Université de Montréal

REGULATION OF CHOLESTEROL INTAKE BY THE CORPUS LUTEUM

par

Leonor Miranda Jiménez

Centre de recherche en reproduction animale

Département de biomédecine vétérinaire

Faculté de médecine vétérinaire

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présentée par

Leonor Miranda Jiménez

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Alan K. Goff, président-rapporteur

Bruce D. Murphy, directeur de recherche

Lawrence C. Smith, membre du jury

Benjamin Tsang, examinateur externe

Paul D. Carrière, représentant du doyen

Résumé

L'approvisionnement en cholestérol est un facteur limitant la stéroïdogenèse ovarienne. Pour cette raison, la majorité du cholestérol requis pour la synthèse des stéroïdes est importé de la circulation via les récepteurs des lipoprotéines de haute (HDL) et de basse densité (LDL) nommés scavenger receptor (SR-BI) et low-density lipoprotein receptor (LDLr). L'ARN messager de SR-BI est exprimé dans les ovaires de porcs durant toutes les étapes de la folliculogenèse ainsi que dans le corps jaune (CL). L'expression de la protéine SR-BI a également été détectée dans les follicules de souris lors du cycle œstral. Chez les deux espèces, l'expression est concentrée dans le cytoplasme et en périphérie des cellules du follicule. Les gonadotrophines induisent l'expression de SR-BI dans les cellules de la granulosa porcines, avec une expression cytoplasmique qui augmente durant la période périovulatoire, et avec une migration aux périphéries cellulaires durant la maturation du CL. Une conformation de 82 kDa de SR-BI est fortement exprimée dans le CL porcin, avec une conformation moins abondante de 57 kDa. Les différences entre les conformations sont attribuables à la glycosylation. La culture in vitro de follicules porcins avec des gonatrophines chorioniques humaines (hCG) a induit une hausse de régulation dépendante du temps du SR-BI de 82 kDa dans les cellules du granulosa. SR-BI et LDLr ont été exprimés réciproquement, avec LDLr étant le plus élévé dans les cellules folliculaires du granulosa et diminuant précipitamment avec la formation du CL. Pour explorer plus en détail les mécanismes d'approvisionnement en cholestérol de la stéroïdogenèse ovarienne, nous avons examiné des souris soumis à un traitement de désaccouplement de l'ovulation, et des souris portant la mutation nulle du gène *Scarb1* (SR-BI$^{-/-}$). Les résultats ont démontré que des ovocytes enfermés dans des structures lutéinisées expriment SR-BI. Les souris SR-BI$^{-/-}$ présentaient de petits CLs, et de large follicules avec des cellules de thèque hypertrophiées et des kystes folliculaires avec des cavités remplies de sang et une diminution de 50% du niveau de progestérone dans le sérum. Les souris SR-BI$^{-/-}$ traitées avec une combinaison de 20 µg / g de mevinoline et 100 µ g / g de chloroquine ont démontré une diminution de 43% du niveau de progestérone sérique chez le type sauvage et de 30% chez les souris SR-BI$^{-/-}$. L'expression protéique de l'enzyme limitant pour la synthèse du cholestérol, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), a augmenté chez les souris SR-BI$^{-/-}$. Nous avons présenté des preuves démontrant que les cellules des follicules expriment le SR-BI durant la

stéroïdogenèse et que la lutéinisation augmente l'expression de SR-BI. La maturation post-transcriptionelle est caractérisée par la glycosylation. Sous des conditions normales, l'expression de LDLr est arrêtée durant la lutéinisation. Ainsi SR-BI devient le facteur principal pour l'importation du cholestérol extracellulaire. En plus, la perturbation extracellulaire du cholestérol synthétisé de novo et l'absorption par les LDLr chez les souris SR-BI$^{-/-}$ diminuent la fonction lutéal. L'homéostasie du cholestérol ovarien est très importante pour une lutéinisation adéquate et sa perturbation mène à une réduction, mais non à un blocage complet, de la fonction lutéal. En conclusion, l'expression de SR-BI est un facteur important, mais non essentiel, pour maintenir l'homéostasie du cholestérol ovarien et la synthèse des stéroïdes, et la lutéinisation. Un réseau de mécanismes complémentaires et compensatoires d'approvisionnement en cholestérol agit en concert pour assurer la synthèse des stéroïdes ovariens.

Mots-clés:
Ovaire, follicule, corps jaune, cholestérol, SR-BI, Récepteurs de lipoprotéines.

Abstract

Ovarian cholesterol supply is rate limiting to ovarian steroidogenesis. For this reason, the majority of cholesterol required for steroid synthesis is imported via scavenger receptor-BI (SR-BI) and the low-density lipoprotein (LDL) receptor from circulating HDL and LDL. SR-BI mRNA is expressed in pig ovaries at all stages of folliculogenesis and in the corpus luteum (CL). SR-BI protein expression in mouse ovary during estrous cycle was also detected. In both species, expression is concentrated in cytoplasm and periphery of follicular cells. Gonadotropins induce SR-BI expression in pig granulosa cells, with cytoplasmic expression increasing through the periovulatory period, with migration to the cell periphery as the CL matured. An 82-kDa form of SR-BI is strongly expressed in the pig CL, with the less abundant 57-kDa form, differences between forms are attributable to glycosylation. In vitro culture of pig follicles with human chorionic gonadotropin (hCG) induced time-dependent upregulation of 82-kDa SR-BI in granulosa cells. SR-BI and LDL receptor were reciprocally expressed, with the latter highest in follicular granulosa cells, declining precipitously with CL formation. To further explore mechanisms of cholesterol supply to ovarian steroidogenesis, we examined mice treated to uncouple ovulation and mice bearing null mutation of the *Scarb1* gene (SR-BI$^{-/-}$). Results show entrapped oocytes in luteinized structures expressed SR-BI. SR-BI$^{-/-}$ mice displayed small corpora lutea, large follicles with theca cells hypertrophied, follicular cysts with blood filled cavities and 50% decreased in plasma progesterone. In SR-BI$^{-/-}$ mice, treatment with a combination of 20 μg/g of mevinolin and 100 μg/g of chloroquine (CHLORO) was employed to disturbed cholesterol sources. Serum progesterone was reduced by 43% in wild type and 30% in SR-BI$^{-/-}$ mice. The protein expression of the rate-limiting enzyme for cholesterol synthesis, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) increased in SR-BI$^{-/-}$ mice. It was concluded that follicular cells express SR-BI during follicle development and luteinization causes upregulation of SR-BI expression. Posttranslational maturation is characterized by glycosylation. Under normal conditions expression of the LDLr (low density lipoprotein recepors) is extinguished during luteinization such that SR-BI becomes the principal means of importation of extracellular cholesterol. Further, perturbation of cholesterol de novo synthesis and uptake from LDLr in SR-BI$^{-/-}$ mice leads to a reduction of luteal function. Ovarian cholesterol homeostasis is central to adequate luteinization, and its perturbation leads to

reduction, but not to complete impairment, of luteal function. We conclude that SR-BI expression is an important but not essential factor in maintaining ovarian cholesterol homeostasis, steroid synthesis and luteinization. A network of complementary and compensatory cholesterol supply mechanisms act in concert to assure ovarian steroid synthesis.

Keywords: Ovary, follicle, corpus luteum, cholesterol, SR-BI, lipoprotein receptors.

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Dedication

Este trabajo se lo dedico con amor y cariño a:

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List of abbreviations

ACTH	adrenocorticotropic hormone	ADAMTS-1	disintegrin and metalloproteinase with thrombospondin motifs
AC	Adenylyl cylase	ACAT	Cholesterol acyl transferase
Ahr	Aryl hydrocarbon receptor	ALAS	5-Aminolevulinate synthase
Ang-1	Angiopoietin 1	Ang-2	Angiopoietin 2
ANOVA	Analysis of variance	Apo-A	apolipoprotein A
Apo-B	apolipoprotein B	Apo-E	apolipoprotein E
Apo-1	apolipoprotein 1	bp	base pares
BSA	Bovine serum albumin	cAMP	cyclic adenosine monophosphate
Ca2 +	Calcium ion	CBR	carbonyl reductase
CCAAT	CCAAT box of about minus 70 bp	CDB-2924	Synthetic progesterone Receptors antagonist
Cdk2	cyclin dependent kinase 2	Cdk4	Cyclin dependent kinase 4
Cdk6	Cyclin dependent kinase 6	CD36	cluster determinant 36 a leukocyte differentiation antigen
Chloro-mevi	Chloroquine-mevinolin	C/EBPβ	CCAAT/enhancer binding proteins
CE	cholesteryl ester	CRHBP	corticotrophin releasing hormone binding protein
CL	corpus luteum	CL-I	postovulatory CL
CL-II	developing CL	CL-III	mid-luteal CL
COX-1	Cyclo-oxygenase-1	COX-2	Cyclo-oxygenase-2
CP45011A	cytochrome p450 cholesterol side-chain cleavage	CRE	cAMP response element
CREB	cAMP response element binding protein	CycE	Cyclin E
CYP11A	cytochome p450 cholesterol side-chain cleavage	cPLA2	phospholipase A2

List of abbreviations (continued)

Dazla	DAZ Like autosomal	DAPI	4'diamino-2-phenyindole dilactate
DAX-1	dosage-sensitive sex reversal-1	DNA	deoxyribonucleic acid
DHT	dehydrotestosterone	EGF	epidermal growth factor
EG-VEGF	endocrine gland VEGF	ET-2	endothelin-2
ET-I	endothelin-I	ERα	estrogen receptor alpha
Erg-1	early growth response protein-1	E2	17ß-estradiol
eCG	Equine chorionic gonadotropin	E2F	transcription factor E2F
FBS	fetal bovine serum	Figla	the factor in the germline alpha
FC	free cholesterol	Foxl2–/–	forkhead box 1/2 knockout mice
FSH	follicle-stimulating hormone	Foxo3a	forkhead transcription factors 3a
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	GCS	g-Glutamylcysteine synthetase
GDP	guanosine diphosphate	GH	growth hormone
GITC	guanidine isothiocyanate buffer	GnRH	gonadotropin releasing hormone
GTPase	Guanosine triphosphate hydrolase	GTP	guanosine triphosphate
GST	glutathione S-Transferase	H	hours
hCG	human chorionic gonadotropin	HB2	HDL-binding proteins
HDL	high-density lipoprotein	HMG-CoA-reductase	3-hydroxy-3-methylglutaryl coenzyme A-reductase
HDL-CE	high-density lipoprotein cholesteryl ester	3α-HSD	3α-hydroxysteroid dehydrogenase
HPS	hematoxylin, phloxine, saffron	17beta HSD-7	17-beta-hydroxysteroid dehydrogenase type 7

List of abbreviations (continued)

3ß-HSD	3ß-hydroxysteroid dehydrogenase	IGF-I	insulin growth factor-1
20α-HSD	20α-hydroxysteroid dehydrogenase	IgG	Immunoglobulin G
IL-Ib	Interleukin-1b	IU	International units
kDa	kilo Daltons	Kg	kilogram
KO	knock-out	LCAT	lecithin cholesterol acyltransferase
LDL	low-density lipoprotein	LDLr	low density lipoprotein receptors
LH	luteinizing hormone	LH-r	Luteinizing hormone receptor
LIM	essential nuclear regulator of myogenic differentiation	LIMPII	lysosomal integral membrane protein II
LMO7	LIM domain only 7	MAPK	mitogen-activated protein kinase
MDP	glycosyl Phosphotidylinositol anchored membrane dipeptidase	min	minute
mg	Milligrams	ml	mililiters
mM	millimolar	mm	Millimeters
mm^3	millimeter cubic	MMP1	matrix metalloproteinase 1
MMP-2	matrix metalloproteinase 2	MMP-9	matrix metalloproteinase 9
MMP-14	matrix metalloproteinase 14	MMP-19	matrix metalloproteinase 19
mRNA	messenger ribonucleic acid	MT-1	metallothionin-1
µl	Microliter	M	molarity
NCI-H295R	human adrenocortical cells	Ngf	nerve growth factor
Nm	Nano moles	*P*	probability
PAP-III	pancreatitis-associated protein-III	PBR	peripheral benzodiazepine receptor

List of abbreviations (continued)

PBS	phosphate buffered saline	PCR	polymerase chain reaction
PDZK1	PDZdomain-containing-1 protein	PDZ	PDZ domain, associated with plasma membrane
PG	prostaglandin	PGE2	prostagandin E2
PGFα	prostaglandin F-2alpha	PGH2	prostaglandin H2
PGI2	prostaglandin I2	PGHS	enzyme PG endoperoxide G/H synthase
PGG2	prostaglandin G2	pH	potential of hydrogen
PKA	protein kinase A	PKC	protein kinase C
P450scc	cytochrome 450side chain cleavage enzyme	P450arom	cytochome P450 aromatase
PMSG	pregnant mare serum gonadotropin	PR	progesterone receptor
pRB	phosphorylated retinoblastoma protein	RGS2	regulator of G-protein signaling protein-2
RNA	ribonucleic acid	RIA	radioimmunoassay
RT	reverse transcriptase	RT-PCR	reverse transcription-polymerase chain reaction
RU486	mifepristone, progesterone receptor inhibitor	SCP2	sterol carrier protein-2
SCARB1	scavenger receptor class B type1	SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SF-1	steroidogenic factor-1	SOD	superoxide dismutase
SREBP	sterol regulatory element binding protein	SR-BI	scavenger receptor class B type1
SR-BII	scavenger receptor class B typeII	SR-BI +/+	scavenger receptor class B type1, wild type mice
SR-BI -/-	scavenger receptor class B type1, knockout mice	SRE	sterol regulatory element

List of abbreviations (continued)

SRE1	sterol regulatory element 1	StAR	steroidogenic-acute regulatory protein
T	testosterone	*Taq* DNA-polymerase	*Thermus aquaticus* DNA polymerase
TBS	Tris buffer saline	TGF-ß	transforming growth factor-ß
TIMP-1	metalloproteinase-1 inhibitor	TNFα	tumor necrosis factor-α
TSG-6	tumor necrosis factor-stimulated gene-6	TTBS	tweenTris-buffered saline
VEGF	vascular endothelial growth factor	VitE	vitamin E
vLDL	very low density lipoproteins	WT	wild type
XL-1 blue	XL Blue competent cells	Y1-BS1	mouse adrenocortical cell line
ZK98299	onapristone, progesterone receptor inhibitor	YTPLL	dileucine base motif
YXXZ	tyrosine-based motif	ZP1	zona pellucida protein 1
ZP2	zona pellucida protein 2	ZP3	zona pellucida protein 3
ZP -/-	ZP knockout mice		

1. INTRODUCTION

In females, the ovaries are the basis for gamete production and hormone synthesis in support of reproductive behavior and gestation. The events of follicular development, ovulation and luteinization are governed by endocrine, paracrine and autocrine factors. In folliculogenesis, some follicles leave their quiescent state to grow, then to ovulate, or die as atretic follicles. The ovulatory follicles collapse to form an ephemeral gland, known as corpus luteum (CL) whose primary function is progesterone production. This hormone regulates the estrous cycle and maintains pregnancy. In humans, the CL synthesizes upwards of 40 mg/day of progesterone (Murphy 2000). The steroidogenic luteal cells are provided with highly efficient machinery to maintain luteal progesterone production. Steroid production by the CL varies remarkably among mammalian species. In humans, monkeys and ruminants, the CL is maintained by pituitary derived luteinizing hormone (LH) acting principally through the cAMP/protein kinase A pathway (Niswender, Juengel et al. 2000). In contrast, it is well established that prolactin and estradiol are critical luteotrophic hormones in rodents and rabbits acting through their respective receptors (Stormshak, Zelinski-Wooten et al. 1987). The substrate for steroid hormone synthesis is cholesterol, derived from multiple sources. Luteal cells can produce cholesterol *de novo*, this method plays a minor role in the normal functioning tissue as evidenced by the low levels of 3-hydroxy-3-methylglutaryl coenzyme A-reductase (HMG-CoA reductase), the rate-limiting enzyme in the cholesterol biosynthetic pathway (Gwynne and Strauss 1982). The major mechanisms for cholesterol delivery to steroidogenic cells are the endocytotic pathway involving low density lipoprotein (LDL) and the selective uptake of cholesterol esters from high-density lipoprotein (HDL) (Brannian and Stouffer 1993). SR-BI is the receptor that mediates the

selective uptake of cholesterol esters from HDL (Acton, Rigotti et al. 1996) and its deletion produces infertility and reduced lipid levels in the CL, as measured by oil red-O staining, suggesting a reduction in cholesterol ester storage (Trigatti, Rayburn et al. 1999). The authors report that progesterone profiles were within the normal range in these animals, suggesting that other sources of cholesterol may have increased in these animals to correct for the absence of HDL delivery.

Recent exploration of the influence of hepatic SR-BI on female fertility by inducing hepatic SR-BI expression in mice with null mutation of the SR-BI-/- gene by adenovirus transduction or stable transgenesis indicated that these treatments restore female fertility by increasing hepatic metabolism of lipids (Yesilaltay, Morales et al. 2006). Given the apparent compensation by alternate sources, the goal of this work was to explore the role SR-BI in ovarian steroidogenesis and to examine possible alternatives sources of cholesterol that maintain normal luteal steroidogenesis in SR-BI -/- mice.

2. LITERATURE REVIEW

In this section the physiological and molecular bases of the reproductive phenomena regulating the presence of reproductive cyclicity in mammals are described, focusing on follicular development, ovulation and corpus luteum formation, events that were studied in this investigation.

2.1. FOLLICLE

2.1.1 Follicular structure

Follicles function to provide suitable conditions to maintain oocyte development, as well as the source of steroid hormones. Follicles produce other substances that act as both autocrine and paracrine modulators of cellular function and include peptides, proteins, glycoproteins, proteoglycans and growth factors whose expression varies according to reproductive and follicular development conditions (Murdoch, Dailey et al. 1981; Lumsden, Kelly et al. 1986; Richards, Hickey et al. 1987; Wise 1987; Prevost, Belanger et al. 1989).

The follicle consists of two cellular compartments. The compartment within the basement membrane of the follicle is known as the granulosa layer, and components of this layer are in direct contact with the oocyte. This layer varies in the number and size of the cells according to the hormonal characteristics (Richards, Jahnsen et al. 1987; Burke, Cardenas et al. 2005) and with the stage of follicular development. For example granulosa cells are flattened in primordial follicles, and this form changes to polyhedral or cubical in primary follicles (Braw-Tal 2002). During early phases of follicular development, the surrounding stromal cells begin to

aggregate concentrically around the follicular basal lamina and form the theca layer (Motta, Makabe et al. 1994). Communication between the two types of follicular cells has been observed in numerous in vivo and in vitro experimental paradigms (Tajima, Orisaka et al. 2006). Despite of the joint function of the follicular cells, for this review they are described separately.

2.1.2 Granulosa cells

As mentioned before, granulosa cells form a continuous layer surrounding the oocyte and can be divided into different subpopulations, based on morphology and response to hormonal stimuli (Redmer, Kirsch et al. 1991). These cells display high proliferative capacity. The *corona radiata* cells maintain an intimate contact and provide nutrients to the oocyte. The *cumulus oophorus* cells support and integrate the oocyte within the follicle, and contribute during the final stage to oocyte maturation, and serve as a barrier to sperm penetration prior to fertilization. The majority of the granulosa cells are known as mural cells, and these populate the follicular cavity with their most basal layer maintaining close contact with the basal membrane (Motlik, Fulka et al. 1986; McGregor, Flaherty et al. 1989).

Granulosa cell proliferation varies according to their localization, to the estrous cycle stage and to the follicular development phase. Proliferation in granulosa cells evaluated by thymidine incorporation showed more elevated proliferative activity in *cumulus oophorus* cells relative to mural granulosa cells (Khamsi and Roberge 2001). Some data suggest that cumulus granulosa cells continue to proliferate for up to 10 h after an ovulatory stimulus, with evidence that cyclin E protein increases after hCG, and there was a tendency of initial decline followed by a transient increase in Cdk2 protein activity 8 h after hCG (Cannon, Cherian-Shaw et al. 2005).

Proliferation is maintained in granulosa cells in the proximity of the oocyte during ovulation of the rat follicle (Cannon, Cherian-Shaw et al. 2005). Observations with electronic microscopy of granulosa cells in co-culture have allowed observation of the morphology and content of cellular elements characteristic of steroidogenic and mitotically active cells (Nottola, Heyn et al. 2006).

2.1.3 Theca cells

The theca layer consists of an internal and external theca. In species as rodents, a special group of cells named interstitial cells capable of steroid production differentiate from the internal theca (Brook and Clarke 1989). This differentiation of theca is an important physiological event occurring during early follicular development and, apparently the theca cell layer develops from stromal cells and is regulated by granulosa cells (Orisaka, Tajima et al. 2006).

The internal theca cell layer consists of undifferentiated fibroblast cells, blood vessels, and cholinergic as well as adrenergic nerve fibers. The structure of these cells is characterized by their elevated concentration of smooth endoplasmic reticulum as well as tubular crest mitochondria, characteristic of active steroidogenic cells (Motta, Nottola et al. 1995). The vascularization of these cells increases as the follicle develops (Macchiarelli, Vizza et al. 1992). Studies on follicular vasculature showed that large vessels are located in the outer theca layer, while smaller vessels are observed neighboring the inner layer, close to the basal membrane (Augustin, Braun et al. 1995; Redmer, Doraiswamy et al. 2001; Feranil, Isobe et al. 2004). The theca is the source of vascularization of the avascular granulosa cells during luteinization. The increase in blood vessels is a response to angiogenic factors including vascular endothelial growth factor (VEGF), angiopoietins 1 and 2 (Ang-1 and Ang-2) and endocrine gland VEGF

(EG-VEGF) originating from the granulosa cells, and regulated by LH (Phan, Rakenius et al. 2006, for review see Fraser 2006).

Within growing follicles, theca cells are stimulated by LH to synthesize androgens that serve as substrate for estrogen synthesis in granulosa cells. There is evidence in ruminants that, after ovulation, theca cells are transformed into the small luteal cells of the corpus luteum (Alila and Hansel 1984; O'Shea 1987) and granulosa cells are believed to be transformed in large luteal cells (Alila and Hansel 1984) but this issue has not been definitively resolved.

2.1.4 Follicular development

The ovary is a primary functional organ of the female reproductive system, and it plays two major physiological roles. First, the ovary is responsible for the differentiation and release of mature oocytes for fertilization (McGee and Hsueh 2000). Second, it is responsible for synthesizing and secreting hormones that are essential for follicle development, menstrual and estrous cyclicity and maintenance of the reproductive tract and its function (Hirshfield 1991).

Ovarian follicle development is a complex process that begins with the establishment of primordial follicle, after birth, depending on the species in a process that involves apoptosis of some of the oocytes and initiation of meiotic division of some others (Gomperts, Garcia-Castro et al. 1994; Pepling and Spradling 1998). The surviving oocytes become enveloped by somatic cells, thereby producing primordial follicles considered to be in a ‘‘quiescent’’ state, since no major morphological changes take place until they enter the pool of growing follicles (Zamboni, Thompson et al. 1972). The resting follicle is a round structure with a diplotene oocyte

surrounded by a single layer of flattened follicular cells (Pepling and Spradling 2001). The primordial follicle population present at birth has long been believed to be predetermined, following a log-linear rate of decline during the reproductive life (Miller, Charleston et al. 1999). Although this idea has recently been challenged (Johnson, Canning et al. 2004; Johnson, Bagley et al. 2005), the weight of the evidence argues against postnatal oogenesis in mammals. Several studies of gene mutation, particularly knockin and knockout mice, have elucidated the importance of genes that control differentiation of germ cells that culminate in alteration of primordial follicular development. Examples of these are the Dazla gene (Ruggiu, Speed et al. 1997; McNeilly, Saunders et al. 2000), the factor in the germline alpha (Figla) (Soyal, Amleh et al. 2000), the nerve growth factor (Ngf) and others (Dissen, Romero et al. 2001).

Recruitment of primordial follicles into the growing pool begins the coordinated and interdependent development of the somatic and germ cell components of the ovarian follicle. The end of reproductive life, or ovarian senescence, occurs when this pool of primordial follicles is depleted by death or through growth, followed by subsequent ovulation and/or atresia (Hirshfield 1991). Although some primordial follicles will be stimulated to grow immediately, the majority will remain dormant, perhaps because of autocrine or paracrine inhibition, until they receive signals to enter the growing pool (McGee and Hsueh 2000). The follicle that is recruited from the pool of resting follicles to enter the growth phase is termed primary follicle. The primary follicle enlarges because of an increase in size of the oocyte (Baca and Zamboni 1967; Zamboni 1974; Motta, Makabe et al. 1994), and conversion of the squamous or flattened granulosa cells into cuboidal granulosa cells (Hirshfield 1991). Another characteristic of the primary follicle is the formation of a zona pellucida, which surrounds the oocyte and is maintained throughout growth

until the oocyte is ovulated (Rankin, Familari et al. 1996; Rankin, Talbot et al. 1999; Rankin, O'Brien et al. 2001). The follicular cells are still arranged in a single layer to surround the oocyte and still changing shape to polyhedral or cubical. In parallel, beginning with the early phases of follicular development, the surrounding stromal cells start to aggregate concentrically around the follicular basal lamina and on time form the theca cells layer (Zamboni 1974; Motta, Makabe et al. 1994).

Conversion of primordial into primary follicles involves participation of genes and proteins that regulate organization of zona pellucida by synthesis of the extracellular zona pellucida proteins ZP1, ZP2 and ZP3 (Rankin, Talbot et al. 1999). Mice $Zp1^{-/-}$ have loosely organized zonae pellucidae and reduced litter sizes. $Zp2^{-/-}$ and $Zp3^{-/-}$ mice are sterile (Rankin, Familari et al. 1996; Rankin, O'Brien et al. 2001).

Forkhead genes have also been shown to play a role in folliculogenesis at the primary follicle stage. In $Foxl2^{-/-}$ ovaries, granulosa cell differentiation is blocked at the squamous to the cuboidal transition (Schmidt, Ovitt et al. 2004). Foxo3a ovaries have a tremendous increase in the number of early growing follicles with enlarged oocytes (Castrillon, Miao et al. 2003).

By the time that the follicle reaches the pre-antral stage, granulosa cells have proliferated to form multiple layers. In addition, the pre-antral follicle acquires an outer layer of thecal cells separated from the follicle by the basal lamina (van den Hurk and Zhao 2005). At the end of this stage, the pre-antral follicle has several layers of granulosa cells and has acquired an extensive network of gap junctions (Hirshfield 1991). These junctions are intercellular membrane channels that allow nutrients, inorganic ions, second messengers and small metabolites to pass from cell to

cell (Kidder and Mhawi 2002). The next step in the developmental sequence is formation of the antral cavity with fluid rich in water, electrolytes, serum proteins and with high concentrations of steroid hormones secreted by the granulosa cells (Hirshfield 1991). In some species, antral cavity formation is not accompanied by notable changes in oocyte morphology, while in others, the oocyte continues to expand during the early antral phase of development (van den Hurk and Zhao 2005). At the antral stage, most of the follicles will undergo atresia, whereas the remaining antral follicles will survive under the influence of FSH and grow to the pre-ovulatory stage (Hirshfield 1991). Factors such as the aryl hydrocarbon receptor (Ahr) and superoxide dismutase (SOD) have been shown to regulate follicular growth to the antral stage (Gupta RK 2006). The Ahr is a ligand-activated transcription factor that functions in mediating the toxicity of various contaminants (Pocar, Fischer et al. 2005). Without this gene, there is a decrease in the number of antral follicles and reduced fertility (Benedict, Lin et al. 2000; Benedict, Miller et al. 2003).

The follicles that survive the massive atresia visited on the preantral and early antral population acquire the capacity to produce estrogen and grow to the pre-ovulatory stage (Hirshfield 1991). They exhibit elevated estradiol concentration, higher granulosa cell number, and elevated LH binding capacity in comparison to non-estrogenic follicles (Ireland and Roche 1983). The pre-ovulatory follicle is capable of releasing the oocyte for fertilization, upon stimulation by LH (McGee and Hsueh 2000). Rupture of follicle basement membrane during ovulation is controlled by several different genes and proteins described in ovulation section (below). After the oocyte is released for fertilization, the remaining theca and granulosa cells differentiate into the CL (Hirshfield 1991; Elvin and Matzuk 1998; McGee and Hsueh 2000)

Folliculogenesis consists of two phases: The first, considered independent of gonadotropin influence, also called basal folliculogenesis, includes the passage from primordial follicle to primary follicle, and is regulated by growth factors. The second, gonadotropin dependent phase, includes follicle development to ovulation, and is also known as tonic folliculogenesis (Peluso, Luttmer et al. 1984; Hillier 1994). Follicular development is highly heterogeneous, due to the fact that follicles of the same size develop at different rates throughout the estrous cycle (Rajakoski 1960; Matton, Adelakoun et al. 1981; Driancourt, Thatcher et al. 1991).

In the complexity of follicular development, recruitment, selection and dominance also need to be considered (Matton, Adelakoun et al. 1981; Hodgen 1982; Goodman and Hodgen 1983). Recruitment considered as independent of gonadotropins, in which a group of follicles initiates growth and acquires the capacity to respond to gonadotropins. The follicles then depend on gonadotrophin support to continue its development; until reaching a preovulatory size (Fortune 1994). At this stage of follicular development not only is gonadotropic action (mainly FSH) necessary but there are also autocrine or paracrine influences, e.g growth factors such as epidermal growth factor (EGF; Dorrington, Bendell et al. 1987; Richards, Russell et al. 2002; Kaczmarek, Schams et al. 2005). Selection is the process by which few follicles, from the total recruited population, escape atresia and continue their development. Dominance is when one or more follicles are selected to undergo ovulation. Dominance may involve interference with follicles in less advanced development in the cohort of the recruited follicles, reducing their FSH support (Thatcher, Driancourt et al. 1991). This effect has been attributed to the secretion of estradiol and inhibin A, coming from the more developed follicles (Andersen and Byskoy 2006)

Specifically, inhibin and estradiol block FSH and favor the increase of LH receptor first in theca cells of tertiary follicles (McNatty, Fidler et al. 2000), an action that is inhibited by progesterone administration. Apparently, the inhibin-estrogen synergism favors LH receptors in granulosa cells and reduce FSH receptors in a feedback process (Lisk 1964; Ramirez, Abrams et al. 1964; Williams and Lipner 1981; Kaneko, Noguchi et al. 2002; Martin, Fogwell et al. 1991; Medan, Nambo et al. 2004; Pak, Chung et al. 2006) It is possible that the selection also occurs in response to the reduced response of small follicles to the FSH stimuli (Driancourt, Thatcher et al. 1991; Driancourt, Webb et al. 1991). Dominance is the mechanism by which one or two follicles (several in mice and pigs) are rescued from atresia (Hodgen 1982). In this process it is considered that the "dominant" follicle becomes hypersensitive to FSH action, which is regulated by autocrine and paracrine factors, such as insulin like growth factor-I (IGF-I) action, (Eden, Jones et al. 1988; Behl and Kaul 2002). Recently Murayama and co-workers suggested that insulin itself may support the maturation of preovulatory follicles and the insulin-induced increase in growth hormone receptor (GHR) in dominant follicles may be a turning point for follicles to enter the preovulatory phase during final follicular development (Shimizu, Murayama et al. 2008).

Differences among species exist. In most domestic animals, two or three waves of follicular activity occur during the estrous cycle (Rajakoski 1960; Savio, Keenan et al. 1988; Fortune 1994). In cattle the first wave has been identified at day 0 (ovulation day) and the second at day 10. During the first wave, the recruitment of several follicles has been observed, but only one reaches the dominant stage, initiating its regression at day 11 of the estrous cycle, this wave is known as non-ovulatory. As in the first wave, second wave is characterized by a varied

number of follicles that initiated to grow, nevertheless, only one becomes dominant and proceeds to ovulation, (for review Ireland, Mihm et al. 2000).

3. OVULATION

Ovulation is the rupture of the ovarian follicle to liberate the oocyte (Espey 1980). Initially, this process was considered to be regulated exclusively by increased follicular pressure; later, the enzymatic action for collagen lysis was added (Espey 1980). More modern concepts consider ovulation as a complex process whereby ovarian follicles reactivate oocyte meiosis, create a rupture pore in the apical follicle wall and initiate tissue restructuring and differentiation to form the CL (Russell and Robker 2007). This complex process is controlled by synchronous participation of endocrine hormones, as well as autocrine, paracrine and intracrine mechanisms from the oocyte, theca, mural and cumulus granulosa cells.

Preovulatory follicles contain two distinct sublineages of granulosa cells with highly different responses to gonadotropin action. Mural granulosa cells respond to LH surge due to greater receptor levels abundance relative to cumulus cells (Peng, Hsueh et al. 1991). Comparing follicle cell contents of LH receptors, theca cells express the highest quantity, while in mural and cumulus granulosa cells LH receptors increase during and after ovulation (Bukovsky, Chen et al. 1993; Goudet, Belin et al. 1999; Robert, Gagne et al. 2003).

The LH surge stimulates G protein-coupled receptors within the plasma membrane of cells in the ovarian follicles, and signals of adenylyl cyclase appear, increasing cyclic adenosine monophosphate (cAMP) which in turns regulates protein kinase A (PKA) (Richards 2001). After the PKA catalytic unit is activated its moves to the nucleus, where it phosphorylates a number of transcription factors such as CREB (Mukherjee, Park-Sarge et al. 1996; Yazawa, Mizutani et al. 2003). The interaction of phosphorylated CREB with the coactivator CREB-binding protein stimulates the expression of gonadotropin/ CRE-regulated ovarian genes (Mukherjee, Park-Sarge et al. 1996). This process is temporally associated with immediate transcriptional regulation of numerous genes, and is presumed to be involved in the synthesis and/or activation of specific proteases that degrade the follicle wall (Ohnishi, Ohnishi et al. 2005).

In recent years, molecular and transgenic techniques have allowed the integration of gonadotropin-regulated effects, steroids and prostanoids that regulate the complex gene cascade that produces ovulation (Robker, Russell et al. 2000; Richards, Russell et al. 2002).

3.1 Role of the granulosa layer during ovulation

There is extensive information about the changes occurring in the granulosa and theca cells during ovulation as well as the role of other follicular tissues, the oocyte and related epithelial, endothelial and connective tissues. Although some changes have their origin within the theca cell layer, or simultaneously within the theca and granulosa layers, the granulosa cell layer undergoes the greater alteration during ovulation, displaying changes as soon as the LH surge occurs, some of these initial changes include activation of the genes taking part in the inflammation, ovulation and cell remodeling processes (Espey 1980).

Within follicular tissue, one enzyme for which transcription is upregulated during the early stages of the ovulatory process is 5-aminolevulinate synthase (ALAS). ALAS increases within the granulosa layer as soon as 30 min after hCG, and declines once the mature ovarian follicles start to rupture (Espey 2006). ALAS expression is involved during the acute inflammatory phase response, one of the characteristics during ovulation, which is an abundance of overlapping pathways activated by tissue damage (Ferreira and Gong 1995; Lentsch and Ward 2000). ALAS has been associated with cytochrome P450scc during steroid metabolism. Also ALAS expression within the corpora lutea as it develops from the ruptured follicles is significant.

Initial changes during the first minutes after LH include the induction of genes for early growth response protein-1 (Egr-1) (Russell, Doyle et al. 2003). Egr-1 is a transcription factor that promotes expression of numerous genes important in inflammation, vascular hyperpermeability, hypoxia, coagulation, and other events associated with tissue damage (Espey, Ujioka et al. 2000; Yan, Fujita et al. 2000; Yan, Lu et al. 2000; Silverman, De Sanctis et al. 2001). In the mouse, Egr-1 mRNA increases in the granulosa layer within 30 min after hCG application, reaches a peak from 2-4 h, remains moderately elevated through 12 h after application, and declines 24 after hCG application (Espey, Ujioka et al. 2000; Russell, Doyle et al. 2003). On the other hand, epiregulin, a member of the epidermal growth factor (EGF) family, has been associated with Egr-1 mRNA expression, as well as with increases of cyclooxygenase 2 (COX-2) mRNA and protein expression (Sasaki, Pai et al. 1998). It has been observed that epiregulin expression presents irregular and temporal expression patterns (Sasaki, Pai et al. 1998; Shirakata, Komurasaki et al. 2000).

COX-2, the key enzyme for prostaglandin synthesis is a component of the ovulatory process (Richards, Russell et al. 1998). COX-2 plays a functional role in cumulus cell expansion and it is induced during the inflammation process, (Sirois and Richards 1993; Williams and DuBois 1996; Song, Sirois et al. 1998). COX-2 null mice do not ovulate (Morham, Langenbach et al. 1995); and cumulus cells do not expand, (Davis, Lennard et al. 1999). In the mouse, COX-2 mRNA, confined to the granulosa layer of the mature follicles, increases 4 h, and declines 8 h after hCG application, (Richards, Russell et al. 1998; Davis, Lennard et al. 1999; Joyce, Pendola et al. 2001). In vitro granulosa cells express COX-2 and VEGF (vascular endothelial growth factor) after stimulation with recombinant adiponectin involving MAPK pathway, indicating that these molecules act to produce tissue remodeling (Ledoux, Campos et al. 2006).

The inflammatory process characterizing ovulation is followed by the imminent elevation of toxic products, which must be neutralized (Buetler 1998). Hence, the granulosa cells trigger the expression of genes such as: g-glutamylcysteine synthetase (GCS), GCS mRNA is distributed within the mature follicle granulosa layer, even before hCG administration (Clague, Sevcik et al. 1992; Jarrell, Sevcik et al. 1992). GCS expression increases with hCG administration in mural granulosa cells, also, hCG permits the expression in theca interna that surrounding the granulosa cells. GCS expression is elevated between 4 and 8 h after hCG application, specifically in the theca tissue as well as in some localized areas of the ovarian stroma. GCS is a metalloprotein that serves as the rate limiting enzyme for synthesis of glutathione. Glutathione is a tripeptide that protects cells against oxidative stress during the acute inflammatory reactions and other conditions promoting cellular damage (Clague, Sevcik et al. 1992; Buetler 1998; Soltaninassab, Sekhar et al. 2000).

Also in mice at 4-8 h after hCG, tumor necrosis factor-stimulated gene-6 (TSG-6) is up-regulated in granulosa cells and goes throughout granulosa cumulus cells 12h after hCG (Yoshioka, Ochsner et al. 2000). TSG-6 is a common component of inflammatory reactions, and may exert a negative feedback action by neutralizing hyaluronan-induced inflammatory stress on the extracellular matrix of the cumulus cell-oocyte complex, during the ovulatory process (Fulop, Kamath et al. 1997; Yoshioka, Ochsner et al. 2000). At the same time, but in different location, the regulator of G-protein signaling protein-2 (RGS2) is expressed, along the antral border of the mural granulosa as well as within the cumulus mass surrounding the oocyte (Ujioka, Russell et al. 2000). RGS2 has been characterized as a GTPase-activating protein, thought to attenuate cell signaling by hydrolyzing GTP to GDP (Ujioka, Russell et al. 2000).

Follicular preparation for oocyte release is accompanied by changes from estradiol synthesis to acute progesterone synthesis, requiring the presence of genes involved in the steroidogenesic process as progesterone progesterone receptors (PR) presence and activity, (Robker, Russell et al. 2000). Progesterone action in ovary regulates follicular granulosa cells during ovulation by increasing its levels and by decreasing in estradiol levels, regulating mitotic or apoptotic activities. Other progesterone actions are regulated by membrane progesterone receptors that decrease cAMP with subsequent inhibition of mitosis (Peluso 2006). Progesterone action during ovulation has been clarified using progesterone receptor null mice that display CL formation with no follicular rupture (Lydon, DeMayo et al. 1995). In addition, anovulation in progesterone receptor null mice has been related with decrease in ADAMTS and cathepsin L, both regulated by FSH and LH in a PR-dependent manner (Robker, Russell et al. 2000). ADAMTS is a protein with strong proteolytic effects and believed to act in follicular wall

degradation, while cathepsin L is a papain family also with proteolytic effects (Robker, Russell et al. 2000). Another element is endothelin which is expressed in granulosa cells and decreases in animals with blockage in progesterone receptors (Palanisamy, Cheon et al. 2006).

A rate limiting factor in steroidogenic activity is maintained is transport of cholesterol into mitochondria by StAR (Ronen-Fuhrmann, Timberg et al. 1998; Chaffin, Dissen et al. 2000). It is coexpressed with adrenodoxin, whose transcript, increases significantly in granulosa cells 4 h after hCG administration, and continues in postovulatory luteinized tissue (Espey and Richards 2002). Adrenodoxin and StAR are also are co-expressed with cytochrome P450scc (Rodgers, Lavranos et al. 1995; Grinberg, Hannemann et al. 2000; Muller, Lapko et al. 2001). StAR mRNA has an initial peak after hCG and a second higher peak once the corpora lutea are synthesizing substantial amounts of progesterone (Ronen-Fuhrmann, Timberg et al. 1998; Chaffin, Dissen et al. 2000).

The presence of 3α-hydroxysteroid dehydrogenase (3α-HSD) is necessary to catalyze the oxydoreductive processes during steroid production (Penning, Pawlowski et al. 1996; Ma and Penning 1999). In rats, 3α-HSD mRNA is detectable within theca interna cells of mature follicles before hCG stimulation, and the protein is translocated to the granulosa layer 2 h after hCG application and continues to be elevated until 8 h after hCG application, declining thereafter, although a limited amount still remains within luteal tissue (Penning, Pawlowski et al. 1996; Espey, Yoshioka et al. 2001; Espey and Richards 2002).

Ovulation requires degradation of follicular wall by the action of proteolytic enzymes that include disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS-1) and cathepsin L for proteolysis of extracellular matrix (Robker, Russell et al. 2000). ADAMTS-1 which increase significantly in the granulosa layer by 4 h after hCG application and reaches a peak at12 h after hCG application (Espey, Yoshioka et al. 2000), at the time when rat follicles begin to rupture. ADAMTS-1 transcription depends on the usual increase in ovarian progesterone synthesis and on the up-regulation of ovarian progesterone receptor during ovulation (Doyle, Russell et al. 2004; Porter, Clark et al. 2005; Shozu, Minami et al. 2005). Cathepsin L is induced in granulosa cells of growing follicle by FSH, but the highest levels of cathepsin L mRNA occur in preovulatory follicles in response to LH (Robker, Russell et al. 2000).

3.2 Role of theca the layer during ovulation

As mentioned previously, the expression of genes at the moment of the ovulation is not exclusive to the granulosa cell layer, as many changes occur simultaneously in theca and granulosa layers (Fig.1).

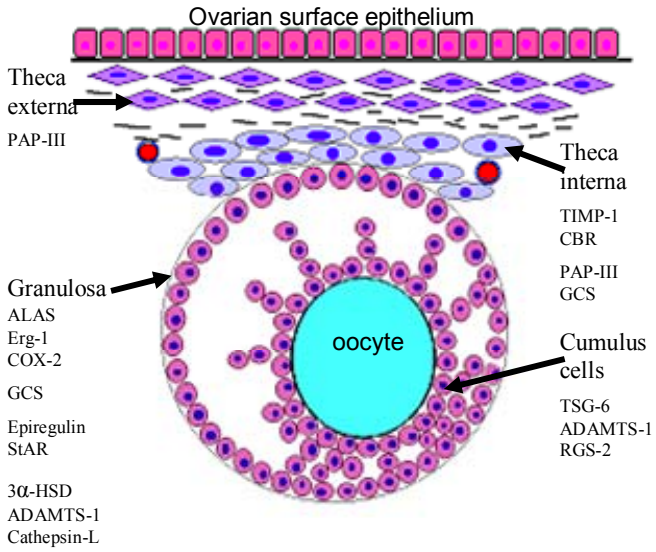

Figure 1. Principal candidate genes involved in ovulation: They are listed according to the follicular compartment; ALAS= amino levulinic acid synthetase, Erg-1= , CoX-2= Cyclooxygenase-2, GCS= γ-glutamilcystein synthetase, epiregulin, StAR (steroidogenic acute regulatory protein), 3a-HSD (3-alpha-hydoxysteroid-dehydrogenase) ADAMST-1(Disintegrin and metalloproteinase with trombospondin motifs), Cathepsin-L, TIMP-1 (Tissue inhibitor of metalloproteinase-1), CBR (Carbonyl reductase), PAP-III (Pancreatitis-associated protein-III), TSG-6 (Tumor necrosis factor-stimulated gene-6), RGS-2 (Regulator of G-protein signaling protein-2) (Adapted from Ohnishi, Ohnishi et al. 2005).

During ovulation, theca cells express multiple matrix metalloproteinases (MMPs) including MMP-1, MMP2, MMP9, MMP14 AND MMP19 (Hagglund, Ny et al. 1999). At least one metalloproteinase-1 inhibitor (tissue inhibitor of metalloproteinase-1 or TIMP-1) mRNA is expressed on a temporal pattern basis. Similar to ADAMTS-1 mRNA, it is present within both thecal and stromal ovarian tissue and increases at the time of ovulation (Simpson, Byers et al. 2001). During ovulation, TIMP-1 regulates ADAMTS-1 degradation as well as other ovarian metalloproteinases (Espey, Ujioka et al. 2003). In the mouse, TIMP-1's protective mechanism results in the presence of carbonyl reductase (CBR) mRNA, detectable before hCG, and with an increase over 2 h after hCG, reaching a peak at 8 h, and declining 24 h after hCG treatment (Espey, Yoshioka et al. 2000). Its spatial distribution is mainly within thecal and stromal regions; however, the granulosa layer shows a little expression (Espey, Yoshioka et al. 2000). CBR increases during ovulation, it might function as a local protective response to the substantial steroid and prostanoid increases within the interstitial tissue of the ovary (Inazu and Fujii 1998; Espey, Yoshioka et al. 2000).

During ovulation, blood vessels degradation may be controlled by pancreatitis-associated protein-III mRNA (PAP-III) that is expressed 8 h after hCG administration in mice (Yoshioka, Fujii et al. 2002), and limited to the hilar region of the ovarian stroma. It is principally found within endothelial cells limiting the inner walls of blood vessels. PAP-III is a macrophage chemoattractant induced in and released from injured tissue (Heller, Fiedler et al. 1999). Ovarian PAP-III mRNA might be expressed in conjunction with a protective response to the hyperemia, exudation, proteolysis, and inflammation characterizing the ovulation event (Yoshioka, Fujii et

al. 2002; Namikawa, Okamoto et al. 2006). As previously noted, theca cell action at ovulation, has not been elucidated; however, Richards et al., have proposed that the theca cell layer may serve as protective anti-inflammatory barrier to prevent inappropriate release of oocytes (Richards, Russell et al. 2002).

4. PROLIFERATION AND DIFFERENTIATION

During and following ovulation, changes at cell cycle activity occur and culminate with final cell differentiation, characterized by arrest in cellular proliferation. These changes include several molecular cascades within the luteal cells. For such a reason, this section attempts to briefly describe the aspects related to these changes, focused on cell proliferation *versus* cell differentiation.

4.1 Hormonal aspects in proliferation versus differentiation

It has been well documented that FSH and estradiol are mitogenic factors, whereas the LH surge terminates granulosa cell proliferation and initiates differentiation by inverting the action of estradiol and FSH over the positive regulators of cell cycle progression in follicular cell types (Richards JS 1980; Rao MC 1978). This action depends on hormones binding to specific receptors and activation of the cAMP and PKA pathway (Jonassen and Richards 1980). Estradiol regulates cell proliferation by inducing Cyclin D1 expression as well as decreasing cdk inhibitor levels (Foster and Wimalasena 1996; Altucci, Addeo et al. 1997). In granulosa cells, estradiol induces cyclin D2 and cyclin E expression, with concurrent p27 reduction (Robker and Richards 1998). Other molecules regulating cell cycle include: a) activin, produced in high levels within preovulatory granulosa cells, has been shown to stimulate DNA synthesis (Miro and

Hillier 1996) b) Insulin-like growth factor-1 (IGF-1) is also implicated in granulosa cell proliferation (Zhou, Refuerzo et al. 1995). Studies in IGF-1 null mice showed that large antral follicles are present within ovaries, indicating that IGF-1 may be more important for differentiation than for proliferation (Baker, Hardy et al. 1996; Chaffin, Schwinof et al. 2001).

4.2 Cell cycle control

During follicular development follicular cells are highly proliferative (Rao, Midgley et al. 1978; Hirshfield 1991), a characteristic that is diminished or lost toward luteinization (Richards, Hedin et al. 1986; Richards 1994). As mentioned before, estradiol activates events that favor proliferation (Hampl, Pachernik et al. 2000; Cannon, Cherian-Shaw et al. 2005). Early, during the G1 phase, D-type cyclin activates cyclin dependent kinase 4 and 6 (cdk4 or cdk6); later, within G1 phase, cyclin E accumulation activates cdk2 (Koepp, et al 2001). Progression through S phase is regulated by cooperation of cdk4/6 and A/D-type cyclins with cdk2/cyclin E complexes (Koff, Cross et al. 1991; Lew, Dulic et al. 1991) followed by the initiation of M phase by cyclin B-cdk2 complexes (Lew, et al. 1996). The S-phase is regulated by cyclin/cdk binding, resulting in a complex requiring phosphorylation to activate, in turn, cellular substrate phosphorylation such as retinoblastoma protein (pRb) which further releases the transcription factor E2F, regulating DNA synthesis and gene expression (Inaba, Matsushime et al. 1992; Xiong, Menninger et al. 1992; Xiong, Zhang et al. 1992; Stevaux and Dyson 2002). Cyclin D2-null mice display impaired granulosa cell proliferation, underdeveloped ovarian follicles, and lack of ovulation (Sicinski, Donaher et al. 1996).

Arrest of the cell cycle in G1 phase with the subsequent CL formation is the result of inhibitors of cell cycle kinases, such as Cip/Kip (p21Cip1, p27Kip1, p57Kip2), as well as Ink4 family, another family of cdk inhibitors (Sherr and Roberts 1999). The Cip/Kips have relatively broad specificity and are able to bind cdk4/6 and cdk2, enabling them to inhibit the activity of several kinase cascades, thereby blocking the cell cycle progression at multiple points, in particular at the G1 phase (Polyak, Lee et al. 1994). Both p27 and p21Cip1, are highly expressed in the corpus luteum, showing slight different patterns of induction (Robker and Richards 1998). Loss of p27 results in impaired differentiation, as has been observed by the inability of granulosa cells to luteinize normally, and produce enough progesterone in order to support pregnancy (Fero, Rivkin et al. 1996).

Analysis of the cell cycle regulators after application of hCG or the LH surge showed that cyclin D2 and p27 are regulated inversely (Robker and Richards 1998). During luteinization, there is down-regulation of cyclin D2 and while p27 is up-regulated, leading to the cell cycle arrest at the G1 phase and at 24h of hCG addition or LH surge (Fero, Rivkin et al. 1996; Nakayama, Ishida et al. 1996). Using *in situ* hybridization in hormone-treated-hypophysectomized rats, it has been shown that cyclin D2 mRNA expression increases and p27 mRNA expression decreases in granulosa cells (Uilenbroek and Richards 1979; Richards 1980). Specifically, in rats treated with estradiol and FSH, cyclin D2 mRNA and protein are expressed at high levels within granulosa cells of preovulatory follicles (Robker and Richards 1998). However, different types of granulosa cells display different proliferative responses: the cells next to the oocyte maintain their mitotic capacity beyond the beginning of luteinization (24h after PMSG), in comparison with mural granulosa cells (Cannon, Cherian-Shaw et al. 2005).

In summary, the major change observed in granulosa cells during the peri-ovulatory transition after gonadotropin action is cdk2 down-regulation, produced by cell cycle inhibitors with a consequent arrest in proliferation.

5. LUTEINIZATION

As described above, follicular cells undergo morphological and biochemical changes in response to the preovulatory surge of LH, resulting in formation of a transitory endocrine ovarian gland, the CL (Zeleznik and Somers 1999). The CL results from a final follicular cell differentiation, termed luteinization, with a shift in the secretion of estradiol to progesterone, an important regulator of the length of reproductive cycle and base for pregnancy maintenance (Uilenbroek 1985; Keyes and Wiltbank 1988; Zeleznik and Somers 1999; Niswender, Juengel et al. 2000; Murphy, Gevry et al. 2001).

During CL formation, one of the major changes is the vasculization of the granulosa cells via elements that invade the follicle from the theca compartment (Bruce and Moor 1976). This invasion also carries the theca cells in a centripetal fashion, and subsequently throughout the CL by lateral branching of centripetal veins and arteries. This is the mechanism by which the steroidogenic theca cells are dispersed throughout the corpus luteum and is a process facilitated by angiogenic factors (Murphy, Gevry et al. 2001).

5.1 Corpus luteum structure

The CL is a heterogeneous gland composed of small and large steroidogenic luteal cells, fibroblasts, endothelial, pericytes, and immune cells (Channing 1969; Channing 1969). These

cells have different morphological, endocrine, and biochemical features. Interactions between the corpus luteum cell components are essential for the maintenance of health and steroidogenic function of the CL (Nelson, McLean et al. 1992; Davis, Rueda et al. 2003; Townson and Liptak 2003). Once luteinization takes place, a defined cell population undergoes extensive hypertrophy and differentiates into large steroidogenic luteal cells, whereas another cell population remains much smaller and comprises small steroidogenic luteal cells (Fitz, Mayan et al. 1982). The primate corpus luteum consists of luteinized granulosa cells, which may correspond to the large steroidogenic luteal cell population in other species (Murphy 2000) and comprise from 25% to 35% of the CL's total volume. The primate luteinized theca cells make up from 12% to 18%. The rest of the CL includes blood vessels 11%, connective tissue 22% to 29%, and fibroblasts 7 to 11% (Gillim, Christensen et al. 1969; Niswender, Juengel et al. 2000; Wulff, Dickson et al. 2001; Davis, Rueda et al. 2003).

The large luteinized cells and the small luteinized cells not only differ in cell population number but also in structure, function and response to different hormonal stimuli (Hoyer and Niswender 1985). Large luteal cells possess abundant mitochondria, rough endoplasmic reticulum, lipid droplets, as well as secretory granules. Large luteal cells contain oxytocin (cow and ewes) and relaxin (rat, pig and cow Taylor and Clark 1993; Bathgate, Moniac et al. 1999). In the mouse, these cells are characterized by a small round nucleus, spherical shape, and abundant cytoplasm (Galosy and Talamantes 1995). In the rat, large luteal cells undergo a dramatic increase in protein content with luteal development, a result of hypertrophy (Gillim, Christensen et al. 1969; Wulff, Dickson et al. 2001) . There is a concomitant increased capacity in the large cells to produce steroids with direct result of the enhanced expression of sterol carrier

protein 2 (SCP2), P450scc, adrenodoxin and adrenodoxin reductase proteins, specifically required to transport and process cholesterol for steroid production (McLean, Nelson et al. 1992).

In contrast to large luteal cells, small luteal cells show less cytoplasm and large oval nuclei (Galosy and Talamantes 1995), moderate mitochondria number, greater amounts of smooth endoplasmic reticulum and ribosomes, minor amounts of Golgi apparatus and no lipid droplets and secretory grains (O'Shea, Wright et al. 1987; Kenny, Farin et al. 1989). These cells produce approximately 15% of the basal progesterone secreted by the CL and have LH receptors (O'Shea, Wright et al. 1987).

In ruminants and rodents, small and large luteal cells differ in many aspects: (a) basal rates of progesterone secretion, with large cells producing more than 80% of the progesterone, (b) an absence of LH receptors, but presence of growth hormone in large luteal cells (Lucy, Collier et al. 1993; Webb, Woad et al. 2002) and PGF2α receptors (Meidan, Girsh et al. 1990), (c) large luteal cells express cycloxygenase 2 (COX-2) in response of PGF2α and participate in luteolysis (Tsai and Wiltbank 1997).

5.2 Corpus luteum classification

Classification of CL is based on a) the female reproductive condition, b) duration of CL or life span and c) progesterone secretion. In the first classification four CL types have been reported in reproductive mammals: 1) normal cycle CL, 2) pregnancy CL, 3) lactation CL, and 4) pseudopregnancy CL. According to its life span, CL development is divided in three phases. In the growing phase, there is hypertrophy of luteal cells. Between estrous cycle days 4-7 in the

cow, the corpus luteum increases in weight and size, acquiring a great capacity to react to LH, causing increases in progesterone secretion. (Kesler, Weston et al. 1981). During the maintenance phase, progesterone is synthesized and secreted by steroidogenic cells. During the regressive phase, there is morpho-physiological death of the endocrine gland (Rothchild 1981). Subnormal corpus luteum function has been associated with infertility and incapacity to maintain gestation. A subnormal corpus luteum function can display short duration of persistence, or of normal duration but low progesterone production (Garverick, Parfet et al. 1988). In ewes, both normal and abnormal CL development are similar until day 4 post-ovulation; nevertheless, at day 5, abnormal CL diminishes in weight and progesterone secretion and regresses during day 6 (Hunter, Southee et al. 1988) .

Abnormal CL may result from a deficiency during the final phases of follicular maturation. Braden et al., (Braden, King et al. 1989) demonstrated that abnormal CL originate from follicles showing more granulosa cells, fewer LH and FSH receptors, and less 17β-estradiol (E2) concentration, in comparison with follicles forming normal corpora lutea (Niswender, Juengel et al. 2000).

5.3 Corpus luteum regulation

It has been known for some time that maintenance of CL depends of luteotropic hormones such as LH (Kaltenbach, Graber et al. 1968; Denamur, Martinet et al. 1973), hCG, and others such as prolactin, E2, prostaglandin, estrogens and hormones derived from maternal or fetal tissues. The importance of these substances depends on the species. LH is considered as the most important luteotropin (Keyes and Wiltbank 1988; Berisha and Schams 2005). One effect of LH as luteotropic hormone is through the activation of protein kinases to produce cyclic AMP

response element binding (CREB) phosphorylation (Mukherjee, Park-Sarge et al. 1996; Yazawa, Mizutani et al. 2003). The interaction of phosphorylated CREB with the coactivator CREB-binding protein (CBP) stimulates the expression of ovarian genes, but its cAMP dependence is lost when luteinization is initiated (Wu and Wiltbank, 2002). Nevertheless, phosphorylation of CREB is higher in luteal cells than in granulosa cells, suggesting that other kinases such as mitogen-activated protein kinase (MAPK) could be implicated (Gonzalez-Robayna, Alliston et al. 1999; Alliston, Gonzalez-Robayna et al. 2000; Hazzalin and Mahadevan 2002).

In some species, such as the rat, rabbit, and pig, estrogens are luteotropic (Stormshak, Zelinski-Wooten et al. 1987). In the rabbit, estradiol has been considered the primary luteotropic hormone (Robson 1937) and LH plays a secondary role. In the rat, estradiol and prolactin appear to be directly involved in stimulation of progesterone secretion. Similarly prolactin is essential to maintain expression of estradiol and LH receptors, and LH stimulates synthesis of estradiol from the corpus luteum. In the pig, the luteotropic role of estrogen is less defined, but the CL growth and increase in progesterone secretion are attributed to the estradiol action (Conley and Ford 1989). Other luteotropic hormones reported are growth hormone (GH) (Liebermann and Schams 1994) and IGF-I (Constantino, Keyes et al. 1991; Parmer, Roberts et al. 1991; Sauerwein, Miyamoto et al. 1992) that increased secretion of progesterone from luteal tissue. GH receptor mRNA has been identified in ovine, bovine, and rat luteal tissue (Carlsson, Nilsson et al. 1993; Lucy, Collier et al. 1993; Juengel, Nett et al. 1997). Growth hormone could have a direct effect on luteal function by increasing secretion of progesterone and oxytocin (Liebermann and Schams 1994). In addition, GH may influence luteal function indirectly by increasing expression of IGF-I (Parmer, Roberts et al. 1991; Obasiolu, Khan-Dawood et al. 1992; Juengel, Nett et al. 1997).

Luteal tissues of primates and domestic animals express IGF-I mRNA and protein (Talavera and Menon 1991; Obasiolu, Khan-Dawood et al. 1992; Perks, Denning-Kendall et al. 1995). IGF-I is thought to activate intrinsic tyrosine kinases in luteal cells, to increase secretion of progesterone (Chakravorty, Joslyn et al. 1993). Binding of IGF-I stimulates phosphorylation of insulin receptor substrate 1 and increases the activity of phosphoinositide 3-kinase. This kinase produces the novel phosphoinositide phosphatidylinositol 3-phosphate, acts as a second messenger (Chakravorty, Joslyn et al. 1993). This substance appears to influence modification of the cytoskeleton and may be involved in prevention of cellular death and apoptosis (Lucy, Collier et al. 1993; Davis, May et al. 1996).

The PGs are of particular interest, because of the involvement of PGF2α in regression of the CL in many species. Production of PGF2α directly by luteal cells has been reported in cows (Milvae and Hansel 1983; Townson and Pate 1994; Townson and Pate 1996) pigs (Guthrie, Rexroad et al. 1978; Diaz, Crenshaw et al. 2000), rhesus monkeys (Johnson, Ottobre et al. 1988), sheep (Rexroad and Guthrie 1979; Tsai and Wiltbank 1997), and rats (Olofsson, Norjavaara et al. 1992). Luteal PGF2α production can be regulated by numerous hormones, including progesterone, uterine PGF2α, and cytokines such as tumor necrosis factor-α(TNFα), interleukin-1b (IL1b) and endothelin (ET-1). On the other hand, PGF2α might play a paracrine role in the modulation of luteolytic cascade and can induce strong vasoconstriction, thereby reducing the blood supply to the CL during spontaneous luteolysis (Shirasuna, Asaoka et al. 2004).

Stimulation of CL also has been proposed by prostaglandins E and I (PGE and PGI) (Milvae and Hansel 1980; Milvae and Hansel 1983). These prostaglandins are produced in higher amounts in the early luteal phase than the late luteal phase and thus are proposed to play a role in luteal development (Milvae and Hansel 1983). In cows, ewes, and humans PGI2 also increased secretion of progesterone (Fitz, Hoyer et al. 1984; Alila, Corradino et al. 1988; Bennegard, Hahlin et al. 1990; Graves, Pierce et al. 1995). In humans, treatment of luteal cells with PGI2 was shown to increase cAMP accumulation, suggesting that PGI2 may increase secretion of progesterone through activation of PKA (Fitz, Hoyer et al. 1984; Bennegard, Hahlin et al. 1990). However the intracellular mechanism by which PGI2 increases secretion of progesterone is not clear.

PGE2 also has been shown to increase progesterone production from luteal cells. These prostaglandins presumably have similar actions as PGI, increasing cAMP and PKA with the final gene transcription (Marsh 1976). Mamluk et al (Mamluk, Defer et al. 1999), have shown that physiological concentrations of Ca2+ ions (up to 5 mM) significantly stimulated cAMP production in luteal cells; and that the effects of Ca2+ on cAMP synthesis and adenyl cyclase (AC) activity. These data may delineate the cross-talk between physiological activators of AC in the CL (such as LH, PGE2, and PGI2) and other ligands (such as PGF2α and endothelin-1), which indirectly modulate AC activity.

Similarly, progesterone seems to be a key factor for adequate CL development, due to the fact that progesterone receptor knockout mice are infertile due to failure of ovulation (Mulac-Jericevic, Mullinax et al. 2000; Drummond 2006). The progesterone receptor inhibitors RU486

and ZK98299 blocked LH-stimulated luteinization of granulosa cells isolated from preovulatory follicles and RU-486 inhibited progesterone action and reduced 3α hydroxysteroid dehydrogenase activity (Dimattina, Albertson et al. 1986; Natraj and Richards 1993).

Cytokines are also potent stimulators of luteal PGF2α production. IL1b and TNFα dramatically stimulated PGF2α production by cultured bovine luteal cells (Nothnick and Pate 1990; Benyo and Pate 1992; Townson and Pate 1994). The acute stimulation of PGF2α production has been postulated to occur through activation of cPLA2 (Townson and Pate 1994; Townson and Pate 1996). In human luteinized granulosa cells IL1b was also found to increase prostanoid synthesis, increase Cox-2 mRNA synthesis, and decrease Cox-2 mRNA degradation (Narko, Ritvos et al. 1997). Treatment with interferon-τ has also been found to stimulate luteal PGF2α production (Fairchild and Pate 1991). Of particular importance, all of the stimulatory effects of these cytokines on PGF2a production could be inhibited by simultaneous treatment with progesterone (Nothnick and Pate 1990; Townson and Pate 1996). Although cytokines increase PGF2α production, it does not appear that PGF2α production is required for cytokine-mediated inhibition of progesterone production or induction of cell death (Fairchild and Pate 1991; Petroff, Petroff et al. 2001).

As mentioned before, ET-1 is also critical in luteolysis (Milvae 2000). Treatment with ET-1 decreases progesterone production in bovine luteal cells in vitro or in vivo, an effect blocked by a specific ET receptor antagonist (Girsh, Milvae et al. 1996; Hinckley and Milvae 2001). Additionally, ET-1 treatment induces PGF2α production by luteal cells (Girsh, Milvae et al. 1996; Miceli, Minici et al. 2001). Thus, ET-1, as well as other cytokine peptides, has complex but critical relationships with PGF2α and progesterone during luteolysis.

5.4 Gene expression in the corpus luteum

Follicular cell transformation into luteinized cells occurs few hours after LH surge, during which there are important genomic changes allowing terminal differentiation also known as luteinization. The purposes of these changes are to arrest cell division and preparing cells for different hormonal secretions. Endocrine/paracrine factors up-regulate genes to initiate and maintain corpus luteum structure and function. These include StAR, PR, ERα, and corticotropin releasing hormone binding protein (CRHBP) (Xu, Stouffer et al. 2005). Similarly, signal transduction factors including CCAAT/enhancer binding proteins (C/EBPβ) are important regulators of luteinization (Sterneck, Tessarollo et al. 1997). While transcripts implicated in gene up-regulation such as LIM domain only 7 (LMO7); cell growth/proliferation factors; as well as genes implicated in tissue remodeling and toxicity e.g. glutathione S-transferase (GST). GST is a soluble enzyme catalyzing unsaturated carbonyls (Hayes and Strange 2000; Al-Gubory, Ceballos-Picot et al. 2005); GST is believed to exert a critical role in cellular protection against oxidative stress and toxic foreign chemicals. They detoxify a variety of electrophilic compounds (Hayes and Strange 2000). In addition to GST action, reduction in toxicity will be favored by metallothionein (MT-1) secretion. These substances are a family of metal binding proteins believed to participate in a cellular defense detoxifying agents of other reactive metals and free radicals (Thornalley and Vasak 1985; Liu, Kershaw et al. 1991; Liu, Liu et al. 1995). It has been speculated that MT-1 functions to regulate steroid synthesis and/or with inflammatory processes (Miles, Hawksworth et al. 2000; Espey, Ujioka et al. 2003).

6. CHOLESTEROL IMPORTATION INTO STEROIDOGENIC TISSUE

Cholesterol is the precursor molecule of steroid hormones, and steroidogenic tissue acquires and synthesizes this molecule. The *de novo* production of cholesterol by steroidogenic cells is achieved by endogenous synthesis from acetate (Carr, MacDonald et al. 1980; Lehoux and Lefebvre 1980), with rate limitation by HMG-CoA reductase. Cells import cholesterol from circulating lipoprotein (Brown and Goldstein 1979; Gwynne and Strauss 1982; Glass, Pittman et al. 1983; Glass, Pittman et al. 1983); and cholesterol can be acquired by hydrolysis of cholesterol ester stored in cytoplasmic lipid droplets (Reaven, Tsai et al. 1996). Figure 2, is adapted from Azhar and Reaven (Azhar and Reaven 2002) and presents the sources of cholesterol supply to cells.

6.1 LDL cholesterol importation

Low density lipoprotein importation is an important method of supplying cholesterol to cells. The following concept is based on a series of reviews on the subject (Brown and Goldstein 1979; Schneider, Beisiegel et al. 1982; Mahley and Innerarity 1983; Brown and Goldstein 1986). LDL is a plasma lipoprotein particle of which its lipid component includes cholesterol, triglycerides and apolipoproteins. In humans, LDL originates from very-low density lipoprotein (vLDL) produced by the liver with the apoprotein B-100. The vLDL is converted to LDL by the action of lipoprotein lipase, an enzyme that hydrolyzes triglycerides in vLDL and releasing free fatty acids.

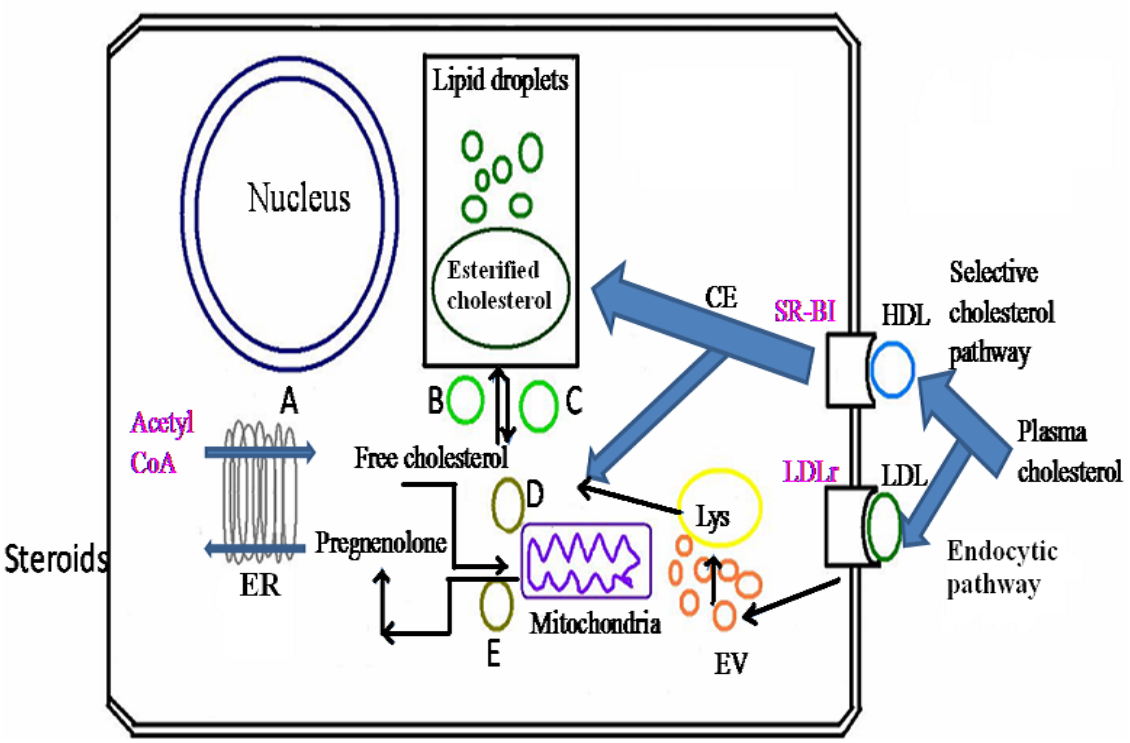

Figure 2. Principal sources of cholesterol, and key enzymes involved in cholesterol metabolism by steroidogenic tissue for steroid biosynthesis. HDL**,** high- density lipoproteins; SR-BI, scavenger receptor class B type I; LDL, low density lipoproteins; LDLr, low density lipoproteins receptors; CE, cholesteryl ester;; CS, Cytoskeleton; EV, Endocytic vesicles; Lis, lysosomes. **A** HMG CoA redustase. **B** Acyl-Coenzyme A: cholesterol acyltransferase (ACAT). **C** Cholesteryl ester (CE) hydrolase. **D** putative cholesterol transport factors (SAP, steroidogenic activator polypeptide; SCP, sterol carrier protein; StAR, steroidogenic acute regulatory protein; PBR, peripheral-type benzodiazepine receptor). **E** P450scc (Azhar and Reaven 2002).

The removal of triglycerides from vLDL by lipoprotein lipase leaves a greater proportion of cholestrol, decreasing the density of the particle. One means of cellular cholesterol intake is by LDL endocytic pathway in which apolipoproteins Apo-B or Apo-E are recognized by the LDL receptor (LDLr), via clathin-coated pit-mediated endocytosis, in which, the entire lipoprotein is internalized in the pits and originate the coated vesicles going to the lysosomes to be degraded, releasing free cholesterol, available to be transported to the mitochondria where it is converted to steroids molecule or other cellular metabolites. The autosomal recessive hypercholesterolemia protein, a cytosolic polypeptide with a phosphotyrosine-binding domain was identified as regulator for clathrin-coat pits and LDLr-mediated endocytosis (Mishra, Watkins et al. 2002). Its presumed influence on LDLr activity is by binding to the NPYX endocytic motif of the cytoplasmic region of the receptors (Garcia, Wilund et al. 2001). Transcriptional regulation of the LDLr pathway helps ensure cellular cholesterol homeostasis via membrane-bound transcription factors known as sterol regulatory element (SRE)-binding proteins (SREBPs; Brown and Goldstein 1999). Liver and steroidogeneic tissues express this transcription factor, where about 70% of the LDLr activity is concentrated. However, whether LDL serves as a source of cholesterol for luteal steroidogenesis, appears to be species-dependent (Brannian and Stouffer 1993).

6.2 HDL cholesterol importation

The HDL receptor, SR-BI, mediates the selective uptake of cholesterol and is highly expressed in cholesterol dependent cells and in steroidogenic cells where SR-BI can influence plasma HDL cholesterol levels, storage, and utilization of cholesterol in steroid hormone-producing cells (Azhar, Nomoto et al. 1998; Li, Peegel et al. 1998). SR-BI brings cholesterol

ester into the cells, by a mechanism that does not involve internalization of ApoAI or ApoAII, the two major protein components of HDL (Sparrow and Pittman 1990; Johnson, Mahlberg et al. 1991; Connelly, Kellner-Weibel et al. 2003). In a process called "selective lipid uptake" observations by ultrasensitive fluorescence microscopy found that HDL particles were endocytosed as clusters and actively transported to the perinuclear region of the cell and the uptake of HDL by an acidic compartment in the Golgi apparatus with a final re-secretion of the HDL particles (Rhode, Breuer et al. 2004). These results were supported by a study using electron microscopy and 125I-labeled HDL (Pagler, Rhodes et al. 2006).

6.3 Generalities about SR-BI

Scavenger receptor class B, type I, SR-BI, is a 509-residue, 82-kDa integral membrane cell surface glycoprotein of the CD36 superfamily that was the first HDL receptor to be characterized in detail (Calvo and Vega 1993; Acton, Scherer et al. 1994). Protein expression was confirmed by Western blot analyses with human SR-BI polypeptide antibodies to show a major 83-kDa band. Confocal immunofluorescence microscopy demonstrated that hSR-BI immunoreactive mass was detectable in a heterogeneous, punctate staining pattern (Hirano, Yamashita et al. 1999).

The chromosomal locations of the rat SR-BI gene and its human homolog, the CD36- and LIMPII analogous-1 (CLA-1) gene, were determined by in situ hybridization, the rat gene was mapped between bands q15 and q16 on rat chromosome 12 (RNO12q15–16), and the human locus, determined by somatic cell hybrids, has been assigned to chromosome 12 between bands 12q24.31 and q24.32 (Johnson, Svensson et al. 1998). The SR-BI gene contains 13 exons, most

of which are small (less than 204 bp) (Cao, Garcia et al. 1997). In the porcine endometrium, SR-BI gene was mapped to 46.3 cM on chromosome 14, and these results show that SR-BI chromosome localization differs among species (Kim, Vallet et al. 2004). There is close identity to hamster, mouse, rat and human sequences (Rajapaksha, McBride et al. 1997), thus SR-BI presents a short cytoplasmic N-terminus of 9 amino acids followed by a first transmembrane domain of 22 amino acids, the extracellular domain of 408 amino acids, the second transmembrane domain of 22 amino acids, and the cytoplasmic C-terminus of 47 amino acids, see fig. 3 (Han and Lee 2002). There is a leucine zipper motif and a peroxisomal targeting sequence (Johnson, Svensson et al. 1998).

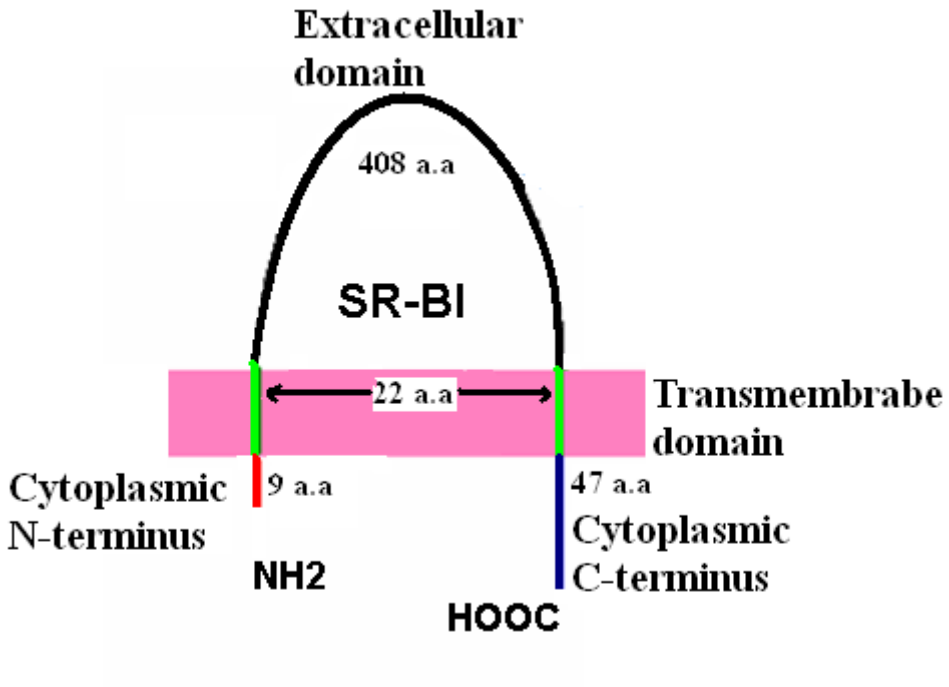

Figure 3. Protein domains and configuration of SR-BI inserted in the plasma membrane.

6.4 SR-BI intracellular location

Immunolocalization at the ultrastructural level showed that SR-BI was associated with microvilli and microvillar channels of the luteal cell surface, suggesting that microvilli and microvillar channels represent a cell surface compartment for the selective uptake of lipoprotein cholesterol into steroidogenic cells. (Reaven, Nomoto et al. 1998). Another structural protein, caveolin, is involved in SR-BI transfer of cholesterol (Azhar, Nomoto et al. 1998; Azhar, Luo et al. 1999), althougth caveolin displays an inverse relationship to selective cholesteryl ester uptake, being most prominent in basal cells and least prominent in luteinized, HDL-incubated cells (Azhar, Nomoto et al. 1998). There were no changes in caveolin expression under activation of selective lipid uptake, suggesting that caveolin may not be functionally involved in this process (Reaven, Nomoto et al. 1998).

6.5 Tissue and cell type-specific expression of SR-BI

Tissue profiles demonstrate highest expression of SR-BI in the ovary, adrenal, liver, spleen and placenta (Acton, Rigotti et al. 1996; Landschulz, Pathak et al. 1996; Rigotti, Edelman et al. 1996; Cao, Garcia et al. 1997). SR-BI was localized by immunofluorescence to the surface of steroidogenic cells in the zona fasciculata and zona reticularis of the adrenal gland and in the CL (Landschulz, Pathak et al. 1996). In the ovary, in situ hybridization revealed expression nearly exclusively in the theca and interstitial cell (Svensson, Johnson et al. 1999; Li, Peegel et al. 2001) compartments of developing follicles. In rat and bovine (Argov, Moallem et al. 2004) ovaries, transcript abundance appears to increase as follicular development ensues (Rajapaksha, McBride et al. 1997). HDL-receptor mRNA levels were low in isolated bovine granulosa cells, but increased by 7-fold during corpus luteum development *in vivo*, and by 5-fold during granulosa cell luteinization in culture, showing that luteinization of bovine granulosa cells is

associated with an increase in HDL-receptor RNA levels along with changes in steroidogenic enzyme activity (Rajapaksha, McBride et al. 1997). A significant increase in SR-BI gene expression was detected in the late phase of corpus luteum formation, and transcripts were abundant in corpus luteum and in thecal cells at all stages of follicular development (Johnson, Svensson et al. 1998).

Unlike in rodents, SR-BI is highly expressed in the human placenta. Apparently, at this level, it is important for lipoprotein importation as a cholesterol source for membrane synthesis, required for tissue growth in the developing embryo and for steroid hormone production in the extraembryonic tissues (Hatzopoulos, Rigotti et al. 1998). In pregnant animals, the mammary gland also expresses high levels of the SR-BI protein (Landschulz, Pathak et al. 1996).

6.6 SR-BI: Hormonal regulation

The evidence that SR-BI is the selective uptake receptor for HDL-cholesterol esters in steroidogenic tissues came from studies showing that SR-BI is upregulated by ACTH in murine adrenal glands and in Y1-BS1 cells where synthesis of SR-BI was induced (Reaven, Nomoto et al. 2006). In the adrenal cortex of mice injected with ACTH, alone or in combination with 17-ethinyl-estradiol SR-BI expression increased (Rigotti, Edelman et al. 1996; Azhar, Nomoto et al. 2002). Steroidogenic factor-1 (SF-1) seems to be necessary for full SR-BI encoding of gene expression in human adrenal cells (Cao, Garcia et al. 1997). In addition, angiotensin II promotes SR-BI-mediated selective uptake of HDL-CE in the bovine adrenal glomerulosa, and in NCI-H295R human adrenocortical cells; apparently, HDL via SR-BI is the major cholesterol source for aldosterone biosynthesis (Pilon, Martin et al. 2003).

In the rat ovary, SR-BI expression is upregulated by gonadotropin stimuli, as SR-BI transcript abundance was increased twofold in the immature rat ovary following eCG administration and in response to stimulation by hCG, resulting from 2.5 to sevenfold increase in the transcript at 3 to 6 h postinjection, respectively (Azhar, Nomoto et al. 1998; McLean and Sandhoff 1998; Rigotti, Miettinen et al. 2003). This increase corresponded to a 58% increase in serum progesterone. The combination of luteinizing hormone (LH) and insulin has a stimulatory effect on the expression of SR-BI in theca-interstitial cells and induces theca cell steroid synthesis (Li, Peegel et al. 2001; Towns, Azhar et al. 2005). Antibody neutralization of SR-BI in rat theca cell cultures reduced gonadotropin-stimulated steroidogenesis by 90% (Wu, Sucheta et al. 2003), attesting to the importance of SR-BI in cholesterol supply in this follicular compartment.

6.7 SR-BI molecular regulation

As previously mentioned, cholesterol homeostasis in mammalian cells is regulated by a unique family of transcription factors known as sterol regulatory element binding proteins (SREBPs). Sterol regulatory elements (SRE) have been identified in the SR-BI gene (Sudhof, Russell et al. 1987). SR-BI presents two SRE, one distal and one proximal (Lopez and McLean 1999), the SRE1 contains the information necessary for mediation of sterol regulation.

Cao (Cao, Garcia et al. 1997) described the structure and subchromosomal location of human SR-BI, providing evidence for its regulation through the steroidogenic factor 1 (SF-1). SF-1, an orphan member of the nuclear hormone receptor gene family, plays a key role in steroidogenesis and is expressed at higher levels in specialized steroidogenic tissues. SF-1 binds

to the SR-BI promoter in a sequence-specific manner, and the efficient transcription from this promoter in the adrenocortical Y1 cells is dependent on an intact SF-1 site. SR-BI contains one SF-I-binding motif (Lopez, Sanchez et al. 2002).

It has been well established that negative factors also play a critical role in gene regulation. One important SR-BI negative controlling factor seems to be the dosage-sensitive sex adrenal hypoplasia congenital critical region on the X chromosome gene-1 (DAX-1) wich is colocalized with SF-1 in steroidogenic tissues (Ikeda, Swain et al. 1996) suggesting that DAX-1 and SF-1 may function assembled or sequentially (Ito, Yu et al. 1997; Crawford, Dorn et al. 1998). DAX-1 can strongly repress steroidogenesis and antagonize SF-1-mediated stimulation of genes such as SR-BI, leading to a decrease in its mRNA (McLean and Sandhoff 1998). In 2003, Kocher et al. (2003) reported a novel mechanism for the physiological and pathophysiological regulation of hepatic SR-BI by PDZK1 (PDZ domain-containing-1 protein) in a tissue-specific and post-transcriptional fashion. SR-BI protein expression in PDZK1 knock-out (KO) mice was reduced by 95% in the liver, 50% in the proximal intestine, and not affected in steroidogenic organs (adrenal, ovary, and testis). The PDZ domain-containing adaptor protein PDZK1 has been shown to bind to and control the activity of SR-BI. Mice deficient in PDZK1 have elevated plasma cholesterol levels due to the virtually complete hepatic ablation of SR-BI (Kocher, Yesilaltay et al. 2003; Lan and Silver 2005; Yesilaltay, Kocher et al. 2005).

Point-mutation analysis demonstrated that PDZK1 is phosphorylated at Ser-509, a process triggered by PKA in vitro (Nakamura, Shibata et al. 2005)). PDZK1 phosphorylation and hepatic SR-BI increase, results in a decrease in plasma HDL levels, indicating that PDZK1

phosphorylation has an important role in the regulation of hepatic SR-BI expression and, influences plasma HDL levels and that the C-terminal region of PDZK1 is crucial for up-regulating SR-BI protein expression. (Nakamura, Shibata et al. 2005).

6.8 Other regulators of SR-BI

Cellular levels of vitamin E (vitE) exert control on SR-BI expression. Witt et al. (Witt, Kolleck et al. 2000) observed that diminishing the amount of vitE in rat diets, resulted in increase of expression of SR-BI protein, increased HDL-binding proteins (HB2) and a glycosyl phosphatidylinositol-anchored membrane dipeptidase (MDP), with a possible role in the selective lipid uptake process, mediated by SR-BI. This effect has been also observed in human SR-BI, increases in vitE were accompanied by reduced protein kinase C (PKC) and SR-BI levels, showing an vitE inhibitory effect on these molecules (Witt, Kolleck et al. 2000).

Taking into account that lecithin-cholesterol acyltransferase (LCAT) is an important HDL component and with the purpose of observing whether LCAT and Apo-I determine SR-BI concentration in adrenal, LCAT and Apo-A knockout mice were developed, which demonstrated 92% reduction in plasmatic lipoproteins as well as 96% in the CE content (Ng, Francone et al. 1997). These results were associated with a 2-fold up-regulation of adrenal SR-BI mRNA, supporting the view that adrenal SR-BI expression is also regulated by the adrenal cholesterol (Ng, Francone et al. 1997).

6.9 SR-BI deficiency

Below, SR-BI expression in the liver as well as in steroidogenic cells, and its regulation by the action of several trophic hormones was addressed. Studies on genetically manipulated mice strains have established SR-BI's key role during lipoprotein metabolism regulation and cholesterol transportation into steroidogenic tissues and into the liver for biliary secretion (Rigotti, Miettinen et al. 2003). Similarly, SR-BI has been defined as lipoprotein receptor responsible for selective CE uptake from HDL and LDL, and in free cholesterol efflux to lipoprotein acceptors (reverse cholesterol transport). These activities depend on lipoprotein binding to its extracellular domain and the subsequent lipid exchange at the plasma membrane (Mardones, Strobel et al. 2002; Out, Kruijt et al. 2004; Lopez and McLean 2006).

HDL may have a particularly important role in mammalian female fertility, because in many species, including humans, HDL is the main class of lipoprotein found in ovarian follicular fluid (Perret, Parinaud et al. 1985; Volpe, Coukos et al. 1991). Mice with targeted homozygous null mutations in the SR-BI gene exhibit approximately 2-fold elevated plasma cholesterol carried in both normal size and abnormally large HDL particles (Rigotti, Trigatti et al. 1997; Trigatti, Rayburn et al. 1999). The ratio of unesterified cholesterol to total cholesterol in plasma is abnormally high in SR-BI-/- mice (Braun, Zhang et al. 2003). SR-BI-/- females are infertile (Trigatti, Rayburn et al. 1999). SR-BI-/- females ovulate normal numbers of oocytes that are dead or defective and thus cannot be fertilized. Although these animals appear to exhibit a normal estrous cycle (Trigatti, Rayburn et al. 1999), when ovaries from SRBI-/- mice are transplanted into otherwise SR-BI-positive recipients or genetic/pharmacological manipulation of lipoprotein, fertility is partially or fully restored (Miettinen, Rayburn et al. 2001). Inducing hepatic SR-BI expression in SR-BI-/- animals by adenovirus transduction or stable transgenesis

substantially reduced levels of abnormally large HDL, normalized the unesterified:total cholesterol ratio and restored female fertility (Yesilaltay et al. 2006).

In addition, analysis of SR-BI -/- mice has shown that SR-BI activity is important during alpha-tocopherol and nitric oxide metabolism, and normal red blood cell maturation. The role of SR-BI/Apo complex has been demonstrated through the knockout mice model for several physiological and pathological conditions (Li, Guo et al. 2005). SR-BI/ApoE double knockout mice developed acute atherosclerosis 4 weeks after birth. Similarly, double SR-BI/ApoA knockout mice developed premature cardiac failure. The up-or-down regulation of other genes in the absence of SR-BI has been documented and the genes affected include those involved in the oxidative process, suggesting that SR-BI is not only important for cholesterol transport to the cell, but also for the regulation of apoptosis (Li, Guo et al. 2006).

6. 10 Alternate splicing of SR-BI and cholesterol importation

As noted above, the mouse SR-BI was the first high-density lipoprotein receptor to be identified (Glass, Pittman et al. 1983). It is highly expressed in tissues with an elevated cholesterol demand where it functions to bind mature HDL particles and selectively remove cholesteryl esters (Murakami, Horiuchi et al. 1987), and to contribute to cellular cholesterol homoeostasis (Graf, Matveev et al. 1999). Alternative splicing of the *SR-B* gene transcript generates two isoforms (types I and II) with identical extracellular regions, but distinct C-terminal cytoplasmic tails (Azhar and Reaven 2002). SR-BII also termed as SR-BI.2 in mice; and CLA-2 in humans, is a high density lipoprotein receptor with an identical extracellular domain that contains a further 40 amino acid residues (Calvo and Vega 1993; Cao, Garcia et al. 1997;

Webb, de Villiers et al. 1997). It has been described in several tissues, including certain cell types in which SR-BI expression is observed (Webb, de Villiers et al. 1997; Reaven, Cortez et al. 2004). Significant proportions of SR-BII mRNA are expressed in the mouse and the receptor binds HDL with high affinity, and mediates both selective lipid uptake through endocytosis via clathrin-dependent pathway and cellular cholesterol efflux , although less efficiently than mouse SR-BI (Eckhardt, Cai et al. 2004). In contrast to SR-BI, SR-BII does not mobilize intracellular chlolesteryl (Webb, de Villiers et al. 1997).

As with SR-BI, SRBII is localized in caveolae (Acton, Rigotti et al. 1996) cholesterol rich plasma membrane microdomains that are implicated in cellular cholesterol homoeostasis and in cell signaling (Krieger 1999; Williams, Connelly et al. 1999). In steroidogenic tissues the cell regions termed as the microvillar compartment (Reaven, Boyles et al. 1988; Reaven, Spicher et al. 1989), and the specialized space created between them are apparently implicated in the selective uptake of lipoprotein CEs into cells regulated by SR-BI and its isoform SR-BII (Reaven, Shi et al. 1990). Electron microscopic and immunocytochemical techniques reveal heavy labeling for SR-BI specifically in these regions (Reaven, Nomoto et al. 1998; Azhar, Nomoto et al. 2002). In Chinese hamster ovary cells the distribution of SR-BII differs from SR-BI, as SR-BI is mainly (approximately 70%) localized on the surface of transfected cells, while the majority of SR-BII (approximately 80-90%) is expressed intracellularly, the distinct C terminus of SR-BII is responsible for its intracellular expression (Eckhardt, Cai et al. 2004). During the process to elucidate the cell distribution of SR-BII, a novel isoform expressed in human macrophages and in human atherosclerotic plaques was described (Svensson, Englund et al. 2005).

The tissue location of SR-BI is considered similar to the SR-BII, but there is currently little information on the SR-BII in the ovary (Akpovi, Yoon et al. 2006).

7. STEROIDOGENESIS

In mammals there are several tissues where elaboration of steroids takes place: ovary (follicles and corpus luteum), testis, adrenal glands, placenta, nervous system (Baulieu 1998; Mellon and Griffin 2002) and in the heart (Kayes-Wandover and White 2000; White 2003). Each tissue synthesizes and secretes different steroid products, however all the steroids have cholesterol as precursor molecule. Herein, only steroidogenesis in the follicles and corpus luteum is mentioned because it fulfills important functions in reproduction.

7.1 Follicular steroidogenesis

Follicles provide a suitable micro-environment for oocyte development. This atmosphere is promoted through hormonal and non-hormonal secretions. The main products secreted within the follicles are estrone, estradiol-17β, androstenedione, testosterone, progesterone and pregnenolone and these play a very important role in mammalian reproductive system (Lunenfeld, Kraiem et al. 1975; Kagawa, Kuwayama et al. 2005).

The estrogen of major importance in follicular steroid synthesis is E2, synthesized in granulosa cells. Its physiologic actions include the regulation of receptivity during the estrus phase, regulation of ovarian cycle, neuroprotection to prevent neuron death (Bains, Cousins et al. 2007), regulation during synaptic communication (Hajszan, Maclusky et al. 2007), protection

against hypertension (Ashraf and Vongpatanasin 2006). Indeed, estrogens regulate multiple reproductive and non-reproductive mechanisms.

The ovarian cells processed cholesterol to steroids (Sasano, Okamoto et al. 1989), the theca cells synthesized androgens, which represents the granulosa substrate for estrogen production. However, Tepenainen et al found that human granulosa displays aromatase activity in response to hCG, FSH and estrogens (Tapanainen, McCamant et al. 1991).

Androgens produced by the theca are aromatized to estradiol in granulosa cells under the influence of FSH, rendering estradiol. Mature rat granulosa cells acquire the capacity to respond to LH by increasing its receptors, an action controlled through FSH (Mondschein and Schomberg 1981). Also, control of estradiol production in granulosa cells is complemented in a complex interaction of growth factors, specially members of TGF-β superfamily (Knight and Glister 2006).

Synergy of FSH with insulin-like growth factor (IGF) induces estradiol production (Hirakawa, Minegishi et al. 1999; Minegishi, Hirakawa et al. 2000; Minegishi, Hirakawa et al. 2004). Estrogen production by granulosa cells is also regulated by inhibin and activin which regulate FSH secretion and by autocrine action, increasing LH receptors in granulosa cells (Glister, Tannetta et al. 2001; Knight and Glister 2001; Welt 2002).

7.2 Key enzymes in steroidogenesis

Considering the importance of cholesterol during steroidogenesis, equilibrium between CE and free cholesterol depends of a number of enzymes, including rate- limiting enzymes in cholesterol biosynthesis, cholesterol acyl transferase (ACAT), the cholesterol esterases and HMG-CoA reductase (Lennernäs 1997). Cellular structures, such as mitochondria and smooth endoplasmic reticulum, are the sites where the enzymatic complex necessary for steroid synthesis is located. Steroidogenic enzymes may be classified into two major classes: the cytochrome P450 heme-containing proteins and the hydroxysteroid dehydrogenases (Payne and Hales 2004; see table 1).

The enzymatic complexes act at different levels in steroid biosynthesis, after cholesterol entrance into the cell the first rate-limiting enzyme is P40scc (also known as cyp11A1) that acts in cleavage of the side-chain of cholesterol, this enzyme is located on the matrix side of the inner mitochondrial membrane (Payne and Hales 2004). The first enzymatic step culminates with elaboration of pregnenolone (Burstein and Gut 1976) a common molecule in steroidogenesis (Miller 1988; Miller 1995). Conversion to progesterone is controlled by Δ^5-3β-hydroxysteroid dehydrogenase (3ßHSD). Androgen synthesis is a rate-limiting step in the follicular estrogen production and is controlled by the 17α-hydroxylase and the C-17, 20-lyase enzymes. The reaction can utilize either pregnenolone by the 5-ene-3ß-hydroxy pathway (Δ^5 pathway); or progesterone through the action of 4-ene-3-oxo or Δ^4 pathway, depending on the species. For example, humans and cattle favor the Δ^4 pathway while rodents use the Δ^5 pathway. The final step in steroidogenesis is the aromatization of androgens to estradiol in granulosa cells by the P450arom. (Graham-Lorence, Khalil et al. 1991; Simpson, Mahendroo et al. 1994).

Gene		Protein	Synonyms	Tissues expression
Human	Mouse			
CYP11A1	Cyp11a1	CYP11A	P450scc, cholesterol side-chain cleavage, cholesterol desmolase,cytochrome P450 cholesterol side-chain cleavage	Ovary
CYP17B1	Cyp17	CYP17	P450c17, 17α-hydroxylase/17,20 lyase, P45017α-hydroxylase/c17-20 lyase	Ovary (theca cells)
CYP19	Cyp19	CYP19	Aromatase, P450arom, cytochrome P450arom, estrogen syntase	Ovary (granulosa cells)
HSD17B1	Hsd17b2	17HSD1	17β-HSD1, estradiol-17β dehydrogenase, estrogen 17-oxidoreductase	Ovary
HSD17B7	Hsd17b7	17HSD7	17β-HSD7, 17βHSD/17-keto steroid reductase 7	Corpus luteum
HSD3B2	Hsd3b1	3β HSDII human 3β HSDI mouse	3-HSD type I (type VI), 3-HSD/5- 4 isomerase, 3-hydroxy-5-steroid dehydrogenase, 3-hydroxy-5-ene steroid dehydrogenase	Ovary

Table 1. Hydroxysteroid deshydrogenase and P450enzymes involved in active ovarian steroid hormone biosynthesis [adapted from Payne and Hales 2004]

7.3 Luteal steroidogenesis

The main steroid synthesized by luteal cells is progesterone. The corpus luteum is composed of a mixture of cells and interaction between these cell types is essential to maintain the luteal steroidogenic function. Progesterone is not the only steroid produced in corpus luteum since androstenedione and E2 have been found in rodents, pigs and primates (Oon and Johnson 2000; Knight and Claire 2006). As in follicular cells mitochondria plays an important role and pregnenolone production takes place by the initial action of P450scc and other enzymes that collaborate in the process increasing progesterone levels. The 3βHSD enzyme increases dramatically in CL (Oonk, Krasnow et al. 1989; Kaynard, Periman et al. 1992). As in follicular steroidogenesis, P450scc catalyzes conversion of cholesterol to pregnenolone.

The involvement of luteal steroidogenesis of stimulatory and inhibitory enzymes are listed in a Table 1. 20α-hydroxysteroid dehydrogenase (20αHSD) catabolizes progesterone into inactive components (Wiest, Kidwell et al. 1968; Stocco, Zhong et al. 2000); 26-cholesterol hydroxylase catalyzes the conversion of cholesterol to the inactive form 26-hydroxycholesterol (Yoshida, Kubota et al. 1999); and 5α-reductase, transforms testosterone to dihydrotestosterone (DHT). These enzymes reduce the activity of the steroidogenic machinery and thus reduce the cellular capacity for steroidogenesis.

8. HYPOTHESIS AND OBJECTIVES

8.1 Experimental goals

There is little information about cholesterol importation during the luteinization. We hypothesize that SR-BI activity is a significant cholesterol source to support luteinization and when SR-BI activity is compromised alternative cholesterol sources will be developed to maintain corpus luteal steroidogenesis. The goal of this work was therefore to determine the importance of the cholesterol importation mechanisms during the luteinization process, by the analysis of LDLr, SR-BI as well as HMG-CoA reductase expression in the porcine and mice ovaries, and by means of experimental paradigms to invoke luteinization in vivo and in vitro.

CHAPTER ONE

Title: LIPOPROTEIN RECEPTOR EXPRESSION DURING LUTEINIZATION OF THE OVARIAN FOLLICLE

Short title: SR-BI and LDL receptor in luteal formation

Authors: Leonor Miranda-Jiménez[1,2] and Bruce D. Murphy[1]

Affiliations: [1]Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal, C.P. 5000, St-Hyacinthe, Québec Canada J2S 7C6

[2]Colegio de Postgraduados-CONACYT, México.

Corresponding author: Bruce D. Murphy, Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal, C.P. 5000, St-Hyacinthe Québec, Canada J2S7C6, telephone 450 773 8521 ext. 18382, facsimile 450 778 8103, email

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ABSTRACT

Ovarian follicles luteinize following ovulation, requiring structural and molecular remodeling along with exponential increases in steroidogenesis. Cholesterol substrates for luteal steroidogenesis are imported via scavenger receptor-BI (SR-BI) and the low density lipoprotein receptor from circulating high and low density lipoproteins (HDL and LDL) respectively. SR-BI mRNA is expressed in pig ovaries at all stages of folliculogenesis and of the corpus luteum (CL). An 82 kDa form of SR-BI predominates throughout, weakly present in granulosa cells, and robustly expressed in the CL, along with the less abundant 57 kDa form. Digestion of N-linked carbohydrates substantially reduced the SR-BI mass in luteal cells, indicating differences between forms is attributable to glycosylation. Immunohistochemistry revealed SR-BI to be concentrated in the cytoplasm of follicular granulosa cells while found mostly at the periphery of luteal cells. To examine receptor dynamics during gonadotropin-induced luteinization, pigs were treated with an ovulatory stimulus, and ovaries were collected at intervals to ovulation. SR-BI in granulosa cell cytoplasm increased through the peri-ovulatory period, with migration to the cell periphery as the CL matured. In vitro culture of follicles with hCG induced time-dependent upregulation of 82 kDa SR-BI in granulosa cells. SR-BI and LDL receptor were reciprocally expressed, with the latter highest in follicular granulosa cells, declining precipitously with CL formation. We conclude that luteinization causes upregulation of SR-BI expression, its post-translational maturation by glycosylation and insertion into luteal cell membranes. Expression of the LDL receptor is extinguished during luteinization, indicating dynamic regulation of cholesterol importation to maintain elevated steroid output by the corpus luteum.

INTRODUCTION

The formation of the corpus luteum (CL) following rupture and collapse of the ovulating follicle requires widespread cell and tissue remodeling, driven by extensive changes in gene expression (17). A significant aspect of luteal remodeling is the shift from estrogen as the principal steroid product to progesterone, a change accompanied by a large-scale increase in total steroid synthesis. At the peak of their output during the late follicular phase, estrogens are synthesized only in μg/day, while the human CL secretes as much as 40 mg/day of progesterone during the luteal phase (13). To achieve this amplification in steroidogenesis, a major increase in the amount of the steroid parent molecule, cholesterol, is required. The quantitatively most important supply of cholesterol for luteal steroid synthesis arrives by importation from extracellular sources, in the form of high and low density lipoprotein (HDL and LDL) borne cholesterol (3, 20).

Importation of lipoprotein-cholesterol from LDL and HDL is mediated by specific membrane receptors. The endocytic LDL receptor system and attendant cholesterol importation has been abundantly characterized in a number of tissues, including the ovary (3, 20). In recent years, it has been shown that HDL importation depends upon a cell surface glycoprotein known as scavenger receptor B1 (SR-BI), a glycoprotein comprised of two transmembrane and two cytoplasmic domains and a large, N-glycosylated extracellular loop (24). The SR-BI gene codes for a protein with a predicted mass of 57 kDa, while immunoblot and immunoprecipitation experiments reveal an apparent mass of 82 kDa (1), suggesting extensive glycosylation. SR-BI is inserted in the plasma membrane as a functional dimer where it serves as high capacity bulk cholesterol delivery system (23).

Tissue profiles demonstrate highest expression of SR-BI in the ovary and adrenal of the rat (10), and in situ hybridization revealed that expression is restricted to the theca compartment of developing follicles (24). In the rat (11) and bovine (2) ovaries, transcript abundance appears to increase as follicular development ensues. In the rat ovary, SR-BI expression is up-regulated by folliculogenetic stimuli, in particular, the combination of luteinizing hormone (LH) and insulin, that induces theca cell steroid synthesis (12). Antibody neutralization of SR-BI in rat theca cell cultures reduces gonadotropin-stimulated steroidogenesis by 90 % (28), attesting to the dominance of SR-BI in cholesterol supply in this follicular compartment. In the rat, SR-BI expression becomes established with the remodeling of the ovary during luteinization, as first shown in vitro by Azhar et al. (4), and evidenced by a large scale increase in expression of the SR-BI message in the developing corpus luteum (11). These changes are evident at 12 h after the ovulatory stimulus, around the time of ovulation. In vitro evidence supports a role for gonadotropin regulation of SR-BI, as treatment of rat luteal cells with the luteinizing hormone analog, human chorionic gonadotropin (hCG) provokes both the expression and dimerization of the mature (82 kDa) form of SR-BI (23). As little is known about kinetics of the changes in gene expression that accompanies the formation of the corpus luteum, we undertook exploration of the mechanisms of importation of cholesterol during the luteinization process by examination expression of the LDL receptor and SR-BI in the porcine ovary and by means of experimental paradigms to invoke luteinization in vivo and in vitro.

MATERIALS AND METHODS

Animals, tissues and treatments

All animal experiments were approved by the University of Montreal Animal Care Committee and performed according to the Canadian Council of Animal Care regulations. To determine the temporal expression and cellular localization of SR-BI protein throughout the ovulatory process, 15 gilts were treated with 500 iu equine chorionic gonadotropin followed by 100 IU human chorionic gonadotropin (hCG) (Intervet Canada Ltd. Whitby, ON) to induce ovulation. Ovaries were recovered at 0 h, 24 h, 30 h, and 38 h after hCG administration. In other trials, pig ovaries were collected at the slaughterhouse. Follicular fluid rich in granulosa cells was aspirated from small (< 3mm diameter), medium (4-7 mm) and large (>8 mm) follicles and washed three times prior to RNA and protein analysis. CL classified according to their developmental morphology (19) as postovulatory (CL-I), developing (CL-II), mid-luteal (CL-III) and regressing (CL-IV). To investigate SR-BI protein expression in situ, whole follicles between 3.5 mm and 5 mm in diameter were dissected from ovaries acquired at the slaughterhouse and cultured in Opti-MEM (Invitrogen, Carlsbad CA) containing 1 % insulin and 10 % fetal bovine serum (FBS, Invitrogen). At 12 h following initiation of incubation, hCG, (100 iu or 500 iu) was added to the culture media and follicle cultures were terminated after a further 12 h or 30 h of incubation. For follicle culture, granulosa cells were aspirated from three independent follicles and rinsed with culture medium to eliminate follicular fluid. We employed a further paradigm of in vitro luteinization of granulosa cells as previously described (22). Briefly granulosa cells from of 3-5 mm diameter follicles were aspirated from slaughterhouse ovaries and seeded at the rate of 7.5 million cells per milliliter of Opti-MEM containing 1% insulin and 10% FBS. Cells were treated with medium alone or medium plus 5 μg/ml actinomyocin-D (Sigma). After 24 h

medium was changed and fresh medium or medium + 100 ng/ml luteinizing hormone (LH NIH USDA), was added. Cultures were terminated 24 or 48 h later. In a second trial, freshly isolated granulosa cells were incubated in medium containing 10 % FBS or 10 % fetal bovine lipoprotein-depleted serum (FBLDS; Gibco BRL) for 48 h. In both the whole follicle and isolated granulosa cell culture trials, results were based on three independent experiments from ovaries acquired on different days.

Pig SR-BI Cloning, sequencing, and RT-PCR

Total RNA extracted from a midcycle porcine CL, quantified by spectrophotometry at 260 nm, and 1 µg sample was used for reverse transcription (RT) with the Omniscript RT kit (Qiagen, Mississauga, ON) according to the manufacturer's instructions. SR-BI primers were designed based on a homologous sequence in GenBank (accession AF467889) as follows: TGA CTC CCG AAT CCT CTC TG (forward) and CTG CTC CTT GCT CTG GGCT (reverse). The consequent 550 bp PCR product was resolved on 1 % agarose gel, excised and purified using a gel extraction kit (Quiagen). The cDNA was ligated into a pGEM-T Easy Vector System I (Promega Corp., Nepean, ON) according to the manufacturer's instructions and transformed into competent *Escherichia coli* strain XL-1 blue. Plasmids were isolated with a QIAprep Spin Miniprep kit (Qiagen) and sequenced (Applied Biosystems, Foster City, CA). Three independent samples were sequenced to verify authenticity. Cell pellet samples and approximately 5 mm^3 of tissue from each CL were homogenized in buffer containing guanidine isothiocyanate (GITC 4.23M) and 0.12 M ß-mercaptoethanol (both from Sigma, St. Louis MO). RNA was purified using an RNeasy Protect Mini kit (Qiagen) as recommended by the manufacturer. Pig specific primers (GTC CAT GCC ATC ACT GCC ACT TG, forward and CCT GCT TCA CCA CCT TCT TG, reverse) for glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) were used as a control. Preliminary investigation indicated that 27 PCR cycles in a final volume of 25 µl, using *Taq* DNA polymerase (Amersham Biosciences Baie d'Urfe, PQ) with a 58 C annealing temperature, addressed the linear portion of the amplification curve for both SR-BI and GAPDH. Semi-quantitative PCR amplification for SRB1 and GAPDH in luteal tissues and granulosa cells was performed tissues from 3-5 animals, and products were resolved on 1 % agarose gel using 1 % ethidium bromide. Optical densities were analyzed using Alpha-imager densitometer (Alpha Innotech, San Leandro, CA).

Western blot analysis

One mg of tissue as described above was homogenized using 300 µl protein loading buffer containing ß-mercaptoethanol and maintained at –20 C until analysis. Protein concentrations were determined by Bradford (Bio-Rad) assay, and an aliquot of 30 µg of protein was loaded per well. Blots were electrophoretically separated using one-dimensional 10% SDS-PAGE at 105 Volts. High molecular weight standards (Amersham Pharmacia, Piscataway, NJ) were used as reference. Resolved proteins were transferred to a 0.45 µm nitrocellulose membrane (Amersham Pharmacia Biotech, Arlington Heights, IL), and stored at 4 C overnight. Membranes were washed with distilled water and stained with Ponceau solution, washed again in 0.1% Tween in Tris-buffered saline (TTBS: 100 mM Tris, 0.9% sodium chloride from Sigma, pH 7.5) and blocked with 1% BSA in TTBS. A polyclonal SR-BI first antibody (Novus Biological, Inc. Littleton, CO) was added at 1:1000 final concentration, followed by 2 h incubation and a second antibody, anti-rabbit IgG, labeled with horseradish peroxidase (Amersham Biosciences UK Limited, Buckinghamshire, UK) was applied at 1:10000 over 30 min. We further examined SR-BII, a splice variant of SR-BI by using a polyclonal antibody (Novus Biological, Inc. Littleton, CO) at 1:1000. The LDL receptor was evaluated by means of

a monoclonal antibody (Abcam Inc. Cambridge, MA), at 1:1000 concentration, incubated for 2 h, and a second antibody, rabbit anti-mouse IgG conjugated to horseradish peroxidase (EMD Biosciences Inc., San Diego, CA) was used at 1:10000 over 30 min. As a control, -tubulin was probed using a monoclonal antibody developed by the Developmental Hybridoma Bank (Iowa City, IA) at 1:1500 concentration under conditions of incubation for 2 h; and the same second antibody and incubation time used for LDL receptors was applied. All incubations were carried out at room temperature. Membranes were rinsed and abundance of all proteins were detected with chemiluminescent substrate (Pierce Biotechnology, Inc. Rockford, IL.), and blots were exposed to Kodak (Rochester, NY) photographic films. The optical density of the protein band was quantified by scanning with an Alpha-image densitometer.

Immunoflourescence

Ovaries, follicles and CL were fixed in Zamboni's solution, embedded in paraffin, and sectioned at 6 μm. Tissue sections were rehydrated and, for antigen recovery, boiled in 10 mM sodium citrate (pH 6.0) for 10 min. Slides were then rinsed once in cold (4 C) PBS (5 min), blocked for 20 min with 1 % BSA, incubated over 2 h at room temperature using the SR-BI polyclonal antibody at 1:50. Tissues were rinsed twice in PBS and incubated for 30 min with a second antibody labeled with a cyanine dye (Cy3, Biocan Scientific Inc. Mississauga, ON). Nuclei were stained with 1:1500 4'diamino-2-phenyindole dilactate, (DAPI, Sigma). All procedures were performed at room temperature. Slides were mounted in Permafluor (Thermo Shando, Pittsburgh, PA,).

Deglycosylation of SR-BI

To establish the basis for SR-BI forms of variant molecular weight, CL III tissues were subject to in vitro deglycosylation using endoglycosidase H (Endo H) and N-glycosidase F

(PNGase, both from New England BioLabs Ipswich, MA), (0.8 to 1 mg) were lysed in 300 µl of protein loading buffer plus ß-mercaptoethanol (14.3 µl/ml), and 50 mg aliquots of total protein (evaluated by Bradford) were denatured at 100 °C over 5 min. Then, 1/10 volume of G5 buffer (0.5 M sodium citrate, pH 5.5 at 25 °C) and 5 units of Endo H were added, and the mixture was incubated overnight at 37 °C. For PNGase digestion 50 mg total protein was treated with 5 units of the glycosidase in ß-mercaptoethanol and G7 buffer followed by overnight incubation at 37 °C. The undigested sample (control) was incubated without enzyme. Samples were subjected to Western blot analysis in one-dimensional 8 % SDS-PAGE at 105 Volts. High molecular standard weight protein markers (Amersham) were used as reference. Samples were evaluated in triplicate.

Statistical analyses

SR-BI mRNA optical density was standardized by comparison to GAPDH. For SR-BI protein expression, optical density data were transformed using square root to obtained normality of distribution, transformed data were analyzed by split plot ANOVA. SR-BI protein deglycosylation was tested using Chi square procedures. LDLR and SR-BI protein optical density data were transformed using square root and analyzed 2 X 2 factorial design ANOVA. The level of significance for all analyses was $P < 0.05$.

RESULTS

Expression of SR-BI in granulosa cells from ovarian follicles and corpora lutea

RT-PCR analysis revealed that SR-BI mRNA was present in granulosa cells aspirated from all classes of ovarian follicles collected, and did not differ in abundance with the stage of follicle development (Fig. 1). Luteal transcript abundance was consistently 1.5 to 2.0 fold

greater, and did not differ through the luteal phase of the estrous cycle (Fig. 1). SR-BI protein quantification of immunoblots indicated that granulosa cells of all follicles express both the 57 and 82 kDa forms of SR-BI (Fig. 2). The abundance of the lower molecular weight form differed from barely detectable in small follicles to more pronounced in corpora lutea. In contrast, the 82 kDa form, also barely detectable in follicles, is robustly expressed in corpora lutea. The relative abundance was consistent with the pattern of mRNA expression, in that it was more strongly expressed in corpora lutea relative to follicles (Fig. 2). The total immunoreactive protein increased 6-7 fold with luteinization, while the proportion of 57 kDa form declined from an average of 10 % in follicles to 5 % in corpora lutea.

Subsequent experiments were undertaken to determine whether the difference between the 82 and 57 kDa forms was related to the carbohydrate moiety of the glycoprotein. Fig. 3 depicts the effects of digestion of protein extracts of midcycle corpora lutea (CL-III) with Endo-H, a glycosidase that cleaves mannose and some hybrid oligosaccharides from N-linked glycoproteins, and with PNGase, an amidase that cleaves N-linked carbohydrates between the innermost GlcNAc and asparagine residues (14). Endo H treatment reduced the 82 kDa form substantially, to a consequent 68 kDa form, with no apparent effects on the 57 kDa version, suggesting partial deglycosylation (Fig. 3). In samples subject to PNGase digestion, the 82 kDa form was entirely eliminated and only the 57 kDa form persisted (Fig. 2). Together, these digestions indicate that the difference between the two forms of SR-BI in corpora lutea (Fig. 2A) is based on glycosylation of the 82 kDA species.

Changes in cholesterol importation receptor abundance and localization during the peri-ovulatory and luteinization periods

Immunolocalization was employed to further characterize the differences in cholesterol transport mechanisms between follicular and luteal cells. As depicted in Fig. 4, the SR-BI signal is present throughout the cytoplasm in theca and granulosa cells, while it is restricted to the periphery of luteal cells. Based on this observation, we examined the changes of SR-BI abundance and localization during the ovulatory process in the pig (Fig. 5). In the largest follicles of untreated pigs, SR-BI distribution conformed to the observations in Fig. 4, with cytoplasmic expression in the theca and granulosa cells, the latter much reduced compared to the former. The first 24 h after the ovulatory stimulus were characterized by a modest increase in cytoplasmic SR-BI expression in granulosa cells that became more intense as ovulation approached (30 and 38 h, Fig. 5A). Early corpora lutea, (CL-I, 24-48 h after ovulation) display both cytoplasmic and membrane localization of SR-BI, while in later stages, the signal was most commonly found on the periphery of the cells (Fig. 5B).

There are multiple mechanisms of cholesterol supply to steroidogenic tissues, and perhaps the second most significant in the ovary is LDL receptor-mediated importation (15). It was therefore of interest to establish whether concurrence, complementarity or some other mechanism characterized the cholesterol import receptors during transformation of the porcine follicle into the corpus luteum. Immunoblotting revealed that the LDL receptor was highly expressed in granulosa cells of small, medium and large follicles (Fig. 6A and 6B) but the signal became undetectable with luteinization. Concurrent analysis of SR-BI demonstrated a pattern of expression identical to that displayed in Fig. 2 (Fig. 6A and 6B), such that the 82 kDa form was virtually absent from follicles and was acquired by the CL following ovulation. Together these

results indicate a temporal sequence in expression of the receptors and complementarity between the two extracellular cholesterol supply processes during the process of CL formation.

Alternative splicing of the SR-BI transcript results in a second form of the protein with a different carboxyl-terminal cytoplasmic tail, apparently conferring predominant intracellular expression (5). As this splice variant represents some 10-15 % of SR-BI in the liver (27), we examined its abundance through the luteal phase by immunoblotting. The results (Fig. 6C) demonstrate that this isoform is present in the CL, but that its relative abundance does not vary across the luteal phase of the estrous cycle.

Gonadotropin induction of the pattern of cholesterol importation in vitro

We then addressed the mechanism by which these modifications in the cholesterol acquisition system might be controlled. We first employed an in vitro model of follicular function in which whole follicles were placed in organ culture for periods up to 30 h. Granulosa cells harvested from follicle culture under conditions expected to induce luteinization, i.e. the presence of 10 % serum, displayed the expected low level of SR-BI and elevated LDL receptor protein abundance (Fig. 7). In granulosa cells aspirated from follicles incubated with serum, but not treated with gonadotropin, there was an increase in SR-BI expression over 12 h relative to freshly isolated granulosa cells. Treatment with the ovulatory stimulus, in the form of 100 and 500 iu hCG, provoked significant increases in the expression of this receptor in the granulosa cell compartment (Fig. 7). These changes persisted through 30 h of treatment. In contrast, and as expected, LDL receptor abundance was greatest in granulosa cells aspirated from newly collected follicles (Fig. 7). Significant declines were induced by both doses of hCG at 12 h, and these persisted through 30 h, recapitulating the observations of luteinization in vivo.

To complement whole follicle studies, and to determine to what extent the increases in SR-BI expression were related to post-transcriptional changes, the model of transcriptional inhibition with Act-D during in vitro luteinization of isolated granulosa cells (18) was employed. In cells incubated with LH, there was an approximate doubling in SR-BI transcript abundance at 24 and 48 h relative to untreated control (Fig. 8A). A similar increase in protein was observed at 24 h, while there was an approximate 5-fold augmentation at 48 h (Fig. 8B). Act-D treatment (Fig. 8) obliterated both the mRNA and protein signals, indicating that increases in abundance of the message and protein are due to new transcription, rather than to post-transcriptional regulation. Progesterone analysis (Fig. 8C) demonstrated that LH induced increases in the accumulation of this steroid at 24 h relative to control, an effect that was abrogated by Act-D treatment. At 48 h, progesterone accumulation was increased in both control and LH treated cultures. There was substantial accumulation in the Act-D treated cells, indicating that the cells remained alive and steroidogenic, in spite of the absence of significant amounts of SR-BI.

We further examined whether lipoprotein content of the medium influenced the expression of SR-BI by incubation with either FBS or lipoprotein depleted FBS (FBLDS). The results (Fig. 9), indicate that, while the overall accumulation of progesterone when FBLDS was present, relative to FBS, no evidence that this treatment affected SR-BI expression, in either transcript or protein abundance.

DISCUSSION

The mammalian corpus luteum is the product of extensive tissue remodeling of the ovarian follicle that begins with the ovulatory stimulus. Its presumed evolutionary advantage, and thus its function, is the large-scale secretion of the progestins required for viviparity. The principal source of cholesterol substrate for this steroid output is importation from extracellular sources (15, 20). The goal of this investigation was to examine the process of luteinization, in terms of the changes in cholesterol importation mechanisms.

Previous studies in the rat suggested that, while SR-BI was present in the theca compartment, it was absent from the granulosa cells of ovarian follicles (12). In contrast, we found transcripts to be present and low levels of protein detectable in granulosa cells of all classes of follicles from the porcine ovary. Immunocytochemical observations in the present study indicate that the localization of SR-BI expression in granulosa cells is, at least in part, cytoplasmic, even up to the period of peri-ovulatory differentiation. This distribution is surprising, and perhaps counterintuitive, given the high concentrations of HDL in porcine follicular fluid (7), and the indications that the function of SR-BI as the HDL receptor requires its insertion in the plasma membrane (24). It is known that porcine granulosa cells do not synthesize steroids from cholesterol, due the absence of steroidogenic acute regulatory protein (StAR) prior to the onset of luteinization (8, 22) and low level expression of cytochrome 450side chain cleavage (CYP11A) (6). Together these observations suggest that HDL uptake is not an essential element of follicular granulosa cell function.

Our findings demonstrate that, in the pig ovary, there are substantial increases in abundance of both SR-BI message and protein associated with luteinization. Qualitative immunohistochemical observations suggest that this is a gradual change, beginning during the

first 24 h after the ovulatory stimulus, and continuing through luteinization. The molecular and microscopy data in the porcine model concur with observations of in situ hybridization findings in the rat ovary, where there is a progressive increase in signal abundance and intensity as the luteinization process ensues (11) and with studies of rat granulosa cells in vitro where SR-BI transcript and protein both gradually increase during luteinization (4). The latter authors demonstrated that SR-BI expression was essential for the uptake of esterified cholesterol by luteinized granulosa cells (4).

Functional luteal regression, i.e. the reduction in steroidogenic capacity, precedes structural regression in most species (25). The continued expression of SR-BI in regressed CL observed in the present investigation suggests uncoupling of the processes of luteal steroidogenesis and the cholesterol intake at the end of the luteal phase. This is in contrast to intracellular steroid transport mechanisms, including the steroidogenic acute regulatory (21) and the Niemann-Pick C-1 proteins (9) that decline rapidly following treatment with a luteolytic stimulus.

From these findings we infer that, during the process of granulosa cell remodeling into large cells of the CL (19), the SR-BI signal is translocated to the cell periphery, and presumably to the cell surface, at a site in which it can function in cholesterol exchange. There is a large-scale increase in the quantity of the 82 kDa form of the protein accompanying luteinization, including a doubling in its abundance during the progression from the post-ovulatory (CL-I) to the midcycle (CL-II and III) corpus luteum. Follicle and isolated cell culture experiments indicate that the expression of SR-BI is induced by gonadotropin treatment, arguing that this event is specific to the luteinization process. The proportion of total immunoreactivity in the larger molecular weight form was double in luteal cells when compared to granulosa cells,

indicating that differentiation involves an alteration in or upregulation of the glycosylation process. This is at odds with the observation that N-glycosylated residues are co-translationally incorporated into the protein during its synthesis (24), but may be a further reflection that the granulosa cell version of the receptor is nonfunctional prior to the ovulatory stimulus. Confirmation awaits further investigation.

A further interesting finding is that HDL and LDL receptors display a complementary pattern of expression during ovarian follicle remodeling. The granulosa cell expression the LDL receptor is marked and invariable in the developing follicle in spite of the apparent absence of LDL in porcine follicular fluid (7). The present investigation indicates that LDL receptor is lost during luteinization and that the signal is virtually undetectable in the CL. The pattern of SR-BI expression is reciprocally related to that of the LDL receptor, at lowest levels in follicle granulosa cells and highest in the CL, in spite of elevated concentrations of HDL in follicular fluid (7).

The question that arises is whether the dramatic switch in cholesterol uptake from LDL receptor to SR-BI is essential, or whether the two importation strategies are redundant in CL function. Mice bearing an inactivating mutation of the SR-BI gene, while infertile, are capable of forming morphologically functional corpora lutea that produce peripheral progesterone at concentrations that do not differ from their wild type counterparts (26). Ovaries from SR-BI null mice transplanted to ovariectomized, immunocompromised mice supported ovulation and gestation (16), further indicating normal luteal function. In fact, sterility in these animals has been recently attributed to dyslipedemia, resulting the absence of normal bi-directional flux of HDL in the liver (30), rather than luteal insufficiency. Nonetheless, there are noticeable differences between the SR-BI knockout and wild type mice in the Oil-Red-O staining of the

corpora lutea, indicating reduction in intracellular cholesterol stores in the former (26). Recent studies suggest that HDL borne cholesterol and cholesterol derived from de novo synthesis contribute approximately equally to the steroid substrate pool in the mouse ovary, while LDL cholesterol has only a minor role in ovarian steroidogenesis (29). It is not clear whether de novo cholesterol synthesis or LDL receptor-mediated importation compensate for the absence of the HDL cholesterol delivered via SR-BI in the mouse.

Less is known about the relative contribution of cholesterol sources in other species. Herein we present evidence to demonstrate that SR-BI, the HDL receptor is strongly expressed during luteinization and this expression is provoked by the ovulatory stimulus. Further, in vitro studies indicate that SR-BI is induced by gonadotropins through new gene transcription, and appears that this process is not influenced by lipoprotein availability. This implicates HDL cholesterol as the major source of substrate for luteal steroidogenesis in the pig.

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DISCLOSURES *The authors have nothing to disclose*

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FIGURE LEGENDS

Figure 1. **Follicles and corpora lutea express SR-BI transcripts. (A)** Representative RT-PCR analysis revealing the occurrence and relative abundance of SR-BI transcripts in small follicles (SF, < 3mm diameter), medium follicles (MF, 4-7 mm) and large follicles (LF, >8 mm) and corpora lutea (CL) across the progression of the luteal cycle (CL-I to CL-IV). GAPDH has been employed as a control. **(B)** Mean ± SD of SR-BI transcript abundance relative to GAPDH for

five follicles and five CL at each stage of development. Means bearing different superscripts are significantly different at $p< 0.05$)

Figure 2. **Developmental sequence of SR-BI protein abundance in ovarian tissues. (A)** Representative immunoblot demonstrating the two species of protein (82 kDa and 57 kDa) detected in porcine ovarian tissues. Tubulin served as loading and constitutive control. Follicle and CL classification as in the legend to Figure 1. **(B)** Mean ± SD of the relative abundance of the 87 kDa version SR-BI in a series of five follicles and five corpora lutea at each stage of development. **(C)** Mean ± SD relative abundance of the 57 kDa form of SR-BI relative to tubulin. Means with different superscripts are different a $p<0.05$.

Figure 3. Effects of enzymatic deglycosylation on SR-BI from corpora lutea as revealed by immunoblot. An aliquot of 50 µg of luteal protein extracts prepared was digested overnight at 37 °C with endoglyosidase-H (Endo-H) or N-glycosidase F (PNGase). Immunoblots demonstrate the relative abundance of the products of digestion in a representative experiment. Tubulin was employed as a control for loading. The graph indicates the percentage of the total sample found in each molecular weight category between control and digested protein samples for each of the enzyme treatments and is representative of three replications of the trial.

Figure 4. Immunocytochemistry demonstrating the divergent cellular localization of SR-BI in cells from compartments of the porcine ovary. The photomicrographs depict representative sections of 3-5 mm follicles showing the theca and granulosa component, along with a representative section from a midcycle CL. Indirect localization was employed using Cy3 as the fluorescent signal for SR-BI, and DAPI as a nuclear marker.

Figure 5. Changes in cellular localization of SR-BI during the periovulatory and luteal phases of the estrous cycle in the pig. Follicular and corpus luteum SR-BI membrane and

cytoplasmic expression. (A). Representative sections demonstrating SR-BI expression and localization in follicles at 0, 24, 30 and 38 h after hCG administration. As in Fig. 4, the Cy3 marker indicates SR-BI, localization, DAPI the nuclear signal. **(B).** Changes in the SR-BI signal across the course of luteal development and during luteal regression in representative corpora lutea, classified according to morphological characteristics. T, theca cells, G, granulosa cells, BM, approximate localization of the basement membrane separating the theca and granulosa compartments.

Figure 6. Comparison of the abundance of the LDL receptor and SR-BI in follicles and corpora lutea by immunoblot. Pig follicles and corpora lutea were classified as in the legend to Fig. 1. **(A)** Representative Western blot demonstrating the occurrence of LDL receptor (LDLR) in the follicular granulosa cells but not corpora lutea, while SR-BI was found, as in previous figures, primarily in corpora lutea. Tubulin expression was used as loading control**. (B)** Mean density of protein bands showing reciprocal expression of the LDL receptor (high in follicles, low in CL, $p<0.01$) and SR-BI (low in follicles, high in CL, $p<0.01$) in tissues representing the events of folliculogenesis and the luteal phase in the pig. **(C)** Immunoblots representative of analysis of porcine corpora lutea across the luteal phase demonstrating the relative abundance of SR-BII, a splice variant of SR-BI.

Figure 7. Induction of the reciprocal expression of the LDL receptor and SR-BI in granulosa cells from porcine follicles in culture. Whole follicles between 3.5 and 5 mm in diameter were dissected from ovaries, incubated for 12h, followed by addition of hCG (100 IU or 500 IU) and incubation until termination of the cultures at 12 or 30 h after initiation of gonadotropin treatment. The granulosa cells were aspirated and subjected to Western analysis. **(A)** is a representative immunoblot while **(B)** and **(C)** depict the mean ± SD of abundance of

each receptor relative to tubulin in triplicate incubations. Means bearing different superscripts are different at $p<0.05$.

Figure 8. Induction of SR-BI expression in isolated luteinized porcine granulosa cells with 100 ng/ml luteinizing hormone (LH) and the effects of transcriptional blockade with actinomyocin-D (Act-D). Granulosa cells isolated from 3-5 mm porcine follicles were treated with culture medium alone or medium plus 5 μg/ml Act-D. After 24 h medium was changed and fresh medium or medium + 100 ng/ml luteinizing hormone (LH NIH USDA), was added. Cultures were terminated 24 or 48 h later and SR-BI transcript **(Panel A)** and protein (**Panel B)** abundance were determined. Progesterone accumulation in culture medium was determined by radioimmunoassay (**Panel C).** Bars represent means ± SD from three replicate experiments. Means bearing different superscripts are different at $p<0.05$.

Figure 9. The effects of lipoproteins in culture medium on expression of SR-BI by luteinized porcine granulosa cells. Cells, isolated from 3-5 mm porcine follicles were cultured for 48 h in medium containing 10 % fetal bovine serum (FBS) or 10 % fetal bovine lipoprotein-depleted serum (FBLDS). SR-BI expression was determined by (**A)** PCR amplification for transcript abundance and **(B)** immunoblot analysis for protein expression. Progesterone accumulation in medium **(C)** was determined by radioimmunoassay. Data are means ± SD from three replicate experiments, and means with different superscripts indicate difference at $p<0.05$.

Figure 1.

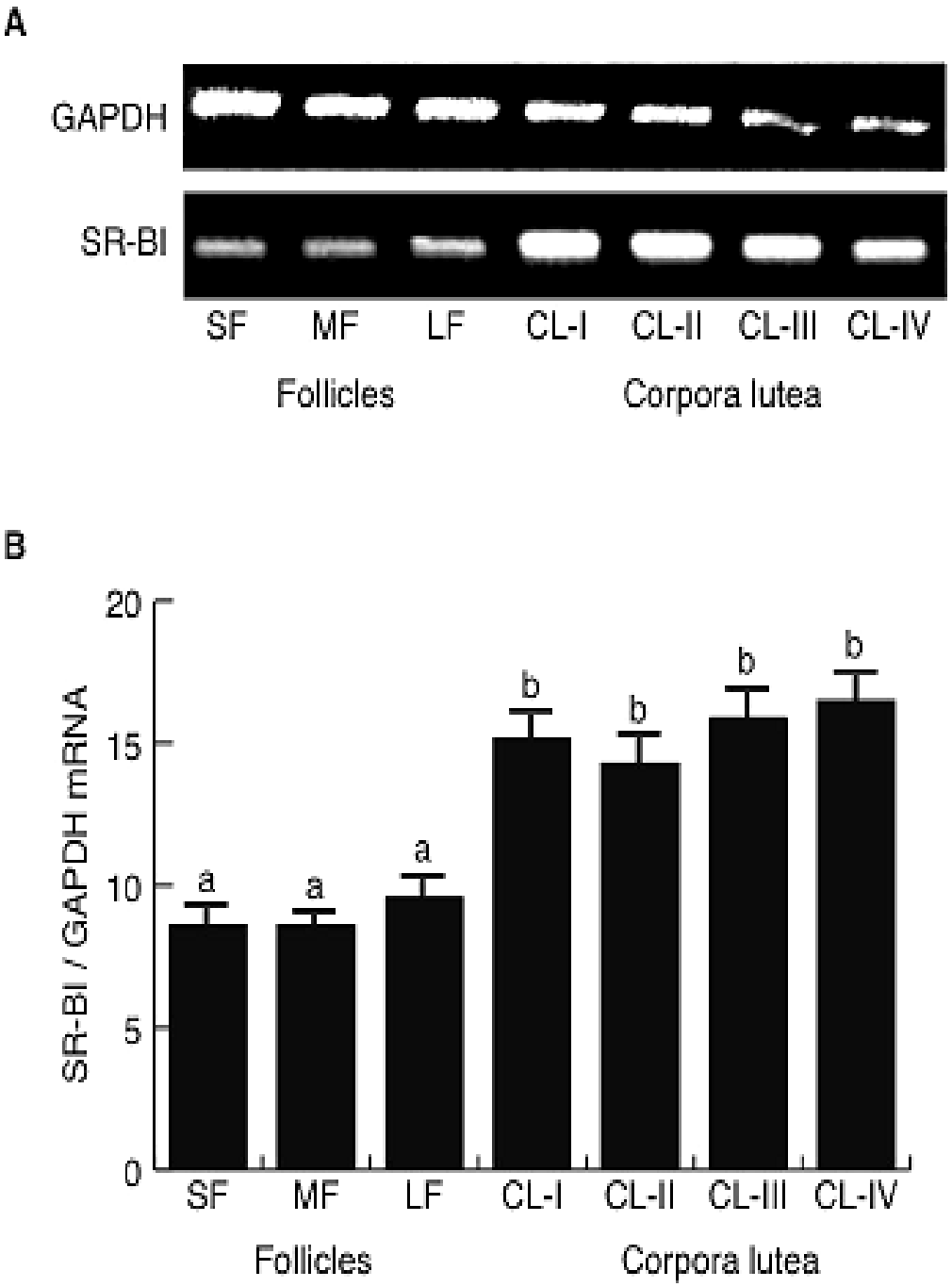

A
GAPDH
SR-BI
SF
MF
LF
CL-I
CL-II
CL-III
CL-IV
Follicles
Corpora lutea
B
SR-BI / GAPDH mRNA
20
15
10
5
0
a
a
a
b
b
b
b
SF
MF
LF
CL-I
CL-II
CL-III
CL-IV
Follicles
Corpora lutea

Figure 2.

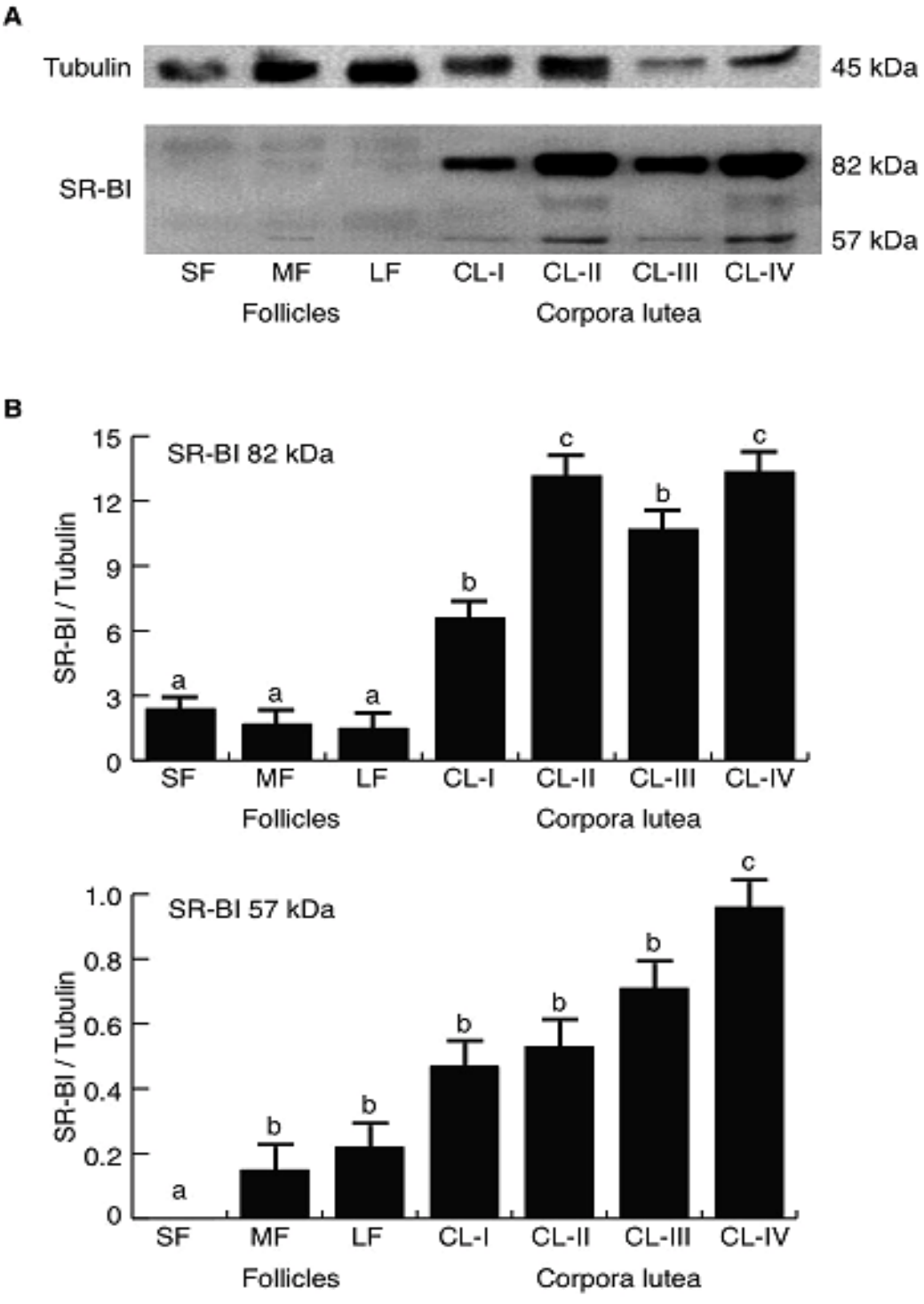
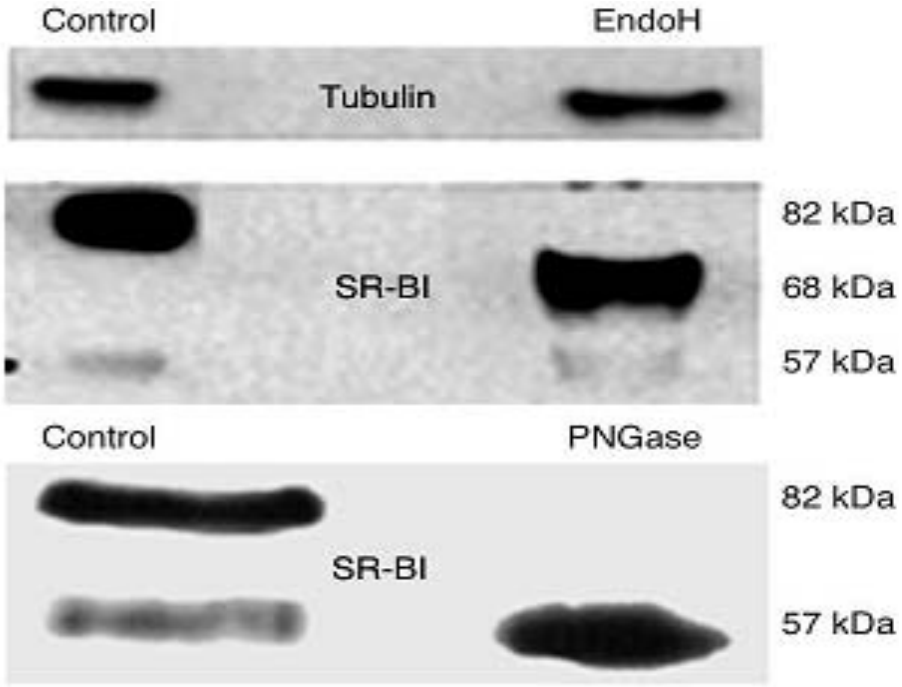

A
Tubulin
45 kDa
SR-BI
82 kDa
57 kDa
SF MF LF CL-I CL-II CL-III CL-IV
Follicles
Corpora lutea
B
SR-BI 82 kDa
SR-BI / Tubulin
15
12
9
6
3
0
a a a b c b c
SF MF LF CL-I CL-II CL-III CL-IV
Follicles
Corpora lutea
SR-BI 57 kDa
SR-BI / Tubulin
1.0
0.8
0.6
0.4
0.2
0
a b b b b b c
SF MF LF CL-I CL-II CL-III CL-IV
Follicles
Corpora lutea

Figure 3.


Control
EndoH
Tubulin
SR-BI
82 kDa
68 kDa
57 kDa
Control
PNGase
SR-BI
82 kDa
57 kDa

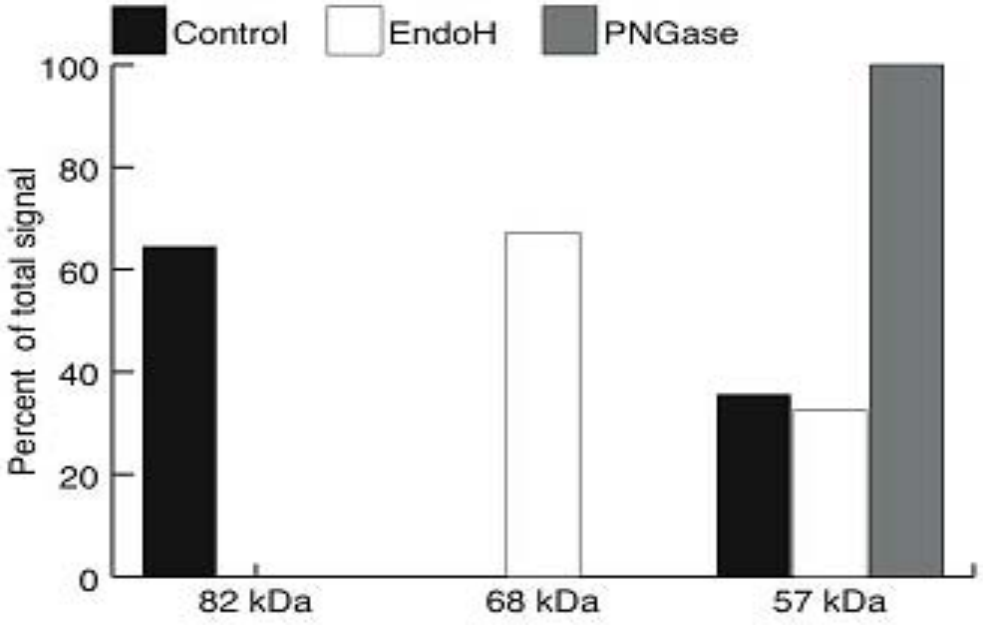

Control
EndoH
PNGase
Percent of total signal
100
80
60
40
20
0
82 kDa
68 kDa
57 kDa

Figure 4.

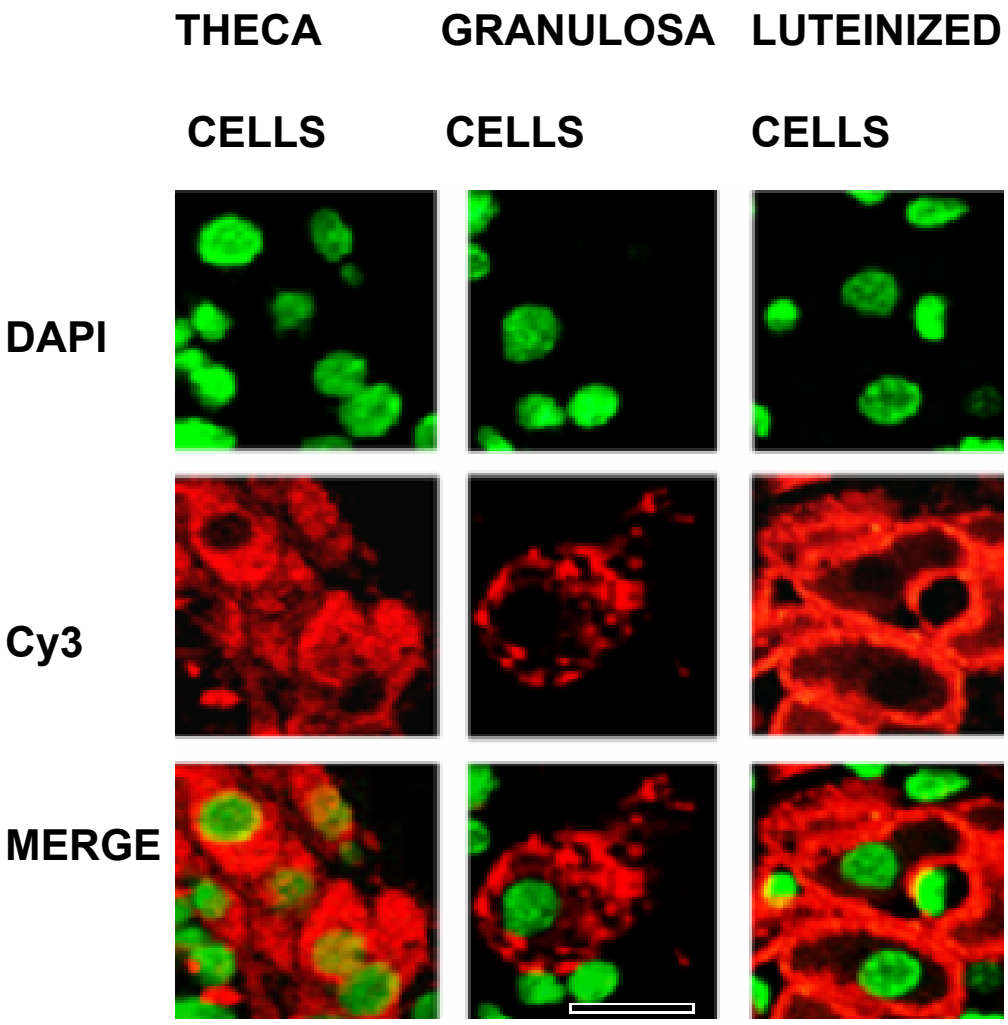

THECA CELLS
GRANULOSA CELLS
LUTEINIZED CELLS
DAPI
Cy3
MERGE

Figure 5.

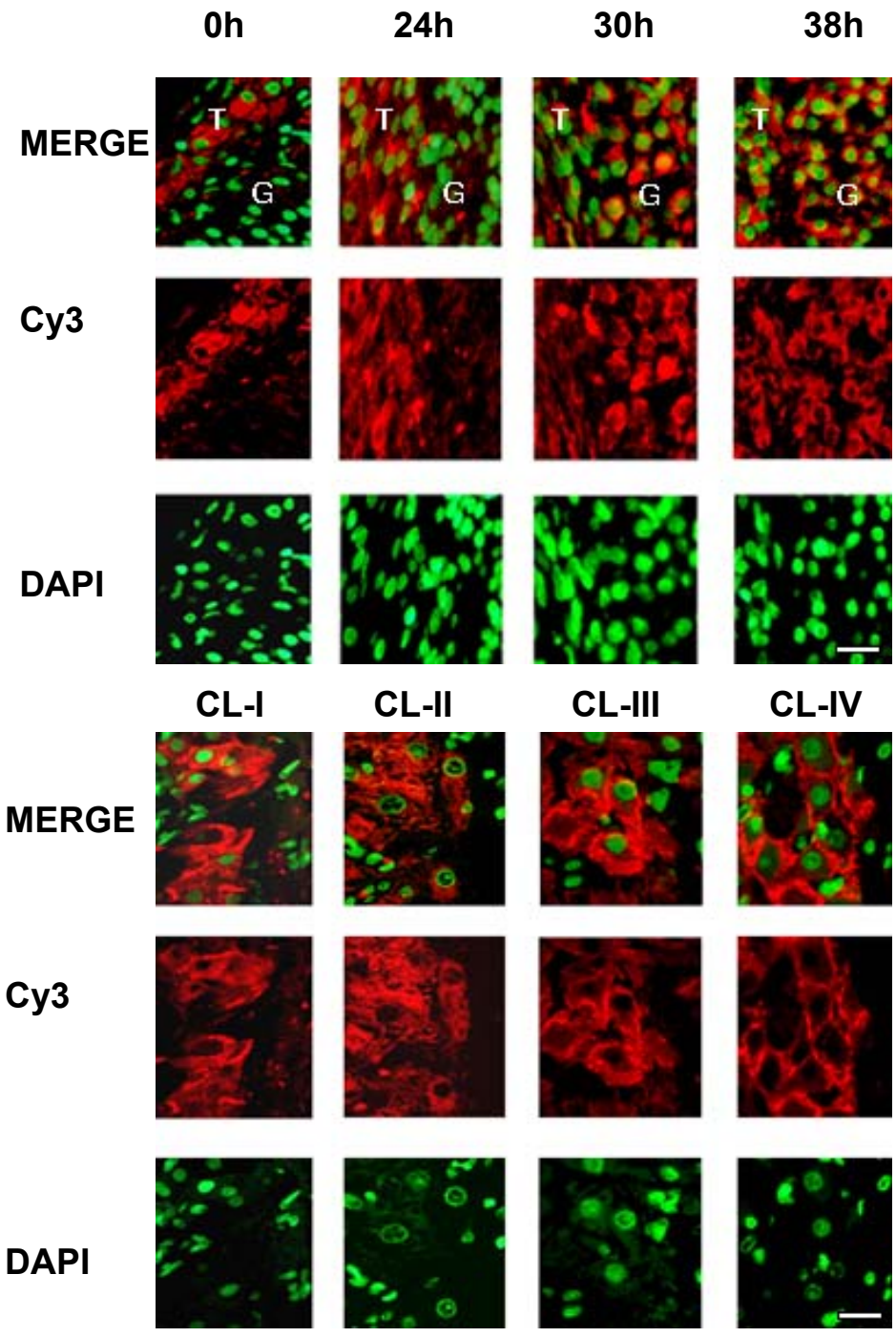

0h
24h
30h
38h
MERGE
T
G
Cy3
DAPI
CL-I
CL-II
CL-III
CL-IV
MERGE
Cy3
DAPI

Figure 6.

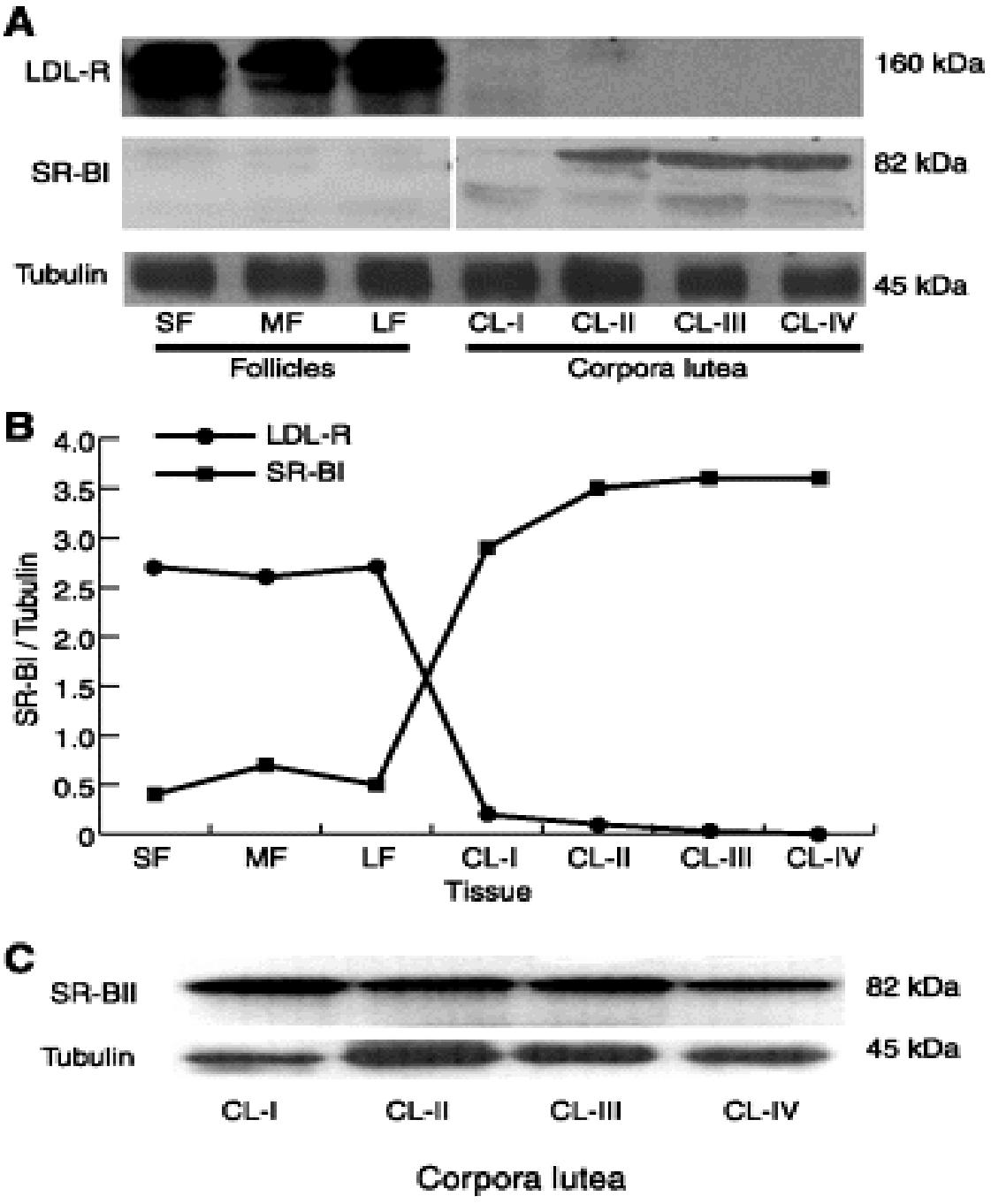

A
LDL-R
160 kDa
SR-BI
82 kDa
Tubulin
45 kDa
SF
MF
LF
CL-I
CL-II
CL-III
CL-IV
Follicles
Corpora lutea
B
LDL-R
SR-BI
SR-BI / Tubulin
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
SF
MF
LF
CL-I
CL-II
CL-III
CL-IV
Tissue
C
SR-BII
82 kDa
Tubulin
45 kDa
CL-I
CL-II
CL-III
CL-IV
Corpora lutea

Figure 7.

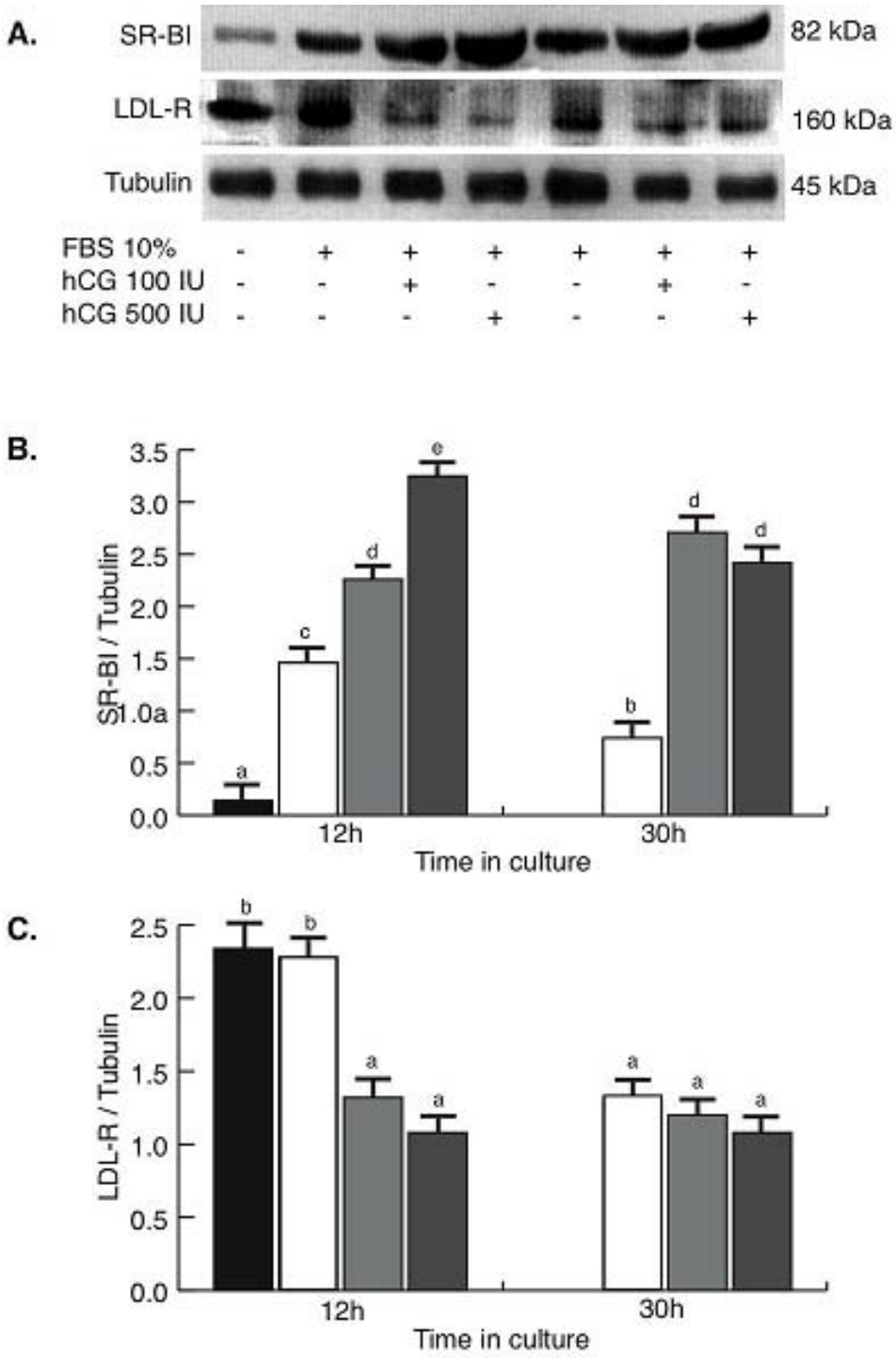

A.
SR-BI
LDL-R
Tubulin
82 kDa
160 kDa
45 kDa
FBS 10%
hCG 100 IU
hCG 500 IU
B.
SR-BI / Tubulin
12h
30h
Time in culture
C.
LDL-R / Tubulin
12h
30h
Time in culture

Figure 8.

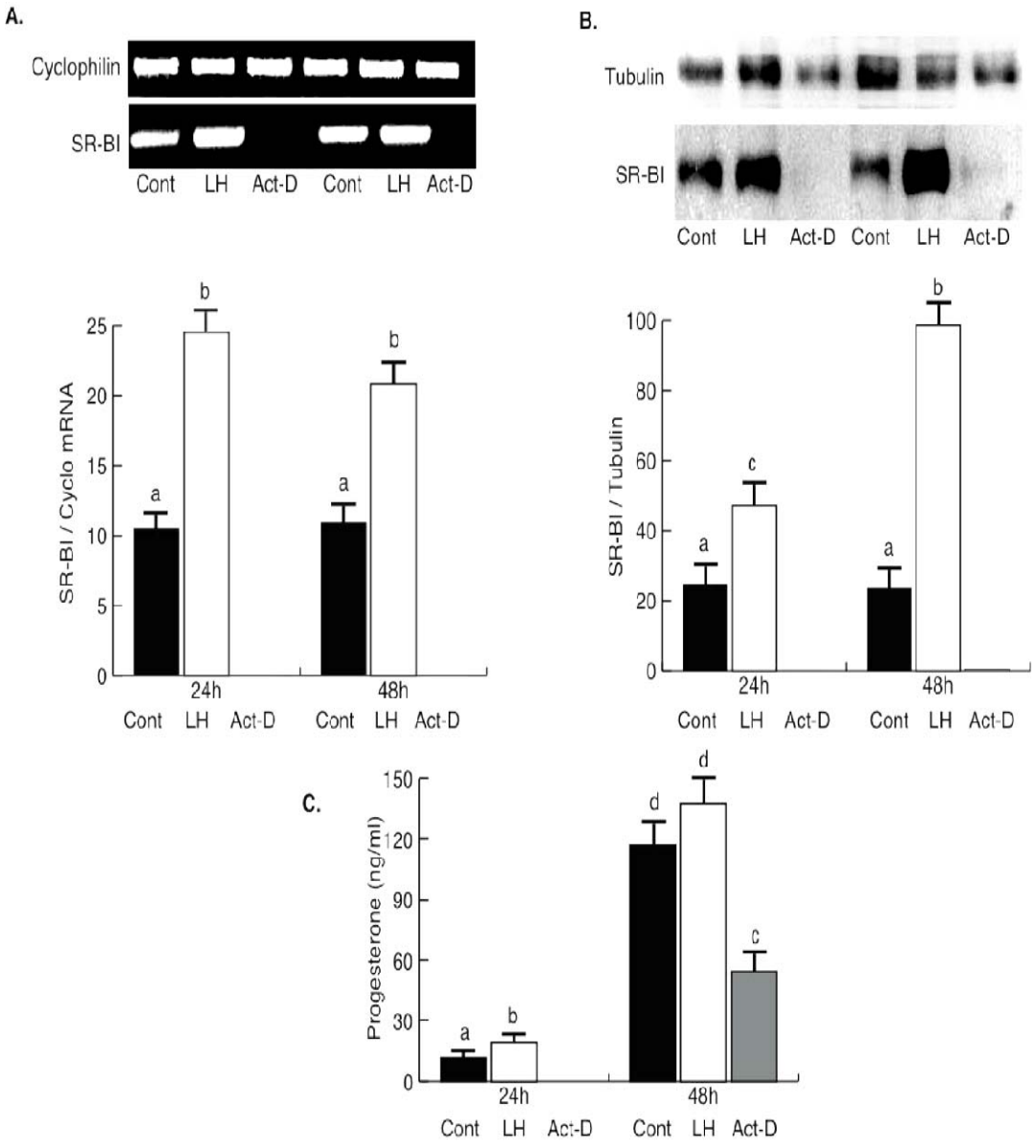
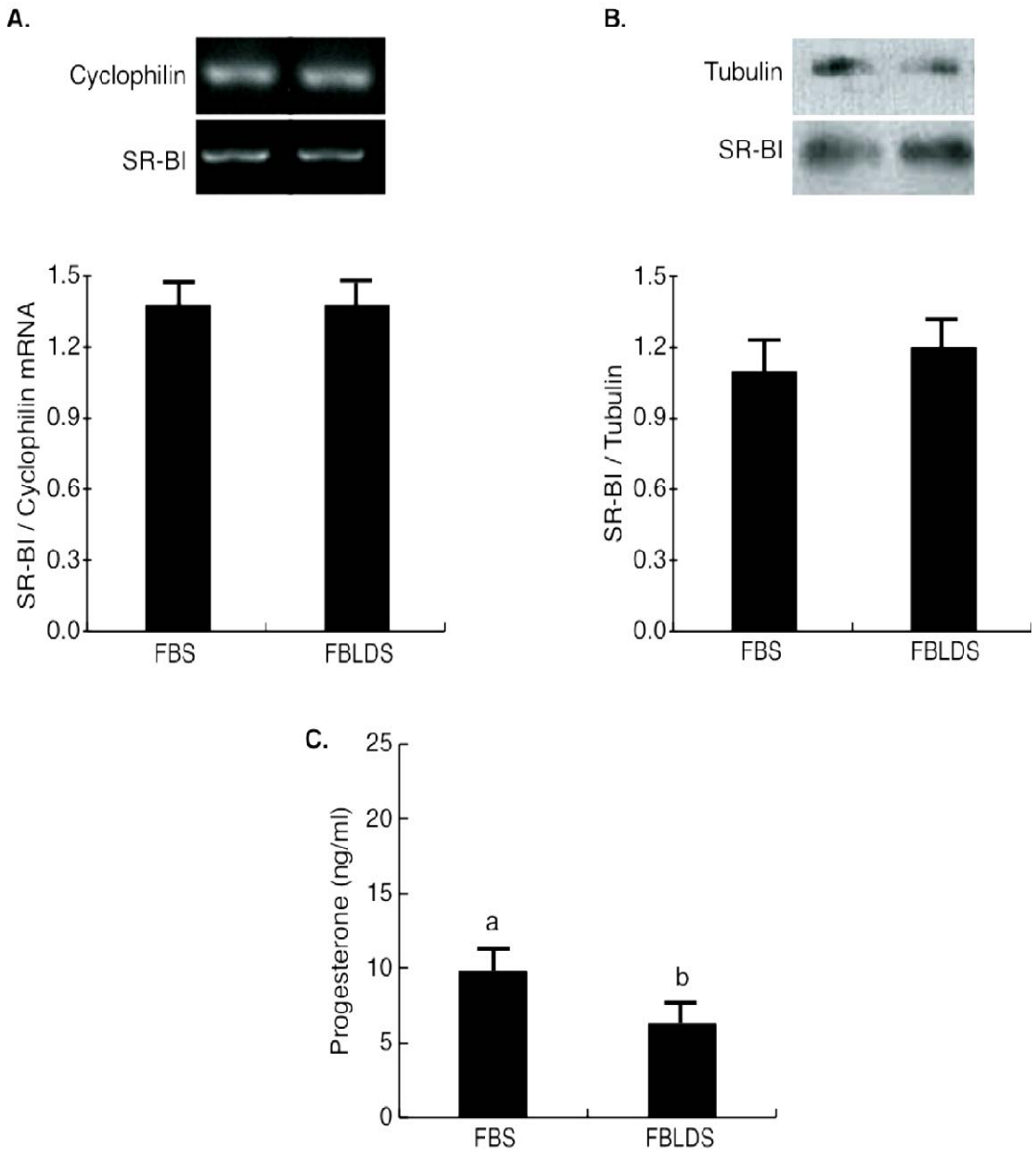

A.
Cyclophilin
SR-BI
Cont LH Act-D Cont LH Act-D
B.
Tubulin
SR-BI
Cont LH Act-D Cont LH Act-D
SR-BI / Cyclo mRNA
25
20
15
10
5
0
a
b
a
b
24h
48h
Cont LH Act-D Cont LH Act-D
SR-BI / Tubulin
100
80
60
40
20
0
a
c
a
b
24h
48h
Cont LH Act-D Cont LH Act-D
C.
Progesterone (ng/ml)
150
120
90
60
30
0
a
b
d
d
c
24h
48h
Cont LH Act-D Cont LH Act-D

Figure 9.


A.
Cyclophilin
SR-BI
SR-BI / Cyclophilin mRNA
1.5
1.2
0.9
0.6
0.3
0.0
FBS
FBLDS
B.
Tubulin
SR-BI
SR-BI / Tubulin
1.5
1.2
0.9
0.6
0.3
0.0
FBS
FBLDS
C.
Progesterone (ng/ml)
25
20
15
10
5
0
a
b
FBS
FBLDS

CHAPTER TWO

In the previous chapter, it was demonstrated that, in the pig model, the expression of SR-B1 characterizes luteinization. Although there is a low level of expression of the transcript and the protein in granulosa cells, there is a major shift increase as the CL develops, indicating the importance of HDL transport to luteal function. The experiments in Chapter Two were developed to further examine this phenomenon in the mouse CL, first by characterizing the pattern of expression of SR-B1, then by exploration of the consequences of null mutation of the SR-B1 gene on luteal structure and function.

Scavenger Receptor B-1 and Luteal Function in the Mouse

Leonor Miranda Jiménez[1,2,] , Mario Binelli[1,4,] , Kalyne Bertolin[1], R. Marc Pelletier[3], and Bruce D. Murphy[1]

Affiliations: [1]Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal, C.P. 5000, St-Hyacinthe, Québec Canada J2S7C6,

[2]Colegio de Postgraduados-CONACYT, México,

[4]Departmente d'Anatomie de Pathobiology, Université de Montréal [4]on leave from the University of Sao Paulo, Brazil.

ABSTRACT

During luteinization, bulk cholesterol uptake to ovarian cells from blood high-density lipoproteins (HDL) is achieved by scavenger receptor-BI (SCARB1). The expression of SCARB1 was detected in the cytoplasm and periphery of theca, granulosa and cumulus cells of developing follicles and increased dramatically in corpora lutea during the mouse estrous cycle. Further, SCARB1 expression is associated with granulosa cell differentiation and is independent of ovulation, as indicated by blockade of ovulation with a prostaglandin synthase-2 inhibitor, meloxicam. The resultant follicles displayed entrapped oocytes in unexpanded cumulus complexes while the granulosa layer expressed SCARB1 and morphology characteristic of luteinization. To further explore mechanisms of cholesterol supply to ovarian steroidogenesis, we examined mice bearing null mutation of the *scarb1* gene (SCARB1$^{-/-}$). The ovaries from SCARB1$^{-/-}$ mice displayed small corpora lutea, large follicles with thecal hypertrophy and follicular cysts with blood filled cavities. Plasma progesterone concentrations were decreased 50% in mice bearing the null SCARB1 mutation. SCARB1$^{-/-}$ mice were treated with a combination of 20 μg/g BW of mevinolin (an inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase, HMGR) and 100 μg/g BW of chloroquine (an inhibitor of lysosomal processing of low density lipoproteins). Serum progesterone was further reduced by this treatment (43% in wild type and 30% in SCARB1$^{-/-}$ mice). HMGR protein expression was increased in SCARB1$^{-/-}$ mice, regardless of CHLORO-MEV treatment. It was concluded that theca, granulosa and cumulus cells express SCARB1 during follicle development, but maximum expression depends on a luteinization stimulus. Knockout of SCARB1$^{-/-}$ leads to ovarian pathology and sub-optimal luteal steroidogenesis. Further perturbation of cholesterol de novo synthesis and uptake from LDL leads to a greater reduction, but not to complete impairment, of luteal function. We

conclude that SCARB1 expression is an important, but not essential, factor in maintaining ovarian cholesterol homeostasis and steroid synthesis.

INTRODUCTION

The cholesterol substrate requirement for most tissues to accomplish steroidogenesis exceeds the capacity for de novo synthesis of this sterol, and the principal means of augmentation of the supply is by importation of lipoprotein bound cholesterol [1]. Low density lipoproteins (LDL) enter cells by receptor-mediated endocytosis of LDL and the LDL receptor (LDLR) [2]. High density lipoproteins interact with a membrane protein known as scavenger receptor-B1 (SCARB1) to effect the selective uptake of cholesterol esters from the HDL molecule into cells and the bi-directional transfer of free cholesterol to and from the cell [3]. The relative contribution of LDL and HDL to the steroidogenic substrate pool varies among species, and may vary among tissues within a species. One of the most active steroidogenic tissues is the corpus luteum (CL), formed from the components of the follicle following ovulation [4]. In humans, circulating LDL is believed to be the major source of cholesterol for luteal steroid synthesis, but it has been shown that luteinized human granulosa cells can derive cholesterol esters from selective uptake via the HDL pathway [5]. The HDL pathway appears to predominate in the CL of rodents [1] and SCARB1 is expressed in theca and luteal cells of the rat ovary [6]. SCARB1 expression in primary cultures of rat granulosa cells is tightly coupled with the uptake of cholesterol esters, again suggesting that it is the major pathway for importation in this tissue [7]. There is a low level of expression of SCARB1 in mouse granulosa cells [8], and recent studies of luteinization in the pig demonstrated insignificant expression of SCARB1 in the granulosa cells of the follicle [9]. The latter study revealed extensive upregulation of SCARB1 in the CL following ovulation, with a high level of expression

persisting through the luteal phase. In the macaque, the ovulatory stimulus causes a rapid increase in expression of both SCARB1 and LDLR, but only LDL can augment steroidogenesis after 24 h in vitro [10].

The inactivating mutation of SCARB1 in the mouse results in infertility, in the presence of a normal rate of ovulation [11], and apparently normal capacity to produce progestational steroids [12]. The observation that non-viable oocytes are produced has led to the implication that the ovulatory process itself is disrupted in some way [11, 12]. It is intriguing that targeted overexpression of SCARB1 in the liver rescues fertility to near wild type levels [13] although mechanisms remain unclear. Further, if the major supply of cholesterol for steroidogenesis in the CL is HDL imported via SCARB1 in mice, the means by which the ovary in the SCARB1 knockout mouse produces a functional CL remains unresolved.

Given the importance of SCARB1 to steroidogenesis and ovarian function, we explored the expression of SCARB1 in the ovary through the estrous cycle in adult mice and following blockade of ovulation in gonadotropin-stimulated immature mice. Finally, to determine possible associations of form and function in the absence of a functional HDL importation system, we examined the morphology of ovarian structures and responses to pharmacological perturbations of the cholesterol metabolism in mice bearing inactivating mutation of SCARB1.

MATERIALS AND METHODS

All animal experiments were approved by the University of Montreal Animal Care Committee and conducted according to guidelines of the Canadian Council of Animal Care.

Experiment 1: Our initial interest was to determine patterns of expression of SCARB1 in the ovary during final stages of follicular development and luteinization. Stages of the estrous cycle were determined in mature wild type (WT) mice of mixed C56/B6 and Balb/c lineage by

examination of exfoliative cytology of the vagina. To determine the distribution of SCARB1, ovaries were collected following euthanasia from three animals at each stage, dissected free from surrounding tissues under a stereoscope, fixed in paraformaldehyde and embedded in paraffin using standard procedures. Ovarian tissue- and cell-specific localization of SCARB1 was verified by fluorescence immunohistochemistry. Briefly, paraffin sections of ovaries were rehydrated, boiled in 10 mM sodium citrate (pH 6.0) for 20 min and cooled to room temperature (RT). Subsequently, sections were blocked in PBS containing 5 % BSA for 30 min at RT, incubated with rabbit polyclonal anti-mouse SCARB1 (Novus Biologicals, Littleton, CO), diluted 1:50 in PBS containing 5 % BSA overnight at 4°C, washed, incubated with Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Lab., Inc.), diluted 1:400 in PBS for 1 h at RT. Slides were then washed, and sections counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO), diluted 1:1000 in PBS, for 5 min. Slides were mounted in Permafluor (Lab Vision Corp.; Fremont, CA). Ovarian distribution of SCARB1 was observed by confocal microscopy using the Olympus Fluoview 1000 system and Fluoview version 1.7 software. Laser sources of 543 nM and 405 nM were employed for detection of CY3 and DAPI signals, respectively. For a given fluorochrome (i.e., CY3 or DAPI), photomultiplier voltage, laser power, pinhole aperture, time of acquisition and image resolution were maintained constant for all confocal images. The Kalman filter mode was used for all image acquisition. For high magnification images (600 x) of follicles, “stacks” of five subsequent images, 1.42 mm thick each, were obtained. Then, for each particular cell type (i.e., theca, mural granulosa or cumulus), the image within the stack showing the strongest signal was selected for analysis.

Experiment 2: To determine whether the elevated expression of SCARB1 that accompanies luteinization depends on ovulation, immature WT female mice received IP injections of equine

chorionic gonadotropin (eCG; 5IU) followed by human chorionic gonadotropin (hCG; 5IU) 48 h later to induce follicular development and ovulation. At 4 h prior to the hCG injection, animals received 0 or 6 mg/g BW meloxicam [MEL; prostaglandin synthase-2, PTGS2) activity inhibitor; Sigma, St. Louis, MO] to block ovulation [14]. Ovaries and oviducts were collected 18, 24 or 36 h after the hCG injection (n=3 animals/time point) and separated by dissection. Oviducts and the right ovary of each mouse were embedded in paraffin as described for Experiment 1. Morphology of ovarian structures and presence of ovulated oocytes in oviducts were observed by light microscopy in hematoxlyn/eosin (HE) stained tissue sections. Ovarian tissue- and cell-specific localization of SCARB1 was verified by fluorescence immunohistochemistry and confocal microscopy, as described for Experiment 1. The left ovary of each mouse was snap frozen in liquid nitrogen and stored at -80C until RNA extraction and real-time PCR (qPCR) analysis of SCARB1 mRNA abundance. Total RNA was isolated from each previously frozen whole ovary using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) as per manufacturer's instructions. For each sample, concentration of isolated total RNA was estimated from the absorbance measured at 260 nm using a a NanoDrop apparatus (NanoDrop Technologies, Wilmington, DE) and the volume of extract containing 1 μg RNA was submitted to DNAse treatment and reverse transcription. The resulting cDNA was used in subsequent qPCR reactions using a 7300 Real-time PCR system (Applied Biosystems, Foster City, CA) conducted in triplicate and each reaction contained 10 μl Power SYBR Green PCR Master Mix (Applied Biosciences, Warrington, UK), 2 μl of a sense-antisense primer mix, 2 μl autoclaved ddH_2O and 6 μl cDNA sample, for a final volume of 20 μl. Common thermal cycling settings were use to amplify each transcript (2 min at 50C, 10 min at 95C, then 40 cycles consisting of 15 sec at 95C and 60 sec at 60C). Melting-curve analyses were performed to verify product

identity, adding a dissociation step to the PCR run (15 sec at 95C, 60 min at 60C, 15 sec at 95C and 15 sec at 60C). The sense and anti-sense sequences of primers for SCARB1 were 5'-tctggcgctttttctatcgt-3' and 5'-acggcccatacctctagctt-3' [15], used in a final concentration of 300 nM each. The ribosomal protein L19 (RPL19) was used for the normalization of SCARB1 abundance. The RPL19 primer sequences for sense and antisense primers were 5'-CTGAAGGTCAAAGGGAATGTG-3'and 5'-GGACAGAGTCTTGATGATCTC-3' [16] and they were each used in a 300 nM final concentration. To test the efficiency of amplification of primers of each gene, a cDNA pool was made using equal volumes of cDNA solution from each sample. The cDNA pool was serially diluted in autoclaved ddH_2O from 1:7.5 to 1:240 and samples analyzed using the qPCR procedure described above. Efficiency of amplification of each sample was estimated using the LinReg software [17] and the efficiency of amplification of the curve obtained from the serially diluted cDNA calculated from the slope of the curve [http://efficiency.gene-quantification.info/]. Calculated efficiencies ranged between 90 and 110% and were therefore considered adequate. Next, cDNA from each sample was diluted 1:15 in autoclaved ddH_2O and analyzed by qPCR for RPL19 and SCARB1. Relative abundance of SCARB1 transcripts was calculated by the Ct method corrected by the efficiency of amplification, as described by Pfaffl [18]. The relative abundance of SCARB1 was analyzed by ANOVA using the procedure GLM from the software SAS [19]. Independent variables were treatment (placebo or MEL), time after hCG injection (18, 24 or 36 hours) and the treatment by time interaction.

Experiment 3: Given the central role of SCARB1 in ovarian steroidogenesis, a SCARB1$^{-/-}$ mouse model provided by Dr. M. Krieger [11], was used to examine the effects of SCARB1 absence on ovarian morphology. Development and ovulation of follicles in adult, wild type

(WT) and SCARB1$^{-/-}$ mice were stimulated with eCG and hCG injections 48 h apart. Cholesterol supply was further perturbed by pharmaceutical intervention with *de novo* synthesis and delivery of cholesterol from LDL. At 4 h prior to hCG injection, animals were divided to receive vehicle or a combination of 20 μg/g BW mevinolin (MEV), an inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR; Sigma, Fair Lawn, NJ) and 100 μg/g BW of chloroquine (CHLORO; Science Lab. Inc., Kingwood, TX)), a weak base that interferes with cholesterol efflux from lysosomes, thereby inhibiting intracellular delivery of cholesterol from LDL [20, 21]. The experiment took the form of a 2x2 factorial (n=3 per group). Ovaries and blood were collected 18 h after the hCG injection, the former fixed in Bouin's solution for 24 h and embedded in paraffin, as described earlier. Morphology of ovarian structures was observed by light microscopy both in HE- and periodic acid-Schiff (PAS)-stained tissue sections. To verify whether inhibiting cholesterol sources affected ovarian tissue- and cell-specific localization of SCARB1 in WT mice, SCARB1 was identified by immunohistochemistry, as described for Experiment 1. Because manipulating cholesterol sources was expected to alter expression of cholesterol synthesizing enzymes, ovarian abundance and distribution of HMGR was analyzed in all animals by immunohistochemistry. The procedure was similar to described above for detection of SCARB1, except that a goat anti-human polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:50 in PBS containing 5% BSA, and a CY3-conjugated donkey anti-goat IgG antibody (Jackson Immunoresearch Lab., Inc.), diluted 1:400 in PBS were employed.

Serum was separated from whole blood and progesterone concentrations in serum were evaluated by radioimmunoassay according to a protocol described previously [22]. Concentrations of progesterone were analyzed by ANOVA using the procedure GLM from the

software SAS [19]. Independent variables were genotype (WT or SCARB1$^{-/-}$), treatment (control or CHLORO and MEV combination) and the genotype by treatment interaction.

RESULTS

Experiment 1: Immunohistochemical analysis revealed that SCARB1 was present in theca and granulosa compartments of large follicles and in CLs (Figure 1). Overall, SCARB1 expression appeared to increase as follicles developed from proestrus to estrus through the estrous cycle and expression was maximum following luteinization. Punctate expression of SCARB1 was observed in theca, mural granulosa, cumulus, and luteal cells (Figure 2). In all cell types, expression was observed throughout the cytoplasm and plasma membrane, but appeared absent from the nucleus. Staining was more intense in the cytoplasm of luteal cells, and, given the larger volume of cytoplasm with respect to follicular cells, it was clear that SCARB1 protein expression per cell is greater in the cells of the CL.

Experiment 2: To further establish the expression patterns of SCARB1 and to determine the role of ovulation in the increase of the SCARB1 gene expression and immunoreactivity, we treated mice with the inhibitor of PTGS2 activity, MEL or with vehicle. Histological analysis of the ovaries (Figure 3) and oviducts (data not shown) revealed that ovulation had occurred in all but a few large follicles in the vehicle treated animals by 18 h after hCG treatment. The expected CL formation was evident in control animals at 24 and 36 h (Figure 3 A-C). In contrast, the ovaries of mice treated with MEL displayed oocytes surrounded by compact layers of cumulus cells in follicles and no corpora lutea at 18 h after hCG treatment, antral follicles with entrapped oocytes and a few corpora lutea at 24 h, and oocytes entrapped in follicles with

reduced or absent antra at 36 h (Figure 3 D-F). In the MEL treated mice, the granulosa layer at 36 h after hCG appeared two or more fold thicker than in preovulatory follicles.

Ovarian abundance of SCARB1 mRNA (Figure 4) increased over time after hCG injection and was maximum at 36 hours (effect of time; $P<0.01$). Increases occurred regardless of treatment (no treatment by time interaction; $P>0.6$), indicating that blocking ovulation did not interfere with whole ovary SCARB1 expression. SCARB1 protein immunolocalization indicated that MEL treatment induced a redistribution of the SCARB1 signal among the different ovarian structures. In the ovaries of vehicle treated mice, the signal for SCARB1 was found in the cytoplasm of granulosa cells at 18 h after the ovulatory stimulus and appeared not to vary at 24 h or 36 h (data not shown). The granulosa cells maintained expression levels not different from proestrus, as seen in Figures 1 and 2. In contrast, immunoreactivity in luteal cells increased across time Figure 5 A-C). Indeed, by 36 h after the hCG injection, luteinization was indicated by increases in the cytoplasm to nuclear volume ratio and luteal cells displayed the luteal phenotype with respect to punctate distribution of SCARB1 immunoreactivity. In ovaries from animals in which ovulation was blocked with MEL, the onset of increased expression of SCARB1 in non-ovulated follicles, as visualized by confocal microscopy, occurred at 18 h (Figure 5 D-F). By 36 h, the cells in the thickened mural granulosa layer had taken on the luteal phenotype with respect to SCARB1 expression in spite of the absence of ovulation and entrapment of the oocyte. In the few follicles that ovulated in MEL treated mice, the signal was similar to that seen in granulosa cells of the vehicle treated controls at 24 h (Figure 5G). At 36 h the luteal phenotype was emerging, but appeared to be delayed relative to the control CL, in that there was an apparently lower expression of SCARB1 (Figure 5H).

Experiment 3: Previous reports suggest that there is no luteal defect in mice bearing null mutation of the SCARB1 gene (Rigotti 1997, Yesilaltay 2005). In contrast, in the present study, we show that circulating progesterone levels in serum samples of superstimulated, ovulated mice were lower in SCARB1 knockout mice by approximately 50 % compared to the WT ($P<0.01$) during the luteal phase, (Figure 6). It was therefore of interest to determine whether there are morphological correlates in ovaries of SCARB1$^{-/-}$ mice that might have caused the reduced progesterone output.

The ovaries in the SCARB1$^{-/-}$ mice examined consistently displayed abnormal structures not found in WT ovaries (Figures 7 and 8). The first was a blood-filled follicular cyst (Figure 8A). This structure was present in various degrees of enlargement throughout the SCARB1$^{-/-}$ ovaries. At 18 h after hCG injection, approximately 6 h after expected ovulation, ovaries from SCARB1$^{-/-}$ mice contained structurally normal corpora lutea that were reduced in size relative to the same structures in WT mice (Figure 8B). A common finding was the presence of follicle-like structures that appeared to have two layers, with the outer comprising hypertrophied, luteal like cells and the interior resembling the granulosa cell compartment of a normal follicle (Figure 8C). To further define this structure, ovarian sections were stained with PAS to reveal polysaccharides. This analysis revealed an intact basement membrane (Figure 8D) between the outer and inner compartments, leading to the conclusion that this structure is an intact follicle with a hypertrophied theca compartment.

We then attempted to further reduce the cholesterol pool in SCARB1$^{-/-}$ and wild type mice by blocking de novo synthesis with MEV and interdicting LDL processing with CHLORO. Overall, this treatment reduced circulating progesterone 42% ($P<0.02$). Reduction was 43% in wild type mice and 30% in SCARB1$^{-/-}$ (Figure 6). No effect of the combined treatment on luteal

morphology was evident in ovaries harvested from either the WT or SCARB1$^{-/-}$ mice (Figure 7). To further explore the means by which SCARB1$^{-/-}$ mice compensate for the deficiency in HDL uptake, we examined the abundance of signal for HMGR in the luteal structure in WT and SCARB1$^{-/-}$ mice by fluorescent immunohistochemistry and confocal microscopy. The intensity of fluorescence indicates that HDL deficiency in SCARB1$^{-/-}$ mice results in increased expression of this enzyme (data not sowed). Blockade of HMGR activity and concurrent interference with LDL metabolism had no apparent effect on the abundance of the HMGR cellular protein in the wild type mice. In contrast, the expression of the cholesterol synthetic enzyme appeared more intense in the MEV-CHLORO treated SCARB1$^{-/-}$ mice. This further supports the view that increased HMGR expression is a major mechanism by which SCARB1$^{-/-}$ mice obtain the cholesterol required for luteal steroid synthesis.

DISCUSSION

The pattern of expression of SCARB1 in the mouse follicle differs from the other species where it has been examined in detail, the pig (Miranda-Jiménez et al. 2007), in that there is substantial signal present in the granulosa cells throughout the estrous cycle. The protein localized to the cytoplasm and to the periphery of both granulosa and luteal cells in the mouse, in contrast to the pig findings, where there was a low level of punctate expression in the cytoplasm and primarily peripheral localization of the protein of the granulosa cells [9]. This difference may reflect, in part, differences in the method of detection, as, in another study, conventional microscopy demonstrated SCARB1 to be localized on the apical membrane of the enterocyte, while confocal microscopy revealed both cytoplasmic and membrane distribution [23].

We further observed strong expression in the cumulus oophorus of the follicle, consistent with the view that the cumulus serves as an important source of cholesterol for the maturing

oocyte [24]. The distribution of the protein suggests SCARB1 may play an important role in uptake of HDL from the follicular fluid and consequent efflux to the oocyte. The SCARB1$^{-/-}$ mouse, while capable of ovulation, is infertile, due to so far unexplained oocyte defects that prevent fertilization [12]. This infertility is reversed by transplant of the ovaries to the WT females [12], or by induction of hepatic SCARB1 expression in the liver alone [13]. These findings are consistent with the conclusion that dyslipidemia, and inappropriate transfer of HDL cholesterol between the cumulus cells and the oocyte impair normal oocyte development, thereby rendering the ovulated ovum not fertilizable.

To explore the role of ovulation and luteinization in SCARB1 expression, we employed a pharmacological blockade of PTGS2, an enzyme well known to be necessary for the ovulatory process [25]. The qPCR results indicate that the process of SCARB1 expression in the ovary occurs in the absence of ovulation, and on a similar time scale. Moreover, dynamics of SCARB1 expression in relation to hCG injection were similar between treatments. This is intriguing because, as observed in Experiment 1, CLs are the ovarian structures with greatest SCARB1 expression, and they were present in a lower number in MEL-treated mice in all time points studied. Fluorescent immunohistochemistry analysis permitted to observe a redistribution of the SCARB1 signal caused by MEL treatment. Specifically, the SCARB1 signal became more intense in non-ovulated follicles in the MEL group, supporting the notion that such follicles luteinized. High expression of SCARB1 in non-ovulated, luteinized follicles probably accounted for less expression of luteal SCARB1 in MEL-treated mice. The rare follicles that ovulated following MEL treatment appeared delayed in expression of SCARB1 suggesting that, in a few cases, the treatment retarded ovulation.

Western analysis confirmed the absence of the SCARB1 protein in the SCARB1$^{-/-}$ mouse. In contrast to previous reports that indicate no differences in ovarian morphology between null and WT mice, and no reduction in circulating progesterone [11], our findings suggest significant pathology in the SCARB1$^{-/-}$ mouse ovary. Corpora lutea were consistently smaller in diameter than those in WT animals. There were large cystic, hemorrhagic follicles which resembled structures in mice with null mutation of the estrogen receptor- gene [26], suggesting that estrogen deficiency may play a role in the phenotype. A further structure represented by an intact granulosa compartment surrounded by a hypertrophied theca was common in the ovaries of the SCARB$^{-/-}$ mouse. The basis for this anomaly is unclear, but follicles with similar morphology can be found in a mouse model engineered to overexpress the luteinizing hormone-ß subunit [27].

We then examined the effects of reducing the availability of cholesterol by concurrent interdiction of de novo synthesis and interference with lyosomal processing of LDL. This combined treatment had no apparent effect on the morphology of the ovaries, and reduced, but did not eliminate, circulating progesterone in both WT and SCARB$^{-/-}$ mice. The increased expression of HMGR in the SCARB1$^{-/-}$ ovary was interpreted to be a feedback response to the reduction in intracellular cholesterol in the transgenic ovaries [28]. The mouse bearing spontaneous mutation in the Niemann-Pick C1 gene is unable to process LDL cholesterol but displays circulating progesterone concentrations that do not differ from WT mice [29]. The dysfunction of the LDL pathway is compensated, presumably by de novo synthesis of cholesterol and its importation via SCARB1. As all three sources were compromised in the present study, it must be concluded that cholesterol stored in the CL in the form of cholestryl esters was the source of the substrate for progesterone synthesis.

The feedback system for cholesterol homeostasis is regulated by the abundance of the transcriptionally active form of SREBP [28]. An important transcriptional target of SREBP is the HMGR gene, and evidence from the present investigation indicates that its expression is elevated in the SCARB1$^{-/-}$ mouse corpus luteum. This concurs with the idea that inactivation of an import pathway, as occurs in the NPC-1 mutant mouse, is compensated by increased de novo cholesterol synthesis. We saw an even greater expression of HMGR when there was interdiction of both the HDL and LDL import pathways. It would appear that increased HMGR expression is a major alternative to supply cholesterol for luteal steroidogenesis.

In summary, we have shown an increasing, predominantly cytoplasmic SCARB1 protein expression in theca and granulosa cells in the mouse ovary during the final stages of follicle growth, with expression becoming maximal as follicular cells luteinize in response to the ovulatory stimulus. Lack of a functional SCARB1 protein resulted in morphological aberrations of follicular structures in the ovary and reduction in progesterone synthesis. Intraovarian cholesterol synthetic mechanisms can compensate, at least in the short term, for a non-functional cholesterol import system.

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FIGURE LEGENDS

Figure 1. Protein expression of SCARB1 in mouse follicles during proestrus (panel A) and estrus (panel D) and in the corpus luteum (panel G) of mature mice undergoing normal estrous cycles. Panels A, D and G: confocal microscopy images of fluorescent immunohistochemistry staining of SCARB1; panels B, E and H: confocal microscopy images of DAPI staining of nuclei; panels C, F and I: merged image of SCARB1 and DAPI staining. The insert in panel G is a negative control for the SCARB1 fluorescent immunohistochemistry staining reaction. Bars: 50mm.

Figure 2. Protein expression of SCARB1 in the ovarian theca (panel A), granulosa (panel B), cumulus oophorus (panel C) and luteal cells (panel D) of mature mice undergoing normal estrous cycles. Panels A, B, C and D are confocal microscopy images of fluorescent immunohistochemistry staining of SCARB1; panels E, F, G and H: confocal microscopy images of DAPI staining of nuclei; panels I, J, K, L: merged image of SCARB1 and DAPI stainings. Bars: 10mm.

Figure 3. Bright field microscopy images of hematoxylin-eosin stained sections of ovaries from immature mice stimulated with equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) and collected 18 (panels A and D), 24 (panels B and E) or 36 h after hCG (panels C and F). Mice received 0 (panels A, B and C) or 6 mg/g BW meloxicam, a prostaglandin synthase-2 activity inhibitor, (panels D, E and F). Bars: 150mm.

Figure 4. Relative abundance of SCARB1 mRNA (LSmeans SEM) in the whole ovary of immature mice stimulated with PMSG and hCG and collected 18, 24 or 36 h after hCG as determined by qPCR. Mice received 0 (Control; open bars) or 6 mg/g BW meloxicam, a cyclooxygenase-2 activity inhibitor (Meloxicam; solid bars).

Figure 5. Protein expression of SCARB1 in the corpus luteum (panels A, B, C, G and H) and in follicles (panels D, E and F) of immature mice stimulated with eCG and hCG and collected 18 (panels A and D), 24 (panels B, E and G) or 36 h after hCG (panels C, F and H). Mice received 0 (panels A, B and C) or 6 mg/g BW meloxicam, a prostaglandin synthase-2 activity inhibitor, (panels D through H). Merged confocal microscopy images of fluorescent immunohistochemistry staining of SCARB1 and DAPI staining of nuclei. Bars: 50mm.

Figure 6. Plasma progesterone concentrations (LS means ± SD) from wild type (WT) or SCARB1$^{-/-}$ mature mice stimulated with eCG and hCG and collected 18 h after hCG. Mice received placebo injections (Control) or a combination of 20 mg/g BW mevinolin (Mev; an HMGR inhibitor) and 100 mg/g BW chloroquine (Chloro; an inhibitor of lysosomal processing of LDL).

Figure 7. Bright field microscopy images of hematoxylin-eosin stained sections of ovaries from wild type (WT; panels A and C) or SCARB1$^{-/-}$ mature mice stimulated with eCG and hCG and collected 18 h after hCG. Mice received placebo injections (panels A and B) or a combination of 20 mg/g BW mevinolin (MEV; an HMGR inhibitor) and 100 mg/g BW chloroquine (CHLORO; an inhibitor of lysosomal processing of LDL; panels C and D). Bars: 200mm.

Figure 8. Bright field microscopy images of atypical structures in hematoxylin-eosin stained (panels A, B and C) or periodic acid-Schiff (PAS) stained (panel D) sections of ovaries from SCARB1$^{-/-}$ mature mice stimulated with eCG and hCG and collected 18 h after hCG. Panel A: follicular cyst-like structure; bar: 200mm. Panel B: small luteinized structure; bar: 100mm. Panel C: partially luteinized follicle; bar: 50mm. Panel D: partially luteinized follicle showing intact basal membrane (arrow); bar: 50mm.

FIGURES

Figure 1.

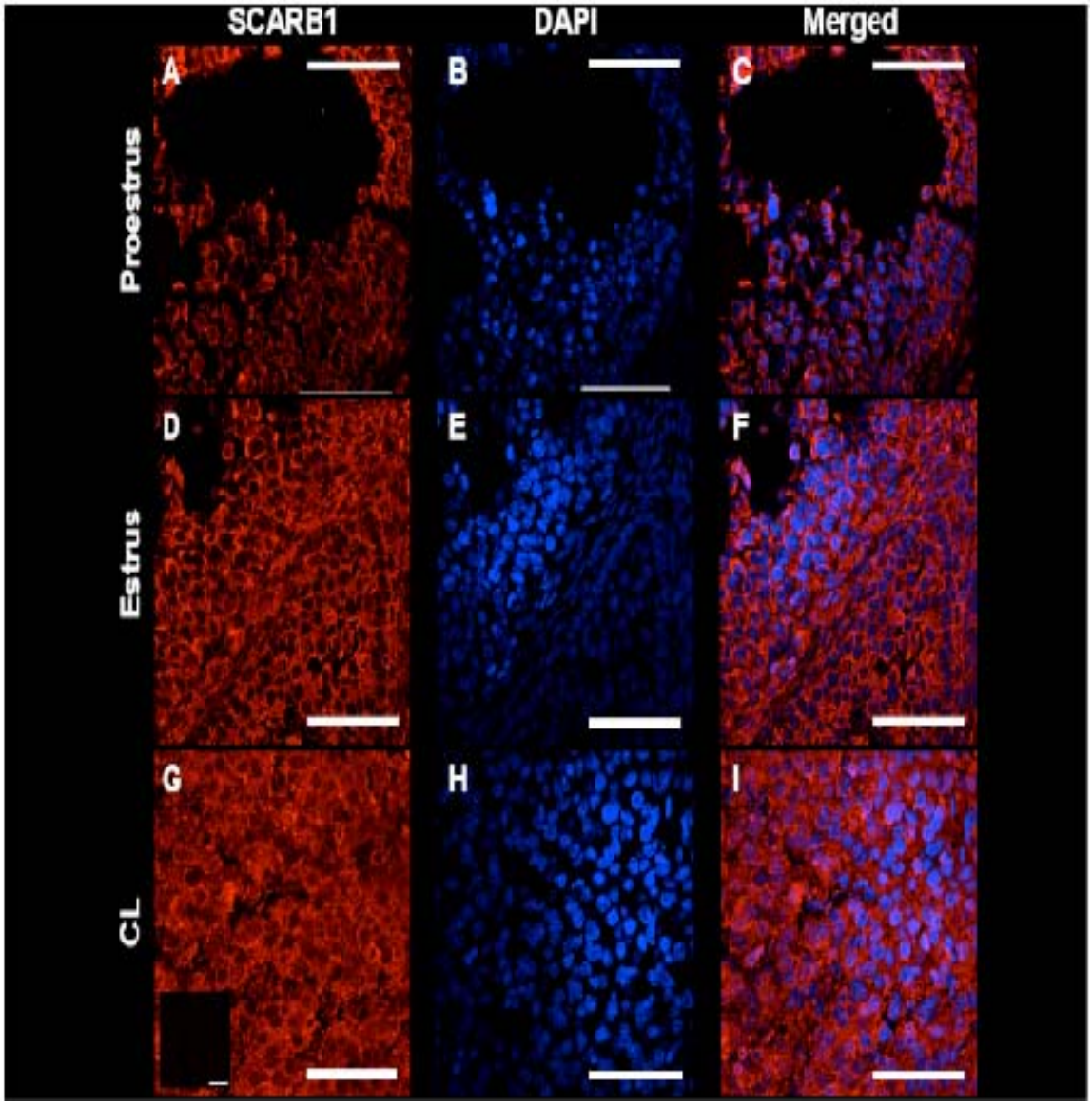

Figure 2.

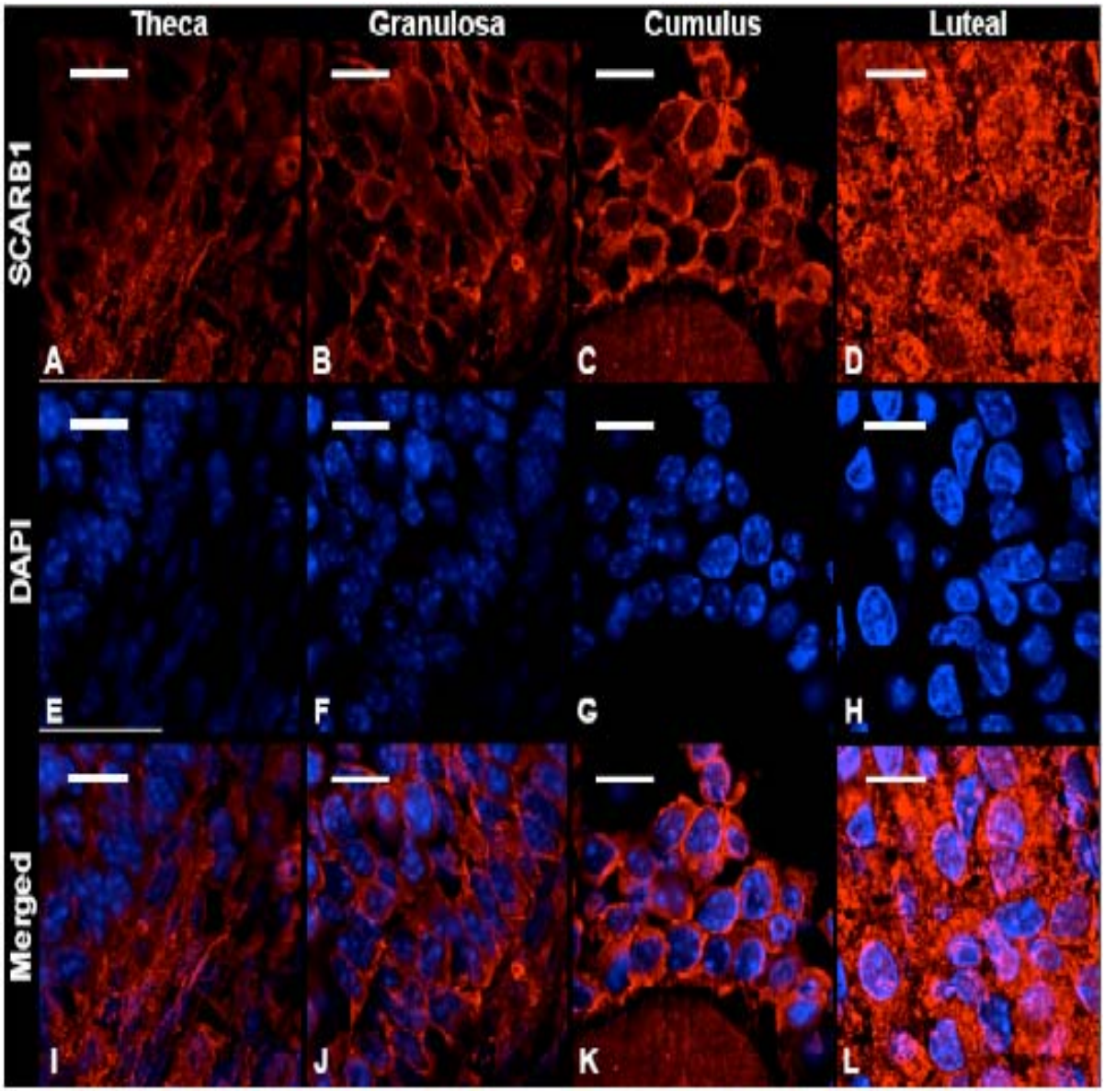
Theca
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Figure 3.

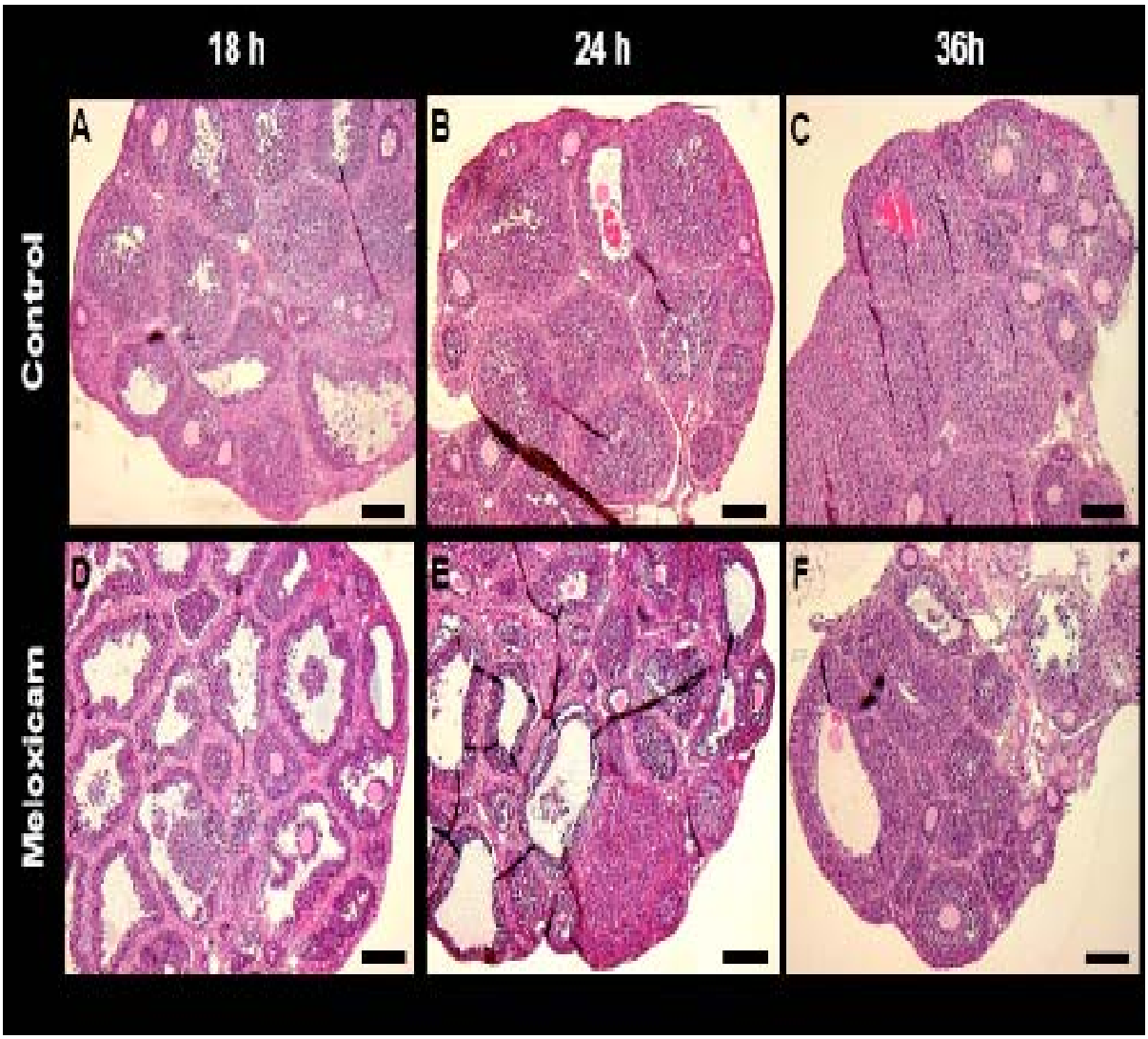
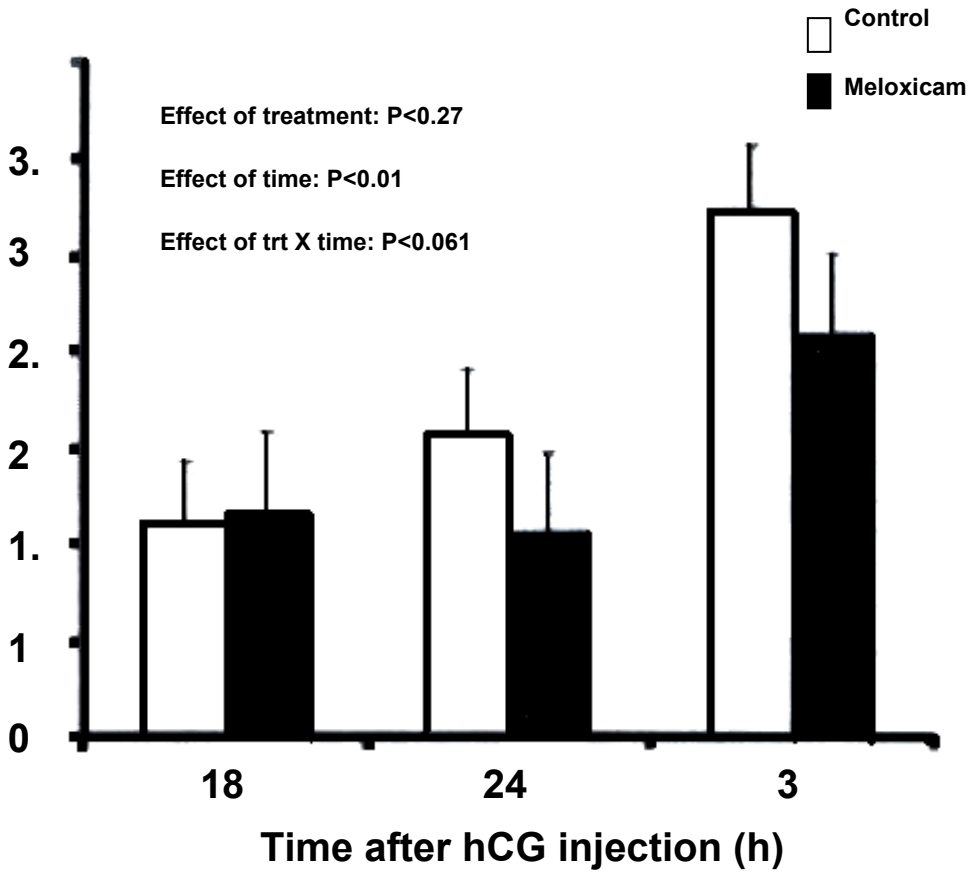


Figure 4.


Control
Meloxicam
Effect of treatment: P<0.27
Effect of time: P<0.01
Effect of trt X time: P<0.061
3.
3
2.
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1.
1
0
18
24
3
Time after hCG injection (h)

Figure 5.

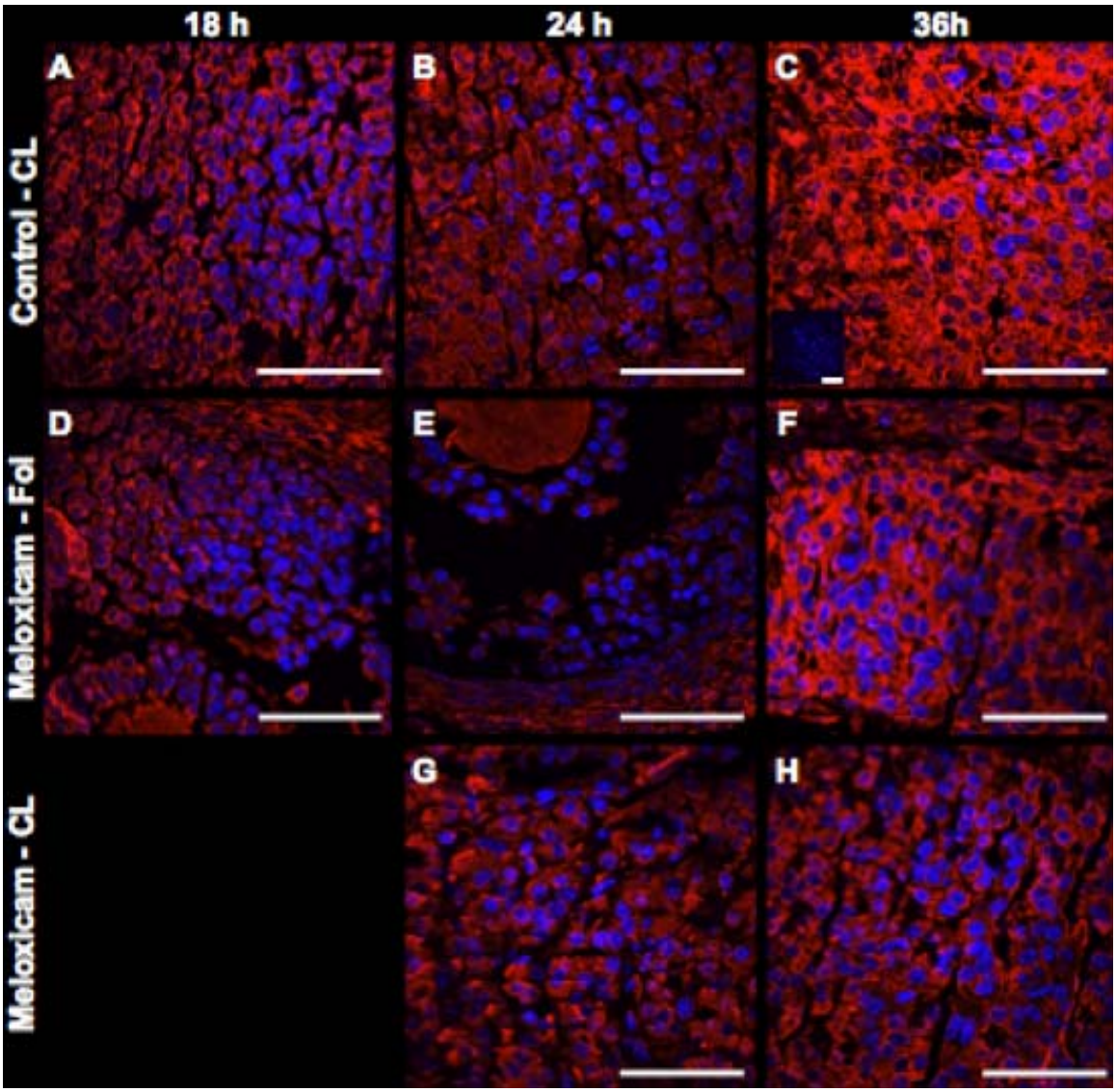


Figure 6.

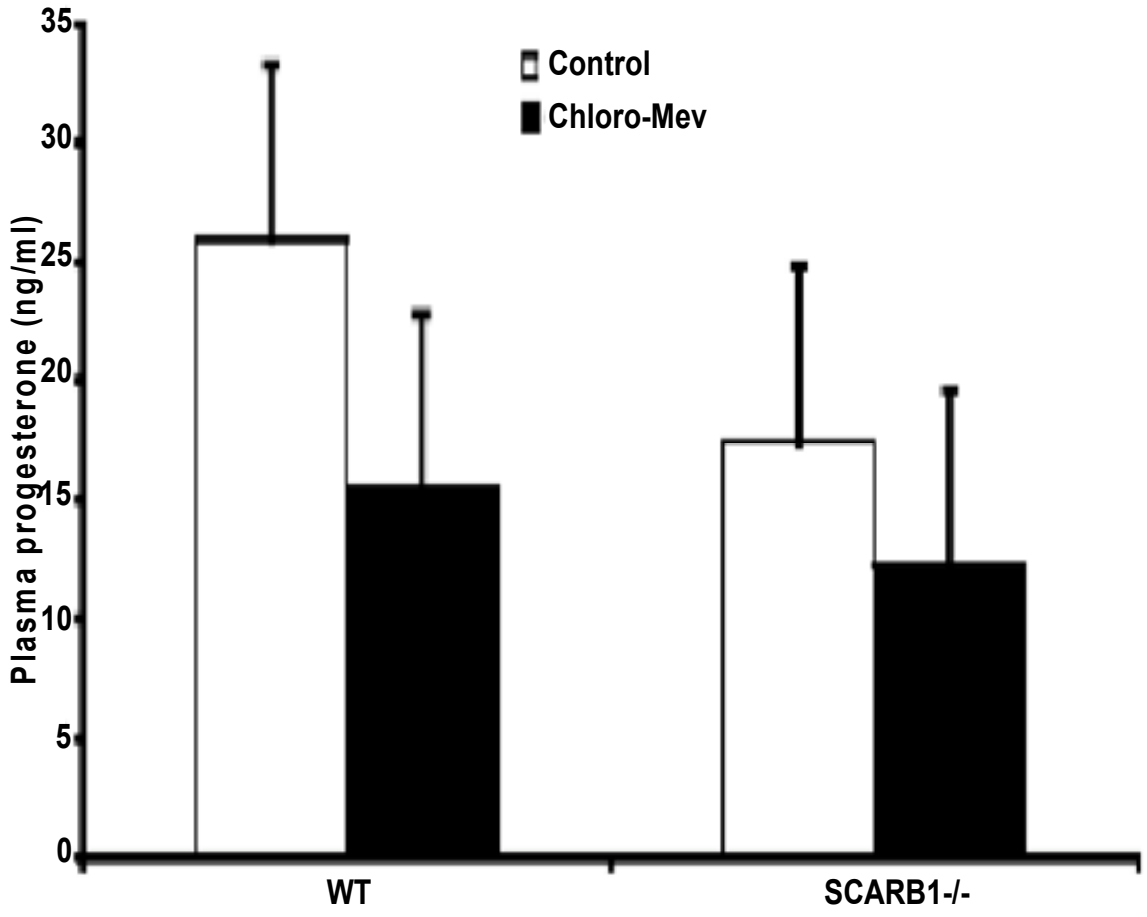

35
30
25
20
15
10
5
0
Plasma progesterone (ng/ml)
Control
Chloro-Mev
WT
SCARB1-/-

Figure 7.

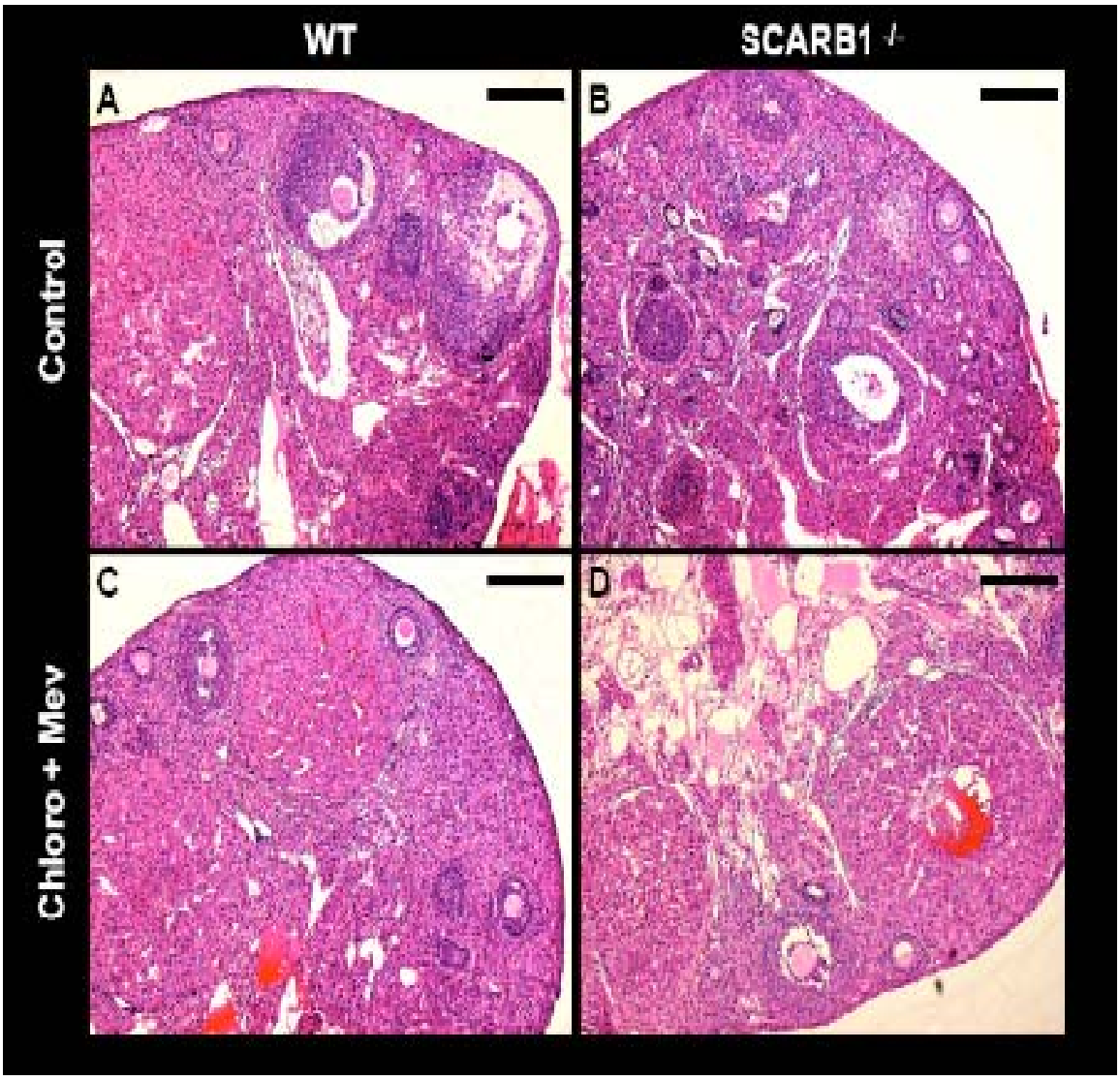
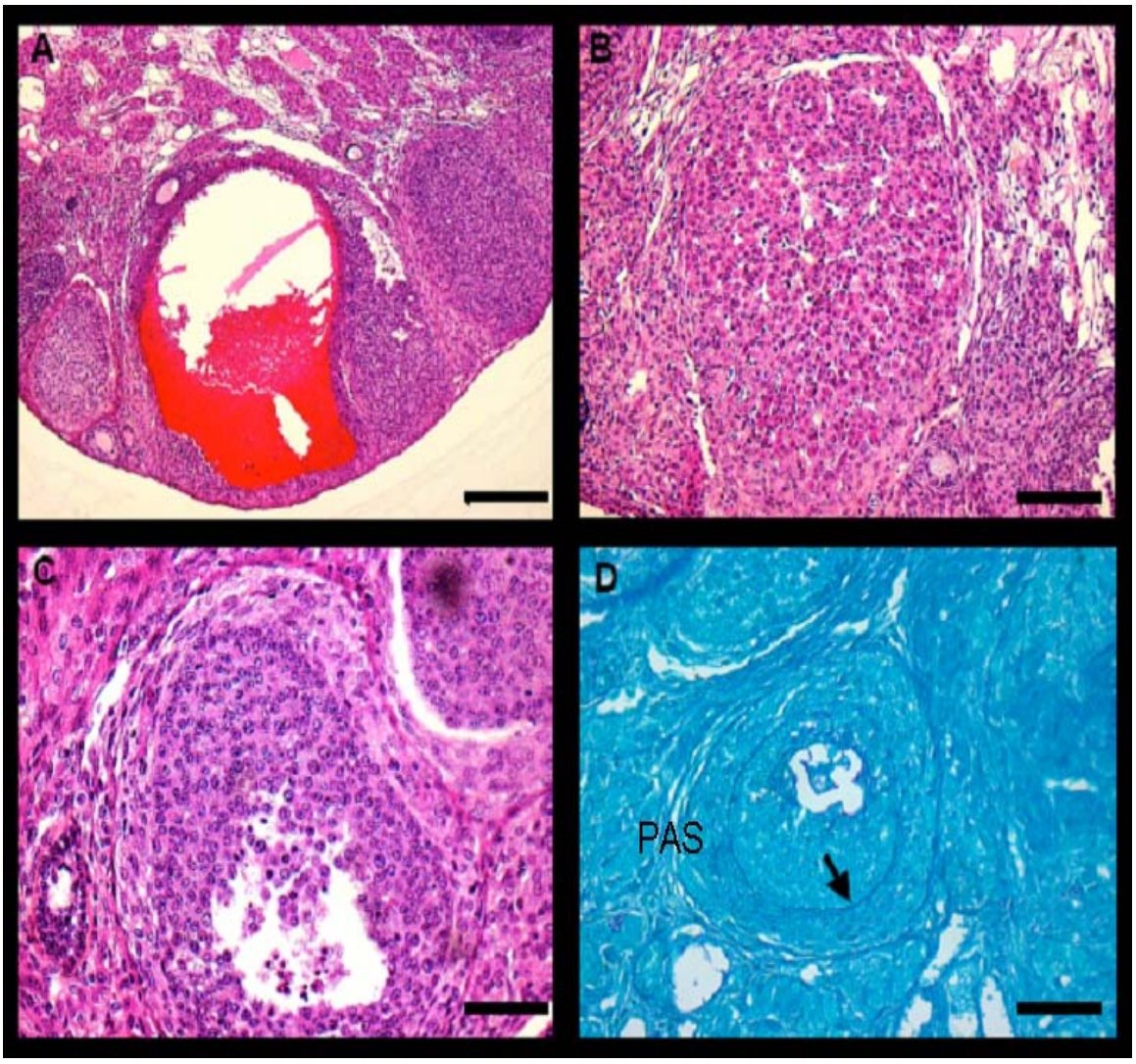


Figure 8.



GENERAL DISCUSSION

Cellular cholesterol supply has been recognized as a fundamental need for adequate steroid production. SR-BI, SR-BII, LDLR, and HMG-CoA-reductase, along with enzymes that esterify and liberate cholesteryl, are molecules that represent the principal mechanistic control of the cholesterol supply for steroidogenic cells. In the ovary, there are reports indicating that the receptors that import cholesterol and lipoproteins from which the cholesterol is derived depend on animal species (Murphy and Silavin 1989). LDL has been reported as the most significant in lipoprotein in humans and pigs, while HDL as the main supply in species such as cattle and rodents. This notwithstanding, studies in the rat suggested that, while SR-BI protein was present in the theca compartment, it was absent from the granulosa cells of ovarian follicles (Driancourt, Quesnel et al. 1998). HMG-CoA is essential for de novo synthesis, but is not believed to supply sufficient cholesterol for adequate ovarian steroidogenesis. A purpose of these studies was to explore the relative significance of cholesterol supply mechanisms to luteal steroidogenesis. Using the pig as animal model, we evaluated SR-BI expression in the follicle and the CL. We concluded that SR-B1 is not an important transport mechanism in follicular granulosa cells, consistent with the view that the substrate for estrogen synthesis in these cells is androgens from the theca compartment of the follicle. In contrast, our results demonstrate that, when cholesterol demand increases as luteinization ensues, the HDL receptor becomes highly expressed. Our protein data concur with studies that show that increases in SR-BI mRNA abundance has been reported previously in the cow CL and attributed to the elevated steroidogenic activity of luteal tissue (Rajapaksha et al. 1997). A new study of rhesus monkey luteinization following controlled ovarian stimulation demonstrates that SR-B1 mRNA abundance increases rapidly,

both in vitro and in vivo, during the first 24 h after treatment with hCG (Cherian-Shaw et al. 2009). In that study, in vitro protein abundance supports this finding, but concurrent increases in the LDL receptor are in contrast to our findings in the pig. Further, Cherian-Shaw et al. (2009) demonstrate that both HDL and LDL support hCG-induced progesterone synthesis in rhesus granulosa cells early in the luteinization process (0-24 h after isolation of cells) while HDL is ineffective from 24-48 h. At these later times, LDL continues to be a significant source of cholesterol to these otherwise substrate-limited cells. This difference further confirms the idea that there are species differences in cholesterol supply to the corpus luteum (Murphy and Silavin 1989).

In chapter 1, we show that SR-BI exists predominately in the cytoplasm of granulosa cells. Associated with ovulation, there appears to be post-transcriptional modification of SR-B1. This translocation was characterized by an apparent transformation of the 57 kDa to 82 kDa, the latter being the active (Cao et al. 2004), glycosylated form. Using enzymatic digestion of carbohydrates we confirmed that the difference between the 57 kDa and the 82 kDa protein in the pig, was in fact, the glycosylation. The larger molecular weight form was twice as abundant in luteal cells when compared to granulosa cells, indicating that differentiation engenders an alteration in or upregulation of the glycosylation process. This is at odds with the observation that N-glycosylated residues are co-translationally incorporated into the protein during its synthesis (Rigotti, Trigatti et al. 1997). In pig granulosa cells, it appeared that SR-B1 localized primarily to the cytoplasm, with predominant expression at the cell periphery, and presumably the membrane in luteal cells. This finding was not confirmed in the mouse, where confocal microscopy revealed abundant expression in the cytoplasm of the mouse luteal cells. While this may be a species difference, it is also likely that it is attributable to increased sensitivity of the

confocal over traditional fluorescence microscopy. Thus, determining whether post-transcriptional changes in the SR-B1 molecule characterize luteinization requires further experimentation for clarification.

Given that SR-B1 is strongly expressed in the CL, the second issue addressed in this thesis was whether ovulation was required to bring about its expression. By immunocytochemistry, the occurrence and relative abundance of the SR-B1 signal was compared in the CL of immature mice induced to ovulate with a combination of eCG and hCG and allowed to ovulate with those treated with gonadotropins in which ovulation was blocked by inhibition of expression of COX-2. The granulosa compartments of the ovaries in which ovulation was inhibited contained entrapped oocytes, and the mural cells took on a luteal phenotype as early as 24 h after hCG administration. The analysis by confocal microscopy, in which the microscopic parameters were held constant, indicated that there was no difference between expression in of SR-B1 between the luteinized follicle and the CL. This was supported by the abundance of of SR-B1 in the ovaries as quantified by realtime PCR. Thus, it would appear that the differentiation program that is initiated by the preovulatory surge does not require the actual extrusion of the oocyte to occur.

To this point, our results have shown that SR-B1 is strongly expressed and characterizes luteal formation and function. We then addressed the puzzle of how the SR-B1 deficient mouse can develop an apparently functional CL in the absence of this important cholesterol importing molecule, as indicated in the report by Trigatti et al. (1999). Careful evaluation of mice with null mutation of the SR-B1 gene revealed that their assertion that luteal function is not different in mice in which this gene is disrupted is not correct. Indeed, we show herein that circulating progesterone is reduced in the SR-B1 knockout animals, and that, in spite of the presence of CLs

in these mice, the ovaries contain a number of abnormal structures. These include hemorrhagic follicles, a pathological condition frequently seen in knockout models in which estrogen synthesis is compromised (Ruiz-Cortez et al. 2005). In further experimentation, we attempted to eliminate two complementary sources of cholesterol substrate, de novo synthesis and LDL-derived cholesterol. To our surprise, these treatments had the effect of only reducing, but not eliminating circulating progesterone. We blocked only the activity of HMGR, and immunocytochemistry strongly suggested that there was robust upregulation of the expression of the HMGR gene. We did not have an adequate means of evaluation of LDL processing following chloroquinone treatment, and it is possible that this source of cholesterol was not entirely interdicted. The only major means to supply cholesterol that remains is liberation of stored cholesteryl esters. Total ovarian storage of cholesterol is reduced in the SR-B1 knockout mouse (Fenske et al. 2008) but our results suggest that a sufficient amount is present to allow for progesterone synthesis, albeit at a reduced level.

The SR-B1 homozygous null mice are infertile, but this infertility is not directly attributable to luteal dysfunction, rather, it appears to be due to oocyte defects. Recently this infertility has been reported as a consequence of dislipidemia which may be resolved through SR-BI repletion at hepatic level (Yesilaltay et al. 2006). This suggests that, HDL particle size may be associated with this phenomenon. The mechanism by which inappropriate oocyte development occurs remains a mystery. Recent reports indicate that oocytes outsource metabolic functions, including the acquisition of cholesterol via SR-B1 (Su et al. 2009)

In overview, this thesis has shown the importance of SR-B1 in luteal function. This was first, by demonstration of its differential expression between the follicular and luteal phase in the pig. Studies in the mouse concurred in general with the distribution and expression patterns in

the pig ovary. Knockout of SR-B1 compromised but did not completely disrupt luteal function, providing further indication of the significance of this molecule in supply of cholesterol for luteal steroidogenesis. Further work could focus on determining the mechanism of cholesterol transfer to the oocyte, to resolve the issue of SR-B1 in fertility.

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