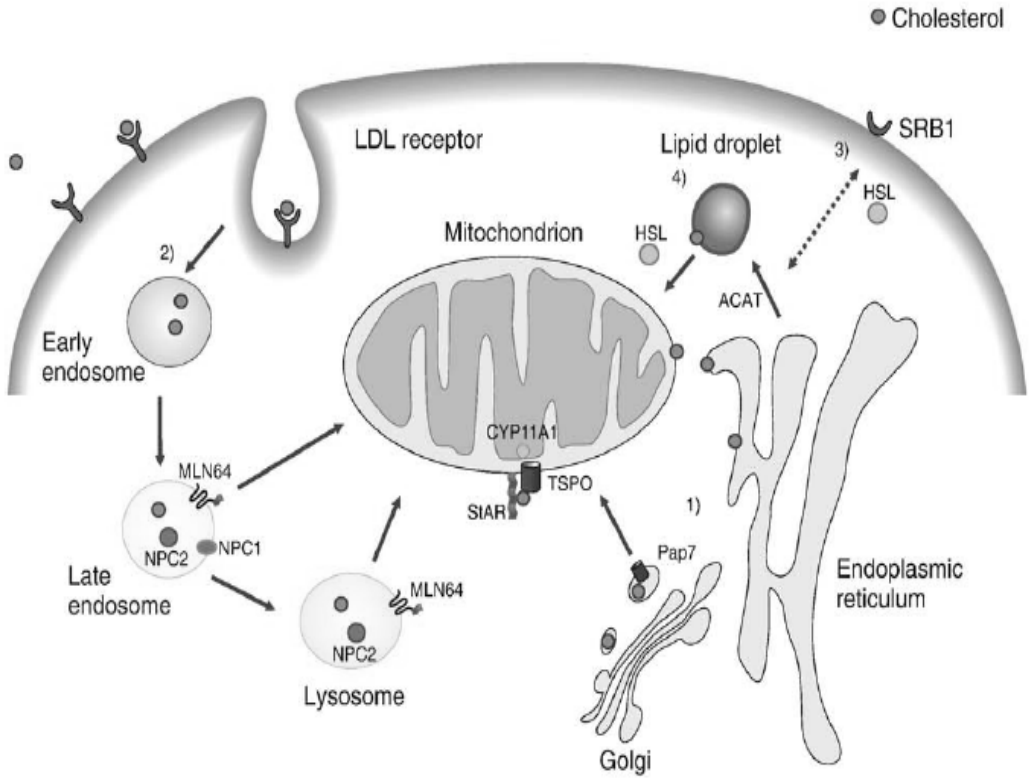

Figure 7. Uptake and transport of cholesterol to the mitochondria for steroidogenesis. Pathway 1 shows the passive diffusion of cholesterol from the endoplasmic reticulum to the mitochondria, via the acyl-CoA binding domain containing (PAP7 or ACBD3) protein. In pathway 2, low density lipoprotein (LDL),binds to the LDLr and cholesterol is trafficked through the endosomes to the mitochondria via the Niemann-Pick type C proteins (NPC) proteins and StAR-related lipid transfer (START) domain containing 3 (MLN64) protein. In pathway 3 cholesterol is transported from high density lipoprotein (HDL) to the plasma membrane by the SR-BI receptor. In pathway 3 and 4 hormone-sensitive lipase (HSL) converts esterified cholesterol from the plasma membrane or from the lipid droplets in free cholesterol. Finally free cholesterol interacts with lipid-binding proteins present in the cytosol for delivery to the mitochondria, where the enzyme cytochrome P450, family 11 subfamily, A polypeptide 1 (CYP11A1) transforms cholesterol into pregnenolone. Taken from (Rone*, et al.*, 2009).

2.3.2. Steroidogenic enzymes

The biosynthesis of steroids involves several enzymes, however all the steroid hormones are synthesized through a common precursor steroid, pregnenolone (Hu*, et al.*, 2010). In the inner mitochondrial membrane, the enzyme CYP11A1 converts the insoluble cholesterol into soluble pregnenolone, by the enzymatic cleavage of a 6-carbon side-chain of the 27-carbon cholesterol molecule (Ghayee & Auchus, 2007). Pregnenolone is next metabolized to progesterone by hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerases (HSD3B). HSD3B isoforms are membrane-bound enzymes, which are found in both mitochondrial and microsomal membranes, depending on the type of cell. Their enzymatic actions are essential to catalyze the conversion of intermediary metabolites into active steroid hormones (see figure 8). The variant isoforms of HSD3B are highly homologous in their amino acid sequence, and are specific for each species (Lavoie & King, 2009). In humans, two isoforms have been identified: HSD3B1 and HSD3B2, and both function as steroid dehydrogenase/isomerases and are involved in the biosynthesis of all active steroid hormones (Payne & Youngblood, 1995). Moreover, the isoforms of HSD3B are expressed in a cell and tissue-specific manner. HSD3B1 is expressed in placenta, skin and breast tissue, whereas HSD3B2 is expressed in adrenal gland, ovary, and testis (Payne & Youngblood, 1995; Pasqualini, 2005). In cows, HSD3B1 is present in ovary and placenta (Takagi*, et al.*, 2007; Vanselow*, et al.*, 2010).

Like HSD3Bs, the hydroxysteroid (17-beta) dehydrogenase enzymes (HSD17B) play essential roles in steroidogenesis, catalyzing the final step in the biosynthesis of active gonadal steroid hormones, estradiol, and testosterone (see figure 8). They are found as either membrane-bound or soluble enzymes and, unlike HSD3B, there is very little homology among different HSD17B isozymes (Payne & Youngblood, 1995). Among the different forms of human HSD17B, only types 1, 3 and 7 participate in the final step of biosynthesis of active steroid hormones in gonads (Payne & Youngblood, 1995). In cattle, Cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1) is expressed in ovary and trophoblast cells (Schuler*, et al.*, 2006; Luo*, et al.*, 2010; Mossa*, et al.*, 2010)

Further, pregnenolone can also be hydroxylated in certain tissues by CYP17A1 to 17-OH-pregnenolone to DHEA (Lavoie & King, 2009). The progesterone and 17-OH-pregnenolone are basic steroids, which can be used as intermediary metabolites for all other steroid hormones (see figures 6 and 8). Table 3 give a general view of the principal steroidogenic proteins and enzymes expressed in bovine.

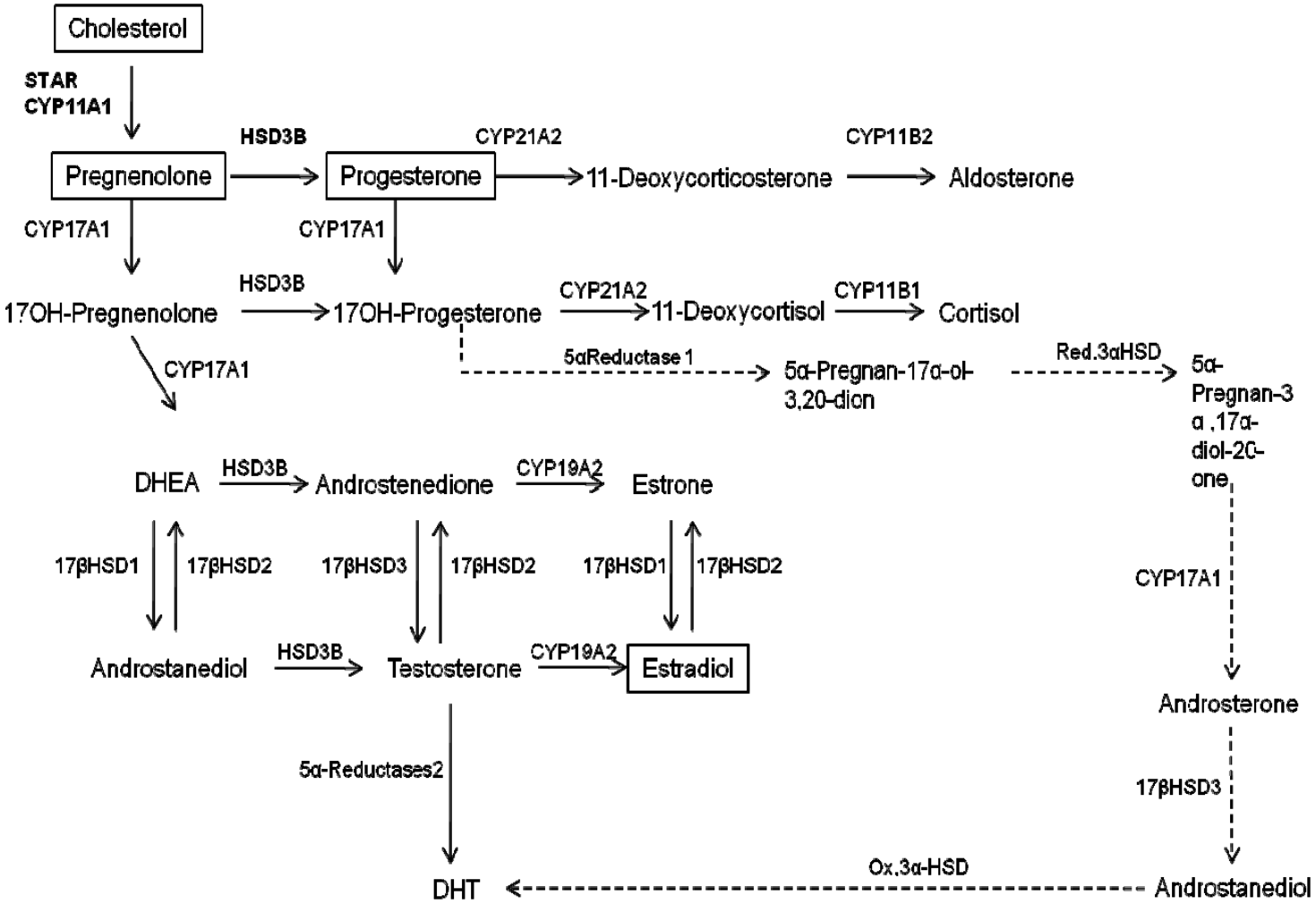
Cholesterol
STAR
CYP11A1
Pregnenolone
HSD3B
Progesterone
CYP21A2
11-Deoxycorticosterone
CYP11B2
Aldosterone
CYP17A1
CYP17A1
17OH-Pregnenolone
HSD3B
17OH-Progesterone
CYP21A2
11-Deoxycortisol
CYP11B1
Cortisol
CYP17A1
5αReductase 1
5α-Pregnan-17α-ol-3,20-dion
Red.3αHSD
5α-Pregnan-3α,17α-diol-20-one
DHEA
HSD3B
Androstenedione
CYP19A2
Estrone
17βHSD1
17βHSD2
17βHSD3
17βHSD2
17βHSD1
17βHSD2
CYP17A1
Androstanediol
HSD3B
Testosterone
CYP19A2
Estradiol
Androsterone
5α-Reductases2
17βHSD3
Ox.3α-HSD
DHT
Androstanediol

Figure 8. Conventional pathways and associated enzymes of steroid biosynthesis. Abbreviations include: DHEA dehydroepiandrosterone and DHT Dihydrotestosterone. Taken from Ghayee and Auchus, 2007 with some modifications.

Table 3. Principal steroidogenic proteins and genes expressed in a different number of bovine tissues and cells.

Protein or gene	Tissue or cells	Reference	Function
STAR			Delivery of cholesterol into the mitochondria.
Gene and protein	Adrenal medulla and cortex of adrenal gland Adrenal mitochondria Adrenal fasciculata cells Adrenocortical cells	(Nishikawa, *et al.*, 1996; Lo, *et al.*, 1998; Ivell, *et al.*, 2000; Yamazaki, *et al.*, 2006)	
Gene	Ovarian theca cells	(Bao, *et al.*, 1998; Ivell, *et al.*, 2000; Braw-Tal & Roth, 2005; Murayama, *et al.*, 2008)	
Gene	Non-luteinizing granulosa cells	(Sahmi, *et al.*, 2004)	
Gene and protein	Corpus luteum	(Hartung, *et al.*, 1995; Pescador, *et al.*, 1996; Mamluk, *et al.*, 1999)	
Gene and protein	Adult testes	(Pilon, *et al.*, 1997; Aspden, *et al.*, 1998)	
Gene and protein	Placenta (260-280 days of gestation)	(Pescador, *et al.*, 1996; Pilon, *et al.*, 1997)	
Gene	Placentomes during gestation and postpartum	(Takagi, *et al.*, 2007)	
CYP11A1			Conversion of cholesterol to pregnenolone
Gene	Non-luteinizing granulosa cells	(Sahmi, *et al.*, 2004)	
Gene	Ovarian theca cells	(Mamluk, *et al.*, 1999; Murayama, *et al.*, 2008)	
Gene and protein	Fetal adrenal gland	(Conley, *et al.*, 1992)	
Gene	Placentomes during gestation and postpartum	(Takagi, *et al.*, 2007)	
Gene and protein	Placenta (100-280 days of gestation)	(Conley, *et al.*, 1992)	
Gene and protein	Adult testes	(Aspden, *et al.*, 1998)	
HSD3B1			Conversion of pregnenolone to progesterone
Gene and protein	Fetal adrenal	(Conley, *et al.*, 1992)	

Gene	Non-luteinizing granulosa cells	(Sahmi, *et al.*, 2004)	
Gene	Theca cells	(Murayama, *et al.*, 2008)	
Enzymatic activity by substrate metabolism	Caruncles and cotyledons (120-280 days of gestation)	(Tsumagari, *et al.*, 1994)	
Gene and protein	Placenta (100-280 days of gestation)	(Conley, *et al.*, 1992)	
Gene	Placentomes during gestation and postpartum	(Takagi, *et al.*, 2007)	
Gene and protein	Adult testes	(Aspden, *et al.*, 1998)	
CYP17A1			Catalysed androgen synthesis
Gene and protein	Placentomes from pregnant (days 80-284), prepartal and parturient period.	(Conley, *et al.*, 1992; Schuler, *et al.*, 2006)	
Gene and protein	Fetal adrenal	(Conley, *et al.*, 1992)	
Gene and protein	Adult testes	(Aspden, *et al.*, 1998)	
Gene and protein	Adrenocortical cells	(McCarthy, *et al.*, 1983; Zuber, *et al.*, 1985)	
CYP19A1			Conversion of androgens into oestrogens
Gene	Granulosa cells	(Vanselow, *et al.*, 2001)	
Gene	Theca cells	(Luo, *et al.*, 2010)	
Gene and protein	Placentomes from pregnant (days 80-284), prepartal and parturient period.	(Schuler, *et al.*, 2006)	
SULT1E1			Sulfation of estrogens
Gene	Villi and caruncle (Day 25-250 of gestation)	(Ushizawa, *et al.*, 2007)	
Gene	Caruncular and cotyledonary tissue at parturition	(Hirayama, *et al.*, 2008)	
STS			Conversion of sulfoconjugated estrogens into estrogens
Gene	Caruncular and cotyledonary tissue at parturition	(Hirayama, *et al.*, 2008)	

HSD17B1 Gene	Granulosa cells	(Zheng*, et al.*, 2008)	Catalyzes the conversion of estrone to estradiol
HSD17B1 Gene	Granulosa cells	(Zheng*, et al.*, 2008)	Oxidizes or reduces estrogens and androgens

2.3.3. Estrogen sulfoconjugation

The activity of estrogens is regulated by sulfoconjugation that controls the availability of free estrogens. The sulfotransferase family 1E, estrogen-preferring, member 1 (SULT1E1) is a cytosolic enzyme with high affinity for estrogens, especially for estradiol; and its presence have been reported in testis, pregnant uterus and placenta of murine (Qian*, et al.*, 2001; Takehara*, et al.*, 2001; Luu-The*, et al.*, 2005; Pasqualini, 2005). SULT1E1 catalyzes the sulfation of estrogens at the 3-hydroxyl position, changing them from hydrophobic to hydrophilic molecules, and the estrone sulfate (E1S) is the principal inactive form of estrogen catalyzed by this enzyme (Song, 2001). Sulfation inhibits the binding of steroids to their nuclear receptors, and these conjugates represent a local reservoir of the hormone after hydrolysis. E_1Ss may serve as substrates for the formation of biologically active estrogens via the action of steroid sulfatase (STS) (Reed*, et al.*, 2005). STS is a member of sulfatase enzymes, and has been found in steroidogenic (testis, ovary, adrenal glands, endometrium, brain and placenta), non-steroidogenic tissues, and as well in physiological processes and pathological conditions (Reed*, et al.*, 2005; Suzuki*, et al.*, 2007). STS is responsible for the hydrolysis of estrone sulfate and

dehydroepiandrosterone sulfate to estrone and dehydroepiandrosterone respectively; both of which subsequently can be converted in active steroids (estradiol and androstenediol) (Reed*, et al.*, 2005). In conclusion, it seems that SULT1E1 and STS work together regulating the activity and the availability of active and inactive estrogens.

2.3.4. Regulation of steroidogenesis and principal steroidogenic pathways

The acute and chronic production of steroids is controlled principally by trophic hormones (LH, FSH or ACTH) and occurs in the order of minutes and hours (Stocco*, et al.*, 2005). The principal trophic hormones that stimulate steroidogenesis are: the adrenocorticotropic hormone (ACTH) in adrenocortical and adrenal fasciculata cells, and LH and follicle-stimulating hormone (FSH) in gonads (Nishikawa*, et al.*, 1996; Sekar*, et al.*, 2000; Rekawiecki*, et al.*, 2005; Wang*, et al.*, 2009).

During acute hormonal stimulation, a rapid mobilization and transport of cholesterol reserves of cholesterol to mitochondria is followed by a rapid synthesis of new steroids by abovementioned steroidogenic proteins and enzymes (Stocco, 2000). Trophic hormones bind to their respective G protein coupled receptors, thus leading, in most cases, to activation of adenylate cyclase, which turn on the cyclic-AMP (cAMP) dependent protein kinase pathway, protein kinase A (PKA), and in the activation of steroidogenic proteins as STAR, CYP11A1, and HSD3B, among others, (Hu*, et al.*, 2010).

LH receptors in bovine CL are located principally in small luteal cells (Okuda*, et al.*, 1999). Binding of LH to its receptor, leads to cAMP dependent PKA pathway activation, and a subsequent increase in P4 production (Rekawiecki*, et al.*, 2008). Moreover LH stimulates Leydig cell testosterone synthesis via cAMP-PKA dependent pathway (Lavoie & King, 2009). In bovine and primate CLs, LH increases the expression and synthesis of the three principal steroidogenic markers (STAR, CYP11A1 and HSD3B) (Ravindranath*, et al.*, 1992; Rekawiecki*, et al.*, 2005). However extensive evidence indicates that trophic hormones can also trigger other second messenger systems. In this respect, in bovine adrenal fasciculata-reticularis cells, ACTH enhances expression of the STAR protein via the cAMP-dependent PKA and protein kinase C (PKC) signaling pathways (Nishikawa*, et al.*, 1996; Morley*, et al.*, 2000). Furthermore, it was reported that in fasciculata cells, calcium (Ca^{2+}) stimulates the expression of STAR, and it is essential for ACTH action on corticoidogenesis (Morley*, et al.*, 2000; Yamazaki*, et al.*, 2006).

It has been found that activation of extracellular-signal-regulated kinases (ERK)/mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinases (P13K) /Akt pathways, modulate the synthesis of steroids in bovine granulosa and theca cells (Tajima*, et al.*, 2005; Fukuda*, et al.*, 2009). Few studies have addressed the principal intracellular pathways implicated in the synthesis of steroidogenic genes and hormone synthesis. In primary cultures of human placental tissue and trophoblast cells, analogues of cAMP stimulate CYP11A1 mRNA and P4 production (Chung*, et al.*, 1986; Ringler*, et al.*, 1989). Conversely, CY11A1 gene regulation in rodent placenta is cAMP independent (Matt*, et al.*, 1986; Sher*, et al.*, 2007).

However, other hormones and factors can also stimulate secretion of steroids in gonads and adrenal glands. In this respect, Rekawiecki et al (2005), found a stimulatory effect of prostaglandin E(2) (PGE(2)) and progesterone on StAR, CYP11A1 and HSD3B1 gene expression in bovine luteal cells. Moreover, previous studies documented a significant role of Ca^{2+}, stimulating the expression of STAR in adrenal cells (Cherradi & Capponi, 1998; Yamazaki*, et al.*, 2006). In concert with the aforementioned factors, insulin or IGF-I in combination with LH regulated the STAR gene expression in porcine granulosa-luteal cells (Sekar*, et al.*, 2000). It was also demonstrated that MA-10 Leydig cells possess highly specific and biologically functional prolactin receptors (PRLR) mediating direct and dose-dependent biphasic effects of PRL on hCG-induced progesterone secretion (Weiss-Messer*, et al.*, 1996). In addition, activated macrophages synthesize pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) that are extremely inhibitory to Leydig cells and appear to act as transcriptional repressors of STAR gene expression (Hales, 2002).

Apparently, placental regulation of steroidogenic genes varies between tissues and species, reflecting in part the placental diversity and placental steroid function in viviparous animals.

2.4. Steroids synthesis during pregnancy

2.4.1. General aspects

During gestation, the steroid hormones, in particular progesterone and estrogens, have important roles in regulating key physiological events such as:

maternal recognition, endometrial receptivity, maintenance of pregnancy, development of glandular tissue and early embryonic development (Graham & Clarke, 1997). As mentioned before, steroidogenesis in the gonads and adrenal glands is predominantly mediated through the interaction of LH, FSH and ACTH. However, there is no report describing the principal intracellular pathways and factors implicated in the regulation of the steroidogenic process in the bovine placenta.

2.4.2. Cholestrol uptake in placenta

Cholesterol plays and essential role in mammalian embryonic development to sustain the rapid growth of the placental and fetal tissues, as well as substrate for steroidogenesis (Pepe & Albrecht, 1995; Plosch*, et al.*, 2007).

In human, de novo synthesis of cholesterol is limited, and cholesterol is obtained principally from plasma low density lipoprotein (Furuhashi*, et al.*, 1989). As we mentioned before (section 2.3.2.1) cholesterol delivery from LDL is mediated by receptor-mediated uptake. In this respect human placenta is able to bind and internalize maternal lipoproteins (Alsat*, et al.*, 1984; Alsat*, et al.*, 1985).

HDL and LDL receptors are expressed in placenta specially at the villous trophoblast and syncytiotrophoblast membrane of rodents and humans; suggesting that the presence of these receptors could serve as an efficient route for lipoproteins transfer from the maternal to foetal tissues (Alsat & Malassine, 1991; Bonet*, et al.*, 1995; Wadsack*, et al.*, 2003; Ethier-Chiasson*, et al.*, 2007; Watanabe*, et al.*, 2010). However the precise mechanism of the uptake of cholesterol in placenta

remains to be elucidated. Moreover the presence of the aforementioned lipoproteins receptors in bovine placenta has not been studied.

2.5. Steroids synthesis in bovine placenta

2.5.1. Placental production of progesterone and estrogens in cattle

In cattle, as in many mammalian species, the placenta produces large amounts of steroids, mainly P4, estrone (E1) and E2 (Hoffmann*, et al.*, 1997; Wood, 1999). But in contrast to other species, where the placenta adopts the role as the main source of progestagens, the CL is the principal source of P4 during gestation, and the placenta does supplement during the third trimester of gestation (Hoffmann & Schuler, 2002). Lutectomy or luteolysis in the cow before day 210 of gestation results in abortion (Day, 1977; Chew*, et al.*, 1979; Johnson*, et al.*, 1981). It would appear that the bovine feto-placental unit generates steroids that reach in the peripheral circulation during the bovine gestation (Gabai*, et al.*, 2004). However, concentrations of P4 in bovine placental tissue increase considerably within the fifth and sixth month of gestation, reaching maximal values in the cotyledons in the eighth and ninth month (Tsumagari*, et al.*, 1994). It must nevertheless be considered that, apart from acting as classical endocrine hormones, sex steroids may also function as autocrine or paracrine regulators, as has been demonstrated in testis and ovary (Zirkin, 1998; Schuler*, et al.*, 1999; Slomczynska & Wozniak, 2001). The presence of both estrogen and progesterone receptors in the caruncular and cotyledonary tissues suggest a functional role for local steroid production in the bovine placenta (Hoffmann & Schuler, 2002; Schuler*, et al.*, 2005).

In addition, there are numerous studies of bovine gestation detailing the synthesis of P4, E2 and E1 by TGC and UTC cell cultures (Reimers*, et al.*, 1985; Wango*, et al.*, 1991; Matamoros*, et al.*, 1994). P4 is synthesized by bovine maternal caruncular cells in a pattern where it was low or undetectable in the first trimester, but increased more than 10-fold in the second trimester of gestation (Shemesh*, et al.*, 1992).

Despite the fact that bovine placental cells produce steroid hormones, localization of most of the enzymes and non-enzymatic proteins that are required for the steroid hormone synthesis has not been well investigated. Studies so far are limited to demonstration of the presence of enzymes needed for the production of steroids (STAR, CYP11A1, CYP17A1, HSD3B1 and SULT1E1) in bovine trophoblast and in caruncular tissue (Nash*, et al.*, 1988; Tsumagari*, et al.*, 1994; Pescador*, et al.*, 1996; Hoffmann*, et al.*, 2001; Takagi*, et al.*, 2007; Schuler*, et al.*, 2008). Although the role of placental steroid hormones in the progression of gestation in ruminants remains indistinct, steroids are known to exert their effect primarily through their cognate nuclear receptors, estradiol receptor 1 (E2R1) and progesterone receptor (PGR), producing paracrine factors that could regulate proliferation and/or differentiated functions of glandular epithelium and, to some extent, endometrial luminal epithelium during pregnancy (Schuler*, et al.*, 2002; Spencer*, et al.*, 2004). Moreover, recent information indicates that placental progestagens could regulate caruncular development, while estrogens could serve as paracrine inducers of differentiation of the fetal trophoblast giant cells (Hoffmann & Schuler, 2002; Schuler*, et al.*, 2008). It was also suggested that bovine UTCs and TGCs exhibit

different steroidogenic capacities constituting a 'two-cell' organization for estrogen synthesis. The aforementioned is based on the fact that CYP17A1 expression appears to be quickly down-regulated and cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1) is up-regulated when UTCs enter the TGCs differentiation pathway (Schuler*, et al.*, 2006). However, most of the aforementioned studies were conducted in advanced gestations. Thus the question for the biological need of steroids production and the expression of steroidogenic factors during early bovine gestation still arises. What is more, the exact steroidogenic homing mechanism and the principal cells implicated in this process in the progression of bovine gestation are not clearly understood.

2.5.2. Bovine steroids and parturition

In addition to the role of P4 and E2 in the progression of gestation, steroids are key factors in the complex cascade of physiologic events in parturition. It is well documented that a considerable increase in E1 and E2, during the periparturient period are necessary in the labor and delivery process (Hoffmann*, et al.*, 1997; Wood, 1999; Senger, 2005). Progesterone promotes myometrial relaxation; and estrogens augment myometrial contractility and excitability (Mesiano & Welsh, 2007). Thus a balance between these two hormones it's pivotal in the parturition process. Limited information exist about the precise mechanism in the parturition process, however it seems very clear that the withdrawal of progesterone is a prerequisite for the uterus to proceed with contraction during parturition (Kindahl*, et al.*, 2004) (Figure 9).

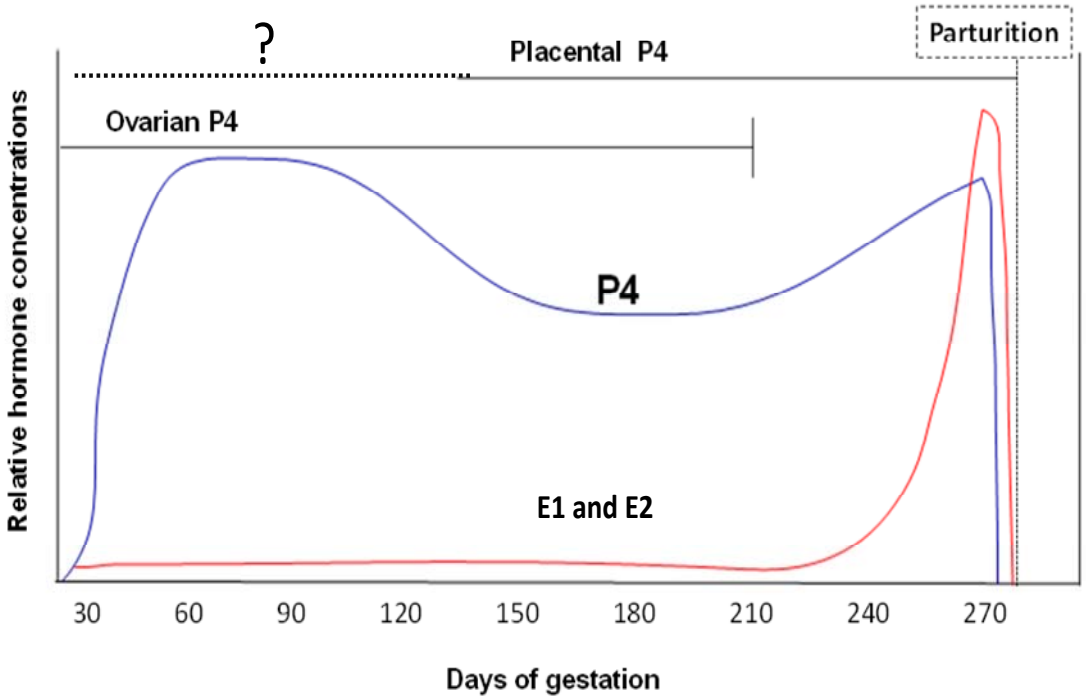

Figure 9. Estrone (E1), estradiol (E2) and progesterone (P4) profiles during gestation in the cow. Taken from Senger, 2005 with some modifications.

2.5.3. Estrogen sulfoconjugation in bovine placenta

To date, studies have demonstrated the presence of STS and SULT1E1 enzymes in bovine placenta (Brown*, et al.*, 1987; Nash*, et al.*, 1988; Janszen*, et al.*, 1995; Hoffmann*, et al.*, 2001; Ushizawa*, et al.*, 2007). It is well known that these enzymes play an essential role regulating the availability of free estrogens. In human placentas, estrogens are formed using fetal and maternal precursors; and most of them circulate in fetal compartment as sulfates. A substantial exchange between the placenta unconjugated estrogens (hydrolysis) and fetal estrogen sulfates in a constant way, has a great biological significance.

The estrogen sulfates may act as an estrogen store, yielding active hormones following hydrolysis; but also controlling the availability of free and active estrogens by the formation of estrogen sulfates (hormonally inactive) (Pasqualini, 2005). E1S has been shown to be a very important parameter of fetoplacental function and placental viability in bovine gestation. (Zhang*, et al.*, 1999; Isobe*, et al.*, 2003; Shah*, et al.*, 2007). The aforementioned is reinforced by the fact that E1S levels are correlated positively with calf birth weight and some placental characteristics (Zhang*, et al.*, 1999; Kindahl*, et al.*, 2004). Furthermore, perturbations in E1S levels may be related to dystocia, retained placenta and small stillborn calves (Isobe*, et al.*, 2003; Kornmatitsuk*, et al.*, 2003; Kindahl*, et al.*, 2004).

In the bovine placenta E1S is secreted in cotyledons, and can be detected as a major conjugated estrogen in the maternal circulation and milk (Zhang*, et al.*, 1999; Shah*, et al.*, 2007). Conversely, considerable STS activity has been previously detected in bovine caruncles (Greven*, et al.*, 2007). The distribution pattern of SULT1E1 and STS enzymes in bovine placenta, points to the existence of a local regulatory system tightly controlling the availability of free, active estrogens in bovine placentomes.

2.6. Calcium

2.6.1. General aspects

Every cell of the body is dependent on the presence of ionic Ca^{2+} for all cellular homeostasis and function and in a variety of structural and functional roles. Ca^{2+} absorption takes place in epithelia of different tissues, including the intestine, kidney, mammary glands, adrenal glands and placenta. The Ca^{2+} transport is regulated by a multifarious array of processes that are mediated by hormonal, developmental, and physiological factors (Khanal & Nemere, 2008). Ca^{2+} is required for skeletal formation, neuromuscular activities, and blood coagulation (Hershberger & Tuan, 1998).

2.6.2. Calcium and bovine placenta

During pregnancy Ca^{2+} absorption is enhanced, and the placenta transports calcium ions actively from mother to fetus against a concentration gradient, rendering the fetus relatively hypercalcemic (Pitkin, 1975; Pitkin, 1983; Pitkin, 1985). The requirements of the fetus for Ca^{2+} increase progressively throughout pregnancy and particularly in late gestation. In late gestation the sheep fetus requires 3 g of Ca^{2+} per day (Wooding*, et al.*, 1996). Thus, the placenta represents the principal site of regulation of fetal Ca^{2+} homeostasis. In the human placenta Ca^{2+} uptake into the trophoblast occurs across the maternal microvillous plasma membrane; and subsequently the Ca^{2+} is translocated from the trophoblast cells to the fetal basal plasma membrane, where it can now enter to the fetal circulation (Belkacemi*, et al.*, 2003). In addition, Ca^{2+} influx into the placenta apparently is mediated by a facilitated diffusion process employing Ca^{2+} binding proteins known as the calbindins (Ca^{2+}BPs) (Belkacemi*, et al.*, 2003). The principal Ca^{2+}BPs that have been reported were: a 57-kDa Ca^{2+}BP for mouse placenta; and a calbindin-D9k (Ca^{2+}BP9k) for human placenta (Hershberger & Tuan, 1998; Belkacemi*, et al.*, 2003). In this regard, one study demonstrated the expression of Ca^{2+}BP9k in the ovine, bovine and caprine placenta. Moreover this study detected the presence of this protein only in the columnar trophoblast cells in sheep and goat (Wooding*, et al.*, 1996). This supports the notion that Ca^{2+}BP9k mediates the high calcium flux by facilitated diffusion.

2.6.2.1. Calcium and placental steroidogenesis

Studies have pointed the Ca^{2+} as key mediator of placental steroid hormone synthesis by cyclic nucleotide independent mechanism (Shemesh*, et al.*, 1988; Weems*, et al.*, 2004). The ionophore A23187 is a Ca^{2+} analogue enhanced progesterone formation (Shemesh*, et al.*, 1988; Weems*, et al.*, 2004). Apparently Ca^{2+} affects mechanisms regulating bovine placental steroidogenesis by stimulating the enzyme CYP11A1 and the PKC pathway (Shalem*, et al.*, 1988). In ovine chorionic cells, modulation of P4 secretion is modulated by Ca^{2+}, calmodulin and protein kinase C (PKC) activators (de la Llosa-Hermier*, et al.*, 1991). Moreover in adrenal glands, Ca^{2+} appears to exert an effect at the intramitochondrial and nuclear level, where it activates transcript of steroidogenic acute regulatory protein (STAR)(Cherradi & Capponi, 1998). Furthermore in bovine adrenal fasciculata–reticularis cells, the dominant second messenger for the stimulation of STAR protein expression is Ca^{2+} (Yamazaki*, et al.*, 2006). However the exact mechanisms and factors involved in Ca^{2+} activatory and activation pathways in bovine placenta are still obscure.

2.7. SCNT pregnancies

2.7.1. General aspects

Over the last decade, a number of species, from farm animals to rodents, have been cloned using the SCNT technology by which the nucleus of a somatic or differentiated cell is introduced into an enucleated oocyte, and the reconstructed oocyte is artificially activated, allowing for nuclear reprogramming to occur. Then at the blastocyst stage, the cloned embryo is transferred to a surrogate mother, allowing the development of the reconstructed embryo into a genetic clone of the animal from which the donor cell was derived (Ross & Cibelli, 2010).

2.7.2. Bovine SCNT pregnancies

The several potential applications of bovine cloning include the generation of homogeneous animal models for experimental purposes, preservation of endangered breeds, multiplication of specific phenotypes, transgenic animals for therapeutic, bio-medicine or for improved animal production, and replication of valuable genitors (Heyman, 2005).

2.7.2.1. Poor efficiency and alterations

Although the most frequent species used for somatic cloning is the cow, the number of births per number of embryos transferred is still low (0-12%) (Wells*, et al.*, 1999; Wells*, et al.*, 2004; Panarace*, et al.*, 2007; Keefer, 2008). This poor efficiency is associated with pre- and postnatal anomalies, including atypical

vascularisation of extra embryonic tissue, abnormal placenta development, prolonged gestation, oversized calves, musculoskeletal deformities, respiratory distress, cardiopathology and abnormal kidneys as well as hypertrophic liver (Cibelli*, et al.*, 1998; Hill*, et al.*, 2000; Bertolini & Anderson, 2002; Chavatte-Palmer*, et al.*, 2002; Lee*, et al.*, 2004). However, a high rate of fetal loss occurs during peri-implantation period and between days 30 and 90 of gestation, which in most cases are associated with anomalies in feto-placental unit (Cibelli*, et al.*, 1998; Hill*, et al.*, 2000; Pace*, et al.*, 2002).

2.7.2.2. Abnormal placental and fetal development

Abnormal placentation in SCNT bovine gestations is often associated with poorly developed placentomes, hypertrophy and fewer cotyledons, increase in caruncular weight, severe edema of the intercotyledonary placental membranes and detachment of placental membranes (Hill*, et al.*, 2000; Hashizume*, et al.*, 2002; Edwards*, et al.*, 2003; Lee*, et al.*, 2004). In addition, many authors have reported abnormal vascularisation of extra-embryonic tissue, lack of placental vascular development, hydrallantois, edematous chorioallantoic membranes, reduced epithelium at the endometrial trophoblast interface, underdeveloped vasculature and a reduced number of TGC (Arnold*, et al.*, 2006; Constant*, et al.*, 2006; Miglino*, et al.*, 2007; Kohan-Ghadr*, et al.*, 2008; Palmieri*, et al.*, 2008).

2.7.2.3. Possible alterations in hormones and factors

Modifications in some factors that promote placental and vascular development have been suggested as possible causes of SCNT failures (Palmieri*, et al.*, 2008). Recent studies have found alterations in the expression of cytokines such as: IGF1, insulin-like growth factor binding protein 1 (IGFBP1) and transforming growth factor, beta 1 (TGFB1) in placenta from bovine SCNT pregnancies (Ravelich*, et al.*, 2004; Hwang*, et al.*, 2009). In this regard, it is well known that cytokines, in particular, members of the TGFB family and members of the IGF family are important factors in determining nutrient partitioning between the fetus and the placenta to promote fetal growth, cellular mitosis and differentiation, cell migration, as well as inhibition of apoptosis (Bauer*, et al.*, 1998; Han & Carter, 2000). In addition, some studies have reported alteration in some angiogenic factors as VEGFA, hypoxia inducible factor 1, alpha subunit (HIF1A), angiopoietin 1 (ANGPT1), placental growth factor (PGF) and fibroblast growth factor 2 (basic) (FGF2) in maternal and embryonic placental tissues from ruminant SCNT gestations (Hoffert-Goeres*, et al.*, 2007; Campos*, et al.*, 2010). Thus, variations in the expression of the aforementioned factors in cloned pregnancies could have a range of effects on fetal growth, placental development and placental neovascularization.

Moreover, Hwang et al. (2009) studying delayed parturition in SCNT pregnancies, detected decreased levels of E2; and elevated levels of TGFB1 and P4 in plasma from SCNT pregnancies compared to the control group. These authors

suggest that delayed parturition in clone calving may be caused by the dysfunction of placentomes and aberrant expressions of steroid hormones and cytokines. In addition to the systemic alterations of above-mentioned hormones, excess of estrogen sulfoconjugation has been found in SCNT during the pre-partum period; and apparently this hormonal imbalance contributes to the lack of overt signs of readiness for parturition in clone pregnancies (Hirayama*, et al.*, 2008).

2.7.2.4. Abnormal expression of genes and transcription factors

In general, many of the defects in cloned embryos are attributed to problems with the reprogramming of a nucleus derived from a differentiated cell. Reprogramming involves changes in the patterns of epigenetic marks, such as DNA methylation and histone modifications, and abnormal patterns of these modifications have been reported in cloned embryos (Dean*, et al.*, 2001; Dean*, et al.*, 2003; Shi*, et al.*, 2003; Yang*, et al.*, 2007). Many of these modifications occur in the later reprogramming, and could affect early placental growth and embryo development, through their influence on gene expression especially on imprinted genes. Consequently, long-term effects from gene modifications would manifest through the progression of gestation, parturition and so forth, in a variety of placental and fetal anomalies (Smith & Murphy, 2004; Jincho*, et al.*, 2008; Couldrey & Lee, 2010).

In this regard, a number of studies have undertaken to characterize abnormal imprinted gene expression in blastocysts and fetal and placental tissues

in SCNT gestations. These studies found altered abundance of achaete-scute complex-like protein 2 (mash2), heart and neural crest derivatives expressed 1 (HAND1), PAGs and PHLDA2, which are critical genes in trophoblast differentiation, proliferation and cell function, in bovine SCNT pregnancies (Arnold*, et al.*, 2006; Guillomot*, et al.*, 2010). Similarly, alteration in DNA methylation pattern on allelic expression of maternally imprinted gene small nuclear ribonucleoprotein polypeptide N (SNRPN) in preimplantation SCNT bovine embryos has been reported (Suzuki*, et al.*, 2009).

Although dysfunctional imprinted and non-imprinted genes may be associated with high rates of fetal loss and abnormal development of the fetus and placenta in SCNT pregnancies, deeper understanding of epigenetic reprogramming will be needed for outlining the molecular mechanisms underlying successful bovine cloning.

2.8. Problem, hypothesis and objectives

The placenta represents a unique organ of the feto-maternal unit and plays a pivotal role in the development and maintenance of pregnancy. In many animal species, including the cow, the placenta produces steroid hormones, principally estrogens, pregnenolone, progesterone and cortisol (Linzell & Heap, 1968; Challis & Patrick, 1981; Matamoros*, et al.*, 1994; Ben-Zimra*, et al.*, 2002; Lia*, et al.*, 2005). As mentioned in the literature review, steroid hormones, especially progesterone, are unequivocally required for establishment and maintenance of pregnancy, as well as maternal support of conceptus survival and development.

Studies *in vitro* have demonstrated that bovine placenta produces steroids, and also is a target for steroid action (Shemesh*, et al.*, 1992; Hoffmann & Schuler, 2002; Schuler*, et al.*, 2005; Schuler*, et al.*, 2008). However, the biological role of placental steroids and the principal target cells in bovine gestations remain unclear; so far very little is known about the onset of placental steroid production. Therefore, the objectives of the first chapter, here included were:

- Determine the occurrence of candidate proteins (STAR, CYP11A1 and HSD3B1), involved in bovine placental steroidogenesis.
- Identify the principal cells implicated in the steroidogenic protein expression through the first third of pregnancy.

The presence of progesterone and estrogen receptors in placenta clearly point to a role of placental steroids as local regulators of placental growth, differentiation and functions in cattle (Schuler*, et al.*, 1999; Schuler*, et al.*, 2002; Schuler*, et al.*, 2005). A reduced capacity to synthesize steroids, is not only associated with implantation failure and miscarriage but also may contribute to abnormal placental and fetal development (Day, 1977; Chew*, et al.*, 1979; Johnson*, et al.*, 1981; Mann*, et al.*, 1999; Daya, 2009).

We therefore hypothesize that placental failure in bovine cloned pregnancies may originate from dysfunction of placentomes as a result of aberrant expression of the principal steroidogenic proteins and genes. Thus, the objectives of the second chapter were:

- To characterize the patterns of expression of mRNA and protein of some of the main steroidogenic factors (*SCARB1, STAR, CYP11A1, HSD3B1, CYP17A1, STS and SULT1E1*) in SCNT and AI early bovine placentas.
- To evaluate the expression of key steroidogenic factors (SCARB1, CYP11A1 and CYP17A1) in early normal pregnancies

Finally, although the regulation of steroidogenic proteins and enzymes have been studied extensively in the gonads and adrenal glands (Nishikawa*, et al.*, 1996; Aspden*, et al.*, 1998; Ivell*, et al.*, 2000; Nishimura*, et al.*, 2004; Rekawiecki*, et al.*, 2005; Fukuda*, et al.*, 2009), further research is needed to better understand the principal intracellular pathways and factors implicated in the regulation of the steroidogenic process in bovine placenta. In view of this statement, the objectives of the third chapter were:

- To develop a bovine placenta explant model to study the synthesis of progesterone in the feto-maternal unit (placentome).
- To assess whether hCG and multiple second messenger systems (dbcAMP, PMA and calcium ionophore) regulate the synthesis of progesterone in bovine placenta.
- To investigate the influence of the aforementioned factors upon the expression of genes (*STAR*, *CYP11A1*, *HSD3B1*, *SULT1E1* and *STS*) implicated in the synthesis of steroid hormones in bovine placenta.

MATERIALS, METHODS AND RESULTS

CHAPTER 3. FIRST ARTICLE

Expression of steroidogenic proteins in bovine placenta during the first half of the gestation

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3.1. Abstract

The aim of the present study was to determine the occurrence and localization of the principal steroidogenic proteins in bovine placenta from day 50 to day 120 of pregnancy. Immunohistochemistry revealed that, at all stages investigated, bovine steroidogenic acute regulatory protein (STAR) and cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1) proteins were found in the chorionic plate (CP) and in the feto-maternal interdigitations: chorionic villi (CV) and maternal septum (MS). Moreover, uninucleated trophoblast cells (UTCs), stromal cells from chorionic villous tree, caruncular epithelial cells (CECs) and caruncular stromal cells (CSCs) were the principal cells detected by both markers. Immunoblot results showed a complementary pattern of STAR protein expression between caruncles and cotyledons. The protein level of hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1) were higher on the caruncle. In contrast *HSD3B1* mRNA expression was more abundant in cotyledon. Thus suggest the existence of *HSD3B1* posttranscriptional regulation. The protein results indicate that caruncular tissue expresses all three steroidogenic markers, suggesting a greater steroidogenic capacity than cotyledons. The variation in expression of steroidogenic proteins between caruncular and cotyledon tissue across gestation in a complementary pattern, indicates that placental steroidogenesis requires cell to cell communication between the maternal and fetal cells.

3.2. Introduction

Proper progression of feto-maternal developmental interactions is critical for positive signalling of maternal recognition of pregnancy, while appropriate placental development is key to maintenance of successful gestation. In many species, the placenta produces steroid hormones, principally estrogens, pregnenolone, progesterone and cortisol, and these play important functions in the establishment and maintenance of pregnancy (Linzell and Heap 1968; Challis and Patrick 1981; Matamoros, Caamano et al. 1994; Ben-Zimra, Koler et al. 2002; Lia, Isomaaa et al. 2005). The contribution of the placenta to gestation varies among species. In swine (Nara, Darmadja et al. 1981) and mink (Douglas, Song et al. 1997) the ovaries are entirely responsible of the steroid support of gestation. In sheep, successful pregnancy and parturition can occur in spite of ovariectomy performed as early as 50 days into gestation (Weems, Vincent et al. 1992; Campbell, Hallford et al. 1994). Studies in pregnant cows have shown that the corpus luteum is not required during the final 70 days of the pregnancy (Day 1977; Chew, Erb et al. 1979). These studies indicate that, in ruminants, placental steroid synthesis contributes to, and, in some cases, can support gestation. It has previously been demonstrated that the bovine placenta produces steroids, and is a target for steroid action, based on the presence of progesterone and estrogen receptors (Shemesh, Izhar et al. 1992; Hoffmann and Schuler 2002; Schuler, Teichmann et al. 2005; Schuler, Greven et al. 2008). Further, it would appear that the bovine feto-placental unit generates steroids that reach the peripheral circulation during gestation

(Gabai, Marinelli et al. 2004). Schuler et al. (2008) have proposed that placental progestagens regulate caruncular development, while estrogens serve as paracrine inducers of differentiation of the fetal trophoblast giant cells.

Steroid hormone synthesis requires a cascade of enzymes and some non-enzymatic proteins. An early step is the transfer of cholesterol into the mitochondrion, and the principal agent that has been implicated in this process is STAR (Miller 2007). STAR is a mitochondrial protein that is rapidly synthesized in response to steroidogenic stimulation. It has been found in most of steroidogenic tissues, including the adrenal cortex, the gonads, and the placenta as well as in the brain of several species (Pescador, Soumano et al. 1996; Pilon, Daneau et al. 1997; Nicol, Wang et al. 1998; Arensburg, Payne et al. 1999; Kim, Kang et al. 2004). The mechanism of action of STAR is not entirely understood (Manna, Dyson et al. 2009) but it appears that STAR interacts with other molecules such as the sterol carrier protein 2 (SCP2), steroidogenic activator polypeptide (SAP) and the outer mitochondrial membrane translocator protein (TSPO), to mediate the transfer of cholesterol from the outer to the inner mitochrondrial membrane (Liu, Rone et al. 2006). The enzyme, CYP11A1, is located on the matrix side of the inner mitochondrial membrane, and catalyzes the conversion of STAR-delivered cholesterol to pregnenolone by cleavage of a six carbon unit from 27-carbon cholesterol molecule (Simpson and Boyd 1966; Stocco 2000). Subsequently, pregnenolone escapes to the cytoplasm where it is converted to progesterone by the enzyme HSD3B1 in the microsomes (Pasqualini 2005).

In ruminant species, the placental structure comprises numerous placentomes in which the trophoblast-derived cotyledon is in apposition to endometrial regions known as the maternal caruncles (Wooding 1992). While it is essentially an epitheliochorial placenta, there is trophoblastic invasion in the form of specialized trophoblast giant cells (TGCs) that inhabit the caruncle (Wooding 1992). These cells are well known for the synthesis of placenta-associated glycoproteins (Hashizume, Ushizawa et al. 2007) and some studies have suggested that they produce placental lactogens and progesterone, contributing to fetal and placental development (Duello 2005). During the early phase of their formation, these TGCs express HSD3B1, but this appears to be extinguished during the latter stages of differentiation (Schuler, Greven et al. 2008)

Previous studies demonstrated that transcripts for *STAR*, *HSD3B1* and *CYP11A1* are present in both caruncle and cotyledon of the bovine placenta, beginning during the first three months of gestation (Takagi, Yamamoto et al. 2007). Thus, it would appear that both components of the ruminant placentome participate in steroidogenesis. Given that the absence of information on the localization of steroid synthesis, and the enigmatic role of the TGCs in the bovine placenta, the aim of this study was to determine the occurrence of candidate proteins (STAR, CYP11A1 and HSD3B1), involved in bovine placental steroidogenesis and to identify the principal cells implicated in the steroidogenic protein expression through the first half of pregnancy.

3.3. Materials and Methods

Sample collection and fixation

Bos taurus bovine placentas were collected at a local slaughterhouse, with four representative samples from each of 50, 70, 90 and 120 days of gestation. Gestational age was determined by fetal crown-rump length (CRL). For the period Day 50 to Day 70, and the period Day 90 to Day 120 of gestation, estimations were calculated based on the Pace et al (2002), and Evans and Sack (1973) developmental charts respectively. From each placenta, three placentomes were collected from the mid-region of the uterine horn that contained the fetus, and were dissected into caruncle and cotyledon and stored at -80°C until analysis. For RNA extraction, the tissues were stabilized with RNA-*later* (Qiagen, Mississauga, ON, Canada) before storage. Entire placentomes were fixed in Bouin's solution for 24 hours. Fixative was then removed and tissues were processed and embedded in paraffin and cut at 7 μm for protein localization studies.

Western blot analysis

For the preparation of tissue homogenates, small pieces of frozen caruncular and cotyledon tissues were powdered individually with pestle and mortar. The samples were homogenized in sonication buffer (20 mM Tris, 50 mM EDTA, 0.1 mM diethyldithiocarbamate (DEDTC), pH8.0; Invitrogen Life Technologies Corporation, Burlington, ON, Canada and Sigma-Aldrich, Oakville, ON, Canada), containing 32 mM octyl glucoside and protease inhibitor (Complete mini, EDTA-free, Roche, Mannheim, Germany). Mitochondrial and cytosolic proteins were obtained by

differential centrifugation (600g for 30 min, then the resultant supernatant was re-centrifuged at 9000g for 30 min)(Pescador, Soumano et al. 1996). The protein concentration was measured by a Bradford protein assay (Bio-Rad Laboratories, California, USA), adjusting the samples to 70 µg of protein. The proteins were then diluted in sample buffer containing 25 mM Tris (pH 6.8), 1 % SDS, 5 % mercaptoethanol, 1 mM EDTA, 4% glycerol, and 0.01% bromophenol blue (Invitrogen and Sigma-Aldrich). The samples were boiled for five minutes, and then subjected to electrophoresis on a 9% and 12% SDS-PAGE gels. The low range molecular weight marker (Amersham Biosciences, UK Limited) was used as reference.

Samples were electroblotted to nitrocellulose membranes (Bio-Rad, Richmond Canada), and the membranes were then blocked with 4% nonfat dry milk in Tris Buffered Saline (TBS) buffer containing 0.5% Tween 20 and incubated with their respective first antibody overnight. The first and second antibodies were diluted in TBS, at dilutions shown in Table 1. The second antibody peroxidase-conjugated Affini Pure Goat anti-rabbit IgG (Jackson Immuno Research Laboratories West Grove, PA, USA), was used at 1:10000 dilution. Mitochondrial and cytosolic proteins from different steroidogenic tissues (mouse adrenal glands and bovine corpus luteum) and non-steroidogenic organs (bovine lung) were used as positive and negative controls respectively to validate the system (Fig.1).

Immunohistochemical staining procedures

An indirect immunoperoxidase staining streptavidin-biotin method (Vectastain Elite ABC Kit PK6101, Vector Laboratories, Burlington ON, Canada) and the immunofluorescence techniques were used for the detection of the three steroidogenic markers (STAR, CYP11A1and HSD3B1). Tissue slides were heated in 10 mM citrate buffer (Fisher Scientific, Ottawa, ON, Canada) for 5 min for antigen retrieval. The sections were blocked with 10% goat serum overnight. The slides were then incubated for 12 h with primary antibodies for the three steroidogenic proteins at the concentrations described in Table 1. For immunoperoxidase staining, the slides were incubated with biotinylated secondary antibody (goat anti-rabbit IgG biotinylated 1:100) and avidin-biotin complex following the manufacturer's instructions (Vector Laboratories). Slides were then incubated with diaminobenzidine (DAB; Sigma-Aldrich) for 40 sec, and counterstained with haematoxylin (Fisher Scientific), for 1 min. For immunofluorescence, the slides were incubated with an appropriate second antibody diluted at 1:1500 (anti-rabbit IgG Alexa 488, Invitrogen, California USA), the nuclei were stained with bis benzimide trihydrochloride (Hoechst, 33342 Sigma-Aldrich). We performed double fluorescence immunolabeling on placentome slides to identify the trophoblast giant cells, using and monoclonal SBU3 antigen antibody, with specificity for the placenta associated glycoproteins produced by the sheep binucleate trophoblast cells (Lee, Gogolin-Ewens et al. 1985; Lee, Ralph et al. 1990). A fluorescein (FITC)-conjugated goat anti-mouse IgG, (Jackson Immuno Research

Laboratories) was used for the double immunofluorescence staining. First and second antibodies were prepared in PBS buffer; dilutions for first antibodies are shown in Table 1. Serum from a non-immunized rabbit or mouse was used as a negative control. For the assessment of immunostaining of the three steroidogenic proteins, regions within the placentome were defined as shown in Figure 2.

Messenger RNA isolation and cDNA synthesis

Total RNA was extracted from 30 mg of caruncular and cotyledon tissues, using an RNeasy Mini Kit (Qiagen Mississauga, ON, Canada). Disruption and homogenization of tissue were achieved with mortar and pestle. Proteinase K digestion (Invitrogen) was used to remove the proteins which could interfere with the RNA isolation. DNase I treatment was applied on the columns to eliminate any contaminating DNA (RNase-free DNase Set, Qiagen). Poly A^+ mRNA was isolated from total RNA using the Oligotex kit (Qiagen). The mRNA extracted was quantified by measuring the absorbance at 260 nm (A260), and mRNA purity was determined by the ratio of 260/280 using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). For each sample, 1 µg of mRNA was reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen).

Quantitative PCR (qPCR)

The transcript abundance for *STAR, CYP11A1, HSD3B1, GAPDH* and actin, beta (*ACTB*) was quantified by qPCR using the 7300 Real Time PCR thermal cycler

(Applied Biosystem). Sequences of the primer pairs and expected product sizes are shown in Table 2.

Bovine *STAR*, *CYP11A1* and *HSD3B1* primers were designed from published bovine sequences (Nishimura, Sakumoto et al. 2006; Orisaka, Tajima et al. 2006; Murayama, Miyazaki et al. 2008). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* and *ACTB* primers were designed based on bovine gene sequences from GenBank, and using the Primer Express software Version 3.0 (PE Applied Biosystem). qPCR products from *GAPDH* and *ACTB* were subcloned into a pDrive cloning vector (Qiagen), and amplified in competent bacteria of XL1-Blue strain. Plasmids were isolated with a QIAprep Spin Miniprep kit (Qiagen) and sequenced (The McGill University and Génome Québec Innovation Centre, Montreal, Qc) to verify that the cDNA amplified fragments corresponded to the target sequences. Primer concentrations were optimized according to the manufacturer's recommendations and dissociation curve analysis and agarose gel electrophoresis were used to verify the specificity of amplified products. The PCR efficiency (E= 1.9-2.0) and linearity of each primer were tested by preparing a series of solutions of the same stock of cDNA used for the qPCR gene analysis. The geometric mean of two reference genes (*GAPDH* and *ACTB*) was used as a normalization factor. Relative quantification method ($\Delta\Delta C_t$) was used to determine changes in target gene expression levels (Livak and Schmittgen 2001; Pfaffl 2001). Data analysis was performed using the Sequence Detection Software version 1.4 (PE Applied Biosystems) and the LinRegPCR Software version 11.1 (Heart Failure Research

Center Academic Medical Centre Amsterdam, the Netherlands). Reactions for qPCR were conducted in sealed 96-well (SARSTEDT INC, Montreal, QC Canada) in triplicate. The total volume of the qPCR reaction was 20 µL, comprising 6 µL cDNA template (diluted 1 in 15 in distilled water), 2 µL each forward and reverse primers and 10 µl Power SYBR green PCR master mix (Applied Biosystems,). Cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C.

Statistical analyses

The optical density of the protein bands was quantified by ImageJ 1.31v software (Wayne Rasband, National Institutes of Health, USA). For STAR, CYP11A1 and HSD3B1 protein expression, optical density data were log-transformed to reduce heterogeneity of variance, and obtain normality of distribution (Shapiro-Wilk, normality test). For qPCR, *STAR* and *CYP11A1* relative gene expression had a normal distribution. In addition, *HSD3B1* expression was log-transformed to achieve normality.

Differences in mRNA and protein relative expression between the groups were statistically evaluated by a two-way analysis of variance, followed by all pairwise multiple comparison procedures (Holm-Sidak method). All statistical analysis was conducted with SigmaPlot (version 11.2, Systat software, Incorporation). Data are expressed as the mean $\pm$ standard error (SE), and values of $P \leq 0.05$ were considered significant.

3.4. Results

Expression of STAR in caruncular and cotyledonary tissues

By Western blot, the polyclonal anti-STAR antiserum identified a single band of 30 kDa in caruncle and cotyledon at all time points studied through pregnancy (Fig. 3 panel A). In caruncles, there was significant reduction in STAR protein around day 70 of gestation compared to other time points (Fig. 3 panel A). Moreover, significant differences were found in cotyledonary tissues between days 50 and 90, compared with days 70 and 120 (Fig. 3 panel B). Significant differences in STAR protein abundance were found between caruncles and cotyledons at 50, 70 and 90 days of gestation, showing a complementary pattern of expression between maternal and fetal components of the placenta (Fig. 3 panel B) such that when cotyledonary expression of STAR was diminished, the abundance of STAR in the caruncle increased and vice versa, suggesting a regulatory mechanism to maintain an optimal level of steroid output by the placenta.

No significant differences were found in the relative abundance of *STAR* transcripts in or between caruncles and cotyledons during the first third of gestation (Fig. 4A). However the mRNA expression between caruncles and cotyledons at all studied periods reflected the same pattern of protein expression between the aforementioned tissues.

Immunohistochemistry revealed that, at all periods investigated, bovine STAR protein staining was mostly restricted to the materno-fetal interdigitations with emphasis in the maternal septum. Occasionally, moderate staining was

observed in the chorionic plate and in the trophoblast of the chorionic villous tree (Fig.5A-B). Cytoplasmic STAR protein expression was clearly detected in the UTCs, stromal cells from chorionic villus tree, CECs and CSCs (Fig. 5 D and G). Expression was never detected in trophoblast giant cells (Fig.5 G-I), suggesting that, at least in the first third of pregnancy, the TGCs cells do not participate in the steroidogenic process.

Expression of CYP11A1 in caruncular and cotyledonary tissues

A 50 kDa band corresponding to the CYP11A1 protein was detected by WB in caruncular and cotyledonary tissues in samples taken during the first half of gestation (Fig. 3 panel A). The abundance of CYP11A1 protein in caruncles was significantly increased at day 90 of pregnancy, compared to the remainder of the times sampled (Fig. 3 panel A and B). In cotyledons, significant differences between 50 and 120 days of gestation were found, showing a decrease in protein abundance as the gestation progress (Fig. 3 panel B). Greatest differences in CYP11A1 protein abundance were found between cotyledon and caruncle at day 50 (Fig.3 panel B). The *CYP11A1* mRNA expression levels showed the same pattern of protein expression at day 70 and 90 of gestation. However no significant mRNA differences were found at any investigated period (Fig. 4B).

CYP11A1 was localized principally in CP, fetal trophoblast and in the interdigitation area: CV and MS (Fig. 6A and B). As with STAR protein, the principal cells that stained positive for CYP11A1 at all periods were the UTCs, stromal cells

from chorionic villus tree, and CSCs (Fig. 5D and G). No CYP11A1 was detected in TGCs at any time studied (Fig. 6 G-I).

Expression of HSD3B1 in caruncular and cotyledonary tissues

By western blot, a band migrating at approximately 42 kDa protein, corresponding to the HSD3B1 protein was identified only in caruncular tissues (Fig. 3 panel A). Statistical analysis revealed HSD3B1 protein abundance in this tissue was significantly more important in the maternal component of the placenta at day 50, compared with day 120 of pregnancy (Fig. 3 panel B).

In contrast to the protein expression pattern, the *HSD3B1* mRNA abundance in cotyledons tended to be higher than in caruncles. However, significant differences between both tissues were found only at day 70 and 120 of gestation ($P \leq .05$). (Fig.4C). *HSD3B1* mRNA abundance in cotyledonary tissue at 70 days of gestation was significantly higher than the rest of the periods in the same tissue ($P \leq .05$). On the other hand, no differences between periods in caruncular tissue were found (Fig. 4C). Differences found between protein expression patterns and mRNA expression could be related to posttranscriptional *HSD3B1* regulation.

By immunohistochemistry, the HSD3B1 protein staining was strongest on the maternal side, where it was found in the feto-maternal interdigitations, predominantly in the maternal septum and the caruncular epithelium (Fig. 7 A and B). On the cotyledonary side, expression was localized in UTCs, and the TGCs exhibited no evidence of HSD3B1 expression (Fig. 7 D-I).

3.5. Discussion

Studies *in vitro* and *in vivo* have demonstrated that the bovine placenta contains the enzymes and proteins to produce steroid hormones in late gestation and at parturition (Evans and Wagner 1981; Gross and Williams 1988; Pescador, Soumano et al. 1996; Pilon, Daneau et al. 1997; Takagi, Yamamoto et al. 2007). Moreover the fact that bovine pregnancy can survive luteolysis and ovariectomy during the last 70 days of gestation (Day 1977; Chew, Erb et al. 1979), indicates that the placenta can provide the steroid support required for the maintenance of gestation. A functional role for local steroid production in the bovine placenta has been demonstrated by the presence of both estrogen and progesterone receptors in the maternal caruncle (Hoffmann and Schuler 2002).

This study provides new information on the cellular origin of bovine placental steroids. It appears that the sites of synthesis of steroids differ from other species in which placental steroidogenesis occurs. In rodents, steroidogenic mRNA markers, including STAR, CYP11A1 and HSD3B1 are expressed in the trophoblast giant cells, while HSD3B1 appears transiently in the maternal decidual (differentiated endometrial stromal) cells (Arensburg, Payne et al. 1999). In primates, steroid synthesis in the placenta appears restricted to the syncytiotrophoblast (Albrecht and Pepe 1999). Results of the present study indicated that in cattle, both the maternal and fetal tissues contribute to hormone synthesis through early gestation. Our results confirm the results from a previous study where transcripts for *STAR*, *HSD3B1* and *CYP11A1* are present in both

caruncular and cotyledonary tissues of the bovine placenta through gestation (Takagi, Yamamoto et al. 2007)

Protein and mRNA of STAR and CYP11A1 were found in caruncles and cotyledons from 50 to 120 days of gestation. HSD3B1 protein expression was only found in caruncles; in contrast the transcript was found in both caruncular and cotyledonary tissues. In the present study the mRNA expression of *HSD3B1* in the caruncle was observed to be significantly reduced compared to cotyledonary tissues. Similarly Takagi et al (Takagi, Yamamoto et al. 2007), observed similar expression patterns for *HSD3B1* transcript in bovine placenta during gestation and postpartum.

The discordance between the mRNA and protein expressions of this enzyme implies the existence of posttranscriptional regulation on *HSD3B1* gene expression in cotyledonary tissue. Such posttranscriptional regulation could indicate a potential regulation of HSD3B1 by RNA binding proteins from the transcription to the translation of this enzyme.

Similar observations were reported for *CYP11A1*, *CYP17A* and *HSD3B1* genes in neonatal pig testes, where mRNA levels of these enzymes was significant increased, but protein levels were lower (Choi, Kim et al. 2009). However the underlying mechanism of this modulation is unknown.

In agreement with earlier reports (Conley, Head et al. 1992; Izhar, Pasmanik et al. 1992; Takagi, Yamamoto et al. 2007), in the present study, the

CYP11A1 protein and mRNA expression remained relatively constant through gestation. In situ analysis concurs in demonstrating *CYP11A1* and *HSD3B1* transcripts in bovine trophoblast cells (Schuler, Greven et al. 2008).

Protein analysis by immunoblotting and immunohistochemistry confirmed the presence of all three steroidogenic proteins on the maternal side. The signal for HSD3B1 on the fetal side is apparently low in tissue sections and below the level of detection by immunoblotting procedure. The paucity of this important steroidogenic enzyme on the fetal side may indicate that progesterone and other downstream steroids are synthesized primarily by the maternal caruncle during early gestation. This notwithstanding, Conley and Ford (1987) demonstrated that, *in vitro*, isolated cotyledonary and caruncular components from bovine placentomes, taken at day 200 of gestation, both produce progesterone from pregnenolone substrate. Furthermore this conversion was abrogated by a HSD3B1 inhibitor. Whether this is a phenomenon of later pregnancy awaits further investigation.

In the ruminant placenta, 20 or more percent of the trophoblast cells are in the binucleate population (Wooding, Morgan et al. 1996). These cells are derived from the mononucleate trophoblast population by a process that includes acytokinetic mitosis (Wimsatt 1951) and endoreduplication such that they can contain a complement of DNA up to 32n (Klisch, Pfarrer et al. 1999). It has been reported that these cells migrate throughout gestation into the maternal endometrial epithelium, where some fuse, resulting in trinucleate feto-maternal

cells (Klisch, Pfarrer et al. 1999). More is known about their structure than their function. They synthesize and secrete the pregnancy associated glycoproteins (PAGs), and placental lactogen (Beckers, Zarrouk et al. 1998). The results of the present study indicate that, in the samples tested during the first third of gestation, the binucleate cells do not express any of the three proteins required for the initiation of steroidogenesis and for progesterone synthesis. In this context, by immune electron microscopy, Ben-David and Shemesh (1990) localized the CYP11A1 in the mitochondria of caruncular epithelial cells and the uninucleate trophoblast cells, but not in trophoblast giant cells during bovine midgestation. Moreover, cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1) has been detected exclusively in uninucleate trophoblast cells, where it was immediately down-regulated when these cells entered the trophoblast giant cell differentiation pathway between days 90-150 (Schuler, Ozalp et al. 2006). *In situ* analysis (Schuler, Greven et al. 2008) demonstrated the presence of *HSD3B1* transcripts in differentiating but not in mature bovine placental binucleate cells. It is believed that binucleate cells continue to differentiate from uninuclear trophoblast cells through gestation (Klisch, Hecht et al. 1999; Klisch, Pfarrer et al. 1999). If the HSD3B1 protein were present, it should have been visible in binucleate cells in our studies. As noted above, while *HSD3B1* mRNA was present in cotyledonary tissue, the immunohistochemical signal was weak; the abundance of this enzyme was below the level of detection by immunoblotting in these cells. Although we have not exhaustively sampled through the period in question, the results from the present investigation suggest that the binuclear cells of the

trophoblast do not participate directly in steroid synthesis during the first third of gestation.

It remains possible that the trophoblast giant cells function in conversion and metabolism of locally produced steroids. Indeed, sulfotransferase family 1E, estrogen-preferring, member 1 (SULT1E1) activity was found in cotyledons and in the trophoblast giant cells (Nash, Glenn et al. 1988; Hoffmann, Falter et al. 2001; Ushizawa, Takahashi et al. 2007), suggesting a two-compartment organisation, where the steroids produced in caruncular tissue are transformed in steroid sulphates by cotyledonary tissue. The sulphate conjugation in cotyledons may inactivate and/or catabolize steroids and contribute to protection of the fetus from effects of the free steroids produced by caruncles. Moreover, the caruncle exhibits pronounced steroid sulphatase activity, indicating that the caruncle is capable of processing steroids from estrogen sulphates, produced by cotyledonary tissue (Hoffmann, Falter et al. 2001; Ushizawa, Takahashi et al. 2007). Confirmation of this hypothesis awaits further experimentation.

The role of placental steroid hormones in cattle is not well understood, presumably due to the lack of a method for the selective interruption of placental steroid production *in vivo*. Apart from acting as classical endocrine hormones, the placental progestins and estrogens may also function as autocrine or paracrine regulators, as has been demonstrated in testis and ovary (Zirkin 1998; Schuler, Wirth et al. 1999; Slomczynska and Wozniak 2001). Not surprisingly, the receptor for progesterone is present in the caruncular stroma and epithelium, and it has

been suggested that this steroid, of ovarian or placental provenance regulates placental development (Hoffmann and Schuler 2002). Given the role of progesterone in gestation in other species, it is presumed that local synthesis serves to inhibit myometrial activity to ensure fetal development.

In conclusion, we have demonstrated the capability of the early bovine placenta to initiate steroidogenesis. It is of interest that both the maternal and fetal components participate in the process, perhaps in a complementary fashion. The caruncular tissue expresses all three steroidogenic proteins (STAR, CYP11A1 and HSD3B1), suggesting that the maternal compartment exhibits greater steroidogenic capacity than cotyledon during the first third of pregnancy. In contrast to other investigations, our studies suggest that the trophoblast giant cells are not a source of placental steroids, at least during the first third of gestation.

Declaration of interest

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

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3.7. TABLES AND FIGURES

Table1. First antibodies and concentrations employed for western blotting (WB) and immunohistochemical staining procedures (IMH) in bovine placenta

Antibody	Specificity	Company	Dilution WB	Dilution IMH
ACTB	Rabbit polyclonal antibody against a synthetic peptide (KLC-coupled) corresponding to amino-terminal residues of human β-Actin	Santa Cruz Biotechnology, Inc. California USA	1: 1 000	
CYP11A1	Rabbit anti-rat cytochrome P450 scc polyclonal antibody	Chemicon, International, Ontario Canada.	1:1 000	1:200
HSD3B1	Rabbit anti-rat 3beta-HSD polyclonal antibody	Santa Cruz Biotechnology, Inc. California USA	1:1 500	1:200
SBU-3	Mouse anti-sheep carbohydrate moiety expressed in trophoblast giant cells	Department of Veterinary Preclinical Sciences. University of Melbourne. Australia		1:50
STAR	Rabbit polyclonal antibody to amino acids 88-98 of mouse StAR protein	Provided by Dr. D.M.Stocco, Texas Tech University	1:1 000	1:100

Table 2. Primer pairs used for mRNA detection in bovine placenta

Genes	qPCR primers sequence (5'–3')	Fragment size (bp)	References/accession no.	Primer concentration
ACTB	FWD GGATGAGGCTCAGAGCAAGAGA	58	NM_173979	FWD 3 µM
	REV TCGTCCCAGTTGGTGACGAT			REV 3 µM
CYP11A1	FWD CTGCAAATGGTCCCACTTCT	209	Murayama et al. (2008)/ NM_176644	FWD 9 µM
	REV CACCTGGTTGGGTCAAACTT			REV 1.5 µM
GAPDH	FWD GGCGTGAACCACGAGAAGTATAA	100	NM_001034034	FWD 9 µM
	REV CCCTCCACGATGCCAAAGT			REV 3 µM
HSD3B1	FWD GCCCAACTCCTACAGGGAGAT	114	Orisaka et al. (2006)/ NM_174343.2	FWD 1.5 µM
	REV TTCAGAGCCCACCCATTAGCT			REV 3 µM
STAR	FWD CCCATGGAGAGGCTTTATGA	115	Nishimura et al. (2006) / NM_174189.2	FWD 9 µM
	REV TGATGACCGTGTCTTTTCCA			REV 3 µM

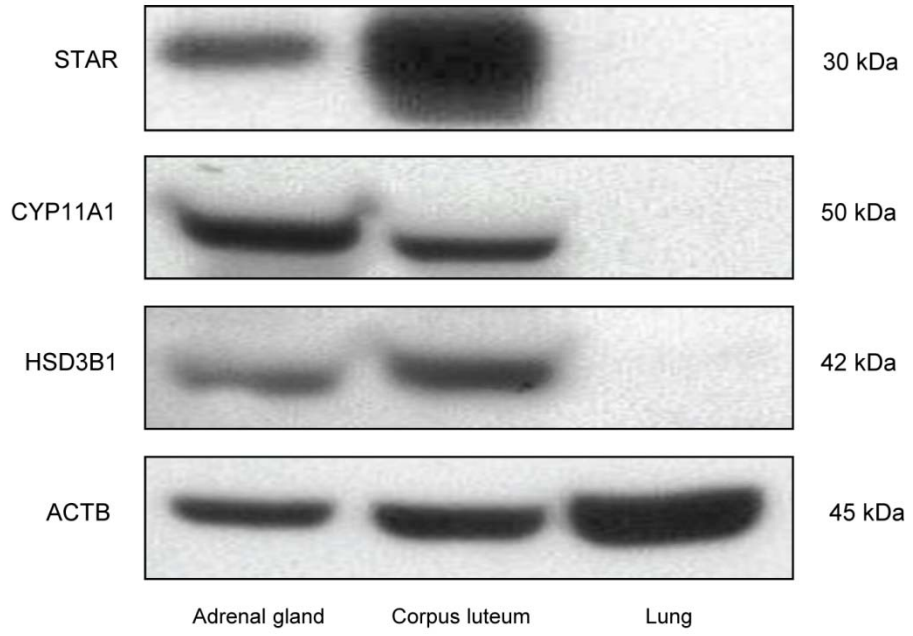
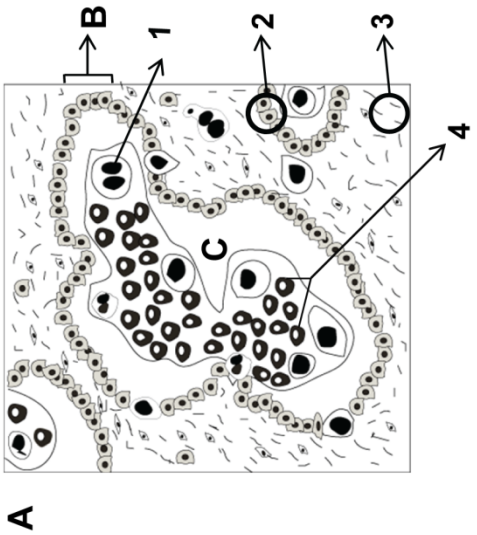


Figure 1. Positive and negative controls to validate the western blot system in bovine placenta. Western blot control of mitochondrial and cytosolic protein (35µg) prepared from mouse adrenal gland (positive control), bovine corpus luteum (positive control), and bovine lung (negative control).


A
B
1
2
3
4
C

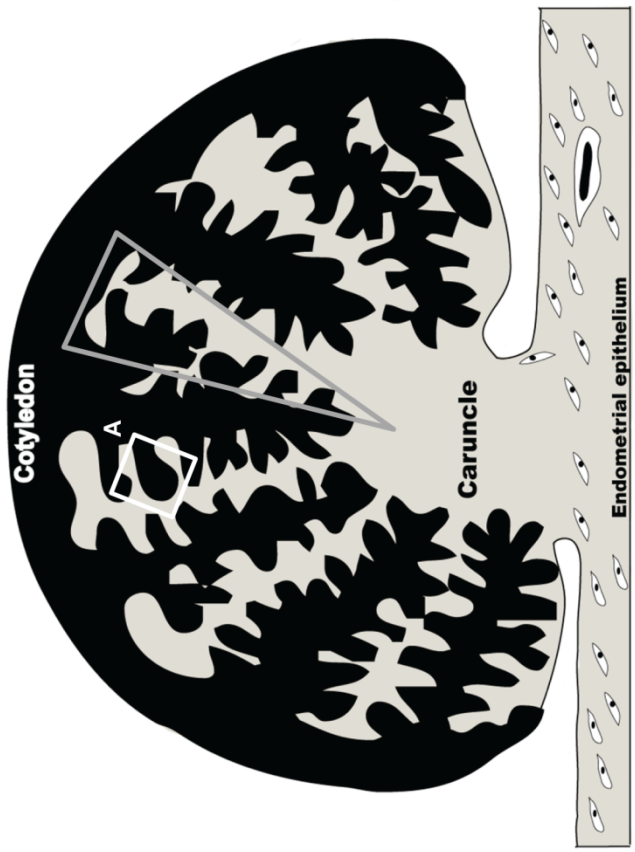

Cotyledon
A
Caruncle
Endometrial epithelium

Figure 2. Diagram representing the bovine placentome. Triangle shows the feto-maternal interface. The white frame (A) is enlarged and depicts an interdigitation. (B) Maternal septum, (C) Chorionic villus, (1) Trophoblast giant cells, (2) Caruncular epithelial cells, (3) Caruncular stromal cells, (4) Uninucleate trophoblast cells.

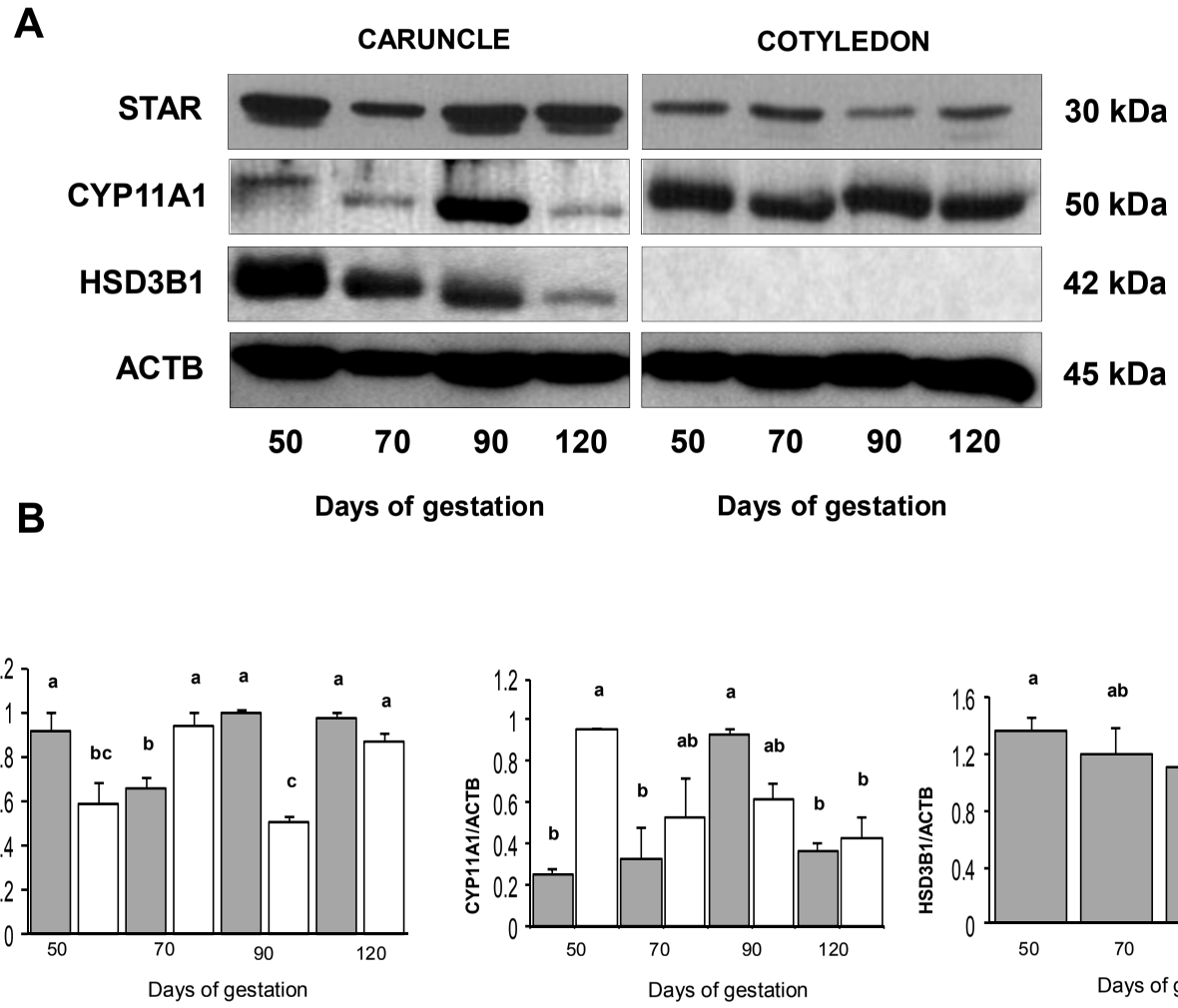

A
CARUNCLE
COTYLEDON
STAR
CYP11A1
HSD3B1
ACTB
30 kDa
50 kDa
42 kDa
45 kDa
50 70 90 120 50 70 90 120
Days of gestation
Days of gestation
B
STAR/ACTB
CYP11A1/ACTB
HSD3B1/ACTB
Days of gestation
Days of gestation
Days of gestation

Figure 3. Characterization of steroidogenic proteins by western blot (WB) analysis (70 µg per lane) prepared from bovine placental tissues. (Panel A) STAR, CYP11A1 and HSD3B1 protein in caruncles and cotyledons at different ages of gestation. (Panel B) Means ± SEM of STAR, CYP11A1 and HSD3B1 protein between caruncles and cotyledons at different periods of gestation. ACTB served as loading and constitutive control. Gray bars represent caruncular tissue, and white bars represent cotyledonary tissue. Means with different superscripts are significantly different ($P<0.05$) (n=4). The WB images correspond to one of the four representative replicates.

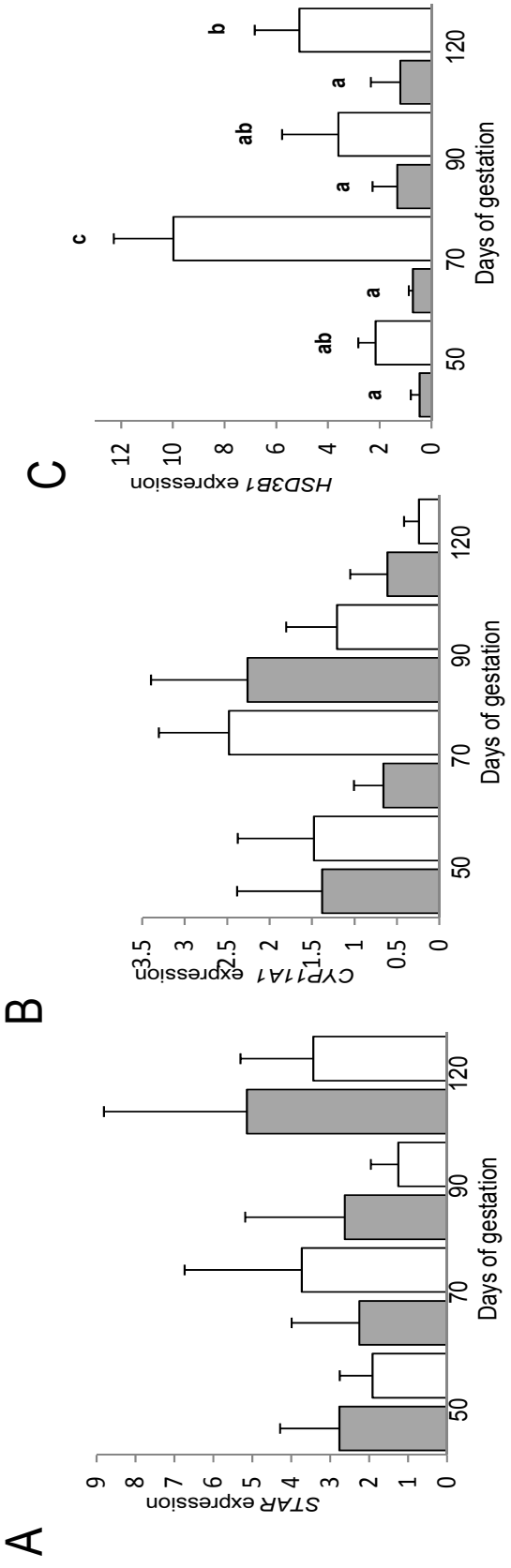

A
STAR expression
0
1
2
3
4
5
6
7
8
9
50
70
90
120
Days of gestation
B
CYP11A1 expression
0
0.5
1
1.5
2
2.5
3
3.5
50
70
90
120
Days of gestation
C
HSD3B1 expression
0
2
4
6
8
10
12
a
ab
a
c
a
ab
a
b
50
70
90
120
Days of gestation

Figure 4. Characterization of mRNA in bovine placental tissues. (A-C). Expression of *STAR*, *CYP11A1* and *HSD3B1* mRNA in caruncles and cotyledons at different ages of gestation. Results are shown as relative mRNA expression for each gene normalized to the reference gene values, and they are expressed as the mean ±SEM. Gray bars represent caruncular tissue, and white bars represent cotyledonary tissue. Means with different superscripts are significantly different ($P<0.05$) (n=4)..

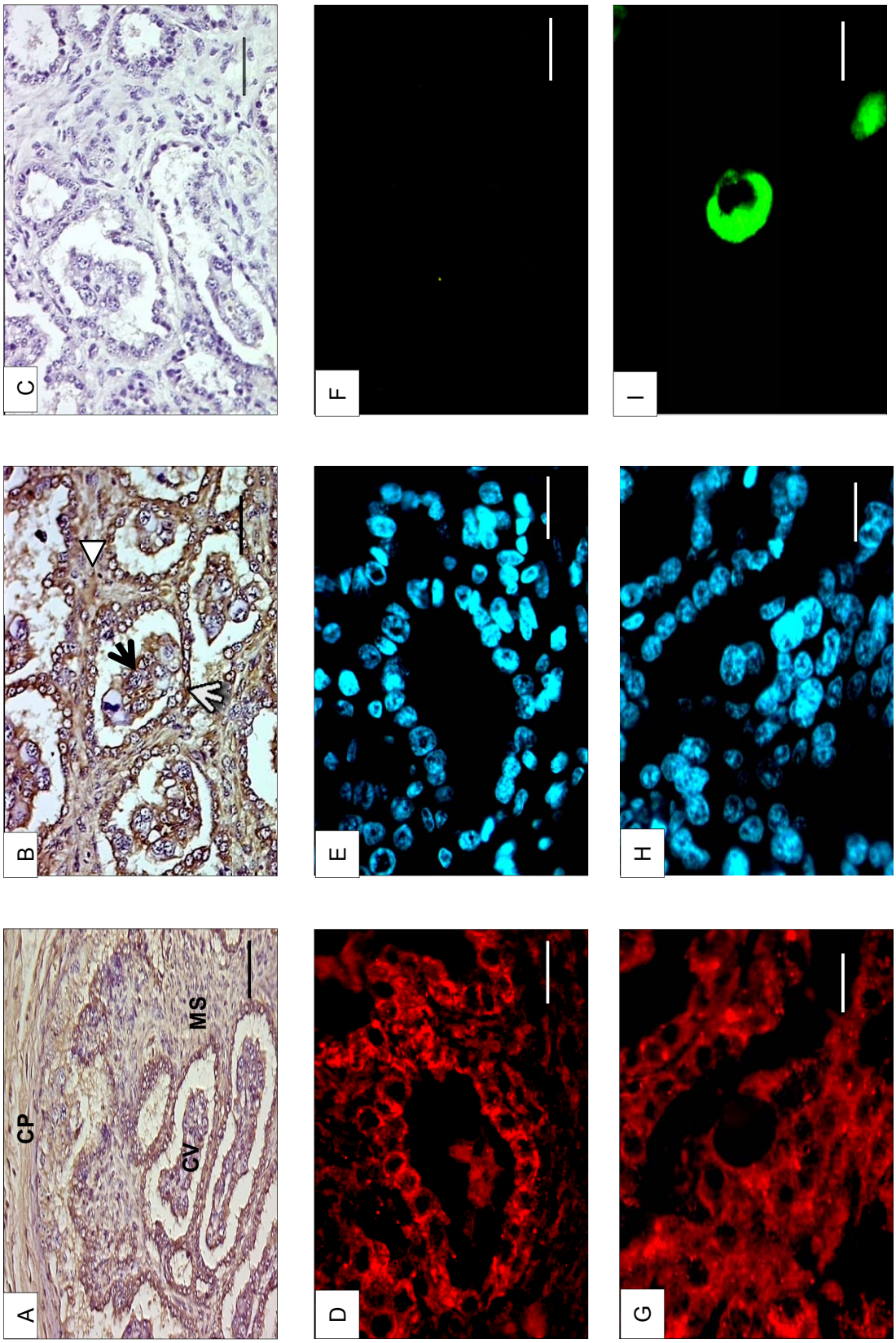

A
CP
MS
CV
B
C
D
E
F
G
H
I

Figure 5. Characterization of STAR expression in bovine placentomes. (Panel A-B) Characteristic staining pattern of STAR protein in a 70 day pregnant cow by immunoperoxidase technique (IP). (A) Considerable staining (brown zones) is localized at chorionic plate (CP) and in the interdigitation area: chorionic villi (CV) and maternal septum (MS). (B) Typical cellular staining pattern in the same cow, shows STAR expression in the feto-maternal interdigitations. The staining is restricted principally to the caruncular epithelial cells (white arrow), caruncular stromal cells (white arrowhead) and the uninucleated trophoblast cells (black arrow), compared to the negative control in panel C. Panel D-I represents immunofluorescence in bovine placentomes at day 90 of gestation. (Panel D) In maternal septum, alexa marker (red) indicates STAR cytoplasmic expression on the caruncular epithelial cells (CECs). (F) CECs were negative to SBU-3 by FITC staining. (G) Uninucleate trophoblast cells (UTCs) stained positive for STAR (red) in chorionic villi. In panel G and I, trophoblast giant cells (TGCs) stained negative for STAR, and positive for SBU-3 (green) respectively. (E and H) Nuclei counterstained with Hoechst (blue). Bars represent .1 mm in panel A, and .05 mm in panels B to I.

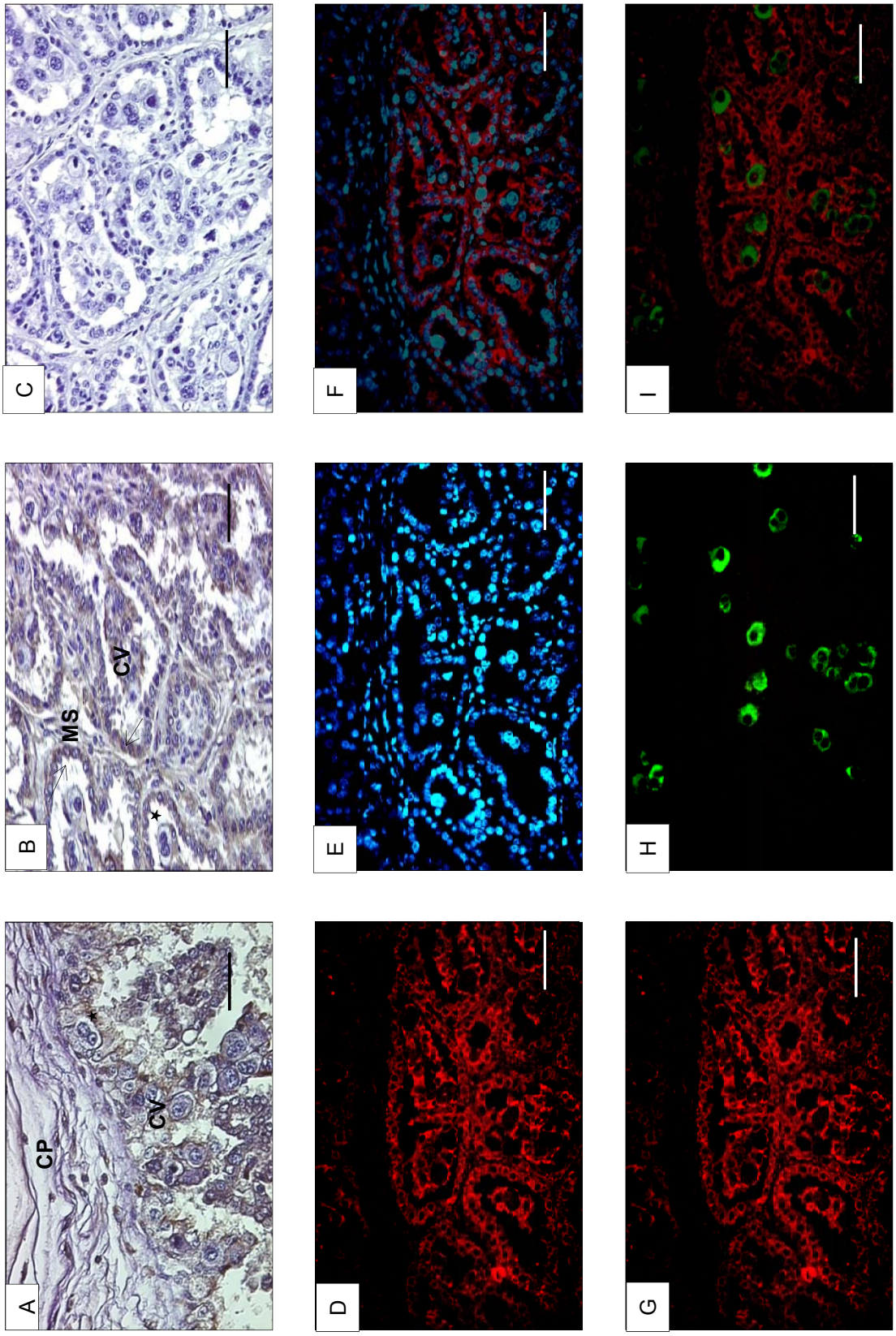
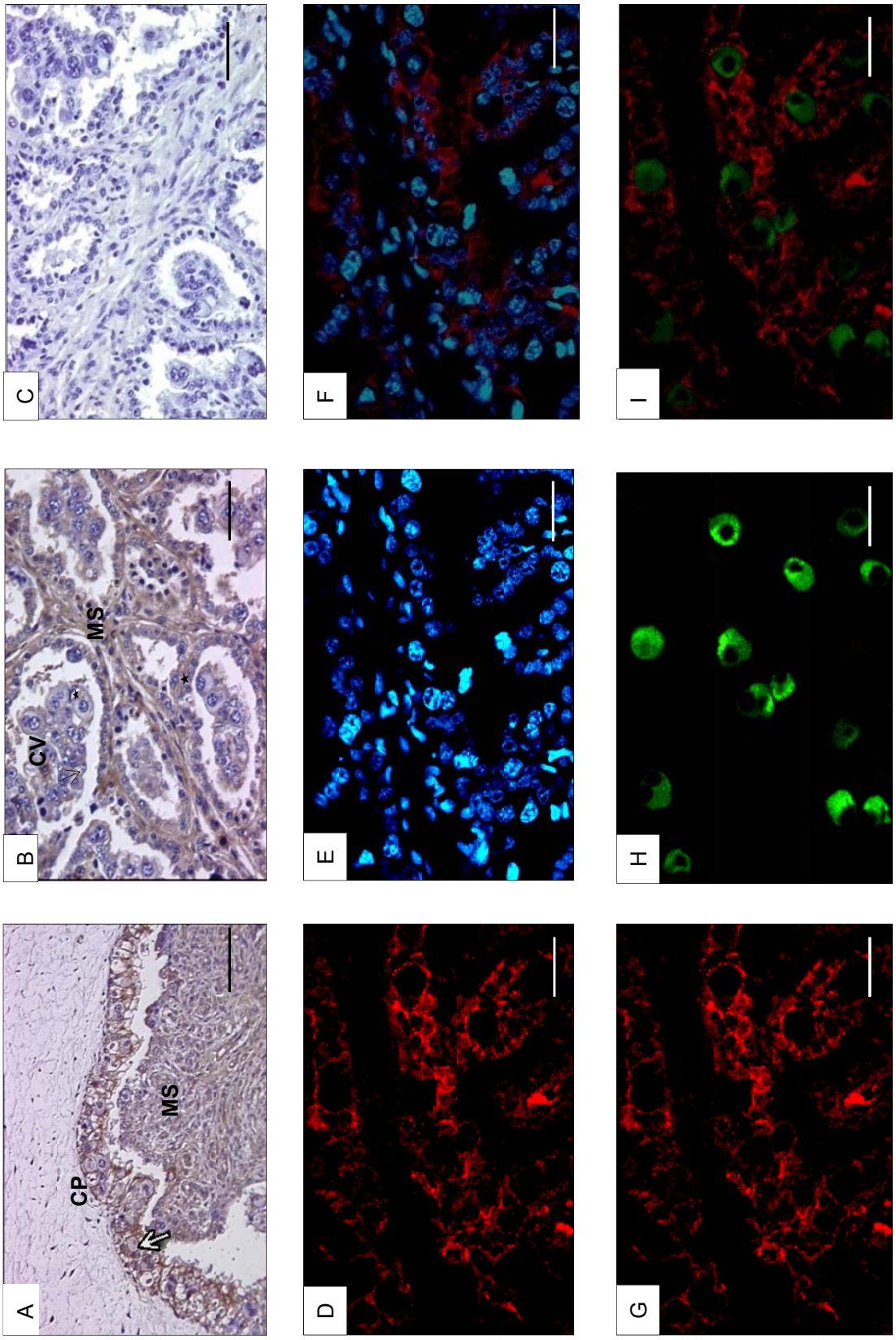

A
CP
CV
B
MS
CV
C
D
E
F
G
H
I

Figure 6. Characterization of CYP11A1 expression in bovine placentomes. (A-C) Characteristic staining pattern in a 50 day pregnant cow by immunoperoxidase technique (IP); significant staining is localized at chorionic plate (CP) and in the interdigitation area: chorionic villi (CV) and maternal septum (MS). (A) Typical cellular staining pattern in the feto-maternal interdigitations, which is restricted principally to the caruncular epithelial cells (CECs) (black arrow). Panel C represents the IP negative control. (Panel A-B) Trophoblast giant cells (TGCs) (star) were negative for CYP11A1 by immunoperoxidase techinque. Panel D-I represents immunofluorescence in bovine placentomes at day 90 of gestation. (Panel D and G) In maternal septum and in chorionic villi, alexa marker (red) shows CYP11A1 cytoplasmic expression on the CECs and on the uninucleate trophoblast cells (UTCs). On the other hand, panel D and G show that TGCs do not stain positive for CYP11A1. Conversely, in panel I, TGCs stained positively for SBU-3 (green). (E) Nuclei were counterstained with Hoechst (blue). Bars, .05 mm.


A
CP
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B
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Figure 7. Characterization of HSD3B1 expression in bovine placentomes. (A-B) Characteristic staining pattern in a 50 day pregnant cow by immunoperoxidase technique (IP). Significant staining is restricted to chorionic plate (CP) and to the interdigitation area: chorionic villi (CV) and maternal septum (MS). Typical cellular HSD3B1 staining pattern in the materno-fetal interdigitations is restricted principally to caruncular epithelial cells (CECs) (black arrow), caruncular stromal cells (CSCs) (black arrowhead), and the uninucleate trophoblast cells (UTCs) (white arrow). (Panel A-B) Trophoblast giant cells (TGCs) were negative for HSD3B1 (black star). Panel C represents the negative control by IP. (D -I) Immunofluorescence in bovine placentomes at day 90 of gestation. (Panel D and G) In the interdigitations, alexa marker (red) shows HSD3B1 cytoplasmic expression only on the UTCs. In Panel H and I, TGCs stained postive for SBU-3 (green) but negative for HSD3B1.Panel E shows nuclei counterstained with Hoechst (blue).

CHAPTER 4. SECOND ARTICLE

Atypical expression of steroidogenic genes in somatic cell nuclear transfer-derived bovine placenta at day 40 of pregnancy

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4.1. Abstract

Pregnancies derived from a somatic cell nuclear transfer (SCNT) cloning have been associated with high rate of fetal loss, especially during the first trimester. The SCNT-derived placentae at term show gross morphological abnormalities, such as large and fused placentomes. The bovine placenta synthesizes steroids that are expected to regulate placental function and fetal development. The objective of this study was to compare expression of a series of genes and proteins coding for steroidogenic enzymes between SCNT and AI placenta, and SCNT and non-clone gestations (NG) respectively. Additionally we investigated by fluorescent immunohistochemistry and confocal microscopy the localization of scavenger receptor class B member I (SCARB1), cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1) and cytochrome P450, subfamily XVII (CYP17A1) in normal pregnancies. Differential expression of SCARB1, steroidogenic acute regulatory protein (*STAR*), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*), *CYP17A1*, *CYP11A1*, steroid sulfatase (*STS*) and sulfotransferase family 1E, estrogen-preferring, member 1 (*SULT1E1*) between AI and SCNT cotyledons was measured by quantitative real time PCR. Protein expression of STAR, CYP11A1 and HSD3B1 was analyzed by western blotting in SCNT and NG. There was a reduction in the abundance of transcripts for *STAR*, *CYP11A1*, *HSD3B1*, *CYP17A1* and *SULT1E1* ($P \leq 0.01$) in placentas from SCNT compared to AI gestations. Protein localization of SCARB1, CYP11A1 and CYP17A1 was restricted to uninucleate trophoblastic cells, indicating that these are the main

cell type responsible for steroid synthesis in day 40 placentae. The reduced abundance of STAR transcripts in placenta derived from cloned animals suggests deficient cellular transfer of cholesterol across the mitochondrial membrane. Altered levels of the CYP11A1, HSD3B1 and CYP17A1 in cloned gestations are expected to lead to a reduction in steroids synthesis by the placenta. A reduced capacity to synthesize steroids may contribute to the abnormal placental and fetal development associated in SCNT pregnancies.

Key words: SCNT gestations, bovine placentae, placental steroids.

4.2. Introduction

Somatic cell nuclear transfer technology has many potential applications in the agricultural and biomedical fields. However, bovine pregnancies derived from SCNT have been associated with high rate of fetal losses, which, in most cases happen during the first trimester of gestation [1]. Some of the alterations reported in these pregnancies are related with defective allantoic vascularisation, edematous chorioallantoic membranes, morphological anomalies of placentomes, dystocia and perinatal loss and anomalies [1-4]. Moreover, the poor survivability of cloned fetuses during the first third of pregnancy is associated with alterations in the chorioallantoic development [5]. Proper progress of placental development is critical for normal development of the fetus. Alterations in some factors that promote placental and vascular development have been pointed as possible causes of SCNT failures [6]. In this regard, alterations in the expression of cytokines such as: insulin-like growth factor 1 (IGF1), insulin-like growth factor binding protein 1 (IGFBP1) and transforming growth factor, beta-1 (TGFB1) were found in placenta from bovine SCNT pregnancies [7, 8]. It is well known that the placenta produces steroid hormones that play important functions in the establishment and maintenance of pregnancy in many species. In normal gestations, the presence of steroidogenic markers have been found in the bovine placenta [9-14].

Recently, we found the principal steroidogenic markers (STAR, CYP11A1 and HSD3B1) in bovine placenta during the first third of pregnancy (Verduzco et al, unpublished). We have also identified the principal regions and cells implicated in

the steroidogenic process in the bovine placenta. However, expression of other steroidogenic factors, such as cholesterol transporters (HDL or LDL) and other steroidogenic enzymes that could contribute to the synthesis of steroids in the bovine placentae, have not been investigated. Moreover, little is known about sub-optimal steroid synthesis in SCNT bovine pregnancies.

Given the importance of steroid hormones for placental and fetal development, the aim of this study was to characterize the patterns of expression of mRNA and protein of some of the main steroidogenic factors (*SCARB1, STAR, CYP11A1, HSD3B1, CYP17A1, STS and SULT1E1*), which could be dysregulated in SCNT placentae, contributing to the placental and fetal developmental abnormalities associated with this technique. We have further evaluated the protein expression of key steroidogenic factors (SCARB1, CYP11A1 and CYP17A1) in early non-clone pregnancies. Additionally, we analyzed the expression and localization of SCARB1, CYP11A1, and CYP17A1 in bovine placenta from AI gestations.

4.3. Results

Messenger RNA abundance of *STAR, CYP11A1, HSD3B1, CYP17A1* and *SULT1E1* were significantly lower in cotelydons from SCNT pregnancies compared to those from AI placenta (Figure 1). No significant differences in transcript abundance of SCARB1 and STS were found. In western blots, the STAR antibody recognised a band with an approximate molecular weight of 30 kDa in NG and

SCNT groups; which corresponds to the mature form of STAR protein [23] (Figure 2). However the intensity of the bands was weak in both experimental groups and only two of four samples in the SCNT group showed the presence of the protein. There was no significant difference between SCNT and NG cows. Anti-CYP11A1 antibody recognized a band of approximately 50 kDa, corresponding to the size of this protein in cattle. CYP11A1 protein abundance did not differ between SCNT and NG cows (Figure 2). As we observed in a previous study (Verduzco et al., unpublished), HSD3B1 protein was not detected in the samples from NG. Surprisingly, the presence of a 42 kDa band, which corresponds to the HSD3B1 bovine protein, was found in two cows from SCNT group (Figure 2). However, only one of the two samples showed band intensity; and no significant differences were found between the two experimental groups.

Immunofluorescence revealed that, in AI gestations, SCARB1 was mostly restricted to uninucleate trophoblast cells, and in vascular smooth muscle form some blood vessels. In contrast no expression or reduced expression was found in trophoblast giant cells (Figure 3). Anti-CYP17A1 antibody yielded scarce expression in uninucleate trophoblast cells; once again in trophoblast giant cells a weak to absent staining was observed (Figure 4). Specific expression of CYP11A1 was observed in fetal stromal cells, uninucleate trophoblast cells and stromal uterine cells. Trophoblast giant cells showed a weak or absent staining (Figure 5).

4.4. Discussion

In bovine SCNT pregnancies, abnormal placentation is a major factor of pregnancy failure in first trimester [5, 24-26]. In domestic animals, the placenta produces a variety of steroid and protein hormones that support conceptus growth and development. Bovine placenta produces steroid hormones; and receptors for these hormones have been identified in this tissue [27, 28]. A putative role of placental steroids as local regulators of placental growth, differentiation and function has been suggested [28-31]. Based on the aforementioned, the present study investigated possible alterations in the expression of the principal steroidogenic factors in placentas from cloned embryos. The placenta is a vital organ for cholesterol transfer from the mother to the fetus.

Low density lipoproteins (LDL) and high density lipoproteins (HDL) are taken up from maternal plasma by LDL and SCARB1 receptors respectively [32]. In the present study, no alterations were found in transcript levels of *SCARBI* in SCNT pregnancies compared to control gestations. This suggests that the selective uptake of HDL cholesteryl ester mechanism is not affected in SCNT gestations. Conversely, we clearly demonstrated that some of the principal steroidogenic genes (*STAR, CYP11A1, HSD3B1, CYP17A1* and *SULT1E1*) in bovine placenta were altered and lower in cloned pregnancies where compared to conventional gestations. It is well known that transcripts of the abovementioned genes, encode proteins which are pivotal in the progesterone, mineralocorticoids, glucocorticoids, androgens, estrogens and oestrone sulphates biosynthesis. The aforementioned hormones,

especially progesterone and estrogens, exert a local, paracrine and autocrine effects in placental and foetal tissues [28, 33-35]. To our knowledge, this is the first study to document clearly, atypical expression of some of the principal steroidogenic genes in bovine placenta from early SCNT pregnancies. Previous studies, reported deregulation of imprinted and non-imprinted genes, essential in the regulation of placentogenesis, fetogenesis, mammogenesis, steroidogenesis, and immune system activity in the SCNT bovine gestations [6, 24, 36-38]. Moreover, some authors suggested that alterations in vascular development of placenta from clones could also be due to modifications in the expression of placental factors [39, 40]. Therefore, it appears that variation in the expression of steroidogenic genes might lead to a wide range of effects on placental and fetal growth that could compromise the proper progression of pregnancy.

In contrast with our results, some authors reported upregulation of *SULT1E1* and *CYP11A1* mRNA expression in preterm and term placentas from SCNT gestations [20, 41]. The discrepancy between the results in advanced pregnancies and our results may be related to the stage of the gestation. The principal function of the SULT1E1 is the sulphoconjugation of estrogens, inhibiting the estrogen activity by conjugating a sulfonate group to estrogens and preventing the estrogen receptor binding [42]. Conversely, the STS enzyme is responsible for the hydrolysis of estrone sulfate and dehydropiandrosterone sulfate to estrone and dehydroepiandrosterone, respectively, both of which can be converted to free steroids [12, 43]. The cooperation of the two aforementioned enzymes controls the

availability of free, active estrogens. In spite of the lack of function and production of estrone sulphates by SULT1E1 in early bovine placenta; we speculate that perturbations of *SULT1E1* in SCNT transcripts could firstly induce an imbalance in the regulatory STS-SULT1E1 system in bovine placenta, affecting the synthesis of estrogen sulphates. However, in the present experiment, no difference in transcript of *STS* gene was found in SCNT pregnancies. Consistent with this view, Hirayama et al [20], found no difference between SCNT and normal placentae at parturition. Alternatively, differences found between early and late gestations, may be associated with individual compensatory mechanisms that could be triggered during gestation in order to overcome disrupted expression of important genes in the placenta, as previously reported [39]. If this is the case, we cannot exclude the possibility that low mRNA *SULT1E1* in SCNT is compensation to the perturbations in the synthesis of steroid hormones. Reduction of the oestrone sulphates synthesis would regulate the availability of free steroids.

In contrast to findings in qPCR, in the present study the western blot results did not show significant differences in STAR, CYP11A1 and HSD3B1 abundance in SCNT gestations versus NG. These results suggest that differences in steroidogenic genes transcription in SCNT gestations did not result in significant protein differences in cotyledonary tissue. In further contrast with our results, previous investigations in late SCNT gestations found higher plasma concentrations of estrone sulphate and progesterone (P4) in cows that aborted or that had prolonged gestations respectively [44]. In this context, Hoffert-Goeres et al [40], studying

genes associated with impaired placental angiogenesis in NT pregnancies, found also discrepant results between transcripts and protein expression. Based on this observation, we propose that the resulting aberrant expression of steroidogenic genes found in this study, might have an impact in the expression of steroidogenic proteins and steroids synthesis in a long term at other developmental stage with effects measurable only at a later time in gestation.

Intriguingly, and contrary with our previous finding [45], where HSD3B1 protein was absent in cotyledonary tissue; in this experiment only one sample from SCNT gestations expressed this enzyme. This fact suggests the possibility that not all cloned embryos have the same disturbances in genome reprogramming after cell nuclear transfer in oocytes. Other authors [46], have noted the importance of individual analyses of cloned animals, since the variation in mean expression levels could mask gene expression abnormalities present in individual clones. However, whether aberrant gene expression is part of the origin of placental dysfunction or just a consequence of abnormal placental development is yet to be established.

We evaluated the expression of SCARB1, CYP17A1 and CYP11A1 in AI gestations by immunohistochemistry. Protein localization of SCARB1, CYP11A1 and CYP17A1 was restricted to uninucleate trophoblastic cells indicating that these are the main cell type responsible for steroid synthesis in day 40 placentae. These results agree with some reports [13, 45, 47] in ovine and bovine placenta, which demonstrated that CYP17A1 and CYP11A1 were exclusively found in the columnar trophoblast cells. A similar pattern of expression was observed for the SCARB1

receptor, where only the uninucleated trophoblast cells expressed the receptor. Human placenta expresses SCARB1 [48]. To our knowledge, this is the first study that localized the expression of SCARB1 in bovine placental tissue. Together this suggests that the principal cells implicated in the transport of cholesterol and the steroid production in cotyledonary tissues are the uninucleate trophoblast cells.

In conclusion, our results suggest that in clone pregnancies, the expression of important steroidogenic markers by placenta are disrupted, and this phenomenon could be traduced in an insufficient biosynthesis of steroid hormones, and also in inadequate placental and fetal growth. Considering the complexity in regulation of steroids biosynthesis in bovine placenta, further studies are necessary for understanding the molecular mechanisms implicated in SCNT failures, and their compensatory effects.

4.5. Materials and methods

Animals and sample collection

Investigations were conducted in accordance with the Canadian Council of Animal Care recommendations and approved by the Université de Montréal Committee on Animal Care and Experimentation. The method to generate somatic cell nuclear transfer embryos have been described previously [15] using a single nuclear donor cell line. Control pregnancies were derived by artificial insemination (AI) of Holstein heifers with semen from a single Holstein bull. SCNT and AI cows were slaughtered on day 40 of gestation (AI, N=8; SCNT, N=8). A second set of

samples from SCNT (N=4) at day 40 of gestation; and non-cloned gestations (N=4) between days 40 – 50 of gestation, were used for the protein analysis. The non-cloned gestations were collected at a local slaughterhouse, and gestational age of placenta was determined by fetal crown-rump length (CRL) as described previously [16]. Cotyledonary tissues from the mid-region of the uterine horn that contained the fetus were collected for each gestation. Cotyledon samples from SCNT and AI were both stabilized with RNA-later (Qiagen, Mississauga, ON, Canada) and stored at -80°C until RNA extraction. For protein extraction, cotyledonary tissues from SCNT (N=4) and NG (N=4) were snap-frozen in liquid nitrogen and stored at -80°C prior to protein extraction. Adjacent sections of cotyledonary tissues (n=4) were collected from AI cows fixed in 4% buffered paraformaldehyde (PAF) solution embedded in paraffin and processed for imunohistochemistry by routine procedures.

The goal in the first instance was to determine the mRNA expression levels of *SCARB1, STAR*, *HSD3B1*, *CYP11A1, CYP17A1, STS,* and *SULT1E1* genes in cotyledonary tissues of SCNT and AI pregnancies. Furthermore, we analyzed protein levels of STAR, HSD3B1 and CYP11A1 proteins in NG and SCNT gestations. Subsequently, fluorescent immunohistochemistry was used to investigate the localization of SCARB1, CYP11A1 and CYP17A1, factors that play an important role in the importation of cholesterol, and steroid biosynthesis, and which have not been thoroughly investigated in bovine placenta.

RNA extraction and cDNA synthesis

Total RNA was isolated from 30 mg of cotyledonary tissues from SCNT and AI pregnancies, using an RNeasy Mini Kit (Qiagen), according to the manufacturer's recommendations. Disruption, homogenization and purification of tissue were achieved with mortar and pestle, and QIAshredder columns (Qiagen). To remove the proteins which could interfere with the RNA isolation, proteinase K digestion (Invitrogen Life Technologies Corporation, Burlington, ON, Canada) was used. DNase I treatment was applied on the columns to eliminate any contaminating DNA (RNase-free DNase Set, Qiagen,).RNA concentrations were measured by spectophotometry, using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and 1 µg of total RNA from each sample was reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen).

Quantitative real time PCR (qPCR)

Gene-specific primers for *SCARB1, STAR, HSD3B1, CYP11A1, CYP17A1, STS, SULT1E1, glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *actin, beta* (*ACTB*) employed to amplify genes in reverse transcribed samples by qPCR using the 7300 Real Time PCR thermal cycler (PE Applied Biosystems, Foster City, Ca, USA). Sequences of the primer pairs and expected product sizes are shown in Table 1. Bovine STAR, CYP11A1, HSD3B1, STS and SULT1E1 primers were designed from published bovine sequences [17-20]. CYP17A1, SCARB1, GAPDH and ACTB primers were designed based on bovine genes sequences from GenBank, and using the Primer Express software Version 3.0 (PE, Applied Biosystems). The qPCR products

from *CYP17A1*, *SCARB1* , *GAPDH* and *ACTB* were subjected to electrophoresis, and then target band was excised and purified using a a QIAquick gel extraction kit (Qiagen) and sequenced (The McGill University and Génome Québec Innovation Centre, Montréal, QC). Primer concentrations were optimized according to the manufacturer's recommendations and dissociation curve analysis and agarose gel electrophoresis were used to verify the specificity of amplified products. The PCR efficiency (E= 1.9-2.0) and linearity of each pair of primers were tested by preparing a series of solutions of the same stock of cDNA used for the qPCR gene analysis. The geometric mean of two reference genes (*GAPDH* and *ACTB*) was used as the normalization factor. The relative quantification method ($\Delta\Delta C_t$) was used to determine changes in target gene expression levels [21, 22]. Data analysis was performed using the Sequence Detection Software version 1.4 (PE, Applied Biosystems) and the LinRegPCR Software version 11.1 (Heart Failure Research Center Academic Medical Centre Amsterdam, the Netherlands). Reactions for qPCR were conducted in sealed 96-well multiply (Sarstedt Inc., Montréal, QC Canada) in triplicate. The total volume of the qPCR reaction was 20 µl, comprising 6 µl cDNA template (diluted 1 in 30 in distilled water), 2 µl each forward and reverse primers, 2 µl of distilled water and 10 µl Power SYBR green PCR master mix (Applied Biosystems). Cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C.

Western Blotting

Homogenates from SCNT and NG pregnancies were obtained by powdering small pieces of frozen cotyledons individually, with mortar and pestle. The samples were homogenized in sonication buffer (20 mM Tris, 50 mM EDTA, 0.1 mM DEDTC, pH8.0; Invitrogen Life Technologies Corporation, Burlington, ON, Canada and Sigma-Aldrich, Oakville, ON, Canada), containing 32mM octyl glucoside and protease inhibitor (Complete mini, EDTA-free, Roche, Mannheim, Germany). The lysates were spun by differential centrifugation (600 x g for 30 min, then the resultant supernatant was re-centrifuged at 9000 x g for 30 min) to obtain mitochondrial and cytosolic proteins [11]. The protein concentration was measured by a Bradford protein assay (Bio-Rad Laboratories, Hercules California, USA), adjusting the samples to 70 µg/µl of protein. The proteins were then diluted in sample buffer containing 25 mM Tris (pH 6.8), 1 % SDS, 5 % mercaptoethanol, 1 mM EDTA, 4% glycerol, and 0.01 % bromophenol blue (Invitrogen and Sigma-Aldrich) and were boiled for five minutes. Microsomal and mitochondrial fractions prepared from cotyledons were size-fractioned on a 12% SDS-polyacrylamide gel and then electrotransferred onto PVDF transfer membrane (GE Healthcare, Piscataway, NJ). The low and high range molecular weight marker (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK Limited) was used as reference. The membranes were then blocked with 4 % nonfat dry milk in TBS buffer containing 0.5 % Tween 20 (TBST) and incubated with their respective first antibody overnight. First and second antibodies were diluted in TBST. The rabbit

anti-human for STAR and ACTB first antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz CA), were applied in a 1:300 and 1: 50 000 dilutions respectively. Rabbit anti-rat first antibodies for CYP11A1 (Chemicon, International, Temecula,CA, USA) and HSD3B1 (Santa Cruz) were used at 1: 1000 and 1:1500 dilutions respectively. The second antibody peroxidase-conjugated Affini Pure Goat anti-rabbit IgG (Jackson Immuno Research Laboratories Inc, West Grove, PA, USA), was used at 1:100000 dilutions. Mitochondrial and cytosolic proteins from different steroidogenic tissues (mouse adrenal glands and bovine corpus luteum) and non steroidogenic organs (bovine lung) were used as positive and negative controls, respectively, to validate the system. The optical density was analyzed using ImageJ 1.31v software (National Institutes of Health, Bethesda MD).

Immunohistochemical staining procedures

Paraffin sections of cotyledons were rehydrated and heated in 10 mM sodium citrate (pH 6.0) for 20 min for antigen retrieval. The sections were blocked with 5% BSA in PBS for 30 min at room temperature, and incubated with the respective first antibody. For SCARB1, rabbit polyclonal anti-mouse antibody (Novus Biologicals, Littleton, CO) diluted 1:50. A CYP11A1 rabbit anti-rat polyclonal antibody (Chemicon, International) diluted 1:200 and a CYP17A1 goat anti-human polyclonal antibody (Santa Cruz Biotechnology) diluted 1:100. Slides were incubated with the respective second antibody Cy3-conjugated goat anti-rabbit IgG and donkey anti-goat IgG (Jackson Immunoresearch.), diluted 1:400 for 1 h at RT. Slides were then washed, and the nuclei were stained with 4',6-diamidino-2-

phenylindole (DAPI, Sigma, St. Louis, MO), diluted 1:1000 in PBS, for 5 min. Slides were mounted in Permafluor (Lab VisionCorp.; Fremont, CA). All antibodies were diluted in PBS, and as a negative control, some sections were incubated with BSA in the place of the first antibody.

Placental distributions of the three factors were observed by confocal microscopy using the Olympus Fluoview 1000 system and Fluoview version 1.7175 software. Laser sources of 543 nM and 405 nM were employed for detection of Cy3 and DAPI signals, respectively. For a given fluorochrome (i.e., CY3 or DAPI), photomultiplier voltage, laser power, pinhole aperture, time of acquisition and image resolution were maintained constant for all confocal images. The Kalman filter mode was used for all image acquisition.

Statistical Analysis

Statistical analysis was carried out using SigmaPlot (version 11.2, Systat software, Inc. Germany). If data had a normal distribution by Shapiro-Wilk test, a parametric (one-way ANOVA and student's t-test) analysis was applied to determine differences between groups. When distribution of the data was not normal, a non parametric analysis was applied (Wilcoxon/Kruskal-Wallis test, Rank Sums). Gene data are presented as mean $\pm$SEM, and differences between samples were considered significant when $p < 0.05$.

Acknowledgments

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4.7. Tables and figures

Table 1. Primer pairs used for RNA detection

Genes	qPCR primers sequence (5'–3')	Fragment size (bp)	References/ accession no.	Primer concentration
ACTB	FWD GGATGAGGCTCAGAGCAAGAGA	58	NM_173979	FWD 3 µM
	REV TCGTCCCAGTTGGTGACGAT			REV 3 µM
CYP11A1	FWD CTGCAAATGGTCCCACTTCT	209	Murayama et al. (2008)/ NM_176644	FWD 9 µM
	REV CACCTGGTTGGGTCAAACTT			REV 1.5 µM
CYP17A1	FWD CCATCAGAGAAGTGCTCCGAAT	79	NM_174304	FWD 3 µM
	REV GCCAATGCTGGAGTCAATGA			REV 3 µM
GAPDH	FWD GGCGTGAACCACGAGAAGTATAA	100	NM_001034034	FWD 9 µM
	REV CCCTCCACGATGCCAAAGT			REV 3 µM
HSD3B1	FWD GCCCAACTCCTACAGGGAGAT	114	Orisaka et al. (2006)/ NM_174343.2	FWD 1.5 µM
	REV TTCAGAGCCCACCCATTAGCT			REV 3 µM
STAR	FWD CCCATGGAGAGGCTTTATGA	115	Nishimura et al. (2006) / NM_174189.2	FWD 9 µM
	REV TGATGACCGTGTCTTTTCCA			REV 3 µM
SCARB1	FWD GACCCCTTAATCCACCTCATCA	99	NM_174597.2	FWD 3 µM
	REV GGCCAGAATCGGAGTTGTTG			REV 3 µM
STS	FWD GCCTCCTGCTCGGCTTTC	65	Greven et al (2007)/DQ532016 .1	FWD 3 µM
	REV TGTTCACGGTTCCGCATCA			REV 9 µM
SULT1E1	FWD CCTGAGTGGGGATTGAAGAA	412	Hirayama et al (2008)/NM_177488	FWD 3 µM
	REV CACAGGCAGGTGAGACTTCA			REV 3 µM

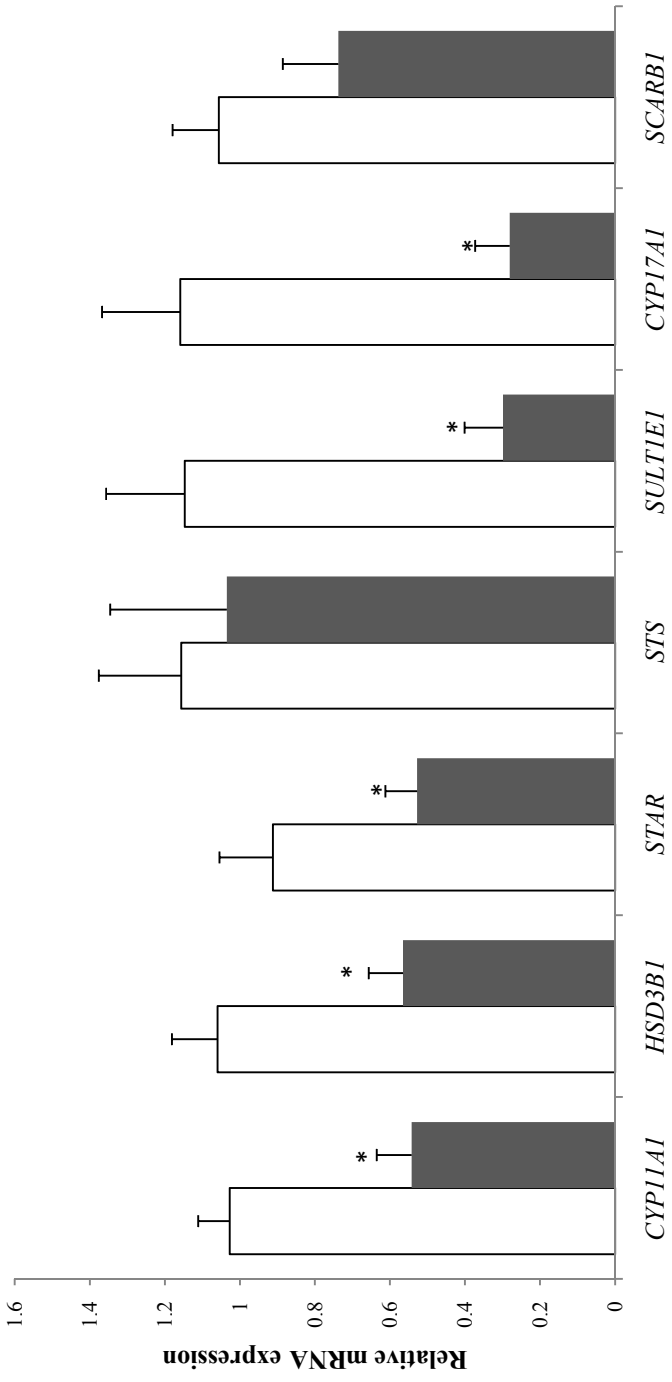
Relative mRNA expression
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
CYP11A1
HSD3B1
STAR
STS
SULT1E1
CYP17A1
SCARB1
*
*
*
*
*

Figure 1. Characterization of mRNA levels for *STAR*, *CYP11A1*, *HSD3B1*, *CYP17A1*, *SCARB1*, *STS* and *SULT1E1* in cotyledonary tissue of artificial insemination (AI) and somatic cell nuclear transfer (SCNT) gestations at day 40. Bovine *ACTB* and *GAPDH* served as loading and constitutive control. Results of qPCR are shown as relative mRNA expression for each gene normalized to the reference genes. Gray bars represent NT group, and white bars represent AI group. Significant differences ($P<0.05$) between NT and AI groups are indicated by an asterisk. (n=4).

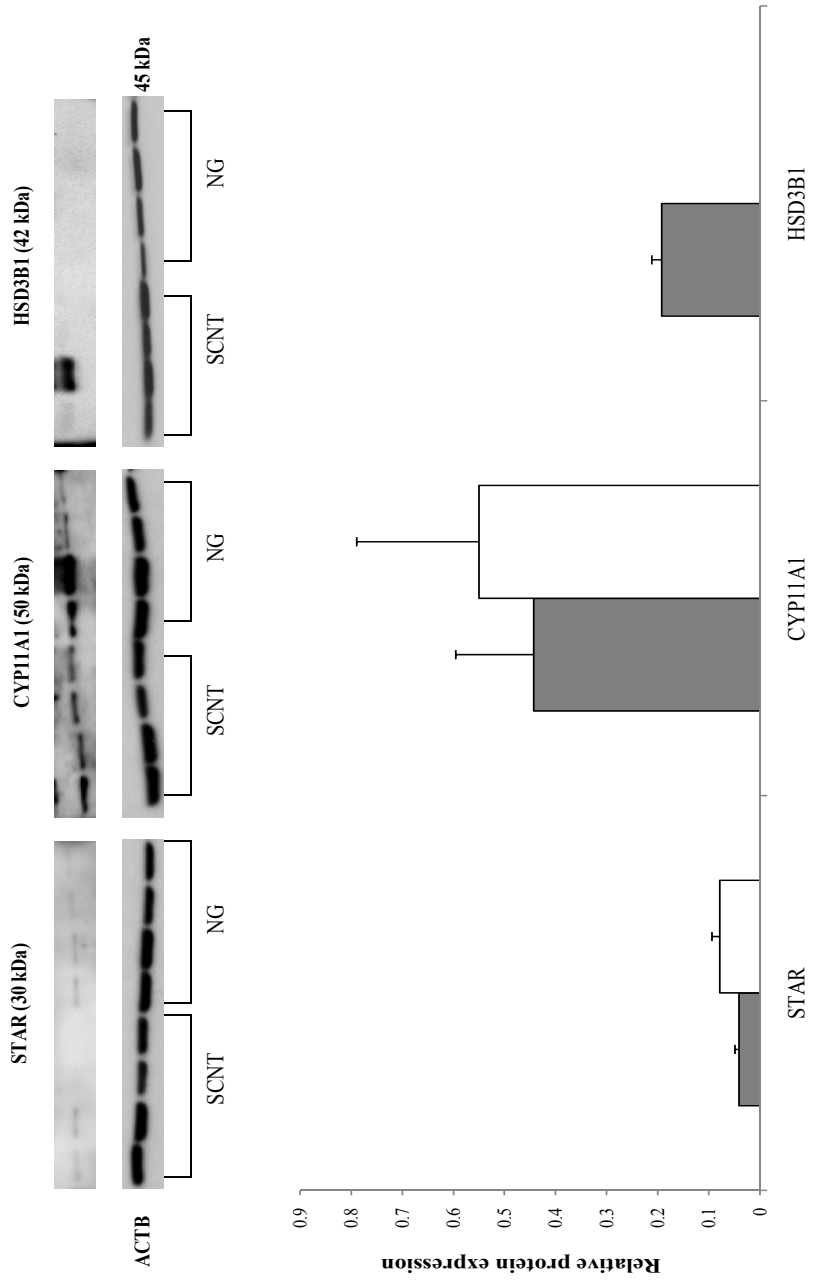
STAR (30 kDa)
CYP11A1 (50 kDa)
HSD3B1 (42 kDa)
ACTB
45 kDa
SCNT
NG
SCNT
NG
SCNT
NG
Relative protein expression
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
STAR
CYP11A1
HSD3B1

Figure 2. Immunoblotting analysis for STAR, CYP11A1, HSD3B1 and ACTB in cotyledonary tissues at day 40 of gestation. White bars represent non-clone gestations (NG), and gray bars represent somatic cell nuclear transfer (SCNT) gestations. Bovine ACTB served as loading and constitutive control. (NG=4 and SCNT=4).

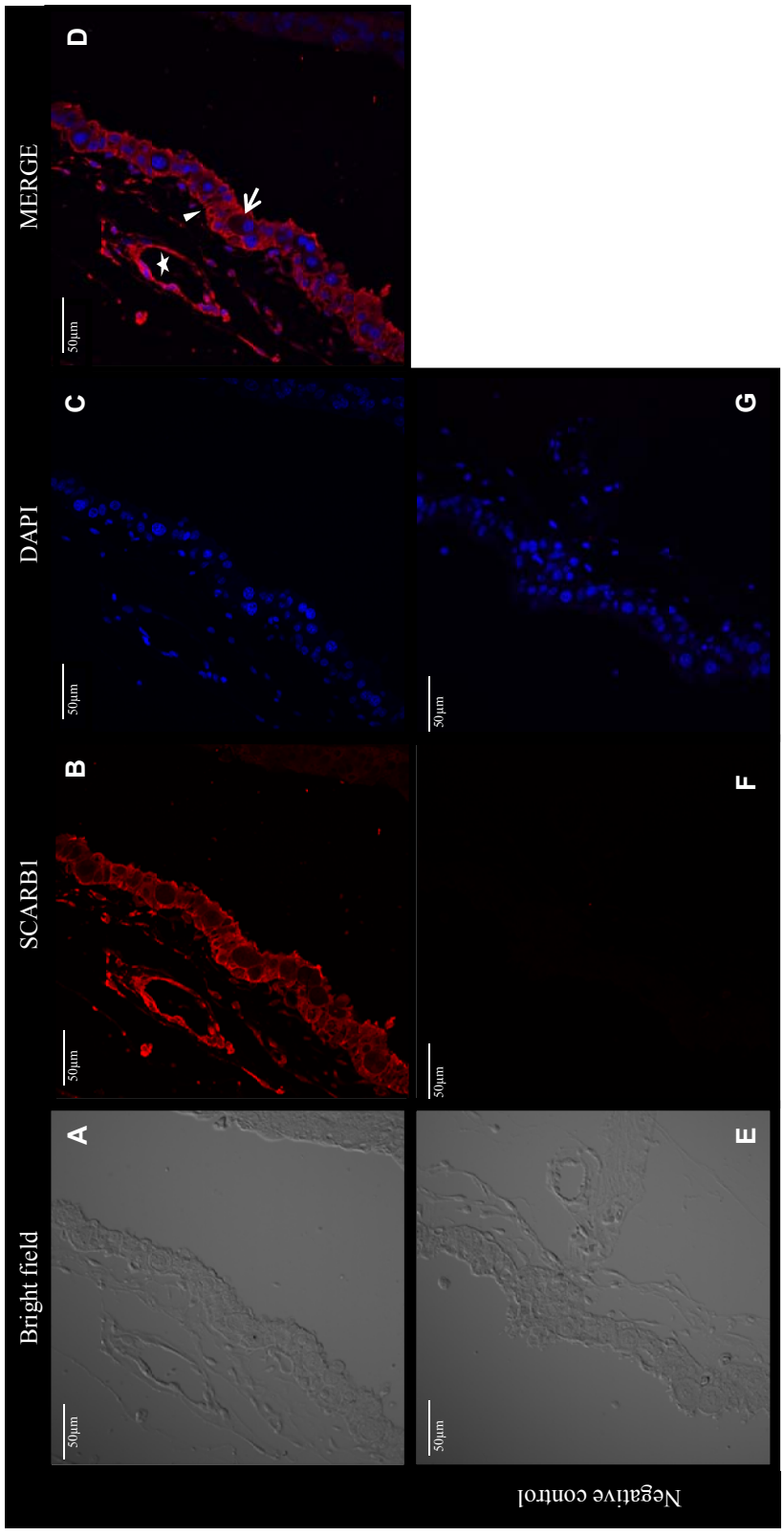
Bright field
SCARB1
DAPI
MERGE
Negative control
A
B
C
D
E
F
G
50µm

Figure 3. Immunofluorescence staining of SCARB1 in bovine cotyledons at day 40 of AI pregnancy. (A and E) Bright field of trophoblast sections. (B and D) SCARB1 expression is present in the uninucleate trophoblast cells (white arrowhead) and in a blood vessel (star). Moreover the SCARB1 expression is greatly reduced or absent in the trophoblast giant cells (white arrow). (C and G) Nuclei counterstained with DAPI. (F) Negative control.

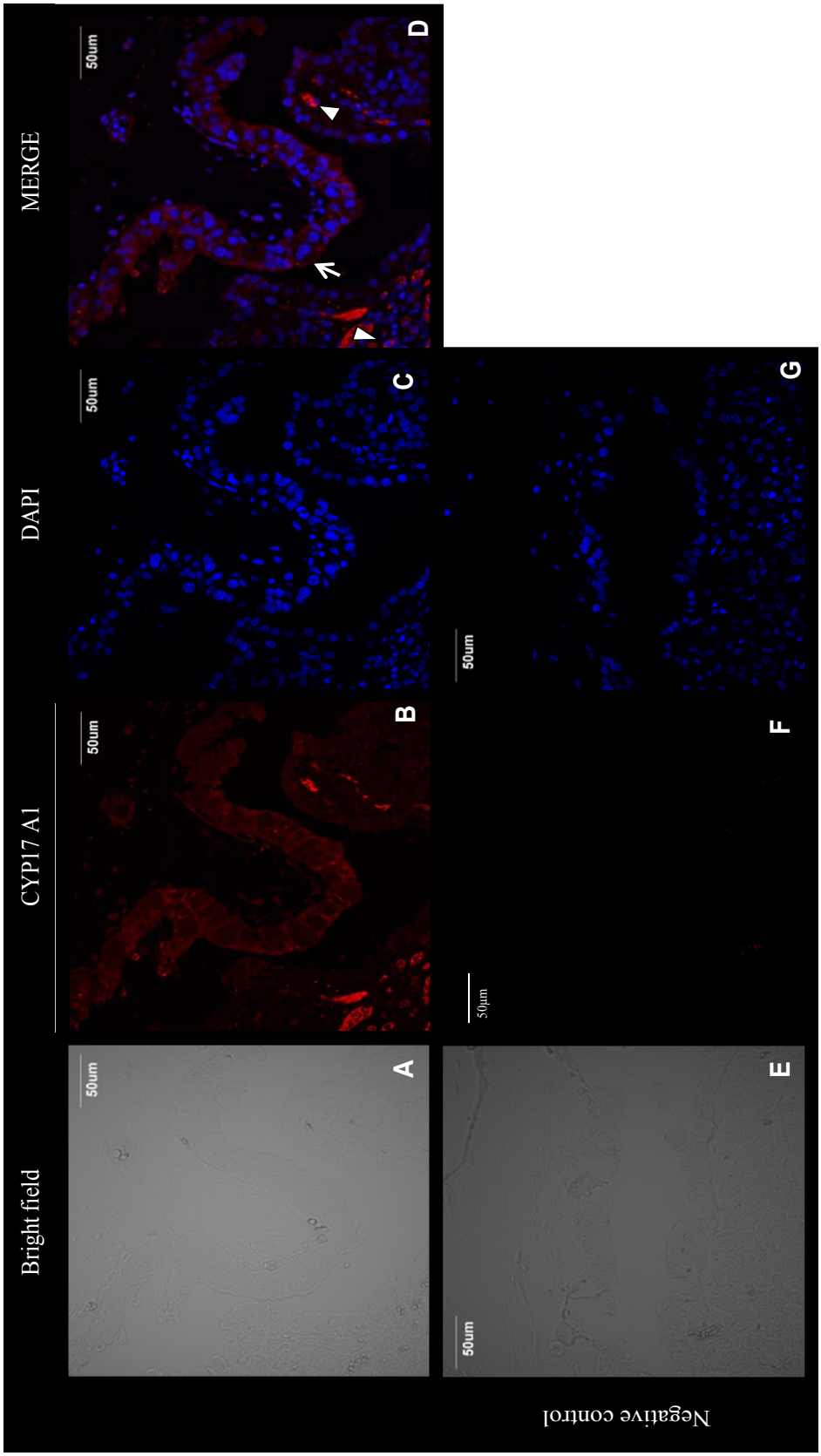
Bright field
CYP17 A1
DAPI
MERGE
Negative control
A
B
C
D
E
F
G
50um
50µm

Figure 4. Immunofluorescence staining of CYP17A1 in bovine cotyledons at day 40 of AI pregnancy. (A and E) Bright field of trophoblast sections. (B and D) CYP17A1 expression is scarcely present in the uninucleate trophoblast cells (white arrowhead) and absent in the trophoblast giant cells (white arrow). (C and G) Nuclei counterstained with DAPI. (F) Negative control.

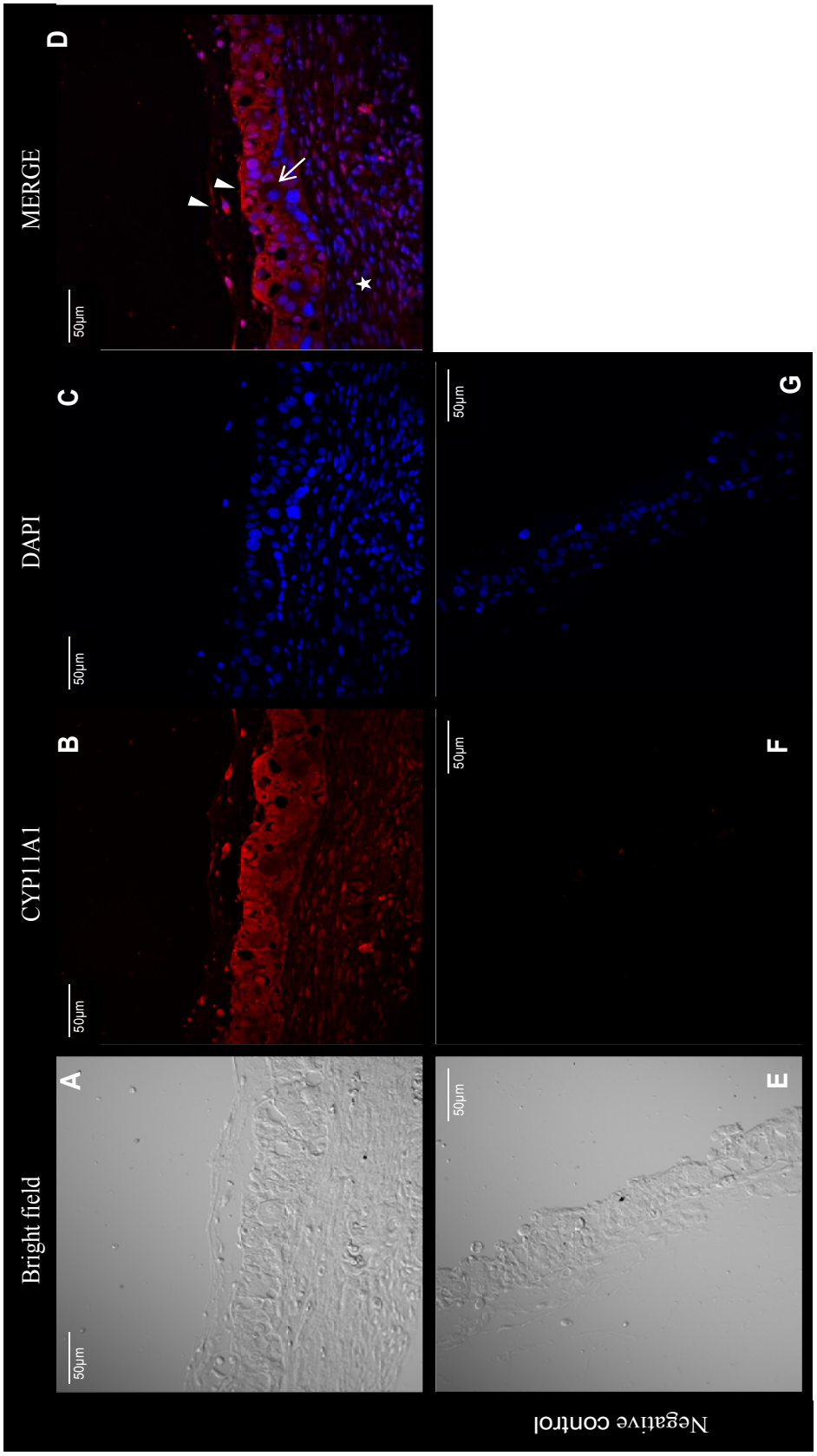
Bright field
CYP11A1
DAPI
MERGE
Negative control
A
B
C
D
E
F
G
50µm

Figure 5. Immunofluorescence staining of CYP11A1 in bovine placentome at day 40 of AI pregnancy. (A and E) Bright field of trophoblast sections. (B and D) CYP11A1 expression is in the fetal stromal cells, uninucleate trophoblast cells (white arrowhead) and uterine stromal cells (white star). Moreover the expression of CYP11A1 is reduced or absent in the trophoblast giant cells (white arrow). (C and G) Nuclei counterstained with DAPI. (F) Negative control.

CHAPTER 5. THIRD ARTICLE

Regulation of placental steroid synthesis in bovine placentome explants during the first half of gestation

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Manuscript in preparation

5.1. Abstract

It has previously been demonstrated that the bovine placenta is capable of steroid production, which in turn is expected to regulate placental and fetal development. The small amount of evidence available suggests that regulation of steroid synthesis differs between the placenta and the ovary in this species. Little is known about the principal factors and intracellular pathways activating the steroidogenic process in bovine placenta. The aim of this study was to investigate the impact of trophic homone and second messengers on steroidogenesis in bovine placenta at 140 ±10 days of gestation. Explants of placentome tissue were incubated for 6, 12 and 18 hours, with human chorionic gonadotrophin (hCG), dibutyryl cAMP (dbcAMP), phorbol 12-myristate 13-acetate (PMA), calcium ionophore (A23187), and a combination of hCG and A23187.

After incubation, progesterone (P4) concentrations in medium were determined by RIA. Semiquantitative RT-PCR was used to measure steroid acute regulatory protein (*STAR*), cytochrome P450, family 11, subfamily A, polypeptide 1 (*CYP11A1*), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*), sulfotransferase family 1E, estrogen-preferring, member 1 (*SULT1E1*) and steroid sulfatase (*STS*) gene expression. Gene expression of *STAR*, *CYP11A1*, *HSD3B1* and *SULT1E1* were increased in placentome explants stimulated with hCG, A23187 and hCG +A23187 in a time-dependent manner. At all periods investigated, calcium ionophore had a stimulatory effect on the P4 levels with respect to the control group ($P<0.05$). Our data suggests that the trophic hormone (LH or hCG) stimulates the expression of steroidogenic genes in bovine placenta. We conclude that in bovine placenta the synthesis of progesterone is modulated principally by intracellular calcium influx, and apparently cyclic nucleotides do not seem to be controlling this process.

5.2. Introduction

Steroid hormones are synthesized in the adrenal gland, gonads, placenta and brain, and are critical for normal reproductive function and body homeostasis (Manna, Dyson et al. 2009). As in many mammalian species, bovine placenta produces estrogens and progesterone (Pimentel, Pimentel et al. 1986; Izhar, Pasmanik et al. 1992). Studies suggest that placental estrogens and P4 are important factors controlling placental and fetal growth in this species (Schuler, Wirth et al. 1999; Hoffmann and Schuler 2002). Steroid hormone synthesis is a

complex process that requires the coordinated expression of several proteins, regulated by the activation of different intracellular pathways. Steroidogenic endocrine tissues such as the adrenal gland and the gonads respond to trophic hormones and other external stimuli with a rapid surge in steroid hormone production (Steele and Leung 1992; Saez 1994; Ghayee and Auchus 2007).

The steroidogenic response is initiated by steroidogenic acute regulatory protein (STAR), which acts as the rate-limiting step in steroid biosynthesis, regulating the delivery of cholesterol from the outer to the inner mitochondrial membrane (Manna, Dyson et al. 2009). Here, the enzyme CYP11A1 is located in the mitochondria matrix associated with the inner membrane, cleaves the side of chain from cholesterol to form pregnenolone, which is next metabolized to P4 by a dehydrogenase, HSD3B1, (Clark and Cochrum 2007; Rekawiecki, Kowalik et al. 2008; Lavoie and King 2009). Pregnenolone and P4 are considered the basic precursors of other steroid hormones as aldosterone, cortisol, testosterone, estrone and estradiol (Lavoie and King 2009). However, the enzyme SULT1E1 regulates the availability of free estrogens by sulfoconjugation; and the STS enzyme produces estrogen sulphates, which may serve as substrates for the production of free estrogens (Reed, Purohit et al. 2005; Pasqualini 2009).

Our previous findings demonstrated the expression of the three principal steroidogenic markers (STAR, CYP11A1 and HSD3B1) in bovine placenta during the first half of the gestation (unpublished). Steroidogenesis in the gonads and adrenal glands is predominantly mediated through the interaction of luteinizing

hormone (LH) and adrenocortocotropic hormone (ACTH), respectively. LH and ACTH bind to specific membrane receptors, which results in the activation of coupled G proteins in their target tissues, with the consequence of intracellular modifications including the activation of cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) pathways (Nishikawa, Sasano et al. 1996; Luo, Chen et al. 1998; Rekawiecki, Nowik et al. 2005; Miller 2007). Nevertheless, other factors such as epidermal growth factor (EGF), insulin-like growth factor 1(IGF1), prolactin (PRL), gonadotrophin-releasing hormone 1 (GNRH1) and macrophage-derived factor (MDF) have been demonstrated to function in the regulation of steroid biosynthesis in gonads and adrenal glands (Weiss-Messer, Ber et al. 1996; Sekar, Lavoie et al. 2000; Hales 2002; Manna, Dyson et al. 2009). In addition, calcium (Ca^{2+}) signaling has been shown to be involved in the modulation of steroidogenesis (Cherradi and Capponi 1998; Yamazaki, Kawasaki et al. 2006; Manna, Dyson et al. 2009). However, there is no report describing the principal intracellular pathways and factors implicated in the regulation of the steroidogenic process in bovine placenta.

Thus, the goal of this current study was to assess whether hCG and multiple second messenger systems (dbcAMP, PMA and calcium ionophore) regulate the steroidogenic process and the synthesis of progesterone in bovine placenta. In the present study, we investigated the influence of the aforementioned factors upon the expression of genes (*STAR*, *CYP11A1*, *HSD3B1*, *SULT1E1* and *STS*) implicated in the synthesis of steroid hormones.

5.3. Material and methods

Placental samples

Bovine placentas at 140 ±10 days of gestation were collected from normal gestations at a local abattoir (n=3). Gestational age was determined by fetal crown-rump length (CRL) based on the Riding (18) and Evans and Sack charts(Evans and Sack 1973; Riding, Lehnert et al. 2008).

Study design

We developed a placentome explant model of bovine gestation around days 140 ±10 based on previous studies with human and equine placental explants (Fuglsang, Moller et al. 2008; Nash, Lane et al. 2008). Moreover, preliminary studies were performed to validate the functionality and viability of placentome explants; and also to establish the optimal culture conditions.

Tissue culture

The pregnant uteri were placed on ice for transport. Placental tissues were washed and dissected under sterile conditions in Dulbecco's Modified Eagle Medium (DMEM) (1X), high glucose (Invitrogen Life Technologies Corporation, Burlington, ON, Canada). Three placentomes per placenta were collected and used as independent replicates, and treatments were performed in triplicate for each treatment. The placentomes were chopped transversely (to maintain the caruncular-cotyledonary union) into small fragments (approximately 300 ± 20 mg

wet weight) using a tissue slicer (Thomas Scientific, Swedesboro, NJ ,U.S.A.). The tissue explants were placed in six-well tissue culture plates, and were incubated in DMEM supplemented with 10% of fetal bovine serum (FBS, Invitrogen-GIBCO) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37 ◦C in a humidified atmosphere of 95% air and 5% CO2. To reduce the influence of growth factors and steroids in the explants, FBS was treated with charcoal/dextran (Lindquist and de Alarcon 1987).

Treatments and sampling

After a 2 hour (h) conditioning period, the medium was removed and replaced with fresh medium (control), or medium containing the appropriate treatment for 18 h. Dimethyl sulphoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA) was used as a carrier agent in all treatments including the control group. Tissues were treated with 1mM of dbcAMP (Sigma-Aldrich,), 10 nM of PMA (Sigma-Aldrich,), 1 µg/ml of hCG (Calbiochem EMD Bosciences Inc, La Jolla, CA, USA), 10 µg/ml of A23187 (Sigma-Aldrich,), and a combination of hCG +A23187 (1 µg/ml and 1 µg/ml respectively). In cattle it was demostrated that hCG mimics the action of LH, inducing ovulation and then the formation of a functional CL (Price & Webb, 1989; Rajamahendran & Sianangama, 1992; Schmitt*, et al.*, 1996). Moreover hCG acts independently of the pituitary gland and has a longer life (16 h) than natural LH (Haour & Saez, 1977; Kinser*, et al.*, 1983).

Before determining the effect of the hCG, on steroid synthesis in placental explants; the presence of mRNA LH receptor in bovine caruncles and cotyledons was evaluated using tissue from bovine corpus luteum (CL) as a positive control. Culture medium and tissue samples from each explant were collected at 0, 6, 12 and 18 h (T0, T6, T12 and T18) after treatment and stored at -20ºC and -80 ºC until measurement of P4 levels and qPCR analysis, respectively. Treatment concentrations and sampling frequency were determined based on previous studies (Sekar, Lavoie et al. 2000; Rekawiecki, Nowik et al. 2005; Bamberger, Briese et al. 2006; Manna, Jo et al. 2007), and in P4 levels found in pilot studies.

Hormone assay

Concentrations of P4 in culture medium were evaluated by radioimmunoassay according to previously described procedure (Pescador, Houde et al. 1997; Ruiz-Cortes, Martel-Kennes et al. 2003). The progesterone concentrations were calculated as pg/ml per 1 mg of tissue. The P4 tracer was purchased from PerkinElmer (NET 381250UC, Waltham, Massachusetts, USA) and the P4 antibody was gently provided by Dr. Gordon Niswender, Colorado State University. The intra-assay and inter-assay were on average 3.38% and 4.75% respectively.

RNA isolation and Quantitative real time PCR (qPCR)

Total RNA was extracted from cultured tissues, using an RNeasy Mini Kit (Qiagen Mississauga, ON, Canada), according to the manufacturer's

recommendations. Disruption, homogenization and purification of tissue were achieved with mortar and pestle, and QIAshredder columns (Qiagen). To remove the proteins which could interfere with the RNA isolation, proteinase K digestion (Invitrogen) was used. DNase I treatment was applied on the columns to eliminate any contaminating DNA (RNase-free DNase Set, Qiagen,). Amounts of RNA were quantified by spectophotometry, using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and 1 μg/sample of total RNA was reverse transcribed to complementary DNA (cDNA) using Superscript II Reverse Transcriptase (Invitrogen).

The abundance of transcripts for luteinizing hormone/choriogonadotropin receptor (*LHCGR*), *STAR*,*HSD3B1*, *CYP11A1*, *STS*, *SULT1E1*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and actin, beta (*ACTB*) were quantified by qPCR using the 7300 Real Time PCR thermal cycler (Applied Biosystems Foster City, Ca, USA). Table 1 shows the sequences of the primer pairs and product sizes. Bovine *LHCGR*, *STAR*, *CYP11A1*, *HSD3B1*, *STS* and *SULT1E1* primers were designed from published bovine sequences (Shemesh, Gurevich et al. 1997; Nishimura, Sakumoto et al. 2006; Orisaka, Tajima et al. 2006; Greven, Kowalewski et al. 2007; Hirayama, Sawai et al. 2008; Murayama, Miyazaki et al. 2008). *CYP17A1*, *GAPDH* and *ACTB* primers were designed based on bovine genes sequences from GenBank, using the Primer Express software Version 3.0 (PE Applied Biosystems).The qPCR products from *CYP17A1*, *GAPDH* and *ACTB* were validated by sequencing (The McGill University and Génome Québec Innovation Centre, Montreal, QC). Primer

concentrations were optimized according to the manufacturer's recommendations (Applied Biosystems) and dissociation curve analysis and validated by electrophoresis. The PCR efficiency (E= 1.9-2.0) and linearity of each primer were tested by preparing a series of solutions of the same stock of cDNA used for the qPCR gene analysis. The geometric mean of two reference genes (*GAPDH* and *ACTB*) was used as a normalization factor. Relative quantification method ($\Delta\Delta C_t$) was used to determine changes in target gene expression levels (Livak and Schmittgen 2001; Pfaffl 2001). Data analysis was performed using the Sequence Detection Software version 1.4 (PE Applied Biosystems) and the LinRegPCR Software version 11.1 (Heart Failure Research Center Academic Medical Centre Amsterdam, the Netherlands). Reactions for qPCR were conducted in sealed 96-well multiply (SARSTEDT INC, Montreal, Canada) in triplicate. The total volume of the qPCR reaction was 20 µl, comprising 6 µl cDNA template (diluted 1 in 30 in distilled water), 2 µl each forward and reverse primers, 2 µl of distilled water and 10 µl Power SYBR green PCR master mix (Applied Biosystems,). Cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C.

Pilot studies

The aims of the pilot studies were to establish the concentrations and timing of sampling collection during experimental treatments, based in the progesterone levels. Placentome explants were treated with three different concentrations of each treatment solution: dbcAMP (10 µM, 100 µM and 1 mM), PMA (1 nM, 10 nM

and 100 nM), hCG (10 ng/ml, 100 ng/ml and 1µg/ml) and A2387 (100 ng/ml, 1µg/ml 10 µg/ml). The timing of sampling collection for the treatments was 1 and 6 h post-treatment and there P4 concentrations in culture medium were determined by RIA. The placentome culture conditions and RIA were done by the above-mentioned methods. Figure 1 shows the treatments, concentrations and sampling times.

Statistical Analysis

Progesterone concentrations and gene expression are represented as means ± standard error (±S.E.M.). Each experiment was performed in triplicate and statistical analysis was carried out using SigmaPlot (version 11.2, Systat software, Inc. Germany). A normal distribution (Shapiro-Wilk test) was performed before a parametric analysis. One-way ANOVA and Dunnett's Method were applied to determine significant differences ($P < 0.05$) between treatments versus control group. For pilot studies a Kruskal-Wallis One Way Analysis of Variance on Raks and Dunn's Method were used.

5.4. Results

In the pilot studies, treatments with dbcAMP, PMA and hCG at different concentrations did not affect the progesterone levels in culture medium with respect to the control group of any studied times. Conversely a high dose (10µg/ml) of A2387 consistently led to increase P4 content in the culture media at

1 and 6 hours after treatment (Figure 1). Based on the aforementioned results we chose the best concentrations for the present study.

Steroidogenic genes expression

PCR demonstrated that the *LHR* mRNA is present in the bovine placenta, (caruncles and cotyledons) (Fig.2). qPCR method was used to test whether dbcAMP, PMA, hCG, A23187 and hCG +A23187 affect the expression of steroidogenic markers in placental explants. Concentrations of mRNA for *CYP11A1* were higher (P<.01) after treatment with hCG, A23187 and hCG +A23187, and with hCG and A23187 at time 6 h and 12h respectively (Figure 3). Moreover mRNA for *HSD3B1* increased (P<.05) after A23187 and hCG +A23187 treatments at 6h; and with hCG, A23187 and hCG +A23187 at 12 h post treatment (Figure 3). Figure 4 illustrates the stimulatory impact of exposure to hCG, A23187 and hCG +A23187 on *SULT1E1* mRNA expression at time 6 h and 12 h; and to hCG at time 18 h. The *CYP11A1*, *HSD3B1* and *SULT1E1* mRNA expression was decreased in the A23187 group, 18 h after treatment (Figure 3 and 4) (P<.05). None of the treatments had an impact in the (mRNA) *STS* expression at all investigated periods (Figure 4). As shown in figure 5-A, hCG, A23187 and hCG +A23187 significantly increased (P<.05) *STAR* mRNA after 18 h of incubation.

Progesterone concentrations

Only calcium ionophore stimulated the secretion of progesterone levels (P<.001) in culture media at all times investigated (Figure 5-B). P4 concentrations

after hCG and hCG +A23187 treatments appeared to be higher compared with control group at all periods, however no significant differences were observed. Apparently, the second messenger analogs dbcAMP and PMA did not alter the progesterone levels or the expression of any of the steroidogenic genes analyzed.

5.5. Discussion

It is well known that bovine placenta produces estrogens and progesterone (Hoffmann and Schuler 2002) but, despite extensive study, the mechanisms of steroidogenesis in the bovine placenta remain unclear. In our previous studies, we investigated the expression of the principal steroidogenic markers (*STAR, CYP11A1* and *HSD3B1)* in bovine placenta, and also the principal cells implicated in the steroidogenic process in bovine placenta during the first half of pregnancy (Verduzco, Fecteau et al. 2010). The purpose of these experiments was to elucidate the principal factors implicated in the control of placental steroidogenesis.

In the present experiment, even if no significant differences were found on P4 concentrations after hCG and hCG +A23187 treatments, the P4 levels tended to be higher compared with control group at all periods. Furthermore a significant increase in *STAR*, *CYP11A1* and *HSD3B1* gene expression stimulated by hCG was observed at different sampling times. These observations are wholly consistent with those reported by other authors, which found that LH stimulated secretion of P4 and increased the *STAR*, *CYP11A1*and *HSD3B* mRNA and protein expression in bovine luteal cells, mouse Leydig tumor cells (MA-10), human luteal cells and

porcine granulosa-luteal cells (Ravindranath, Little-Ihrig et al. 1992; Ivell, Tillmann et al. 2000; Sekar, Lavoie et al. 2000; Hauet, Yao et al. 2005; Rekawiecki, Nowik et al. 2005).

In cattle, the preovulatory surge of LH is necessary for the luteinization of follicular cells and for CL maintenance (Rekawiecki, Kowalik et al. 2008). The presence of LHR has been shown in a very wide range of bovine tissues such: ovary, adrenal glands, endometrium, myometrium, uterine blood vessels, cervix and uterine veins (Little, Rahe et al. 1982; Schallenberger, Rampp et al. 1985; Shemesh, Gurevich et al. 1997), but the presence of (mRNA) for LH receptors in placenta appear not to have previously been shown. In human, placental expression of the LH receptors in trophoblast cells, cytotrophoblasts and syncytiotrophoblast have been reported (Pidoux, Gerbaud et al. 2007). In our understanding the present work demonstrated for the first time that the *LHR* mRNA is present in bovine placenta, however, western blot and binding assays will be required to authenticate the presence of the LHR in bovine placenta.

Surprisingly, and contrary to the notion that binding of LH to its cell-surface G protein-coupled receptor activates adenylyl cyclase resulting in activation of cAMP-dependent protein kinase (PKA) to stimulate P4 synthesis (Rekawiecki, Kowalik et al. 2008); in the present study hCG treatment increased the expression of steroidogenic markers but not the P4 levels. Moreover, in the present report placental explants treated with dbcAMP did not have any impact in the P4 levels and neither in the expression of steroidogenic genes. These findings suggest that

LH and its second messenger cAMP, apparently are not the principal factors that control the steroidogenic process in bovine placenta. In this regard, some authors found that during bovine pregnancy LH concentrations decreased as pregnancy progress (Little, Rahe et al. 1982; Schallenberger, Rampp et al. 1985), and apparently luteal progesterone secretion at day 200 of gestation, is done via prostaglandin E_1 (PTG E_1) and prostaglandin E_1 (PTG E_2) , and not via LH (Weems, Lammoglia et al. 1998).

An extensive amount of evidence indicates that trophic hormones can also activate the protein kinase C (PKC) signaling pathway (Manna, Jo et al. 2007; Manna, Dyson et al. 2009; Manna, Huhtaniemi et al. 2009) (Morley, Hobkirk et al. 2000; Rekawiecki, Kowalik et al. 2008). Nonetheless, in the present study we observed that P4 concentrations and genes involved in steroidogenesis were not affected by PMA treatment. PMA is a phorbol ester activator that acts as a second messenger analogue in the PKC pathway (Niedel, Kuhn et al. 1983; Ballester and Rosen 1985).

These results imply that in bovine placenta, P4 synthesis could be modulated by cAMP and PKC independent pathways. In this context, some authors found that activation of extracellular-signal-regulated kinases (ERK)/mitogen activated protein kinase (MAPK) and P13K/Akt pathways, modulate the synthesis of steroids in bovine granulosa and theca cells (Tajima, Yoshii et al. 2005; Fukuda, Orisaka et al. 2009). Moreover LH has been shown to stimulate PLC- inositol phosphate pathway by inositol phosphate accumulation and intracellular Ca^{2+} in

bovine luteal cells (Nishimura, Shibaya et al. 2004) (Davis, May et al. 1996). In this regard, in our experiment, calcium ionophore was the only treatment that significantly increased P4 concentrations with respect to the control group. We also observed an increase in mRNA abundance of *STAR* at 18 h of treatment and in mRNA abundance of *CYP11A1* and *HSD3B1* after 6 h and 12 h of treatment in the explants cultures treated with A23187. These findings concur with a previous investigation in bovine placenta, where authors examined the effects of the calcium ionophore on P4 formation, and found that this drug enhanced progesterone formation (Shemesh, Hansel et al. 1984). Our data support the hypothesis proposed by the aforementioned authors, that progesterone synthesis in the bovine placentome is calcium dependent and cyclic nucleotide independent. In human placenta, calcitriol, which results from the conversion of 25-hydroxyvitamin D_3 (25-OHD_3) into 1,25-dihydroxyvitamin D_3 (calcitriol) promotes calcium transport in the placenta and is a physiological regulator of steroidogenic enzymes, and consequently stimulates estrogens and P4 synthesis (Dusso, Brown et al. 2005; Barrera, Avila et al. 2007) .

It would be conceivable that calcium influx either directly or indirectly is regulated by other factors and mechanisms. In fact, PGE(1), PGE(2), epidermal growth factor (EGF), insulin-like growth factor (IGF), prolactin (PRL), gonadotrophin-releasing hormone (GnRH) and macrophage-derived factors (MDFs) have been demonstrated to function in the regulation of steroid

biosynthesis in gonads and adrenal glands (Weiss-Messer, Ber et al. 1996; Sekar, Lavoie et al. 2000; Hales 2002; Manna, Dyson et al. 2009).

In our studies, we did not observe a synergistic or additive effect of hCG and calcium ionophore (hCG +A23187), however, we used a lower dose of calcium ionophore for the hCG +A23187 than in A23187 treatment alone. For that reason we cannot exclude the possibility that LH could act concomitantly with calcium, increasing the expression of stereroidogenic genes and the P4 synthesis.

Treatment of explants cultures with hCG, A23187 and hCG +A23187 increased mRNA abundance for *SULT1E1* for almost all studied periods. This is the first evidence linking increased *SULT1E1* expression to LH and calcium influx. Estrogen sulfotransferase is a metabolic enzyme that transforms active estrogens into sulfoconjugated estrogens which then lose their ability to interact with the estrogen receptor and are hormonally inactive. Sulfated estrogens act as regulators to attenuate the local estrogen response, and they could also be precursors of the production of free estrogens depending, on estrogen sulfatase activity (Rossier and Pierrepoint 1974*).* Non-specific stimulatory effects on (mRNA) STS expression were observed at any treatment. Previously, it was demonstrated that SULT1E1 is expressed in cotyledonary tissue from midgestation to parturition (Hoffmann, Falter et al. 2001), however, there is lack of data about the mechanisms that control this enzyme in bovine placenta. In mouse Leydig cells estrogen sulfotransferase expression is under control of LH (Song, Qian et al. 1997; Qian and Song 1999), but the regulation of this enzyme by LH in placenta is not clear. In addition, in guinea

pigs, estrogen sulfotransferase is detectable in uterus and chorion, and is stimulated by the presence of Mg2+, Ca2+ or Mn2+ (Freeman, Saidi et al. 1983). Interestingly, in the present experiment the stimulatory influence of A23187 on *CYP11A1*, *HSD3B1* and *SULT1E1* gene expression decreased significantly at time 18 h compared to the control group. The abundance and stability of mRNA is determined by multiple processes, including fluctuations in their half-lives in response to developmental environmental stimuli like nutrient levels, cytokines, hormones and temperature shifts as well as environmental stresses such as hypoxia, hypocalcemia, viral infection, and tissue injury (Guhaniyogi and Brewer 2001). In the present study, the decline in the mRNA might have been due to half-life in the target mRNAs, whose expression may have been controlled by regulating mRNA turnover of the steroidogenic genes in response to calcium stimulus. Further critical analysis of the data along with the performance of additional analyses of different samples will be required to corroborate this phenomenon confidently.

Although we did not measure the protein abundance of each steroidogenic protein, the mRNA expression levels of *StAR*, *CYP11A1* and *HSD3B1* stimulated by calcium ionophore are expected to be a gross indicator of the changes in progesterone found in this experiment, because these three proteins are fundamental to P4 synthesis. Conversely, in the present study, we did not measure concentrations of estrone sulphate in culture media, and for that reason additional studies will be required to confirm the stimulatory action of LH and calcium in bovine placenta to produce sulfoconjugated estrogens.

In conclusion, hCG alone or in combination with calcium influx, increases mRNA of steroidogenic genes but not P4 levels. The second messenger analogues dbcAMP and PMA did not have an impact in the steroidogenic process in bovine placental explants, but calcium ionophore exerted a genomic regulatory effect by increasing the transcript abundance of steroidogenic genes, and stimulating P4 biosynthesis in placental explants. Taken together, the data point that calcium has a pivotal role for intracellular signaling in the steroidogenic biosynthesis in bovine placenta. Additional studies will be necessary to unravel the exact mechanism by Ca^{2+} the steroid synthesis in bovine placenta.

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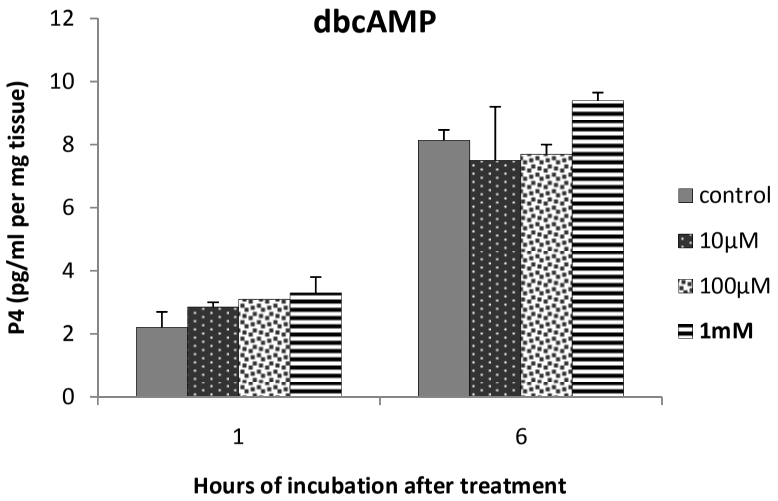
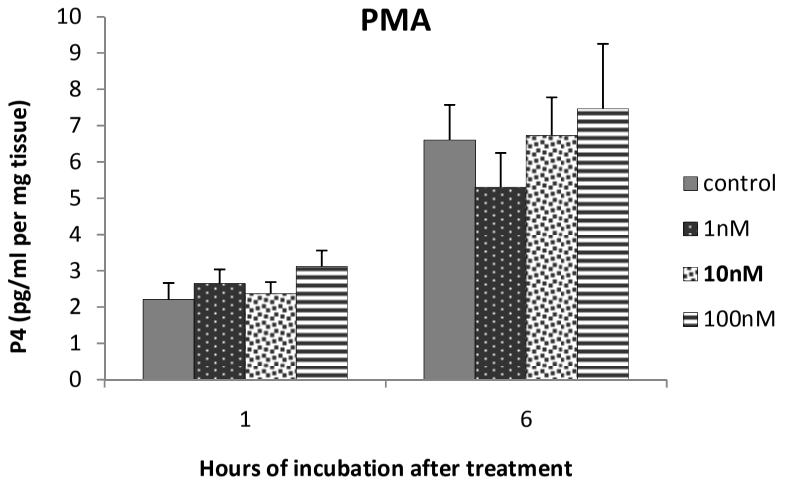
5.7. Tables and figures

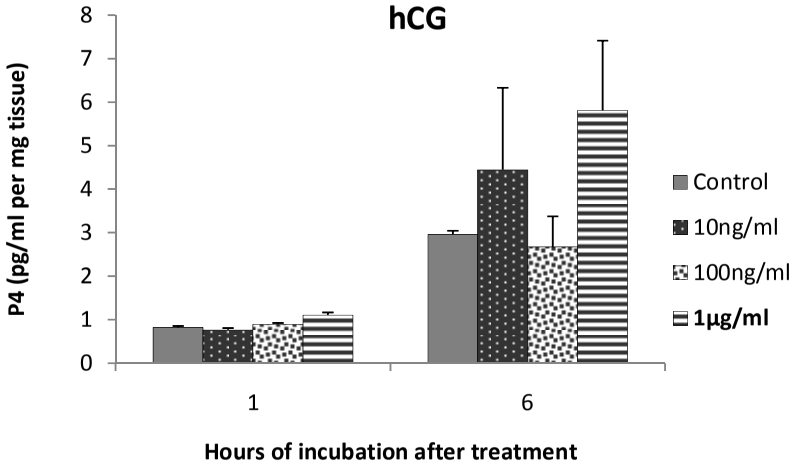
Table 1. Primer pairs used for RNA detection

Genes	qPCR primers sequence (5'–3')	Fragment size (base pairs)	References/accession no.	Primer concentration
ACTB	FWD GGATGAGGCTCAGAGCAAGAGA	58	NM_173979	FWD 3 µM
	REV TCGTCCCAGTTGGTGACGAT			REV 3 µM
CYP11A1	FWD CTGCAAATGGTCCCACTTCT	209	Murayama et al. (2008)/ NM_176644	FWD 9 µM
	REV CACCTGGTTGGGTCAAACTT			REV 1.5 µM
CYP17A1	FWD CCATCAGAGAAGTGCTCCGAAT	79	NM_174304	FWD 3 µM
	REV GCCAATGCTGGAGTCAATGA			REV 3 µM
GAPDH	FWD GGCGTGAACCACGAGAAGTATAA	100	NM_001034034	FWD 9 µM
	REV CCCTCCACGATGCCAAAGT			REV 3 µM
HSD3B1	FWD GCCCAACTCCTACAGGGAGAT	114	Orisaka et al. (2006)/ NM_174343.2	FWD 1.5 µM
	REV TTCAGAGCCCACCCATTAGCT			REV 3 µM
LHCGR	FWD CACCCTCACAGTCATCACAC	552	Shemesh et al. (1997)/NM_174381.1	FWD 10 µM
	REV CTCAGCAACAGAAAGAAATC			REV 10 µM
STAR	FWD CCCATGGAGAGGCTTTATGA	115	Nishimura et al. (2006) / NM_174189.2	FWD 9 µM
	REV TGATGACCGTGTCTTTTCCA			REV 3 µM
STS	FWD GCCTCCTGCTCGGCTTTC	65	Greven et al (2007)/DQ532016.1	FWD 3 µM
	REV TGTTCACGGTTCCGCATCA			REV 9 µM
SULT1E1	FWD CCTGAGTGGGGATTGAAGAA	412	Hirayama et al (2008)/NM_177488	FWD 3 µM
	REV CACAGGCAGGTGAGACTTCA			REV 3 µM

FWD: forward. REV: reverse.


dbcAMP
P4 (pg/ml per mg tissue)
12
10
8
6
4
2
0
1
6
Hours of incubation after treatment
control
10µM
100µM
1mM


PMA
P4 (pg/ml per mg tissue)
10
9
8
7
6
5
4
3
2
1
0
1
6
Hours of incubation after treatment
control
1nM
10nM
100nM


hCG
P4 (pg/ml per mg tissue)
8
7
6
5
4
3
2
1
0
1
6
Hours of incubation after treatment
Control
10ng/ml
100ng/ml
1µg/ml

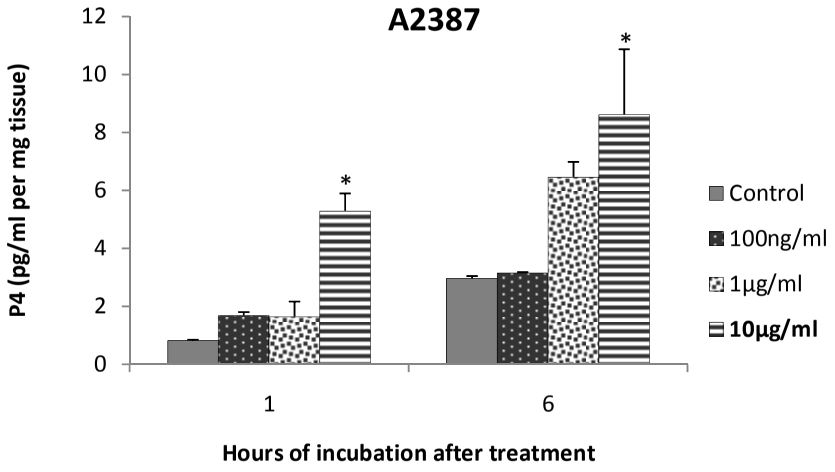

A2387
P4 (pg/ml per mg tissue)
12
10
8
6
4
2
0
*
*
1
6
Hours of incubation after treatment
Control
100ng/ml
1µg/ml
10µg/ml

Figure 1. Pilot studies to determine the influence of different concentrations of dbcAMP, PMA, hCG and A2387 on P4 concentrations in bovine placental explants after 1 and 6 hours post-treatment. Values are expressed as means ± S.E.M for P4 synthesis by two placentomes per each placenta (one placenta per treatment) around day 140 ±10 of gestation. Significant differences ($P<0.05$) between control group and treatments are indicated by an asterisk. Bold letters in treatments legends represent the best concentration.

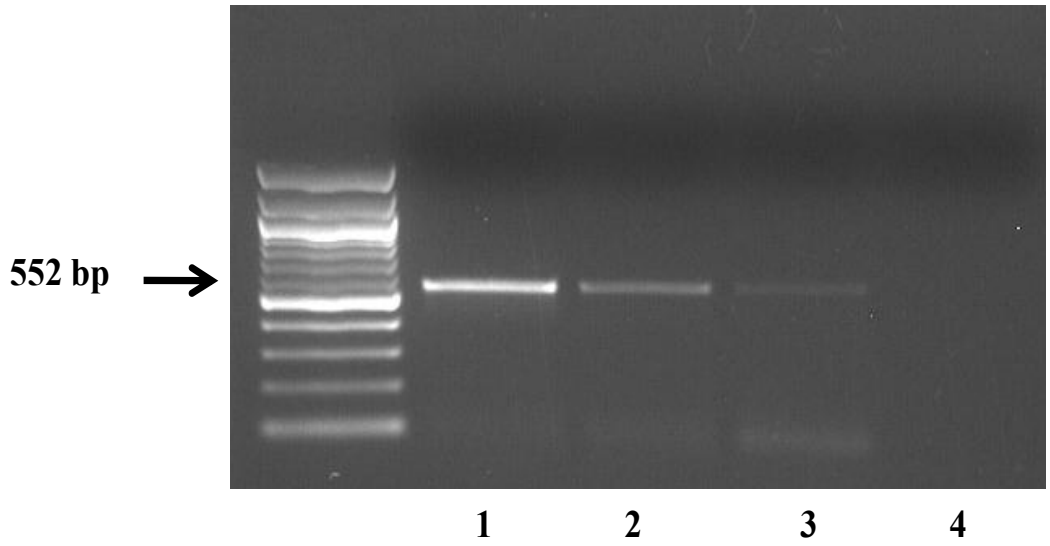


Figure 2. Quantification of *LHR* gene expression. RT-PCR was performed using pool of mRNA extracted from corpus luteum (lane 1), caruncular (lane 2) and cotyledonary tissues (lane 3) from 3 cows. Placental samples were obtained from gestations around day 50 to 120. Lanes 1 and 4 represent positive and negative control respectively. PCR products of qPCR amplification (estimated 552 bp) were stained with ethidium bromide and separated on agarose gel. bp: base pairs.

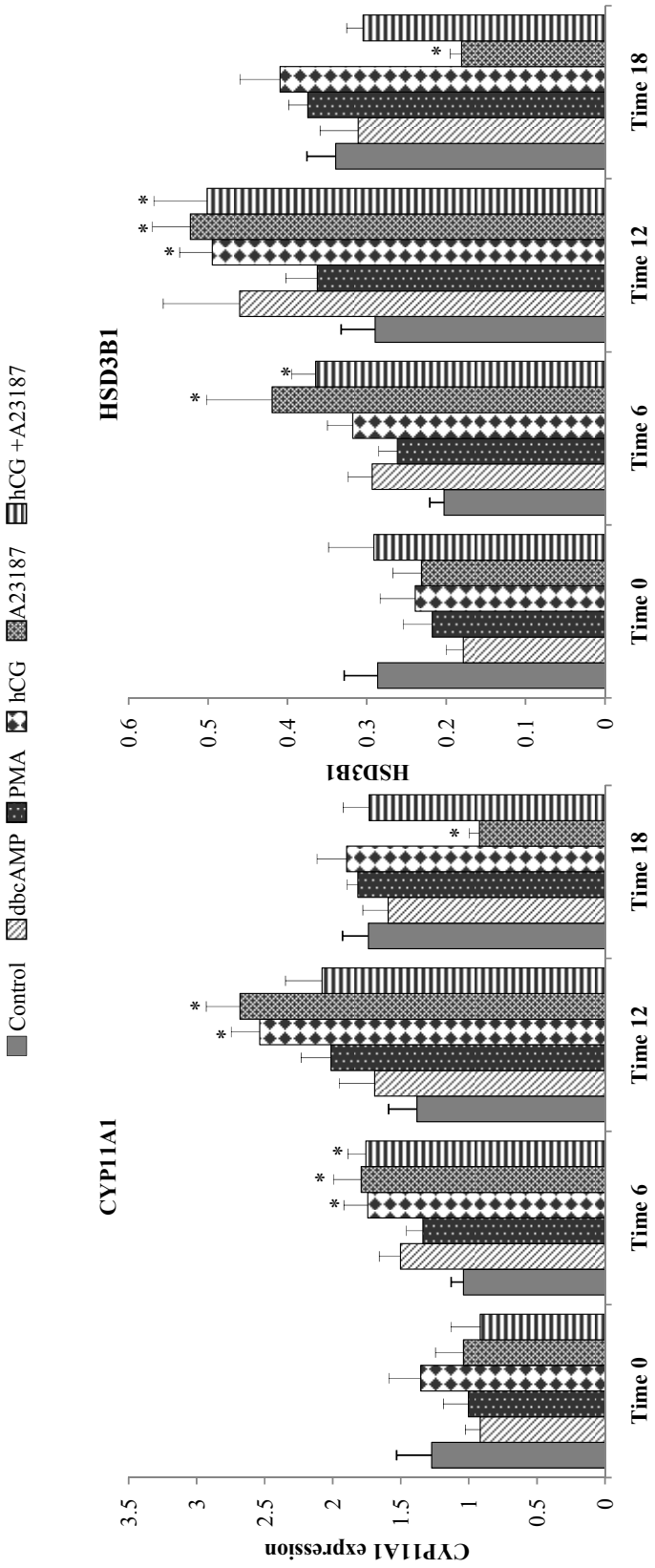

Control
dbcAMP
PMA
hCG
A23187
hCG +A23187
CYP11A1
HSD3B1
CYP11A1 expression
HSD3B1
Time 0
Time 6
Time 12
Time 18
0
0.5
1
1.5
2
2.5
3
3.5
0.1
0.2
0.3
0.4
0.5
0.6
*

Figure 3. Expression of CYP11A1 and HSD3B1 mRNA in bovine placental explants around day 140 ±10 of gestation. Explants were treated for 6, 12 and 18 h with dbcAMP (1 mM), PMA (10 nM), hCG (1 μg/ml), A23187 (10 μg/ml) and hCG +A23187 (1 μg/ml and 10 μg/ml). Significant differences ($P<0.05$) between control group and treatments are indicated by an asterisk. The data are expressed as the mean ± S.E.M for CYP11A1 and HSD3B1 mRNA expression in three placentomes per each placenta (3 placentas per treatment). Results are shown as relative mRNA expression for each gene normalized to the reference genes (ACTB and bovine GAPDH).

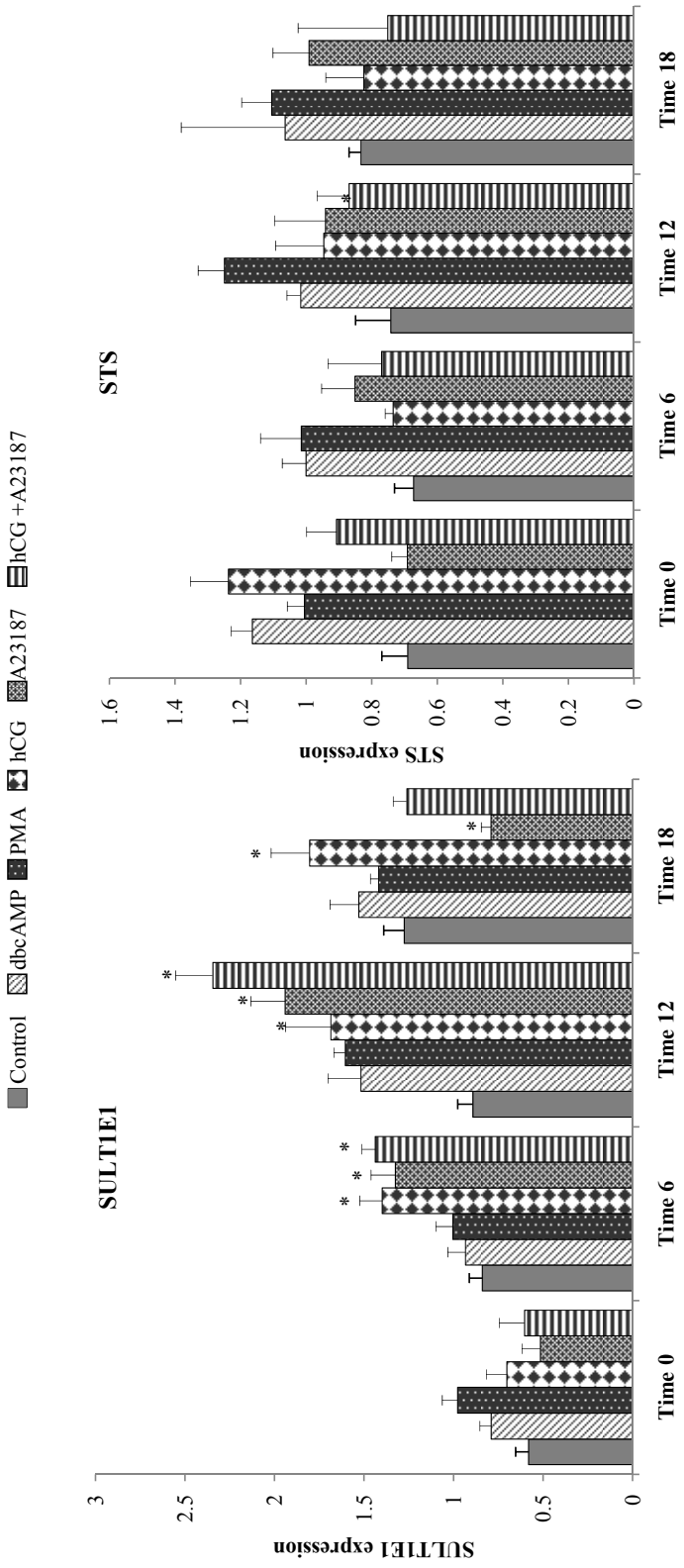
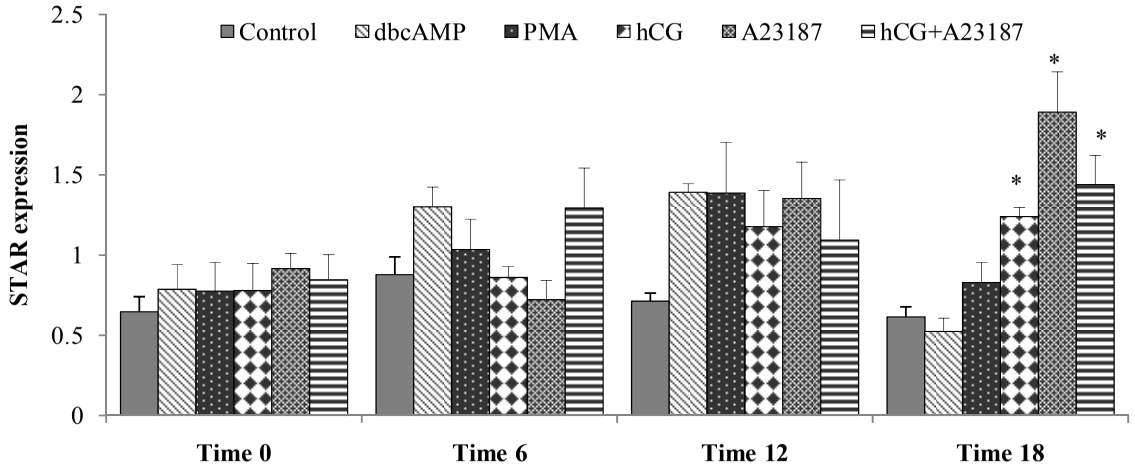

Control
dbcAMP
PMA
hCG
A23187
hCG +A23187
SULT1E1
SULT1E1 expression
3
2.5
2
1.5
1
0.5
0
Time 0
Time 6
Time 12
Time 18
STS
STS expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Time 0
Time 6
Time 12
Time 18

Figure 4. Expression of SULT1E1 and STS mRNA in bovine placental explants around day 140 ±10 of gestation. Explants were treated for 6, 12 and 18 h with dbcAMP (1 mM), PMA (10 nM), hCG (1 μg/ml), A23187 (10 μg/ml) and hCG +A23187 (1 μg/ml and 10 μg/ml). Significant differences ($P<0.05$) between control group and treatments are indicated by an asterisk. The data are expressed as the mean ± S.E.M for SULT1E1 and STS mRNA in three placentomes per each placenta (3 placentas per treatment). Results are shown as relative mRNA expression for each gene normalized to the reference genes (bovine ACTB and bovine GAPDH).

A


Control
dbcAMP
PMA
hCG
A23187
hCG+A23187
STAR expression
2.5
2
1.5
1
0.5
0
Time 0
Time 6
Time 12
Time 18
*

B

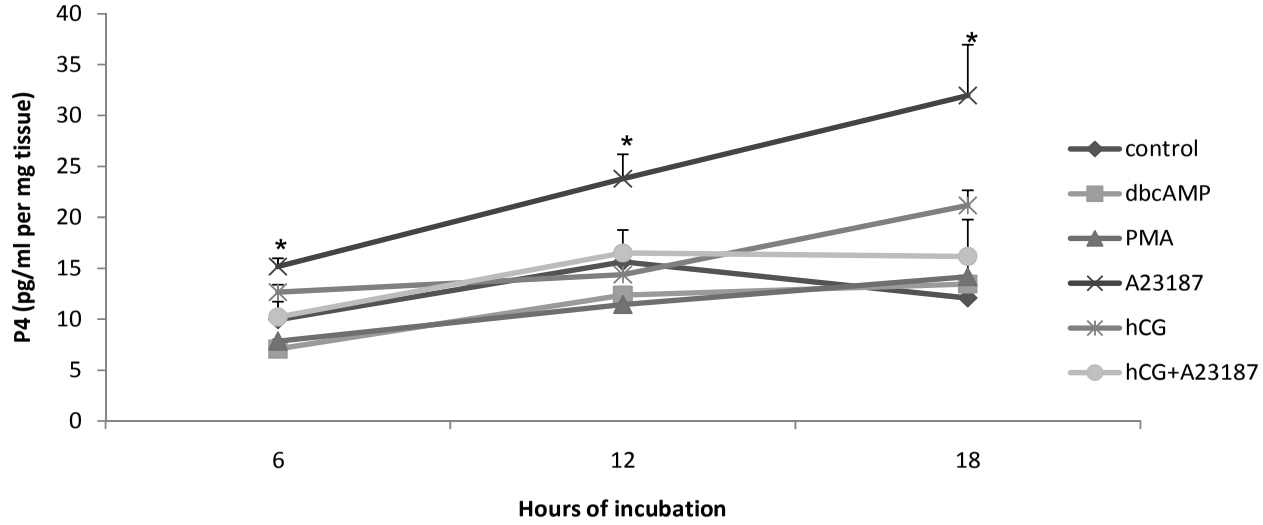

P4 (pg/ml per mg tissue)
40
35
30
25
20
15
10
5
0
6
12
18
Hours of incubation
control
dbcAMP
PMA
A23187
hCG
hCG+A23187
*

Figure 5. STAR mRNA abundance and P4 concentrations in placentome explants around day 140 ±10 of gestation. STAR mRNA abundance (Panel A) and P4 levels (Panel B) at 6, 12 and 18 h post-treatment .Placentome explants from 3 bovine gestations around day 140 ±10 treated with dbcAMP (1 mM), PMA (10 nM), hCG (1 μg/ml), A23187 (10 μg/ml) and hCG +A23187 (1 μg/ml and 10 μg/ml). The data are expressed as the mean ± S.E.M for STAR mRNA expression and P4 synthesis in three placentomes per each placenta (3 placentas per treatment). Significant differences ($P<0.05$) between control group and treatments are indicated by an asterisk. Results of qPCR (5-A) are shown as relative mRNA expression for each gene normalized to the reference genes. Bovine ACTB and GAPDH served as loading and constitutive control.

CHAPTER 6. GENERAL DISCUSSION

Although the physiological role of steroids in gonads and adrenal glands has been studied extensively in cattle, the biological role of placental steroids and the principal factors and intracellular pathways implicated in the steroidogenic process in bovine placenta are still not completely understood.

In the cow, corpus luteum is the main source of progesterone, which is essential for establishing and maintaining pregnancy (Estergreen*, et al.*, 1967; Chew*, et al.*, 1979). Nevertheless, some studies demonstrated that bovine pregnancy could survive luteolysis and ovariectomy during the last 70 days of gestation (Day, 1977; Chew*, et al.*, 1979), indicating that the placenta could provide the steroid support required for the maintenance of gestation. For many years this concept was discarded, because levels of progesterone in uterine arteriovenous system in bovine placenta apparently had not been altered (Comline*, et al.*, 1974; Ferrell*, et al.*, 1983). Subsequent in vitro studies demonstrated that bovine placental cells were capable of producing estradiol, estrone and progesterone in late gestation (Evans & Wagner, 1981; Gross & Williams, 1988; Matamoros*, et al.*, 1994). Furthermore, some enzymes and proteins, that are pivotal in the production of steroid hormones have been detected in bovine placental tissue in late gestation and at parturition (Gross & Williams, 1988; Pescador*, et al.*, 1996; Pilon*, et al.*, 1997; Takagi*, et al.*, 2007). However, very little is known about the production of these hormones in early pregnancy. In addition, placental abnormalities in cattle following inappropriate steroid synthesis in bovine placenta have not been yet demonstrated.

The overall purpose of this research was to better understand the regulatory mechanisms of steroidogenesis in bovine placenta during early gestation. In addition, we investigated whether inappropriate synthesis of steroid hormones could lead to placental abnormalities reported in SCNT pregnancies.

The major findings of this thesis are summarized as follows:

1. The maternal and fetal cells involved in steroid hormone biosynthesis from day 50 to day 120 of pregnancy were identified.
2. Alterations in the expression of some steroidogenic proteins and genes in SCNT derived placenta at day 40 of pregnancy were described.
3. The factors controlling the synthesis of progesterone in bovine placentome explants at 140 $\pm$10 days of gestation were investigated.

Identification of the maternal and fetal cells involved in steroid hormone biosynthesis from day 50 to day 120 of pregnancy (Chapter III and IV)

In the chapter III (first article) we determined the principal cells implicated in the placental steroid synthesis and we investigated the expression of candidate steroidogenic proteins (STAR, CYP11A1and HSD3B1) in bovine placentomes from day 50 to day 120 of pregnancy. The expression of the aforementioned proteins led us to determine, if progesterone or its precursor, pregnenolone, could be produced by bovine placenta in early gestations. We used techniques that included immunohistochemistry, quantitative PCR (qPCR) and immunoblot to achieve this objective.

Immunohistochemistry results showed the presence of the three steroidogenic proteins at interdigitations of the feto-maternal interface at all periods investigated. Moreover, the principal cells that expressed all the markers were the uninucleate trophoblast cells and the caruncular epithelial cells. In contrast to the notion that in bovine placenta the steroids are produced by the trophoblast giant cells (Reimers, *et al.*, 1985; Wooding, 1992), our results demonstrated that in early gestation these cells do not participate directly in pregnenolone or progesterone synthesis. The evidence for this conclusion was the lack of expression of any of the three steroidogenic markers. Transcripts and protein abundance of the steroidogenic markers were analyzed separately in caruncular and cotyledonary tissues. Protein and mRNA of STAR and CYP11A1 were found in caruncles and cotyledons from 50 to 120 days of gestation. In contrast, HSD3B1 protein expression was only found in caruncles. It therefore seems that the sites of synthesis of placental steroids differ from other species. In rodents and mice, steroidogenic markers (STAR, CYP11A1 and HSD3B1) are expressed in the fetal side specifically in the trophoblast giant cells, while HSD3B1 appears only transiently in the maternal deciduas (Arensburg, *et al.*, 1999).

Our results suggest that even if the two placental compartments have the capacity to synthesize pregnenolone, the only compartment capable to produce progesterone is the maternal (caruncle). Moreover, a complementary pattern in expression of STAR and CYP11A1 proteins between caruncular and cotyledonary tissues across gestation was observed in the present study. These data show that apparently there are clear functional distinctions in terms of steroidogenic capacity

of caruncles and cotyledons of the bovine placenta. The findings of our experiment open new perspectives on the requirement of cell to cell communication between the maternal and fetal cells to produce steroid hormones, and further research is needed to identify the underlying processes on a molecular level.

In Chapter IV (second article), we evaluated the presence of SCARB1, CYP11A1 and CYP17A1 in bovine cotyledons at day 40 of gestation using immunohistochemistry. The overall purpose of this evaluation was to determine the presence at cellular level of the abovementioned receptor and enzymes, all of which are involved in the selective absorption of cholesterol, and in the synthesis of pregnenolone, androgens and some steroid precursors.

Protein localization of SCARB1, CYP11A1 and CYP17A1 was restricted to uninucleate trophoblast cells indicating that in cotyledons, these are the main cell type responsible for selective absorption of cholesterol into the cell, and also for pregnenolone and estrogen precursor synthesis in early gestation. These results agree partially with some reports (Ben-David & Shemesh, 1990; Schuler*, et al.*, 2006) in bovine placenta, which demonstrated that CYP17A1 and CYP11A1 were exclusively found in the uninucleate trophoblast cells. To our knowledge, this is the first time that SCARB1 was examined in bovine placenta. Furthermore, the results of the present study are of interest in advancing our current understanding of how cholesterol absorption and steroid synthesis is achieved in cotyledonary tissue at day 40 of pregnancy.

Taken together, this study provides new information into the cellular origin of placental steroid synthesis, and the steroidogenic capacity of the feto-materno-placental unit from day 50 to day 120 of pregnancy.

Alterations in the expression of some steroidogenic proteins and genes in SCNT derived placenta at day 40 of pregnancy (Chapter IV)

The cloning of cattle by SCNT has been strongly associated to placental abnormalities, which can cause early and fetal losses (Hill*, et al.*, 2000; Heyman*, et al.*, 2002; Kohan-Ghadr*, et al.*, 2008). Despite the fact that placental steroids are important factors controlling placental and fetal growth in different species, no reports have appeared documenting altered expression of steroidogenic proteins and enzymes in SCNT bovine placenta.

In the second manuscript (Chapter IV), we compared the expression of a series of genes and proteins coding for steroidogenic proteins between SCNT and non-clone pregnancies in cotyledonary tissue at day 40. Using qPCR, we identified a significant reduction in the abundance of transcripts for STAR, CYP11A1, HSD3B1, CYP17A1 and SULT1E1 in placentas from SCNT compared to AI gestations. Previous studies, reported deregulation of imprinted and non-imprinted genes, essential in the regulation of placentogenesis, fetogenesis, angiogenesis, mammogenesis, steroidogenesis, and immune system activity in the SCNT bovine gestations (Hashizume*, et al.*, 2002; Patel*, et al.*, 2004; Constant*, et al.*, 2006; Hoffert-Goeres*, et al.*, 2007; Palmieri*, et al.*, 2008; Ledgard*, et al.*, 2009; Guillomot*, et al.*, 2010).

To our knowledge, this is the first study to document clearly the atypical expression of some of principal steroidogenic genes in bovine placenta from early SCNT pregnancies. The aberrant expression of the above mentioned genes would cause insufficiency in some proteins and enzymes, which participate in the synthesis of diverse steroids as: pregnenolone, progesterone, estrogen precursors and estrone sulfate. Thereby, steroidogenic dysfunctional genes might lead to a wide range of effects on placental and fetal growth that could compromise the proper progression of pregnancy at short or long term. However, the western blot results did not show significant differences in STAR, CYP11A1 and HSD3B1 abundance in SCNT gestations versus non-clone pregnancies. Although no significant difference in expression of HSD3B1 protein was found, one sample from SCNT group expressed this enzyme strongly. This fact suggests the possibility that not all cloned embryos have the same disturbances, since the variation in mean expression levels could mask gene expression abnormalities present in individual clones. Some authors (Campos*, et al.*, 2010), have noted the importance of individual analyses of cloned animals. Moreover, differences found between early and late gestations, may be associated with individual compensatory mechanisms that could be triggered during gestation in order to overcome disrupted expression of important genes in the placenta, as previously reported (Constant*, et al.*, 2006).

Interestingly, and in disparity with our results, some authors reported upregulation of SULT1E1 and CYP11A1 mRNA expression in SCNT preterm and term placentas (Everts*, et al.*, 2008; Hirayama*, et al.*, 2008). The differences found between SCNT early and late gestation might be associated with individual

compensatory mechanisms that could be triggered during gestation in order to overcome disrupted expression of important genes in the placenta, as previously reported (Constant*, et al.*, 2006).

In summary, disruption in the expression of important steroidogenic markers in SCNT placenta could be translated into insufficient biosynthesis of steroid hormones, and also in inadequate placental and fetal growth. However, whether trigger of the aberrant gene expression is part of combination of insufficient reprogramming that could be key origin of placental dysfunction, or just a consequence of abnormal placental development is yet to be established.

In chapter V, we elucidated of the factors, which control the synthesis of progesterone in bovine placentome explants at 140 ±10 days of gestation.

Although previous studies have identified the principal factors and intracellular pathways involved in regulation of steroidogenesis in the gonads and adrenal glands, very little is known about the regulation of such steroidogenic factors in bovine placenta. Owing to the results obtained in chapter III, regarding the bovine feto-maternal unit as a complex system controlling the steroidogenesis process, we sought an in vitro culture system that would allow us to maintain together the fetal and maternal compartments to study more precisely the mechanisms for the control of placental steroidogenesis. We tried several approaches in vitro for the isolation and culture of different placental cell populations (density separation by Percoll and flow cytometry). However these approaches did not allow us to study the bovine placenta as a feto-maternal unit, as

happens in vivo conditions. We therefore developed a placentome explant model of bovine gestation around days 140 ±10, based on previous studies with human and equine placental explants (Fuglsang*, et al.*, 2008; Nash*, et al.*, 2008). Our objective in the chapter V was to examine the principal intracellular pathways and factors implicated in the regulation of the steroidogenic process in bovine placenta at 140 ±10 days of gestation. The effect of chorionic gonadotrophin and second messengers on progesterone synthesis and steroidogenic proteins from cultured placental explants was studied. We measured progesterone levels in culture media by RIA, and steroidogenic transcripts and protein by qPCR and western blot. However in the present chapter only transcript results are shown, protein analyses are still ongoing; and once these analyses have been finished, the final manuscript will be submitted to a scientific journal.

Before investigating the effect of the hCG on cultured placental explants, we determined the presence of luteinizing hormone receptor transcript in caruncular and cotyledonary tissues. In our understanding the present work demonstrated for the first time the presence of mRNA for LH receptor in bovine placenta.

In the present study, progesterone production by placentome explants was only significantly stimulated by calcium ionophore at all periods investigated. Moreover steroidogenic transcripts of the principal protein (STAR) and enzymes (STAR, CYP11A1, HSD3B1 and SULT1E1) involved in the synthesis of steroids and steroids sulfates were also stimulated by this calcium analogue at different periods. This indicates that in bovine placenta, apparently the calcium influx stimulates the

progesterone production by increasing the cholesterol transport into the mitochondria and the rate of conversion cholesterol-pregnenolone, pregnenolone-progesterone, and estrogen-estrone sulfates by the activation of the aforementioned enzymes. Previous investigation in bovine placenta found the same stimulatory effect of the calcium analogue on progesterone formation; however these authors did not evaluate the effect of calcium on the principal steroidogenic enzymes (Shemesh*, et al.*, 1984; Shemesh*, et al.*, 1988). The factors that could regulate calcium metabolism cells are not known, but studies on adrenal cells demonstrated that adrenal glucocorticoid synthesis is stimulated by the trophic hormone ACTH; and that its dominant second messenger for the stimulation of STAR protein expression is calcium (Yamazaki*, et al.*, 2006).

In gonads and adrenal gland, the trophic hormones were identified as the principal factors responsible for acute steroid stimulation (Steele & Leung, 1992; Saez, 1994; Ghayee & Auchus, 2007). Therefore, in this study we decided to explore the potential stimulatory effect of LH on progesterone synthesis in bovine placenta using the human placental LH analog, hCG.

Contrary to what we expected, in our study, the progesterone levels were not affected by hCG or hCG +A23187 treatment, however the mRNA expression of STAR, CYP11A1, HSD3B1 and SULT1E1 was increased at different sampling times.

In this experiment hCG, calcium ionophore and a combination of both increased mRNA abundance for SULT1E1 enzyme for almost all studied periods.

SULT1E1 is a metabolic enzyme that acts as estrogen regulator, transforming the active estrogens into sulfoconjugated estrogens which then lose their ability to interact with the estrogen receptor and are hormonally inactive (Rossier & Pierrepoint, 1974). Importantly, in bovine gestation an increase in estrogens is necessary for the labor and delivery process, and this augment is accompanied by a decrease in the sulfoconjugation activity (Hoffmann*, et al.*, 2001; Senger, 2005). Nevertheless, there is a lack of information about the mechanisms that control the sulfoconjugation in bovine placenta. In mouse Leydig cells, estrogen sulfotransferase expression is under control of LH (Song*, et al.*, 1997; Qian & Song, 1999). Our findings may have significant implications for understanding the role of trophic hormones and calcium in the regulation of estrogen sulfoconjugation in bovine placenta. However, additional studies will be required to more precisely describe this regulation.

On another front, even if hCG had an impact in the steroidogenic transcripts in placentome cultures, this stimulus was not of sufficient magnitude to increase considerably the synthesis of progesterone. This led us to speculate that this trophic hormone might not be considered the principal factor of regulation of steroidogenesis in bovine placenta. Even though the principal signaling pathways regulating steroidogenesis in adrenals and ovary are the activation of cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) pathways (Nishikawa*, et al.*, 1996; Luo*, et al.*, 1998; Rekawiecki*, et al.*, 2005; Miller, 2007), our findings suggest that in bovine placenta the synthesis of progesterone is modulated principally by intracellular calcium influx, and apparently cyclic nucleotides do not

seem to be controlling this process. It would be conceivable that calcium influx either directly or indirectly is regulated by other factors and mechanisms. Previous studies have demonstrated that PGE (1) Prostaglandin E(1) (PGE(1)), PGE (2), EGF, insulin-like growth factor (IGF), PRL, gonadotrophin-releasing hormone (GnRH) and MDFs participate in the regulation of steroid biosynthesis in gonads and adrenal glands (Weiss-Messer*, et al.*, 1996; Sekar*, et al.*, 2000; Hales, 2002; Manna*, et al.*, 2009). Further research is necessary to understand the mechanisms and the molecular actions and the possible intracellular pathways in regulating calcium signaling and steroidogenesis in bovine placenta. Moreover we do not exclude the possibility that multiple factors and second messenger systems may cross-talk with one another in placental steroidogenesis. Finally, the placentome explants used here, provided a good model to mimic in vivo conditions to explore specific mechanisms underlying placental steroidogenesis, and possibly other process or pathologies in bovine placenta.

CHAPTER 7. GENERAL CONCLUSIONS AND PERSPECTIVES

General Conclusions

1. In this thesis, we demonstrated the capability of the early bovine placenta to initiate steroidogenesis, and also we determined the principal cells implicated in this process. Moreover, in contrast to the notion that in bovine placenta the steroids are produced by the trophoblast giant cells, our studies propose that during early pregnancy these cells are not a source of placental steroids, because they did not express the indispensable proteins for the steroidogenic process. In more detail, we demonstrated that the caruncles expressed the full steroidogenic proteins needed for pregnenolone and progesterone synthesis. In contrast, the cotyledons only expressed the steroidogenic proteins required for pregnenolone production. The aforementioned observations suggest that in early pregnancies, the maternal compartment exhibits greater steroidogenic capacity than fetal component, particularly for the synthesis of progesterone. However, we also observed than in early gestations the cotyledonary cells expressed a receptor and steroidogenic enzymes, which are responsible for the selective absorption of cholesterol into the cell, and also for pregnenolone and estrogen precursors' synthesis in early gestations. In addition we found a complementary pattern of steroidogenic protein expression found between fetal and maternal compartments.

   Taking together our data suggest that synthesis of steroids is very distinctly partitioned at the cellular level within the placenta, but apparently cell to cell communication between the maternal and fetal cells is required for placental steroidogenesis.

2. In this study, we indentified, for the first time, significant alterations in the expression of a wide range of steroidogenic genes in bovine placenta from SCNT gestations. Thus, we postulate that reduced expression of steroidogenic genes may cause an insufficient biosynthesis of steroid hormones, which potentially might lead to placental and fetal anomalies in SCNT gestations at short or long term. A better understanding of failures in physiological and/or genetic mechanisms essential for the placental and fetal development will be vital for developing new strategies to improve assisted reproductive techniques.

3. To our knowledge, this thesis had demonstrated for the first time the presence of luteinizing hormone receptor transcript in bovine placenta; and also the stimulatory effect of hCG on steroidogenic genes in bovine placentomes. Moreover, calcium ionophore had a stimulatory effect on the progesterone levels and on the steroidogenic transcripts of the principal proteins involved in the synthesis of steroids and steroids sulfates. Based on these findings, we conclude that in bovine placenta the synthesis of progesterone is modulated principally by intracellular calcium influx, and apparently cyclic nucleotides do not seem to be controlling this process. In the present study, we developed an efficient placentome explants culture model that allow to mimic in vivo conditions to explore specific mechanisms underlying placental steroidogenesis, and possibly other process or pathology in bovine placenta.

4. Finally, understanding of the regulatory mechanisms of steroid synthesis and the role of placental steroids in the regulation of placental and fetal development could be a powerful tool in the development of potential preventive treatments to improve pregnancy outcomes after assisted reproductive techniques.

Future perspectives

The results shown in this thesis contribute to the elucidation of the principal hormonal, cellular and molecular mechanism in placenta controlling establishment and maintenance of bovine clone and non-clone pregnancies. Strategic manipulation of those physiological mechanisms may help to develop better therapeutic strategies to improve uterine capacity, conceptus survival, and reproductive health in domestic animals as well as humans. Moreover, these new findings open up great opportunities to identify the origin and consequences of SCNT placental and fetal anomalies. Understanding the origin and consequences of SCNT placental and fetal anomalies, open up a great opportunity to develop potential preventive treatments to enable a better gestational outcome for cloned animals.

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CHAPTER 9. ANNEXES

ANNEX 1. Separation of subpopulation of bovine placental cells by size, using fluorescence activated cell sorting method (FACS)

Methodology

Bovine gestations ranged 140 ±10 days of gestation based on crown-rump lengths were collected from a local slaughterhouse. Caruncles and cotyledons were separated manually and cut into small sections (5 mm). Tissue pieces from caruncles and cotyledons, were incubated and digested separately in a shaker for 30 min at 37°C in a solution of collagenase (0.5%) diluted in Dulbecco's Modified Eagle Medium (DMEM) (1X), high glucose (Invitrogen Life Technologies Corporation, Burlington, ON, Canada) supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Enzymatic dispersion and trophoblast cells isolation was performed as described by (Nakano, Takahashi et al. 2001). The cell suspension for each tissue was sedimented by centrifugation at 800 x g for 20 min. The cells were washed and resuspended in DMEM and transported to the Flow Cytometry laboratory at the CRRA; to be separated by size, using the FACS method.

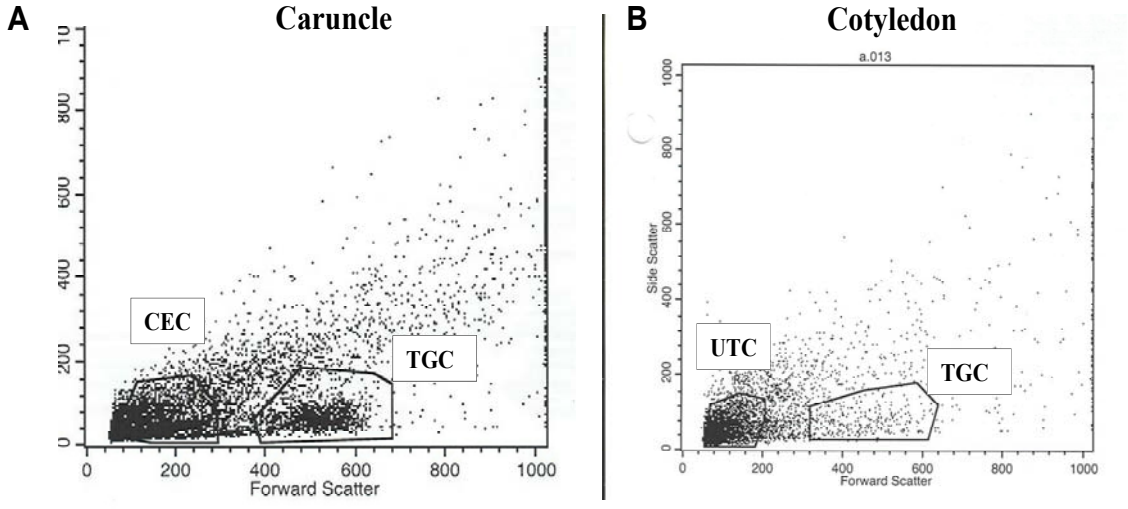

Figure 1. Isolation of different bovine placental cells by FACS in caruncular and cotyledonary tissues. Abbreviations: CEC caruncular ephithelial cells, TGC trophoblast giant cells and UTC uninucleate trophoblast cells.

ANNEX 2. Preliminary studies to determine the viability and sampling time of bovine placentome explants

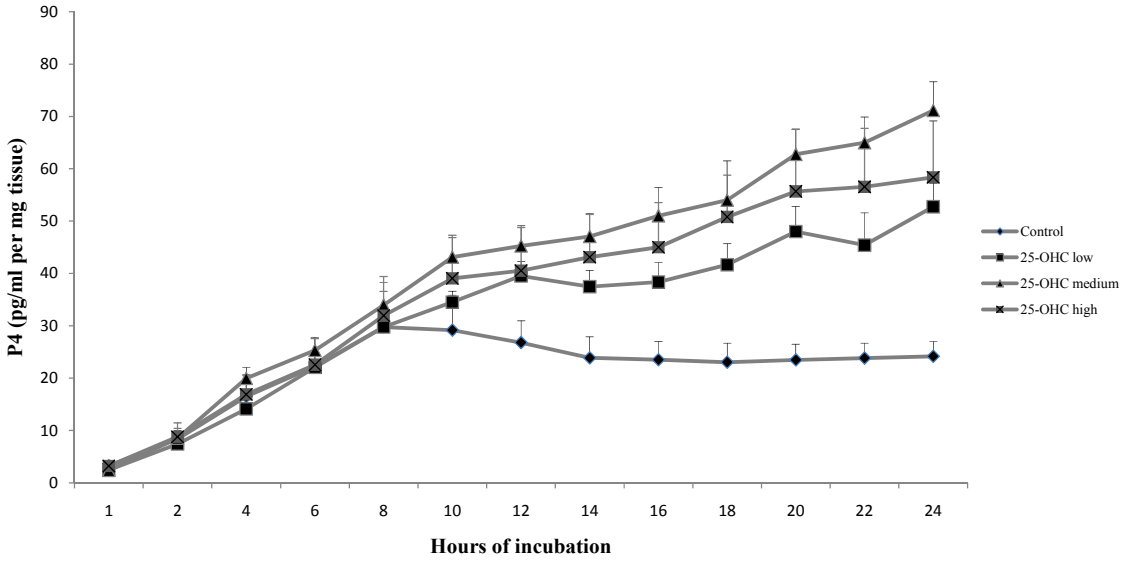

Figure 1. Progesterone (P4) concentrations after 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h of incubation to determine the viability and sampling time of placenta explants. Placentome explants from five bovine gestations around day 140 ± 10 were treated with high (15 µg/ml), medium (5 µg/ml) and low (1 µg/ml) doses of 25-hydroxycholesterol (25OHC) as external source of cholesterol. The data are expressed as the mean ± S.M.E.

Nakano, H., T. Takahashi, et al. (2001). "Expression of placental lactogen and cytokeratin in bovine placental binucleate cells in culture." Cell Tissue Res **303**(2): 263-270.