Université de Montréal

ANALYSE DE L'EXPRESSION DE GÈNES INDUITS DANS LES CELLULES DE GRANULOSA DU FOLLICULE OVULATOIRE BOVIN

par

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ANALYSE DE L'EXPRESSION DE GÈNES INDUITS DANS LES CELLULES DE GRANULOSA DU FOLLICULE OVULATOIRE BOVIN

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RESUME

Chez les mammifères, l'ovulation dépend étroitement des évènements moléculaires qui surviennent dans les cellules de granulosa (CG), du complexe cumulus-ovocyte et de la thèque sous l'influence des gonadotropines, des cytokines et des facteurs de croissance. L'hypothèse de cette étude sous-tend que l'ovulation et la lutéinisation du follicule préovulatoire résultent de l'induction de certains gènes dans les CG suite à la relâche préovulatoire de l'hormone lutéinisante (LH). Les objectifs visaient à caractériser et à étudier le patron d'expression de cinq gènes potentiellement stimulés par l'hormone chorionique gonadotropine (hCG) dans les CG en période périovulatoire. Les ADNc de la phospholipase A2 cytosolique alpha (cPLA2α ou PLA2G4A; 2863 pb), de la cavéoline-1 (CAV1; 2455 pb), du KIAA-1798 (3892 pb), du "trypsin-like inhibitor" (4020 pb) et de deux "ankyrin and socs-box protein 9" (ASB9-1: 1604 pb ; ASB9-2: 1435 pb) ont été clonés à partir de génothèques d'ADNc de CG bovines induites à l'hCG puis caractérisés par séquençage. Le profil d'expression de ces ARNm a été étudié par RT-PCR dans différents types de follicules ovariens bovins: petits follicules de 2-4 mm, follicules dominants à J5 du cycle oestral, follicules ovulatoires (FO) 24 h post-hCG et de corps jaune (CL) à J5, puis à différents temps (0, 6, 12, 18, 24 h) suivant l'injection d'hCG. La distribution tissulaire de KIAA-1798, du "trypsin-like inhibitor" et des ASB9 a été comparée dans différents tissus. De plus, l'expression protéique de PLA2G4A et CAV1 a été analysée par immunobuvardage et immunohistochimie à différentes étapes du développement folliculaire. L'ARNm de PLA2G4A est induit dans les CG de FO ; l'expression maximale est atteinte à 18 h post-hCG. La protéine PLA2G4A est localisée

dans les CG murales des FO. Aussi, le traitement de CG en culture par la forskoline induit l'expression de PLA2G4A. Le rôle de PLA2G4A est de permettre la libération d'acide arachidonique à partir des phospholipides membranaires pour la formation des prostaglandines nécessaires à l'ovulation. L'ARNm de CAV1 est induit dans les CG de FO dès 12 h post-hCG ; l'expression maximale a été atteinte à 24 h post-hCG. La protéine CAV1 est aussi détectée dans les CG de FO et une expression élevée dans les cellules endothéliales de la thèque et du CL est observée. Le rôle de CAV1 lors de l'ovulation serait de contribuer à l'augmentation du trafic moléculaire et à la désensibilisation des mécanismes de transduction activés par la relâche préovulatoire de LH. L'ARNm de KIAA-1798 est augmenté dans les CG 24 h post-hCG ; le rôle de ce gène serait de contrôler l'activité transcriptionnelle lors de la différentiation des CG en cellules lutéales. L'ARNm du "trypsin-like inhibitor"est augmenté dans les CG 24 h post-hCG ; il a été détecté dans plusieurs tissus bovins. Le rôle de cette protéine doit être élucidé ; elle interviendrait dans l'atténuation des actions des sérines protéases activées lors de l'ovulation. Les ARNm d'ASB9-1 ainsi que d'ASB9-2 sont fortement induits dans les CG de FO à partir de 12 h suivant l'injection d'hCG. L'expression des ASB9 est plus élevée dans les CG induites à l'hCG et les testicules comparativement aux autres tissus. Lors de l'ovulation, le rôle des ASB9 serait d'inhiber les mécanismes de transduction membranaires activés par les cytokines. Cette étude rapporte pour la première fois la caractérisation de cinq ADNc bovins dont l'expression est sous le contrôle de la relâche préovulatoire de LH/hCG.

Mots-clés: bovin, ovaire, follicule, granulosa, ovulation, expression génique, PLA2G4A, CAV1, KIAA-1798, ASB9, trypsin-like inhibitor.

ABSTRACT

In mammals, ovulation is intimately linked to molecular events that occur in granulosa cells (GC), cumulus-oocyte complex and theca cells under the control of gonadotropins, cytokines and growth factors. The hypothesis of this study is that ovulation and luteinization of the preovulatory follicle result from the induction of specific genes in GC following the preovulatory luteinizing hormone (LH) surge. The objectives were to characterize and study the spatio-temporal expression profile of five genes potentially stimulated by human chorionic gonadotrophin (hCG) in GC during the periovulatory period. Full-length bovine cDNAs corresponding to cytosolic phospholipase A2 alpha (cPLA2α or PLA2G4A; 2863 bp), caveolin-1 (CAV1; 2455 bp), bovine KIAA-1798 (3892 bp), a trypsin-like inhibitor (4020 bp) and two ankyrin and SOCS-box containing proteins 9 (ASB9-1: 1604 bp; ASB9-2: 1435 bp) were cloned by screening size-selected cDNA libraries derived from hCG-stimulated GC. Their mRNA expression profiles were compared by RT-PCR in different bovine ovarian follicles: small follicles of 2-4 mm, dominant follicles at D5 of the estrous cycle, ovulatory follicles (OF) 24 h post-hCG and corpus luteum (CL), and in OF collected at different times (0, 6, 12, 18, 24 h) following the injection of hCG. Expression of KIAA-1798, trypsin-like inhibitor and ASB9 mRNAs were also analyzed in various bovine tissues. Protein expression of PLA2G4A and CAV1 were analyzed by immunoblotting and immunohistochemistry at different stages of follicular development. Induction of PLA2G4A mRNA was observed in GC of OF, and reached a maximal expression level 18 h post-hCG. PLA2G4A protein was mainly localized in mural GC of hCG-induced OF. Furthermore, forskolin stimulation of cultured bovine GC induced the expression of PLA2G4A. The function of PLA2G4A at

ovulation would contribute to the release of arachidonic acid from membrane phospholipids, required for the production of prostaglandins. Induction of CAV1 mRNA was initially observed in GC of OF at 12 h post-hCG, reaching a maximum at 24 h post-hCG. Induction of CAV1 protein was also observed in GC of OF, and was shown to be highly expressed in endothelial cells of the theca layer and CL. During ovulation, CAV1 expression in GC may contribute to increase molecular trafficking and to desensitize the transduction mechanism activated by the preovulatory LH surge. The mRNA for KIAA-1798 was increased in GC of OF 24 h post-hCG, and may be involved at the transcriptional level in the differentiation of GC into luteal cells. The mRNA for trypsin-like inhibitor was increased in GC of OF 24 h post-hCG. The mRNA level was highest in GC of OF when compared to other bovine tissues. The function of this protein remains to be determined but could intervene in the inhibition of activated proteases that occurs at ovulation. Alternative splicing of ASB9-1 mRNA results in ASB9-2 mRNA. Both ASB9 mRNAs are strongly induced in GC of OF already at 12 h post-hCG. Expression of ASB9-1 mRNA is strongest in hCG-stimulated GC and testis compared to other tissues, whereas ASB9-2 is only detected in GC and testis. The function of ASB9 during ovulation may modulate negatively the transduction mechanisms induced by cytokines. This study reports for the first time the characterization of five bovine cDNAs, and shows that the LH/hCG preovulatory surge stimulates their expression.

Keywords : bovine, ovary, follicle, granulosa, ovulation, gene expression, PLA2G4A, CAV1, KIAA-1798, ASB9, trypsin-like inhibitor.

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LISTE DES ABREVIATIONS

(Note : la définition anglophone des termes a été conservée par souci de cohérence à la nomenclature disponible par le « Hugo gene nomenclature committee » (www.gene.ucl.ac.uk/nomenclature)

α2M :	α2 macroglobulin
3 α-HSD :	3 alpha hydroxy dehydrogenase
12/15-HPETE :	12/15-hydropéroxyeicosatetraenoïque
17βHSD4 :	17 beta hydroxysteroid dehydrogenase type 4
AC :	Adenylate cyclase
ADAMTS :	A disintegrin and metalloproteinase with thrombospondin motifs
ADAMTS1 :	A disintegrin and metalloproteinase with thrombospondin motifs 1
ADAMTS4 :	A disintegrin and metalloproteinase with thrombospondin motifs 4
AGT2 :	Angiotensine II
ADN (DNA) :	Acide désoxyribonucléique
ADNc :	Acide désoxyribonucléique complémentaire
AEG :	Acidic epididymal glycoprotein
ALAS :	5-aminolevulinate synthase
AMH :	Anti-mullerian hormone = Mullerian inhibiting substance (MIS)
AMPc :	Adénosine monophosphate cyclique
Apo ER2 :	Apolipoprotein E receptor 2
ARF6 :	Adenosine diphosphate (ADP) ribosylation factor 6
ARF6GAP3 :	ADP-ribosylation factor GTPase activating protein 3
ARNm :	Acide ribonucléique messager
ASB :	Ankyrin and SOCS box containing protein
ASB1 :	Ankyrin and SOCS box containing protein 1
ASB2 :	Ankyrin and SOCS box containing protein 2
ASB3 :	Ankyrin and SOCS box containing protein 3

ASB4 : Ankyrin and SOCS box containing protein 4

ASB8 : Ankyrin and SOCS box containing protein 8

ASB9 : Ankyrin and SOCS box containing protein 9

ASB9-1 : Ankyrin and SOCS box containing protein 9 isoform 1

ASB9-2 : Ankyrin and SOCS box containing protein 9 isoform 2

ASB17 : Ankyrin and SOCS box containing protein 17

ATK : Arachidonyl trifluoromethyl ketone

BMP15 : Bone morphogenetic protein 15

CAV1 : Cavéoline 1

CAV2 : Cavéoline 2

CAV3 : Cavéoline 3

CBR : Carbonyl reductase

CD62P : P selectin

CD63 : Cell surface antigen antigen CD63

CDK 1 : Cyclin-dependant kinase 1 (kinase p34 ^{cdc2})

CEBPB : CAAT-enhancer binding protein beta

CEEF : Cumulus expansion-enabling factor

CG : Cellules de la granulosa

CIS : Cytokine-inducible SH2-containing

CL : Corpus luteum (corps jaune)

CJA1 : Connexine 43 (Cx43)

CRE : cAMP response element

CREB : cAMP response element binding protein

COC : Complexe ovocyte cumulus

COX-2 : Cyclooxygenase-2

CPD : Carboxypeptidase D

CTS4 : Cathepsine L

CYB : Cycline B

CYP11A1 : Cytochrome P_{450} side chain cleavage enzyme (P450 scc)

CYP17A1 : Cytochrome P_{450} 17-alpha hydroxylase

CYP19A1 : Cytochrome P_{450} aromatase enzyme (P450 arom)

DAG :	Diacylglycerol
DDRT-PCR :	Differential display reverse transcriptase-polymerase chain reaction
DF :	Follicule dominant à jour 5 du cycle œstral
ECM :	Extracellular matrix
EGF :	Epithelial growth factor
EGR1 :	Early growth regulatory factor 1
eNOS :	Endothelial nitric oxide synthase
EPAC :	Exchange protein directly activated by cAMP
Erb A :	Protooncogene homologous to avian erythroblastosis
EREG :	Epireguline
ESR1 :	Estrogen receptor alpha
ESR2 :	Estrogen receptor beta
FGF2 :	Fibroblast growth factor 2
FGIF :	Follicle growth inhibitory factor
FKHRL1 :	Forkhead in Rhabdomyosarcoma-like 1 (Foxo3a)
FKHR :	Foxo-1
FOXO	Forkhead in Rhabdomyosarcoma-like
FSH :	Follicle-stimulating hormone
FSHR :	Follicle-stimulating hormone receptor
Fz-1 :	Frizzled receptor 1
GDF9 :	Growth differentiation factor 9
GH :	Growth hormone
GnRH :	Gonadotropin releasing hormone
GPRC :	G protein receptor coupled
GPRCK :	G protein receptor coupled kinase
Gq :	Sous unité "q" de la protéine G
Gs :	Sous unité "s" de la protéine G
GST :	Glutathione S-transferase
GVBD :	Germinal vesicle breakdown
h :	heure
H1F00 :	Histone ovocyte specific linker H100

HA :	Hyaluronic acid
HAS2 :	Hyaluronan synthase-2
hCG :	human chorionic gonadotropin
HRas :	Harvey rat sarcoma viral oncogene homolog
HSD3B2 :	3 beta-hydroxysteroid dehydrogenase (3 ß-HSD)
IL1A :	Interleukin 1 alpha
IL1B :	Interleukin 1 beta
IL6 :	Interleukin 6
IL1R1 :	Interleukin 1 receptor type 1
ILR2 :	Interleukin 1 receptor type 2
IL1RN :	Interleukin 1 receptor antagonist
IαI :	Serum-derived inter alpha-inhibitor
IGF :	Insulin-like growth factor
IGF1 :	Insulin-like growth factor 1
IGF2 :	Insulin-like growth factor 2
IGFBP :	Insulin-like growth factor binding protein
IGFBP2 :	Insulin-like growth factor binding protein 2
IGFBP4 :	Insulin-like growth factor binding protein 4
IGFBP5 :	Insulin-like growth factor binding protein 5
IP3 :	Phosphatidyl inositol triphosphate
J0 :	Jour 0 (jour de l'oestrus)
J5 :	Jour 5 (5 jours après l'oestrus)
JAK :	Janus kinase
kDa :	Kilo dalton
KIAA-1798 :	"KI" stands for Kazuka DNA Research Institute and "AA" are reference characters
KITLG :	Kit ligand
LDL :	Low-density lipoprotein
Lgl1 :	Late gestation lung protein 1
LH :	Luteinizing hormone
LHBP :	Luteinizing hormone receptor binding protein

LHCGR :	Luteinizing hormone/ choriogonadotropin receptor
LPS :	Lipopolysaccharides
LRP8 :	Low–density lipoprotein related protein 8
MAPK :	Mitogen-activating protein kinase
MBT :	Malignant brain tumor domain
MAS :	Meiosis activating substance
MIS :	Mullerian inhibiting substance
MMP :	Matrix metalloproteinase
MMP1 :	Matrix metalloproteinase 1
MMP2 :	Matrix metalloproteinase 2
MT1A :	Metallothionein-1
MPF :	Maturation promoting factor
MYC	Protooncogene homologous to myelocytomatosis virus
NGF :	Nerve growth factor
NOS :	Nitric oxide synthase
NRIP :	Nuclear receptor-interacting protein (RIP 140)
NTRK1 :	Neurotrophic tyrosine kinase receptor-type 1
FO :	Follicule ovulatoire
OMI :	Oocyte maturation inhibitor
PA :	Plasminogen activator
PACAP :	Pituitary adenylate cyclase-activating polypeptide
PAF :	Platelet-activating factor
PAI :	Plasminogen activator–inhibitor
PAI1 :	Plasminogen activator–inhibitor type 1
PAP-III :	Pancreatitis-associated protein III
PAPPA :	Pregnancy-associated plasma protein A
PCR :	Polymerase chain reaction
PDE :	Phosphodiesterase
PDE3 :	Phosphodiesterase 3
PDE4 :	Phosphodiesterase 4
PGE :	Prostaglandine E

PGE2 :	Prostaglandine E2
PGF2α :	Prostaglandine F2α
PGR :	Progesterone receptor
PGTS2 :	Prostaglandin endoperoxide synthase 2 (COX-2)
PIAS :	Protein inhibitor of activated STAT
PKA :	Protéine kinase dépendante de l'AMPc
PKC :	Protéine kinase C
PLA2 :	Phospholipase A2
PLA2G1B :	Phospholipase A 2 pancréatique
PLA2G4A :	Phospholipase A 2 cytosolique alpha
PLA2G4B :	Phospholipase A 2 cytosolique beta
PLA2G4C :	Phospholipase A 2 cytosolique gamma
PLA2S :	Phospholipase A 2 synovial
PLC :	Phospholipase C
PN1 :	Protéase nexin1 (ou SERPINE2)
PTGER2 :	Prostaglandin E receptor 2
PTGS1 :	Prostaglandin endoperoxide synthase 1
PTGS2 :	Prostaglandin endoperoxide synthase 2
PRC1 :	Protein regulator of cytokinesis 1
PRG1 :	Proteoglycan 1
PRKO :	Progesterone receptor knock out
RACE :	Rapid amplification of cDNA ends
RE :	Response element
RGS2 :	Regulator of G-protein signaling protein-2
RT-PCR :	Reverse transcription-polymerase chain reaction
RPA2 :	Replication protein A 2
SAGE :	Serial analysis of gene expression
SAM :	Sterile alpha motif
SCD :	Stearoyl-coenzyme A desaturase
SCP2 :	Sterol carrier protein 2
SERPINE 1 :	Serine protease inhibitor E1 (PAI1)

SERPINE 2 :	Serine protease inhibitor E2
sFRP :	Secreted frizzled related proteins
sFRP4 :	Secreted frizzled related protein 4
SSB :	SPRY domain-containing protein
Src :	Avian sarcoma (Schmidt-Ruppin A-2) viral oncogene
SSH :	Subtractive suppression hybridization
STAR :	Steroidogenic acute regulatory protein
STAT :	Signal transducers and activators of transcription
TGFα :	Transforming growth factor alpha
TGFß :	Transforming growth factor beta
TIMP :	Tissue inhibitor of metalloproteinase
TIMP1 :	Tissue inhibitor of metalloproteinase 1
TIMP2 :	Tissue inhibitor of metalloproteinase 2
TIMP3 :	Tissue inhibitor of metalloproteinase 3
TNF :	Tumor necrosis factor alpha
TNFAIP6 :	Tumor necrosis factor alpha induced protein 6 (TSG6)
tPA :	Tissue plasminogen activator
TPX1 :	Testis-specific protein 1
TRIB2 :	Tribbles homolog 2
uPA :	Urokinase-plasminogen activator
uPAR :	Urokinase-plasminogen activator receptor
VEGF :	Vascular endothelial growth factor
Wnt :	wingless-type mmtv integration site family
Wnt4 :	Wingless-type mmtv integration site family, member 4
WSB	WD-40-repeat containing proteins
WT1 :	Wilm's tumor gene 1

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CHAPITRE 1

1. INTRODUCTION

La fertilité de la femelle, à l'inverse de celle du mâle, n'est pas continue durant la vie de l'individu. Elle est tributaire de la réserve de follicules primordiaux qui s'est constituée de manière définitive durant la vie fœtale. De la puberté à la réforme de l'animal ou la ménopause chez la femme, une faible proportion estimée à environ un pour cent des follicules de la réserve ovulera; la majorité va subir inexorablement l'atrésie ou la mort cellulaire programmée (Gougeon, 1996; Christin-Maitre & Bouchard, 2002).

La croissance folliculaire est un phénomène complexe minutieusement contrôlée par les interactions entre des signaux systémiques émis par les gonadotropines et des facteurs locaux intra-ovariens tels les stéroïdes, les cytokines et les facteurs de croissance (Roche, 1996; McGee & Hsueh, 2000). A l'image de leur sécrétion, les hormones hypophysaires "follicle-stimulating hormone" (FSH) et "luteinizing hormone" (LH) n'agissent pas en synchronie mais par complémentarité; *in vivo,* les effets biologiques de la FSH sont antérieurs et nécessaires à ceux de la LH. Cette succession hormonale est non seulement synergique mais elle est aussi importante pour un développement folliculaire adéquat. Dans les petits follicules, la FSH et plusieurs facteurs intra-ovariens induisent la prolifération cellulaire et la maturation des follicules antraux sélectionnés en follicules préovulatoires (Robker & Richards, 1998; Richards et al., 1998). Dans ces derniers, la relâche préovulatoire de LH déclenche les événements

conduisant à l'ovulation et à la lutéinisation. Par la suite, la formation du corps jaune (*corpus luteum :* CL) cyclique résultera de la différenciation des cellules de la granulosa et des thèques (Richards, 1994; Richards, 2001a).

L'ovulation est un processus biologique important à considérer pour la pérennité des espèces et sur le plan économique pour les animaux de rente. Une meilleure connaissance de son mécanisme d'action permettrait de comprendre certaines infertilités afin de mieux y remédier et de contribuer à l'augmentation de la productivité des élevages. Le processus ovulatoire implique une série complexe d'événements biochimiques et biophysiques sous-jacents qui conduiront à la rupture du follicule ovulatoire et l'extrusion d'un ovocyte apte à la fécondation (Espey & Lipner, 1994). De nos jours, l'ovulation est assimilée à une "réaction inflammatoire contrôlée" incluant l'hyperhémie, la perméabilité vasculaire, l'œdème, la migration leucocytaire et les activités protéolytiques (Espey, 1980; Espey & Lipner, 1994). Ces événements sont la résultante de l'activation ou de l'inhibition de plusieurs gènes (Richards, 1994; Espey & Richards, 2002).

Etudier les gènes impliqués dans le mécanisme ovulatoire serait sans nul doute une contribution à une meilleure connaissance de ce phénomène. Pour ce faire, il faudra au préalable identifier et caractériser les gènes impliqués dans ce processus. La présente étude s'inscrit dans cette optique. Afin de mener à bien cette investigation, il est nécessaire de distinguer parmi tous les gènes exprimés dans les cellules de la granulosa du follicule ovulatoire, ceux qui sont spécifiquement associés à l'ovulation. Dans cette

recherche, les innovations de la biologie moléculaire et le développement de la bioinformatique constituent des outils essentiels. En effet, durant cette décade plusieurs techniques ont permis d'identifier des gènes différenciellement exprimés entre deux types de cellules données (Martin & Pardee, 2000; Hennebold, 2004). Parmi ces innovations, on pourrait citer le "differential display reverse" (DDRT-PCR, Liang & Pardee 1992; Liang 2002; Bédard et al., 2003), le "serial analysis of gene expression" (SAGE, Velculescu et al., 1995; Yamamoto et al., 2001; Ye et al., 2002), le "suppression subtractive hybridization" (SSH; von Stein et al., 1997; Diatchenko et al., 1999; Fayad et al., 2004; Ndiaye et al., 2005) et le "cDNA microarray" (Pennie, 2002; Hennebold, 2004). Hormis le "cDNA microarray", ces techniques ne génèrent que des fragments d'ADNc. Les ADNc pleine longueur sont obtenus par des techniques complémentaires tels le "rapid amplification of cDNA ends" (RACE; Das et al., 2001) ou le criblage de génothèque d'ADNc (Bédard et al., 2003; Lévesque et al., 2003). Suite à l'identification des ADNc différentiellement exprimés, les conclusions des études des souris ayant une mutation nulle pour l'expression des gènes spécifiques (souris "knock out") permettront de présager la fonction de ces gènes (Burns & Matzuk, 2002).

La vache est un modèle expérimental de choix car chez cette espèce, parmi les vagues folliculaires qui se succèdent au cours du cycle oestral, un seul follicule ovulera (Fortune et al., 2001). De plus, le cycle oestral peut être aisément induit, la croissance folliculaire suivie par échographie et la récolte en quantité suffisante de matériel biologique pour l'extraction de l'ARN et des protéines peut être réalisée. De ce fait, le

follicule ovulatoire peut être comparé individuellement à un autre follicule ovarien à un stade de développement bien caractérisé.

2. RECENSION DE LA LITTERATURE

2.1. Introduction à la folliculogénèse

Les cellules germinales primordiales sont originaires d'une région de l'épiblaste adjacente à l'ectoderme extra-embryonnaire (Magre & Vigier, 2001). Ces futurs ovocytes vont coloniser la crête génitale et ils se retrouveront dans l'ovaire fœtal après migrations (Wassarman & Albertini, 1994; Suh et al., 2002). Après leur progression dans les différents stades de la prophase méïotique (leptotène, zygotène, pachytène et diplotène), les ovocytes se regroupent avec des cellules somatiques (futures cellules de la granulosa) au cours du développement de l'ovaire fœtal (Magre & Vigier, 2001). Ainsi, se forme le follicule primordial qui est constitué par un ovocyte (arrêté au stade diplotène de la prophase I) entouré par des cellules de la granulosa. A la naissance, le nombre de follicules formant la réserve folliculaire varie selon l'espèce animale et l'individu : 400 000-1 000 000 chez la femme, 100 000 -200 000 chez la vache, 160 000 chez la brebis et 20 000 chez la ratte (Driancourt et al., 2001). La période de fertilité de la femelle est déterminée par la taille de cette réserve et son taux de déplétion après la naissance (Gougeon, 1996). Ce dogme est ébranlé par l'étude récente de Johnson et al. (2004) qui rapporte l'existence de cellules germinales souches dans l'ovaire de souris adulte capable de reconstituer la réserve folliculaire.

La folliculogénèse est le processus de croissance et de maturation des follicules ovariens, du stade de follicule primordial jusqu'à l'ovulation d'où sera libéré l'ovocyte fécondable (**Fig.1**). La durée de la croissance folliculaire varie selon l'espèce animale soit : 25-30 jours chez la ratte (Driancourt et al., 2001), 180 jours chez la femme (Gougeon, 1996), 100-120 jours chez la vache et la brebis (Lussier et al., 1987). Le développement folliculaire du stade primordial jusqu'à l'ovulation concerne une faible proportion de la réserve folliculaire car 99 % des follicules vont dégénérer par atrésie ou mort cellulaire programmée (Gougeon, 1996). L'atrésie peut survenir à n'importe quelle étape de la croissance folliculaire (Tilly, 1996; Johnson & Bridgham, 2002). Elle est massive pendant la vie fœtale et se poursuit de la naissance jusqu'à la fin de la vie reproductive de l'animal ou la ménopause chez la femme (Driancourt et al., 2001). Une fois le développement folliculaire initié, le follicule ovarien croît et évolue soit vers l'ovulation ou l'atrésie (Hsueh et al., 1994; Markström et al., 2002). La folliculogénèse peut être subdivisée en deux étapes consécutives : une phase initiale de recrutement et une phase terminale de maturation. Considérant la problématique traitée dans ce document, l'accent sera mis sur la maturation finale du follicule qui se termine par l'ovulation et la lutéinisation.

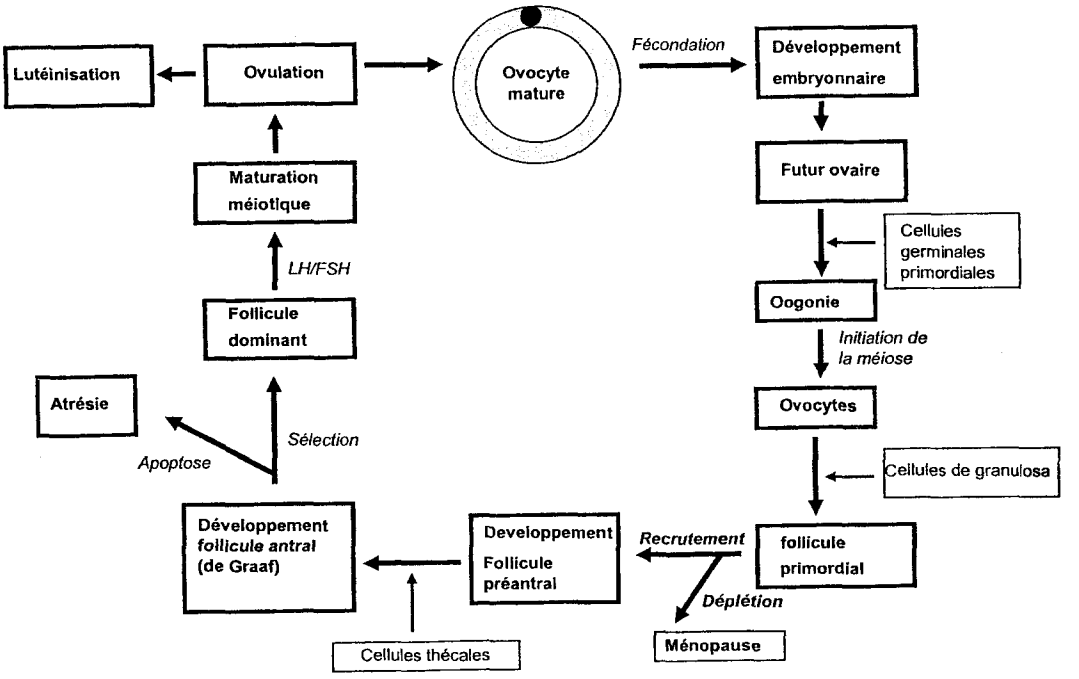

Figure 1. Schéma de la folliculogénèse (adapté de Suh et al., 2002)

2.2. Recrutement folliculaire initial

2.2.1. Définition

Il a été longtemps admis que les follicules primordiaux de la réserve sont au "repos" mais plusieurs études ont démontré que les cellules de la granulosa entourant l'ovocyte entraient occasionnellement dans le cycle cellulaire, toutefois la croissance du follicule ne continuait pas (Hirshfield, 1989; Oktay et al., 1995; Lundy et al., 1999; Meredith et al., 2000). La croissance folliculaire basale est initiée continuellement sur des follicules primordiaux de la réserve, on parle de *recrutement initial* ou *initiation*. Elle se caractérise par la prolifération et le changement morphologique des cellules de la granulosa (Braw-Tal, 1994), l'accroissement de l'ovocyte (Braw-Tal, 1994; 2002) et la formation de la zone pellucide (Braw-Tal & Yossefi, 1997). Au cours de la croissance initiale, les follicules ovariens sont classés en plusieurs types selon le stade évolutif qui tient compte du nombre de couches des cellules de la granulosa, du développement de la thèque interne et de l'apparition de l'antre (McNatty et al., 1999; Smitz & Corvrindt, 2002).

2.2.2. Mécanismes impliqués

Les mécanismes qui régissent la sélection des follicules à partir de la réserve ainsi que l'initiation de leur croissance spontanée sont encore inconnus. Toutefois, il est unanimement admis que le recrutement initial ne nécessite pas de gonadotropines car l'invalidation des récepteurs de ces hormones et l'hypophysectomie n'inhibent pas le recrutement (Cattanach et al., 1977; Kendall et al., 1995; Dierich et al. 1998; McGee & Hsueh, 2000). L'ARNm du récepteur de la FSH ("follicle-stimulating hormone

receptor"; FSHR) a été détecté dans les cellules de la granulosa du follicule pré-antral (Xu et al., 1995a) tandis que ceux du récepteur de l'hormone lutéinisante ("luteinizing hormone/choriogonadotropin receptor"; LHCGR) et des enzymes stéroïdogéniques tel que : le "cytochrome P450 side chain cleavage" (P450scc ou CYP11A1), le "cytochrome P450 17α-hydroxylase" (P450c17 ou CYP17A1) et le "3β-hydroxysteroid dehydrogenase" (3β-HSD ou HSD3B2) ont été observés dans les cellules thécales (Bao et al., 1997a). Le rôle des gonadotropines dans l'induction de l'expression de ces gènes dans les follicules préantraux et au stade initial de la formation de l'antre n'est pas connu (Webb et al., 1999).

Divers facteurs intra-ovariens contrôlent le recrutement initial à partir de la réserve de follicules primordiaux comme le démontrent de nombreuses études *in vitro* et l'invalidation de certains gènes. Les facteurs associés à l'initiation de la croissance sont : le "kit ligand" (KITLG) encore nommé "stem cell factor" (Kuroda et al., 1988; Parrot & Skinner, 1999; Driancourt et al., 2000), le "growth differentiation factor 9" (GDF9; McGrath et al., 1995; Aaltonen et al., 1999), les activines, les inhibines (Schwall et al., 1990; Miro & Hillier, 1996; McGee & Hsueh, 2000), le "transforming growth factor beta" (TGFβ; McNatty et al., 1999; Knight & Glister, 2003), le "bone morphogenetic protein 15" (BMP15; Yan & Matzuk, 2001), "fibroblast growth factor 2" (FGF2; Nilsson & Skinner, 2001; 2004), les oncogènes "protooncogene homologous to myelocytomatosis virus" (MYC) et Erb A (Sato et al., 1994; Smitz & Cortvrindt, 2002). Chez la souris, l'invalidation de GDF9 provoque un arrêt du développement folliculaire au stade de follicule primaire (Dong et al., 1996). Dans le même ordre d'idée,

l'inhibition de l'interaction de KITLG à son récepteur c-kit empêche la transformation des follicules primordiaux en follicules primaires (Huang et al., 1993; Yoshida et al., 1997). Ces expérimentations parmi tant d'autres, ont mis en exergue l'importance des facteurs sécrétés par l'ovocyte dans l'initiation de la folliculogénèse. Aussi, les interactions entre les cellules somatiques et germinales permettant la circulation de divers facteurs biologiques, sont fondamentales dans l'initiation et le contrôle subséquent de la folliculogénèse (Nilsson & Skinner, 2001; Eppig, 2001). En effet, l'absence de connexine 43 et 37, protéines nécessaires à la formation des jonctions de type gap, affecte respectivement les premières et les dernières étapes de la folliculogénèse associée avec un impact négatif sur les cellules germinales (Simon et al., 1997; Ackert et al., 2001; Epifano & Dean, 2002).

A l'inverse des facteurs cités ci avant, d'autres freinent le recrutement initial. Il s'agit de l'"anti-Mullerian hormone" (AMH; Durlinger et al., 1999; 2002) et le "Wilm's tumor-1" (WT1; Hsu et al., 1995; Chun et al., 1999). WT1 réprime l'activité des promoteurs de l'inhibine α et du FSHR (McGee & Hsueh, 2000). Le Foxo3a ou FKHRL1, membre de sous-famille FOXO des "forkhead transcription factors" est un régulateur négatif (suppresseur) de l'activation du développement du follicule. Cette conclusion a été déduite des études de Castrillon et al. (2003) qui ont montré que l'absence de la fonction de Foxo3a chez la souris ayant une mutation nulle de ce gène occasionne une activation des cellules primordiales avant la maturité sexuelle des souris. L'environnement tissulaire (stroma) des follicules primordiaux semble jouer un rôle inhibiteur car en son absence les cellules de la granulosa prolifèrent *in vitro* (Wandji et

al., 1997; Fortune et al., 2000); toutefois, le ou les facteurs impliqués n'ont pas été élucidés.

En résumé, le recrutement initial est un processus continu contrôlé par le déséquilibre entre les facteurs stimulateurs (ou permissifs) et inhibiteurs dans l'environnement du follicule primordial (Fortune et al., 2004). Chez les ruminants, il débute au cours de la vie fœtale et continue sans interruption durant toute la vie reproductive de l'animal (McNatty et al., 1999). Avant la puberté, tous les follicules chez lesquels la croissance a été initiée subissent inexorablement l'atrésie car le développement folliculaire au delà du stade antral requiert une concentration donnée les gonadotropines (McGee & Hsueh, 2000).

2.3. Recrutement folliculaire cyclique

2.3.1. Définition

A la puberté, l'élévation du taux de FSH dans la circulation sanguine permet la croissance des follicules antraux qui peuvent poursuivre leur développement jusqu'à l'ovulation si toutes les conditions sont réunies; on parle de *recrutement cyclique* (McGee & Hsueh, 2000). La FSH est donc l'hormone-clé à laquelle tout follicule antral sain doit répondre pour continuer à se développer et par conséquent échapper à l'atrésie (Fortune, 1994). En effet, l'hypophysectomie et la suppression de la sécrétion des gonadotropines par l'injection d'antagoniste à la GnRH inhibent le recrutement cyclique (Edwards et al., 1977; Eckery et al., 1997).

Après la formation de l'antre, même si les gonadotropines demeurent pour les follicules un facteur de survie crucial, des mécanismes hormonaux intra-folliculaires jouent un rôle non moins important (Erickson & Shimasaki, 2001; Christin-Maitre et al., 2002). Généralement, les facteurs intra-ovariens (activines, insulin growth factor (IGF), etc...) impliqués dans ces mécanismes agissent en synergie avec les gonadotropines (Knight & Glister, 2001; Webb et al., 2003).

2.3.2. Notion de vagues folliculaires

Le recrutement cyclique concerne un groupe de follicules antraux qui se développent de manière synchrone, on parle de *cohorte* ou de *vague folliculaire* (**Fig. 2**). Les vagues folliculaires se développent régulièrement au cours du cycle oestral (Sirois & Fortune, 1988; Fortune et al., 1991), durant la gestation (Ginther et al., 1989, 1996) et en période post-partum (Evans, 2003). Chaque vague folliculaire est précédée d'une augmentation de la relâche en FSH (Adams et al., 1992; Ginther et al., 1996; Evans, 2003). En outre, il existe une corrélation positive entre l'augmentation de l'amplitude de la sécrétion de la FSH et le nombre de follicules dans la vague (Adams et al., 1994; Evans et al., 1994; Macklon & Fauser, 1999).

Chez la vache, les follicules sont recrutés à partir de 3 mm de diamètre (Lussier et al., 1994; Gong et al., 1996; Driancourt et al., 2001) et une vague folliculaire est composée de 3 à 6 follicules qui croissent au delà de 5 mm (Fortune, 1994).

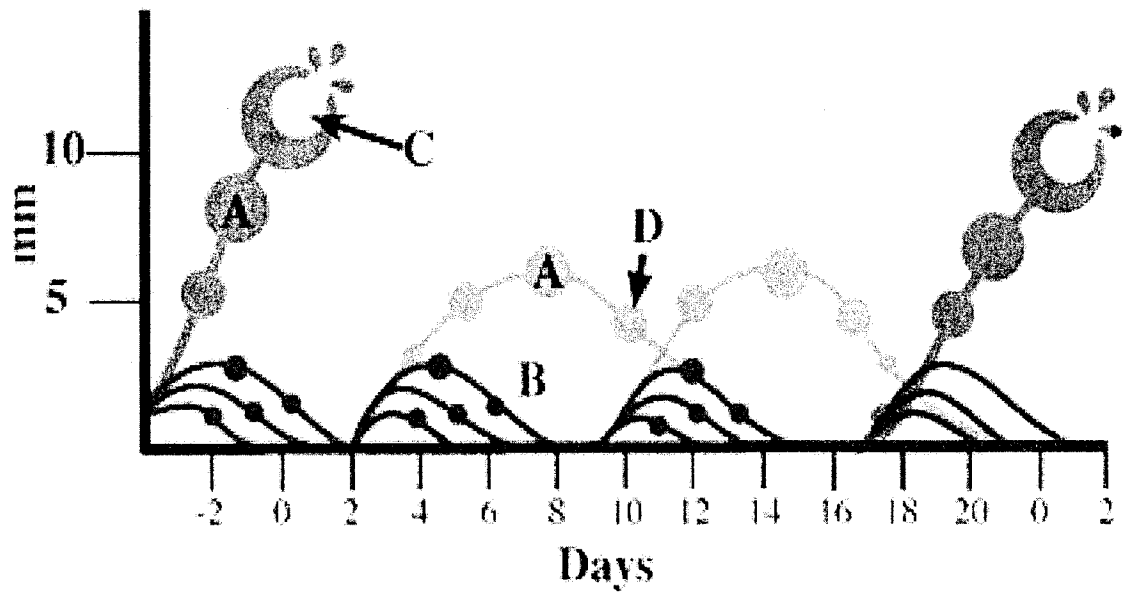

Figure 2. Vagues et structures folliculaires durant le cycle oestral chez la vache (Hall et al., 2002). Day=0 jour des chaleurs; **A** follicule dominant; **B** follicules subordonnés; **C** follicule en ovulation; et **D** follicule en régression.

(www.ext.vt.edu/pubs/beef/400-013)

Au cours d'un cycle oestral, 2 ou 3 vagues folliculaires ont été observées chez la vache (Sirois & Fortune, 1988) tandis que la majorité des juments n'ont qu'une seule vague (Sirois et al., 1989 cités par Fortune, 1994). Les vagues folliculaires ont été détectées par échographie aux jours 2, 9 et 16 (jour 0 étant le jour de l'ovulation) du cycle oestral chez les vaches à 3 vagues folliculaires et aux jours 2 et 11 pour les vaches à 2 vagues (Sirois & Fortune, 1988).

2.3.3. Déviation et sélection folliculaires

Au cours de la croissance, les différents compartiments du follicule (cellules de la granulosa, thèque interne, complexe cumulus-ovocyte) synthétisent, sous l'influence des gonadotropines, divers facteurs biochimiques qui vont à leur tour influencer le développement folliculaire. Ces facteurs dits intra-ovariens peuvent agir selon les modes paracrine et autocrine (Gougeon, 1996). La FSH en synergie avec des facteurs intra-ovariens tels les activines et l'IGF, augmentent le nombre des FSHR, induit les LHCGR, stimule la prolifération cellulaire, l'activité de la CYP19A1 ou aromatase et la synthèse protéique de la cellule (Hsueh et al., 1989; Webb et al., 1999). Cette dynamique de synthèse est responsable du fait qu'au sein de chaque vague folliculaire, à un moment donné, certains follicules se développent au détriment de leurs congénères. Ce phénomène appelé *déviation* à cause de la divergence des follicules (aussi bien en terme de croissance que la capacité de synthèse) va se terminer par la *sélection* finale d'un ou des follicules qui pourraient ovuler selon que l'espèce soit mono ou poly ovulatoire (Ginther et al., 2001a; Webb, et al., 1999; Fortune et al., 2004). La déviation coïncide avec la diminution de la concentration de FSH causée par le rétrocontrôle négatif exercé

par l'oestradiol et potentiellement l'inhibine, tous deux synthétisés par le follicule en développement (Erickson & Shimasaki, 2001; Fortune et al., 2004).

Les causes de la déviation ne sont pas entièrement élucidées mais il est indéniable que les follicules sélectionnés sont plus aptes (que leurs congénères) à répondre aux stimuli favorisant leur croissance. Selon les études de Xu et al. (1995b) et Bao et al. (1997b), la sélection est associée à l'initiation de l'expression du LHCGR et la HSD3B2 dans les cellules de la granulosa. Bao & Garverick (1998) par hybridation *in situ* ont comparé l'expression des ARNm des récepteurs des gonadotropines dans les follicules sélectionnés et ceux qui ne l'ont pas été. Ces auteurs concluent que les ARNm du LHCGR sont plus élevés dans les cellules thécales et celles de la granulosa du follicule dominant par contre aucune différence d'expression du FSHR n'a été observée dans les deux types de follicule comparés. Selon Baker & Spear (1999), le degré de vascularisation du follicule jouerait un rôle critique dans la sélection du follicule. Dans le même sens, Zeleznik et al. (1981) et Hunter et al., (2004) ont détecté des concentrations élevées de "vascular endothelial growth factor" (VEGF) dans le follicule dominant.

2.3.4. Dominance folliculaire

Le follicule est qualifié de *dominant* lorsqu'il poursuit son développement tandis que les autres dits *subordonnés* régressent en sa présence (**Fig. 2**). La dominance ainsi décrite peut être considérée comme l'étape finale du processus de sélection car le follicule dominant peut se développer en follicule ovulatoire si les conditions lui sont

favorables, notamment la relâche préovulatoire de LH (Sirois & Fortune, 1988; Fortune, 1994; Baker & Spears, 1999; Driancourt, 2001; Evans, 2003). Si le follicule dominant se caractérise par sa grande capacité à produire l'oestradiol (Ireland & Roche, 1983; Fortune et al., 2001) grâce à une augmentation de CYP19A1 (Sisco et al., 2003; Fayad et al; 2004; Ndiaye et al., 2005), il a aussi la faculté de répondre à la stimulation de la LH au moment où la concentration circulante de la FSH décline (Adams, 1999).

Malgré d'innombrables études, les mécanismes impliqués dans la sélection du follicule dominant ne sont pas encore entièrement élucidés. Cependant, deux théories ont été émises (Baker & Spears, 1999; Driancourt, 2001). La première stipule que la sélection folliculaire est contrôlée par des mécanismes endocrinien,s notamment la baisse de la concentration circulante en FSH. Selon la deuxième théorie, le follicule de plus grande taille inhiberait le développement de ses congénères au sein de la cohorte. Dans ce sens, Armstrong & Webb (1997) ont émis l'hypothèse selon laquelle la sécrétion d'inhibiteurs de croissance des follicules ("follicular growth inhibitory factors"; FGIFs) par le follicule dominant empêcherait le développement des follicules subordonnés par un mécanisme inconnu. Les deux théories citées ci-avant n'étant pas mutuellement exclusives, elles peuvent toutes s'appliquer chez les espèces monovulatoires telle la vache (Campbell et al., 1995), tandis que la première théorie prévaut chez la brebis (Fortune et al., 1991).

La taille du follicule bien qu'étant un facteur déterminant n'est pas un indicateur précoce de la dominance car les changements biochimiques précèdent la différence

morphologique entre les follicules sélectionnés et non sélectionnés (Ginther et al., 2001b). Concernant les changements biochimiques, plusieurs molécules sont impliquées dans le processus de la dominance. Chez la vache, les études de Beg et al. (2002) et Mihm et al. (2000) ont montré que les concentrations d'oestradiol, d'estrone et d'IGF libre augmentent dans le liquide folliculaire, tandis que celles des "insulin-like growth factor binding protein 2" (IGFBP2) et IGFBP4 diminuent. Les concentrations d'androstènedione, de progestérone, des inhibines totales et d'inhibine A quant à elles ne changent pas durant les 12 h d'établissement de la dominance. En utilisant l'hybridation *in situ*, l'expression des ARNm des enzymes stéroïdogéniques et des récepteurs de gonadotropines a été comparée entre les follicules dominant et subordonnés. Dans le follicule dominant, les concentrations d'ARNm du LHCGR et de la CYP17A1 sont élevées dans la thèque interne et il en est de même pour ceux du FSHR et de la CYP19A1 dans les cellules de la granulosa (Bao et al., 1997b; Fortune et al., 2001; Fortune et al., 2004; Fayad et al., 2004; Ndiaye et al., 2005). Les études de Evans & Fortune (1997) et Bao et al. (1997b) contrairement aux observations de Bao & Garverick (1998) n'ont pas détecté la présence du LHCGR dans les cellules de la granulosa, amenant ces auteurs à conclure que l'acquisition du dit récepteur surviendrait au cours de la dernière étape de la dominance. Selon Robert et al. (2003) dont les travaux récents par RT-PCR et immunobuvardage ont détecté le LHCGR dans les cellules de granulosa de follicules < 4 mm et ceux > 5mm, cette divergence de résultats est imputable à la sensibilité de la technique d'hybridation in situ (utilisée dans les études précédentes) par rapport à celle de la RT-PCR.

De plus en plus, le système IGF est impliqué dans les modèles de sélection du follicule dominant. Dans les cellules de la granulosa, les liaisons fonctionnelles entre les voies de signalisation de l'IGF1 et de la FSH ont été décrites par Zhou et al. (1997) et Richards et al. (2002a). Dans ces cellules du follicule antral, les IGF1 et IGF2 induisent principalement en synergie avec la FSH, la synthèse d'oestradiol et la prolifération cellulaire. Ces actions sont inhibées lorsque les IGFs sont liées à leurs protéines de liaison dénommées IGFBPs (Poretsky et al., 1999; Schams et al., 2002; Spicer et al., 2002; Spicer, 2004). Aussi, il existe des protéases qui clivent les IGFBPs libérant ainsi les IGFs (Webb et al., 1999; Adashi, 1998). Parmi celles-ci, la protéase d'IGFBP4 nommée "pregnancy-associated plasma protein A" (PAPPA), secrétée par les cellules de la granulosa a été reconnue comme un marqueur de la dominance folliculaire chez plusieurs espèces : la ratte (Hourvitz et al., 2002), la femme (Erickson & Shimasaki, 2001), les ruminants, porcins et équins (Mazerbourg et al., 2001; Spicer 2004; Fortune et al., 2004). En outre, l'expression de l'ARNm de PAPPA est positivement corrélée à celle de la CYP19A1 et du LHCGR (Monget et al., 2002; Fayad et al., 2004). Fortune et al. (2004) suggèrent que le premier follicule qui acquiert une activité protéolytique contre IGFBP4 et IGFBP5 aura un taux élevé d'IGF libre, d'oestradiol et par conséquent sera le follicule dominant. Les travaux de Fayad et al. (2004) ont montré une augmentation significative de l'ARNm dans le follicule dominant comparativement aux petits follicules ($\leq$ 4 mm), par contre aucune différence significative n'a été notée entre le follicule dominant et le follicule ovulatoire. Les études récentes de Santiago et al. (2005) chez la vache ont démontré que l'expression de l'ARNm de PAPPA dans les cellules de la granulosa du follicule dominant n'est pas significativement différente de

celle des follicules subordonnés. Aussi, ces auteurs ne trouvent aucune corrélation entre les concentrations de l'ARNm de PAPPA et le taux d'oestradiol dans le liquide folliculaire. De ces observations, Santiago et al. (2005) émettent l'hypothèse que chez la vache, il se pourrait que les changements dans l'activité de PAPPA surviennent sans un changement de la concentration de l'ARNm. A la lumière des ces études réalisées chez la vache, seule l'augmentation de l'activité protéolytique de PAPPA pourrait être utilisée comme un critère de dominance.

Les techniques du DDRT-PCR et de la SSH ont démontré que plusieurs gènes présentent un taux d'expression plus élevé dans le follicule dominant (> 8mm) comparativement aux follicules ovulatoires et les petits follicules antraux de 2-4 mm de diamètre (Fayad et al., 2004; Hennebold, 2004; Ndiaye et al., 2005). On pourrait citer plusieurs gènes dont celui de la "serine protease inhibitor-E2" (SERPINE 2; Bédard et al., 2003), de l'"apolipoprotein E receptor 2" (Apo ER2) aussi connu sous le nom de "low-density lipoprotein receptor related protein 8" (LRP8; Sisco et al., 2003; Fayad et al., 2004) et de la "carbopeptidase D" (CPD, Sisco et al., 2003; Ndiaye et al., 2005) toutefois leur rôle précis dans l'établissement de la dominance n'est pas été encore établi.

Considérant les conclusions de nombreuses études menées sur la dominance folliculaire chez la vache, il apparaît que ce processus semble être contrôlé par plusieurs mécanismes qui agissent de concert afin de permettre la poursuite du développement du follicule sélectionné.

2.4. Croissance du follicule dominant

Le follicule dominant va poursuivre sa croissance et il aura la chance d'ovuler si le corps jaune cyclique régresse. Dans le cas échéant, ce follicule subira l'atrésie. Ce fait est pris en compte dans les principes de la synchronisation du cycle oestral chez la vache. Le qualificatif "préovulatoire" est utilisé dans ce document pour définir le follicule mature n'ayant pas été stimulé par l'hormone lutéinisante (LH/hCG). Dans ce follicule préovulatoire, les LHCGR sont plus nombreux que les FSHR (Hillier, 2001) ce qui lui confère la capacité de produire des quantités élevées d'oestradiol.

Dans les follicules en croissance, les cellules de la granulosa constituent le type cellulaire qui prolifère le plus. Dans la maturation du follicule préovulatoire, ces cellules passent par des étapes de différenciation en acquérant les capacités à déclencher et répondre à la décharge ovulatoire de la LH. C'est dans ce cadre que s'inscrit l'augmentation de la CYP19A1 nécessaire pour la production de l'oestradiol qui est un facteur essentiel au déclenchement du pic ovulatoire de LH (Richards 2001a ; Richards et al., 2002a). Le récepteur nucléaire bêta ou récepteur 2 de l'œstrogène (ESR2) contrairement à son analogue le récepteur alpha ou un (ESR1) semble nécessaire pour la croissance du follicule mais aussi sa différenciation (Couse et al., 2005). La souris dont le gène ESR2 a été invalidé présente un faible taux de rupture folliculaire à cause d'une induction insuffisante de la "prostaglandin endoperoxide synthase 2" (PTGS2) aussi connue sous le nom de cyclooxygénase 2 (COX-2), du récepteur nucléaire de la

progestérone (PGR), de l'incomplète expansion du complexe cumulus ovocyte, d'une aberrante augmentation de la CYP19A1 et de l'oestradiol plasmatique (Couse et al., 2005). Les cellules du cumulus ont des caractéristiques différentes des cellules de la granulosa murale. En effet, elles démontrent un taux élevé de prolifération, une faible capacité stéroïdogénique, une faible expression du LHCGR, la capacité de sécréter de la matrice extracellulaire ("extracellular matrix"; ECM) (Li et al., 2000). Plusieurs études ont montré que cette différence phénotypique est promue par l'ovocyte (Eppig et al., 1997; Li et al., 2000).

Avant la relâche préovulatoire de LH, le noyau de l'ovocyte, arrêté au stade diplotène de la prophase I, est entouré d'une membrane nucléaire. Les facteurs qui maintiennent cet état de l'ovocyte ont été l'objet de plusieurs études. Le premier constat est que les cellules somatiques du follicule antral jouent un rôle important dans ce phénomène car la maturation spontanée de l'ovocyte survient lorsqu'il est soustrait à l'environnement de ces cellules (Richard & Sirard 1996; Eppig et al., 2004). Si la présence des jonctions de type gap est primordiale dans ce processus (Cecconi et al., 2004; Salustri et al., 2004), d'autres facteurs sont aussi impliqués. En effet, plusieurs études ont rapporté que le liquide folliculaire inhibait la reprise de la méiose (Tsafari & Dekel, 1994 cités par Conti et al., 2002). Dans ce fluide antral, trois substances produites par les cellules de la granulosa sont responsables de cet effet. Ce sont : l'"ovocyte meiotic inhibitor" (OMI; Conti et al., 2002) dont la nature biochimique n'est pas connue, l'hypoxantine (Downs et al., 1985; Eppig, 1991) et dans le fluide folliculaire porcin le "mullerian inhibiting substance" (MIS; Sirard et al., 1998).

Le maintien de l'arrêt méiotique est corrélé avec une élévation de l'AMPc dans l'ovocyte (Eppig et al., 1983; Aktas et al., 2003) et inversement une diminution de l'AMPc est associée à la reprise de la méiose (Sirard et al., 1998). Si le passage de l'AMPc à travers les jonctions de type gap a été rapporté (Lawrence et al., 1978), son mécanisme d'action n'a pas été clairement défini (Conti et al., 2002; Eppig et al., 2004) bien qu'une protéine Gs ait été identifiée au niveau de l'ovocyte de souris (Mehlmann et al., 2002). Aussi, chez la vache, l'adénylate cyclase (AC) a été localisée dans les cellules du cumulus (Kuyt et al., 1988) et son activité a été démontrée dans la membrane plasmatique de l'ovocyte (Bilodeau et al., 1993). La stimulation par la forskoline de l'ovocyte dénudé des cellules du cumulus de ratte retarde la maturation nucléaire (Dekel et al., 1984). Le facteur qui active l'AC est inconnu; s'il provenait des cellules de la granulosa/cumulus, l'augmentation de l'AMPc de l'ovocyte serait due au transfert de l'AMPc des cellules de la granulosa/cumulus et l'activation de AC (Eppig et al. 2004). Chez la ratte et la souris, la phosphodiestérase 3 (PDE3) exprimée exclusivement par l'ovocyte est inhibée par l'hypoxanthine; elle joue un rôle important dans la reprise de la méiose (Downs et al., 1989; van den Hurk & Zhao, 2005).

2.5. Maturation finale du follicule ovarien

2.5.1. Définitions et historique

Chez les mammifères, l'ovulation peut se définir comme étant un phénomène biologique qui libère l'ovocyte suite à la rupture du follicule (tissu sain) à la surface de l'ovaire (Espey & Lipner, 1994). La décharge ovulatoire de LH provenant de

l'hypophyse ou l'injection de l'hormone chorionique gonadotropine humaine (hCG) est responsable de l'ovulation et de la lutéinisation du follicule préovulatoire (Espey & Lipner, 1994). L'intervalle de temps qui s'écoule entre la stimulation par LH/hCG et l'ovulation est variable selon les espèces (Sirois & Doré 1997). Toutefois, l'intervalle entre l'induction de PTGS2 et l'ovulation est estimé à 10 h chez la ratte, la vache et la jument; ce facteur est donc constant entre les espèces considérées bien que l'induction de PTGS2 ait lieu à 4, 18 et 30 h après injection de hCG respectivement chez la ratte, la vache et la jument (Sirois & Doré, 1997).

L'ovulation a été l'objet de plusieurs études comme en témoigne les différentes théories explicatives du phénomène rapportées par Espey & Lipner (1994). En effet, au cours du siècle dernier, pour beaucoup d'auteurs, l'ovulation était due à une contraction des muscles lisses (Von Winiwarter & Saintmont, 1909; Thomson, 1919; tous cités par Espey & Lipner, 1994), pour d'autres l'augmentation de la pression du liquide folliculaire en était la cause (Heape, 1905; Zacharia & Jensen, 1958, auteurs cités par Espey & Lipner, 1994). Aussi, l'activité des enzymes protéolytiques a été suspectée comme agent causal (Schochet, 1916; Moricard & Gothie, 1946; cités par Espey & Lipner, 1994). Actuellement, ces théories sont obsolètes, toutefois celle suggérant l'implication des enzymes protéolytiques est en partie véridique.

De nos jours, l'ovulation est assimilée à une réaction inflammatoire (Parr, 1974; Espey, 1980) durant laquelle la relâche préovulatoire de LH induit la synthèse d'ARNm et de protéines dans les différents compartiments du follicule (Richards, 1994). Des

expériences antérieures avaient montré que l'injection d'inhibiteurs de synthèse d'ARNm (l'actinomycine) ou de protéines (la cycloheximide) empêche l'ovulation (Espey & Lipner, 1994). Les néo-synthèses d'ARNm et de protéines au sein du follicule sont variables dans le temps selon le gène et l'espèce animale considérés; elles débutent tôt (1h après la stimulation de LH chez la ratte) et peuvent persister quelques heures avant l'ovulation (Espey & Lipner, 1994). Ainsi, l'expression d'une multitude de gènes est augmentée ou induite durant le processus ovulatoire (Espey & Richards, 2002); ce nombre continuera indéniablement de s'accroître avec le temps et les innovations technologiques.

Sur le plan physiologique, les conséquences de ces néo-synthèses se traduisent au sein du follicule par les événements suivants : l'augmentation de la perméabilité vasculaire (Acosta & Miyamoto, 2004), l'arrêt de la prolifération et la différenciation des cellules de la granulosa et des thèques en cellules thécales (Chaffin & Stouffer, 2002), l'orientation de la stéroïdogénèse en faveur de la sécrétion de la progestérone (Chaffin & Stouffer, 2002) et la synthèse de prostaglandines (Eppig, 2001; Sirois et al., 2004a).

Les termes *processus ovulatoire et période périovulatoire* réfèrent respectivement aux événements et à la période survenant avant la rupture du follicule. Le terme *ovulation* quant à lui est utilisé aussi bien pour la libération de l'ovocyte que la rupture du follicule. Cependant, la locution *rupture folliculaire* est utilisée par souci de clarté pour insister sur cet aspect en cas de nécessité.

2.5.2. Mécanismes d'action de l'hormone lutéinisante

Les actions biologiques de la LH s'effectuent après sa liaison au domaine extracellulaire de son récepteur. La glycosylation des hormones LH et hCG est nécessaire pour l'obtention d'une réponse biologique (Davis, 1994). Le LHCGR est un polypeptide codé par un gène de 11 exons, il appartient à la famille des récepteurs couplés aux protéines G (GPCR) avec leurs 7 domaines transmembranaires caractéristiques (Ascoli et al., 2002). Plusieurs isoformes du dit récepteur ont été identifiés. Chez le rat, 4 variantes ont été rapportées; ils lient tous l'hCG (McFarland et al., 1989; Ascoli et al., 2002; Robert et al., 2003). Provenant du même gène, ils sont issus de l'épissage alternatif et des différents sites d'initiation et/ou de polyadénylation (Minegishi, 2004). La fonction biologique de ces différents isoformes n'est pas connue. Chez la vache, plusieurs isoformes d'ARNm du récepteur complet ont été caractérisés et jugés fonctionnels (Kawate & Okuda, 1998). Les études de Muller et al. (2003) ont montré que l'action biologique de la LH était réduite avec l'isoforme du LHCGR dépourvu de l'exon 10 à cause de la production insuffisante de l'AMPc, par contre l'action de hCG n'était pas affectée.

Les mutations et l'invalidation du gène LHCGR occasionnent l'infertilité dans les deux sexes (Lei et al., 2001; Rao & Lei, 2002; Burns & Matzuk, 2002). Les études de Menon et al. (2004) chez la ratte ont démontré l'existence d'une protéine de liaison du LHCGR (LH "receptor binding protein", LHBP) qui contrôlerait la stabilité de l'ARNm

du dit récepteur. Ces mêmes auteurs ont rapporté que la diminution de l'expression de l'ARNm du LHCGR en réponse à l'élévation de l'AMPc est corrélée à l'augmentation de LHBP.

2.5.2.1. Voies de signalisation activées

La relâche préovulatoire de LH active plusieurs voies de signalisation bien que celle de la protéine kinase A (PKA) soit la plus fréquemment citée (Richards et al., 1998 ; Richards 2001b). La LH/hCG, en se liant à son récepteur, active la sous-unité Gs de la protéine G hétérotrimérique, puis l'adénylate cyclase. Celle-ci favorise la production d'AMPc qui stimule à son tour la PKA. Les unités catalytiques de PKA migrent dans le noyau et phosphorylent plusieurs facteurs de transcription. La protéine de liaison "cAMP response element binding protein" (CREB) se lie sur le "cAMP response element" (CRE) du promoteur de plusieurs gènes afin de les activer (De Cesare & Sassone-Corsi, 2000). De plus, l'expression de plusieurs gènes ne possédant pas de site CRE dans la partie proximale de leur promoteur, peut être induite par des facteurs de croissance, d'autres "response elements" (RE) et voies de signalisation (De Cesare & Sassone-Corsi, 2000).

Le LHCGR en activant la sous-unité Gq de la protéine G stimule aussi la phospholipase C (PLC) qui à son tour active la protéine kinase C (PKC) (Davis, 1994; Espey & Lipner, 1994). Il occasionne l'augmentation intracellulaire d'inositol triphosphates (IP3), du diaglycerol (DAG) et du calcium (Davis, 1994; Richards, 1994). Les voies de signalisation de "Janus kinase" (JAK ; Luttrell & Lefkowitz, 2002;

Carvalho et al., 2003) et du "mitogen activated protein kinase" (MAPK; Salvador et al., 2002; Gutkind, 1998) sont aussi activées par le LHCGR. La signalisation MAPK est stimulée aussi bien par la PKA que les "exchange protein directly activated by cAMP" (EPAC) qui bien que n'étant pas des kinases se lient à l'AMPc (Springett et al., 2004). Les interactions nommées aussi "cross-talk" entre ces différentes cascades de signalisation ne sont pas entièrement élucidées.

2.5.2.2. Désensibilisation du récepteur LHCGR

La désensibilisation est un processus physiologique qui protège contre l'hyperstimulation aiguë et chronique. Elle est la conséquence de plusieurs mécanismes : i) le découplage du récepteur à la protéine G suite à la phosphorylation du récepteur; ii) l'internalisation du récepteur et iii) la diminution du nombre de récepteurs ("down regulation") suite à la réduction du taux transcriptionnel et traductionnel ainsi que la dégradation des récepteurs (Minegishi, 2004). La durée de ces processus est variable, elle est de quelques secondes à quelques minutes pour la phosphorylation et des heures pour la diminution des récepteurs. L'activation des MAPK est impliquée dans la désensibilisation (Luttrell, 2002; Amsterdam et al., 2002; McArdle et al., 2002).

La phosphorylation des GPRC par des kinases nommées GPRCK augmente leur affinité de liaison aux β-arrestines (Zhang et al., 1999; Minegishi, 2004). En absence de stimulation, la protéine G monomérique "adenosine diphosphate ribosylation factor 6" (ARF6) est ancrée à la membrane cytoplasmique. Stimulée par l'activation du LHCGR, elle va libérer l'arrestine 2 encore nommée β-arrestine 1 qui va se fixer au GPRC

(Hunzicker-Dunn et al., 2002; Ascoli et al., 2002). Le complexe formé par GPRC-β-arrestine découple le récepteur à la protéine G et favorise son internalisation dans les puits de clathrine ("clathrin coated pits"; Minegishi, 2004; Luttrell & Lefkowitz, 2002) ou les cavéoles (Pelkmans & Helenius, 2002; Nabi & Le, 2003). D'autres voies de signalisation telles Src ("avian sarcoma (Schmidt-Ruppin A-2) viral oncogene") et MAPK peuvent être activées durant ce processus (Hunzicker-Dunn et al., 2002; Ascoli et al., 2002). La liaison de l'arrestine 2 à la 3^{ième} boucle intracellulaire du LHCGR est essentielle pour l'internalisation par endocytose (Hunzicker-Dunn et al., 2002). Après l'internalisation, plusieurs types de GPCRs ne sont pas recyclés parce qu'ils sont acheminés et dégradés dans les lysosomes (e.g. cas du récepteur à l'endotheline) ou demeurent dans les endosomes (e.g. le récepteur Angiotensine1A; Ferguson, 2001). Le LHCGR du rat est recyclé à la surface alors que celui de l'humain est dégradé dans les lysosomes (Ascoli et al., 2002). En cas de re-sensibilisation, les GPCRs sont déphosphorylés dans les endosomes par les phosphatases puis recyclés au niveau de la membrane plasmatique (Ferguson, 2001).

Par différents mécanismes d'action impliquant plusieurs voies de signalisation, la LH stimule la synthèse de protéines codées par des gènes spécifiques qui initient ou altèrent des voies de signalisation préexistantes. Il en résultera les événements suivants : l'arrêt de la croissance folliculaire, la différenciation des cellules de la granulosa et des thèques en cellules lutéales, la maturation finale du complexe cumulus-ovocyte, la rupture de la paroi folliculaire libérant ainsi l'ovocyte et enfin le remodelage tissulaire,

un prélude à la formation du CL. Ces événements physiologiques sont concomitants et peuvent être indépendants (Espey & Lipner, 1994; Espey & Richards, 2002).

2.5.3. Changements dans les différents compartiments du follicule ovarien

Les changements biochimiques initiés par la LH dans les cellules somatiques du follicule ovarien se traduisent par une expression génique reprogrammée et irréversible. D'une manière générale, le taux d'expression de nombreux gènes importants pour l'ovulation et la lutéinisation augmente au détriment de celui des gènes qui étaient essentiels pour la croissance folliculaire. Les gènes induits et ceux dont l'expression augmente vont favoriser : la différenciation des cellules de la granulosa et de la thèque, la sécrétion de progestérone et de prostaglandines, la protéolyse de l'apex pour la libération de l'ovocyte (**Fig. 3**).

Bon nombre de gènes induits durant le processus ovulatoire ont été rapportés dans les réactions inflammatoires d'où l'analogie entre l'ovulation et l'inflammation. Dans un souci de concision, une citation sommaire de certains gènes importants, voire essentiels, sera faite. Il faut souligner que la plupart des études d'expression ont été réalisées chez la souris et la ratte (Espey & Richards, 2002), la variation entre les espèces animales est à considérer.

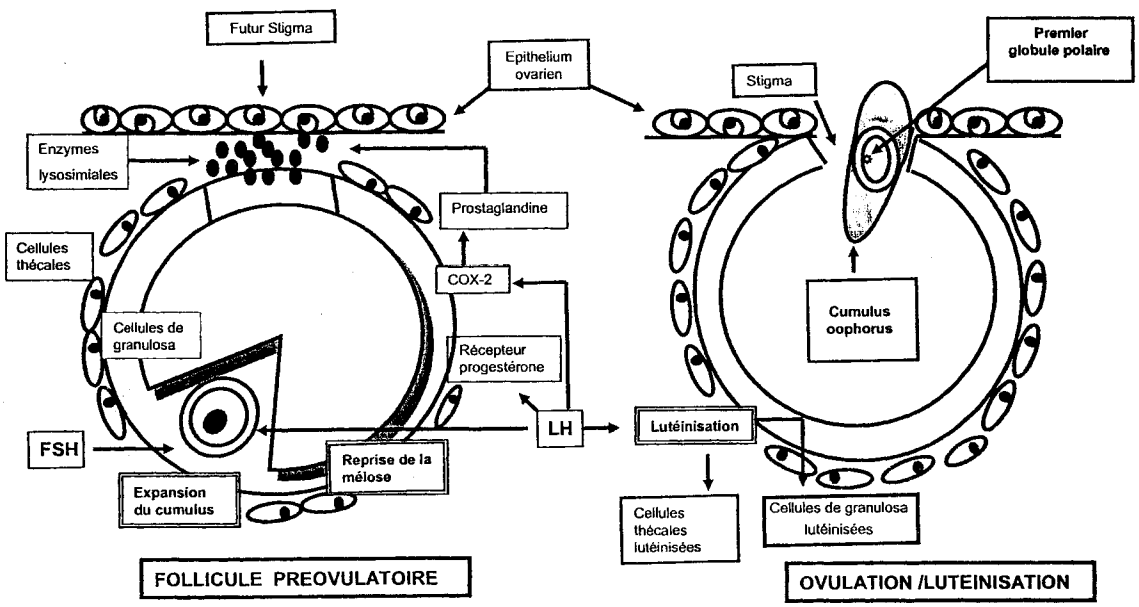

Figure 3. Mécanismes cellulaires induits durant le processus ovulatoire dans le follicule ovarien (adapté de Erickson, 1995).

(www.endotext.org/female)

2.5.3.1. Changements dans les cellules de la granulosa

2.5.3.1.1 Les gènes réprimés

Selon le gène considéré, le taux d'expression peut varier de la diminution significative à la suppression totale aussi bien pour l'ARNm que la protéine. Tel est le cas de plusieurs gènes impliqués dans la croissance du follicule préovulatoire incluant la prolifération cellulaire et la synthèse des oestrogènes. Ces gènes sont : la cycline D2 (Richards, 2001c), Foxo-1 ou FKHR (Richards et al., 2002a), la connexine 43 (Cx43 ou CJA1; Nuttinck et al., 2000; Kalma et al., 2004; Ndiaye et al., 2005), le FSHR, la follistatine et le CYP19A1. Une réduction significative de l'expression du facteur de ribosylation ARFGAP3, le "protein regulator of cytokinesis 1" (PRC1), le "proteoglycan 1" (PRG1), le "replication protein A 2" (RPA2), l'enzyme "stearoyl-coenzyme A desaturase" (SCD), le récepteur LRP8/ApoER2, la CPD, la SERPINE2, la sous unité bêta A de l'inhibine et la kinase "tribbles homolog 2", (TRIB2) ont été rapportés récemment par Ndiaye et al. (2005) suite à la caractérisation d'une génothèque soustraite d'ADNc de cellules de granulosa (CG) de follicule préovulatoire bovin précédant et après la stimulation de hCG.

2.5.3.1.2. Les gènes induits ou surexprimés

S'il est indéniable que tous les gènes induits ou surexprimés jouent un rôle dans le processus ovulatoire et/ou la lutéinisation, la fonction biologique et le mécanisme d'action sont méconnus pour bon nombre d'entre eux. Ces gènes pourraient être regroupés en 4 groupes en tenant compte de la précocité de leur expression par rapport à la relâche préovulatoire de LH ou de leurs fonctions dans d'autres événements

biologiques connus. Ainsi, on distingue : les gènes induits précocement ("immediate–early genes"), ceux qui sont impliqués dans la stéroïdogénèse, la réaction inflammatoire et enfin ceux dont le rôle dans l'ovulation est inconnu.

a) Gènes induits précocement

Induits transitoirement durant les premières étapes du processus ovulatoire (maximum d'expression atteint dans les 4 heures chez la ratte), ces gènes sont impliqués dans l'expression d'autres gènes associés eux aussi au processus ovulatoire (Richards et al., 2002a). Il s'agit de l'"early growth regulatory factor-1" (EGR1, Espey et al., 2000a; Russell et al., 2003a), le récepteur de la progestérone (PGR; Richards 2001a), de l'interleukine-1 beta (IL1B) et IL6 (Gerard et al., 2004; Brannström, 2004), la PTGS2 (Sirois, 1994; Espey & Richards, 2002), le "CAAT-enhancer binding protein beta" (CEBPB; Sirois & Richards, 1993), la "5-aminolevulinate synthase" (ALAS; Espey & Richards, 2002), le "tumor necrosis factor-α" (TNF; Brannström et al., 1995; Espey et al., 2004).

L'"early growth regulatory factor-1" augmente l'expression de plusieurs gènes dont IL1B, TNF et CEBPB (Espey & Richards, 2002; Russell et al., 2003a). Ces trois gènes à leur tour stimulent l'expression d'autres gènes dont PTGS2 (Sterneck et al., 1997; Richards, 1994; Kol et al., 2002) qui induit le VEGF (Espey et al., 2004). Les gènes PTGS2, CEBPB, EGR1 jouent un rôle important dans les follicules ovulatoires car les souris invalidées pour chacun de ces gènes sont infertiles (Robker et al., 2000). En effet, l'invalidation de PTGS2 (Davis et al., 1999) et celle de CEBPB (Sterneck et

al., 1997) empêchent l'expulsion des oocytes dans le follicule. L'infertilité de la souris ayant une mutation nulle pour l'EGR1 est causée par l'absence de la sous unité β de la LH (Lee et al., 1996).

b) Gènes impliqués dans la stéroïdogénèse

Suite à une stimulation par la LH, la synthèse des œstrogènes diminue au profit de celle de la progestérone. Les enzymes impliquées dans la biosynthèse de cette hormone augmentent non seulement dans la thèque mais aussi dans les cellules de la granulosa chez la ratte; il s'agit de : la CYP11A1 (Kaynard et al., 1992; Boerboom & Sirois, 2001), la 3α-HSD, la "steroidogenic acute regulatory protein" (STAR; Espey & Richards, 2002; Chaffin & Stouffer, 2002) et l'adrénodoxine (Espey & Richards, 2002). Chez la vache, l'expression de la CYP11A1 diminue dans les cellules de la granulosa 24 h après l'injection de hCG (Ndiaye et al., 2005).

Le PGR qui est aussi un facteur de transcription (Park & Mayo, 1991; Natraj & Richards, 1993; Cassar et al., 2002) est induit dans les cellules de la granulosa murale et du cumulus. La souris invalidée pour ce gène (PRKO) ne peut ovuler (Richards, 1994; Robker et al., 2000). Du PGR, dépendent l'expression de deux protéases associées à l'ovulation/lutéinisation : la "disintegrin and metalloproteinase with thrombospondin-like repeats" 1 (ADAMTS1); Espey et al., 2000b; Russell et al., 2003b; Boerboom et al., 2003) et la cathepsine L (CTS4; membre de la famille des Papaïnes; Sriraman & Richards, 2004). En effet, chez la souris PRKO, l'expression de ces deux gènes est significativement réduite (Robker et al., 2000). De plus, l'expression du gène 17β-

hydroxystéroïde déhydrogénase de type 4 (17βHSD4) qui contribue à la réduction des concentrations des oestrogènes est augmentée dans le follicule ovulatoire équin (Brown et al., 2004).

c) Gènes impliqués dans la réaction inflammatoire

Comme énoncé précédemment, beaucoup de gènes induits dans le processus ovulatoire sont impliqués dans la réaction inflammatoire. Dans cette entité qu'est la réaction inflammatoire, il sera distingué des gènes pro-inflammatoires favorisant l'atteinte tissulaire et d'autres anti-inflammatoires qui sont associés au contrôle de l'inflammation et à la phase de réparation tissulaire.

La mise en place de la réaction inflammatoire nécessite l'induction de l'expression des gènes pro-inflammatoires telles les prostaglandines qui sont nécessaires pour l'ovulation. Les concentrations de prostaglandine E2 (PGE2) et de PGF2α augmentent en période périovulatoire (Richards, 1994). L'invalidation du récepteur "prostaglandin E receptor 2" (PTGER2) de la PGE2 occasionne des troubles de reproduction (Ushikubi et al., 2000). La PTGS2, l'enzyme-clé dans la production des prostaglandines est aussi un marqueur de l'ovulation car elle est induite environ 10 h avant l'ovulation chez la ratte, la vache et la jument (Sirois et al., 2004b). Si chez la ratte l'expression de ce gène est précoce (4 h post LH; Espey & Richards, 2002), chez la vache, elle débute dans les cellules de la granulosa murale et du cumulus à 18 h post-LH/hCG et le maximum survient à 24 h (Sirois, 1994; Liu et al., 1997a; Nuttinck et al.,

2002). L'isoforme "prostaglandin synthase 1" (PTGS1) quant à lui est exprimé de manière constitutive dans la thèque interne du follicule bovin (Sirois et al., 2004b).

D'autres gènes pro-inflammatoires sont aussi associés à l'ovulation : le "tumor necrosis factor-alpha-induced protein 6" (TNFAIP6 ou TSG6) induit par TNF (Yoshioka et al., 2000; Mukhopadhyay et al., 2001; Milner & Day, 2003), le "nerve growth factor" (NGF) et son récepteur "neurotrophic tyrosine kinase receptor-type 1" (NTRK1; Dissen et al., 2000; Barboni et al., 2002), l'épiréguline (EREG; Espey & Richards, 2002), le VEGF (Garrido et al., 1993), la phosphodiestérase 4 (PDE4; Espey & Richards, 2002; Richards, 2001a; Park et al., 2003), le "nuclear receptor-interacting protein" (NRIP ou RIP140; Parker et al., 2003), le "nitric oxide synthase" (NOS; Espey et al., 2004), le "platelet-activating factor" (PAF; Narahara et al., 1996; Kordan et al., 2003), le "cell surface antigen CD63" (CD63; Espey & Richard, 2002) et le plasminogène et ses activateurs (Murdoch, 1999; Espey et al., 2004). A ces gènes s'ajoutent les interleukines, PTGS2, CEBPB, EGR1, TNF, ADAMTS1 et CTSL cités précédemment.

Plusieurs gènes impliqués dans le contrôle de la réaction inflammatoire sont associés au processus ovulatoire. On peut citer : le "regulator of G-protein signaling protein-2" (RGS2; Ujioka et al., 2000), l'"α2-macroglobulin" (α2M; Richards, 1994), les "tissue inhibitors of metalloproteinases" (TIMPs; Simpson et al., 2001; 2003; Espey et al., 2003), le "tumor suppressor p53" (Yaron et al., 1999), le "pituitary adenylate cyclase-activing peptide" (PACAP) et un des ses récepteurs PAC1 (PACAP récepteur de

type 1) sont induits aussi par la décharge ovulatoire (Richards et al., 2002b). Le PACAP ne semble pas dépendre de la stimulation du PGR car son expression n'est pas modifiée chez la souris PRKO. Outre l'action anti-inflammatoire, PACAP stimule la production de progestérone et la maturation de l'ovocyte (Gras et al., 1999; Ko & Park-Sarge, 2000; Chaffin & Stouffer, 2002).

d) Autres gènes

Plusieurs gènes induits au cours du processus ovulatoire démontrent une activité protéolytique, tels les métalloprotéases ADAMTS1 et ADAMTS4. Ces enzymes possèdent plusieurs fonctions dont celle de dégrader les protéoglycanes telles la versicane, l'aggricane (Kuno et al., 2000; Tortorella et al., 2000) et la brévicane (Nakamura et al., 2000). Cette protéolyse permet d'activer de nombreux facteurs de croissance dont GDF9, FGF2, EGF, TGFα qui étaient inhibés par leur liaison aux protéoglycanes (Park et al., 2000).

La fonction de la cathepsine L contrairement aux ADAMTS est moins bien connue. Selon Sambrano et al. (2000), dans les plaquettes sanguines, les cathepsines L et G contrôleraient l'activité de certains récepteurs en stimulant des protéases. L'expression de l'ocytocine et de son récepteur augmente dans les follicules après la stimulation de la LH. Leur rôle dans l'ovulation demeure inconnu, toutefois cette hormone interviendra au cours de la lutéolyse (Ivell et al., 1999; Jo & Fortune, 2003).

L'expression de "wingless-type mmtv integration site family, member 4" (Wnt4, membre de la famille des Wnts) augmente après la relâche préovulatoire de LH, la concentration maximale est détectée dans le corps jaune (Richards et al., 2002a). La signalisation des Wnts est importante lors du développement embryonnaire de l'ovaire et au cours de l'ensemble des différentes étapes de la folliculogénèse. Cependant, la fonction des ces glycoprotéines dans l'ovaire n'est pas connue. Il semble que Wnt4 ait des fonctions différentes dans la granulosa et les cellules lutéales en fonction de la présence du type de récepteur serpentin ("frizzled"; Fz) et de l'élément régulateur "secreted frizzled related proteins" (sFRP; Richards et al., 2002b; Jones & Jomary, 2002). Avec la stimulation de LH, en plus de Wnt4, sFRP4, Fz1 ont été détectés dans les cellules de la granulosa de la ratte (Hsieh et al., 2002, 2003; Ricken et al., 2002).

2.5.3.2. Changements dans les cellules thécales et le stroma ovarien

Le rôle des cellules thécales dans le processus ovulatoire n'est pas bien défini. Ces cellules étant le siège de la régulation de diverses enzymes aldo-kéto réductases, Richards et al. (2002a) suggèrent que la thèque interne aurait un rôle protecteur en empêchant les substances toxiques d'atteindre les cellules de la granulosa et l'ovocyte. Comme dans les cellules de la granulosa, la LH stimule dans les cellules thécales l'expression de plusieurs gènes dont le "carbonyl reductase" (CBR; Espey & Richards, 2002), des MMP et leurs inhibiteurs TIMPs qui démontrent un profil d'expression variable dans l'ovaire. TIMP1 est induit exclusivement par la relâche ovulante de LH (Espey & Richards, 2002), tandis que MMP2 est exprimée dans la thèque des follicules préantraux, préovulatoires et ovulatoires (Liu et al., 1998). Les enzymes

stéroïdogéniques (CYP11A1, 3α-HSD, STAR) et le CD63 sont aussi exprimés dans la thèque (Espey & Richards, 2002). Il en est de même pour le gène "pancreatitis-associated protein-III" (PAP-III) qui est induit au niveau du hile de l'ovaire et des cellules endothéliales (Yoshioka, et al., 2002). Le gène "P selectin" (CD62P) est induit dans les cellules endothéliales des vaisseaux des thèques; il est impliqué dans la migration leucocytaire vers les sites d'inflammation (Sayasith et al., 2005).

2.5.3.3. Changements dans les cellules du cumulus

En plus des gènes communs aux cellules de la granulosa, la relâche préovulatoire de LH induit dans les cellules du cumulus via l'AMPc, des gènes spécifiques responsables de la réaction d'*expansion* (Gilchrist et al., 2004; Richards, 2005). L'expansion se définit comme la résultante de l'accroissement des espaces intercellulaires suite à la production d'une ECM viscoélastique abondante par les cellules du cumulus (Mermillod, 2001; Richards et al., 2002b). L'ECM est composé principalement d'acide hyaluronique ("hyaluronan"; HA) synthétisé par la "hyaluronan synthase 2" (HAS2). Cette matrice est stabilisée par des protéines de liaison de HA dont les protéoglycanes telle la versicane (Richards, 2005), la glycoprotéine sérique "inter-α trypsin inhibitor" (IαI; Fries & Kaczmarczyk, 2003) et le TNFAIP6 (Carrette et al., 2001; Fries & Kaczmarczyk, 2003). L'expression de tous ces gènes est induite par la relâche préovulatoire de LH (Richards et al., 2002a; Stock et al., 2002).

L'ovocyte participe à l'expansion du cumulus par la sécrétion du "cumulus expansion–enabling factor" (CEEF) qui est identique à GDF9 selon Eppig (2001). Ce

gène stimule les gènes HAS2, PTGS2, PGE2 et son récepteur PTGER2 qui sont impliqués dans l'expansion du cumulus (Eppig, 2001; Gui & Joyce, 2005; Zhuo & Kimata, 2001). Chez les souris invalidées respectivement pour PTGS2 et PTGER2, l'expression de TNFAIP6 est réduite dans les cellules du cumulus et pas dans la granulosa murale (Ochsner et al., 2003). TNFAIP6 et IαI démontrent une activité inhibitrice dirigée contre plusieurs sérine protéases comme la cathepsine G et la plasmine, par conséquent leur présence dans l'ECM du "cumulus oocyte complexe" (COC) crée une enveloppe protectrice contre les protéases (Smith et al., 1999).

L'activité protéolytique dans l'ECM est sous l'influence de deux grands systèmes: le "plasminogen activator" (PA) et le MMP. En d'autres termes, elle est contrôlée par l'équilibre entre les protéases et leurs inhibiteurs respectifs (Ny et al., 2002). L'expression spatio-temporelle de ces protéases qui sont sécrétées sous forme de proenzymes est variable selon le stade développement folliculaire et l'espèce animale (Russell et al., 2003b; Madan et al., 2003; Boerboom et al., 2003; Richards et al., 2005). En effet, les gènes "PA–inhibitor type 1" (PAI1 ou SERPIN1) et "tissue-type PA" (tPA) augmentent chez la ratte avant l'ovulation alors que chez la souris l'urokinase–PA (uPA) et la protéase "nexin1" (PN1 ou SERPINE2) sont impliquées dans l'ovulation (Ny et al., 2002; Smith et al., 1999). Chez la vache, l'expression de la SERPINE2, l'inhibiteur de la serine protéase le plus efficace dirigée contre la uPA et le tPA, est réduite après la relâche préovulatoire de LH (Bédard et al., 2003). Cette baisse de l'expression de SERPINE2 permettrait la levée de l'inhibition sur uPA et tPA qui auront une activité accrue. La résultante combinée de la plasmine et d'autres protéases sera un remodelage

tissulaire contrôlé. L'expression spatio-temporelle des MMPs et leurs inhibiteurs respectifs suggère leur redondance pour un meilleur contrôle du processus de dégradation de la paroi folliculaire lors de l'ovulation (Smith et al., 1999; Richards et al., 2005). Après la stimulation de LH, les composantes de l'ECM favorisent le détachement du COC du follicule, son expulsion après l'affaissement de la paroi folliculaire occasionné par le remodelage tissulaire et sa capture par le pavillon de l'oviducte (*fimbria*).

2.5.3.4. Changements au niveau de l'ovocyte

Les changements sont importants à considérer pour une transition adéquate du gamète en embryon puis en individu. La stimulation par la LH permettra une maturation de l'ovocyte qui se terminera après la fécondation. Les changements qui surviennent au cours de cette maturation peuvent être regroupés sous trois grandes rubriques: la maturation nucléaire, la maturation épigénétique et enfin la maturation cytoplasmique. Les ovocytes sont dits *compétents* lorsqu'ils peuvent compléter l'ensemble de ces transformations qui sont des préalables à la fécondation et au développement embryonnaire. Cette compétence n'est acquise qu'au cours des derniers stades de la croissance du follicule antral précédant la relâche préovulatoire de LH (Eppig et al., 2004; Perry et al., 2005).

2.5.3.4.1. Maturation nucléaire : reprise de la méiose

La maturation nucléaire se caractérise par la reprise de la méiose. Il en résultera, un ovocyte dont la division est arrêtée en métaphase II et l'expulsion d'un globule

polaire. *In vivo*, la reprise de la méiose est observée après la relâche ovulatoire de LH. Elle se caractérise par la disparition de la membrane nucléaire ou "germinal vesicle breakdown" (GVB), la condensation des chromosomes et la formation des fuseaux de chromatine. Les mécanismes précis qui déclenchent cette induction de la maturation nucléaire ne sont pas entièrement élucidés. En effet, la question centrale est de savoir si la reprise de la méiose est due à une stimulation positive provenant des cellules somatiques ou la levée de l'inhibition qu'elles exerçaient sur l'ovocyte.

De nombreuses études ont démontré que les gonadotropines initient la maturation nucléaire des ovocytes en culture (Wassarman & Albertini, 1994). Selon Dekel (1988) cité par Eppig et al. (2004), la relâche ovulatoire de LH en bloquant le transfert des signaux inhibiteurs provenant des cellules somatiques via les jonctions de type gap permet la maturation nucléaire. Des études ont rapporté que la phosphorylation de la CJA1 occasionnée par la LH était corrélée à la GVB (Granot & Dekel, 1994; Ndiaye et al., 2005). Il en est de même pour l'augmentation de calcium occasionnée par la relâche ovulatoire de LH (Mattioli et al., 1998; 2000). Chez la souris, un stérol appelé "meiosis activating substance" (MAS) sécrété par les cellules de la granulosa dans le liquide folliculaire a été identifié comme un facteur stimulant la reprise de la méiose (Sirard et al. 1998; Donnay et al., 2004).

Les expériences *in vitro* sur les facteurs impliqués dans le maintien de l'arrêt de la méiose tels l'hypoxanthine, les analogues de l'AMPc ou les inhibiteurs des phosphodiesterase (PDE) indiquent que la maturation nucléaire est induite par un signal

positif provenant des cellules de la granulosa stimulées par les gonadotropines (Downs et al., 1988). Cette induction requiert des jonctions de type gap fonctionnelles (Fagbohun & Downs, 1991; Downs, 1995). Les voies de signalisation des MAPK (Su et al., 2001, 2002) et de l'AMPc (Tsafriri et al., 1996) sont aussi associées à la GVB. En se basant sur de nombreuses études, Eppig et al. (2004) émettent l'hypothèse selon laquelle les gonadotropines stimuleraient l'augmentation de l'AMPc et activeraient les concentrations de MAPK dans les cellules de la granulosa. Par ces actions, les cellules de la granulosa génèreraient le signal de la reprise de la méiose qui est transmis à l'ovocyte par les jonctions de type gap. Ce signal activerait PDE3 de l'ovocyte qui diminuerait le niveau de l'AMPc au niveau de l'ovocyte. Par la suite, après plusieurs étapes de cette cascade de signalisation non encore élucidées, la phosphatase CDC25b et le "maturation-promoting factor" (MPF) seraient activés. Il faut rappeler que le MPF est constituée par l'association de la kinase $p34^{cdc2}$ (CDK1) et la cycline B (CYB). La CDC25b quant à elle est essentielle pour l'activation de CDK1 dans la transition G2/M dans les ovocytes de souris (Eppig et al., 2004). Chez la souris dont le gène CDC25b a été invalidé, les ovocytes sont incapables de subir la GVB ou la méiose (Lincoln et al., 2002). Après avoir repris la méiose, l'ovocyte arrêtera sa division au stade métaphase II jusqu'à la fécondation. Selon Gordo et al., (2001), l'activation de la MAPK est requise pour l'arrêt de l'ovocyte à ce stade.

2.5.3.4.2. Maturation épigénétique et cytoplasmique

La maturation épigénétique est l'ensemble des modifications qui influencent l'expression des gènes sans changer la séquence d'ADN. Durant l'ovogénèse, les

modifications épigénétiques de la chromatine servent non seulement à inclure des empreintes spécifiques dans le génome mais elles contrôlent aussi l'expression des gènes au cours de la maturation ovocytaire (Bao et al., 2000; Eppig et al., 2004). Plusieurs voies de signalisation induisent les modifications sur les protéines des histones. Les changements apportés aux histones de l'ovocyte entraînent des modifications transcriptionelles de protéines spécifiques (Eppig et al., 2004). Tanaka et al. (2001) rapportent une "histone ovocyte specific linker H100" (H1F00) spécifique à l'ovocyte qui semble être impliquée dans l'organisation de la chromatine et la transition du follicule primordial en follicule primaire (Tanaka et al., 2005).

La maturation cytoplasmique peut être définie comme l'ensemble des évènements qui surviennent dans les ovocytes en croissance. Elle est essentielle pour la fécondation et les premières étapes du développement embryonnaire (Eppig et al., 2004). La migration des granules corticaux est le trait le plus marquant de la maturation cytoplasmique. En effet, dès la reprise de la méiose, ces granules qui avaient une localisation diffuse dans le cytoplasme de l'ovocyte immature migrent vers la zone corticale et s'associent au cytosquelette (Mermillod, 2001). Leur exocytose surviendra après la pénétration du spermatozoïde dans l'ovocyte. Cette migration est synchrone au rassemblement d'autres organelles tels les mitochondries et le golgi dans la région périnucléaire (Mermillod, 2001).

S'il est généralement admis que la maturation nucléaire et cytoplasmique se déroulent parallèlement, certaines étapes surviennent durant la croissance de l'ovocyte

avant la reprise de la méiose (Wassarman & Albertini, 1994; Eppig et al., 2004). Des niveaux de transcription élevés permettent l'accumulation des messages maternels, des ribosomes et des autres organelles dans l'ovocyte en croissance. Ces changements sont primordiaux pour l'accomplissement de la méiose, la fécondation et le développement préimplantatoire (Wassarman & Kinloch, 1992; Schickler et al., 1992; Albertini et al., 2003). De plus, l'ovocyte exprime aussi des gènes qui lui sont propres (Albertini et al., 2003; Hennebold, 2004; Vallée et al., 2005).

2.5.4. Ovulation

Il s'agit en fait de la succession de deux événements chronologiques : la rupture de la paroi folliculaire suivie de l'expulsion de l'ovocyte (**Fig. 3**). La rupture folliculaire est la résultante de l'activité des prostaglandines et de plusieurs enzymes protéolytiques. Elle est étroitement associée aux familles des MMP (ADAMTS1, MMP1, TIMP1) et des activateurs du plasminogène soit tPA et uPA (Murdoch, 1999; Chaffin & Stouffer, 2002; Liu et al., 2004; Richards et al., 2005). Les gélatinases, les collagénases (Smith et al., 1999) et la cathepsine L dégradent les composantes de la paroi folliculaire que sont l'élastine, la fibronectine, le collagène de type I et IV (Robker et al., 2000). Les prostaglandines quant à elles jouent un rôle aussi bien dans la rupture du follicule que dans la réparation tissulaire au site d'ovulation (Chaffin & Stouffer, 2002). La dégradation des couches de collagène de la paroi folliculaire s'accompagne d'une vasodilatation et d'une augmentation perméabilité vasculaire (Acosta & Miyamoto, 2004). Par la suite, le flux sanguin diminue au niveau de l'apex du follicule pour faciliter la rupture à cet endroit (Brannström et al., 1998; Ny et al., 2002).

L'ovocyte est entouré par la "corona radiata" qui est constituée de cellules de cumulus rattachées à la zone pellucide. L'ovocyte est expulsé avec toutes ces cellules annexes qui interviendront dans la fécondation (Yanagimachi, 1994). Il sera capté par le pavillon de l'oviducte. L'uPA serait impliquée dans le remodelage tissulaire des cellules du cumulus après l'ovulation (Eppig, 2001).

2.6. La formation du corps jaune

Après l'ovulation, le CL se forme rapidement à partir des cellules de la paroi du follicule rompu avec un accroissement de la sécrétion de progestérone : c'est la lutéinisation. Elle est la différentiation terminale des cellules somatiques en une glande endocrine (Niswender & Nett, 1994). La progestérone joue un rôle indispensable dans le développement du CL; sa concentration est corrélée au nombre de LHCGR et à l'augmentation de l'expression d'un récepteur de "low density lipoprotein" (LDL), STAR, CYP11A1 et HSD3B (Niswender & Nett, 1994; Juengel & Niswender; 1999; Chaffin & Stouffer, 2002). En plus de la sécrétion de la progestérone, un CL en formation se caractérise par un développement intense de la vascularisation, des mitoses répétées des cellules stéroïdogéniques, une faible activité de la 20α-hydroxysteroide deshydrogenase (qui convertit la progestérone en 20α-hydroxyprogestérone) et le rétablissement des connections intercellulaires (Acosta & Miyamoto, 2004; Murphy, 2004). Chez la vache l'expression de la CJA1 reste faible dans le CL comparativement aux cellules de la granulosa (Ndiaye et al., 2005). Chez la plupart des espèces, les cellules de la granulosa et de la thèque deviennent les cellules stéroïdogéniques du CL.

Les grandes et petites cellules lutéales proviendraient respectivement des cellules de la granulosa et des cellules thécales chez la brebis et la vache (Arosh et al., 2004). Par contre chez la jument, les cellules thécales ne contribuent pas à la formation du CL (Murphy, 2004). L'expression de plusieurs gènes est augmentée lors de la lutéinisation on pourrait citer les gènes impliqués dans la stéroïdogénèse (STAR, CYP11A1, HSD3B2), la cathepsine L, les intégrines (Murphy, 2004; McRae et al., 2005). Pour bons nombre d'auteurs dont Espey & Lipner (1994), l'ovulation a lieu durant les premières étapes de la lutéinisation. Il faut par contre rappeler que la lutéinisation a lieu même en l'absence de la rupture folliculaire et de la non extrusion de l'ovocyte.

En plus des hormones lutéotrophiques que sont la LH, la progestérone et la "growth hormone"(GH) (Juengel et al., 1997), des facteurs sécrétés localement supportent la fonction lutéale: l'IGF1, les prostaglandines E et I peuvent être cités en exemple (Juengel & Niswender, 1999). L'action lutéotrophique de la PGE2 se fait via l'AMPc et stimule la sécrétion de progestérone (Arosh et al., 2004). Des études *in vitro* ont montré que les facteurs de croissance angiogéniques et endotheliaux comme le FGF2 et le VEGF stimulent l'angiotensine II (AGT2), la PGF2α et la sécrétion de la progestérone dans le CL en croissance (Kobayashi et al., 2001). De plus, les inhibiteurs de métalloprotéases (TIMP1, TIMP2 et TIMP3) et les PA (PAI1) sont impliqués dans le remodelage tissulaire et dans l'angiogénèse (Smith et al., 1999; Murdoch, 1999). L'α2M (Richards, 1994), la "metallothionein-1" (MT1A; Espey & Richards, 2002; Espey et al., 2003), la glutathione S-transferase (GST; Rabahi et al., 1999; Espey & Richards, 2002) et plusieurs types d'ADAMTS sont présents dans le CL (Madan et al., 2003).

Le CL en l'absence de gestation régresse à cause des facteurs lutéolytiques que sont la PGF2α (Wiltbank et al., 1991; Pate, 1994; Niswender & Nett, 1994) et le TNF (Meidan et al., 1999). L'induction de la lutéolyse par la PGF2α n'est possible que sur un CL de 8 à 15 jours d'âge. Elle se fait par l'intermédiaire de la PKC après l'activation de la PLC (Meidan et al., 1999). Selon Sen et al. (2004), le fait que les isoenzymes de PKC ne s'expriment pas de manière synchrone dans le CL bovin expliquerait que l'action lutéolytique de PGF2α soit inhibée sur un CL de moins de 5 jours. La régression lutéale est la résultante de l'augmentation de la PGF2α, l'apoptose, la vasoconstriction, l'activité de la 20α-hydroxysteroide deshydrogenase, des cellules autoimmunes et la diminution de la progestérone (Knickerbocker et al., 1988; Arosh et al., 2004).

Lors de gestation, la lutéolyse est inhibée par des facteurs provenant de l'embryon tel l'interféron-tau chez les ruminants : on parle de "reconnaissance de la gestation" (Hansen et al., 1999). Ce facteur inhibe le développement des récepteurs de l'ocytocine et la sécrétion de PGF2α par l'endomètre (Mann et al., 1999).

3. HYPOTHESE ET OBJECTIFS

3.1. Hypothèse et modèle d'étude

L'ovulation représente une étape cruciale dans la reproduction chez l'ensemble des mammifères. Du fait qu'elle libère un gamète femelle fécondable, elle est essentielle pour la pérennité des espèces. De plus, elle a un impact économique sur la croissance

des populations d'élevage. Expliquer le processus de l'ovulation et les mécanismes qui le sous-tendent ont été et demeurent encore de nos jours une préoccupation scientifique comme en témoigne les nombreuses études réalisées. La compréhension des mécanismes moléculaires et biochimiques impliqués contribuerait non seulement à lutter contre certaines infertilités mais aussi à augmenter le taux de conception des femelles. De plus, l'ovulation constitue un bon modèle pour étudier une réaction inflammatoire "contrôlée". Aussi, cette investigation permettrait d'élucider les mécanismes associés à certaines pathologies inflammatoires (guérison des plaies, arthrite), le remodelage tissulaire, la différenciation et la signalisation cellulaire.

Comme énoncé dans la section 2.5.1, l'ovulation à l'instar des autres étapes de la folliculogénèse dépend étroitement des évènements qui surviennent dans les CG, du COC et de la thèque interne sous l'influence des gonadotropines, des facteurs de croissance, des cytokines et des signaux provenant de l'ovocyte. La relâche préovulatoire de l'hormone lutéinisante (LH/hCG), principal élément déclenchant, agit via l'AMPc et des protéines kinases en induisant la synthèse d'ARNm et des protéines nécessaires aux processus de l'ovulation et de la lutéinisation (Richards, 1994 ; Espey & Lipner, 1994). Sur ce constat se fonde l'hypothèse de la présente étude qui stipule que l'ovulation et la lutéinisation du follicule résultent de l'induction ou de la répression de l'activité transcriptionnelle de certains gènes dans les CG, du COC et de la thèque interne suite à la relâche préovulatoire de la LH. L'hypothèse spécifique de nos travaux sous-tend que plusieurs gènes sont induits ou leur expression est accrue dans les cellules de la granulosa au cours du processus ovulatoire.

La vache, espèce monovulatoire a été notre modèle d'étude car son cycle oestral peut être maîtrisé par la synchronisation d'une part par l'injection de PGF2α et le suivi des chaleurs chez l'animal. D'autre part, l'échographie transrectale permet un suivi du développement folliculaire. De plus, les différentes composantes du follicule (liquide folliculaire, CG et les thèques) peuvent être isolées séparément et en quantité suffisante pour permettre une étude plus spécifique des compartiments folliculaires.

Une étude préliminaire de Lévesque et al. (2002) a comparé à l'aide de la technique SSH, l'expression des gènes induits ou augmentés dans les CG et le COC d'un follicule 24 h suivant l'injection de hCG (qualifié de "follicule ovulatoire") comparativement aux mêmes types cellulaires d'un follicule dominant obtenu à J5 du cycle oestral. De l'analyse des données, il en a résulté une liste de gènes qualifiés de "différentiellement" exprimés dans le follicule ovulatoire. A partir de cette liste, cinq gènes ont été choisis car ils n'avaient jamais été associés ni décrits dans le processus ovulatoire.

3.2. Objectifs

Les objectifs de cette présente recherche seront de caractériser et d'étudier le patron d'expression de cinq gènes induits par la LH/hCG dans les CG en période périovulatoire chez la vache. Pour chacun des cinq gènes choisis, les objectifs spécifiques suivants seront réalisés:

1. Caractériser et cloner l'ADNc entier à partir d'une génothèque d'ADNc de CG induit par l'hCG;
2. Etudier et comparer l'expression de l'ADNc dans les CG de follicules à différents stades de développement, soit : petits follicules de 2-4 mm, follicule dominant à J5 du cycle oestral (J0 étant le jour de l'ovulation), le follicule ovulatoire induit à l'hCG et du CL à J5;
3. Etudier et comparer l'expression de l'ADNc dans les parois folliculaires (thèque interne et CG) de follicules ovulatoires récoltées à 0, 6, 12, 18, 24 h après l'injection d'hCG;
4. Comparer l'expression de l'ARNm des gènes dont la fonction est inconnue dans différents tissus bovins;
5. Etudier et localiser la protéine correspondante dans les follicules ovulatoires et le CL pour les protéines dont les anticorps sont disponibles.

CHAPITRE 2

Expression of phospholipase A2 group IVA (PLA2G4A) is up-regulated by human chorionic gonadotropin in bovine granulosa cells of ovulatory follicles

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Running title: Regulation of PLA2G4A in ovulatory follicles

Key words: ovary, follicle, granulosa cells, gene expression, ovulation, PLA2G4A, cPLA2α, PLA2G1B, PLA2A, phospholipase A2.

ABSTRACT

Prostaglandins (PG) are required for the ovulatory process, and their biosynthesis depends on the initial release of arachidonic acid (AA) from membrane phospholipids. We hypothesized that phospholipase A2 group IVA (PLA2G4A) expression is up-regulated in granulosa cells (GC) and may contribute to the increase in PG synthesis during ovulation. The objectives of this study were to characterize bovine PLA2G4A cDNA, and to investigate its spatio-temporal regulation at the mRNA and protein levels in human chorionic gonadotropin (hCG)-induced ovulatory follicles and *in vitro*, using forskolin (FSK)-stimulated GC. The full-length bovine PLA2G4A cDNA is composed of a 5'-untranslated region (UTR) of 140 bp, a 3'-UTR of 476 bp and an open reading frame of 2247 bp encoding a putative Mr protein of 85.3. The bovine PLA2G4A protein is highly conserved (83%-95% identity) compared with other mammalian orthologs. Regulation of PLA2G4A mRNA expression was studied in GC obtained from bovine follicles collected at different developmental stages: small follicles (2-4 mm), dominant follicles at day 5 (D5) of the estrous cycle, ovulatory follicles 24 h following injection of an ovulatory dose of hCG, and corpus luteum at D5. A transcript of 3.1 kb for PLA2G4A was observed by virtual Northern analysis. Semiquantitative RT-PCR analysis showed a 14-fold increase in PLA2G4A mRNA in hCG-stimulated versus dominant follicles ($P < 0.0001$). Follicular walls obtained from ovulatory follicles recovered at 0, 6, 12, 18 and 24 h post-hCG injection showed a marked regulation of PLA2G4A mRNA, with an initial 16-fold increase in PLA2G4A transcript at 12 h that reached a 45-fold increase at 24 h, as compared to 0 h ($P < 0.0001$). Immunoblots of GC extracts showed an initial induction of the PLA2G4A protein at 18 h post-hCG reaching

a maximum at 24 h, which mirrored mRNA results. Immunohistochemical localization confirmed induction of PLA2G4A in GC at 24 h post-hCG. Immunolabelling was mainly observed in mural GC compared to antral GC, and was focalized and associated with perinuclear vesicular structures. Stimulation of cultured bovine GC with 10 μM of FSK, caused an increase in PLA2G4A mRNA that was initially detected at 6 h and maximal at 24 h post-FSK. Induction of the protein *in vitro* was confirmed at 24 h post-FSK. This study reports the characterization of bovine PLA2G4A, and demonstrates that PLA2G4A mRNA and protein expression are induced by hCG in GC of ovulatory follicles via the induction of adenylyl cyclase/cAMP pathway, which suggests that induction of PLA2G4A likely contributes to the release of AA required for PG synthesis at the time of ovulation.

INTRODUCTION

In mammals, the ovulatory process begins at the time when the endogenous luteinizing hormone (LH) surge stimulates the G protein-coupled LH/human chorionic gonadotropin receptors (LHCGR) that are distributed on the surface of granulosa and theca cells of a mature preovulatory follicle. This gonadotropin–induced process is controlled by temporal and spatial expression of specific genes, and presents features reminiscent of an acute inflammatory reaction [1,2]. Modern methods in molecular biology such as mRNA differential display, cDNA array and suppression subtractive hybridization (SSH) allow the detection of differentially expressed genes during the process of follicular growth and ovulation [2-4]. Transcription of genes in granulosa cells (GC) that controls the growth of a bovine dominant or preovulatory follicle is rapidly down-regulated as a consequence of LH-mediated increases in intracellular signaling [5,6]. In conjunction with the termination of specific gene expression in preovulatory follicles, LH/hCG induces expression of genes involved in ovulation and luteinization as shown in rodents [2]. For ovulation, many genes were found to be induced or up-regulated by the LH surge using rodent models, such as: the prostaglandin-endoperoxide synthase 2 (PTGS2) [7], the progesterone receptor (PGR) [8], the CCAAT/enhancer-binding protein beta (CEBPB) [9,10], and the early growth response protein-1 (EGR1) [11]. These were shown to be obligatory for ovulation to occur since the process is impaired in mice null for these genes. Most data on the temporal pattern of gene expression arising during ovulation have been obtained using whole ovarian RNA extracts from the immature eCG- and hCG-stimulated rodent ovaries, which may conceal identification of genes specifically induced in GC. Using

GC isolated from hCG-induced ovulatory follicles from bovine adult female, SSH was applied to identify up-regulated expression of specific genes that occurs during ovulation [12]. The latter study identified a cDNA fragment that corresponded to the 3'-untranslated region (3'-UTR) of the bovine phospholipase A2 group IVA (PLA2G4A), also known as the cytosolic PLA2 alpha (cPLA2α). Thus, PLA2G4A was identified as a potential candidate gene induced in GC following stimulation by the LH/hCG preovulatory surge.

The PLA2 family is subdivided on the basis of size, secretory capabilities and calcium requirements into three subfamilies: the secreted low molecular weight Ca^{2+}-dependent enzymes (sPLA2-IB, sPLA2-IIA), the cytosolic high molecular weight Ca^{2+}-dependent enzymes (cPLA2s), and the Ca^{2+}-independent isoforms (iPLA2) [13-15]. The cPLA2 subfamily is composed of three enzymes who share some structural features without being functionally redundant : PLA2G4A (cPLA2α), PLA2G4B (cPLA2β) and PLA2G4C (cPLA2γ) [16,17]. Most of the PLA2s are widely expressed in different tissues, where they are involved in different biological processes such as inflammation, tissue repair and apoptosis [18,19]. The biological function of PLA2 enzymes is to catalyze hydrolysis of the sn-2 position of membrane glycerophospholipids, leading to production of free fatty acids and lysophospholipids, two precursors of potent bioactive mediators. This reaction is of particular importance if the esterified fatty acid is arachidonic acid (AA). Thus, the first regulated step involved in prostaglandin formation is the release of AA from membrane glycerophospholipids which is selectively done by cPLA2s [20]. Conversion of AA is further processed by downstream metabolic enzymes

like cyclooxygenases to eicosanoids including prostaglandins (PGs) and leukotrienes (LTs) [17] known to impact ovulatory mechanisms.

In relation to follicular growth and ovulation, increase in ovarian PLA2 activity in response to LH/hCG injection was first reported in rodents using whole protein ovarian extract [21]. Since a fragment of PLA2G4A cDNA was identified as up-regulated during ovulation in the bovine species [12], we hypothesized that expression of the PLA2G4A gene may be induced by the LH/hCG preovulatory surge in GC, thereby contributing to increase AA substrate required for the synthesis of PGs involved in the ovulatory process. In the present study, the bovine ovulatory follicle was used as the *in vivo* model to investigate the regulation of PLA2G4A during hCG-induced ovulation/luteinization. The regulation of PLA2G4A was then further studied *in vitro* by analyzing the stimulation of forskolin (FSK) on PLA2G4A expression using an established bovine GC culture system. The specific objectives of this study were to clone the bovine PLA2G4A cDNA, and to determine the spatio-temporal expression profile of PLA2G4A mRNA and protein in ovulatory follicles following hCG treatment and in FSK-stimulated GC.

MATERIALS AND METHODS

Cloning of the bovine PLA2G4A cDNA

Isolation of the full-length bovine PLA2G4A cDNA was performed by screening a size-selected cDNA library. Initially, the size of the full-length bovine PLA2G4A cDNA was estimated by performing a virtual Northern blot analysis. Briefly, total RNA

was isolated from GC that were obtained from bovine ovulatory follicles 24 h following an hCG injection [6], and transformed into cDNA by the SMART cDNA synthesis method (BD Biosciences Clontech, Mississauga, ON) as previously described [22]. The cDNAs were separated by gel electrophoresis, transferred onto a nylon membrane and hybridized with a bovine PLA2G4A radioactive probe (760 bp) generated from a previous SSH screening experiment [12]. Once the size of the full-length bovine PLA2G4A cDNA was determined, total SMART cDNAs from hCG-stimulated GC were size fractionated by agarose gel electrophoresis, cDNAs from 2.5 to 3.5 kb were purified and used to construct a size-selected cDNA library based on the pDrive plasmid (Qiagen PCR cloning kit; Qiagen, Mississauga, ON) that was then screened by radioactive hybridization as previously described [22]. Positive PLA2G4A hybridizing bacterial colonies were grown, their plasmid contents were isolated (QIA-prep, Qiagen), and the size of the cloned cDNA was analyzed following an *EcoR*1 digestion and gel electrophoresis analysis. The cDNAs were sequenced via the dideoxy sequencing method (Big Dye Terminator 3.0; ABI Prism, Applied BioSystem, PE, Branchburg, NJ) that were analyzed on an ABI Prism 310 sequencer (Applied Biosystem). Nucleic acid sequences were analyzed by BLAST (Basic Local Alignment Search Tool) against GenBank data banks.

Bovine tissues and RNA extraction

Bovine ovarian follicles and corpora lutea (CL) were isolated at specific stages of the estrous cycle from normal cyclic crossbred heifers, as previously described [6,23]. Briefly, estrous cycle was synchronized with $PGF_{2\alpha}$ (25 mg, im; Lutalyse, Upjohn,

Kalamazoo, MI) and ovarian follicular development was monitored by daily transrectal ultrasonography until ovariectomy. Dominant follicles (DF; n = 4 cows) were defined as > 8 mm and growing, by ultrasonographic measurement while subordinate follicles were either static or regressing. They were obtained on the morning of day 5 of the estrous cycle (day 0 = day of estrus) by ovariectomy (via colpotomy). Ovulatory follicles (OF) were obtained following an injection of 25 mg of $PGF_{2\alpha}$ on day 7 of the synchronized estrus cycle to induce luteolysis, thereby maintaining the development of the DF of the first follicular wave into a preovulatory follicle. An ovulatory dose of hCG (3000 IU, iv; APL, Ayerst Lab, Montréal, QC) was injected 36 h after the induction of luteolysis, and the ovary bearing the hCG-induced OF was collected by ovariectomy at 0, 6, 12, 18 and 24 h after hCG injection (n = 2-4 cows/time point). Follicles were dissected into preparations of follicular wall (theca interna with attached GC) [23] or further dissected into separate isolates of GC [6]. Ovariectomies were also performed on day 5 of the synchronized estrous cycle to obtain CL (n = 3 cows). Additionally, GC were collected from 2-4 mm follicles that were obtained from slaughterhouse ovaries representing a total of three pools of 20 small follicles (SF). These experiments were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine of the Université de Montréal. Total RNA was isolated from tissues as previously described [24]. The concentration of total RNA was quantified by measuring optical density at 260 nm, and quality was evaluated by visualizing the 28S and 18S ribosomal bands following electrophoretic separation on a formaldehyde denaturing 1% agarose gel with ethidium bromide.

Semiquantitative RT-PCR analysis

Total RNA (1 μg) from individual follicles or CL was reverse-transcribed with an oligo-dT30 primer and PowerScript (BD Biosciences Clontech) to generate the first strand cDNA using the SMART PCR cDNA synthesis kit (User manual: PT3041-1; BD Biosciences Clontech, Mississauga, ON) [6]. Second cDNA strands were produced with the SMART II 5'-anchored oligo, and PCR-amplified for 15 cycles using Advantage 2 DNA polymerase (BD Biosciences Clontech). SMART cDNA pools were then diluted to 50 μl in 10 mM Tris pH 8 buffer. To perform semiquantitative RT-PCR, SMART cDNA pools were diluted 10-fold in H_2O, and 1 to 2 μl were used in a 25 μl PCR reaction using the Advantage 2 DNA polymerase kit (BD Biosciences Clontech). Gene-specific PCR primers were designed in the open reading frame of the cDNA sequence for *PLA2G4A* (sense: 5'-CTTGCATTCTACACGTGATGTGCC-3' ; anti-sense: 5'-GATGTATTGAGATTCAAGCCCAGC-3'; GenBank: AY363688), phospholipase A2 group IB (*PLA2G1B*; also known as pancreatic PLA2A; sense: 5'-GAGACTCCTGGTGTTGGCTGCTC-3'; anti-sense: 5'-TTCTTGTCCAGGTTCTTGTGCTCC-3'; GenBank: NM_174646), cytochrome P450 family 19 subfamily 1 (*CYP19A1*; sense: 5'-GTCCGAAGTTGTGCCTATTGCCAGC-3' ; anti-sense: 5'-CCTCCAGCCTGTCCAGATGCTTGG-3'; GenBank: NM_174305), prostaglandin-endoperoxide synthase 2 (*PTGS2*; sense: 5'-GCATTCTTTGCCCAGCACTTCACCC-3'; anti-sense: 5'-CTATCAGGATTAGCCTGCTTGTCTGG-3'; GenBank: AF031698), and glyceraldehyde-3-phosphate dehydrogenase (*GAPD*; sense: 5'-TGTTCCAGTATGATTCCACCCACG-3'; anti-sense: 5'-

CTGTTGAAGTCGCAGGAGACAACC-3') [6]. For all these genes, PCR conditions were as follows: 95 C for 1 min, 95 C for 30 s, 64 C for 45 s, and 68 C for 90 s. The number of PCR cycles was limited (14-20 cycles) and optimized for each gene to be analyzed. The PCR reactions (20 μl/reaction) were resolved on a 2% TAE-agarose gel (40 mM Tris acetate pH 8, 1 mM EDTA) with ethidium bromide (0.5 μg/ml); PCR products were visualized by UV and the images were digitized. The digitized signals for each gene were analyzed by densitometry using ImageQuant software (Amersham Pharmacia Biotech, Pointe-Claire, QC).

Cell extracts and immunoblot analysis

Tissues and cells were homogenized in M-PER buffer (Pierce, Rockford, IL) that was supplemented with a mix of protease inhibitors (Complete; Roche Applied Science, Laval, QC) as recommended by the manufacturer's protocol. Complete lysis of GC was achieved with multiple passages through a 25G needle attached to a 3-ml syringe. CL were homogenized with a polytron PT1300D (7,000 rpm; Kinematica AG, Littau-Lucerne, SW). The protein extracts were centrifuged at 16,000 x g for 15 min at 4 C, and the recovered supernatant (whole cell extract) was stored at –80 C until electrophoretic analyses were performed. Protein concentration was determined by the Bradford method (Bio-Rad Protein assay) [25]. Protein extracts (100 μg proteins/sample) were heat-treated (5 min, 100 C), size-fractionated via one-dimensional SDS-PAGE, and electrophoretically transferred onto polyvinylidene difluoride membranes (PVDF; Hybond-P, Amersham Pharmacia Biotech). Immunoblots were performed as previously described [26]. Membranes were incubated with the

monoclonal antibody against human PLA2G4A (1:100; SC-454; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and detected with a horseradish peroxidase-linked sheep antimouse secondary antibody (1: 20,000 dilution; Amersham Pharmacia Biotech). To analyse PTGS2 protein, membranes were incubated with the rabbit antihuman PTGS2 antibody (1:5,000; MF243) [27] and detected with a horseradish peroxidase-linked donkey antirabbit secondary antibody (1: 20,000 dilution; Amersham Pharmacia Biotech). Detection of immunoreactive proteins was performed by the enhanced chemiluminescence system (ECL Plus, Amersham Pharmacia Biotech) following the manufacturer's protocol, and exposed to Hyperfilm (Amersham Pharmacia Biotech).The expression of α-glutathione S-transferase was used as control protein [28]. Autoradiograph images were digitized using a ScanMaker 9800XL flatbed scanner (Microtek lab, Inc., Redondo Beach, CA).

Primary Granulosa Cell Cultures

Primary cultures of bovine GC were performed as previously described [29]. Briefly, cells were seeded in cultures at a density of 2 x 10^6/100 mm plates in minimal essential medium (MEM) supplemented with L-glutamine, non-essential amino acids, 2% fetal bovine serum, insulin (1 μg/ml), transferin (5 μg/ml), and penicillin (100 units/ml)-streptomycin (100 μg/ml), and incubated at 37 C in a humidified atmosphere of 5% CO_2. Prior to addition of forskolin (FSK), cells were starved overnight in fresh culture media in the absence of serum. After the incubation for various times in the presence of FSK (10 μM), cells were harvested and submitted to total RNA and protein extractions. The extraction of total RNA from GC was performed using TRIzol reagent

(Invitrogen Life Technologies). RNA samples (100 ng) served for the RT-PCR analysis using the One-Step RT-PCR System in accordance with the manufacturer's instructions (Qiagen, Mississauga), with sense and anti-sense primers specific for PLA2G4A and GAPD as described above. The reaction conditions were one cycle at 48 C for 30 min and 95 C for 15 min, followed by 35 PCR-cycles of 94 C for 30 s, 59 C for 1 min and 72 C for 2 min. Following PCR amplification, reaction products were electrophoresed on 1% TAE-agarose gels. Whole cell protein extracts were isolated as described previously, and protein concentrations were determined as described above. Samples (50 μg of protein/well) were resolved by one-dimensional SDS-PAGE, and electrophoretically transferred to PVDF membranes. Membranes were immunoblotted and revealed as described above.

Immunohistochemical localization of PLA2G4A

Immunohistochemical staining was performed on PBS-buffered formalin-fixed tissues as previously described [26]. Paraffin-embedded tissues were cut to 3 μm thickness, mounted on SuperfrostPlus slides (Fisher Scientific, Pittsburgh, PA), deparaffinized and then rehydrated. Antigenicity lost during the fixation process was retrieved by heat treatment in a pressure cooker for 14 min as previously described [26]. Non-specific binding sites were saturated by a 30 min incubation in blocking buffer: TBS (100 mM Tris pH 7.5, 150 mM NaCl), 1% bovine serum albumin and 1% fat-free skim milk. Tissue sections were incubated overnight at 4 C with a monoclonal antibody against human PLA2G4A (SC-454; Santa Cruz Biotechnology Inc.) diluted to 1:100 in blocking buffer. Negative control tissue sections were incubated similarly with or

without normal mouse serum. After three 5 min washes in TBS, primary antibody-PLA2G4A complexes were detected by incubation for 2 h at room temperature with a rabbit anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich, Oakville, ON) diluted to 1:40 in blocking buffer. Tissue sections were washed three time in TBS, and incubated with the NBT/BCIP alkaline phosphatase substrate (Roche Applied Science, Laval, QC). Sections were mounted in 5% gelatin, 27% glycerol, and 0.1% sodium azide. Photographs were taken under bright field illumination using a Nikon Eclipse E800 microscope equipped with a digital camera (Nikon DXM 1200). Digital images were processed by Photoshop software (Adobe Systems Inc., San Jose, CA) and assembled by Illustrator software (Adobe Systems Inc.).

Statistical analysis

Gene-specific signals were normalized with corresponding *GAPD* signals for each sample. Homogeneity of variance between follicular groups and CL was verified by O'Brien and Brown-Forsythe tests. Corrected values of gene-specific mRNA levels were compared between follicular or CL groups by one-way ANOVA. When ANOVA indicated significant differences ($P < 0.05$), the Tukey-Kramer test was used for multiple comparisons of individual means. Data were presented as least-square means ± SEM. Statistical analyses were performed using JMP software (SAS Institute, Inc., Carry, NC).

RESULTS

Characterization of bovine PLA2G4A cDNA

A bovine PLA2G4A cDNA fragment (760 bp) was initially obtained from a previous gene expression profiling experiment using an SSH screening that aimed at identifying differentially expressed genes in hCG-stimulated GC [12]. When this fragment was used as a probe to screen a size-selected cDNA library of 2.5 to 3.5 kb generated from hCG-induced bovine GC, 26 positive bacterial colonies were obtained from a screen of 768 colonies, of which two clones were selected for plasmid DNA purification and sequencing. Results revealed that the full-length bovine PLA2G4A cDNA consisting of 2863 bp had been obtained. It was shown to be composed of a 5'-untranslated region (UTR) of 140 bp, an open reading frame of 2247 bp (including the stop codon), and a 3'-UTR of 476 bp containing four copies of an AU-rich element (ATTTA), a motif known to contribute to short-lived mRNAs [30], as well as a poly-adenylation signal (AATAAA) followed by a poly(A)$^+$ tail (Fig. 1). The coding region of bovine PLA2G4A encodes a 749-amino acid protein, with a theoretical molecular mass (Mr) of 85,300 and an isoelectric point (pI) of 5.2. Amino acid homology search in GenBank by PsiBlast revealed orthologous proteins with an overall identity level of 95% (equine: O77793), 94% (mouse: B39898; rabbit: AAF15299), 93% (human: NP_077734; rat: NP_598235) and 83% (chicken: NP_990754). When compared to the aforementioned species, protein domains of importance are conserved in bovine PLA2G4A (Fig. 1), and include a calcium binding domain (C2-domain; Phe^{20}-Thr^{106}) with the essential calcium-binding residues D^{43} and D^{93}, a lysophospholipase catalytic

site (PLA2-B domain; Val^{190}-Glu^{675}), and consensus phosphorylation sites (Ser^{505}, Ser^{515}, Ser^{727}).

Expression of PLA2G4A and PLA2G1B mRNAs in granulosa cells and CL

The expression profile of *PLA2G4A* mRNA was compared by virtual Northern analysis using GC collected from follicles obtained at different developmental stages including: small follicles (SF; 2-4 mm), dominant follicles (DF) at day 5 of the estrous cycle, ovulatory follicles (OF) 24 h following injection of an ovulatory dose of hCG, and corpus luteum (CL) at day 5. A single transcript estimated at 3.1 kb for *PLA2G4A* was observed and was shown to be expressed predominantly in GC of OF (data not shown). The semiquantitative RT-PCR demonstrated low expression in SF, DF and CL whereas a 14-fold increase in *PLA2G4A* mRNA was observed following hCG treament in GC of OF compared to DF ($P < 0.0001$; Fig. 2). In a parallel analysis, *PLA2G1B* mRNA was shown to be expressed in all samples but at a variable level; its highest expression was observed in SF, which showed a decrease of 3-fold when compared to OF ($P < 0.0001$; Fig 2).

Regulation of PLA2G4A and PLA2G1B mRNAs in follicles during the periovulatory period

Since hCG treatment clearly caused an induction of *PLA2G4A* mRNA in GC 24 h after its injection, semi-quantitative RT-PCR was used to study the regulation of *PLA2G4A* and *PLA2G1B* mRNAs in ovulatory follicles isolated at different times between 0 and 24 h after hCG injection. Levels of bovine *PLA2G4A* mRNA were low,

but detectable, in follicles preceding hCG treatment (0 h). An initial 16-fold up-regulation of *PLA2G4A* mRNA was apparent 12 h after hCG treatment, and a maximum 45-fold increase was reached 24 h following hCG injection when compared to 0 h ($P < 0.0001$; Fig. 3A). Conversely, *PLA2G1B* mRNA expression decreased during estrus from 0 to 24 h but the difference was not statistically significant ($P < 0.076$; Fig. 3A). The expression profile of *CYP19A1* and *PTGS2* mRNAs was investigated in order to validate the physiological status of follicles collected during the periovulatory period. Results showed that *CYP19A1* mRNA was elevated at 0 h then declined to undetectable levels 24 h post-hCG ($P < 0.0002$; Fig. 3B). Conversely, *PTGS2* mRNA was undetectable at 0 h, significantly increased at 18 h, and reached a maximum level at 24 h post-hCG ($P < 0.0002$; Fig. 3B). In light of these results, we conclude that *PLA2G4A* transcript is up-regulated by hCG and precedes the expression of *PTGS2*.

Regulation of PLA2G4A protein in ovulatory follicles

To determine whether the increase in *PLA2G4A* mRNA in bovine ovulatory follicles after hCG treatment was associated with changes in protein level, the regulation of PLA2G4A protein was studied in GC protein extracts isolated from follicles collected between 0 and 24 h after hCG. Immunoblot analysis showed that the antibody recognized a 100,000 Mr band that was induced 18 h after hCG and reached the highest level of expression at 24 h post-hCG (Fig. 4). The 72,000 Mr band corresponding to PTGS2 was faint at 18 h and reached a maximum at 24 h (Fig. 4). The expression of α-glutathione S-transferase-1 [28], used as a control protein, showed no significant difference between samples (data not shown). To determine the cellular localization of

PLA2G4A protein expression in ovulatory follicles, immunohistochemistry was performed on sections of bovine follicles isolated 0 and 24 h after hCG treatment (two follicles per time point). Immunolabeling was undetectable in GC of dominant or preovulatory follicles isolated before hCG treatment (Fig. 5A,B). Immunoreactive staining of PLA2G4A protein was observed mainly in mural GC compared to antral GC of follicles obtained at 24 h post-hCG. Moreover, mural GC presented a variable signal intensity of labeling. Intracellular immunolocalization of PLA2G4A protein in GC appeared well focalized and associated with perinuclear vesicular structures (Fig. 5E). Theca cells were unstained (Fig. 5D,E).

Regulation of PLA2G4A expression in bovine granulosa in culture

The LHCGR exerts its effect in GC of preovulatory follicles at least through the activation of the adenylyl cyclase/cAMP/cAMP-dependent protein kinase (PKA) pathway. We investigated if activation of the PKA pathway would stimulate the expression of PLA2G4A at the mRNA and protein levels. Bovine GC were treated with 10 μM of FSK, a concentration known to stimulate the PKA pathway in these cells [29]. An increase in *PLA2G4A* mRNA expression was observed 6 h following incubation with FSK, with maximum levels of expression reached at 24 h (Fig 6A). An increase in PLA2G4A protein expression was observed 24 h following the addition of FSK (Fig. 6B), which paralleled the maximum increase observed at the mRNA level. These results showed that PLA2G4A mRNA and protein expression in bovine GC are correlated with the increase in cAMP.

DISCUSSION

This study demonstrates that the process of ovulation induced by the preovulatory LH/hCG surge is preceded by the induction of PLA2G4A (also known as cPLA2α), at the mRNA and protein levels, in GC of bovine ovulatory follicles. Using primary bovine GC in culture, PLA2G4A mRNA and protein were also shown to be up-regulated via the FSK-stimulated adenylyl cyclase/cAMP pathway. Conversely, the mRNA of the secreted phospholipase A2, PLA2G1B (also known as pancreatic PLA2A), was shown to be expressed in GC of small follicles but its level of expression decreased in GC of preovulatory or LH/hCG-induced ovulatory follicles. In rodents, a previous study using total ovarian protein extracts showed that total PLA2 enzymatic activity changes during estrus [21], however, no reference to the type of PLA2 family member was mentioned. A gene expression profiling study using subtractive suppression hybridization identified *PLA2G4A* as a candidate gene that may be induced in bovine GC by the LH/hCG preovulatory surge [12]. Since only a cDNA fragment was identified in the latter study, and multiple phospholipases are expressed in various tissues [31], the full-length cloning of bovine *PLA2G4A* cDNA was achieved using cDNAs derived from hCG-stimulated GC.

Bovine PLA2G4A amino acid sequence was found to be highly conserved when compared to that of other species. The Ca^{+2} binding domain (C2-domain; Phe^{20}-Thr^{106}) is responsible for the interaction of PLA2G4A to membranes and phospholipid vesicles [32,33] for which the Ca^{+2}-binding residues D^{43} and D^{93} are essential [34,35]. Calcium-mobilizing agonists induce AA release from membrane phospholipids by promoting the

translocation of PLA2G4A from the cytosol to primarily the nuclear envelope and the endoplasmic reticulum [36-38], and by stabilizing its interaction to membranes [38,39]. Several serines (Ser^{505}, Ser^{727}, Ser^{515}) are also conserved in bovine PLA2G4A, and were shown to be phosphorylated in orthologous proteins [40] of which Ser^{505} was the most important for enzymatic activity [13]. However, phosphorylated PLA2G4A failed to release AA in the absence of an increase in intracellular Ca^{+2}, which indicated that phosphorylation alone is insufficient for enzyme activation but rather promotes its activity [39]. Thus, an increase in free intracellular Ca^{+2} concentration is the primary mechanism triggering enzymatic activation of PLA2G4A. Interestingly, LH/hCG treatment of cultured porcine GC was shown to elicit a biphasic rise in intracellular Ca^{+2} levels via immediate intracellular Ca^{+2} mobilization and delayed transmembrane Ca^{+2} influx through the activation of phospholipase C [41,42]. Thus, the LH/hCG preovulatory surge may activate PLA2G4A in bovine GC through increased intracellular concentration of Ca^{+2} at the time of ovulation.

In cyclic cows, injection of an ovulatory dose of hCG up-regulated *PLA2G4A* mRNA expression in GC initially at 12 h post-hCG. The GC samples used in this study were also validated for mRNA expression of two genes known to be either down-regulated (*CYP19A1*) or up-regulated (*PTGS2*) by the preovulatory LH/hCG surge. The initial induction of *PTGS2* mRNA in GC was detected 18 h post-hCG, which corroborates previous observations in cattle [43]. Thus, in bovine GC the LH/hCG preovulatory surge induces *PLA2G4A* expression prior to *PTGS2* expression. Results obtained for *PLA2G4A* in cyclic cows corroborate and extend previous observations

made in rats using either gonadotropin-primed immature or adult rats, where PLA2G4A enzymatic activity and immunoreactivity were reported to increase at proestrus [44,45]. Immunoblotting and immunohistochemical results obtained in GC of ovulatory follicles mirrored the induction of *PLA2G4A* mRNA by LH/hCG whereas PLA2G4A protein was undetectable in GC of dominant or preovulatory follicles obtained at day 5 or 7 of the estrous cycle. The immunostaining pattern observed in GC for PLA2G4A indicated a well localized vesicular and perinuclear pattern, which concords with the described translocation of the PLA2G4A protein from the cytoplasm to the nuclear membrane following the increase in free intracellular Ca^{+2} concentration [36-39]. The vesicular and perinuclear localization of activated PLA2G4A also match the described perinuclear localization of PTGS2 [46]. Furthermore, we observed that mural GC stained more intensely for PLA2G4A when compared to antral GC. Interestingly, it is known that mural and antral GC differ in the level of LHCGR with higher levels of expression in mural GC [47,48]. Thus, the higher expression of LHCGR by mural GC could also explain the higher induction of PLA2G4A in these cells, thereby allowing an effective diffusion of released PG to the blood vessels of the theca layer.

The PLA2G4A preferentially releases AA from phospholipids, thereby providing the upstream substrate for PTGS2 to produce PG [49]. In the bovine ovarian follicle, *PTGS1* is constitutively expressed in the theca cells but absent in GC, whereas *PTGS2* is inducible by LH/hCG only in GC [43]. Following the LH/hCG surge, the expression of *PLA2G4A* mRNA in GC precedes that of *PTGS2*; both enzymes are temporally regulated in a similar fashion, and once activated, they co-localize in perinuclear

vesicular structures. These observations underscore that the two enzymes are metabolically coupled in GC during ovulation to insure PG synthesis as required for ovulation and oocyte maturation. Recently, *PLA2G4A* and *PTGS2* mRNAs were also observed to be co-induced in the proresolving phase of the acute inflammatory process [50]. Since ovulation is considered to be an inflammatory reaction [2], the expression of PLA2GA4 may contribute to the release of the AA substrate necessary for the release of PTGS2-derived proinflammatory mediators such as PGs and leukotrienes. Interestingly, in the acute inflammatory reaction, interleukin-1β (IL1B) stimulates the expression of PLA2G4A [50]. Similarly, IL1B stimulated the expression of PLA2G4A in cultured rat GC [51,52] while LH stimulated the expression of IL1B in human GC [53]. Furthermore, the receptor IL1R1 is synthesized in GC of different species [54]. LH and IL1B were shown to synergistically stimulate PG synthesis by GC [55]. Collectively, these results strongly support a model where LH/hCG co-induces the expression of PLA2G4A and PTGS2 in GC of ovulatory follicles, as well as the expression of IL1B. The latter acts as a paracrine factor that synergizes with LH/hCG signaling to promote maximal expression and activity of PLA2G4A and PTGS2.

Cyclic AMP is a major intracellular second messenger following activation of the LHCGR. We have demonstrated that FSK treatment stimulated PLA2G4A mRNA and protein expression in bovine GC in vitro. Using the same bovine GC model, we previously showed that FSK also induced the expression of PTGS2 [29]. In GC, increase in cAMP leads to the activation of signaling mechanisms such as PKA and guanine nucleotide-exchange factors (GEF; also known as exchange protein directly

activated by cAMP or EPAC) [56,57]. Interestingly, the promoter sequence of human *PLA2G4A* contains a cAMP-responsive element (CRE), which suggests that CRE-binding proteins may promote the expression of *PLA2G4A* mRNA in GC.

We investigated whether other PLA2 isoforms were expressed by bovine GC. Since the availability of cDNA sequences for different PLA2 isoforms is limited in the bovine species, the expression of a secreted form of PLA2, PLA2G1B, was investigated. The expression of *PLA2G1B* mRNA was higher in GC of small follicles and reached the lowest level in GC of hCG-stimulated ovulatory follicles. Thus, expression of *PLA2G1B* mRNA in GC was inversely related to *PLA2G4A* during ovulation. We concluded that PLA2G1B does not supply AA to PTGS2 during ovulation.

Ovulation and oocyte maturation were shown to be inhibited in female mice null for *PTGS2*, suggesting an essential role for PG in these processes [58]. Conversely, *PLA2G4A* null mice showed a decrease in fertility that was mainly associated with modifications at the embryo uterine interaction level but ovulation was modestly reduced [59,60]. However, we have clearly shown an induction of PLA2G4A mRNA and protein in bovine GC by LH/hCG, which supports a role for PLA2G4A in providing AA substrate for PTGS2. Interestingly, intra-ovarian bursal injection of arachidonyl trifluoromethyl ketone, a specific PLA2G4A inhibitor, significantly decreased ovulation and total ovarian PGE2 synthesis in rats [45], which underscores a role for PLA2G4A activity in the release of AA necessary for PTGS2. Discrepancies between our observations made in the bovine species and in the *PLA2G4A* knockout mouse model

suggest that compensatory mechanisms may develop with other PLA2 isoforms contributing to the release of AA substrate. Thus, the temporal expression of other PLA2 isoforms should be investigated, as well as their relative contribution to ovulation which may differ among species.

In summary, this report demonstrates that the ovulatory process is associated with an LH/hCG-dependent induction of PLA2G4A in granulosa cells in vivo. Furthermore, the induction of the PLA2G4A enzyme is dependent on activation by the LHCGR of adenylyl cyclase/cAMP pathway. Combined with previous work showing the induction of PTGS2 in bovine preovulatory follicles [49], the present study supports the premise that the coordinated induction and localization of PLA2G4A and PTGS2 enzymes contribute to maximize PG synthesis prior to ovulation.

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Fig. 1. Nucleotide and amino acid sequences of bovine PLA2G4A.

The bovine *PLA2G4A* cDNA was cloned by library screening, as described under *Materials and Methods*, and amino acids deduced from cDNA. Nucleotides and amino acids are numbered at the *left*. The bovine *PLA2G4A* is composed of 2863 bp, a 5'-UTR of 140 bp, an open reading frame (ORF) of 2247 bp and a 3'-UTR of 476 bp followed by a poly(A)$^+$ tail. Amino acid numbering begins at the first methionine of the ORF, which encodes for 749 amino acids representing a putative protein of 85,300 Mr and a pI of 5.2. In the 3'-UTR region, *asterisks* represent the stop codon, the four AU-rich motifs (ATTTA) are *overlined*, and the polyadenylation signal is *underlined*. The poly(A)$^+$ tail is not represented. The nucleotide sequence was submitted to Genbank (Genbank accession number: AY363688).

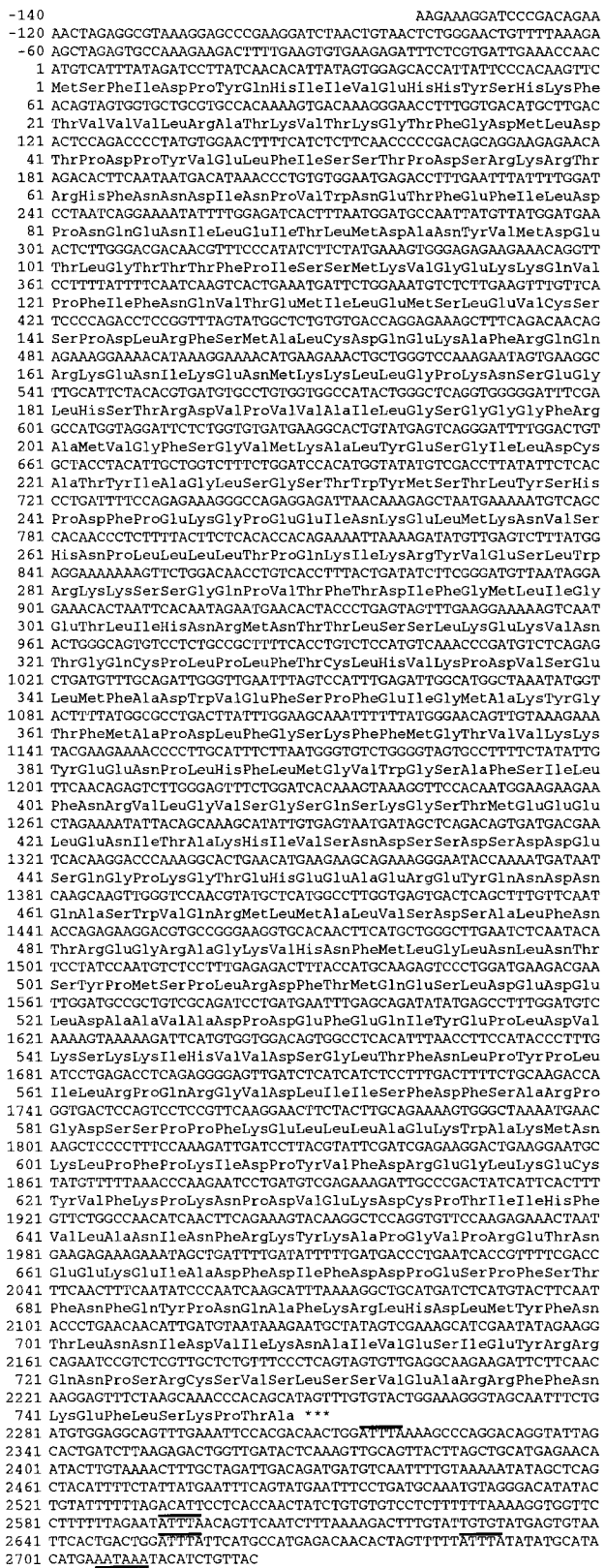

Figure 1

Fig. 2. Expression of PLA2G4A and PLA2G1B transcripts in bovine granulosa cells and CL.

Total RNA was extracted from bovine granulosa cells collected from 2-4 mm follicles (SF), dominant follicles (DF) at day 5 of the estrous cycle, ovulatory follicles (OF) 24 h after injection of hCG (OF) and corpora lutea (CL) from day 5 of the estrous cycle, then used in mRNA expression analyses using semiquantitative RT-PCR as described under *Materials and Methods*. The control gene was *GAPD* (710 bp); it showed no significant difference in mRNA expression levels between groups. The *PLA2G4A* (960 bp) and *PLA2G1B* (437 bp) PCR fragments were normalized with their corresponding *GAPD* signal, and the results are presented as a relative change in ratio between groups. Different letters denote samples that differed significantly ($P < 0.05$) when Tukey-Kramer multiple comparison tests were performed to compare group means for a specific gene. Data are presented as least-square means ± SEM, and the number of independent samples, *i.e.* animals, per group is indicated in parenthesis.

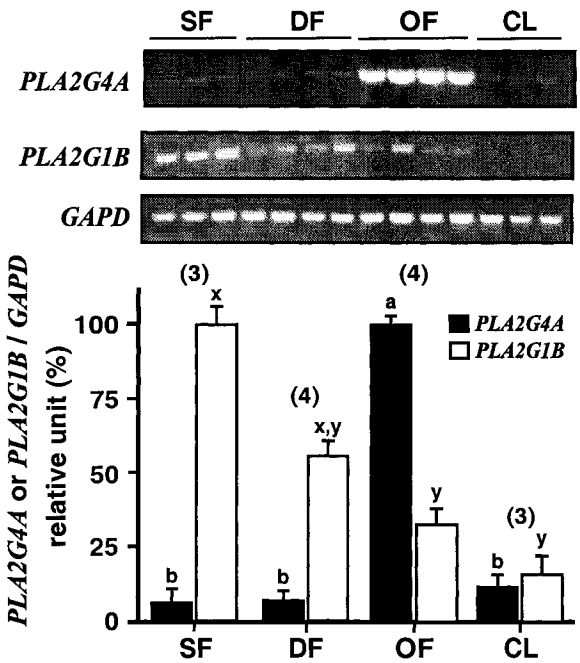

Figure 2

Fig. 3. Regulation of PLA2G4A and PLA2G1B transcripts by hCG in bovine follicles during the periovulatory period.

Total RNA was extracted from preparations of bovine follicular wall obtained from ovulatory follicles isolated between 0 and 24 h after hCG then used in mRNA expression analyses by semiquantitative RT-PCR as described under *Materials and Methods*. The control gene was *GAPD* (710 bp); it showed no significant differences in mRNA expression levels between groups. **A**, The *PLA2G4A* (960 bp) and *PLA2G1B* (437 bp) PCR signals were normalized with their corresponding *GAPD* signals, and the results are presented as a relative change in ratio between groups. **B**, The *CYP19A1* (520 bp) and *PTGS2* (418 bp) PCR signals were normalized with their corresponding *GAPD* signals, and the results are presented as a relative change in ratio between groups. Different letters denote samples that differed significantly ($P < 0.05$) when Tukey-Kramer multiple comparison tests were performed to compare group means for a specific gene. Data are presented as least-square means ± SEM, and represent two distinct follicles, *i.e.* animals, per time point.

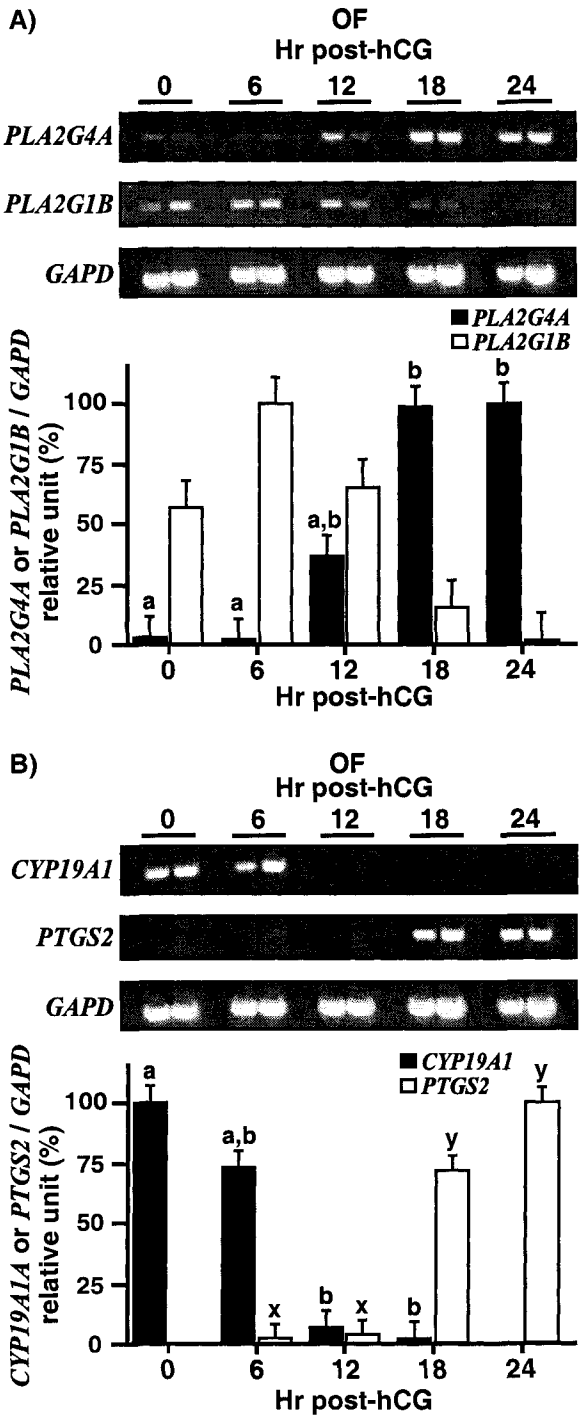

Figure 3

Figure 4. Regulation of PLA2G4B and PTGS2 proteins by hCG in bovine follicles.

Granulosa cells were isolated from bovine dominant follicles (DF) at day 5 of the estrous cycle and from ovulatory follicles between 0 and 24 h after hCG treatment. Protein extracts (100 μg) were analyzed by 5-18% gradient one-dimensional SDS-PAGE and immunoblotted as described under *Materials and Methods.* **A)** Immunoblotting was performed using a monoclonal antibody against human PLA2G4A (1:100; SC-454) and revealed a 100,000 Mr protein band. Two distinct follicles, *i.e.* animals, per time point are represented. **B)** Immunoblotting was performed using a rabbit anti-human PTGS2 antibody (1:5,000; MF243) and revealed a 72,000 Mr protein band. One representative follicle per time point is shown.

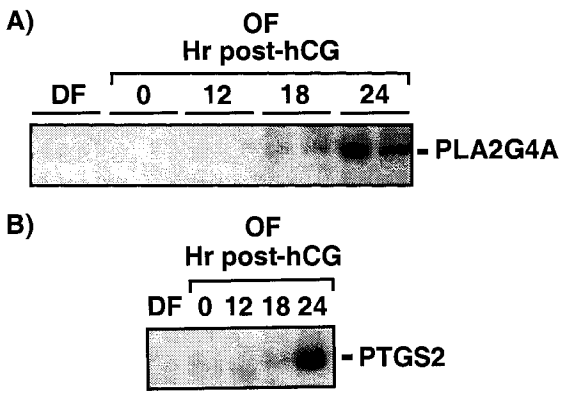

Figure 4

Figure 5. Immunohistochemical localization of PLA2G4A in bovine ovulatory follicles.

Immunohistochemistry was performed on formalin-fixed sections of ovulatory follicles isolated at 0 h (**A-C**) and 24 h (**D-F**) after hCG treatment, as described under *Materials and Methods*. The scale bar is equal to 0.05 mm in all figures. Staining was absent when the primary antibody was omitted (**C, F**). Staining is undetectable in GC of preovulatory follicles obtained preceding hCG treatment (0 h; **A, B**). A clear induction of PLA2G4A immunoreactivity is observed in GC of ovulatory follicles obtained 24 h post-hCG (**D, E**). Mural GC located near the basal membrane demonstrated a stronger labeling pattern (arrows) when compared to antral GC.

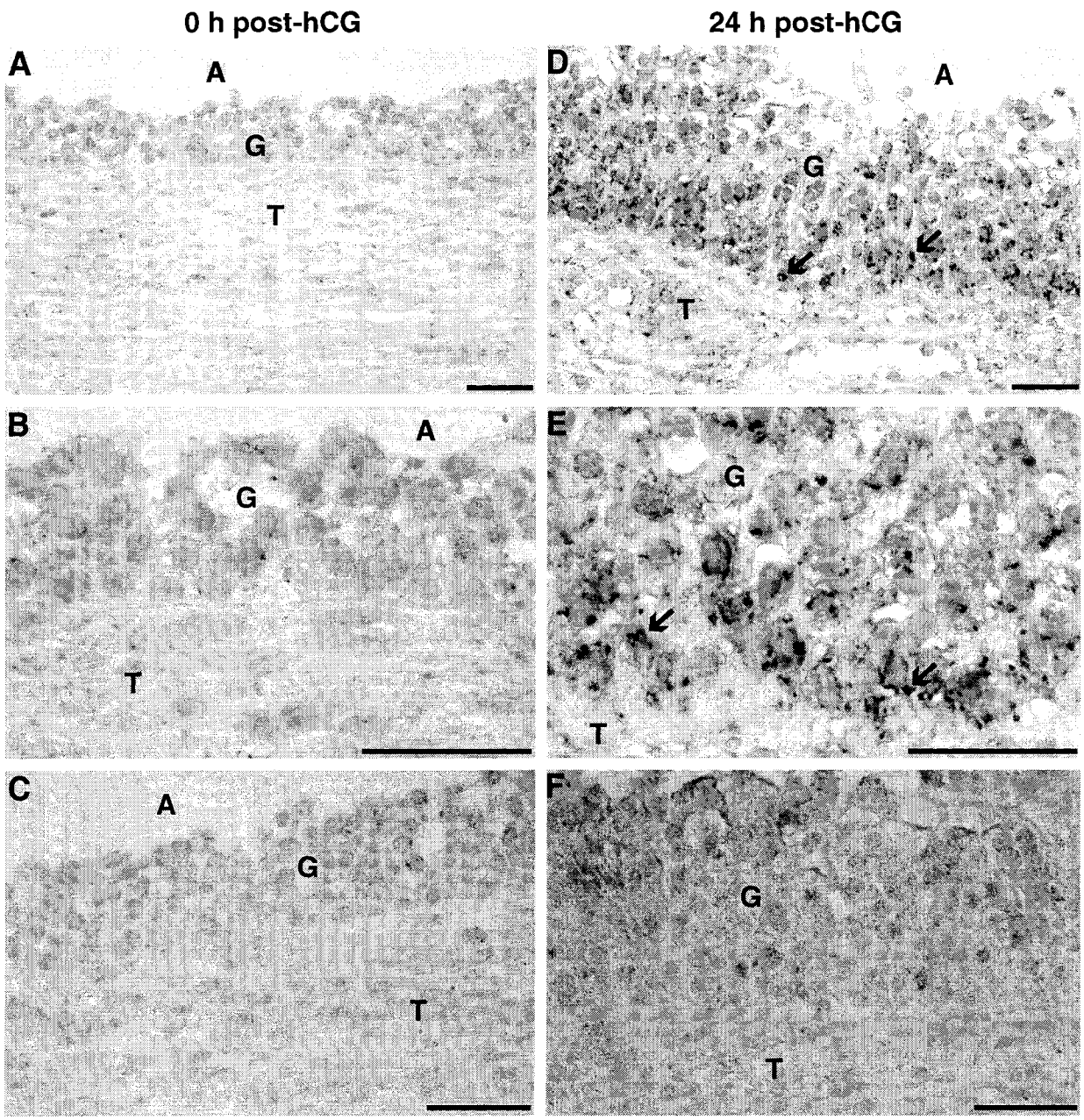

Figure 5

Figure 6. Regulation of PLA2G4A expression in bovine granulosa cells in culture. Bovine GC were cultured as described under *Materials and Methods* in the presence of 10 μM FSK, and cells were collected at different time points (0 to 48 h post-FSK) for extraction of total RNA and protein. Representative results obtained from two experiments are presented. **A)** Results show that FSK stimulates *PLA2G4A* mRNA expression by 6 h which then reaches a maximum level at 24 h post-FSK. No difference in *GAPD* mRNA was detected. **B)** PLA2G4A protein was also induced 24 h post-FSK.

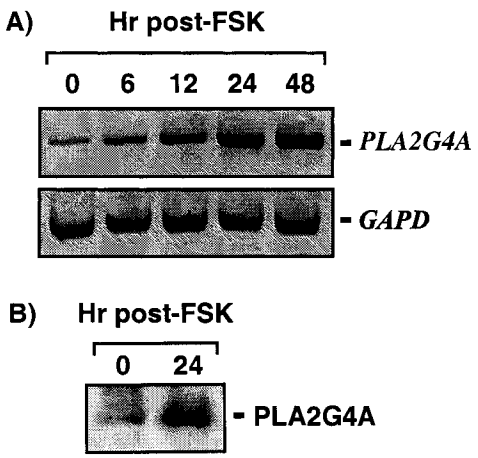

Figure 6

CHAPITRE 3

Induction of alpha-caveolin-1 (αCAV1) expression in bovine granulosa cells in response to an ovulatory dose of human chorionic gonadotropin

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Running title: Regulation of α-caveolin-1 in ovulatory follicles.

Key words: ovary, follicle, granulosa cell, gene expression, ovulation, caveolin, CAV1, CAV2.

ABSTRACT

Caveolae and caveolins (protein markers of caveolea) are implicated in endocytosis, cholesterol trafficking and signal transduction. A cDNA fragment corresponding to caveolin-1 (*CAV1*) was identified in a mRNA profiling expression study as being potentially up-regulated in bovine granulosa cells (GC) following human chorionic gonadotropin (hCG)-induced ovulation. The objectives of the present study were to characterize *CAV1* cDNA and study its spatio-temporal expression pattern in bovine ovarian follicles. The full-length bovine alpha-*CAV1* cDNA was cloned by screening a size-selected cDNA library (2.3-2.7 kb) derived from hCG-stimulated GC. The α*CAV1* cDNA is composed of a 5'-untranslated region (UTR) of 62 bp, a 3'-UTR of 1852 bp and an open reading frame of 541 bp encoding a putative 22 kDa protein that is highly conserved when compared to orthologous proteins. Expression of CAV1 was studied in bovine GC obtained from follicles at different developmental stages including: small follicles (SF: 2-4 mm), dominant follicles (DF: at day 5 of the estrous cycle), ovulatory follicles (OF: 24 h following injection of an ovulatory dose of hCG), and corpus luteum (CL: at day 5). A transcript of 2.7 kb for α*CAV1* was observed by virtual Northern analysis. Semiquantitative RT-PCR analysis showed a 6.5-fold increase in α*CAV1* mRNA in GC of OF versus DF ($P < 0.0001$), whereas *CAV2* mRNA (another caveolin family member) was increased by only 2-fold ($P < 0.0007$). Temporal expression of α*CAV1* mRNA was studied in follicular walls obtained from OF recovered at 0, 6, 12, 18, and 24 h after hCG injection. Results showed an 8.5-fold increase of α*CAV1* mRNA after 24 h compared to 0 h ($P < 0.0018$). Conversely, no significant variation in mRNA level was detected for *CAV2* during this time ($P < 0.16$).

Immunoblot of GC extracts demonstrated an initial increase in αCAV1 protein level 12 h post-hCG, reaching a maximum at 24 h, which mirrored mRNA results. Immunohistochemical localization of CAV1 protein was observed in GC of OF isolated 18 h and 24 h after hCG injection, whereas no signal was detected in GC of DF and SF. The strongest immunostaining pattern for CAV1 was observed in endothelial cells of the theca layer of all follicles irrespective of size and in endothelial cells of CL. This study is the first to demonstrate the induction of αCAV1 mRNA and protein expression in GC of ovulatory follicles, and suggests that the induction of αCAV1 likely contributes to control the increase in membrane signaling that occurs at the time of ovulation and luteinization.

INTRODUCTION

Caveolae are 50-100 nm vesicular invaginations of the plasma membrane that can be singular or found in detached grapelike clusters. They have been identified in a wide variety of tissue and cell types such as adipocytes, fibroblasts, endothelial cells, type I pneumocytes, as well as striated and smooth muscle cells [1]. Caveolins are found predominantly at the plasma membrane but also in Golgi, endoplasmic reticulum (ER), vesicles, and in cytosolic locations such as ER lumen and secretory vesicles [2-4]. Caveolae contain several proteins but only the caveolins can serve as protein markers of caveolae [4], and are known to act as scaffolding proteins. Three members of the caveolin gene family have been identified: caveolin 1 (*CAV1*), caveolin 2 (*CAV2*) and caveolin 3 (*CAV3*). These caveolin genes and proteins share significant homology, and proteins are highly conserved throughout evolution [1]. CAV1, also termed vesicular integral-membrane protein of 21 kDa (VIP21), and CAV3 proteins are both independently necessary and sufficient to drive caveolae formation whereas CAV2 requires the presence of CAV1 for proper membrane targeting and stabilization [1]. CAV1 is implicated in vesicular transport [5-7], cholesterol trafficking [8-10] and signal transduction [1].

The ovulatory and luteinization processes in mammals begin at the time when the endogenous luteinizing hormone (LH) surge stimulates the G protein-coupled LH/human chorionic gonadotropin receptors (LHCGR) that are distributed on the surface of granulosa and theca cells of a mature preovulatory follicle. Gonadotropin–stimulated ovulation and luteinization are controlled by the induction [11-14] and the

down-regulation of specific genes [15,16] that are spatially and temporally regulated in the different follicular compartments during the periovulatory period. Based on a previous mRNA expression profiling study applying subtractive suppression hybridization, a cDNA fragment that corresponded to bovine *CAV1* was identified as a candidate gene that would be induced in GC by the LH/hCG preovulatory surge [12]. Thus, we hypothesized that expression of the CAV1 in GC of ovulatory follicles is induced by the LH/hCG preovulatory surge, which would contribute to ovulation and luteinization. The specific objectives of this study were to clone the corresponding full-length bovine *CAV1* cDNA, and to study the spatio-temporal expression profile of CAV1 mRNA and protein in bovine ovulatory follicles following hCG treatment. Bovine ovulatory follicles were used as the *in vivo* model to investigate the regulation of CAV1 expression during hCG-induced ovulation and luteinization.

MATERIALS AND METHODS

Cloning of bovine caveolin-1 cDNA

Isolation of the full-length bovine *CAV1* cDNA was performed by screening a size-selected cDNA library. Initially, the size of the full-length bovine *CAV1* cDNA was estimated by performing a virtual Northern blot analysis. Briefly, total RNA was isolated from GC that were obtained from bovine ovulatory follicles 24 h following an injection of an ovulatory dose of hCG (3000 IU; APL Ayerst Lab, Montréal, PQ) [16]; total RNA was transformed into cDNA by the SMART cDNA synthesis method (BD Biosciences Clontech, Mississauga, ON) as described [17]. The cDNAs were separated by gel electrophoresis, transferred onto a nylon membrane and hybridized with a bovine

CAV1 radioactive probe (570 bp) generated from a previous SSH screening experiment [12]. Once the size of the full-length bovine *CAV1* cDNA was determined, total SMART cDNAs from hCG-stimulated GC were size fractionated by agarose gel electrophoresis, and cDNAs from 2.3 to 2.7 kb were purified to construct a size-selected cDNA library based on the pDrive plasmid (Qiagen PCR cloning kit; Qiagen, Mississauga, ON). The cDNA library was then screened by radioactive hybridization as described [17]. Positive CAV1 hybridizing bacterial colonies were grown, their plasmid content was isolated (QIA-prep, Qiagen), and the size of the cloned cDNA was analyzed following *EcoR*1 digestion and gel electrophoresis analysis. The cDNAs were sequenced via the dideoxy sequencing method (Big Dye Terminator 3.0; ABI Prism, Applied BioSystem, PE, Branchburg, NJ) and analyzed on an ABI Prism 310 sequencer (Applied Biosystem). Nucleic acid sequences were analyzed by BLAST (Basic Local Alignment Search Tool) against GenBank data banks.

Bovine tissues and RNA extraction

Bovine ovarian follicles and corpora lutea (CL) were isolated at specific stages of the estrous cycle from normal cycling crossbred heifers, as described [16]. Briefly, estrous cycle was synchronized with $PGF_{2\alpha}$ (25 mg, im; Lutalyse, Upjohn, Kalamazoo, MI) and ovarian follicular development was monitored by daily transrectal ultrasonography until ovariectomy. Dominant follicles (DF; n = 4 cows) were defined by ultrasonographic measurement as > 8 mm and growing while subordinate follicles were either static or regressing. Dominant follicles were obtained on the morning of day 5 of the estrous cycle (day 0 = day of estrus) by ovariectomy (via colpotomy). Ovulatory

follicles (OF) were obtained following an injection of 25 mg of $PGF_{2\alpha}$ on day 7 of the synchronized estrous cycle to induce luteolysis, thereby maintaining the development of the DF of the first follicular wave into a preovulatory follicle. An ovulatory dose of hCG (3000 IU, iv; APL, Ayerst Lab, Montréal, QC) was injected 36 h after the induction of luteolysis, and the ovary bearing the hCG-induced OF was collected by ovariectomy at 0, 6, 12, 18 and 24 h after hCG injection (n = 2-4 cows/time point). Follicles were dissected into preparations of follicular wall (theca interna with attached GC) [18] or further dissected into separate isolates of GC [16]. Ovariectomies were also performed on day 5 of the synchronized estrous cycle to obtain CL (n = 3 cows). Additionally, GC were collected from 2-4 mm follicles that were obtained from slaughterhouse ovaries representing a total of three pools of 20 small follicles (SF). These experiments were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine of the Université de Montréal. Total RNA was isolated from tissues as described [19]. The concentration of total RNA was quantified by measuring the optical density at 260 nm, and quality was evaluated by visualizing the 28S and 18S ribosomal bands following electrophoretic separation on a formaldehyde denaturing 1% agarose gel with ethidium bromide [19].

Semiquantitative RT-PCR analysis

Total RNA (1 μg) from individual follicles or CL was reverse-transcribed with an oligo-dT30 primer and PowerScript (BD Biosciences Clontech) to generate the first strand cDNA, using the SMART PCR cDNA synthesis kit (User manual: PT3041-1; BD Biosciences Clontech, Mississauga, ON) [16]. Second cDNA strands were produced

with the SMART II 5'-anchored oligo, and PCR-amplified for 15 cycles using Advantage 2 DNA polymerase (BD Biosciences Clontech). SMART cDNA pools were then diluted to 50 µl in 10 mM Tris pH 8 buffer. To perform semiquantitative RT-PCR, SMART cDNA pools were diluted 10-fold in H_2O, and 1 to 2 µl were used in a 25 µl PCR reaction using the Advantage 2 DNA polymerase kit (BD Biosciences Clontech). Gene-specific PCR primers were designed in the open reading frame of the bovine cDNA sequence for: *CAV1* (sense: 5'-GGCAAATACGTAGACTCAGAGGGAC-3'; anti-sense: 5'-CTGCGTGTTGATGCGGATATTGCTG-3'; GenBank: AY823915), *CAV2* (sense: 5'-GATGTCCAGCTCTTCATGGACGAC-3'; anti-sense: 5'- GACGCAGCTTCTCTTCTGTCAG-3'; GenBank: AY699947), and glyceraldehyde-3-phosphate dehydrogenase (*GAPD*; sense: 5'-TGTTCCAGTATGATTCCACCCACG-3'; anti-sense: 5'-CTGTTGAAGTCGCAGGAGACAACC-3') [16]. For all these genes, PCR conditions were as follows: 95 °C for 1 min, 95 °C for 30 s, 64 °C for 45 s, and 68 °C for 90 s. The number of PCR cycles was limited (14 to 21 cycles) and optimized for each gene to be analyzed. The PCR reactions (20 µl/reaction) were resolved on a 2% TAE-agarose gel (40 mM Tris acetate pH 8, 1 Mm EDTA) with ethidium bromide (0.5 µg/ml); PCR products were visualized by UV and the images were digitized. The digitized signals for each gene were analyzed by densitometry using ImageQuant software (Amersham Pharmacia Biotech, Pointe-Claire, QC).

Cell extracts and immunoblot analysis

Tissues and cells were homogenized in M-PER buffer (Pierce, Rockford, IL) that was supplemented with a mix of protease inhibitors (Complete; Roche Applied Science,

Laval, QC) as recommended by the manufacturer's protocol. Complete lysis of GC was achieved with multiple passages through a 25G needle attached to a 3-ml syringe. CL were homogeneized at 7,000 rpm with a polytron PT1300D (Kinematica AG, Littau-Lucerne, SW). The protein extracts were centrifuged at 16,000 x g for 15 min at 4 °C, and the recovered supernatant (whole cell extract) was stored at –80 °C until electrophoretic analyses were performed. Protein concentration was determined by the Bradford method (Bio-Rad Protein assay) [20]. Protein extracts (50 μg proteins/sample) were heat-treated (5 min, 100 °C), size-fractionated via a one-dimensional 18% SDS-PAGE, and electrophoretically transferred onto polyvinylidene difluoride membranes (PVDF; Hybond-P, Amersham Pharmacia Biotech). Immunoblots were performed as described [21]. Membranes were incubated with rabbit polyclonal antibody against human CAV1 (1:500 dilution; SC-894; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and detected with a horseradish peroxidase-linked sheep anti-rabbit secondary antibody (1:20,000 dilution; Amersham Pharmacia Biotech). Detection of immunoreactive proteins was performed by the enhanced chemiluminescence system (ECL Plus, Amersham Pharmacia Biotech) following the manufacturer's protocol, and exposed to Hyperfilm (Amersham Pharmacia Biotech). The expression of α-glutathione S-transferase-1 (GSTA1) was used as control [22]. Membranes were incubated with a rabbit polyclonal antibody against bovine GSTA1 (1:3,000 dilution) and detected with an alkaline phosphatase-linked goat anti-rabbit secondary antibody (1:20,000 dilution; Sigma Chemicals) using NBT/BCIP as alkaline phosphatase substrate (Roche Molecular Biochemicals). Images were digitized using a ScanMaker 9800XL flatbed scanner (Microtek lab, Inc., Redondo Beach, CA).

Immunohistochemical localization of Caveolin-1

Immunohistochemical staining was performed on PBS-buffered formalin-fixed tissues as described [21]. Paraffin-embedded tissues were cut to 3 μm thickness, mounted on SuperfrostPlus slides (Fisher Scientific, Pittsburgh, PA), deparaffinized and then rehydrated. Antigenicity lost during the fixation process was retrieved by heat treatment in a pressure cooker for 14 min as described [21]. Non-specific binding sites were saturated by a 30 min incubation in blocking buffer: TBS (100 mM Tris pH 7.5, 150 mM NaCl), 1% bovine serum albumin and 1% fat-free skim milk. Tissue sections were incubated overnight at 4 °C with a rabbit polyclonal antibody against human CAV1 (SC-894; Santa Cruz Biotechnology Inc.) diluted to 1:100 in blocking buffer. Negative control tissue sections were incubated similarly with or without normal mouse serum. After three 5 min washes in TBS, primary antibody-CAV1 complexes were detected by incubation for 2 h at room temperature with a rabbit anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich, Oakville, ON) diluted to 1:200 in blocking buffer. Tissue sections were washed three times in TBS, and incubated with the NBT/BCIP alkaline phosphatase substrate (Roche Applied Science, Laval, QC). Sections were mounted in 5% gelatin, 27% glycerol, and 0.1% sodium azide. Photographs were taken under bright field illumination using a Nikon Eclipse E800 microscope equipped with a digital camera (Nikon DXM 1200). Digital images were processed by Photoshop software (Adobe Systems Inc., San Jose, CA) and assembled by Illustrator software (Adobe Systems Inc.).

Statistical analysis

Gene-specific signals (*CAV1*, *CAV2*) were normalized with corresponding *GAPD* signals for each sample. Homogeneity of variance between follicular groups and CL was verified by O'Brien and Brown-Forsythe tests. Corrected values of gene-specific mRNA levels were compared between follicular and CL groups by one-way ANOVA. When ANOVA indicated significant differences ($P < 0.05$), multiple comparisons of individual means for SF, DF, OF and CL groups were compared by the Tukey-Kramer test ($P < 0.05$). Dunnett's test ($P < 0.05$) was used to compare individual means for the temporal hCG-induced follicular wall samples. Statistical analyses were performed using JMP software (SAS Institute, Inc., Carry, NC). Data were presented as least-square means ± SEM.

RESULTS

Characterization of bovine alpha-caveolin-1 cDNA

A bovine *CAV1* cDNA fragment (570 bp) was obtained from a previous gene expression profiling experiment using SSH screening that aimed at identifying differentially expressed genes in hCG-stimulated GC [12]. This cDNA fragment was used as a probe to screen by hybridization a size-selected cDNA library of 2.3 to 2.7 kb generated from bovine GC that were collected 24 h following injection of hCG. Positive bacterial colonies were obtained, of which two clones were randomly selected for plasmid DNA purification and sequencing. The full-length bovine α*CAV1* cDNA was cloned and consisted of 2455 bp that included a 5'-untranslated region (UTR) of 62 bp, an open reading frame of 534 bp, and a 3'-UTR of 1859 bp containing four

polyadenylation signals (AATAAA) followed by a poly(A)$^+$ tail (Fig. 1). The coding region of bovine α*CAV1* cDNA encodes a 178-amino acid protein, with a theoretical molecular mass (Mr) of 20,593 and an isoelectric point (pI) of 5.6. Amino acid homology search in GenBank by PsiBlast revealed orthologous proteins with an overall identity level of 100 % to ovine (NP_001009477), 99 % to porcine (AAR16300), 97 % to rat (AAR16308), and 96 % to human (AAP36880) and mouse (AAR16290) proteins. When compared to these species, protein domains of importance are conserved in bovine αCAV1 (Fig. 1), including: a caveolin–1 consensus motif (Phe^{68}-Pro^{75}), a caveolin-scaffolding domain (CSD; Asp^{82}-Arg^{101}), a potential hairpin loop structure that inserts into the membrane (Ala^{105}-Leu^{125}), a potential phosphorylation site (Tyr^{14}), and potential S-palmitoylated cysteines (Cys^{133}, Cys^{143}, Cys^{156}) located at the carboxyl-terminus.

***Expression of* α*CAV1* and *CAV2* mRNAs in granulosa cells and CL**

The expression profile of α*CAV1* mRNA was studied first by virtual Northern analysis using GC collected from follicles obtained at different developmental stages: small follicles (SF; 2-4 mm), dominant follicles (DF) at day 5 of the estrous cycle, ovulatory follicles (OF) 24 h following injection of an ovulatory dose of hCG, and corpus luteum (CL) at day 5. A single transcript estimated at 2.7 kb for α*CAV1* was observed and was shown to be expressed predominantly in GC of hCG-induced OF (data not shown). Semiquantitative RT-PCR demonstrated a 6.5-fold increase in α*CAV1* mRNA in GC of hCG-stimulated OF compared to DF ($P < 0.0001$; Fig. 2); α*CAV1* mRNA was also detected in CL albeit at a low level. In a parallel analysis, *CAV2* mRNA

showed a similar pattern of expression as *CAV1* with a 2-fold increase in GC of OF compared to DF ($P < 0.0007$; Fig. 2) whereas the transcript level was lower in GC of SF. These results underscore that α*CAV1* and *CAV2* mRNA levels are regulated in GC in association with the stage of follicular development, however, the level of induction by the LH/hCG surge in GC of OF is more intense for α*CAV1* than *CAV2*.

Regulation of αCAV1 and CAV2 mRNAs in follicles during the periovulatory period

Since hCG induced α*CAV1* mRNA in GC of OF 24 h following its injection, semiquantitative RT-PCR was used to study the regulation of α*CAV1* and *CAV2* mRNAs in follicular walls of OF isolated at different times between 0 and 24 h post-hCG treatment. The RT-PCR analysis of α*CAV1* mRNA showed a significant up-regulation of the transcript level after hCG treatment, with an initial increase of 1.6-fold at 12 h that reached a maximum of 8.5-fold at 24 h when compared to mRNA level preceeding hCG injection at 0 h ($P < 0.0018$; Fig. 3). This result was confirmed by virtual Northern blot analysis (data not shown). In contrast, *CAV2* transcript level did not vary significantly in follicles collected at various times during the periovulatory period ($P < 0.16$; Fig. 3). Quality of follicular wall samples during the periovulatory period was validated by RT-PCR analysis of control genes including cytochrome P450 family 19 subfamily 1 (*CYP19A1*), a gene known to be down-regulated by LH/hCG, and prostaglandin-endoperoxide synthase 2 (*PTGS2*), a gene known to be up-regulated by LH/hCG [23].

Regulation and localization of αCAV1 protein in ovulatory follicles

To determine whether the increase in *αCAV1* mRNA in bovine ovulatory follicles after hCG treatment was associated with changes in protein level, the regulation of αCAV1 was studied in GC protein extracts isolated from follicles collected between 0 and 24 h post-hCG treatment. Immunoblot analysis showed that the antibody recognized a 22,000 Mr band that was weakly expressed in GC preceeding hCG treatment. The increase in protein level was detected initially at 12 h post-hCG and reached a maximum at 24 h (Fig. 4A). The expression of GSTA1, used as a control protein, showed no significant difference between samples (Fig. 4B). Cellular localization of αCAV1 protein expression in follicles was determined by immunohistochemistry. Strong immunostaining for CAV1 was detected in endothelial cells of all antral follicles and CL (Fig. 5 A-F). No immunoreactive signal was detected in GC of dominant or preovulatory follicles collected at day 5 or 7 of the estrous cycle (Fig. 5 A, B) and small antral follicles (2-4 mm; Fig. 5 E). At 18 and 24 h after hCG injection, αCAV1 protein was clearly induced in GC that presented heterogeneity in the labeling pattern (Fig. 5 C, D). Granulosa cells of small antral follicles adjacent to the ovulatory follicle were not stained (Fig.5 E). Theca and luteal cells did not stain for αCAV1 (Fig. 5A-E).

DISCUSSION

This study is the first to demonstrate that the process of ovulation and lutenization promoted by hCG injection induces CAV1 gene expression, as shown by an acute increase of αCAV1 mRNA and protein levels in GC of bovine ovulatory follicles. Expression of αCAV1 was not detected in GC of SF (2-4 mm) or DF at days 5 and 7 of

the estrous cycle. Furthermore, CAV1 was shown to be highly expressed in endothelial cells of capillaries perfusing the theca layer, irrespective of the size or status (healthy or atretic) of antral follicles, and in endothelial cells of CL blood vessels. Conversely, *CAV2* mRNA was shown to be expressed in GC of SF and DF, and its concentration was increased by hCG stimulation albeit to a moderate degree when compared to α*CAV1* mRNA expression.

A preliminary mRNA gene expression profiling study identified a cDNA fragment that corresponded to *CAV1*, which suggested that *CAV1* expression would be up-regulated by the LH/hCG preovulatory surge in bovine GC [12]. Since the cDNA fragment corresponded to the 3'UTR of *CAV1* mRNA, we characterized the full-length bovine *CAV1* cDNA from hCG-stimulated GC to ascertain its identity. Two isoforms of CAV1 have been identified in other species: the αCAV1 consists of 178 amino acids whereas the βCAV1 lacks the first 31 amino acids of the αCAV1 [24-26]. The α and βCAV1 derived from *CAV1* gene are generated from distinct mRNAs that are regulated independently at the transcriptional level by alternative promoters [27]. In the present study, RT-PCR analysis of *CAV1* mRNA using oligos spanning amino acid G^4 to Q^{175} of bovine αCAV1 revealed a single amplicon corresponding to the α-isoform. Furthermore, a single protein band of 22 kDa was observed in immunoblotting analysis of bovine GC, which corresponds to αCAV1, confirming the RT-PCR observations. Characterization of α*CAV1* mRNA revealed four polyadenylation signals in its 3'-UTR segment. The precise biological reason of these multiple adenylation signals in αCAV1 are unknown, but as suggested for other genes, they may increase the stability of α*CAV1*

mRNA or the efficacy of translation through tissue-specific polyadenylation factors [28]. As observed for orthologous proteins, the bovine CAV1 protein contains a conserved hydrophobic membrane spanning domain (A^{105}-L^{125}), which adopts a hairpin structure within membrane lipids resulting in amino- and carboxy-termini located in the cytoplasm [24]. The three cysteines at the C-terminus (C^{133}, C^{143}, C^{156}) are conserved and may be palmitoylated thus helping to stabilize the protein at the membrane [24,29]. The CAV1 scaffolding domain (D^{82}-R^{101}) allows interactions with diverse proteins by recognizing a specific caveolin binding motifs (AXAXXXXA and AXXXXAXXA; where A is an aromatic amino acid) localized in intracellular signaling proteins [30]. The protein-protein interaction capacity enables the formation of high molecular weight homo-and hetero-oligomers. CAV1 may also be phosphorylated on Y^{14} in response to a number of growth factors that regulate its interaction with signal transducing proteins [24,31-33-].

Expression of CAV1 was induced in GC following injection of an ovulatory dose of hCG. The increase in *CAV1* mRNA were mirrored by observations at the protein level, where an initial increase was detected 12 h post-hCG and was sustained until 24 h post-hCG. Although CAV1 is induced by hCG in GC, its role at the time of ovulation remains hypothetical. The functional roles attributed to CAV1 are diverse and range from vesicular transport such as endocytosis and transcytosis, to cholesterol homeostasis, regulation of signal transduction and suppression of cellular transformation [24,34-36]. A role for CAV1 in cholesterol transport in GC cannot be supported since dominant or preovulatory follicles known to synthesize high concentration of estradiol

do not express CAV1 [15,16,19,37,38]. Furthermore, at the time of ovulation, steroid hormone synthesis decreases in GC when CAV1 expression increases [16, 37,38], and despite the high progesterone synthesis by luteal cells [39], CAV1 expression in bovine luteal cells was not detected by immunohistochemistry. Interestingly, CAV1 was shown to interact with phospholipase A2 group IVA (PLA2G4A also known as cPLA2α) [40] and prostaglandin-endoperoxide synthase 2 (PTGS2 also known as COX2) [41], two enzymes involved in prostaglandin synthesis. PLA2G4A releases arachidonic acid from membrane phospholipids, which is then converted to PGH2 by PTGS2 and then further to PGE by PGE synthase [18]. PLA2G4A and PTGS2 are induced by the LH/hCG preovulatory surge in bovine GC [16,23]. CAV1 was shown to inhibit PLA2G4A enzymatic activity [40] and to co-localize with PTGS2 [41]. Collectively, these results suggest that CAV1, through its compartmentalization and interaction with PLA2G4A and PTGS2, could modulate prostaglandin production during ovulation.

Since the preovulatory LH/hCG surge induced CAV1 expression in GC, a role for CAV1 in modulating LHCG receptor (LHCGR) signaling is possible. It is known that upon ligand activation, LHCGR cluster at the cytoplasmic membrane [42], are phosphorylated and complexed with β-arrestin 2, and are internalized via clathrin-coated pits [43,44]. These complexes are then targeted to lysosomes for degradation [45]. However, several G-protein coupled receptors such as endothelin, bradykinin, muscarinic acetylcholine and β-adrenergic receptors have been localized to caveolae, and CAV1 is known to directly interact with the α-subunit of G-proteins [24]. It is thus plausible that an increase in CAV1 following the LH/hCG surge may inhibit LHCGR

signaling through clathrin-independent endocytosis [46]. The recent description of CAV1-null mice further suggest, that CAV1 may act as a tumor suppressor and regulator of cell proliferation by modulating the signaling of various growth factors [35]. These observations suggest that CAV1, at the time of ovulation, may be involved in regulating growth factor signaling, thereby modulating GC division.

The expression of both *CAV1* and *CAV2* mRNA was detected in GC but the expression of *CAV1* is acutely regulated in comparison to *CAV2*. Expression of *CAV2* mRNA was detected in GC of SF and DF, and the increase following hCG injection was less pronounced when compared to *CAV1*. CAV2 is unable to form caveolae by itself and requires interaction with CAV1 to be incorporated into caveolae [4]. The formation of caveolae is thus driven by the presence of CAV1. The presence of CAV1 protein in GC extracts of small antral follicles was detected by immunoblotting and may have resulted from minor contamination of endothelial cells when GC were mechanically dislodged from follicular walls. Similarly, the detection of basal levels of *CAV1* mRNA in follicular wall samples at time 0 and 6 h post-hCG likely reflects the endothelial cell contribution of CAV1 *mRNA* to these samples. This explanation is supported by the immunohistochemical observations which clearly demonstrate the high expression of CAV1 in endothelial cells of capillaries adjacent to the basal membrane of the granulosa layer, and the absence of CAV1 in GC of SF and DF. The high expression of CAV1 in endothelial cells is well described in the literature [24], and may serve in transcytosis of blood-born nutrients reaching the GC layer. The expression of CAV1 mRNA and

proteins in CL was associated with endothelial cells of capillaries since luteal cells were not immunolabelled for CAV1.

In summary, this report demonstrates for the first time an LH/hCG-dependent induction of CAV1 mRNA and protein in GC in vivo. Since CAV1 can associate through its scaffolding domain to many proteins thus inhibiting their biological action, the up-regulation of CAV1 expression is likely to be important for controlling the increase in membrane signaling occurring at the time of ovulation and luteinization.

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Fig. 1. Nucleotide and amino acid sequences of bovine αCAV1.

The bovine *αCAV1* full-length cDNA was cloned by library screening, as described under *Materials and Methods*, and amino acids deduced from cDNA. Nucleotides and amino acids are numbered at the left; amino acid numbering begins at the first methionine. The bovine *αCAV1* is composed of 2455 bp, including a 5'-UTR of 62 bp, an open reading frame (ORF) of 534 bp and a 3'-UTR of 1859 bp followed by a poly(A)$^+$ tail. The ORF codes for 178 amino acids representing a putative protein of 20,593 Mr with a pI of 5.6. In the 3'-UTR region, *asterisks* represent the stop codon, and the four polyadenylation signals are underlined. The poly(A)$^+$ tail is not represented. The nucleotide sequence was submitted to Genbank (accession number: AY823915).

Figure 1

Fig. 2. Expression of αCAV1 and CAV2 transcripts in bovine granulosa cells and CL. Total RNA was extracted from bovine granulosa cells collected from 2-4 mm follicles (SF), dominant follicles (DF) at day 5 of the estrous cycle, ovulatory follicles (OF) 24 h after injection of hCG (OF) and corpora lutea (CL) from day 5 of the estrous cycle, then served for mRNA expression analyses using semiquantitative RT-PCR as described under *Materials and Methods*. The control gene *GAPD* (710 bp) showed no significant difference in mRNA expression levels between groups. The *αCAV1* (437 bp) and *CAV2* (960 bp) PCR fragments were normalized with their corresponding *GAPD* signal, and the results are presented as a relative change in ratio between groups. Tukey-Kramer multiple comparison tests were performed to compare group means for *αCAV1* or *CAV2* mRNA; different letters denote samples that differed significantly ($P < 0.05$). Data are presented as least-square means ± SEM, and the number of independent samples (*i.e.* animals per group) is indicated in parenthesis.

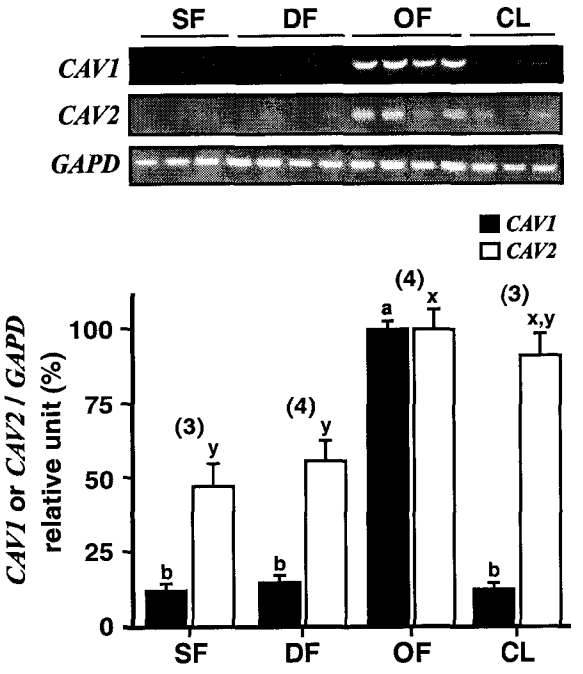

Figure 2

Fig. 3. Regulation of αCAV1 and CAV2 transcripts by hCG in bovine follicles during the periovulatory period.

Total RNA was extracted from preparations of bovine follicular wall obtained from ovulatory follicles isolated between 0 and 24 h after injection of hCG then used in mRNA expression analyses by semiquantitative RT-PCR as described under *Materials and Methods*. The control gene *GAPD* (710 bp) showed no significant differences in mRNA expression levels between groups. The *αCAV1* (437 bp) and *CAV2* (960 bp) PCR signals were normalized with their corresponding *GAPD* signals, and the results are presented as a relative change in ratio between groups. Dunnett's test was used for comparisons of group means of *αCAV1* or *CAV2* mRNAs; bars marked with an asterisk are significantly different ($P < 0.05$) from 0 h post-hCG. Data are presented as least-square means ± SEM, representing two independent animals per time point.

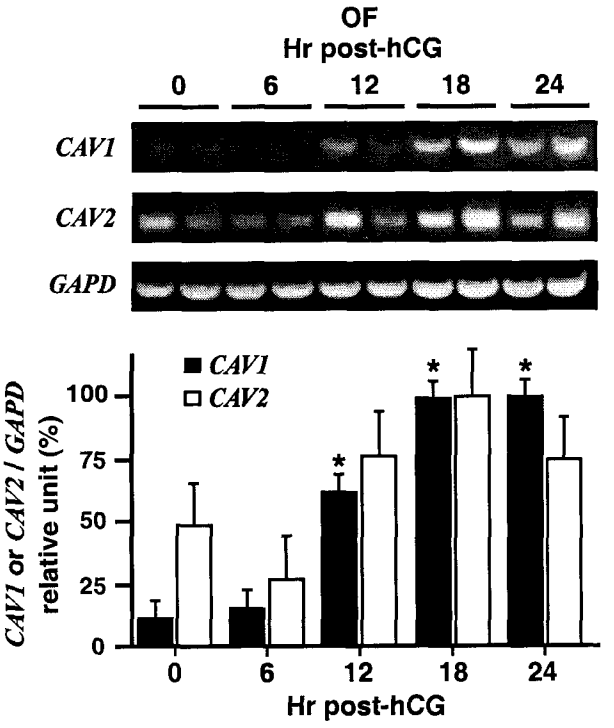

Figure 3

Figure 4. Regulation of αCAV1 protein by hCG in bovine follicles.

Granulosa cells were isolated from bovine ovulatory follicles between 0 and 24 h after hCG treatment. Protein extracts (50 μg) were analyzed on a one-dimensional 18% SDS-PAGE and immunoblotted as described under *Materials and Methods*. Immunoblotting was performed using (**A**) a polyclonal antibody against human CAV1 (1:500; SC-894) revealing a 22,000 Mr protein band or (**B**) a polyclonal antibody against bovine GSTA1 (1:3000) revaling a 28,000 Mr protein band. Two distinct follicles from independent animals per time point are represented.

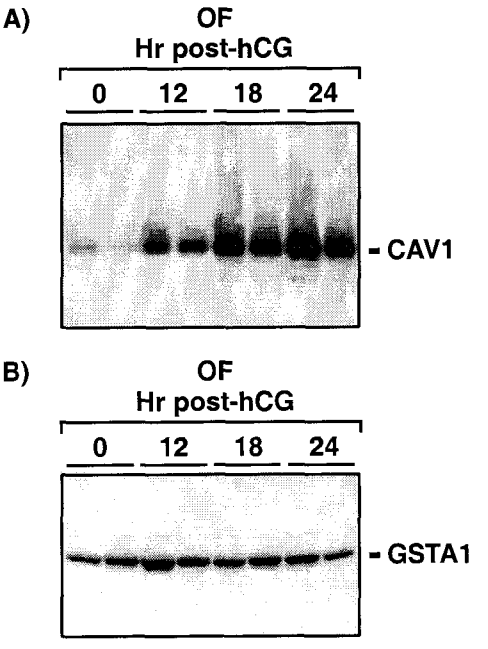

Figure 4

Figure 5. Immunohistochemical localization of αCAV1 in bovine ovulatory follicles and CL.

Immunohistochemistry was performed on formalin-fixed sections of ovulatory follicles isolated preceeding and following hCG injection, as described under *Materials and Methods*. The scale bar is equal to 0.05 mm in all figures. Staining was absent when the primary antibody was omitted (data not shown). **A)** DF at day 5 of the estrous cycle; **B)** preovulatory follicle at day 7; **C)** ovulatory follicle 24 h post-hCG; **D)** enlargement of OF presented in C; **E)** small antral follicle (2 mm) adjacent to the ovulatory follicle at 24 h post-hCG; **F)** CL at day 5 of the estrous cycle. Alpha-CAV1 staining is undetectable in GC of dominant or preovulatory follicles preceeding hCG treatment (A,B), and in GC of small follicles (E) and luteal cells (F). A clear induction of αCAV1 immunoreactivity is observed in GC of ovulatory follicles obtained 24 h post-hCG (C, D). Endothelial cells in all antral follicles and CL are stained for CAV1. (A: antrum; BM: basal membrane; En: endothelial cells; G: granulosa; T: theca).

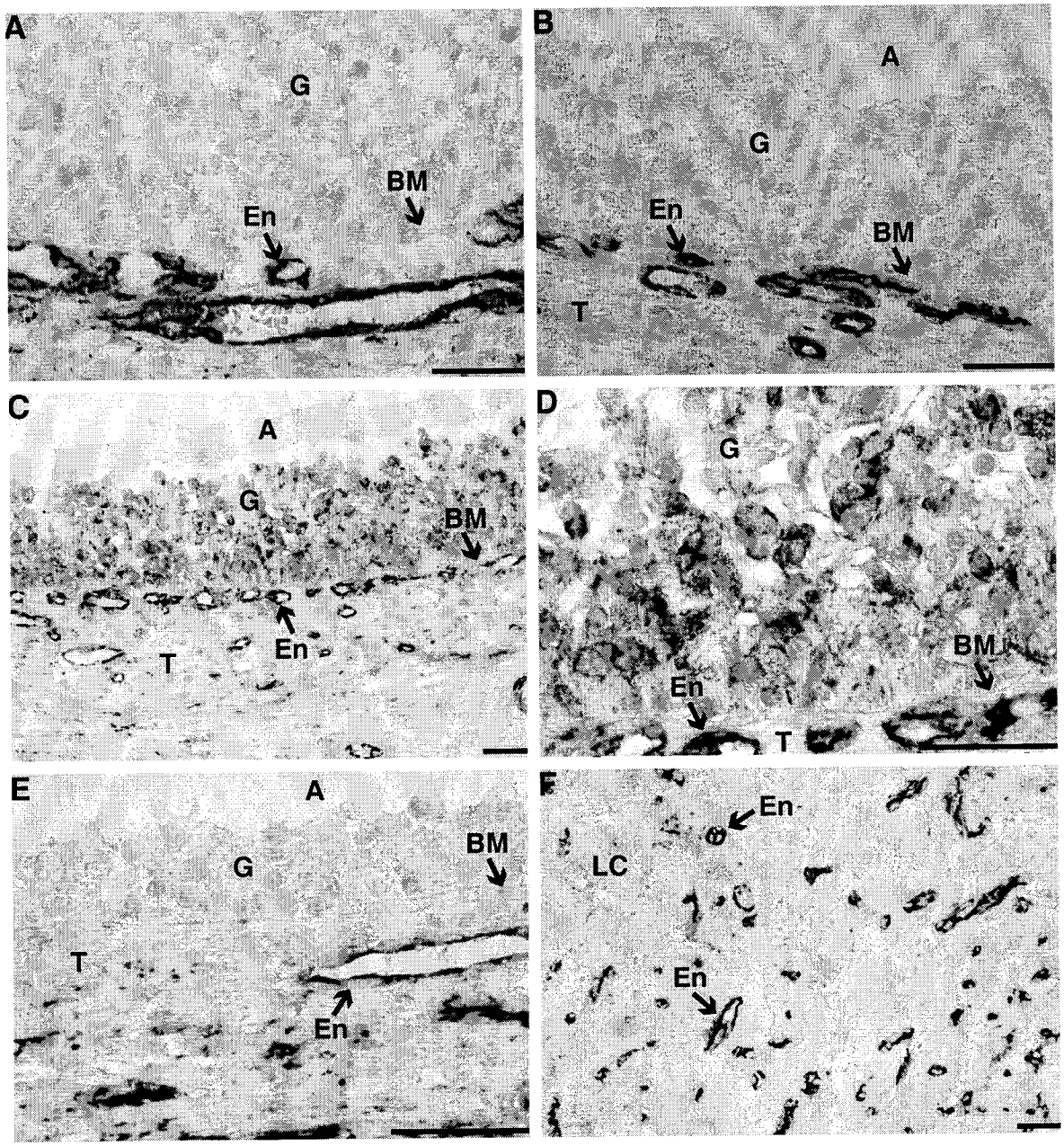

Figure 5

CHAPITRE 4

Characterization of novels genes induced by human chorionic gonadotropin in granulosa cells of bovine ovulatory follicles: molecular cloning and spatio-temporal expression studies

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Key words: ovary, follicle, granulosa, gene expression, ovulation, KIAA-1798,

Trypsin-like inhibitor, ASB9

ABSTRACT

In mammals, the ovulatory process begins at the time when the endogenous luteinizing hormone (LH) surge stimulates the G protein-coupled LH/human chorionic gonadotropin receptors (LHCGR) that are distributed on the surface of granulosa and theca cells of a mature preovulatory follicle. The LH/hCG–induced ovulatory process is controlled by temporal and spatial expression of specific genes, and present features reminiscent of an acute inflammatory reaction. Based on a previous mRNAs profiling expression study in granulosa cells (GC), cDNA fragments corresponding to KIAA-1798, trypsin-like inhibitor and ASB9 were identified as being up-regulated by LH/hCG in GC during ovulation. The objectives of this study were to characterize these bovine cDNAs and to study their spatio-temporal expression pattern in ovarian follicles and tissues. Full-length bovine cDNAs were cloned by screening size-selected cDNA libraries derived from hCG-stimulated GC. The bovine KIAA-1798 cDNA consists of 3892 bp with an open reading frame (ORF) of 2376 bp encoding a putative 80.5 kDa protein that is 96% identical to human KIAA-1798. The bovine trypsin-like inhibitor cDNA possesses 4020 bp, its ORF of 1491 bp encodes a putative 55.6 kDa protein that is 85% identical to human trypsin-like inhibitor and 83% to rat late gestation protein-1. Two ASB9 cDNAs isoforms were cloned that consist of 1604 bp and 1435 bp for ASB9-1 and ASB9-2, respectively. The ORF of the ASB9-1 has 764 bp encoding a putative 31.4 kDa protein while ASB9-2 protein has an ORF of 675 bp encoding a putative protein of 24.7 kDa. Bovine ASB9-1 is 75% identical to human (BC013172), 74% to rat (XM_346301) and 73% to mouse ASB9. Expression of these mRNAs was studied in bovine GC obtained from follicles at different developmental stages: small

follicles (SF: 2-4 mm), dominant follicles (DF: at day 5 of the estrous cycle), ovulatory follicles (OF: 24 h following injection of an ovulatory dose of hCG), and corpus luteum (CL: at day 5). A transcript of 4.7 kb for KIAA-1798, 5.2 kb for trypsin-like inhibitor and of 1.6 kb and 1.5 kb for ASB9-1 and ASB9-2, respectively, were observed by virtual Northern analysis. Semiquantitative RT-PCR analyses showed a significant increase in mRNAs in GC of OF versus DF for all 3 genes ($P< 0.0001$). Temporal expression of mRNAs was studied in follicular walls obtained from OF recovered at 0, 6, 12, 18, or 24 h after hCG injection. Results showed a 10.4 fold increase for KIAA-1798 ($P < 0.041$), a 2.2-fold increase for ASB9-1 ($P< 0.006$) and a 3.6-fold increase for ASB9-2 ($P < 0.021$) compared to follicular wall samples preceeding hCG-injection. Analyses of mRNA expression by RT-PCR/Southern blot in various bovine tissues revealed a wide but variable pattern of KIAA-1798 and trypsin-like inhibitor transcripts across tissues. Conversely, ASB9 transcripts were detected mainly in male and female gonads. ASB9-1 was the most highly expressed isoform in GC and testis whereas ASB9-2 was detected only in GC. Furthermore, both ASB9 transcripts were highly expressed in GC of OF compared to DF. This study reports for the first time the characterization of three bovine cDNAs, and demonstrates that their mRNA expression in GC is induced by hCG. The results suggest that up-regulation of KIAA-1798, trypsin-like inhibitor and ASB9 mRNAs by the LH/hCG preovulatory surge likely intervenes in GC during ovulation and luteinization. The biological role played by each of these three genes remains to be determined.

INTRODUCTION

In mammals, the ovulatory process begins at the time when the endogenous luteinizing hormone (LH) surge stimulates the G protein-coupled LH/human chorionic gonadotropin receptors (LHCGR) that are distributed on the surface of granulosa and theca cells of a mature preovulatory follicle. This gonadotropin–induced process is controlled by temporal and spatial expression of specific genes, and present features reminiscent of an acute inflammatory reaction (Robker et al., 2000; Espey & Richards, 2002). Modern methods in molecular biology such as mRNA differential display, cDNA array and suppression subtractive hybridization (SSH) have allowed the detection of differentially expressed genes during the process of follicular growth and ovulation (Espey & Richards, 2002; Leo et al., 2001; Hennebold, 2004). Transcription of genes in granulosa cells (GC) that control the growth of a bovine dominant or preovulatory follicle is rapidly down-regulated as a consequence of LH-mediated increases in intracellular cAMP (Fayad et al., 2004; Ndiaye et al., 2005). In conjunction with the termination of specific gene expression in preovulatory follicles, the LH/hCG preovulatory surge induces expression of genes involved in ovulation and luteinization as shown in rodents (Espey & Richards, 2002). For ovulation to occur, many genes were found to be induced or up-regulated by the LH surge, such as prostaglandin-endoperoxide synthase 2 (PTGS2) (Sirois et al., 1992), progesterone receptor (PGR) (Pack and Mayo, 1991), CCAAT/enhancer-binding protein beta (C/EBPB) (Sirois & Richards, 1993; Sterneck et al., 1997), and early growth response 1 protein (EGR1) (Espey et al., 2000). These genes were shown to be critical to ovulation since the process is impaired in mice null for these genes.

Most data on the temporal pattern of gene expression arising during ovulation have been obtained using whole ovarian RNA extracts from immature PMSG- and hCG-stimulated rodent ovaries, which conceals the identification of gene specifically induced in GC by LH/hCG. Using GC isolated from hCG-induced ovulatory follicles from bovine adult females, SSH was applied to identify expression of specific genes that are up-regulated during ovulation (Lévesque et al., 2002). The latter study identified three bovine cDNA fragments that corresponded to the 3'-untranslated region (3'-UTR) of human cDNAs, since found to be: KIAA-1798, trypsin-like inhibitor, and ankyrin-repeat-containing protein 9 (ASB9). We hypothesized that the expression of these genes would be induced by the LH/hCG preovulatory surge in GC and that they might be potential molecular determinants that intervene during ovulation. Information on these cDNAs is currently sparse. Furthermore, their expression has never been associated with ovarian physiology. The specific objectives of this study were to clone these three bovine cDNAs and to determine the spatio-temporal expression profile of their mRNA in ovulatory follicles following hCG treatment. Bovine ovulatory follicles were used as the *in vivo* model to investigate the regulation of these cDNAs in hCG-induced ovulation/luteinization.

MATERIALS AND METHODS

Cloning of the bovine KIAA-1798, trypsin-like inhibitor and ASB9

Isolation of the full-length bovine KIAA-1798, trypsin-like inhibitor and ASB9 cDNAs was performed by screening a size-selected cDNA library for each of the cDNA. Initially, the respective sizes of the full-length cDNAs were estimated by performing a

virtual Northern blot analysis. Briefly, total RNA was isolated from GC obtained from bovine ovulatory follicles 24 h following injection of hCG (Ndiaye et al., 2005), and transformed into cDNA by the SMART PCR cDNA synthesis method (BD Biosciences Clontech, Mississauga, ON) as previously described (Lévesque et al., 2003). The cDNA pool was separated by gel electrophoresis, transferred onto a nylon membrane and hybridized with the bovine KIAA-1798, trypsin-like inhibitor and ASB9 radioactive probes generated from a previous SSH screening experiment (Lévesque et al., 2002). The size of the corresponding full-length cDNA was determined.

Total SMART cDNAs from hCG-stimulated GC were size-fractionated by agarose gel electrophoresis and cDNAs ranging from 5 to 6 Kb for KIAA-1798 and trypsin-like inhibitor, and 1.8 to 1.6 Kb for ASB9 were purified and used to construct size-selected cDNA libraries into pDrive plasmid (Qiagen PCR cloning kit; Qiagen, Mississauga, ON). These cDNA libraries were then screened with their respective radioactive probe by hybridization of bacterial colonies as previously described (Lévesque et al., 2003). Positively hybridizing bacterial colonies were grown, their plasmid contents were isolated (QIA-prep, Qiagen), and the size of the cloned cDNA was analyzed following an *EcoR*1 digestion and gel electrophoresis analysis. The cDNAs were sequenced via the dideoxy sequencing method (Big Dye Terminator 3.0; ABI Prism, Applied BioSystem, PE, Branchburg, NJ) and analyzed on an ABI Prism 310 sequencer (Applied Biosystem). Nucleic acid sequences were analyzed by BLAST (Basic Local Alignment Search Tool) against GenBank database.

Bovine tissues and RNA extraction

Bovine ovarian follicles and corpora lutea (CL) were isolated at specific stages of the estrous cycle from normal cycling crossbreed heifers, as previously described (Filion et al., 2001; Ndiaye et al., 2005). Briefly, estrous cycle was synchronized with $PGF_{2\alpha}$ (25 mg, im; Lutalyse, Upjohn, Kalamazoo, MI) and ovarian follicular development was monitored by daily transrectal ultrasonography until ovariectomy. Dominant follicles (DF; n = 4 cows) were defined by ultrasonographic measurement as measuring > 8 mm and growing while subordinate follicles were either static or regressing. They were obtained on the morning of day 5 of the estrous cycle (day 0 = day of estrus) by ovariectomy (via colpotomy). Ovulatory follicles (OF) were obtained following an injection of 25 mg of $PGF_{2\alpha}$ on day 7 of the synchronized estrous cycle to induce luteolysis, thereby maintaining the development of the DF of the first follicular wave into a preovulatory follicle. An ovulatory dose of hCG (3000 IU, iv; APL, Ayerst Lab, Montréal, QC) was injected 36 h after the induction of luteolysis, and the ovary bearing the hCG-induced OF was collected by ovariectomy at 0, 6, 12, 18 or 24 h after hCG injection (n = 2-4 cows/time point). Follicles were dissected into preparations of follicle wall (theca interna with attached GC; Filion et al., 2001) or further dissected into separate isolates of GC (Ndiaye et al., 2005). Ovariectomies were also performed on day 5 of the synchronized estrous cycle to obtain CL (n = 3 cows). Additionally, GC were collected from 2-4 mm follicles that were obtained from slaughterhouse ovaries representing a total of three pools of 20 small follicles (SF). These experiments were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine of the Université de Montréal. Total RNA was isolated from tissues as previously described

(Bédard et al., 2003). Concentration of total RNA was quantified by measuring optical density at 260 nm, and quality was evaluated by visualizing the 28S and 18S ribosomal bands following electrophoretic separation on a formaldehyde denaturing 1% agarose gel with ethidium bromide.

Semiquantitative RT-PCR analyses

Total RNA (1 μg) from individual follicles and CL was reverse-transcribed using an oligo-dT30 primer and PowerScript (BD Biosciences Clontech) to generate the first strand cDNA using the SMART PCR cDNA synthesis kit (User manual: PT3041-1; BD Biosciences Clontech, Mississauga, ON; Ndiaye et al., 2005). Second cDNA strands were produced with the SMART II 5'-anchored oligo, and PCR-amplified for 15 cycles using the Advantage 2 DNA polymerase (BD Biosciences Clontech). SMART cDNA pools were then diluted to 50 μl in 10 mM Tris pH 8 buffer. To perform semiquantitative RT-PCR, SMART cDNA pools were diluted 10-fold in H_2O, and 1 to 2 μl were used in a 25 μl PCR reaction using the Advantage 2 DNA polymerase kit (BD Biosciences Clontech). Gene-specific PCR primers were designed in the open-reading frame of each cDNA sequence for: KIAA-1798 (sense: 5'-GTTATTACAGATGAGAGTGAGATGG-3'; anti-sense: 5'-ACTTAAAGGACAGAAAGACGGC-3'; GenBank: AY437805), trypsin-like inhibitor (sense: 5'-CTATGGGATCCTGGACGACAGG-3'; anti-sense: 5'-CCTGCAAGTTCACTGCCTGACG-3'; GenBank: AY369781), ASB9 (sense: 5'-CAGCCATTCATGGACGTCTGC-3'; anti-sense: 5'-GCTTGATCCCAAAGCACTTCCG-3'; GenBank: AY438595) and glyceraldehyde-3-phosphate dehydrogenase (*GAPD*; sense: 5'-TGTTCCAGTATGATTCCACCCACG-3'; anti-

sense: 5'-CTGTTGAAGTCGCAGGAGACAACC-3'; Ndiaye et al., 2005). For all these genes, PCR conditions were as follows: 95 °C for 1 min, 95 °C for 30 s, 64 °C for 45 s, and 68 °C for 90s. The number of PCR cycles was limited (19-22 cycles) and optimized for each gene to be analyzed. Twenty μl of the PCR reactions were resolved on a 2% TAE-agarose gel (40 mM Tris acetate pH 8, 1 Mm EDTA) with ethidium bromide (0.5 μg/ml), PCR products were visualized by UV and the images were digitized. The digitized signals for each gene were analyzed by densitometry using ImageQuant software (Amersham Pharmacia Biotech, Pointe-Claire, QC).

Various bovine tissues were obtained from a slaughterhouse and total RNA was extracted as described (Bédard et al., 2003). The QIAgen one-step RT-PCR kit was used for semiquantitative/Southern blotting analysis of KIAA-1798, trypsin-like inhibitor, ASB9 and GAPD expression in various bovine tissues. Reactions were performed as directed by the manufacturer using sense and antisense primers specific for bovine KIAA-1798, trypsin-like inhibitor, ASB9 and GAPD as described above. These reactions resulted in the generation of DNA fragments of 800 bp for KIAA-1798, 420 bp for trypsin-like inhibitor, 500 bp for ASB9 isoform 1 (ASB9-1), 649 bp for ASB9 isoform 2 (ASB9-2) and 710 bp for GAPD. Each reaction was performed using 100 ng of total RNA, and cycling conditions were one cycle of 48 °C for 45 min and 94 °C for 2 min, followed by a variable number of cycles of 94 °C for 30 s, 64 °C for 1 min, and 68 °C for 2 min. The number of PCR cycles was optimized for each gene to fall within the linear range of PCR amplification: 30 cycles for KIAA-1798; 27 cycles for trypsin-like inhibitor; 22 cycles for ASB9 and 22 cycles for GAPD. Following PCR

amplification, samples were resolved on a 2% TAE-agarose gel, transferred onto nylon membranes, and hybridized with corresponding radiolabeled KIAA-1798, trypsin-like inhibitor, ASB9 and GAPD cDNA fragments as described (Ndiaye et al., 2005). Membranes were exposed to a phosphor screen, and signals were quantified on a Storm imaging system using the ImageQuant software as described above.

Statistical analysis

Gene-specific signals were normalized with corresponding *GAPD* signals for each sample. Homogeneity of variance between follicular groups and CL was verified by O'Brien and Brown-Forsythe tests. Corrected values of gene-specific mRNA levels were compared between follicular or CL groups by one-way ANOVA. When ANOVA indicated significant differences ($P < 0.05$), multiple comparisons of SF, DF, OF and CL individual means were compared by the Tukey-Kramer test, whereas the Dunnett's was used to compare individual means of the temporal hCG-induced follicular wall samples. Data are presented as least-square means ± SEM. Statistical analyses were performed using JMP software (SAS Institute, Inc., Carry, NC).

RESULTS

Characterization of bovine KIAA-1798, trypsin-like inhibitor and ASB9 cDNAs

The full-length bovine KIAA-1798 cDNA was cloned and consists of 3892 bp (Genbank: AY437805). It is composed of a 5'-UTR of 205 bp, an open reading frame (ORF) of 2373 bp, and a 3'-UTR of 1314 bp containing three AU-rich elements (ATTTA) as well as three polyadenylation signals (AATAAA) followed by a poly(A)$^+$

tail (Fig. 1A). The coding region of bovine KIAA-1798 cDNA encodes a protein of 773 amino acids, with a theoretical molecular mass (Mr) of 80,500 and an isoelectric point (pI) of 6.06. Amino acid homology search in GenBank by PsiBlast revealed orthologs with an overall identity level of 96% to human KIAA-1798 (GenBank: BAB47427) protein (Fig. 1B) also called lethal(3) malignant brain tumor-like protein (L3MBTL; GenBank: NP_115814). Analysis of bovine KIAA-1798 amino acid *in silico* by pFam and Interpro softwares revealed it to contain three malignant brain tumor (MBT) repeat domains (MBT-1: M^{268}-N^{341}; MBT-2: M^{375}-F^{448}; MBT-3: M^{479}-A^{552}), one sterile alpha motif (SAM; S^{706}-K^{770}), and a probable intracellular localization.

The full-length bovine trypsin-like inhibitor cDNA was cloned and consists of 4020 bp (Genbank: AY369781). It is composed of a 5'-UTR of 210 bp, an ORF of 1488 bp, and a 3'-UTR of 2322 bp containing six AU-rich elements (ATTTA) as well as two polyadenylation signals followed by a poly(A)$^+$ tail (Fig. 2A). The coding region of bovine trypsin-like inhibitor cDNA encodes a protein of 496 amino acids, with a theoretical Mr of 55,600 and pI of 8.2. Amino acid homology search in GenBank by PsiBlast revealed orthologs (Fig. 2B) with an overall identity level of 83% to a human trypsin inhibitor (AAQ89150), 78% to rat (XP_346524) and 76% to mouse protein (NP_084485). Bovine trypsin-like inhibitor protein possesses a peptide signal (M^1-G^{22}) that may be cleaved between G^{22}-F^{23}, a sterol-carrier protein-like domain (SCP-like; L^{60}-N^{199}) and two LCCL domains (LCCL-1: V^{287}-F^{378}; LCCL-2: L^{388}-K^{487}).

Two cDNAs corresponding to bovine ASB9 isoforms were cloned from LH/hCG-stimulated GC. The full length bovine ASB9 isoform 1 (ASB9-1; Genbank: AY438595) is composed of 1604 bp with a 5'-UTR of 241 bp, an ORF of 762 bp, and a 3'-UTR of 562 bp containing a polyadenylation signal (ATTAAA) followed by a poly(A)$^+$ tail (Fig. 3A). The bovine ASB9 isoform 2 (ASB9-2; AY442176) has 1435 bp and is composed of a 5'-UTR of 261 bp, an ORF of 672 bp, and a 3'-UTR of 502 bp containing a polyadenylation signal (ATTAAA) followed by a poly(A)$^+$ tail (Fig. 3B). The coding regions of bovine ASB9-1 and ASB9-2 encode for proteins of 287 and 224 amino acids, respectively. The Mr of ASB9-1 is 31,400 with a pI of 6.8 whereas the ASB9-2 has a Mr of 24,700 with a pI of 7.2. Amino acid homology search in GenBank by PsiBlast revealed orthologs for ASB9-1 with an overall identity level of 75% to human (BC013172), 74% to rat (XM_346301) and 73% to mouse ASB9 (NM_027027; Fig. 3C). Bovine ASB9-1 contains six ankyrin domains (ANK-1: S^{29}-A^{61}; ANK-2: D^{62}-V^{94}; ANK-3: D^{95}-D^{127}; ANK-4: S^{130}-H^{159}; ANK-5: H^{160}-G^{192}; ANK-6: D^{194}-A^{224}) whereas ASB9-2 contains five ankyrin domains (ANK-1: S^{29}-A^{61}; ANK-2: D^{62}-V^{94}; ANK-3: D^{95}-D^{127}; ANK-4: S^{130}-H^{159}; ANK-5: H^{160}-C^{192}). At their C-terminus, ASB9-1 and ASB9-2 share a consensus motif of suppressors of cytokine signaling, named SOCS-box; ASB9-1: P^{248}-I^{287}; ASB9-2: P^{185}-I^{224}).

Analyses of mRNA expression in granulosa cells and CL

The expression profile of each mRNA was compared by virtual Northern analysis using GC collected from small follicles (SF; 2-4 mm), dominant follicles (DF) at day 5 of the estrous cycle, ovulatory follicles (OF) 24 h following injection of an ovulatory

dose of hCG, and from corpus luteum (CL) at day 5. A transcript estimated at 4.7 kb for KIAA-1798, 5.2 kb for trypsin-like inhibitor, 1.6 and 1.5 Kb for ASB9-1 and ASB9-2, respectively, were observed. They were shown to be expressed predominantly in OF (data not shown). Semiquantitative RT-PCR analyses showed low expression in SF, DF and CL for KIAA-1798 and trypsin-like inhibitor whereas 3.2-fold and 5-fold increases in mRNA levels were observed in GC of OF compared to DF ($P < 0.0001$ for both mRNAs; Fig. 4A,B). Furthermore, 14.8-fold and 6-fold increases in mRNA levels were observed for ASB9-1 and ASB9-2 in GC of OF compared to DF ($P < 0.0001$ for both mRNAs; Fig. 4C).

Analyses of mRNA expression in follicles during the periovulatory period

Since KIAA-1798, trypsin-like inhibitor, ASB9-1 and ASB9-2 mRNAs were clearly induced in GC 24 h following hCG injection, semiquantitative RT-PCR analyses were used to study the temporal expression of these four mRNAs in follicular wall preparations collected from ovulatory follicles isolated at 0, 6, 12, 18 or 24 h post- hCG. Levels of bovine KIAA-1798 mRNA were low, but detectable, in follicles preceding hCG treatment (0 h). An initial up-regulation of 3.7-fold for KIAA-1798 mRNA was apparent 6 h after hCG treatment, that reached a maximum 10.4-fold at 18 h post-hCG when compared to 0 h ($P < 0.041$; Fig. 5A). Conversely, trypsin-like inhibitor mRNA expression varied from 0 to 24 h but the difference was not statistically significant ($P < 0.15$; Fig. 5B). Both ASB9 mRNAs increased significantly during the periovulatory period (ASB9-1: $P < 0.006$; ASB9-2: $P < 0.021$; Fig. 5C); for the ASB9-1, a maximun

2.2-fold increase was observed 12 h post-hCG, whereas a maximum of 3.6-fold increase was dectected 18 h post-hCG for ASB9-2.

Tissue distribution of bovine mRNAs

We then investigated if these genes were uniquely expressed in follicles and if their level of expression varied between bovines tissues. Results revealed a wide distribution of expression of the KIAA-1798 and trypsin-like inhibitor transcripts, however, mRNA levels varied across tissues (Fig. 6). Highest levels of mRNAs for KIAA-1798 and trypsin-like inhibitor were observed in GC of OF obtained at 24 h post-hCG. High levels of KIAA-1798 mRNA were detected in pituitary, thyroid, liver, spleen, kidney, CL, adrenal gland, testis and epidymis. Low levels of mRNA were detected in GC of DF at day 5, placentome, uterus, fetal ovary, muscle and brain cortex. For trypsin-like inhibitor, low level of mRNA was detected in uterus, placentome and epidymis, whereas a detectable signal was observable in other tissues. Highest levels of mRNA for ASB9 isoforms were detected in GC of OF obtained 24 h post-hCG. High expression of ASB9 was also found in testis whereas expression was low in other tissues. Expression of ASB9-1 was higher than ASB9-2 in all tissues analyzed.

DISCUSSION

This study demonstrates that the processes of ovulation and luteinization promoted by the preovulatory LH/hCG surge are accompanied by the induction of multiple genes, of which for the first time, KIAA-1798, trypsin-like inhibitor and ASB9 isoforms were shown to be up-regulated in bovine GC of hCG-induced OF when

compared to DF and SF. The temporal mRNA expression analyses using follicular walls obtained from 0 h to 24 h following hCG injection confirmed up-regulation of KIAA-1798, trypsin-like inhibitor, ASB9-1 and ASB9-2 mRNAs in GC. The follicular wall preparations that include thecal cells, endothelial cells and fibroblasts, contributed to the basal expression of KIAA-1798, trypsin-like inhibitor and ASB9 mRNA detected preparation preceeding hCG injection. Despite the contribution of the theca layers, the same trend in the expression profile of KIAA-1798 and ASB9 isoforms was observed in follicular wall samples at different times after hCG injection. However, the induction of trypsin-like mRNA in follicular wall samples did not reach statistical significance. This may be explained by the contribution of various cells in theca layers but also by the bimodal expression of the trypsin-like gene throughout this 24 h period; the first increase was observed at 6 h post-hCG, followed by a decrease at 12 h then an increase up to 24 hr. Comparison of mRNA expression profiles for these three genes in various bovine tissues revealed that their level of expression was highest in hCG-stimulated GC of OF. While KIAA-1798 and trypsin-like inhibitor genes are widely expressed in different tissues, their level of expression varies between tissues. Conversely, ASB9 is expressed mainly in gonads. Furthermore, the present study documents the existence of a novel ASB9-2 isoform expressed mainly in GC. Collectively, these results support potential contributions of KIAA-1798, trypsin-like inhibitor and ASB9 as molecular determinants that may impact ovulation. However, the physiological contributions of these genes during ovulation and luteinization are still unclear due to the lack of information on their respective biological action. The consequences of overexpression,

complete deletion or tissue targeted deletion of these genes will be determined only upon the construction of transgenic mouse lines.

The KIAA-1798

The systematic characterization of large cDNAs expressed in brain was realized through the development of the KIAA project ("KI" stands for Kazuka DNA Research Institute and "AA" are reference characters; Kikuno et al., 2002; Koga et al., 2004). While, this project provides nucleic acid sequences and a general comparative gene expression profile between tissues, the biological function of some of the cDNAs are unknown. Since large proteins such as those encoded by KIAA cDNAs frequently display multiple domains, they are probably involved in various interactions with other molecules *in vivo* (Kikuno et al., 2004; Koga et al., 2004). The bovine KIAA-1798 cDNA characterized in the present study that was similar to human KIAA-1798, also called lethal(3) malignant brain tumor-like protein (L3MBTL), contains three MBT repeats and one SAM domains. The role of this protein is unknown, and it has not been reported to be expressed in ovary.

The SAM domain is found in a variety of signaling molecules including Ephrin (Eph) family of receptor tyrosine kinases (Tessier-Lavigne, 1995), serine/threonine protein kinases (Tu et al., 1997), diacylglycerol kinases (Sakane et al., 1996), Src homology 2 (SH2) domain–containing adapter proteins (Shultz et al., 1997), cytoplasmic scaffolding and adaptor protein kinases, regulators of lipid metabolism, GTPases, and members of E26 transformation-specific (ETS) family of transcription

factors. The SAM domain can potentially function as a protein interaction module through its ability to homo-or hetero-oligomerize with other SAM domains or also proteins devoid of SAM domains, and even complexing to RNA (Bornemann et al., 1996; Stapleton et al., 1999; Thanos et al., 1999). Such versatility of the SAM domain contained in different proteins earns them functional roles in myriad of biological processes, from signal transduction to transcriptional and translational regulation (Qiao & Bowie, 2005). The paucity of information available at the present time enables us to hypothesize, based only on structural domains of the protein, that KIAA-1798 protein interacts with other proteins via its SAM domain in GC at the time of ovulation/luteinization.

The MBT repeat domain is found in a number of nuclear proteins such as the drosophila sex comb on midleg protein (Bornemann et al., 1996), and is predicted to possess a strong transcription-repressing activity (Boccuni et al., 2003). Recently, a mouse paralog of bovine and human KIAA-1798/L3MBTL was characterized and named MBT-1. In human, KIAA-1798/L3MBTL is located on human chromosome 20q12 whereas MBT-1 is located on human chromosome 6q23. MBT-1 structurally ressembles the KIAA-1798/L3MBTL since it is composed of 883 amino acids and contains a unique 100 amino acid glutamine-rich domain that is absent in the bovine and human KIAA-1798/L3MBTL proteins (Arai & Miyazaki, 2005). MBT-1 was shown to localize strictly to the nucleus, and was induced transiently by 2-*O*-tetradecanoylphorpbol 13-acetate (TPA) in a myeloid progenitor cell line in response to induction of differentiation (Arai & Miyazaki, 2005). MBT-1 studies revealed that it

regulates the maturational advancement of myeloid progenitors during differentiation, and was proposed to act in this process by inducing cell cycle arrest via the enhancement of cyclin-dependent kinase inhibitor activities preceding differentiation (Arai & Miyazaki, 2005). These recent biochemical observations pertaining to MBT-1 suggest that KIAA-1798/L3MBTL may act in a similar fashion by localizing to the nucleus and regulating cell differentiation. Based on this analogy, we could hypothesize that the induction of KIAA-1798/L3MBTL in GC at the time of ovulation is likely to participate in the control of the final differentiation of GC into luteal cells.

Trypsin-like inhibitor

The trypsin-like inhibitor identified in GC of OF possesses one SCP2-like and two LCCL domains. These two domains are found in other mammalian proteins, which possess different functions. The SCP2-like domain is found in rodent sperm–coating glycoprotein (or acidic epididymal glycoprotein; AEG), which is thought to be involved in sperm maturation (Mizuki & Kasahara, 1992), mammalian testis–specific protein (Tpx-1; Kasahara et al., 1989) and 17-beta hydroxysteroid dehydrogenase type 4 (17βHSD4) that is involved in 17β-estradiol inactivation (Brown et al., 2004). The name of the LCCL domain is derived from the name of proteins in which this domain was found: Limulus factor C, Coch-5b2 and mammalian late gestation lung protein. The protein Limulus factor C is a lipopolysaccaride endotoxin-sensitive trypsin type serine protease that serves to protect the organism from bacterial infection (Trexler et al., 2000). Coch-5b2 is a vertebrate cochlear protein and when the LCCL domain is mutated it causes deafness (Robertson et al., 1998; Liepinsh et al., 2001). The mammalian late

gestation lung protein (Lgl1) is thought to participate in the degradation of pulmonary surfactant (Oyewumi et al., 2003; Kaplan et al., 1999).

Trypsin is a serine protease mainly secreted by panceatic cells but it has an extrapancreatic localization such as in lung and kidney (Koshikawa et al., 1998). In addition to its capacity to degrade proteins, it can activate latent forms of many metalloproteases (MMP; Paju et al., 2001; Descamps et al., 2004). The proteases including serine proteases are important for many biological process such as extracellular matrix remodeling (Honda et al., 2002). Protease inhibitors are a major form of control of secreted proteases (Drapkin et al., 2002; Honda et al., 2002). So, many trypsin-inhibitors are identified in animal tissues such as protease nexin-1 (PN-1 or SERPINE2; Ny et al. 2002; Bédard et al., 2003), pancreatic secretory trypsin inhibitor (PSTI) also called Tumor–associated trypsin inhibitor (TATI) used as a marker in the diagnosis of ovarian cancer (Stenman, 2002; Uchima et al., 2003), and plasminogen activor inhibitor-1 (PAI-1 or SERPINE1; Cao et al., 2005). During the ovulatory process, many proteases including serine proteases are activated and implicated mainly in extracellular matrix remodeling (Smith et al., 1999; Cao et al., 2005). Their enzymatic activity is regulated by specific protein inhibitors (Drapkin et al., 2002; Honda et al., 2002; Stenman, 2002). By analogy to these inhibitors of serine proteases, we hypothesize that the secreted trypsin-like inhibitor protein characterized herein shares characteristics common to other inhibitors of serine proteases found in inflammatory reactions. In the ovulatory follicle, the trypsin-like inhibitor is likely involved in the control of tissue repair by abolishing or attenuating the extent of degradation of the

extracellular matrix induced by some proteases. Further biochemical studies should demonstrate the capacity of this trypsin-like inhibitor at inhibiting serine protease as well as its extracellular secretion.

ASB9

Many *ASB* mRNAs have been identified in various tissues (Kile et al., 2000, 2001; McDaneld & Moody, 2003; Liu et al., 2003). *ASB3* was the most widely expressed of the ASB family (Kile et al., 2001). In contrast to the other family members, *ASB4*, *ASB9* and *ASB17* mRNA expression is limited exclusively to the testes (Kile et al., 2000, 2001; Kim et al., 2004; Guo et al., 2004). In the present study two transcripts were detected in the ovary, we have confirmed that *ASB9* mRNA was exclusively expressed in both male and female gonads, but the level of expression was highest in GC of OF whereas in testis, only one transcript has been detected (Kile et al., 2000, 2001). Moreover, our results demonstrate that the expression of *ASB9* mRNAs is regulated in a cellular and stimulus specific fashion. This is well illustrated by the LH/hCG induction of *ASB9* in GC of OF when compared to preovulatory GC. The induction of *ASB9* expression can result directly from LH/HCG stimulation or indirectly by other factors induced by LH/hCG. The novel isoform ASB9-2 characterized herein lacks one ankyrin domain when compared to ASB9-1, which results from alternative splicing of *ASB9-1* since only one gene corresponds to *ASB9* in the human and mouse genomes. The biological significance of this difference between male and female gonads as well as the difference of expression between these two ASB9 isoforms during the periovulatory period is unknown.

ASB9-1 and ASB9-2 posseses a SOCS-box that is a well conserved motif localized to the carboxyl-terminal region of proteins that constitute the SOCS protein family member. The conserved SOCS-box suggests that in each protein it plays a similar role. SOCS-box containing proteins possess other domains such as ankyrin repeat for the ASB protein family members, the WD-40-repeat for WD-40-repeat containing proteins (WSB), the SPRY domain for SPRY domain-containing proteins (SSB), and the GTPase domain for the RAR-like GTPases (Nicholson & Hilton, 1998; Hilton et al., 1998; Yasukawa et al., 2000; Kile et al., 2002). Many data have shown that SOCS-box containing proteins can be regulated by cytokines or growth factors (Larsen & Ropke, 2002; Krebs et al., 2000; Yasukawa et al., 2000; Nicholson & Hilton, 1998; Tollet-Egnell et al., 1999). If it is well known that members of the SOCS protein family inhibit the signaling of several cytokine pathways through turning off the receptor–associated tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathways, little is known about the biological function of ASB, WSB and SSB proteins (Nicholson & Hilton, 1998; Liu et al., 2003).

The ASB9-1 and ASB9-2 contain 5 and 4 ankyrin domains respectively. Ankyrin domain is known to mediate protein-protein interactions (Kile et al., 2000; Liu et al., 2003; Hryniewicz-jankowska et al., 2002). For many authors, it seems possible that the ASB proteins will act in a manner similar to the SOCS-box family of proteins, which interact with protein parteners and target them for degradation (Nicholson & Hilton, 1998; Kile et al., 2000). Deletion or overexpression of *ASB1* had no obvious deleterious effects on normal mouse development and hematopoiesis. However, an increased

frequency of testicular anomalies was observed by the loss of *ASB1*. Expression of the others ASB was not altered in *ASB1* knockout mice (Kile et al., 2001). Liu et al. (2003) suggested that ASB8 might interact with target protein by ankyrin repeats and then coupled to Elongin BC-based E3 machinery for ubiquitination. Considering all this information, we hypothezise that ASB9-1 and ASB9-2 may act like other SOCS-box containing proteins by regulating negatively and transiently the JAK/STAT pathway. This fact may be important for the ovulatory process since cytokine concentrations increase in the ovary during the periovulatory period (Kol et al., 1999).

In summary, the KIAA-1798/L3MBTL, trypsin-like inhibitor and ASB9 cDNAs characterized herein were shown to be induced by LH/hCG in GC of bovine OF. They are likely important contributors to the ovulatory and luteinization processes. However, little is known about their biological function since their cDNAs were just recently characterized in other species. However, to elucidate their specific roles in biological processes such as ovulation and luteinization, physiological, developmental and genetic studies are necessary.

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Fig. 1A. Nucleotide and amino acid sequences of bovine KIAA-1798.

The bovine *KIAA-1798* cDNA was cloned by library screening, as described under *Materials and Methods*, and amino acids deduced from cDNA. Nucleotides and amino acids are numbered at the left. The bovine *KIAA-1798* is composed of 3892 bp, a 5'-UTR of 205 bp, an open reading frame (ORF) of 2373 bp and a 3'-UTR of 1314 bp followed by a poly(A)$^+$ tail. Amino acid numbering begins at the first methionine of the ORF, which encodes for 773 amino acids representing a putative protein of 80,500 Mr and a pI of 6.06. In the 3'-UTR region, asterisks represent the stop codon, the three AU-rich motifs (ATTTA) are overlined, and the two polyadenylation signals (AATAAA) are underlined. The poly(A)$^+$ tail is not represented. The nucleotide sequence was submitted to Genbank (Genbank accession number: AY437805).

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   1                                                AGGGGTAGGGGTATCTGTTTGTCCG
  26 TCTGCGCTCTGAGAGACGTGGGGGTAACGAGCCGCCCTCCGGGGTCGCGAAGATCGCGGC
  86 TCTCACCCCACCCTTCCACGGGGAGACGAAGACCTCCAGGAGAAGTCTTGAGGGTGAACC
 146 ACAGTCTTAAAGACCATCAGATAATTATTTCTTCACTGCCACTAGGTTAAAAAATAAATC
 206 ATGACTGAATCTGCCTCTAGCACAAGTGGTCAAGAATTTGATGTATTCAGTGTTATGGAT
   1 MetThrGluSerAlaSerSerThrSerGlyGlnGluPheAspValPheSerValMetAsp
 266 TGGAAGGATGGAGTAGGTACATTACCAGGAAGTGACTTAAAGTTCCGTGTAAATGAGTTT
  21 TrpLysAspGlyValGlyThrLeuProGlySerAspLeuLysPheArgValAsnGluPhe
 326 GGAGCCCTGGAAGTTATTACAGATGAGAGTGAGATGGAAAATGTTAAAAAAGCAACAGCA
  41 GlyAlaLeuGluValIleThrAspGluSerGluMetGluAsnValLysLysAlaThrAla
 386 ACTACAACTTGGATGGTACCAACTGCTCAAGAAGCCCCGACCTCTCCCCCGAGCTCCAGG
  61 ThrThrThrTrpMetValProThrAlaGlnGluAlaProThrSerProProSerSerArg
 446 CCCGTATTTCCACCTGCCTACTGGACATCTCCACCTGGATGTCCCACAGTCTTTTCTGAG
  81 ProValPheProProAlaTyrTrpThrSerProProGlyCysProThrValPheSerGlu
 506 AAGACTGGAATGCCTTTCAGGCTGAAGGACCCAGTGAAAGTAGAAGGGCTTCAATTCTGT
 101 LysThrGlyMetProPheArgLeuLysAspProValLysValGluGlyLeuGlnPheCys
 566 GAGAACTGTTGTCAGTATGGCAACGTAGATGAGTGTCTTTCTGGAGGGAACCACTGCAGC
 121 GluAsnCysCysGlnTyrGlyAsnValAspGluCysLeuSerGlyGlyAsnHisCysSer
 626 CAGAACTGTGCTCGACATATTAAAGACAACAGAGATCAGAAGGAAGAAAGGGACATGGGA
 141 GlnAsnCysAlaArgHisIleLysAspAsnArgAspGlnLysGluGluArgAspMetGly
 686 GAAGACAACGAGGAAGAAGATTCTAAGTGTAGTAGGAAGAAAAAGCCAAAGTTATCTCTG
 161 GluAspAsnGluGluGluAspSerLysCysSerArgLysLysLysProLysLeuSerLeu
 746 AAAGCTGACTCCAAGGAGGAAGAAGAGCGGGATGATGAGATGGAAAACAAACAAGATGGG
 181 LysAlaAspSerLysGluGluGluGluArgAspAspGluMetGluAsnLysGlnAspGly
 806 AGAATCCTGAGGGGTTCACAGAGAGCCCGAAGGAAAAGACGAGGTGATTCGGTTGTATTA
 201 ArgIleLeuArgGlySerGlnArgAlaArgArgLysArgArgGlyAspSerValValLeu
 866 AAGCAGGGTTTGCCTCCTAAAGGAAAGAAAGCTTGGTGCTGGGCATCCTACCTGGAAGAG
 221 LysGlnGlyLeuProProLysGlyLysLysAlaTrpCysTrpAlaSerTyrLeuGluGlu
 926 GAGAAAGCTGTGGCCGTGCCGGCAAAGCTGTTCAAGGAGTATCAGTCTTTTCCATATAAC
 241 GluLysAlaValAlaValProAlaLysLeuPheLysGluTyrGlnSerPheProTyrAsn
 986 AAAAATGGGTTTAAAGTTGGCATGAAACTAGAAGGCGTGGACCCTGAACACCAGTCTGTG
 261 LysAsnGlyPheLysValGlyMetLysLeuGluGlyValAspProGluHisGlnSerVal
1046 TATTGCGTCCTCACTGTGGCTGAGGTTTGTGGATACCGGATAAAGCTGCACTTTGATGGA
 281 TyrCysValLeuThrValAlaGluValCysGlyTyrArgIleLysLeuHisPheAspGly
1106 TACTCTGATTGTTACGATTTCTGGGTAAATGCCGATGCTCTGGATATCCACCCAGTTGGG
 301 TyrSerAspCysTyrAspPheTrpValAsnAlaAspAlaLeuAspIleHisProValGly
1166 TGGTGTGAGAAAACTGGCCATAAACTCCATCCTCCAAAAGGATATAAAGAAGAAGAATTT
 321 TrpCysGluLysThrGlyHisLysLeuHisProProLysGlyTyrLysGluGluGluPhe
1226 AATTGGCAGACCTATCTCAAGACATGCAAAGCTCAGGCTGCTCCCAAGTCATTATTTGAA
 341 AsnTrpGlnThrTyrLeuLysThrCysLysAlaGlnAlaAlaProLysSerLeuPheGlu
1286 AATCAGAATATAACAGTGATCCCATCAGGCTTTCGAGTTGGAATGAAGCTTGAAGCTGTA
 361 AsnGlnAsnIleThrValIleProSerGlyPheArgValGlyMetLysLeuGluAlaVal
1346 GACAAAAAGAACCCTGCATTCATCTGTGTTGCTACGGTAACAGATATGGTGGACAATCGT
 381 AspLysLysAsnProAlaPheIleCysValAlaThrValThrAspMetValAspAsnArg
1406 TTCCTTGTACATTTTGACAACTGGGATGAGAGCTATGACTATTGGTGTGAAGCCTCTAGT
 401 PheLeuValHisPheAspAsnTrpAspGluSerTyrAspTyrTrpCysGluAlaSerSer
1466 CCACACATTCATCCAGTTGGTTGGTGTAAAGAACATCGAAGAACGCTCATTACTCCTCCA
 421 ProHisIleHisProValGlyTrpCysLysGluHisArgArgThrLeuIleThrProPro
1526 GGTTATCCAAATGTGAAACATTTTTCTTGGGATAAATACTTAGAAGAAACCAATTCTTTA
 441 GlyTyrProAsnValLysHisPheSerTrpAspLysTyrLeuGluGluThrAsnSerLeu
```

Figure 1A

```
1586 CCTGCTCCTGCAAGAGCTTTCAAAGTGAAGCCACCACATGGATTCCAGAAAAAAATGAAG
 461 ProAlaProAlaArgAlaPheLysValLysProProHisGlyPheGlnLysLysMetLys
1646 CTTGAGGTCATAGACAAAAGAAATCCCATGTTTATTAGAGTAGCAACAGTTGCAGACACA
 481 LeuGluValIleAspLysArgAsnProMetPheIleArgValAlaThrValAlaAspThr
1706 GATGATCACCGAATAAAAGTTCACTTTGATGGCTGGAATAGTTGCTATGATTACTGGATA
 501 AspAspHisArgIleLysValHisPheAspGlyTrpAsnSerCysTyrAspTyrTrpIle
1766 GATGCAGATTCTCCTGACATTCACCCTGTTGGTTGGTGTTCAAAAACTGGGCATCCTCTT
 521 AspAlaAspSerProAspIleHisProValGlyTrpCysSerLysThrGlyHisProLeu
1826 CAACCTCCTTTGAGCCCCTTAGAACTGATGGAAGCCTCAGAACACGGTGGCTGCACAACC
 541 GlnProProLeuSerProLeuGluLeuMetGluAlaSerGluHisGlyGlyCysThrThr
1886 CCAGGATGTAAAGGGATTGGCCATTTTAAGAGAGCGAGACACCTGGGCCCTCACAGTGCT
 561 ProGlyCysLysGlyIleGlyHisPheLysArgAlaArgHisLeuGlyProHisSerAla
1946 GCCAACTGTCCCTATTCAGAAATCAATTTGAATAAAGACCGAATTTTCCCAGATCGTTTA
 581 AlaAsnCysProTyrSerGluIleAsnLeuAsnLysAspArgIlePheProAspArgLeu
2006 AGTGGTGAAATGCCTCCAGCTAGTCCATCATTTCCAAGAAGTAAAAGAGCCGACACAAAT
 601 SerGlyGluMetProProAlaSerProSerPheProArgSerLysArgAlaAspThrAsn
2066 GAAATCTCTTCGTCTCCTGAAACCAGAGACCAGCGTGCTGAGGATGTCAAAGAAGACTTT
 621 GluIleSerSerSerProGluThrArgAspGlnArgAlaGluAspValLysGluAspPhe
2126 GAAGAGAGAACAGAGAGTGAGCTGAGGACACCACATGAAGCCAGAGGTGCCCCGGGAAGAA
 641 GluGluArgThrGluSerGluLeuArgThrProHisGluAlaArgGlyAlaArgGluGlu
2186 AGCAGCGTACAGCAGGCGCAGCGTCGGTCGGCCGTCTTTCTGTCCTTTAAGTCCCCAATT
 661 SerSerValGlnGlnAlaGlnArgArgSerAlaValPheLeuSerPheLysSerProIle
2246 CCATGTCTGCCCCTGCGCTGGGAGCAGCAGAGCAAACTTCTCCCCACAGTGGCTGGCATT
 681 ProCysLeuProLeuArgTrpGluGlnGlnSerLysLeuLeuProThrValAlaGlyIle
2306 CCTGCCAGTAAAGTTTCTAAATGGAGTACGGATGAGGTGTCAGAATTCATACAGAGCTTA
 701 ProAlaSerLysValSerLysTrpSerThrAspGluValSerGluPheIleGlnSerLeu
2366 CCTGGATGTGAAGAACATGGAAAGGTATTTAAAGATGAACAAATTGATGGAGAGGCATTT
 721 ProGlyCysGluGluHisGlyLysValPheLysAspGluGlnIleAspGlyGluAlaPhe
2426 CTGCTTATGACCCAAACAGACATCGTGAAAATTATGAGCATTAAGTTGGGCCCAGCGCTC
 741 LeuLeuMetThrGlnThrAspIleValLysIleMetSerIleLysLeuGlyProAlaLeu
2486 AAAATTTTCAATTCTATCTTGATGTTCAAAGCGGCAGAGTAAGAACTTTTGGCATTTTGT
 761 LysIlePheAsnSerIleLeuMetPheLysAlaAlaGlu***
2546 AAACATTGTTGAATTCTCTTTCATGTACACTGTTTCTTTTTCAATACTTTCAGGAACCTT
2606 TTTTATATAATCAAACTTCAACTTAAACCAAATTAGTCAAAACCTGGCTAAGTGTAGCCA
2666 GTGGGACTGTTACCTGTATTGTTAATTCTGTGCCATATTTGAATTGCAACATTATGCTAA
2726 GTATAACTTTGCAAGTCATGATATTGCCTAACTGCTTGTCCTGATTCATTGAGGCTGAAT
2786 AGTTGTAAAAATTACCTTCCTTTAAATCAGTGTTTTTACTTTTGCACGCAATTATGAAAT
2846 GTTAAACAAATTACTTTCACCAGCATCTTGACTATAATTACCAAAAACGTGTATATAAAT
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2966 AGTCTCATGCATTGTGGTACCCTGTGTTTACAGGATTCCAGTGATATCAAAGGAATACAC
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3086 GCTTTTTCTGTAATTGTCATTGAAATAACTGTGGTGCTTTGCAGAGGTGAAGGGATCGTT
3146 CTGTCGATGTAAACACTAATCCCAAAAGAGCTCTGTCTTAGACCTTGCAGTGTCCTCCAA
3206 ACCCAAAGGTTTTCTTCAGTGTTGGCTAAAGAGAGGGTGTATAGATTTTACTGAAGAGGC
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3326 TGACTTCCTAAAGCTGCATTTTGGAGAGACCCTCTTCTCCATAGAAGTGTTTGCCTCAGG
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3446 GTAGTGTTTCTTTTGCAAGAATGCATTTCCAACGCAAAAGGTAAATTATTTTATCAGCCT
3506 ATGTATGTAACTTCTATTTCCAGGATTGGATATTTATCCAAGGACTATTTTAAAAGTAGA
3566 AAGTAAGTTTGTAGCATGTTGTCTGGATTCTGGGGAAAGTTTCACTTTCTCTGTAGTTGC
3626 TCTCATTTGTTCTCATGTAGGATTCCCTTGCATCTGCCCTGCCCCACACACCCTGTCGGA
3686 GTCTGTCGTGGTCCTTTGCTTTTGTTGTAGTTCAAGGTAAAACAGGGCCCATGAAATCTG
3746 CCATGGCCCCTTCGTTTCTTTGCATGAGTCTGCTTAAAAAAAGTTTTTCGTTGTTAGTTT
3806 TTCTTTTCTTTATGTTTTGTTCAGAGTTTGTACGTTCAAGTTGCACTTGTTATACCTGAC
3866 ATGAATAAAATAAAATCTTAATTGCAG
```

Figure 1A (continued)

Fig. 1B. Predicted amino acid sequence of bovine KIAA-1798 and comparison with other mammalian orthologs.

The amino acid sequence of bovine KIAA-1798 (GenBank: AY437805) is aligned with the human ortholog (BAB47427; NP_115814). Dots represent identical amino acid residues and hyphens indicate gaps in protein sequences created to optimize alignment. The bovine KIAA-1798 protein is 96% identical to human KIAA-1798 also called lethal (3) malignant brain tumor-like 3 proteins (LMBL3; GenBank: NP_115814). The three malignant brain tumor (MBT) repeat domains are overlined and the sterile alpha motif (SAM) is boxed. Amino acid are numbered at the left.

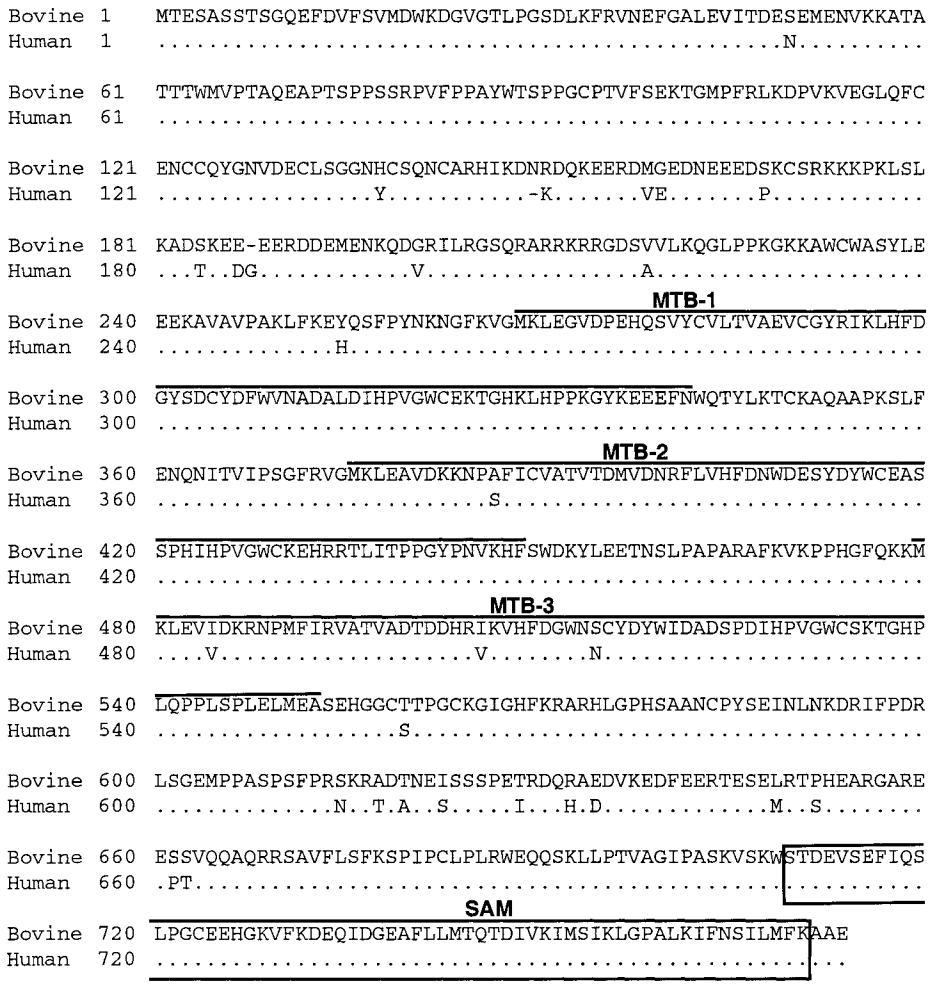

Figure 1B

Fig. 2A. Nucleotide and amino acid sequences of bovine trypsin-like inhibitor.

The bovine *trypsin-like inhibitor* cDNA was cloned by library screening, as described under *Materials and Methods*, and amino acids deduced from cDNA. Nucleotides and amino acids are numbered at the left. The bovine *trypsin-like inhibitor* is composed of 4020 bp, a 5'-UTR of 210 bp, an open reading frame (ORF) of 1488 bp and a 3'-UTR of 2322 bp followed by a poly(A)$^+$ tail. Amino acid numbering begins at the first methionine of the ORF, which encodes for 496 amino acids representing a putative protein of 55,600 Mr and a pI of 8.2. In the 3'-UTR region, asterisks represent the stop codon, the six AU-rich motifs (ATTTA) are overlined, and the two polyadenylation signals are underlined. The poly(A)$^+$ tail is not represented. The nucleotide sequence was submitted to Genbank (Genbank accession number: AY369781).

```
   1                                  GACTGCATCCTTCGGCGGCCGGAGAGTTCC
  31 TCATCCCGCGATCTGCCGGCTGTGTCTGCTGTGCTTGCGCTGCTGTCGCTGCCACTCCGC
  91 GCAGGAGACGCCGGCAAGGAGGTGGTTGAGCCCTGCGGAGGGAGAGCACGAGATCGCCCA
 151 GCTCTGCCTCAGGCGCCCAGGCCGCCCGTCATTCCCTGCCCTCGTTGCCGGCGTGGAGCC
 211 ATGAGCTGTGTCCTGACTGGCGTCGTGCCCTTGGGGCTGGTGCTGCTGCTCTGCGGAGCC
   1 MetSerCysValLeuThrGlyValValProLeuGlyLeuValLeuLeuLeuCysGlyAla
 271 CAAGGCTTTTTCCTGCCCAATGTCACACAGTTAGAGAAGCTGCTCAGCAAATACCAGGGG
  21 GlnGlyPhePheLeuProAsnValThrGlnLeuGluLysLeuLeuSerLysTyrGlnGly
 331 GACCAGCCCCACTCACGGACTCGGAGGGCCATCTCCAGGGCGGACCGGGAGGAGATCCTC
  41 AspGlnProHisSerArgThrArgArgAlaIleSerArgAlaAspArgGluGluIleLeu
 391 ACACTTCACAACAAGCTGCGGGGCCAGGTCTCGCCTCCCGCCTCCAACATGGAGTACATG
  61 ThrLeuHisAsnLysLeuArgGlyGlnValSerProProAlaSerAsnMetGluTyrMet
 451 ACCTGGGATGAAGAGCTGGAGAAGTCAGCGGTGGCCTGGGCCCGGGAATGCATCTGGGAG
  81 ThrTrpAspGluGluLeuGluLysSerAlaValAlaTrpAlaArgGluCysIleTrpGlu
 511 CATGGGCCCACCAGCCTGCTGGTGTCCATCGGGCAGAACCTGGCCGTGCACTGGGGCAGG
 101 HisGlyProThrSerLeuLeuValSerIleGlyGlnAsnLeuAlaValHisTrpGlyArg
 571 CCTCGTTCCCCTGGCTCCCATGTGCAGTCCTGGTATGATGAGGTGAAGGACTACACCTAC
 121 ProArgSerProGlySerHisValGlnSerTrpTyrAspGluValLysAspTyrThrTyr
 631 CCCTACCCCCACGAATGCAACCCTTGGTGTCCGGAGAGGTGCTCCGGGCCCATGTGCACC
 141 ProTyrProHisGluCysAsnProTrpCysProGluArgCysSerGlyProMetCysThr
 691 CACTACACCCAGATAGTTTGGGCCACAACCAACAGGATCGGCTGTGCCGTGAACACCTGC
 161 HisTyrThrGlnIleValTrpAlaThrThrAsnArgIleGlyCysAlaValAsnThrCys
 751 CCGAGGATGAATGTCTGGGGAGATGTTTGGGAGAATGCGGTCTACCTCGTCTGCAATTAT
 181 ProArgMetAsnValTrpGlyAspValTrpGluAsnAlaValTyrLeuValCysAsnTyr
 811 TTTCCAAAGGGCAACTGGATCGGAGAAGCCCCCTACAAAACCGGCCAGCCTTGCTCCGAG
 201 PheProLysGlyAsnTrpIleGlyGluAlaProTyrLysThrGlyGlnProCysSerGlu
 871 TGCCCGGGCAAGTACCGCGGTGGCTGCAAGAACAACCTCTGTTACCAAGAGACTTATGGC
 221 CysProGlyLysTyrArgGlyGlyCysLysAsnAsnLeuCysTyrGlnGluThrTyrGly
 931 CAGGAAACTGAAACGGACGACATGAACGAGGTGGAAGCGGCCCCTATTCCGGATGAGAAG
 241 GlnGluThrGluThrAspAspMetAsnGluValGluAlaAlaProIleProAspGluLys
 991 CACGTCTGGGTCACCCCGAGGGTGATCAAACCCAAGAAGCCCAAGAAAGACTCTCCTGTG
 261 HisValTrpValThrProArgValIleLysProLysLysProLysLysAspSerProVal
1051 AACTACATGACCCAAGTCGTCAAGTGTGACACCAAGATGAAGGACAAGTGTAAAGGGTCC
 281 AsnTyrMetThrGlnValValLysCysAspThrLysMetLysAspLysCysLysGlySer
1111 ACGTGCAACAGGTACCAGTGCCCCGCAGGCTGCCTGCACAGTGGAGCAAAGATCTTTGGG
 301 ThrCysAsnArgTyrGlnCysProAlaGlyCysLeuHisSerGlyAlaLysIlePheGly
1171 ACTCTCTTTTATGAAAGCGCGTCCAGCATCTGCCGGGCCGCCATTCACTATGGGATCCTG
 321 ThrLeuPheTyrGluSerAlaSerSerIleCysArgAlaAlaIleHisTyrGlyIleLeu
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1291 TCAGAGAGAAACGGCGTGCAGTCTCTGAGCAAATACAAGGCTTCCAGCTCATTCACGGTG
 361 SerGluArgAsnGlyValGlnSerLeuSerLysTyrLysAlaSerSerSerPheThrVal
1351 TCAAAAGTAAAAGTGCAAGACCTGGACTGCTACACCACCGTGGCCCAGCTCTGCCCGTAC
 381 SerLysValLysValGlnAspLeuAspCysTyrThrThrValAlaGlnLeuCysProTyr
1411 GAGAAGCCGGGGACACACTGCCCAAGAGTTCGCTGTCCAGCGCACTGCAAAGACGAGCCA
 401 GluLysProGlyThrHisCysProArgValArgCysProAlaHisCysLysAspGluPro
1471 TCCTACTGGGCTCCCGTGTTTGGAAGCAACATCTATGCAGACACTTCCAGCATCTGTAAG
 421 SerTyrTrpAlaProValPheGlySerAsnIleTyrAlaAspThrSerSerIleCysLys
1531 ACTGCCGTGCACGCAGGGGTCATCCGGAACGAGAGTGGGGGCTACGTGGACGTGATGCCC
 441 ThrAlaValHisAlaGlyValIleArgAsnGluSerGlyGlyTyrValAspValMetPro
1591 GTGGATAAAAAGAAGACCTACGTGGCCTCGCTCAGGAATGGAGTCCAGTCTGAAAGCTTG
 461 ValAspLysLysLysThrTyrValAlaSerLeuArgAsnGlyValGlnSerGluSerLeu
1651 AGGACCCCTAGAGACGGAAAGGCCTTCCGCATCTTTGCCGTCAGGCAGTGAACTTGCAGG
 481 ArgThrProArgAspGlyLysAlaPheArgIlePheAlaValArgGln***
```

Figure 2A

1711 GCTGGGGAGAAGGGCGCGTCCTCAAGACGGCTTCAGGGGTTCTCTTTTTATTTTTATTTT
1771 GTCATGGGGCGGGGTGGGCCGGGAATGTGGGGAGTCGGGAAGCTTCCTTTGATTGGCATC
1831 CAGCGTCCCATCTGCCGTGCCTTGGGCTCAGCTCCTCGCACCTGGAAGAGAGAGCATCCC
1891 TGGGGTCCTGAGCAGACAGCTGGTGATCATCTTGCCGAGGCCCGGGGGTCTCCCCCGCAGA
1951 GGCGTCCTTCTATGGACGGAGAGAGGATTCCTACCGGAGGAGAGAATGAAGGATTTGGAG
2011 CTGATACCGCGAGGCACTGGGGCCATGGGAAGAGGCGCACGGAGAACACTCGGGGGGCGG
2071 GGGAGTTTCAGGGATGGAGTAGAGGTAGCTATTTAAAACATTCAGAAACACAATCTGTCC
2131 CTACCAATGGTGAAAATGGGATTAATGTTTGCTGGTCAGACAAACGGGCTGGTCTGGGGG
2191 ACTCTGCCCTGGCGCCTGTCCTGCTCCCGTCCTGCTGCATGCGGGCTGGTTCTATACACA
2251 GCAGTGCTGGTTGACTACAAGTTCAGCCCTAATTCCAGAAGATGTGTCTTGTCTTGGTCC
2311 TTCCCTACGTCCACGTGGCCTGTCTGTCTGCTCGACCTTTGGTCTCATTGAGGACTAACA
2371 AACCAGGACCCTTCTTTGATCCCCTCCTCACTGCGGCTCACACTCCACCCAGACTAGTTT
2431 TTTTGTCTCCCACCAGGGTCTTTGCTTAGTAAGTGATTCTGTATGAAGCCCACCTTTTAA
2491 TTTCAACCCCTCGTTCTCTGTGGGTCCCACCAAGATGTCTAAAAACAGCCAAAGAAGGGC
2551 TGTTTAGTTGCTGCCTTTAAAATGGCACTGAAGTGTGCAGATTCCCCATCCGTCAGATGA
2611 CCTATTTTTCTGCAGTGGGAGGAATGGCTTCATTCTTCAGTACATTCCTCGCCGAGGTTA
2671 GCAGCTCTGGAAGGAGACACAGCCTGTGGTTATGAAACCATCTTGCAGCATAATAACAAG
2731 AGATGGTAACGCGCGGGGGATTTCTCTTCCTGTTTTTGTGGAACGGTTCTTGCCAAATGT
2791 TTCTGAGGCTCCGAGGAGCGTGGTGCTCTCTGGCTCCCATTGCCATATAAAACTTCATAA
2851 GCCTCGACCCGAACCCACAGTGAAATAAAAAACCCTTCTGTAAATAGTGTTTCTTTGCAG
2911 AAGGTAAAATTCCACCTTCCACCACCAGACTGGCCAAAAGATCACTTTCGTGAATGCCAG
2971 TTTCAAAGTTGATTTCACCCTCAATCAGCATATTTCGAAGGCAAAAGAGCTAGCAAAAAG
3031 TTAAAAAGAAAAAACTAAGATAGTCCGGACAAGGAACTCTACCTGAGGAACCAAGCTGGT
3091 TGCTTCCGTTAGTGTATCCGTAATTAGAGCAGAAACAGAAGTTAAATACTATTCCCAGCT
3151 AGAGGGAGTTTGTAGAAGTTTGTTAACCTTGACAGAGCTGAAATGAGCAGGACTGTTTTT
3211 GCTTTGCACACTATGGGCTTGTTATAATTCATAAGTACTGACTGTCCGACTCCAAATGTC
3271 AGAGTGGAAGAATTTGGTCAGACTGTCAGATACTCATCTTGTCCCTGCCAAAGATCTGAG
3331 TCCTTGGATCAGATGCTAGCTAGGTATTACACTGTTGGGCTTCTGCTTGTCACCAGGCTT
3391 TGGTGGCTGTGACCATGTCCAAACTCTGAGTTTGGGTTTTCAGCATTAGTGACAGTACAG
3451 GGGAATTTCAGGTTTGCTTTTGAAGCATTCTGCCCGAGGGAGTCTGAAACATCCAATAGG
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3571 GGACTAGAAACTCCTTGCCCAGAACCAGGTCTTGCTATCTCTTTCACTCTCACAAACATT
3631 TCGAAACTGGAACCATTTATCTTCCAGAAATGAACACAGGGCTTCAAAGGAGAAGCTCTC
3691 CTACTAATCAGGGACCTCCTTCTCATTTAATGTTTAACAGTGCTTACGGCGCCTTCAGCA
3751 CCTCACCCATAAGATGATAAATCTGGCCGACAGTGTTACTGGGACCCCCAAAATAGAAAG
3811 TTTTGGTTTCCTTCCGGTTTTTCCCGTCATGTTCTGGTTTTTCATACTGTCTGTAGGCTC
3871 ACTCAGCCCGCAGCTTACATGTGTGCTTTTTTCTATGAAAAATGATGTATTTTACTACTT
3931 TCTATGTACAAAAATTTATTGTAAATTTATGTGTGCTTCGCTTGAACCGGGACCACGTTG
3991 CTGTCATTTCAATAAAACTGATTTTATTTC

Figure 2A (continued)

Fig. 2B. Predicted amino acid sequence of bovine trypsin-like inhibitor and comparison with other mammalian orthologs.

The amino acid sequence of bovine trypsin-like inhibitor (GenBank: AY369781) is aligned to human (AAQ89150), rat (XP_346524) and mouse (NP_084485) orthologs. Dots represent identical amino acid residues and hyphens indicate gaps in protein sequences created to optimize alignment. The bovine trypsin-like inhibitor is 83% identical to human, 78% to rat and 76% to mouse proteins. The amino terminal arrow indicates the cleavage site of the hydrophobic signal peptide. The SCP-like domain is boxed and the two LCCL domains are overlined. Amino acid are numbered at the left.

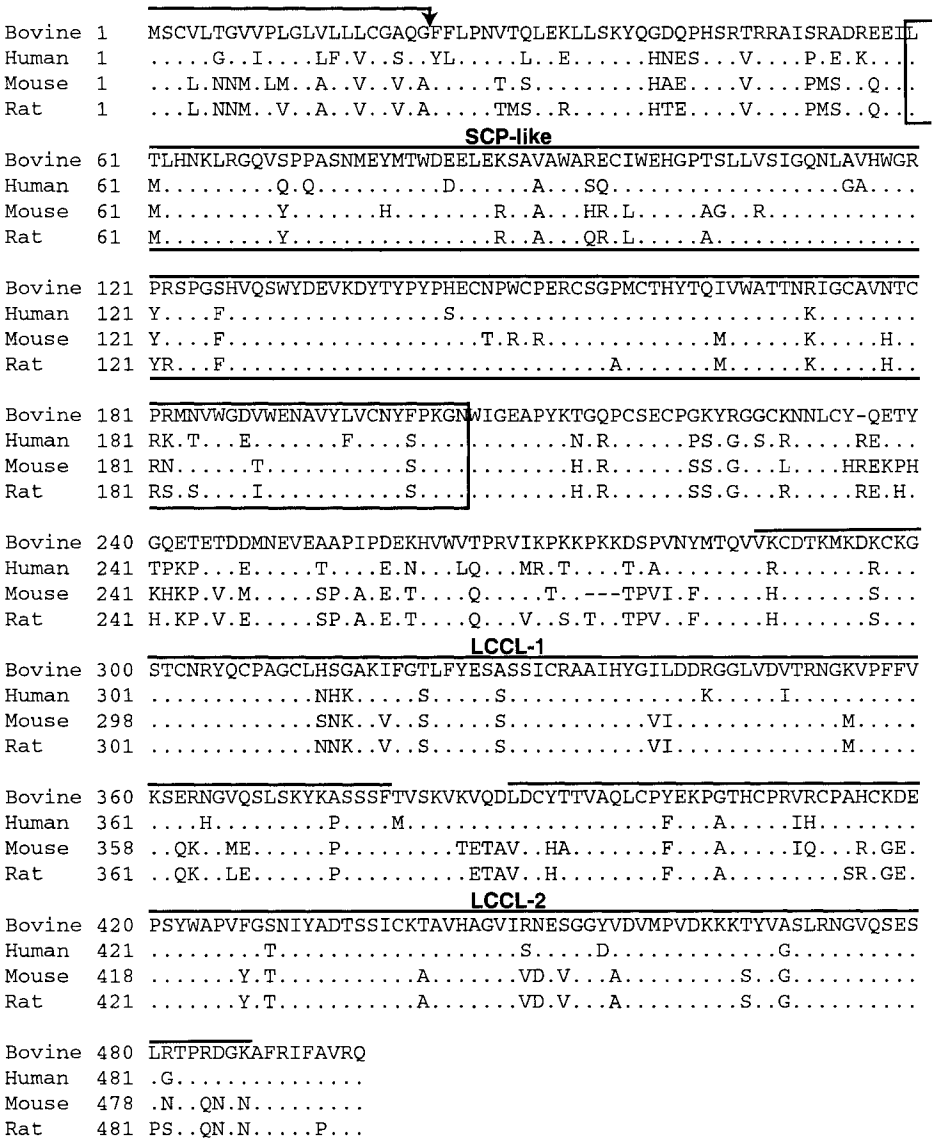

Figure 2B

Fig. 3A. Nucleotide and amino acid sequences of bovine ASB9 isoform 1.

The bovine *ASB9-1* cDNA was cloned by library screening, as described under *Materials and Methods*, and amino acids deduced from cDNA. Nucleotides and amino acids are numbered at the left. The bovine *ASB9-1* is composed of 1604 bp, a 5'-UTR of 241 bp, an open reading frame (ORF) of 762 bp and a 3'-UTR of 562 bp followed by a poly(A)$^+$ tail. Amino acid numbering begins at the first methionine of the ORF, which encodes for 287 amino acids representing a putative protein of 31,400 Mr and a pI of 6.8. In the 3'-UTR region, asterisks represent the stop codon, and the polyadenylation signal (ATTAAA) is underlined. The poly(A)$^+$ tail is not represented. The nucleotide sequence was submitted to Genbank (Genbank accession number: AY438595).

```
   1                                                                   A
   2 GATCGTGCGTGTGTGCGTGTGTGTGTATGTGTGTGTGTGTGTCCCCGTGTGCGCGTGTCT
  62 GTTTGACCACAGGTAGTACTCAGATTACCCTGCCTGTCCTGCTCTCTGCCATCTGACCAG
 122 GAGGGTGCCGCTCAGCCACTCACAGCCCTGGAATTTCCCAGGACCCGGGGGAGCATGGAA
 182 TTTGGACTTTCCTGAACAGCTGAAGTGGAGTGTGGAGCTTGCCCAGCCCAGGAGAGCACC
 242 ATGGATGGTGAACGACCAGGCAGAAACGGCAGCAAGCCCCCGGACATGAGGCTCTTTTCC
   1 MetAspGlyGluArgProGlyArgAsnGlySerLysProProAspMetArgLeuPheSer
 302 AACCCATTGATGGGGGACTTTGTATCTGATTGGTCTCCTGTGCATGAAGCAGCCATTCAT
  21 AsnProLeuMetGlyAspPheValSerAspTrpSerProValHisGluAlaAlaIleHis
 362 GGACGTCTGCTGTCTTTGAGGAGCCTCATCAACCAGGGGTGGCCGGTGAACCTCATCACT
  41 GlyArgLeuLeuSerLeuArgSerLeuIleAsnGlnGlyTrpProValAsnLeuIleThr
 422 GCAGATCGTGTGTCTCCACTCCACGAAGCCTGTCTTGGAGGTCATCCCTCCTGTGTAAAA
  61 AlaAspArgValSerProLeuHisGluAlaCysLeuGlyGlyHisProSerCysValLys
 482 ATATTACTGAGACATGGAGCTAATGTGAACAGTGTCACTGTAGACTGGCACACTCCCTTG
  81 IleLeuLeuArgHisGlyAlaAsnValAsnSerValThrValAspTrpHisThrProLeu
 542 TTTAATGCTTGTGTCAGCGGCAGCCACGAGTGTGTGAATTTGCTTCTGCAATATGGAGCC
 101 PheAsnAlaCysValSerGlySerHisGluCysValAsnLeuLeuLeuGlnTyrGlyAla
 602 AGTCCCCACCCGGAGAATGACCTGGCGTCCCCCATCCACGAAGCTGCTAAGAGAGGCCAC
 121 SerProHisProGluAsnAspLeuAlaSerProIleHisGluAlaAlaLysArgGlyHis
 662 ATGCAGTGCATCGAGTCTCTTGTGGCTCACGGGGGCGACATTGACCAAAACATCCACCAC
 141 MetGlnCysIleGluSerLeuValAlaHisGlyGlyAspIleAspGlnAsnIleHisHis
 722 CTGGGAACACCGTTATATATGGCTTGTGAAAACCTGCAGGTGGCCTGTGCCAAGAAACTT
 161 LeuGlyThrProLeuTyrMetAlaCysGluAsnLeuGlnValAlaCysAlaLysLysLeu
 782 CTGGAGTCAGGAGTGAATGTGAACCAGGGCCGGGGGCTGGACTCCCCCCTTCACGCAGTG
 181 LeuGluSerGlyValAsnValAsnGlnGlyArgGlyLeuAspSerProLeuHisAlaVal
 842 GCCAGGGCATCTAGCGGGGAGCTGGTCAGTCTGCTCCTGGACTTTGGAGCAGACACCCAG
 201 AlaArgAlaSerSerGlyGluLeuValSerLeuLeuLeuAspPheGlyAlaAspThrGln
 902 GCCAGGAACGCTGAAGGCAAACGACCCTTGGAAGTGGTGCCTCCAGAGAGCCCCCTGATC
 221 AlaArgAsnAlaGluGlyLysArgProLeuGluValValProProGluSerProLeuIle
 962 CAGCTCTTGCAGAGAGAAGGGCCCTCTTCTTTGATGCAGTTATGCCGCCTTAGAATTCGG
 241 GlnLeuLeuGlnArgGluGlyProSerSerLeuMetGlnLeuCysArgLeuArgIleArg
1022 AAGTGCTTTGGGATCAAGCAGCATCATAAGATCACTGAGCTCAACCTCCCTGAGGAGCTG
 261 LysCysPheGlyIleLysGlnHisHisLysIleThrGluLeuAsnLeuProGluGluLeu
1082 AAACGGTTTCTCCTCCACATTTAAATGTGTCAAGCGAATGGCGACACAGACAACAGACAA
 281 LysArgPheLeuLeuHisIle***
1142 ATGTTATTGAGTGTTGAGACCACTGGGATTTTCAAGTTAAGTCAGGTTTATAGAGTTCAG
1202 CTAAGTTTTTGTTGTTTGCAGTGAGACGTTTATTGTAGCTTCGTACTAGGTTCTTTTGCG
1262 CTGTTGGTTTGGAGGGTATGAAAAATTATCTCCCCTGCCTGGAAGAGGGTGGCTAAGATA
1322 TCCATGGTGTTGAATATCTTACCCAGCACTGAGCTGGGAACCCTTTATGCTTTGTCTAAT
1382 TTAGTCCCACTCTTCCTCCCCTCTTCCCTAGGCGATACCCTAAGACTTGGGGAGGTCAGT
1442 TTGTTTAGTTACCCACAGTGAGGCTAGGCAGCCAGATTTAAGTGTAGGTTTTCTGATGCT
1502 AACCTGGGGGCTTGTGGGGAGCAGAATTTTCCACTATCGCTATCCTGGCTCTGTCTCCAC
1562 TTCGTGGGGAAAAAAGATTAAAGCAGTGTGACATTTCTGTGAG
```

Figure 3A

Fig. 3B. Nucleotide and amino acid sequences of bovine ASB9 isoform 2.

The bovine *ASB9-2* cDNA was cloned by library screening, as described under *Materials and Methods*, and amino acids deduced from cDNA. Nucleotides and amino acids are numbered at the left. The bovine *ASB9-2* is composed of 1435 bp, a 5'-UTR of 261 bp, an open reading frame (ORF) of 672 bp and a 3'-UTR of 502 bp followed by a poly(A)$^+$ tail. Amino acid numbering begins at the first methionine of the ORF, which encodes for 224 amino acids representing a putative protein of 24,700 Mr and a pI of 7.2. In the 3'-UTR region, asterisks represent the stop codon, and the polyadenylation signal (ATTAAA) is underlined. The poly(A)$^+$ tail is not represented. The nucleotide sequence was submitted to Genbank (Genbank accession number: AY442176).

```
   1                                                  CAGCCTCCAGCCTGTGAGGTA
  22  GATCGTGCGTGTGTGCGTGTGTGTGTATGTGTGTGTGTGTCCCCGTGTGCGCGTGTCT
  82  GTTTGACCACAGGTAGTACTCAGATTACCCTGCCTGTCCTGTTCTCTGCCATCTGACCAG
 142  GAGGGTGCCGCTCAGCCACTCACAGCCCTGGAATTTCCCAGGACCCGGGGGAGCATGGAA
 202  TTTGGACTTTCCTGAACAGCTGAAGTGGAGTGTGGAGCTTGCCCAGCCCAGGAGAGCACC
 262  ATGGATGGTGAACGACCAGGCAGAAACGGCAGCAAGCCCCCGGACATGAGGCTCTTTTCC
   1  MetAspGlyGluArgProGlyArgAsnGlySerLysProProAspMetArgLeuPheSer
 322  AACCCATTGATGGGGGACTTTGTATCTGATTGGTCTCCTGTGCATGAAGCAGCCATTCAT
  21  AsnProLeuMetGlyAspPheValSerAspTrpSerProValHisGluAlaAlaIleHis
 382  GGACGTCTGCTGTCTTTGAGGAGCCTCATCAACCAGGGGTGGCCGGTGAACCTCATCACT
  41  GlyArgLeuLeuSerLeuArgSerLeuIleAsnGlnGlyTrpProValAsnLeuIleThr
 442  GCAGATCGTGTGTCTCCACTCCACGAAGCCTGTCTTGGAGGTCATCCCTCCTGTGTAAAA
  61  AlaAspArgValSerProLeuHisGluAlaCysLeuGlyGlyHisProSerCysValLys
 502  ATATTACTGAGACATGGAGCTAATGTGAACAGTGTCACTGTAGACTGGCACACTCCCTTG
  81  IleLeuLeuArgHisGlyAlaAsnValAsnSerValThrValAspTrpHisThrProLeu
 562  TTTAATGCTTGTGTCAGCGGCAGCCACGAGTGTGTGAATTTGCTTCTGCAATATGGAGCC
 101  PheAsnAlaCysValSerGlySerHisGluCysValAsnLeuLeuLeuGlnTyrGlyAla
 622  AGTCCCCACCCGGAGAATGACCTGGCGTCCCCCATCCACGAAGCTGCTAAGAGAGGCCAC
 121  SerProHisProGluAsnAspLeuAlaSerProIleHisGluAlaAlaLysArgGlyHis
 682  ATGCAGTGCATCGAGTCTCTTGTGGCTCACGGGGGCGACATTGACCAAAACATCCACCAC
 141  MetGlnCysIleGluSerLeuValAlaHisGlyGlyAspIleAspGlnAsnIleHisHis
 742  CTGGGAACACCGTTATATATGGCTTGTGAAAACCTGCAGGTGGCCTGTGCCAAGAAACTT
 161  LeuGlyThrProLeuTyrMetAlaCysGluAsnLeuGlnValAlaCysAlaLysLysLeu
 802  CTGGAGTCAGGGCCCTCTTCTTTGATGCAGTTATGCCGCCTTAGAATTCGGAAGTGCTTT
 181  LeuGluSerGlyProSerSerLeuMetGlnLeuCysArgLeuArgIleArgLysCysPhe
 862  GGGATCAAGCAGCATCATAAGATCACTGAGCTCAACCTCCCTGAGGAGCTGAAACGGTTT
 201  GlyIleLysGlnHisHisLysIleThrGluLeuAsnLeuProGluGluLeuLysArgPhe
 922  CTCCTCCACATTTAAATGTGTCAAGCGAATGGCGACACAGACAACAGACAAATGTTATTG
 221  LeuLeuHisIle***
 982  AGTGTTGAGACCACTGGGATTTTCAAGTTAAGTCAGGTTTATAGAGTTCAGCTAAGTTTT
1042  TGTTGTTTGCAGTGAGACGTTTATTGTAGCTTCGTACTAGGTTCTTTTGCGCTGTTGGTT
1102  TGGAGGGTATGAAAAATTATCTCCCCTGCCTGGAAGAGGGTGGCTAAGATATCCATGGTG
1162  TTGAATATCTTACCCAGCACTGAGCTGGGAACCCTTTATGCTTTGTCTAATTTAGTCCCA
1222  CTCTTCCTCCCCCTCTTCCCTAGGCGATACCCTAAGACTTGGGGAGGTCAGTTTGTTTAGT
1282  TACCCACAGTGAGGCTAGGCAGCCAGATTTAAGTGTAGGTTTTCTGATGCTAACCTGGGG
1342  GCTTGTGGGGAGCAGAATTTTCCACTATCGCTATCCTGGCTCTGTCTCCACTTCGTGGGG
1402  AAAAAAGATTAAAGCAGTGTGACATTTCTGTGAC
```

Figure 3B

Fig. 3C. Predicted amino acid sequence of bovine ASB9 isoforms 1 and 2 compared with other mammalian orthologs.

The amino acid sequence of bovine ASB9-1 (GenBank: AY438595) and ASB9-2 (GenBank: AY442176) are aligned to human (BC013172), mouse (NM_027027) and rat (XM_346301) proteins. Dots represent identical amino acid residues and hyphens indicate gaps in protein sequences created to optimize alignment. The bovine ASB9-1 is 75% identical to human, 73% to mouse and 74% to rat proteins. The six ankyrin domains (ANK) of ASB9-1 are overlined and the SOCS-box located at the C-terminus is boxed. Amino acid are numbered at the left.

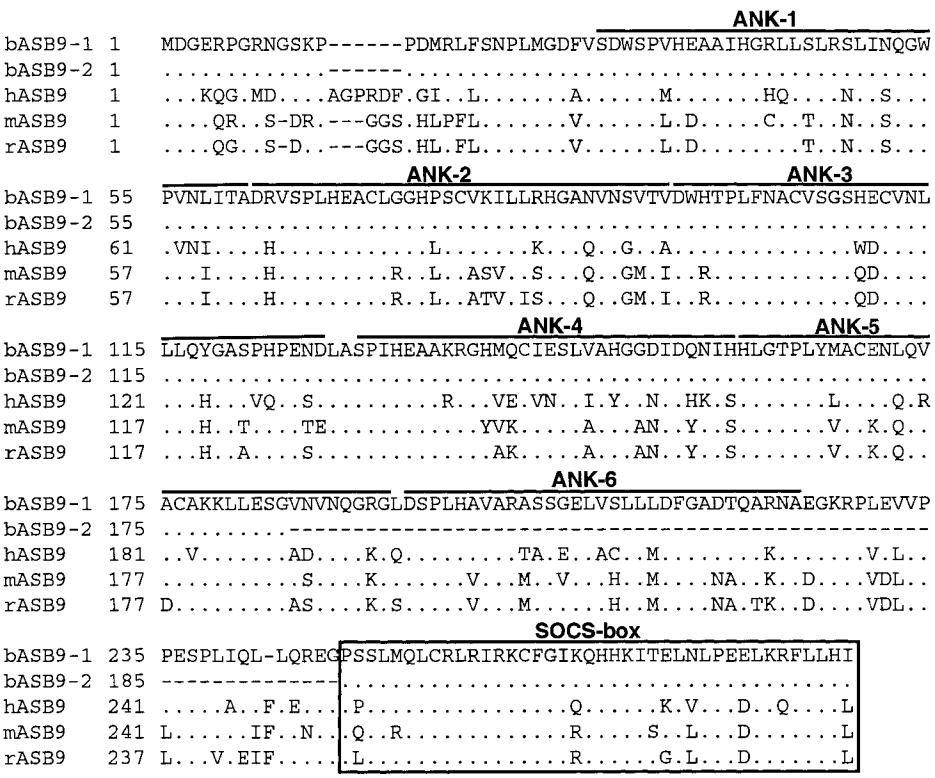

Figure 3C

Fig. 4. Expression of KIAA-1798, trypsin-like inhibitor and ASB9 transcripts in bovine granulosa cells and CL.

Total RNA was extracted from bovine granulosa cells collected from 2-4 mm follicles (SF), dominant follicles (DF) at day 5 of the estrous cycle, ovulatory follicles (OF) 24 h after injection of hCG (OF) and corpora lutea (CL) from day 5 of the estrous cycle, then used in mRNA expression analyses with semiquantitative RT-PCR as described under *Materials and Methods*. The control gene was *GAPD* (710 bp); it showed no significant difference in mRNA expression levels between groups. **A**, the *KIAA-1798* (800 bp) **B**, *trypsin-like inhibitor* (420 bp) and **C**, ASB9 isoforms (649 bp and 500 bp) PCR fragments were normalized with their corresponding *GAPD* signal, and the results are presented as a relative change in ratio between groups. Different letters denote samples that differed significantly ($P < 0.05$) when Tukey-Kramer multiple comparison tests were performed to compare group means for a specific gene. Data are presented as least-square means ± SEM, and the number of independent samples, *i.e.* animals, per group is indicated in parenthesis.

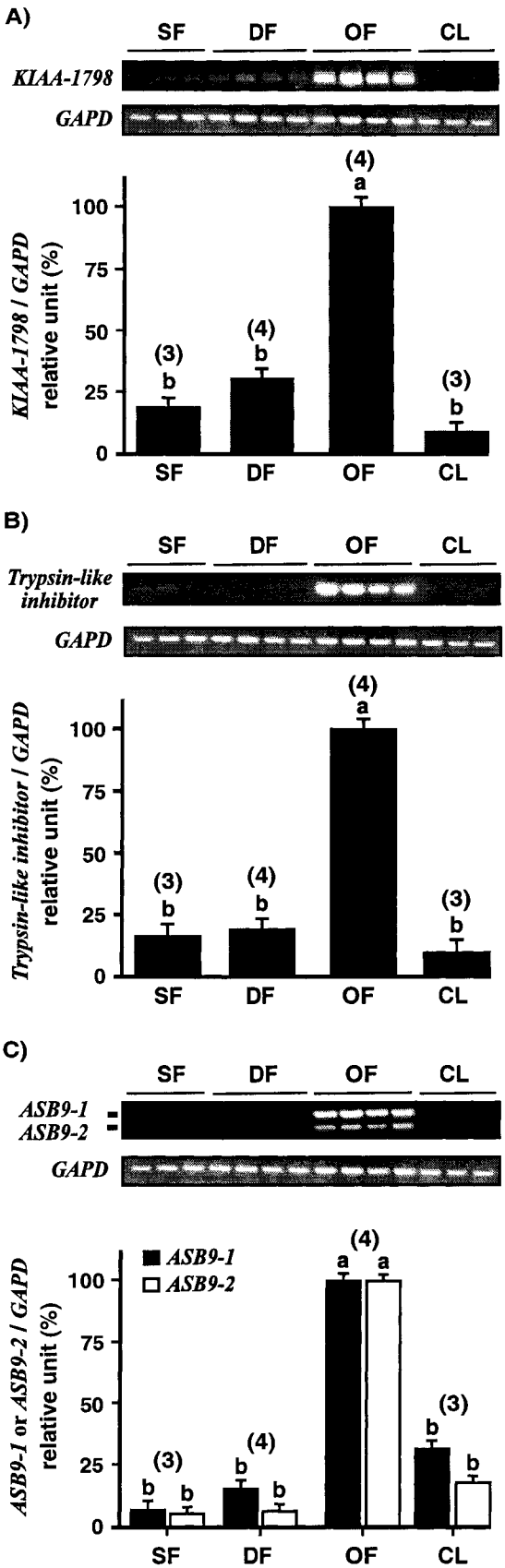

Figure 4

Fig. 5. Regulation of KIAA-1798, trypsin-like inhibitor and ASB9 transcripts by hCG in bovine follicles during the periovulatory period.

Total RNA was extracted from preparations of bovine follicular wall obtained from ovulatory follicles isolated between 0 and 24 h after hCG then used in mRNA expression analyses by semiquantitative RT-PCR as described under *Materials and Methods*. The control gene was *GAPD* (710 bp); it showed no significant differences in mRNA expression levels between groups. **A**, the *KIAA-1798* (800 bp), **B**, the trypsin-like inhibitor (420 bp) and **C**, the ASB9 isoforms (649 bp and 500 bp) PCR signals were normalized with their corresponding *GAPD* signals, and the results are presented as a relative change in ratio between groups. Bars marked with an asterisk are significantly different ($P < 0.05$) from 0 h post-hCG when Dunnett's test was used for comparisons of group means for a specific gene. Data are presented as least-square means ± SEM, and represent two distinct follicles, *i.e.* animals, per time point.

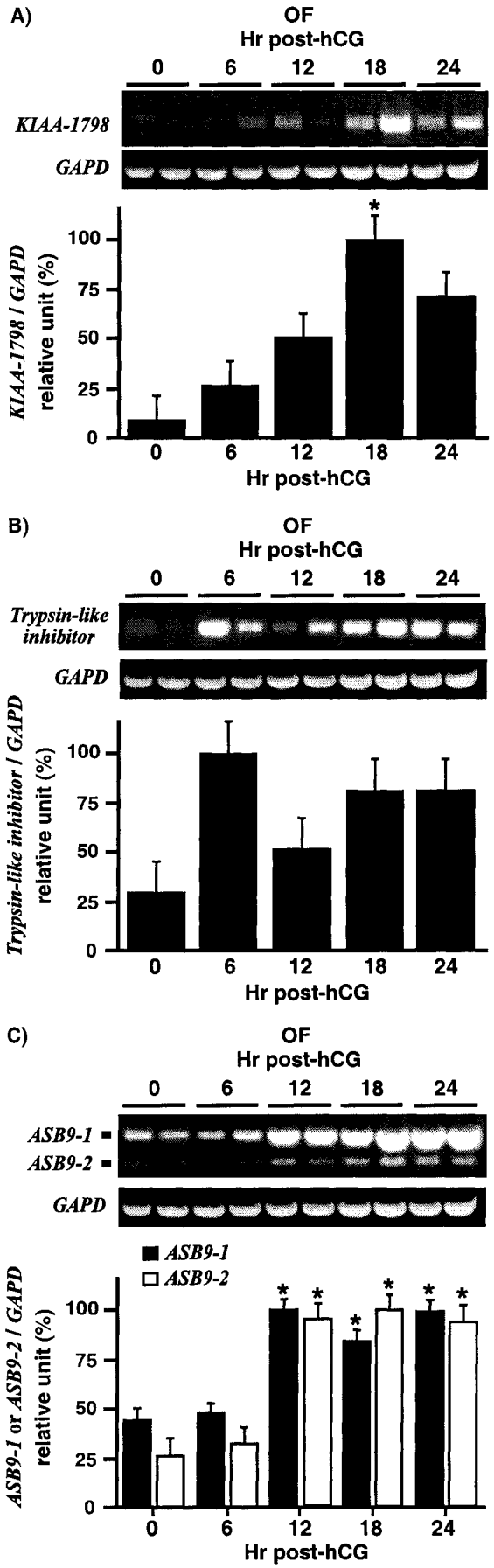

Figure 5

Fig. 6. Expression of KIAA-1798, trypsin-like inhibitor and ASB9 transcripts in bovine tissues.

Total RNA was extracted from various bovines tissues, and samples (100 ng) were analyzed for *KIAA 1798-like*, *trypsin-like inhibitor*, *ASB9-1*, *ASB9-2* and *GAPD* (control gene) content by a semiquantitative RT-PCR/Southern blotting technique, as described under *Materials and Methods*. Comparison of expression of mRNA in different bovine tissues for *KIAA-1798* (800 bp), *trypsin-like inhibitor* (420 bp), *ASB9-1* and *ASB9-2* (649 bp, 500 bp) and GAPD (710 bp) is presented.

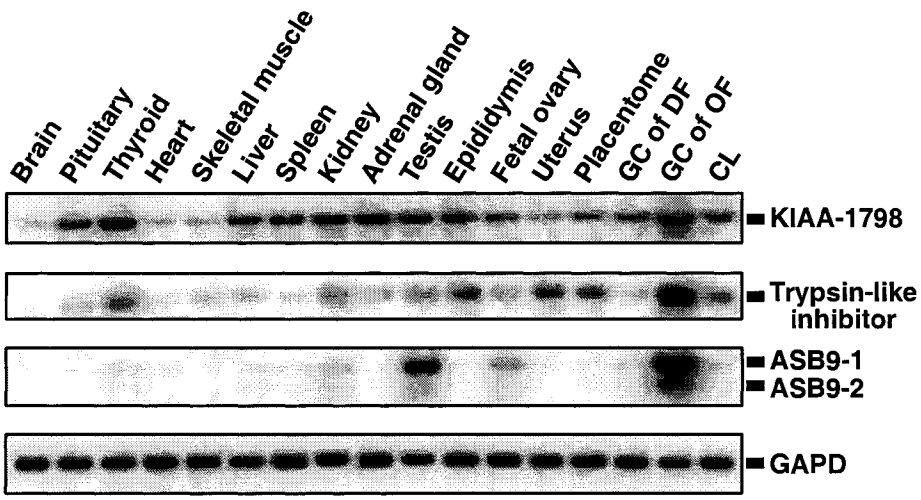

Figure 6

CHAPITRE 5

5. DISCUSSION GÉNÉRALE ET PERSPECTIVES

L'intérêt de la présente étude porte sur la caractérisation et la détermination du profil d'expression de gènes induits par la relâche préovulatoire de LH/hCG dans les cellules de granulosa lors de l'ovulation chez la vache. Jusqu'à ce jour, l'expression de ces gènes n'avait pas été associée à ce phénomène biologique. Comme la plupart des gènes induits ou dont l'expression est accrue lors de l'ovulation, PLA2G4A est associée à la réaction inflammatoire (Kudo & Murakami, 2002; Scott et al., 1999); tel n'est pas le cas des autres gènes étudiés, en l'occurrence CAV1, KIAA-1798, ASB9 et l'inhibiteur potentiel de sérine protéase possédant un domaine d'inhibiteur de trypsine, nommé "trypsin-like inhibitor". Peu d'informations relatives aux trois derniers gènes précités sont disponibles dans la littérature. Dans cette partie du document, les aspects sur chacun des gènes n'ayant pas été pris en compte dans les chapitres précédents, seront commentés. Tout en apportant des données complémentaires à cette étude, ces informations nous permettront d'entrevoir les perspectives à mettre en œuvre pour compléter l'étude de chaque gène.

5.1. Phospholipase A2 cytosolique alpha (PLA2G4A)

Des études, menées pour la plupart chez la ratte, ont montré que plusieurs membres de la grande famille des phospholipases A2 (PLA2) tels la PLA2G4A, la PLA2 pancréatique (PLA2G1B) et la PLA2 synoviale (PLA2S) sont exprimées dans l'ovaire (Bonney & Wilson, 1993; Nomura et al., 1994; Kurusu et al., 1998a) et elles sont retrouvées dans d'autres tissus reproducteurs dont le placenta et les membranes

fœtales (Rosenthal et al., 1998; Johansen et al., 2000). La régulation de l'expression des PLA2 peut être sous le contrôle hormonal (Bonney & Wilson, 1993; Kurusu et al., 1998a; Kol et al., 1997b; Ben-Shlomo et al., 1997) et des facteurs de croissance (Kol et al., 1997a; Miyashita et al., 1995; Li et al., 1997). Des études ont montré que la stimulation de la synthèse des PG par IL1B est réalisée par l'intermédiaire des PLA2 (Kol et al., 1997a; Ben Shlomo et al., 1997).

Le PLA2G4A est le principal membre de la famille de PLA2 dont l'expression est induite par la relâche préovulatoire de LH/hCG dans les cellules de la granulosa du follicule bovin durant le processus ovulatoire. De plus, nous avons démontré que cette augmentation de l'ARNm et de la protéine précède l'induction de PTGS2. Ces résultats sous-tendent qu'au cours de la période périovulatoire chez le bovin, PLA2G4A serait la principale enzyme libérant de l'acide arachidonique des phospholipides membranaires. L'acide arachidonique est le substrat de l'enzyme PTGS2 pour la synthèse des eicosanoïdes, parmi lesquels les PG sont obligatoires pour l'ovulation. Ces dernières permettent les changements de la matrice extracellulaire à l'origine de la rupture de la paroi folliculaire ainsi que la maturation ovocytaire. Notre conclusion est en accord avec les observations de Kurusu et al. (1998b) rapportant une augmentation de l'activité enzymatique de PLA2G4A en période périovulatoire chez la ratte. Ces travaux ont démontré qu'une injection locale dans la bourse ovarienne d'un inhibiteur spécifique de PLA2G4A, soit l'"arachidonyl trifluoromethyl ketone" (ATK), empêcherait la production de PGE et par conséquent l'ovulation. En considérant l'ensemble de ces

données, il est indéniable que PLA2G4A induit par la relâche ovulatoire de LH/hCG contribue à la synthèse des PG au cours du processus ovulatoire chez la vache.

Les observations réalisées chez la souris dont le gène PLA2G4A a été invalidé ont démontré une baisse légère du nombre d'ovulations et des difficultés marquées lors de la parturition (Uozumi & Shimizu, 1997; Bonventre et al., 1997; Sapirstein & Bonventre, 2000). Ce constat indique le rôle important joué par PLA2G4A dans le déroulement de la mise-bas (Song et al., 2002; Lappas & Rice, 2004). De plus, si nos travaux et ceux de Kurusu et al. (1998b) ont démontré une régulation spatio-temporelle de PLA2G4A lors du processus ovulatoire, les observations obtenues à partir du modèle de souris ayant une mutation nulle pour PLA2G4A suggèrent l'existence d'une voie alternative de production d'écosanoïdes en cas de défaillance de celle de PLA2G4A. En effet, bien que les PLA2 cytosoliques (PLA2G4A, PLA2G4B, PLA2G4C) soient plus spécifiques à la synthèse d'acide arachidonique, les autres PLA2 possèdent aussi la capacité d'en produire (Murakami & Kudo, 2001; Kudo & Murakami, 2002). En outre, des interactions existent entre les différentes enzymes PLA2. En effet, plusieurs travaux ont rapporté que les interactions entre les PLA2 des groupes I, II et III encore nommées PLA2 sécrétées et PLA2G4A, se font par l'intermédiaire du métabolite hydropéroxyeicosatetraenoique (12/15-HPETE) issu de l'action de l'enzyme 12/15-lipoxygénase sur l'acide arachidonique libre (Kuwata et al., 2002; Balboa et al., 2003). Les PLA2 sécrétées qui hydrolysent les glycérophospholipides à la position *sn-2* sans être sélectif sur l'acide gras agissent par les modes paracrine et autocrine (Murakami & Kudo, 2002; Balboa et al., 2003) alors que PLA2G4A est intracellulaire. Des études

rapportent que l'activation de PLA2G4A non seulement précède mais semble être requise pour l'activation des PLA2 sécrétées (Balsinde & Dennis, 1996; Balsinde et al., 1998). En d'autres termes, ces auteurs préconisent que PLA2G4A semble être l'initiateur et le régulateur de la réponse tandis que les PLA2 sécrétées amplifient le signal généré par leur mode d'action (Balboa et al., 2003). Cette hypothèse n'a pu être vérifiée dans notre étude car la comparaison entre PLA2G4A et une PLA2 sécrétée, en l'occurrence PLA2G1B, s'est limitée à l'expression de l'ARNm dans les GC et des parois folliculaires. L'analyse comparative du profil d'expression des protéines correspondantes pourrait confirmer ou infirmer les conclusions des auteurs précités.

En plus de son implication dans la synthèse d'acide arachidonique, la protéine PLA2G4A pourrait être associée à l'involution et le remodelage tissulaire car elle est présente dans les sites phagocytaires et les tissus fibreux (Kurusu et al., 2001). Le rôle de PLA2G4A dans l'apoptose est controversé. Pour certains auteurs, les PLA2 sont impliquées dans l'initiation de ce processus (Pate, 1994; Kronke & Adam-Klages, 2002) tandis que d'autres stipulent que leur présence est une conséquence et non une cause de l'apoptose (Taketo & Sonoshita, 2002). De cette controverse, il sera retenu que PLA2G4A est exprimée lors de l'apoptose. Cette observation expliquerait la présence de la PLA2G4A détectée par l'immunobuvardage des échantillons issus de cellules de la granulosa de petits follicules. En fait, ces échantillons étaient constitués d'un pool de petits follicules dont certains auraient pu être atrésiques. Cette hypothèse fut vérifiée par le fait que l'analyse immunohistochimique des petits follicules sains n'a pas permis d'observer la présence de la protéine dans les cellules de la granulosa. Cette observation

corrobore les travaux de Kurusu et al. (1998b) qui démontrèrent la présence de PLA2G4A dans les follicules atrésiques. De plus, la protéine PLA2G4A a aussi été détectée dans le CL en régression (Kurusu et al., 1998a; Pate, 1994).

5.2. Cavéoline-1 (CAV1)

Les cavéoles sont des invaginations de la membrane plasmatique impliquées dans le transport des molécules (endocytose, transcytose) et les événements de mécanotransduction (Fan et al., 1983; Cohen et al., 2004). Ces organelles hébergent plusieurs protéines dont les cavéolines. Ces dernières sont considérées comme les marqueurs des cavéoles (Williams & Lisanti, 2004). Parmi les trois cavéolines identifiées, seules CAV1 et CAV3 possèdent la capacité de former des cavéoles (Liu et al., 1997b; Galbiati et al., 2001). Quant à CAV2, elle nécessite la présence de CAV1 pour permettre sa stabilité à la membrane plasmatique (Razani et al., 2002a). CAV1 et CAV2 sont exprimées dans diverses cellules, tandis que l'expression de CAV3 est spécifique au muscle squelettique (Way & Parton, 1995; Tang et al., 1996).

Dans les cellules de la granulosa du follicule ovulatoire bovin, notre étude a démontré l'induction de l'isoforme α de CAV1 aussi bien pour l'ARNm que la protéine. Il faut souligner que l'isoforme β de CAV1 code pour une protéine d'environ 3 kDa de moins que la protéine codée par l'isoforme α. L'isoforme β est issu d'un épissage alternatif du gène CAV1 (Scherer et al., 1995). À l'image de l'expression de l'ARNm de CAV1 dans les cellules de granulosa, l'expression de l'ARNm de CAV2 a aussi augmenté, cependant cette élévation fut moins prononcée que celle de CAV1. Cette

similarité de profil d'expression des deux ARNm confirme que les transcrits dans le même type cellulaire répondent tous les deux aux mêmes stimuli mais à des intensités différentes. En effet, l'expression de CAV1 semble plus finement contrôlée car nous avons observé une augmentation graduelle et importante du transcrit en période périovulatoire. Toutefois, les observations réalisées sur CAV2 n'ont pas été statistiquement significatives au cours de cette période, possiblement à cause de la contribution des cellules thécales et endothéliales des échantillons de parois folliculaires. La co-expression de CAV1 et CAV2 dans la même cellule a été rapportée par des études précédentes (Scherer et al., 1997; Mora et al., 1999; Parolini et al.,1999), toutefois la signification biologique de cette association est méconnue. Est-ce que CAV2 aurait un rôle différent de celui de CAV1 ou contribuerait- il à l'action de CAV1? Cette interrogation soulevée par Boyd et al. (2004) demeure sans réponse.

Dans notre étude, l'immunobuvardage a détecté la protéine CAV1 dans les cellules de la granulosa des petits follicules antraux (2-4 mm). Cette présence est vraisemblablement due à une contamination des échantillons biologiques par les cellules endothéliales des capillaires sous-jacents à la membrane basale. Cette interprétation est corroborée par les observations immunohistochimiques démontrant une absence de la protéine CAV1 dans les cellules de la granulosa des dits follicules (Annexe). Il faut souligner que les cavéolines sont abondamment exprimées dans les cellules endothéliales (Cohen et al., 2004).

Plusieurs études ont démontré que CAV1 est impliquée dans le transport de diverses molécules (Razani & Lisanti, 2001; Razani et al., 2002) incluant celui du cholestérol (Schlegel et al., 2000; Uittenbogaard & Smart, 2000) ainsi que dans la régulation de la signalisation cellulaire. En effet, CAV1 est associée à diverses molécules dont : la connexine 43 (Schubert et al., 2002), la PLA2G4A (Smart et al., 1999; Balboa et al., 2003; Graziani et al., 2004), les protéines associées à l'angiogénèse dont le récepteur de VEGF, eNOS, uPAR (Frank et al., 2003) et les molécules de signalisation intracellulaire dont HRas, "Src-like tyrosine kinase" et les sous-unités alpha des protéines G (Couet et al., 2001; Sargiacomo et al., 1993; Li et al., 1995). Hormis le récepteur de l'insuline, CAV1 induit une régulation négative de nombreuses molécules signalétiques auxquelles elle se lie (Yamamoto et al., 1998). Ces observations suggèrent que l'augmentation d'expression de CAV1 dans les cellules de granulosa favoriserait le contrôle et/ou l'inhibition des mécanismes de transduction stimulés par les récepteurs LHCG activés. CAV1 contribuerait au mécanisme de désensibilisation du récepteur LHCG (Hunzicker-Dunn et al., 2002). De plus, l'interaction démontrée entre CAV1 et PLA2G4A suggère que CAV1 inhiberait l'activité principale de cette enzyme occasionnant ainsi une diminution de la libération d'acide arachidonique, substrat essentiel de la PTGS2. Par ce biais, CAV1 empêcherait une réponse inflammatoire exacerbée. L'induction de CAV1 ne semble pas être une condition *sine qua none* pour l'ovulation, car les souris invalidées pour CAV1, CAV2, CAV3 et la double invalidation CAV1/3 sont viables et fertiles (Razani et al., 2002b, 2002c; Cohen et al., 2004). L'invalidation de CAV1/3 a été le premier modèle animal qui a permis de soustraire un organite cellulaire soit, les vésicules d'endocytose ou cavéoles localisées à la membrane

cytoplasmique et responsable de l'importation ou de l'exportation de matériel biologique. Ainsi, la vie sans cavéole est donc possible. Il a donc été proposé que chez ces souris invalidées pour les différents gènes CAV, les rôles dévolus aux cavéolines dans la formation des cavéoles pourraient être compensés par les radeaux lipidiques ("lipid rafts"; Abrami et al., 2002) permettant entre autre le transport du cholestérol. De plus, d'autres molécules comme les clathrines, voie alternative contribuant à l'endocytose, seraient impliquées dans les mécanismes compensatoires (Minegishi, 2004; Luttrell & Lefkowitz, 2002).

5.3. "Ankyrin SOCS Box 9" (ASB9)

Caractérisée par la présence de la boîte SOCS à l'extrémité carboxy-terminale, la grande famille des protéines SOCS est subdivisée en plusieurs sous-familles: les "cytokine-inducible SH2-containing" (CIS), les SOCS, les ASB, les "WD-40-repeat containing proteins" (WSB), les "SPRY domain-containing proteins" (SSB) et les "RAR-like GTPases" (Nicholson & Hilton 1998; Hilton et al., 1998; Yasukawa et al., 2000; Kile et al., 2002). Ces sous-familles comportent plusieurs membres, mais seuls les SOCS ont fait l'objet d'études exhaustives. L'expression de l'ARNm des SOCS est contrôlée par les cytokines et les facteurs de croissance (Larsen & Ropke, 2002; Krebs et Hilton, 2000; Yasukawa et al., 2000); cette régulation varie d'un tissu à un autre (Starr et al., 1997; Yasukawa et al., 2000; Tollet-Egnell et al., 1999). L'activation transcriptionnelle des gènes SOCS s'effectue via les protéines "signal transducers and activators of transcription" (STATs) (Krebs & Hilton, 2000; Cooney, 2002) et la dégradation des protéines SOCS est fonction des interactions entre la boîte SOCS, le

système ubiquitine–protéasome et les élonguines B et C (Kamura et al., 1998; Matsumoto et al., 1997). La principale voie de signalisation des cytokines et des facteurs de croissance est la voie JAK/STAT. La fonction biologique principale reconnue des SOCS est d'inactiver la signalisation JAK/STAT (Nicholson & Hilton, 1998; Larsen & Ropke, 2002). La régulation négative de cette voie de signalisation se fait au moins par deux mécanismes: les "protein inhibitors of activated STAT" (PIAS) qui diminuent l'affinité de liaison des STAT à leur séquence d'ADN (Shuai, 2000; Chung et al., 1997) et les protéines SOCS qui inhibent l'activité des JAK kinases ou leur liaison avec les récepteurs des cytokines (Nicholson & Hilton, 1998; Cooney, 2002). La régulation de la voie JAK-STAT est importante car son dérèglement est associé à des troubles hématopoïétiques, des cancers, des maladies autoimmunes et inflammatoires (Ihle, 2001; O'Shea et al., 2002).

A ce jour, bien que 18 gènes ASB aient été caractérisés chez la souris et l'homme, leur fonction biologique est toujours méconnue. En plus de la boîte SOCS, les ASB possèdent plusieurs domaines ankyrines ("ankyrin repeat domain") qui peuvent être unique ou répété. Ce domaine est présent dans divers types de protéines par exemple des facteurs de transcription, des enzymes et des régulateurs du cycle cellulaire. La fonction reconnue du domaine ankyrine est de permettre les interactions protéine-protéine (Rubtsov & Lopina, 2000; Hryniewicz-Jankowska et al., 2002; Kile et al., 2003; Liu et al., 2003).

Les protéines ASB ont été récemment caractérisées et deux modèles de souris transgéniques sont disponibles. En effet, la délétion ou la surexpression d'ASB1 n'ont pas influencé le développement normal de la souris ainsi que sa fertilité. Toutefois, des anomalies testiculaires ont été observées (Kile et al., 2001; 2002). De plus, l'expression des autres ASB n'a pas été altérée chez la souris dont le gène ASB1 a été invalidé (Kile et al., 2001). Si les transcrits des ASB1, ASB2, ASB3 (Kile et al., 2000; 2001), ASB8 (Liu et al., 2003) sont détectés dans plusieurs tissus, ASB4 (Kile et al., 2001), ASB17 (Kim et al., 2004; Guo et al., 2004) et ASB9 (Kile et al., 2001) sont exprimés exclusivement dans les testicules. Notre étude a confirmé cette expression spécifique de l'ASB9 aux gonades mâle et femelle. Elle a aussi permis la caractérisation d'un nouvel isoforme. En effet, la présence de deux transcrits d'ASB9 a été démontrée comme étant fortement exprimés dans les cellules de la granulosa du follicule ovulatoire bovin. L'expression de l'ASB9-2 semble exclusive aux cellules de granulosa. L'induction est importante pour les deux isoformes puisqu' aucun signal n'a été détecté dans les cellules de granulosa du follicule dominant. Cette expression spatio-temporelle suggère que ce gène jouerait incontestablement un rôle dans le processus ovulatoire. L'ASB9-2, seul transcrit détecté dans le testicule bovin et tel que rapporté chez la souris par Kile et al. (2001) met en question le rôle biologique de cet isoforme dans les cellules de la granulosa. Puisqu'un seul gène ASB9 est présent dans la séquence génomique humaine et murine, l'ASB9-2 serait issu d'un épissage alternatif de l'ASB9-1. Cet épissage alternatif occasionnerait la perte d'un domaine ankyrine à l'ASB9-2.

La fonction biologique des ASB bien qu'inconnue à ce jour est assimilée à celle des membres de la grande famille SOCS, soit de cibler les protéines afin de promouvoir leur dégradation (Kile et al., 2000). En effet, plusieurs études ont démontré que les protéines pourvues d'une boîte SOCS co-précipitent avec les élonguines B et C formant ainsi un complexe qui interagit avec une ubiquitine E3 ligase. La protéolyse qui en résulte, en détruisant le complexe protéine–élonguine B et C, permettra à la cellule de répondre à nouveau au stimulus (Larsen & Ropke, 2002; Liu et al., 2003). Considérant ces informations, on peut émettre l'hypothèse selon laquelle ASB9-1 et ASB9-2 seraient induites par les différentes cytokines et facteurs de croissance dont l'expression est stimulée par la relâche préovulatoire de LH/hCG dans les cellules de la granulosa du follicule ovulatoire. Parmi les cytokines induites, le système interleukine 1 composé de IL1A, IL1B, leurs récepteurs IL1R1 et ILR2 et l'antagoniste des récepteurs IL1RA qui atténue l'action des interleukines, mérite une attention particulière. Les interleukines sont importantes durant la folliculogénèse car elles promouvoient la prolifération et la suppression de la différenciation cellulaire. De plus, au cours de l'ovulation, elles augmentent la production locale d'eicosanoïdes, des stéroïdes, des MMPs et d'agents vasoactifs tel le VEGF (Brannstrom, 2004). Plusieurs études ont démontré que la LH stimule l'expression de IL1B et ses récepteurs dans les cellules de la granulosa chez la femme (Chen et al., 2000), la jument (Caillaud et al., 2005)) et la ratte (Brannstrom, 2004). Il est fort probable que l'augmentation de l'IL1B stimulé par la LH/hCG occasionne l'induction de l'expression de l'ASB9. Ce dernier à son tour inhiberait et cela précocement dans les premières étapes de la voie de signalisation du système interleukine intra-ovarien. En d'autres termes, les protéines ASB9 par rétrocontrôle

négatif inhiberaient transitoirement la voie de signalisation JAK/STAT des facteurs qui ont induit leur transcription. Cette hypothèse est compatible à la fonction biologique attribuée au domaine ankyrine des protéines. Pour vérifier cette hypothèse, l'expression de l'ASB9 pourrait être étudiée à l'aide de cultures cellulaires traitées avec différentes doses d'IL1B. D'autres rôles pourraient être envisageables si des études d'immunoprécipitation et de co-localisation avec d'autres molécules cibles des ASB9 sont réalisées. La disponibilité d'un anticorps est nécessaire afin de réaliser de telles investigations. De plus, le profil d'expression de la protéine pourrait être étudié dans différents types de follicule bovin et comparé à celui des ARNm.

5.4. Le "trypsin-like inhibitor"

L'expression de l'ARNm du "trypsin-like inhibitor" augmente dans les cellules granulosa du follicule ovulatoire comparativement au follicule dominant. Toutefois, au cours de la période périovulatoire, l'induction du transcrit dans les échantillons de parois folliculaires est masquée d'une part par la contribution des divers types cellulaires présents dans les thèques et d'autre part par l'expression bimodale du transcrit qui augmente à 6 h après hCG pour décroître à 12 h avant d'augmenter à nouveau à 18 h après hCG. Ces événements expliquent le fait que l'analyse statistique n'a pas démontré de différence significative au cours de la période périovulatoire. On ne pourrait être affirmatif pour la protéine correspondante car l'expression protéique n'a pas fait l'objet d'étude du fait de l'indisponibilité d'un anticorps. Pour les mêmes raisons, la localisation n'a pu être réalisée mais on présume que la protéine pourvue d'un peptide signal serait secrétée dans le liquide folliculaire et le tissu interstitiel.

La fonction biologique de la protéine "trypsin-like inhibitor" n'est pas connue. Les domaines qui composent cet inhibiteur potentiel de sérine protéase, en l'occurrence les domaines "sterol carrier protein 2" (SCP2)-like et le LCCL, sont aussi présents dans d'autres protéines démontrant des rôles biologiques variés. En effet, SCP2-like est retrouvé dans : la protéine "acidic epididymal glycoprotein" (AEG) des rongeurs chez lesquels elle est impliquée dans la maturation du sperme (Mizuki & Kasahara, 1992), une protéine spécifique au testicule, TPX1 (Kasahara et al., 1989) et dans l'enzyme "17-beta hydroxysteroid dehydrogenase type 4" (17βHSD4) qui inactive le 17β-estradiol (Brown et al., 2004). Ce domaine est aussi présent dans les protéines des procaryotes. Le domaine LCCL est très conservé au cours de l'évolution mais peu de données biologiques sont disponibles. Selon Trexler et al. (2000), il pourrait être impliqué dans la liaison avec les lipopolysaccharides (LPS). Le domaine LCCL est retrouvé dans la protéine Lgl1 ("late gestation lung protein 1") induite lors du développement du poumon (Oyewumi et al., 2003; Kaplan et al., 1999) et Coch-5b2, une protéine cochléaire chez laquelle la mutation de ce domaine occasionne la surdité (Robertson et al., 1998; Liepinsh et al., 2001).

Les informations disponibles sur les domaines de cette protéine nous apportent peu d'indications sur le rôle probable de cet inhibiteur potentiel de sérine protéase. Nous suggérons que le "trypsin-like inhibitor" contribue à atténuer, voire abolir, l'activité des multiples protéases présentes dans le follicule ovulatoire (voir section 2.5.3.3). Un préalable aux expérimentations visant à démontrer cette hypothèse est l'obtention d'un

anticorps spécifique. Dès lors, l'expression de la protéine en fonction de la relâche préovulatoire de LH pourrait être analysée et sa localisation cellulaire précisée. Pour vérifier l'hypothèse émise précédemment, des cultures de cellules eucaryotes chez lesquelles la protéine est surexprimée doivent être envisagées. Par la suite, du fait que la protéine de "trypsin-like inhibitor" soit sécrétée, le surnageant de culture cellulaire pourrait être analysé par zymographie (Cao et al., 2004) afin d'analyser sa capacité à inhiber différentes protéases associées au processus ovulatoire.

5.5. KIAA-1798

KIAA est une appellation donnée au projet de caractérisation de longs ADNc (> 4 kb) générés à partir d'ARNm du cerveau et codant pour des protéines > 50 kDa (Kikuno et al., 2002, 2004). Les protéines KIAA possèdent souvent plusieurs domaines qui interagissent avec d'autres molécules *in vivo* (Kikuno et al., 2004; Koga et al., 2004). La complexité de ce groupe de protéines fait que les fonctions biologiques des KIAA ne sont pas bien élucidées. La protéine bovine KIAA-1798 (aussi nommée L3MBTL) induite dans les cellules de la granulosa du follicule ovulatoire bovin n'échappe pas à cette règle avec ses domaines "malignant brain tumor (MBT)" et "sterile alpha motif (SAM)" qui se retrouvent dans des protéines nucléaires et de signalisation (Bornemann et al., 1996; Tessier-Lavigne, 1995; Schultz et al., 1997; Stapleton et al., 1999).

Plusieurs auteurs suggèrent que le domaine SAM soit impliqué dans les interactions protéine-protéine car il a la capacité de former des homo- et hétéro-

oligomères avec d'autres protéines possédant ou non un domaine SAM (Bornemann et al., 1996; Stapleton et al., 1999;Thanos et al., 1999; Qiao & Bowie, 2005). Une étude récente a permis la caractérisation de MBT-1 chez la souris, un paralogue à KIAA-1798/L3MBTL. MBT-1 s'apparente aux protéines bovine et humaine KIAA-1798/L3MBTL par la présence de ces trois domaines MBT et du domaine SAM mais possède en plus un domaine de 100 acides aminés riche en glutamine (Arai & Miyazaki, 2005). Les souris ayant une mutation nulle pour le gène MBT-1 meurent *in utero* à cause d'une anémie sévère, démontrant ainsi l'importance du rôle joué par MBT-1 dans l'hématopoïèse. On a démontré que MBT-1 se localise dans les noyaux et qu'elle agirait comme point de contrôle dans la différentiation des cellules myéloïdes en modulant l'activité des certaines cyclines. Par analogie, nous suggérons que l'induction de KIAA-1798/L3MBTL dans les cellules de granulosa suite à la relâche préovulatoire de LH participerait aux mécanismes de contrôle intervenant dans la différentiation finale des cellules de granulosa en cellules lutéales.

5.6. Résumé

Les travaux de recherche présentés dans cette thèse ont permis de caractériser pour la première fois les ADNc pleine longueur de PLA2G4A, CAV1, ASB9-1, ASB9-2, KIAA-1798 et du "trypsin-like inhibitor" chez l'espèce bovine. De plus, les résultats ont bien démontré que la relâche préovulatoire de LH/hCG induit l'expression de leurs ARNms. Cette expression spatio-temporelle a certainement son importance dans le processus ovulatoire. Si le rôle de PLA2G4A est mieux connue, des fonctions biologiques ont été suggérées pour les autres gènes étudiés considérant les domaines

protéiques et l'analogie à d'autres molécules apparentées. Ainsi, il apparaît que CAV1 et ASB9 seraient impliqués à diminuer la réactivité des cellules par rapport à certains stimuli après la relâche préovulatoire de LH/hCG. Le "trypsin-like inhibitor" pourrait être impliqué dans le contrôle de la modification tissulaire en inactivant les protéases tandis que le KIAA-1798/L3MBTL ferait partie du mécanisme de contrôle de la différentiation finale des cellules de la granulosa en cellules lutéales. En considérant l'ensemble de ces informations, on peut souligner que si la fonction de stimulation de la relâche préovulatoire de LH/hCG sur les cellules de la granulosa a été étudiée, le contrôle des éléments activés par ce stimulus est par contre peu élucidé. En d'autres termes, en se référant à nos travaux, PLA2G4A est associée aux mécanismes de stimulation de l'ovulation, CAV1, ASB9 et le "trypsin-like inhibitor" semblent être impliqués dans l'inhibition ou la régulation à la baisse des stimuli induits lors de l'ovulation, tandis que KIAA-1798 contrôlerait en partie la différentiation finale des cellules de granulosa en cellules lutéales.

L'expression protéique n'a pas pu être étudiée pour ASB9, KIAA-1798 et le "trypsin-like inhibitor" faute de la disponibilité d'un anticorps. L'obtention d'anticorps spécifiques permettrait par analyses d'immunoprécipitation de définir si ces protéines s'associent à d'autres protéines et d'entrevoir à leur tour leur caractérisation afin de définir les interactions protéiques. Les analyses d'immunobuvardage et d'immunohistochimie révèleraient leur profil d'expression et leur localisation cellulaire. De plus, des études de transgénèse (délétion et surexpression) devraient être entreprises.

L'ensemble de ces approches contribuerait à l'élucidation de la fonction biologique respective de KIAA-1798, d'ASB9 et du "trypsin-like inhibitor".

6. CONCLUSION GÉNÉRALE

L'ovulation a toujours été un phénomène biologique intriguant pour les scientifiques tant pour la libération du gamète femelle fécondable que les mécanismes responsables de la rupture folliculaire. Au fil des décennies, cette curiosité scientifique a conduit à l'élaboration de plusieurs théories explicatives du phénomène et en se basant sur différentes hypothèses et expérimentations. Malgré les innombrables informations recueillies, les mécanismes qui sous-tendent le processus ovulatoire demeurent d'actualité et ils constituent encore des sujets de recherche. Les résultats des études rapportés dans le présent document s'inscrivent dans ce cadre et ils constituent une contribution à cette préoccupation majeure.

Les outils de la biologie moléculaire combinés aux techniques d'immunobuvardage et d'immunohistochimie ont permis la caractérisation et les études de l'expression de l'ARNm et des protéines correspondantes (selon le cas) de cinq gènes différentiellement exprimés dans les cellules de la granulosa du follicule ovulatoire bovin. Antérieurement à la présente étude, les gènes ASB9, KIAA-1798 et le "trypsin-like inhibitor" dont on a démontré l'induction par la relâche ovulatoire de LH/hCG n'avaient jamais été associés jusque là au processus ovulatoire.

Au terme de cette recherche, s'il est irréfutable que l'expression de ces gènes soit induit dans les cellules de granulosa du follicule ovulatoire par la relâche ovulatoire de LH/hCG, leur rôle biologique dans l'ovulation n'est pas entièrement élucidé hormis

pour PLA2G4A. En effet, PLA2G4A s'est avérée être l'enzyme responsable de la libération de l'acide arachidonique qui servira de substrat à plusieurs enzymes dont la PTGS2 pour la production de prostaglandines si indispensables au processus ovulatoire. CAV1 quant à elle semble jouer un rôle dans le trafic des molécules et la terminaison de certaines signalisations intracellulaires. Dans le même sens, ASB9 semble être impliqué dans le rétrocontrôle négatif de la voie de signalisation JAK. Des interrogations portent sur les supposés rôles du KIAA-1798 et du "trypsin-like inhibitor" induits dans les cellules de la granulosa des follicules ovulatoires. Nul doute que des études complémentaires seront nécessaires pour définir l'expression protéique et la fonction biologique des protéines correspondantes à ASB9, du KIAA-1798 et du "trypsin-like inhibitor".

ANNEXE

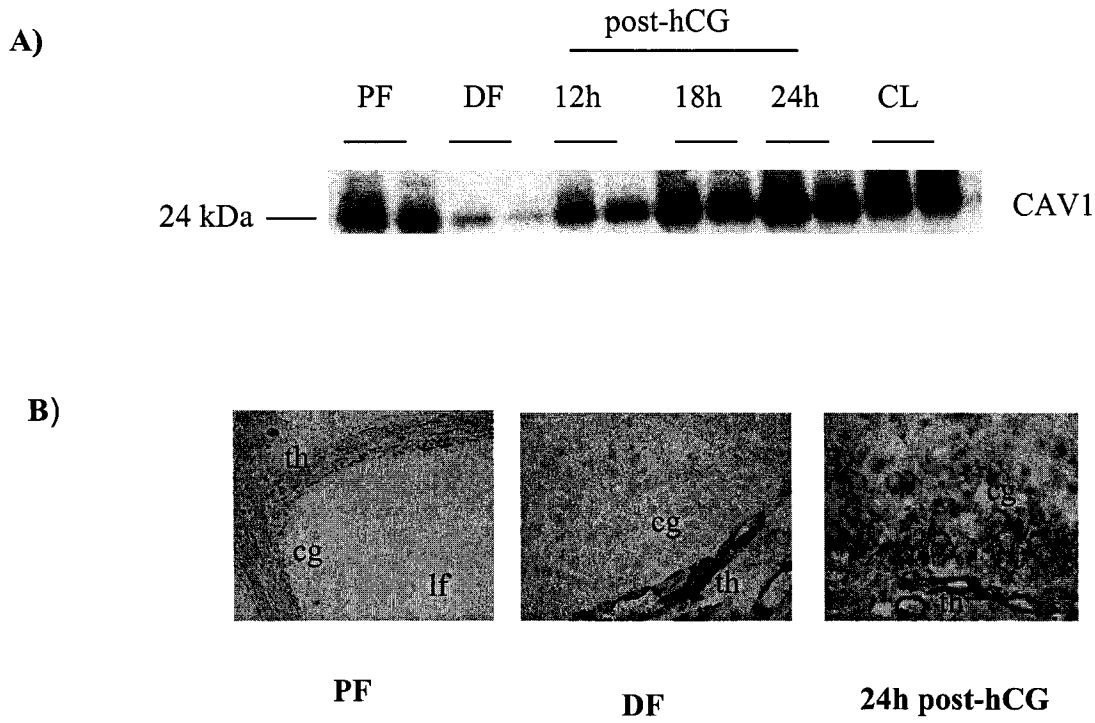

Figure 1. Immunodétection de la protéine de CAV1 dans les cellules de granulosa de follicules et corps jaune bovin. A) Immunobuvardage de 50 µg d'extraits protéiques de cellules de la granulosa provenant de petits follicules (PF; 2-4 mm), follicule dominant à jour 5 (DF), follicules ovulatoires récoltés 12, 18 et 24 h après injection d'hCG et corps jaune à jour 5 (CL). L'anticorps anti-CAV1 humain (1:500; SC-894) a été utilisé comme décrit au chapitre 3. **B)** Immunohistochimie comparative de la protéine CAV1 dans les cellules de la granulosa de petit follicule (**PF**; 2-4 mm), follicule dominant à jour 5 (**DF**), follicule ovulatoire 24 h post-hCG (**OF**) qui démontre l'absence de signal dans les cellules de la granulosa dans les PF et DF mais l'expression de la protéine CAV1 dans le follicule ovulatoire 24 h après l'injection de hCG (**cg**: cellules de granulosa; **lf** : liquide folliculaire; **th**: thèque interne).

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