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OOCYTE COMPETENCE AND CUMULUS CELLS GENE EXPRESSION

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RÉSUMÉ

La compétence au développement de l'ovocyte consiste en sa capacité à réussir les étapes successives de maturation, de fécondation pour atteindre le stade blastocyste et donner une progéniture en santé. Facteur limitant du développement embryonnaire, la maturation de l'ovule est le fruit d'interactions optimales avec son environnement somatique et hormonal notamment les cellules de cumulus. Ces dernières étant essentielles pour la maturation de l'ovule et considérées comme un miroir pouvant renseigner sur sa qualité. L'apparence de ces cellules figure parmi les principaux critères morphologiques utilisés lors de la sélection du complexe cumulus-ovocyte. Comme de tels critères sont subjectifs et qualitatifs, on envisage ici d'identifier des biomarqueurs exprimés dans le cumulus et permettant une sélection efficace et objective d'ovocytes compétents via une approche génomique. Par conséquent, les cellules du cumulus d'un ovocyte compétent sont considérées comme étant le site privilégié d'évènements spécifiques dont l'activation des voies de signalisation et des cascades d'expression génique reflétant la qualité de l'ovule. En faisant recours aux technologies de pointe utilisées en biologie moléculaire telles que les biopuces et la PCR quantitative, et en se servant de logiciels performants pour l'analyse des voies de signalisation, nous nous sommes concentrés sur l'étude des profils d'expression des cellules du cumulus en relation avec la compétence au développement des ovocytes.

Le premier objectif de cette thèse était de trouver des gènes exprimés différentiellement dans des conditions de culture qui influencent la compétence et donc associés à de taux de blastocystes élevés. Outre la confirmation de la voie PKC comme principal acteur impliqué dans la compétence ovocytaire, on rapporte ici une liste de candidats communément surexprimés entre les trois traitements de MIV (FSH, PMA, FSH+PMA) et donc ayant de meilleures chances d'être associés au processus moléculaires de l'acquisition de compétence.

Le deuxième objectif était l'analyse des gènes exprimés dans les cellules du cumulus suite à l'action du pic de LH *in vivo*. Comme la LH est essentielle pour la maturation finale *in vivo* et à la production d'ovocytes de qualité, on s'est intéressé aux gènes différentiellement exprimés dans les cellules de cumulus et associés à la compétence ovocytaire à 6 heures après le pic de LH. Par la suite, l'expression des gènes à ce stade (GVBD) a été comparée à son homologue *in vitro* (objectif #1) pour générer une liste commune des marqueurs potentiels de compétence des

ovocytes à la fois *in vivo* et *in vitro*. L'identification de ces biomarqueurs pourrait accroître les connaissances sur les profils d'expression génique dans les cellules du cumulus et servira de préambule à comprendre des séquences d'événements cruciaux du processus moléculaire d'acquisition de la compétence au développement de l'ovocyte. Ces résultats pourraient être un outil précieux pour accroître les rendements de fécondation *in vitro* et pourraient permettre d'optimiser la stimulation ovarienne *in vivo* aussi bien chez le bovin que l'humain. Par la suite, on a procédé à une analyse comparative des effets génomiques des deux gonadotrophines autour de la GVBD: FSH *in vitro* versus LH *in vivo*. Nous avons mis en évidence, pour la première fois chez le bovin, une possibilité de compensation ou de substitution de la LH par la FSH *in vitro*.

La troisième partie de cette thèse a été l'analyse de l'expression des gènes dans les cellules du cumulus humains collectés juste avant l'ICSI (intracytoplasmic sperm injection) afin d'identifier des biomarqueurs génomiques fiables permettant de prédire de façon précise et non invasive la compétence au développement des ovocytes et de renforcer les critères morphologiques existants. Pour des cellules de cumulus associés à des ovocytes de morphologies similaires et ayant la même biréfringence de la zona pellucida, on a identifié 7 gènes différentiellement associés à des ovules ayant mené à une grossesse. Ces biomarqueurs pourraient devenir un outil quantitatif et non-invasif permettant de distinguer le bon embryon à transférer parmi d'autres ayant des propriétés morphologiques similaires. Un tel embryon devrait faciliter la pratique du transfert mono-embryonnaire, évitant ainsi les grossesses multiples.

Finalement, la présente étude a permis d'identifier plusieurs gènes marqueurs et d'explorer leurs voies de signalisation potentielles aussi bien chez le bovin que l'humain. Ces résultats devraient contribuer à enrichir les connaissances actuelles des profils d'expression génique des cellules du cumulus en plus de donner un aperçu des voies moléculaires qui régissent la compétence au développement des ovocytes. Ils pourraient aussi servir à l'identification et la compréhension des voies moléculaires reliés aux subséquents événements de fécondation et de développement embryonnaire précoce dont les mécanismes demeurent encore méconnus.

ABSTRACT

Oocyte competence is the ability of the oocyte to fulfil maturation, undergo successful fertilization, reach the blastocyst stage and yield a healthy baby. Cumulus cells are indispensable for this process. Their removal significantly impairs oocyte maturation and therefore the development outcome. Moreover, the properties and functions of cumulus cells are regulated by the oocyte and thought to reflect its degree of maturation. We believe that the cumulus compartment of a competent oocyte is the site of specific signalling and gene expression events which support and reflect the gamete's quality. Using state of the art molecular biology technologies as microarrays and quantitative real time PCR, as well as gene pathways software analysis, we have focused on the study of cumulus cells gene expression and their relationship with oocyte competence.

The first objective of my thesis was to find differentially expressed genes in bovine CCs between three IVM treatments (FSH, PMA, FSH+PMA) collected at 6h of IVM *in vitro* (GVBD) and associated to variable blastocyst yields. Besides the confirmation of PKC pathway as main player involved in oocyte competence, we report herein a list of commonly overexpressed candidates between the three treatments that have serious chances to be associated with the molecular process of acquisition of competence.

The second objective was the analysis of cumulus cells' gene expression induced by LH *in vivo*. Since the LH surge is crucial to the *in vivo* final maturation and improves oocyte quality, we have analyzed differentially expressed genes associated with oocyte final maturation at 6 hours after the LH surge (around GVBD). Afterwards, the gene expression at this stage was compared to its corresponding stage *in vitro* and a common list of potential markers of oocyte competency both *in vivo* and *in vitro* was established. The identification of these biomarkers would increase knowledge about gene expression patterns in cumulus cells and would serve as a preamble to understand the crucial events of the oocyte competence acquisition process. These findings could be valuable tool to increase IVF yields *in vitro* and to optimize ovarian stimulation *in vivo* for both the bovine and human species. Interestingly and following a comparative analysis of the transcriptional effects of FSH *in vitro* versus LH *in vivo*, we report for the first time a possibility of compensation/substitution of LH action by FSH *in vitro*.

The third part of this thesis concern the analysis of gene expression in human cumulus cells harvested prior to ICSI in order to identify reliable genomic biomarkers that accurately and non-invasively predict the oocyte developmental competence and reinforce the already used morphological criteria. Seven genes were differentially ($p \leq 0.05$) associated to both successful pregnancy and implantation under similar morphological and zona pellucidae birefringence parameters. These biomarkers are quantitative and non-invasive tools that could be used to discriminate amongst good morphology embryos the one that has best chances to achieve a successful pregnancy.

Ultimately, these biochemical markers and their associated signalling pathways in both bovine and human species increased the knowledge of gene expression patterns in cumulus cells, which would yield insights into the molecular pathways controlling oocyte competence and would serve as a preamble to the understanding of crucial events in subsequent fertilization and early embryonic development.

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بسم الله الرحمن الرحيم،

الحمد لله جلّ جلاله، وعزّ جاهه، وتقدّست أسماؤه، وعَظُم عطاؤه، له الحمد على كل حال وفي كل آن، وله الحمد ما دامت السماوات والأرض، حمدا يليق بجلاله وعظيم سلطانه، ويكافئ فضله وإنعامه. وأشهد أن لا إله إلا الله وحده لا شريك له، له الملك وله الحمد يحيي ويميت وهو على كل شيء قدير، وأشهد أن نبينا وقائدنا وقدوتنا وسيّدنا محمدا عبد الله ورسوله، وصفيه من خلقه وخليله، خاتم الأنبياء والمرسلين، وسيّد الأولين والآخرين،المبعوث رحمة للعالمين، المرسل إلى الناس كافة أجمعين. قال العزيز الحكيم: (وَمَا بِكُم مِّن نِّعْمَةٍ فَمِنَ اللّهِ) النحل/53، وقال تعالى : (يرفع الله الذين آمنوا منكم والذين أوتوا العلم درجات) المجادلة/11 . وقد صحّ في الحديث عن رسول الله صلى الله عليه وسلم أنه قال: " من التمس طريقا يلتمس فيه علما سهل الله له طريقا إلى الجنة " رواه مسلم/ وقال عليه الصلاة والسلام : (إذا مات الإنسان انقطع عمله إلا من ثلاثة صدقة جارية ، أو علم ينتفع به ، أو ولد صالح يدعو له) رواه مسلم/، فأسأل الله الكريم رب العرش العظيم أن يتقبل مني هذا العمل خالصاً لوجهه وأن يرفع به درجاتي في الدنيا والآخرة. هذا، وما كان من توفيق فمن الله وحده، وما كان من خطأ أو سهو أو زلل أو نسيان فمني ومن الشيطان، والله ورسوله منه براء، وأخر دعوانا أن الحمد لله رب العالمين

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«وَلَقَدْ خَلَقْنَا الْإِنْسَانَ مِنْ سُلَالَةٍ مِنْ طِينٍ - ثُمَّ جَعَلْنَاهُ نُطْفَةً فِي قَرَارٍ مَكِينٍ - ثُمَّ خَلَقْنَا النُّطْفَةَ عَلَقَةً فَخَلَقْنَا الْعَلَقَةَ مُضْغَةً فَخَلَقْنَا الْمُضْغَةَ عِظَامًا فَكَسَوْنَا الْعِظَامَ لَحْمًا ثُمَّ أَنْشَأْنَاهُ خَلْقًا آخَرَ فَتَبَارَكَ اللَّهُ أَحْسَنُ الْخَالِقِينَ - ثُمَّ إِنَّكُمْ بَعْدَ ذَلِكَ لَمَيِّتُونَ - ثُمَّ إِنَّكُمْ يَوْمَ الْقِيَامَةِ تُبْعَثُونَ» (سورة المؤمنون 12-16)

« Nous avons certes créé l'homme d'un extrait d'argile * puis Nous en fîmes un zygote dans un reposoir solide * Ensuite, Nous avons fait de l'œuf une morula; et de la morula Nous avons créé un blastocyste; puis, de cet embryon Nous avons créé des os et Nous avons revêtu les os de chair. Ensuite, Nous l'avons transformé en une tout autre création. Gloire à Allah le Meilleur des créateurs !* Et puis, après cela vous mourrez. * Et puis au Jour de la Résurrection vous serez ressuscités» *(Coran, chapitre 23 (Les Croyants), 12-16)*

«Verily We created man from a product of wet earth; (12) Then placed him as a drop (of seed/zygote) in a safe lodging; (13) Then We fashioned the drop a morulae /clot, then We fashioned the clot a little blastocyst/lump, then We fashioned the little blastocyst/lump bones, then clothed the bones with flesh, and then produced it as another creation. So blessed be Allah, the Best of creators! (14) Then after that you will most surely die. (15) Then surely on the day of resurrection you shall be raised (again) (16) » *(Quran, chapter 23 (The Believers), 12-16)*

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%	percentage
^{0}C	degree celsius
µg	micrograms
µl	microliter
µm	micrometer
µM	microMole
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
2PN	two pronuclei
AA	arachidonic acid
AC	adenylate cyclase
ACTB	β-actin
ADAMTS 1	a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 1
AI	anaphase I
Akt / PKB	protein kinase B
AMFR	autocrine motility factor receptor
AP1	transcription factor AP-1
AR	androgen receptor
AREG	amphiregulin
aRNA	antisens RNA
ART	assisted reproductive technologies
ATF2	activating *transcription* factor 2
BAMBI	BMP and activin membrane-bound inhibitor homolog
Bax	BCL2-associated X protein
BCL	B-cell lymphoma
bFGF	basic fibroblast growth factor
BLR	binary logistic regression
BMP	bone morphogenetic protein
bp	base pairs
BSA	bovine serum albumin
BSA-FAF	bovine serum albumin fatty acid free
BTC	betacellulin
Ca	calcium
CALM1	calmodulin 1
CALU	calumenin
cAMP	cyclic adenosine monophosphate
CASP9	caspase 9
CAV1	caveolin 1
CCNA2	cyclin A2
CCs	cumulus cells
CD44	CD44 antigen /gene
CDC25	cell division cycle 25
CDC2L	cell division cycle 2-like
CDH11	cadherin 11
CDK	cyclin-dependent kinase
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CHGB	chromogranin B
CHSY1	chondroitin sulfate synthase 1
CIDEC	cell death-inducing DFFA-like effector c
CKAP4	cytoskeleton-associated protein 4
c-kit	C-kit receptor

CL	corpus luteum
CMYC	C-Myc transcription factor (proto-oncogene)
COC	cumulus-oocyte complex
COCs	cumulus-oocyte complexes
COL4A1	collagen, type IV, alpha 1
COX	cyclooxygenase
CPE	cytoplasmic polyadenylation element
CPEBP	cytoplasmic polyadenylation element binding protein
CPSF	cleavage and polyadenylation specificity factor
CRCs	corona radiata cells
CRE	cAMP-responsive-element
CREB	CRE-binding protein
CSPG	Chondroitin sulfate proteoglycans
CTRL	control
CTSL	cathepsine L
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
Da	Dalton
DAD1	defender against cell death 1
DAG	Diacyl glycerol
DFP	dominant follicle punction
DH5α-T1	*Escherichia coli* cell line
DHCR24	24-dehydrocholesterol reductase
DMSO	dimethyl sulfoxide
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6
dNTPs	deoxynucleotide triphosphates
dpf	days post-fertilization
dpp	days post-partum
DPP8	Dipeptidyl peptidase 8
E2	estradiol / oestradiol
EB	Elution buffer
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EEF1A1	eukaryotic translation elongation factor 1 alpha 1
EGF	epidermal growth factor
EGFR	Epidermal Growth Factor Receptor
eIF-4E	Eukaryotic initiation factor 4E
ENO1	enolase 1
Epac	exchange protein activated
ER	Estrogen receptor
EREG	epiregulin
ERK	extracellular signal-regulated kinase
ERRFI1	ERBB receptor feedback inhibitor 1
eSET	elective single embryo transfer
F-actin	Actin filament
FBN2	fibrillin 2
FDR	false discovery rate
FF	follicular fluid
FGF	fibroblast growth factors
FOXO3	forkhead box O3
FP	false positive
FSH	follicle stimulating hormone
FSHR	FSH receptor
FTH1	ferritin, heavy polypeptide 1
GAG	glycosaminoglycan

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCs	Granulosa cells
GDF	growth differentiation factor
GFP	green fluorescent protein
GH	growth hormone
GJA1	gap junction protein alpha 1, 43 kDa
GJB1	gap junction protein beta 1, 32 kDa
GJC	gap junctions genes/proteins
GnRH	gonadotropin-releasing hormone
GnRHa	gonadotrophin releasing hormone agonist
GPCR	G-protein coupled receptor
GREM	Gremlin
GSH	glutathione
GSTA1	glutathione S-transferase A1
GTF2A2	general transcription factor IIA
GV	germinal vesicle stage
GVBD	Germinal Vesicle Breakdown
h / hr/ hrs	hours
HA	hyaluronan / hyaluronic acid
HAS2	Hyaluronan synthase 2
Hat	histone acetylase or acetyltransferase
HB-EGF	heparin-binding EGF
hCG	human chorionic gonadotropin
HCl	hydrogen chloride
HEPES	culture medium
HIST1H4C	histone cluster 1, H4c
HPGD	hydroxyprostaglandin dehydrogenase
HSD3B2	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2
HSP70	heat shock protein 70 kDa
HSP90	heat shock protein 90 kDa
HSP90AA1	heat shock protein 90kDa alpha, class A member 1
HSP90B1	heat shock protein 90kDa beta 1
HSPA13	heat shock protein 70kDa family, member 13
HSPD1	heat shock 60kDa protein 1
HZB	high ZP birefringence
ICSI	intracytoplasmic sperm injection
IFITM3	interferon induced transmembrane protein 3
IFN	Interferon
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IgG	Immunoglobulin G
IL1	interleukin 1
IL6	interleukin 6
INHBA	inhibin, beta A
IP3	inositol 1,4,5-triphosphate
IPA	Ingenuity Pathway Analysis
IU	International Unit
IVC	*In vitro* culture
IVF	*In vitro* fecondation
IVM	*in vitro* maturation
IVT	*in vitro* transcription
IαI	inter-α-trypsin inhibitor
JNK	*c-jun*terminal kinase
K^+	potassium

KDa	kilo Daltons
KL	Kit-ligand
KO	knock out
Ldh	Lactate Dehydrogenase
LH	luteinizing hormone
LHCGR	luteinizing hormone/choriogonadotropin receptor
LHR	luteinizing hormone receptor
LZB	low zona birefringence
MAPK	mitogen-activated protein kinase
MEK	MAP kinase kinase
mer	the number of nucleotides
MET	maternal to embryonic transition
mg	milligram
MGCs	mural granulosa cells
MII	metaphase II
min	Minutes
ml	millilitre
mm	millimeter
mM	milliMole
MOS	Proto-oncogene serine/threonine-protein kinase
MPF	maturation promoting factor
mRNA	messenger ribonucleic acid
mRNP	Messenger ribonucleoprotein
MYO1D	myosin ID
NCBI	National Center for Biotechnology Information
ng	nanogram
NIA	National Institute of Aging
NO	nitric oxide
NRP1	neuropilin 1
O_2	oxygen
oFSH	ovine FSH
OR	Odds ratio
P450arom	enzyme P450 aromatase
PABP	poly (A) binding proteins
PAK1	protein -activated kinase 1
PAP	poly (A) polymerase
PBS	phosphate buffered saline
PC	principal components
PCA	principal components analysis
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent kinase
p-f	post-fertilization
PG	prostaglandin
PGC	primordial germ cells
PGE2	prostaglandin E2
PGR	progesterone receptor
PGRMC1	progesterone receptor membrane component 1
PI	phosphatidyl inositol
PI3K	phosphatidyl inositol 3-kinase
PI3K	phosphoinositide-3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A

PKB	protein kinase B
PKC	protein kinase C
Pkm	pyruvate kinase
PKN2	protein kinase N2
PLC	Phospholipase C
PLCB2	phospholipase C, beta 2
PLOD1	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
PMA	phorbol myristate acetate
PMSG	pregnant mare's serum gonadotropin
POF	preovulatory follicle
POLR2G	polymerase (RNA) II (DNA directed) polypeptide G
poly (A)	tail of adenine nucleotides sequence
PSMD6	proteasome (prosome, macropain) 26S subunit, non-ATPase, 6
PTGER2	prostaglandin E receptor 2
PTSG2	prostaglandin-endoperoxide synthase 2
PTX3	pentraxin-related gene 3
PVA	Polyvinyl alcohol
PVP	polyvinylpyrrolidone
PXN	paxillin
QPCR	Quantitative real-time PCR
qRT-PCR	Quantitative real-time PCR
Raf	proto-oncogene serine/threonine-protein kinase
Rap	Ras-like related protein
RAS	Small GTPase protein
rhFSH	recombinant human FSH
RHOA	ras homolog gene family, member A
RNases	Ribonucleases
RPL9	ribosomal protein L9
rRNA	ribosomal RNA
RT	reverse transcriptase
S60	ribosomal subunit 60
SAPK	stress-activated protein kinases
SCF	stem cell factor
SDHA	succinate dehydrogenase complex, subunit A
SDS	Sodium dodecyl sulfate
sec	second
SELENBP1	selenium binding protein 1
SERPINE2	serpin peptidase inhibitor, clade E
SET	single embryo transfer
SGK1	Serine/threonine-protein kinase 1
SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5
SNAP	synaptosomal-associated protein
SOF	synthetic oviduct fluid medium
SPRY 2	sprouty homolog 2
SSH	supression and subtractive hybridization
SYNPO	synaptopodin
SYT11	synaptotagmin XI
TGF	transforming growth factors
TGFB1	transforming growth factor, beta 1
THBS	thrombospondin
THOC2	THO complex subunit 2
TLH	Hepes-buffered Tyrode medium

Tm	melting temperatures
TMF1	TATA element modulatory factor 1
TMR	transmembrane receptor
TMSB10	thymosin beta 10
TNF	tumor necrosis factor
TNFAIP6	tumor necrosis factor-induced protein-6
TOM1	target of myb1
TP	true positive
tPA	tissue plasminogen activator
TRA1	tumor rejection antigen 1
TRIB2	tribbles homolog 2
TUBA1B	Tubulin alpha-1B
TUG1	taurine upregulated 1 gene
TZP	trans-zonal projections
UBE2N	ubiquitin-conjugating enzyme E2N)
UBE3C	ubiquitin protein ligase E3C
UBQLN1	ubiquilin1
ULS	Universal Linkage System
ULS	Universal Linkage System
UTMP	Uterine milk protein
UTP	uridine triphosphate
UTR	Untranslated region
UV	ultraviolet rays
VEGF	vascular endothelial growth factor
XB	Extraction Buffer
ZBNP	zona bad non pregnant
ZG	zona good
ZGNP	zona good non pregnant
ZGP	zona good pregnant
ZNF330	zinc finger protein 330
ZP	zona pellucida
β-ME	beta-mercaptoethanol

GENERAL INTRODUCTION

The mammalian ovary is an organ that ensures the reproductive function including both endocrine and germ cell production activities. This crucial function for the species' perpetuation involved many endocrine factors that regulate and synchronize the hypothalamic-pituitary-ovarian- endometrial axis and allow the ovulation of mature oocyte(s) able to achieve subsequent fertilization and embryo development. Significant progress has been made over the years to understand the ovarian function and to provide tools to control and/or improve its regulation. In this context, assisted reproductive technologies (ART) are providing hopeful techniques and strategies to improve the livestock genetic and productivity. In human and with the quick rise of the infertility incidence, ART are nowadays used worldwide to help infertile couples to procreate. Despite valuable ART advantages, the subsequent challenge is how to increase qualitatively and quantitatively the embryo outcomes. The oocyte quality has been recognized as the major limiting step and is therefore the target of numerous works to elucidate its intricate process (Schultz *et al.* 1983, Hyttel *et al.* 1997, Mermillod 2001, Hunter 2003, Sirard & Trounson 2003). Bidirectional interplay between the oocyte and its somatic neighbourhood mainly cumulus cells (CCs) were shown to be indispensable to oocyte competence acquisition. Their removal significantly affects the blastocyst rates (Ali *et al.* 2005). Moreover, the properties and functions of CCs are to some extent regulated by the oocyte and reflect the oocyte maturation degree (Tanghe *et al.* 2002, Gilchrist *et al.* 2008). This competency may be defined as the ability of the oocyte to successfully achieve successive steps of maturation, fertilization and early embryo development, and to deliver a healthy newborn. Although several parameters were used to assess the oocyte competence, they lack enough accuracy and remain therefore subjective (Coticchio *et al.* 2004, Krisher 2004) due to our poor understanding of its molecular process. This study aimed at understanding signalling and gene expression pathways in CCs involved in the molecular process of oocyte maturation. The possibility of finding quantitative and non-invasive biomarkers that could efficiently predict the oocyte developmental competence and make the single embryo transfer (SET) more successful in human IVF clinics is also in the scope of this work.

The literature review has focused on mammalian ovarian functions (mainly mouse, bovine and human species), the CCs differentiation and possible/potential roles as well as a quick overview of human ART. The first result chapter describes how we have used the bovine model *in vitro* to explore possible pathways associated to the oocyte competence *in vitro* and associated to the FSH

and/or PKC pathway. In the following chapter, these findings were compared to their *in vivo* counterparts 6h following the LH surge and common gene candidates were suggested as possible biomarkers of oocyte competence.

In the last part, we have looked for non-invasive biomarkers expressed in human CCs and associated to good morphological criteria, successful pregnancy and subsequent implantation. Somme gene candidates that would have an interesting prognostic role in high-quality oocyte selection in IVF clinics were suggested. These molecular tools should be helpful to minimizing the risk of multiples for the infertile couple.

CHAPTER 1:

LITERATURE REVIEW

1.1. SECTION # 1: OVARIAN FUNCTION

1.1.1. Embryonic origin of oocyte and ovary in mammals

Few embryonic stem cells, named primordial germ cells (PGC), are the origin of the whole germ cell compartment of the ovary. Having an extragonadal origin (the epiblast) confirmed through Oct-4 expression (Pesce *et al.* 1998), these PGC are diploid and are detected in the yolk sac endoderm near the caudal end of the primitive streak and the allantoids, at the third to fourth week post-fertilization in human embryo (Motta *et al.* 1997). They are reported to be round shape and contain enough energy reserves (glycogen particles and lipid droplets) particularly at the beginning of their migration from the epiblast to the genital ridges (Wassarman & Albertini 1994). During their transit, human PGC start mitosis and move first passively to the gut epithelium, and then actively by amoeboid movements and/or chemotactic guidance by their microenvironment in human five-week embryos as well as in most mammals (Makabe & Motta 1989, Pereda & Motta 1991). Once they reach the ventromedial face of the mesonephros, PGC lose their motility, become more proliferative, colonize the coelomic epithelium and form therefore the gonadal primordium. Somatic compartment of the developing genital ridge seems to be formed by segregating cells of the mesonephros and the coelomic epithelium. The active mitosis and morphodynamic changes of the presumptive gonad is sex independent until the seventh week of the human early embryo (Pereda *et al.* 2006). Thereafter, the primordial gonadal tissue gets an oval shape and shows two different zones. While the central zone is dense, the cortex becomes clearer showing the first signs of sexual differentiation (Motta *et al.* 2003, Pereda *et al.* 2006). It is to note that the generation of the PGC from the mouse epiblast was reported to be governed by some of the transforming growth factor β (TGFβ) family members as bone morphogenetic protein BMP-2, -4 and -8 (Ying *et al.* 2001).

1.1.2. Folliculogenesis chronology

Following their arrival to the presumptive gonad, PGC (named also at this stage gonocytes) show intensive mitotic division and then start differentiation either in oogonia or spermatogonia depending on the embryo genotype. In the differentiated ovary, dividing oogonia are clustered and interconnected giving rise to the cortical sex cords. Despite the estrogen rise during oogonia proliferation and its decrease when meiosis occurs, little is known about the exact factors responsible of the conversion of oogonia to oocytes through the meiosis induction (reviewed in

(van den Hurk & Zhao 2005)). Some studies suggested that the meiosis entrance of oogonia is genetically programmed, and therefore independent of the gonadal environment (Wassarman & Albertini 1994, Magre & Vigier 2001). Germ cells are connected by intercellular bridges that join their cell membranes. In fact, oogonia are organized inside the inner ovarian cortex into dividing clusters with identical chromosomal configurations, and coordinate mitosis and differentiation (Pereda *et al.* 2006).

In human, the first differentiation events of the PGC to oogonia was observed the 9th week post-fertilization (p-f). The cordlike somatic structures, derived from both coelomic (mesothelial) and mesonephric cells, finally start fragmentation, surround oogonia and give origin to pre-granuolsa cells. The outer somatic cells of mesenchymal origin will thereafter associate to give the pre-theca. Following this "pre-follicular structure" formation, the first events of folliculogenesis begins in the human embryo at 17 to 20 weeks p-f (Motta *et al.* 1997). The first meiosis events marked the beginning of folliculogenesis. Folliculogenesis is the set of physiological and molecular events inside the follicle that drive the development of the small primordial follicles since leaving the ovarian germ cell reserve into preovulatory follicles that undergo atresia or exceptionally ovulation (Driancourt *et al.* 2001).

Following the intensive mitotic activity of oogonia that prepares the whole oocyte reserve during all the reproductive life, part of oogonia start meiosis through the preleptotene stage. This step of DNA replication and meiosis preparation marked the conversion of oogonia to primary oocytes. Thereafter, the oocyte moves through the events of the prophase I stage including leptotene, zygotene, pachytene before arresting at diplotene (van den Hurk & Zhao 2005). It is to note that the beginning of the prophase I is variable among mammalian species. It occurs during the fetal stage at the 14th and 17th day p-f respectively for mouse and rat. It is at the 9 to 10th week p-f for woman and cow (Magre & Vigier 2001).

Coincident with the meiosis's initiation, primary oocytes surrounded by one layer of compacted pre-granulosa cells become enclosed in a basement membrane and form an independent entity named the primordial follicle. These follicles occupy the periphery of the ovarian cortex and are considered as the structural and functional unit of the mammalian ovary, from which will derive all the subsequent follicular development stages pre- and post-natally. In cattle, their diameter is equal or less than 40 µm (Braw-Tal & Yossefi 1997). It is established that the oocyte growth starts before and continues during the follicular formation. In fetal sheep for example, the

oogonia diameter ranges from 13 to 17 µm. After the primordial follicle formation, the oocyte and the follicle have respectively 17 to 22 µm and 23 to 53 µm of diameter (van den Hurk & Zhao 2005).

The follicular growth is associated to an active mitosis of GCs which become cuboidal. When a single layer of GCs surrounds the oocyte, the follicle reaches the primary stage. At this stage, the establishment of first gap junctions between the corona radiata cells and the oocyte was reported. The origin and the type of factors that induce the development of some primordial follicle are still undetermined although some candidate genes like the epidermal growth factor (EGF), the stem cell factor (SCF), the c-kit protein and its ligand (KL), bone morphogenetic protein-15 (BMP-15) and the growth differentiation factor-9 (GDF-9) were suggested in the mouse model (reviewed by (Picton 2001)).

Secondary follicle is obtained by the presence of at least two layers of GCs surrounding the oocyte, a peripheral theca cell layer, a basement membrane and the zona pellucida formation. This follicular stage is marked by a growing oocyte I, still at the dictyate stage, encapsulated by mitotically active GCs (Fair *et al.* 1997, Picton *et al.* 1998). Antral or tertiary follicles are marked by the formation of a follicular fluid-filled antrum, the differentiation of granulosa cells, and the proliferation of gap junctions between corona radiata cells and oocyte. These events are mainly induced by FSH stimulation (Wassarman & Albertini 1994, Abel *et al.* 2000). The antrum volume pursues its increase concomitantly with an active mitosis in the somatic compartment (theca, granulosa, cumulus). Knowing that antral follicles were found at birth in women and cattle, follicular recruitment and follicle growth look to begin in the fetal life (Russe 1983). These early tertiary follicles are ready for follicular waves' recruitment. During a cattle's oestrus cycle (discussed later), these follicular dynamics may include one to four waves of follicular synchronized cohorts that lead to the development and the selection of the dominant preovulatory follicle (Celik *et al.* 2005). Just prior to ovulation, GCs acquire the LH receptor and the perivitelline space between the oocyte and its zona pellucida is formed (Driancourt *et al.* 2001). Table 1.1 summarizes a comparative morphological analysis of some characterizations of folliculogenesis between mouse, cattle and human. By combination of several studies in these three species, we tried to focus on the follicle diameter increase and the approximate timepoint of the first formation of each follicular stage.

Table 1.1: Comparative analysis of follicular stages in mouse, cow and woman: morphological and chronological aspects. Abbreviations: dpf: days post-fertilization; dpp: days post-partum (Erickson 1966, Russe 1983, Forabosco *et al.* 1991, Gordon 1994, Fair *et al.* 1995, Braw-Tal & Yossefi 1997, Fair *et al.* 1997, Hyttel *et al.* 1997, Motta *et al.* 1997a, Salha *et al.* 1998, Fortune *et al.* 2000, Picton 2001, Aerts & Bols 2008, Baerwald *et al.* 2009)

Follicle	**Morphology**	**Follicle diameter (µm)**			**Female age (dpf)**		
		mouse	**cow**	**woman**	**mouse**	**cow**	**woman**
Primordial	Follicle made up of a immature oocyte surrounded by limited number (<10) of flattened pregranulosa cells Located in the ovary's peripheral cortex No zona pellucida	≈ 17	≥ 40	≈ 30	7	90	≈ 60
Primary	Immature oocyte surrounded by one layer of actively dividing cubic granulosa cells First steps of zona pellucida (ZP) deposition Initiation of gap junctions development	≤ 20	40-80	30-60	13	140	77-156
Secondary	Growing oocyte independent of gonadotropins Presence of at least two layers of granulosa cells Intensive mitosis of granulosa cells ZP formation continues Theca cells differentiation	100	80-250	100-200	10-12 dpp	210	≈ 200
Early antral	Growing oocyte bordered by multiple layers of cumulus cells Antrum cavity formation and fillfulfilment with follicular fluid Final differentiation of ZP	250	≥ 280	≥ 500	21 dpp	230	≥ 210
Graafian/ ovulatory	Perivitelline space formation High E2 production Presence of LHR on granulosa cells Important cumulus-cells expansion and ECM formation	≥ 600	18-25 mm	18 -23 mm	After puberty		

1.1.3. Follicular waves and atresia

1.1.3.1. Follicular waves:

The traditional theory of mammal folliculogenesis, developed over the past 50 years, holds that a single cohort of antral follicles is recruited to grow in each ovary during the late luteal phase of the estrus cycle. A single dominant follicle was believed to be selected to achieve final maturation and ovulate. Recent studies in woman reported that menstrual cycle exhibits 2 to 3 follicular waves. Only the final wave (named also major wave) of each cycle was ovulatory. The three-waves woman has longer menstrual cycles and shorter interwave period compared to the two waves ones (Baerwald *et al.* 2003a).

Follicular dynamics in cow was also extensively studied. During the estrus cycle, two or three follicular waves are discernible. Each wave is a cohort of 3-to-5-synchronously-developing follicles (Gordon 1994, Ginther *et al.* 1996, Roche 1996).

Final follicular growth in mammals is a gonadotropin-dependant process that includes three main steps: recruitment, selection and dominance. Recruitment is the entrance into the gonadotropin-dependent phase of a pool of follicles. It looks to be mainly dependent on the follicle size and the availability of functional gonadotropin receptors in its somatic compartment. The selection phase describes the emergence of one or more follicles from the recruited pool. This involves an atretic process that reduces the number of follicles potentially able to ovulate. For dominance phase, it is the consequence of a final and rigorous selection of the ovulatory follicle(s) that is specie- and breed- dependent. (Ginther *et al.* 2000, Driancourt *et al.* 2001, Fortune *et al.* 2001, Baerwald *et al.* 2003a). It is reported to be associated with a decline of FSH and a rise of estradiol concentrations, as well as the GCs acquisition of LH receptors (Ginther *et al.* 1996, Ginther *et al.* 2000). A progressive switch of the dominant follicle from FSH to LH dependence in the preovulatory phase was also suggested as an explanation of this process (Adams 1999). While many other factors were discussed to be involved in the follicular dynamics (figure 1.1), the molecular involvement of oocyte to the selection and recruitment steps is not yet fully understood. The hypothesis concerning growth differentiation 9 (GDF9) and bone morphogenetic protein 15 (BMP15) potential involvements remains unclear.

It is important to distinguish between the initial recruitment, which cover the follicular growth from the primordial and/or primary to the antral follicles, and the cyclic follicle recruitment which is discussed herein and called 'recruitment' (McGee & Hsueh 2000).

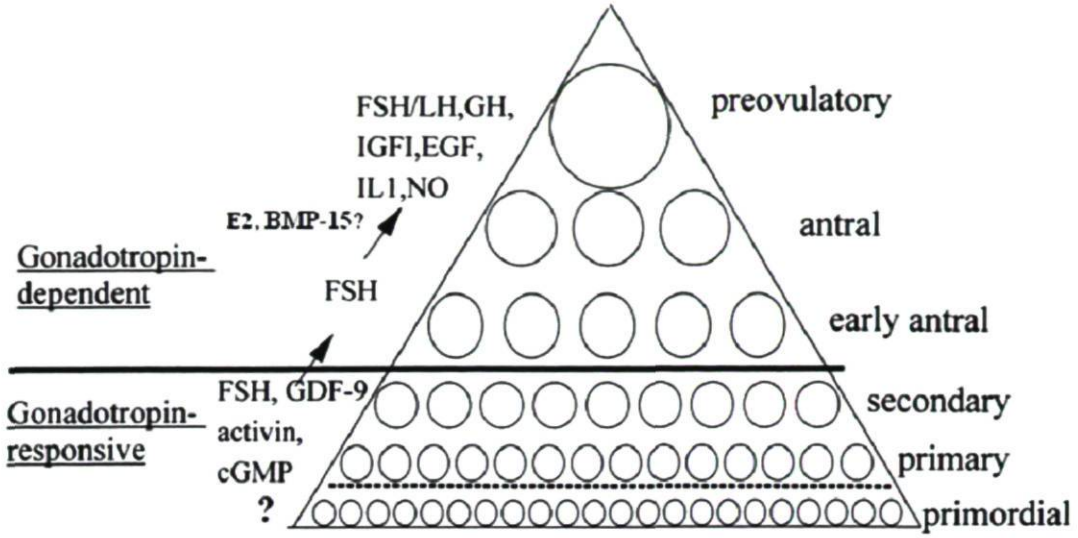

Figure 1.1: Some potential and stage-specific factors involved in follicular dynamics (McGee & Hsueh 2000).

1.1.3.2. Follicular atresia:

Among the large initial number of primordial follicles, approximatively fewer than 500 oocytes ovulate during women reproductive life since puberty and until 40 years (assuming regular menstrual cycles and no pregnancy). The atresia rate exceeds 99 %. This programmed-cell death is a devastating molecular process that can occur at any stage of the lifespan. Once the initial pool of follicles is established, any inadequate exchanges between the oocyte and the somatic compartment could be lethal. It is established that the somatic compartment is in continuous interactions with the surrounding environment, including oxygen and nutrients support. Any imbalance in these inputs could affect this compartment's crucial role and may leads to apoptosis. This molecular process is the consequence of the activation of specific effectors that cause the reduction of theca cells vascular supply and consequently the oxygen diffusion. Then, this affects firstly the GC and their aromatase activity, then the fragmentation of the basal lamina, the hypertrophy of theca cells and consequently the oocyte (Gordon & Lu 1990, Monniaux *et al.* 1999). The oocyte apoptosis occurs only at the last stages of the follicular atresia process (Kruip & Dieleman 1982). This may be considered as a strive mechanism to preserve the oocyte as a maternal legacy of vital importance to the species' next generations (Wassarman & Albertini 1994). Unless not fully understood, some factors were reported to be involved in apoptosis. This process is regulated by members of the Bcl-2 family and executed by caspases enzymes

(reviewed by (Driancourt *et al.* 1998, Monniaux *et al.* 1999, Manabe *et al.* 2004)). Table 1.2 includes a comparison between the follicular atresia rates at birth between mouse, cow and our own specie.

Table 1.2: Comparative summary of some physiological parameters in mouse, cattle and woman (Russe 1983, Gordon 1994, Wassarman & Albertini 1994, Marieb 1999, Driancourt *et al.* 2001, Hunter 2003, van den Hurk & Zhao 2005)

Specie	Germ cell number		Atresia at birth (%)	Oestrus cycle (d)	Ovulation (hours post-LH)	Pregnancy (days)
	Initial (X 10^6)	At birth ((X 10^3)				
Mouse	0.15	8	95	4-5	12	20
Cow	2.7	135	95	22	24	280
Woman	7	700	90	28	35-40	280

1.1.4. Oogenesis

Oogenesis describes the full steps of oocyte development and differentiation ranging from the migration of the first germ cells (embryonic ectoderm origin) to the genital ridge during the first weeks of gestation / pregnancy until the formation of haploid, mature and therefore potentially fertilizable oocyte (Russe 1983). Given that the oocyte is the germinal (central) compartment of the follicle, oogenesis and folliculogenesis are two interdependent processes that occur in the ovary and continue throughout the reproductive life of the female. As mentioned before, the PGC (2n) start active mitosis once they attain the genital ridge and become oogonia. This mitotic activity is crucial for the size of the initial oocyte stockpile that will serve during the whole reproductive career of mammalian species. The key process in oogenesis is meiosis which marked the transformation of the oogonia (surrounded by few pregranulosa cells) into oocytes. The mechanism and molecular factors involved in triggering the meiotic initiation are still poorly understood (Byskov & Nielsen 2003). Starting with a DNA synthesis phase, the oocytes progress with duplicated chromosomes through the transitory stages (leptotene, zygotene and pachytene) of the prophase I, which is characterized mainly by the genetic material exchange between maternal and paternal chromatids (crossing-over). Each chromosome start to condense, becomes thicker (visible to light microscope) and lines up with its homologous to form a tetrad structure (4 chromatids). Following the synaptonemal complex degradation at the diplotene stage, the bivalents remain firmly attached via the recombination sites or chiasmata. All the oocyte stockpile become arrested at the diplotene, named also the germinal vesicle (GV) stage, prior to birth for both woman and cow (Byskov 1985, Gordon 1994, Wassarman & Albertini 1994). Meiosis arrest is maintained for many years (more than 40 years in woman) and oocytes are unable to spontaneously resume meiosis during oocyte growth and follicle development. Concomitantly with the follicle growth, the oocyte grows and is able to achieve active DNA transcription. Following the LH surge and prior to ovulation, the oocyte resumes meiosis (germinal vesicle breakdown (GVBD)) and achieves its first meiotic division (the reductional division) and continues into the second meiotic division (the equational division) and arrests again at MII. The transcriptional activities during this period are very reduced (Hyttel *et al.* 1997, Driancourt *et al.* 2001). We should wait until fertilization to see the final achievement of the meiosis and the second polar body emission. The mature oocyte herein is the haploid female gamete or pronucleus (n) involved in fertilization. Besides its role in polyploidy prevention,

meiosis allows genotype diversity by the recombination and the independent assortment of homologous chromosomes.

We should highlight that the oocyte diameter increases from 35 to at least 120 μm in woman preovulatory follicles, and its volume with more than 100-fold in mouse. The transcriptional and translational activities are the most active during the oocytes of growing follicles. The synthetic activities continue during follicular development until growth fulfillment or atresia (Picton *et al.* 1998, Gougeon 2003). It is also established that the achievement of nuclear maturation does not guarantee competence (Sirard *et al.* 2006). Common characteristics classified according to the oocyte diameter and its fine structure in murine, bovine and human species are shown in table 1.3.

Table 1.3: Comparative summary of oocyte diameter increase during follicular growth and some common characteristics in mouse, bovine and woman (Russe 1983, Espey 1994, Braw-Tal & Yossefi 1997, Fair *et al.* 1997, Hyttel *et al.* 1997, Fortune *et al.* 2000, Picton & Gosden 2000, Picton 2001, Gougeon 2003, Hunter 2003, Aerts & Bols 2008)

Female germ cell class	Specie	Diameter (µm)	Characteristics
Primordial germ cells	mouse	≤ 12	Round and oval-shaped mitochondria Golgi apparatus close to nucleus Free ribosomes
	cow	≈30	
	woman	35	
Oogonia	mouse	12-15	High frequency of mitotic division More ribosomes associated to ER Few lipid droplets
	cow	≈30	
	woman	≥ 35	
Non-growing oocytes (Prophase I)	mouse	≈20	Cessation of mitosis Meiosis initiation & arrest at dictyate stage (GV) Biosynthesis of cortical granules ZP formation Active transcription & translation
	cow	50-60	
	woman	≈60	
Fully-grown oocytes/mature (MII)	mouse	≥ 80	Denser and thicker ZP Peripheral cortical granules First polar body emission Resume of meiosis (GVBD) and new arrest at MII
	cow	≥ 110	
	woman	≥ 120	

1.1.5. Human and bovine estrous cycle

Estrous cycle consists of a follicular phase (proliferative) and a luteal (secretory) phase separated by ovulation. As mentioned above, two to three follicular waves were detected during an estrous cycle both in cow and woman (Ginther *et al.* 1996, Baerwald *et al.* 2003a). Their number in each cycle looks to be independent of age, the breed as well as the presence of a corpus luteum (CL) in the ipsilateral or contralateral position; leading to the assumption that both ovaries work in synergy as a single unit (Ginther *et al.* 2000, Baerwald *et al.* 2003a).

Estrous cycle starts at puberty; i.e. between 8 to 12 months in heifers and 8 to 12 years in woman. It included four stages: proestrus (CL regression, progesterone declines, estradiol increases, a preovulatory follicle starts its final growth step), estrus (male receptivity & ovulation), metestrus (CL development) and diestrus (CL optimal secretory function). While the proestrus and estrus formed the follicular phase, metestrus combined with the diestrus constituted the luteal phase (Sjaastad *et al.* 2005).

In woman, her first-day cycle corresponds to day one in the 28-day menstrual cycle. The duration of both follicular and luteal phases is about 14 days each. In longer or shorter cycles, only the follicular phase time-span and the ovulation timepoint fluctuate (Baerwald *et al.* 2009). For cow, the estrous cycle lasts on average 21 days starting by the estrus phase. Woman ovulation occurs around the 14^{th} day, i.e 12 hours following a 12-hour estrus period, and the CL reaches its maximal growth on day 16^{th} (Hunter 2003). For both species, the progesterone is the main hormone produced during the luteal phase. In the absence of fertilization, the CL regresses and the level of FSH increases (suppression of progesterone negative feed-back) inducing a new follicular wave (figure 1.2). Comparative analysis of estrous cycle length, estrus time point and duration, as well as the pregnancy duration between the three mammalian species: mice, bovine and human are summarized in table 1.2.

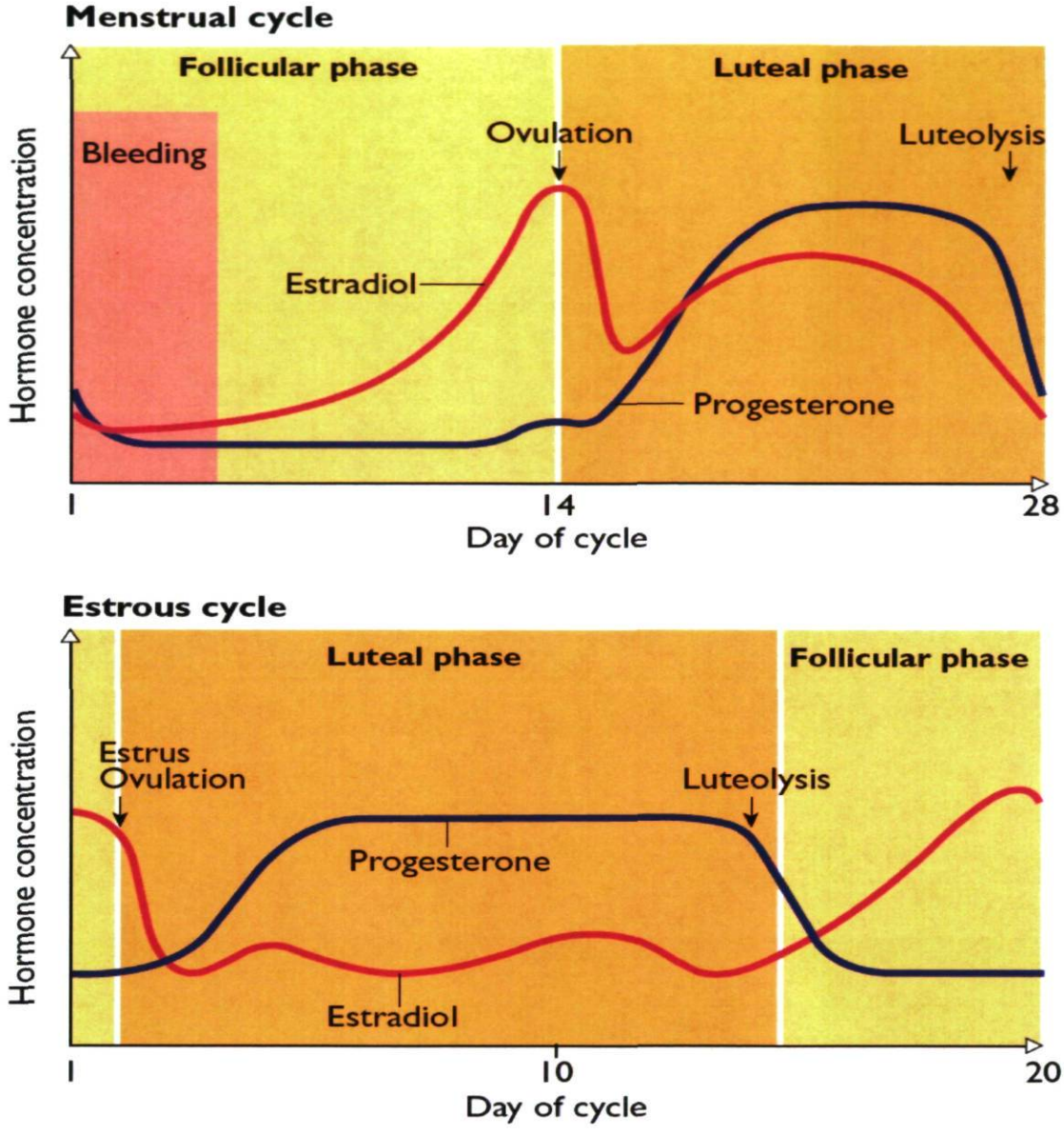

Figure 1.2: Superposition of the main endocrine patterns of the menstrual cycle (day 1= bleeding; day 14= ovulation) in women and the estrous cycle in cow (day 1= first day of estrus) (Sjaastad et al. *2005)*

1.1.6. Regulation of ovarian function

Ovarian function, including oogenesis and folliculogenesis, is finely regulated by gonadotropins as well as many intraovarian factors. Meticulous communications between theca cells, GCs, follicular fluid (FF), cumulus cells (CCs) as well as oocyte during the prolonged period of oocyte growth and development are necessary to the optimal achievement of the oocyte maturation.

Consequently, the oocyte acquires gradually the required molecular and cellular machinery and reaches a competent stage able to sustain the early embryo development. This competence is not only dependent on the intrinsic properties of the oocyte but also on the follicular environment and the follicle health (Simon *et al.* 1997, Sirard *et al.* 2006). The bi-directional communications between oocyte and its somatic cell environment is achieved mainly by paracrine and gap-junctional signalling pathways (Albertini *et al.* 2001, Eppig *et al.* 2002).

To date, two major oocyte-secreted factors are confirmed as crucial in the very earliest and the late preovulatory follicular development. These two molecules are the GDF9 and BMP15. They are involved in the GCs differentiation, CCs expansion and the expression of many key genes involved in follicular metabolism and growth, the oocyte maturation as well as ovulation (Moore *et al.* 2004, Pangas & Matzuk 2005, Su *et al.* 2008). This regulatory capacity of the oocyte on GCs varies depending on its growth and maturation stage, as well as the differentiation state of the somatic compartment. While oocyte induces mainly the mitotic activity of GCs in early mammalian follicular stages, it regulates more functions as Kit-ligand (KL) and cyclooxygenase 2 (COX2) expression, cumulus expansion and matrix stabilization in antral follicles (Joyce *et al.* 2000, Joyce *et al.* 2001, Gilchrist *et al.* 2004). In mouse, this KL expression induction is conversed into inhibition when the oocyte reaches its full size (Joyce *et al.* 1999).

Gonadotropins are also key exogenous factors that orchestrate the oocyte-somatic cells' interactions. In fact and during the regression of the corpus luteum (CL) in the late luteal phase of the estrous cycle, the increase of FSH concentrations allows synchronous growth of a group of gonadotropin-dependent follicles. Concomitant with this follicular growth, an active mitosis in bovine GCs, an increase in the aromatase activity and therefore estrogens production, the rise of the concentration of inhibin in the follicular fluid (FF) and the appearance of LH receptors were reported (Gordon 1994, Greenwald & Roy 1994, Hunter 2003). By negative feedback, estradiol and inhibin cause of the decline in the FSH levels. Thus, all the follicles of the cohort move towards atresia because they are still FSH-dependent except the dominant follicle. The reasons that the dominant follicle continues its growth are not fully elucidated. Indeed some authors suggested the reason that the dominant follicle has time and diameter advantages. Therefore, it has already expressed sufficient LH receptors and is able to switch into LH-dependency growth mode which drives the dominance stage (Ginther *et al.* 2000, Zeleznik 2004). Another hypothesis involves the secretion of a regulatory factor by the dominant follicle that inhibits the rest of the

cohort. Estradiol, Insulin-like growth factor (IGF), inhibin and the IGF binding proteins (IGFBP) were suggested to be involved in this process (Salustri 2000, Driancourt *et al.* 2001, Armstrong *et al.* 2002). We assume that this rigorous selection mechanism involves a "limiting" follicular endocrine environment that only the most resistant follicle will survive. The dominant follicle is thought to be the most equipped to overcome this selection pressure and pursue its itinerary toward ovulation.

The other follicles begin a process of atresia resulting from their early stages by the synthesis of apoptotic elements (IGFBP, p53, B-cell lymphoma 2 (BCL2) family, as BCL2-associated X protein (Bax) and caspases, and the loss of synthesis of survival factors (Bcl-xL) (Quirk *et al.* 2004). After that, the final development of the ovulatory follicle is under the exclusive control of LH pulses. In addition to the effect of activin, the amount of estradiol reaches a threshold and excerts positive feedback on the hypothalamus to induce the surge of pituitary gonadotropins mainly the LH. In the few hours after the ovulatory peak, the follicular fluid pressure increases, a localized vasoconstriction at the apex (or stigma), where the antral follicle leans the surface of the ovary, which will allow the ovulation of the mature follicle (Fortune 1994, Hunter 2003, Russell & Robker 2007). Besides FSH, the LH is a key factor of follicular dominance and a major inducer of the somatic cell steroidgenesis in the follicle. Theca cells express LH receptors and use cholesterol to produce progesterone and testosterone. In contrast, granulosa cells express FSH receptors and converts androgens to estrogen (E2) through the enzyme aromatase (P450arom) (Roberts & Skinner 1990, Robel 2001). If estradiol is considered as the main hormone secreted by the dominant follicle, six hours following the LH surge are marked by a loss of the aromatization potential (and estradiol levels decrease) of granulosa cells and a gradually increase of progesterone reflecting a switch to the luteinisation process. Concomitant with these hormonal pattern changes, the bovine oocyte resumes its meiosis (GVBD) (Kruip *et al.* 1983). Progesterone production continues to increase with the CL proliferation and is reported to induce the expression of proteolytic enzymes essential to ovulation (Richards 2007).

In addition to gonadotropins, locally produced members of growth factors families such as fibroblast growth factors (FGF), epidermal growth factors (EGF), transforming growth factors (TGF) and IGF are involved in follicular growth and oocyte maturation regulation. While GDF9 and BMP15 are produced by the oocyte to control the somatic cells proliferation and differentiation (Diaz *et al.* 2007a, Su *et al.* 2008), other TGFβ factors, mainly inhibin and activin,

contribute to the follicle development and the oocyte maturation. Inhibin is synthesized in GCs and exerts a specific negative control on FSH secretion without influencing the LH one. However, its antagonist, the activin, induces the FSH receptor (FSHR) expression and proliferation of rat and human GCs (Li *et al.* 1995, Hunter 2003). Inhibin expression in human and bovine GCs is induced by LH or human chorionic gonadotropin (hCG) (Glister *et al.* 2001). Once secreted, activin regulation is achieved through follistatin by a sequestration mechanism (Shintani *et al.* 1997). Measurement of inhibin β serum levels during women follicle growth had basal level secretion that increases 1-2 days throughout FSH treatment during maturation progression. Inhibin β serum levels was suggested as a predictive value in monitoring ovarian stimulation treatment for IVF and to assess the rate of healthy oocytes to collect later (Eldar-Geva *et al.* 2002). An other study reported that inhibin beta expression is differentially upregulated in GCs of bovine competent follicles (Fayad *et al.* 2004).

For the EGF family, they are part of a large family of peptide growth factors that includes the TGF-α and heparin-binding EGF (HB-EGF). *In vitro* studies documented that EGF stimulated GCs proliferation and protein phosphorylation. The expression of both EGF and its receptor EGFR are induced by FSH in most mammalian granulosa cells. EGF concentration in follicular fluid (FF) decreased at the late stages of follicular development (De La Fuente *et al.* 1999, Glister *et al.* 2001, Hunter 2003). Other EGF-like factors as epiregulin (EREG), amphiregulin (AREG) and betacellulin (BTC) are triggered also by gonadotropins. Incubation of follicles with these growth factors induced some morphological and biochemical events initiated by pituitary hormones, including CCs expansion and oocyte maturation (Freimann *et al.* 2004, Park *et al.* 2004). These EGF-related growth factors are, therefore, paracrine mediators that disseminate the FSH and LH signal throughout the mammalian ovulatory follicles. The upregulation of these factors in GCs was associated to dominant follicles and are part of the signal transduction pathway which leads to ovulation and luteinisation in the human ovary (Freimann *et al.* 2004).

One of the main members of TGF family is basic FGF (bFGF). It acts as a mitogen factor in bovine granulosa cells and may stimulate theca cells proliferation and inhibits their steroidogenesis. It is also an angiogenic factor that modulates steroidogenesis and increases the tissue plasminogen activator (tPA) expression in GC prior to ovulation (LaPolt *et al.* 1990).

IGF (IGF-1 and -2) are other extensively studied growth factors. They are polypeptide factors that promote cell growth and proliferation, and acts therefore as apoptosis inhibitors. They are

involved in many molecular processes such as gene expression, protein synthesis and amino acid transport (Cheatham & Kahn 1995). That's why they are used as common supplement in cell and tissue culture. They are reported to act through the protein kinase pathway (PKB) to induce steroidogenesis and FSHR expression, and to enhance GCs response to FSH (Gonzalez-Robayna *et al.* 2000, Glister *et al.* 2001, Armstrong *et al.* 2002). IGF-I knock out (KO) mice were infertile and unable to overcome the secondary follicle stage (Baker *et al.* 1996). One of the main players involved in IGF regulation are their family of binding protein (IGFBPs) which affected their bioavailability (Ingman *et al.* 2000). For example, IGFBP-2 was reported be underexpressed in cattle dominant follicles compared to the subordinates which leads to change in the free IGF concentrations (Roberts & Echternkamp 2003).

We should highlight here that the FF, where bathes the COC, is a repository with dynamic composition depending on the follicular stage, the oocyte status, and the intra-ovarian and the pituitary signals. Granulosa cells are distanced by around 20 nm-diameter channels that ease the passage of up-to-Mr-500 000 molecules into the antrum (Gosden *et al.* 1988). The porous structure of the follicular epithelium (wall) allows similar exchanges between the FF and the venous plasma. FF is reported to contribute to the meiotic arrest of oocyte at the GV until the oocyte cytoplasmic maturation achievement (Fukui *et al.* 1987, Sirard *et al.* 1988, Ali *et al.* 2004). It ensured also a buffering role and may also inhibit the early luteinisation of granulosa cells (Hunter 2003).

Other players could also be involved in this complex paracrine/autocrine regulatory loop between oocyte, granulosa and theca cells, confirming a fine degree of the oocyte-somatic cell communication regulations (Figure 1.3).

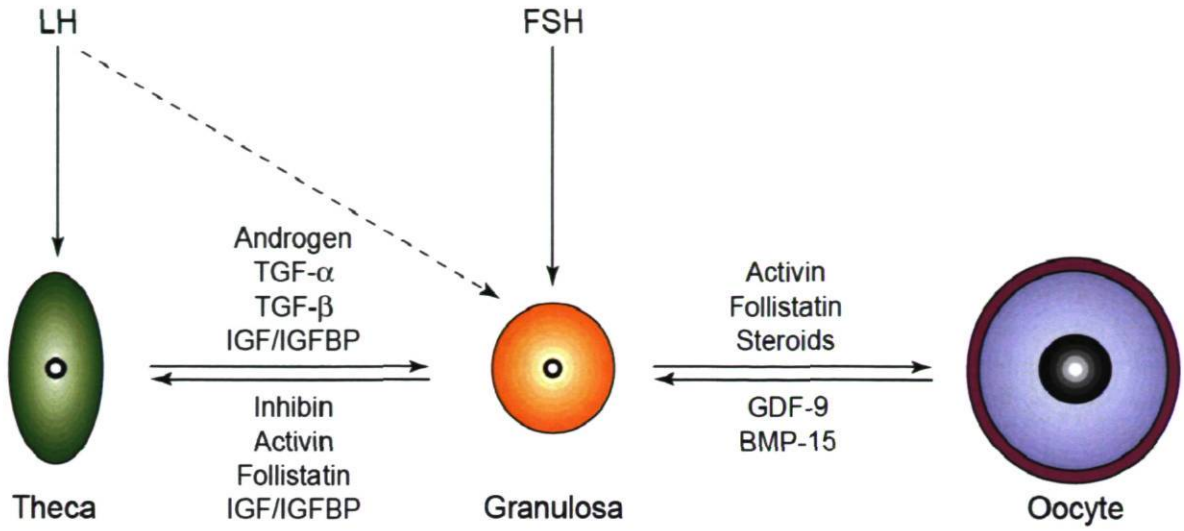

Figure 1.3: Summary of the main factors involved in the follicle development and oocyte maturation. In addition to the primary extrinsic factors (gonadotropins), several intrafollicular factors likely to be involved in cell–cell communications between theca cells, granulosa cells and the oocyte (Knight & Glister 2001)

1.1.7. Oocyte maturation & competence

Oocyte competence is the ability of the oocyte to complete successful maturation, to be fertilized and to produce a good quality and transferable blastocyst able to give viable and healthy progeny. Many studies and reviews documented that maturation is the crucial step in the competence acquisition (Hyttel *et al.* 1997, Mermillod 2001, Sirard *et al.* 2006, Sirard *et al.* 2007). Thus, the oocyte maturation at the nuclear, cytoplasmic and molecular levels are discussed herein. An optimal maturation at the three levels is determinant to the global successful competence. These processes are concomitant and interdependent.

1.1.7.1. Nuclear maturation:

The main event in nuclear maturation is mainly the meiotic dynamics which was detailed in previous paragraph about oogenesis. It refers to the progression of the oocyte nucleus from the germinal vesicle stage (GV), and through the GVBD, the association of the homologous chromosomes with the first polar body extrusion, until the second meiotic arrest at MII. The second meiotic resumption is induced by the male pronucleus and leads to the fulfillment of meiosis and the second polar body extrusion (Hyttel *et al.* 1986). This meiotic progression is very exceptional in terms of its duration as well as its multiple arrests.

The nucleus at GV is separated from the cytoplasm by porous nuclear membrane allowing a selective exchange of molecules. This inner membrane face contains the nuclear lamina which have high affinity to chromatin and whose phosphorylation by the maturation promoting factor (MPF) (a complex cyclin B1 / cyclin dependent kinase 1 (Cdk 1)) causes their depolymerisation and therefore disruption of the nuclear envelope into small vesicles (GVBD) (Isaji *et al.* 2004).

As for histones (H2A, H2B, H3, H4 and H1), they are particularly abundant in the nucleus of the oocyte and are characterized by their small size and positive charges. By means of electrostatic forces, they interact with the phosphoric acid groups of the negatively charged DNA. These histones are highly conserved and are involved in nuclear maturation by structural arrangement of DNA within the chromatin as well as transcription regulators mainly when are phosphorylated. Histone H1 phosphorylation is associated mainly with the solenoid formation and chromatin condensation (Wassarman & Albertini 1994, Liu *et al.* 2004).

As the follicle grow (GV), the oocyte nucleolus increases and become dense and very fibrous (filamentous) indicating and intense synthesis of ribosomal RNA (rRNA) (Wassarman & Albertini 1994). Moreover, during its growth until around 110 µm of diameter (case of bovine and human oocytes), the oocyte produces increasing amounts of mRNAs stabilized by a poly(A) tail. The GVBD is associated with an arrest of most if not all the transcriptional activity until the maternal-to-embryonic transition (MET) in the early embryo development (Fair *et al.* 1997, Tremblay *et al.* 2005).

Finally, it should be mentioned that the oocyte is the only cell that undergoes the longest meiotic division marked by a first locking prophase I (GV) which can last for years, a second arrest in MII after ovulation, and yielded only one ovule (mature oocyte) that inherited almost the whole cytoplasm of the mother oocyte by means of unequal meiosis.

1.1.7.2. Cytoplasmic maturation:

The cytoplasmic maturation includes all the ultrastructural modifications that occur in the ooplasma during oocyte prematuration (few days before the LH surge) and final maturation to allow successful fertilization, cleavage and early embryo development (Kruip *et al.* 1983, Hyttel *et al.* 1986, Hyttel *et al.* 1997). Unfortunately, there is no available direct measure of this maturation. That's why the meiotic maturation is commonly used as a reference to the cytoplasmic one. Besides this indirect approach, cytoplasmic maturation could be also assessed retroactively through the early embryo development outcome. That's why it is difficult to

distinguish between the oocyte maturation and competence at both the cytoplasmic and molecular levels.

The cytoplasmic maturation focuses essentially on ultrastructural and morphological aspects. In fact, the elongated mitochondria were concentrated at the center of oocytes in early growth stages. With the oocyte maturation progression, their number increased and became oval-shaped and closely associated with smooth endoplasmic reticulum in the oolema periphery (Michaels *et al.* 1982). As the oocyte grow and similarly to mitochondria, the Golgi apparatus become more active through spaced lamella associated to numerous lipid vesicles, and move to the subcortical region of the oocyte. It looks to contribute actively in the formation of cortical granules, and the secretion and concentration of zona pellucida (ZP) glycoproteins. At final maturation (MII), there is an important decrease in Golgi membranes and a peripheral migration of the cortical granules prior to fertilization to achieve their polyspermybarrier role (Wassarman & Albertini 1994, Fair *et al.* 1995, Sun 2003). For nucleolus and concomitant with oocyte growth, they increased in diameter and switched from diffuse to dense and uniform structure indicative of active rRNA synthesis (Mirre & Stahl 1981). Consistent with nucleolar morphology, the number of ribosomes, whether free or associated to polysomes, increased signifying an important protein synthesis activity (Wassarman & Albertini 1994). We should highlight also that the ZP, which appears and differentiate in the secondary follicles, the nucleus diameter and the first polar body emission are also other criteria used to assess progression of oocyte cytoplasmic maturation. ZP thickness and differentiation increased and were maximal at final maturation step prior to fertilization (Hyttel *et al.* 1986).

1.1.7.3. Molecular maturation:

During oocyte maturation, many molecules that may contribute in oocyte fertilization and early embryo development were produced and accumulated. They include all the molecular processes that occur both in the nucleus and ooplasma during the oocyte maturation. Unless still poorly defined, molecular maturation is needed to accurately assess the oocyte maturation status (Sirard *et al.* 2006). Its importance increases especially when we know the limits of the morphological criteria used to identify the competent oocyte. For similar visible properties of oocytes and COC, some oocytes were more competent than others. We proposed herein to discuss some molecular events that, although invisible, were reported to be crucial in the oocyte maturation fulfillment and further competence. In fact, the oocyte volume is reported to increase until 300-fold in

mouse. This tremendous cell enlargement is a synonym of intense metabolic activities (Wassarman & Albertini 1994, Mermillod & Marchal 1999). Thanks to its reserves (mRNA, ribosomes, proteins…), the mature oocyte is therefore fertilizable and able to support the early embryonic development stages. Among the major molecular mechanisms of oocyte maturation, we will focus on the RNA transcription and protein synthesis that will allow the oocyte to acquire its competence and contribute to support the early embryo progression until the embryo genome activation (Barnes & First 1991).

One of the main events in molecular maturation is mRNA transcription and storage. RNA transcription is essential for the resumption of meiosis in cattle (Sirard *et al.* 1989). Several studies have shown a significant synthesis of mRNA during maturation of bovine oocyte. Measured through uridine triphosphate 35S-UTP incorporation, the transcription level is reported to be high during the bovine GV stage and fall around the M II (Memili *et al.* 1998) . The transcription decrease occurred sharply when the diameter of the oocyte reached 110µm (Fair *et al.* 1995). According to Memili and collaborators (1998), the active transcription machinery at diplotene stage (GV) is promoted by a permissive structure of chromatin to the transcription factors. Moreover, it is well established that the oocyte is able to store its maternal mRNAs in an inactive translational state for long periods without being degraded. Despite its very small fraction compared to total RNA, oocyte's mRNA has the property to have a variable length of the tail Poly (A) and binding proteins (RNA binding protein), which protect them from the RNases enzymes. For the same transcript, different poly (A) tails could be found which is synonym of post-transcriptional regulation of the gene expression (Lequarre *et al.* 2004, Tremblay *et al.* 2005). It is reported that the poly (A) tail is crucial to translation initiation and necessitates a cytoplasmic polyadenylation element (CPE) at the 3' UTR (Jackson & Standart 1990, Dai *et al.* 2005). The CPE will bind to the CPEBP that recruits the cleavage and polyadenylation specifity factor (CPSF). The CPSF initiates the polyadenylation process with the contribution of the poly (A) polymerase (PAP). The poly (A) binding proteins (PABP) are therefore linked to the tail to support the PAP action until the addition of 200 to 300 adenosine nucleotides (Keller *et al.* 2000). Transcripts with short poly (A) tails (less than 90) were associated to dormant translational state whereas those with longer ones (more than 150) were translated (Bachvarova 1992). The PABP are also involved in the initiation of translation through their interactions with the translation initiation factors. Other studies reported transcripts deadenylation during the oocyte maturation

whether for storage or degradation (Brevini *et al.* 2002, Lequarre *et al.* 2004, de Moor *et al.* 2005). Messenger ribonucleoprotein complexes (mRNP) were required to a safe store of the mRNA in the xenopus oocyte. This complex is assumed to protect the transcript from both the translational and degradation machineries (Weston & Sommerville 2006).

For translation, it was demonstrated that protein synthesis is necessary for the resumption of meiosis of the oocyte. Its inhibition with cycloheximide prevents meiosis resumption. This protein synthesis is achieved through a selective process based mainly on the poly (A) tail length (Kastrop *et al.* 1991b, Levesque & Sirard 1996).Untranslated region (3'UTR) appears to influence the stability, storage and translation of mRNA (Henrion *et al.* 2000). The cap molecule binds the 5' end to the mRNA and recruits the translation initiation factors like the eIF-4E. The ribosome S60 subunit is therefore bound to this complex to initiate translation (Tomek *et al.* 2002, Tomek *et al.* 2002a). Protein synthesis in bovine oocyte *in vitro* was maximum around the GVBD stage (approximately 6 h of MIV), three times more than its level at GV, before decreasing again around the MII stage (Tomek *et al.* 2002a). Other studies have identified additional proteins that were synthesized and accumulated around the GVBD as ribosomal and mitochondrial proteins, the ZP glycoproteins, histones and kinases (Wassarman & Albertini 1994, Massicotte *et al.* 2006). Besides translation of new proteins, post-translational modifications mainly phosphorylation were reported during the transition G2 / M of the bovine oocyte (Kastrop *et al.* 1991a). Phosphatase (1 and 2A) (Kallous et al., 1993) and several kinases are involved in this process mainly Mos Kinases, the phosphatidylinositol 3 (PI3)-kinase, PKA, PKB, PKC and MAP kinases.

Metabolic activity within the oocyte is characterized by a significant increase in glycolysis including pyruvate, glutamine and lactate, which seems to have a direct effect on nuclear maturation and competence development of the oocyte (Coffer *et al.* 1998, Cetica *et al.* 1999, Krisher & Bavister 1999, Fan & Sun 2004).

1.1.7.4. Oocyte competence:

The oocyte competence is its ability to properly achieve nuclear, cytoplasmic and molecular maturation, to get fertilized and go across early stages of embryo development until the blastocyst stage, and to lead to a viable and healthy offspring (reviewed by (Sirard *et al.* 2006)). We assume that an optimal molecular maturation should provide the ideal and well-orchestrated sequences of molecules (and molecular events) in a precise space, time and magnitude orders. This should be

synonym of successful nuclear and cytoplasmic maturation, as well as suitable acquisition of the developmental competence. Partial or total failure of any maturation type should affect the competence achievement. On the other hand, successful achievement of previous events of oocyte maturation doesn't guarantee the followings. Due to its complexity, there is currently no available way to measure the oocyte competence. The approaches used and based whether on oocyte morphology or markers are still predictive.

1.2. SECTION # 2: CUMULUS CELLS: FACTS & FICTIONS

1.2.1. Introduction

To reach its competence, the mammalian oocyte should successfully pursue consecutive steps of growth, maturation, fertilization and early embryonic development. During the long journey of folliculogenesis, the oocyte is surrounded by follicular somatic cells that differentiate into mural granulosa cells (MGCs) and from the tertiary stage of follicular growth into cumulus cells (CCs). The CCs are highly specialized follicular cells occupying the immediate vicinity of the oocyte and maintaining intimate cell-to-cell connections supported by gap junction communications. The CCs removal or disruption of these intercellular communications (Isobe & Terada 2001, Modina *et al.* 2001, Matzuk *et al.* 2002, Sutton *et al.* 2003, Ali *et al.* 2005, Mese *et al.* 2007), or the inhibition of their normal metabolic, transcriptional and/or translational activities (Motlik *et al.* 1989, Haghighat & Van Winkle 1990, Tatemoto & Terada 1995) deeply affect both oocyte nuclear and cytoplasmic maturation, and therefore its competence. In addition to the follicular and gonadotropins environments, the CCs functions are influenced by some oocyte-secreted factors mainly the transforming growth factors (TGFs) (Elvin *et al.* 2000a, Eppig 2001, Gilchrist *et al.* 2004, Su *et al.* 2004) and the EGF-like peptides (Tsafriri *et al.* 2005, Downs & Chen 2008, Panigone *et al.* 2008). Several pertinent studies were achieved to investigate the CCs roles in the oocyte maturation, ovulation, fertilization and early embryo development. Using physiological (Sutovsky *et al.* 1993, Ali *et al.* 2005, Sasseville *et al.* 2009b), metabolic (Haghighat & Van Winkle 1990, Goldberg *et al.* 2002, Eppig *et al.* 2005) and recently genomic approaches in livestock and human species (Feuerstein *et al.* 2007, Assidi *et al.* 2008, Assou *et al.* 2008, van Montfoort *et al.* 2008, Tesfaye *et al.* 2009, Huang & Wells 2010), wealthy findings about the CCs functions have been reported. CCs are evermore used as a main non-invasive criteria (morphologically or using molecular biomarkers) when assessing the oocyte developmental competence in mammalian species as human and bovine (Blondin & Sirard 1995, Assou *et al.* 2010, Seli *et al.* 2010). However, the exact mechanism of cumulus-oocyte communications (including zona pellucida (ZP) & the trans-zonal projections (TZP)), and the complete molecular pathway of CCs contributions into the oocyte developmental competence remain to be elucidated. This section focuses on the CCs differentiation in mammalian species and their relationships with the oocyte from the preovulatory stage to the early embryonic development. New roles of CCs

are suggested based on the recent physiological, metabolic and genomic findings reported in ovarian or other tissues.

1.2.2. Origin of cumulus cells

Besides their endocrine role, the ovarian follicle ensures germ cell line housing by providing them with a suitable environment for their multiplication and storage from the prenatal life until when the germ line reserve is exhausted. Granulosa cells (GCs) are a pseudostratified internal epithelium that undergoes deep morphological and physiological differentiation from the secondary follicle stage until ovulation and luteinisation (Lu *et al.* 2005). It is widely accepted that GCs derived from the mesonephric precursor cells (Byskov & Høyer 1994). However, this assumption was recently challenged by a recent study suggesting a mesothelium origin for sheep GCs (Sawyer *et al.* 2002). This means that GCs might also derive from the presumptive surface epithelium of the ovary. Since the cumulus cell line derives later from GCs, they have therefore the same embryonic origin.

CCs are also the result of the differentiation of preantral GCs into two separated antrum cell sub-types: MGCs localized close to the basal lamina of the ovarian follicle and CCs that surround the oocyte. The squamous pregranulosa cells of the primordial follicle become cuboidal and start to actively proliferate until the secondary follicle stage. From secondary follicle GCs becomes FSH-responsive in most mammal species (Greenwald & Roy 1994). Both oocyte and follicular growths are firstly occurring in a linear fashion and positively correlated until the oocyte is close to its maximal size. Thereafter, the growth of the ovarian follicle becomes faster with quick cell proliferation (GCs mitosis) and the beginning formation of islets filled with follicular fluid (FF) within the GCs intercellular space (Byskov & Høyer 1994). The fusion of FF pockets leads to the antrum cavity, synonym of tertiary follicle. At this stage, the cumulus-oocyte complex (COC) occupies a more peripheral position compared with the rest of the follicle possibly as a preparation for subsequent ovulation (Hunter 2003). Concomitant with GCs proliferation in mouse, GCs transcriptional activity increases and reaches its maximum just prior the antrum formation (Moore *et al.* 1974). GCs final differentiation is marked by a centripetal gradient increasing meiotic index in follicles of cyclic mouse (Hirshfield 1986). Interestingly, this gradient is deeply affected following a surgical oocytectomy (Vanderhyden *et al.* 1992) supporting an important role of the oocyte in the centripetal gradient of GCs differentiation. While the GCs of rat preovulatory follicles contain well-developed mitochondria, smooth RE and lipid droplets, the

CCs show large rough RE with/without lipid droplets (Hillensjo *et al.* 1981). CCs show therefore several ultrastructural and molecular signs of differentiation and high specialization that might be associated to specific functions. Despite the presence of the LH receptor (LHR) transcripts in both cumulus and granulosa (Robert *et al.* 2003, Shimada *et al.* 2003, Fu *et al.* 2007), immunoassays using the anti-LHR in the pig ovary revealed the absence of LHR protein at the surface of both cumulus cells and oocyte compared to mural GC (Meduri *et al.* 1992).

Whilst MGCs represent the major part of the somatic compartment, the neo-formed CCs continue to differentiate and to be influenced by the oocyte-derived factors (Vanderhyden *et al.* 1992, Eppig *et al.* 2002, Gilchrist *et al.* 2004, Su *et al.* 2004, Pangas & Matzuk 2005). The innermost layers of CCs, also identified as the corona radiata, maintain intimate contact with the zona pellucida (ZP) and make specific exchanges required to the oocyte maturation. These exchanges are thought to occur particularly between the oolemma and the CCs transzonal cytoplasmic projections (TZPs) (Albertini *et al.* 2001). During subsequent ovulation, most of CCs and follicular fluid (FF) will accompany the oocyte to the oviduct while the MGCs and theca cells will contribute to the corpus luteum formation.

It has long been established that somatic cells are essential to properly support oocyte maturation, meiosis resumption and competence acquisition. More persuasive facts during the last few years documented crucial roles of the oocyte in folliculogenesis by promotion of the follicular cells proliferation and differentiation. The TGF-β family was suggested as one of the main players triggering these effects (Eppig 2001, Matzuk *et al.* 2002). In fact, the TGF-β family (mainly GDF9 and BMP15) are two important oocyte-derived paracrine factors that have been shown to drive CCs final differentiation prior to ovulation. These factors are reported to allow CCs acquisition of its ability to expand and to express key genes needed for final maturation and ovulation (Diaz *et al.* 2007b). While the main distinctive morphological criteria of CCs is mucification, transcriptomic studies showed that MGCs and CCs differentially respond to FSH by the expression of different gene sets (Ingman *et al.* 2000, Li *et al.* 2000, Salustri 2000). Recently, significant differences in gene expression patterns between the two somatic cell types were demonstrated in women undergoing IVF or ICSI (Koks *et al.* 2010). These major functional differences between MGCs and CCs are not only due to the oocyte effect but also to intrinsic molecular pathways that allow differential gene expression and therefore distinctive functions between the two somatic compartments (Diaz *et al.* 2007a). These findings were also confirmed

by a comparative proteomic approach between CCs and GCs using the 2-dimensional PAGE (2D-PAGE). The GCs co-cultured with the oocyte showed a reduced of both synthesized proteins patterns and developmental competence compared to CCs matured in COCs *in vivo*. Authors suggested that CCs would be more helpful to the oocyte competence acquisition than GCs (Latham *et al.* 1999).

Looking at the effect of both gonadotropins and oocyte-derived paracrine factors on follicular cells, one can observe a morphogen gradient given the unique characteristics of CCs such as repression of LH-R (Peng *et al.* 1991, Eppig *et al.* 1997b, Park *et al.* 2004) and ability of progesterone secretion (Schuetz & Dubin 1981, Shimada & Terada 2002, Shimada *et al.* 2006b, Guidobaldi *et al.* 2008). Two different gradients could be described. Based on the sphere-like structure of the mammalian ovarian follicle, centrifugal effects would refer to the influence of oocyte-derived factors and centripetal effects would describe the gonadotropins actions. The gonadotropins actions predominate in theca and MGCs and decrease progressively closer to the oocyte. However, the oocyte-derived paracrine factors are more efficient in CCs, and seem to have less influence thereafter (Erickson & Shimasaki 2000) (Figure 1.4). These two opposite morphogen gradients are key regulators of follicular cells differentiation and specialization mainly observed at the preovulatory stage. Further exploration of the differential gene expression between GCs and CCs should reveal additional functional differences.

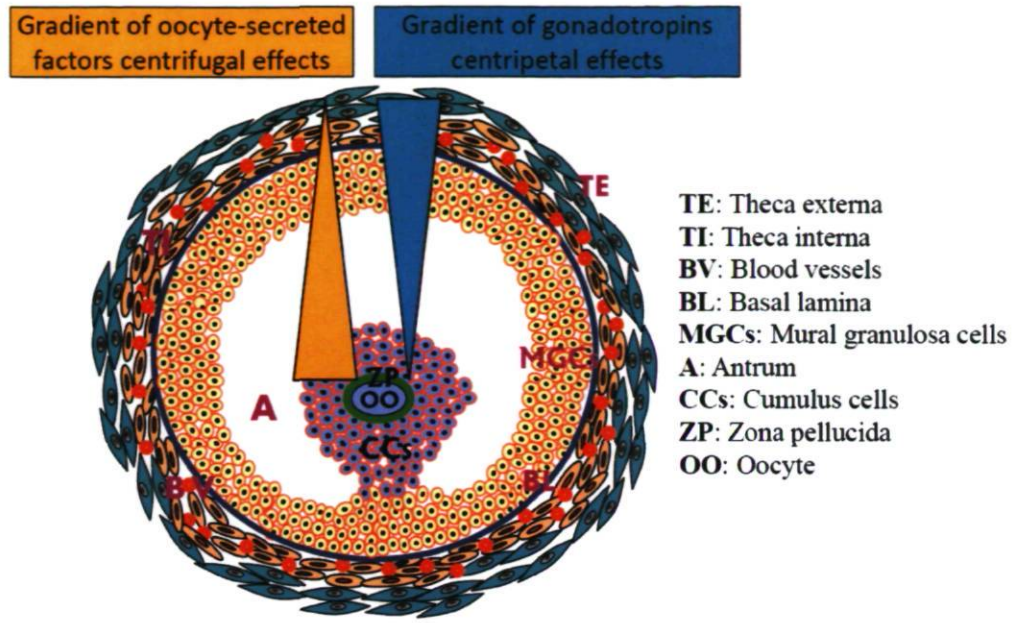

Figure 1.4: Schematic illustration of two main gradients that drive CCs differentiation and their reciprocal communications with oocyte: the gonadotropins centripetal signal versus the oocyte-derived paracrine factors.

1.2.3. Cumulus cells mucification

Before the onset of the gonadotropins preovulatory surge, CCs of a preovulatory follicle form a compact mass that enclosed the oocyte and maintained cell-cell communications both between each other and with the oocyte. The process of cumulus mucification, named also expansion, describes the dramatic morpho-structural changes induced *in vivo* by the LH surge and leading to the synthesis and the deposit of a mucoelastic matrix in the intercellular space between cumulus cells. This extracellular matrix (ECM) is formed following intensive secretion of an enriched network of glycoproteins, proteoglycans and hyaluronic acid (HA). The expansion of this cell mass in the immediate vicinity of the oocyte is a crucial step that lies between the LH surge and ovulation. *In vivo*, CCs expansion is induced by LH or by HCG (LH-like effect) in PMSG-primed animals (Dekel *et al.* 1979, Eppig *et al.* 1997a). It is a crucial step required for oocyte maturation and ovulation (Tanghe *et al.* 2002, Yokoo & Sato 2004). However, CCs expansion may be induced by many factors *in vitro* such as FSH, EGF superfamily (EGF, AR, BTC, EREG…), IL6, and the adenylyl cyclase activator, forskolin (Racowsky 1985, Tirone *et al.* 1997, Mattioli & Barboni 2000, Ali & Sirard 2005, Ashkenazi *et al.* 2005, Liu *et al.* 2009).

After exposing preovulatory follicles to the LH surge, cumulus mucification starts with rapid expression of HAS2 and, intensive synthesis and secretion of HA-rich matrix leading to CCs distancing. FSH stimulation of mouse COC *in vitro* increased the HA biosynthesis around 20 folds within 3-12 h(Salustri *et al.* 1989). The serum-derived factor inter-α trypsin inhibitor (IαI) is another component of the ECM that was reported to form protein complexes and to link them covalently to the HA. It has a HA's retention capacity, which is considered as an essential step in the ECM stabilization (Carrette *et al.* 2001).

HA, which represents the backbone of this matrix, binds with high affinity the TNFAIP6's module domain (Kahmann *et al.* 2000). Similar domains that recognize HA were reported also in other extracellular matrix proteins mainly versican (CSPG2) and CD44. The interaction of HA with its ubiquitous receptor CD44 is involved in mediating a wide-range of biological functions notably its anchoring to the surface of cells, and therefore the whole ECM. This interaction is modulated by TNFAIP6 (Knudson *et al.* 1996, Lesley *et al.* 2004). In addition to the IαI, the matrix stabilization is also promoted by an immune-cell related gene: pentraxin 3 (PTX3). It is a multifunctional protein playing an essential role in the ECM assembly by possible covalent link with TNFAIP6 (Bottazzi *et al.* 2006). CCs mucification is also under the oocyte governance through the oocyte-secreted factors mainly the GDF9 and BMP15 (Su *et al.* 2004, Richards 2007, Diaz *et al.* 2008). In fact, these TGFβ factors are crucial for successful ovulation by triggering the expression of hyaluronan synthase 2 (HAS2), pentraxin 3 (PTX3) and tumor necrosis factor–induced factor 6 (TNFAIP6), required for the formation and the stabilization of the COC matrix (Figure 1.5) (Elvin *et al.* 1999).

Laminin, type IV collagen and their actin-linked membrane receptors (integrin α-6 and β-1) were also overexpressed during cumulus mucification and involved in ECM structure (Sutovsky *et al.* 1995). Other proteases are also described as matrix-associated factor as ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin- like repeats 1). The ADAMTS1 binds to HA and can specifically cleave versican (CSPG2) (Russell *et al.* 2003). Cumulus expansion seems to be achieved and amplified through the EGF-like factors. Phenotypes of impaired ECM structure and ovulation failure were reported in each one of these three TNFAIP6-, PTX3- and ADAMTS1-null female mice ((Zhuo & Kimata 2001, Richards 2007, Edson *et al.* 2009) for reviews).

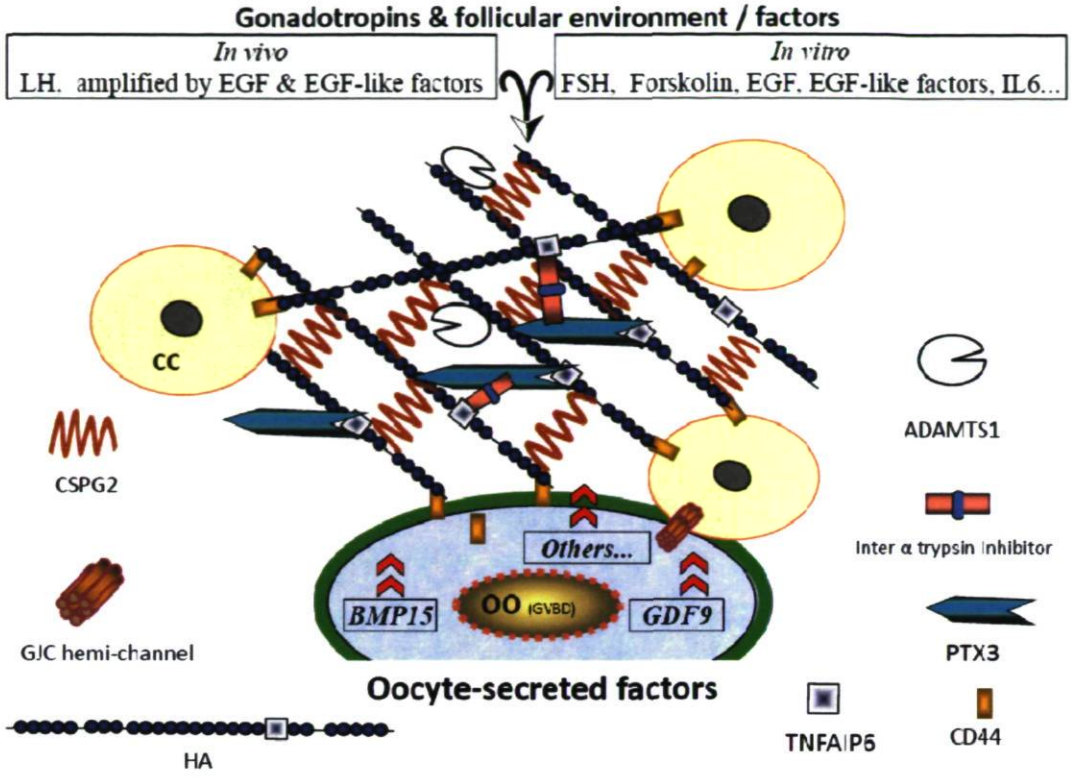

Figure 1.5: Schematic representation of the main factors involved in ECM structure following CCs expansion in vivo/vitro.

Using microinjection of fluorescent dyes and immunocytochemistry, Sutovsky and collaborators investigated the cytoskeletal components distribution during bovine CCs mucification. They reported that despite expansion, CCs remain joined via the same number of gap junctions (GJC) during the entire culture time. They also denoted that there is remaining GJC between CCs and the oocyte even after 24 h of culture (Sutovsky *et al.* 1993, Sutovsky *et al.* 1994). This decrease of GJC during *in vitro* culture was also reported by Gilchrist Group elsewhere (Thomas *et al.* 2004). Concerning the importance of these cell-cell junctions, previous report in our laboratory have highlighted the importance of this functional coupling during the bovine COC IVM in the oocyte developmental competence acquisition (Ali *et al.* 2005). Moreover, cumulus expansion is preceded by particular dynamics of the cytoskeleton concomitant with the extension and elongation of newly formed cytoplasmic projections with CCs. Therefore, CCs contacts within each other and with the oocyte are maintained despite distension. That's why, the incubation with

cytochalasin B, a microfilament disruptor, inhibited these CCs cytoplasmic extensions as well as subsequent cumulus expansion. Actin filament (F-actin) is therefore a mediator of the gonadotropins-induced CCs mucification (Sutovsky *et al.* 1995).

We should highlight that despite worthy information available about CCs expansion, additional yet unknown factors could be also involved in this process.

1.2.4. Zonae pellucidae

Although the focus of this section is CCs, I have chosen to add a brief description of ZP and its main functions here mainly because of its strategic position between CCs and oocyte. Therefore, it is thought to be involved in some way in the CCs-oocyte interplay.

1.2.4.1. Ultrastructure

Since the secondary follicle stage, the mammalian oocyte starts active growth with the accumulation of discontinuous patches of fine fibres in the perivitelline space leading the ZP formation. This ZP deposit is considered as a sign of the oocyte initiation growth in most mammals (Wassarman & Albertini 1994). However, other authors reported the existence ZP proteins in primordial follicles of some species including rabbit, monkey and human (Grootenhuis *et al.* 1996).

ZP is a filamentous matrix secreted around the oocyte and composed of well-structured and highly- glycosylated glycoproteins. This glycosylation gradient decreases from the periphery to the inner, more compact and less porous region (Jimenez-Movilla *et al.* 2004, Lunn & Wright 2006). Most mammalian ZP includes mainly three proteins encoded by three different genes ZP1, ZP2 and ZP3 (Wassarman 1988). An additional protein expressed by a ZP1-paralogue gene was recently reported in the human ZP, the ZP4 (Hughes & Barratt 1999, Lefièvre *et al.* 2004). In most mammals, ZP exhibits layers of long filaments with repetitive protein heterodimers ZP2-ZP3 cross-linked by ZP1 homodimers (Green 1997, Wassarman & Litscher 2008). The distribution of ZP1 binding sites on ZP2–ZP3 dimers as well as their frequency is not fully established.

ZP genes transcription and their glycoprotein synthesis are reported in both the oocyte and CCs of most mammals (Gook *et al.* 2008). The thickness of this coat increases with the oocyte maturation progression to reach around 17 µm at the human MII stage. The oocyte ZP proteins production decreases slightly until ovulation where the ZP diameter remains relatively stable to the first steps of cleavage (Goyanes *et al.* 1990, Gook *et al.* 2008). Concomitant with embryo

cleavage progression, ZP thickness decreases due to blastomeres expansion which makes it harder until the hatching stage (Montag *et al.* 2000a). For ZP involvement in blastocyst hatching process, it is still a debate. While some authors suggested that the hatching process is a consequence of cell number/water volume increase and therefore a mechanical intrinsic pressure, others suggested the action of uterine lytic enzymes (Orsini & McLaren 1967, Montag *et al.* 2000a). *In vitro*-observed hatching in some mammals blastocysts as mouse, cow, pig and human raised other doubts of trophectodermal lytic actions (Perona & Wassarman 1986).

Prior to ovulation and thereafter, ZP microfilament networks arrangement becomes more fibrous (filamentous structure) (Familiari *et al.* 2006a).The molecular variation of ZP composition and structure since the preovulatory stage and thereafter needs additional works (Gook *et al.* 2008). In human and mice, ZP3 is considered as the putative sperm receptor glycoprotein and the inducer of the acrosome reaction (Brewis *et al.* 1996, Familiari *et al.* 2006, Ni *et al.* 2007, Wassarman & Litscher 2008). Moreover, $ZP3^{-/-}$ mouse fail to assemble their ZP and are infertile. Sperm recognition and acrosome reaction are also affected (Wassarman & Litscher 2008). Interestingly, some studies have reported positive correlation between ZP Thickness and uniformity, with the human oocyte developmental potential (Chan 1987, Montag *et al.* 2008). That's why many human IVF clinics used the ZP thickness as a morphological criterion for oocyte and early embryo selection (Montag *et al.* 2008, Ebner *et al.* 2009, Madaschi *et al.* 2009).

It is to note that despite interspecies similarities in ZP structure, there seems to be enough diversity and species' particularities to avoid cross-species fertilization (Familiari *et al.* 2006). Interestingly, the mammalian ZP is traversed by the cytoplasmic foots or extensions of the corona radiata cells which interact with the oocyte cytoplasm (Hunter 2003) (Figure 1.6). These trans-zonal projections (TZP) raise several questions concerning the communication possibilities between the two cell sub-types.

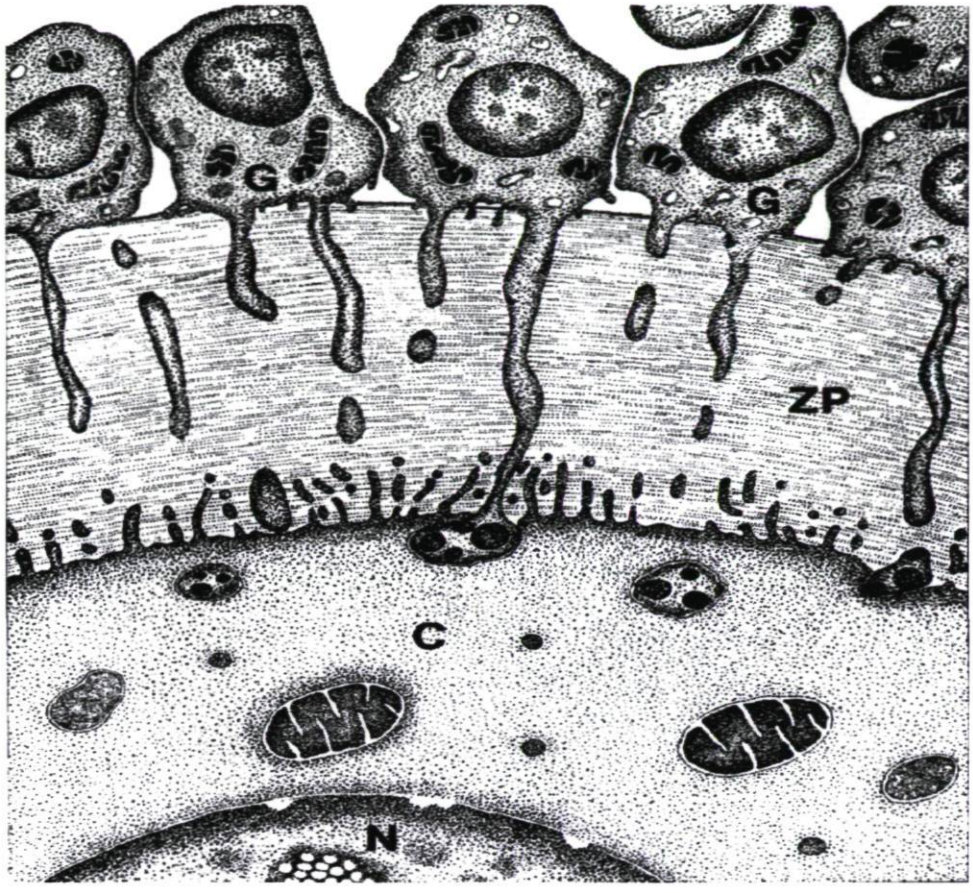

Figure 1.6: Transzonal cytoplasmic extensions of CCs that traverse the zona pellucida to interact with the oocyte. G= corona radiata cells; ZP= zona pellucida; C= oolema; N= oocyte nucleus (Hunter 2003).

1.2.4.2.Main established roles

The ZP accomplishes many crucial functions. These functions include:

- Providing a permeable but selective vestment to the oocyte that encloses the transzonal cytoplasmic protrusions (Hunter 2003). This corona radiata-oocyte communication is essential in the maturation progression and final oocyte developmental competence.
- Driving the fertilization events by sperm recognition, acrosome reaction and reacting to cortical granule exocytosis (Wassarman 2005, Ni *et al.* 2007, Wassarman & Litscher 2008).
- Maintaining the species-specifity sperm binding. This function prevents cross-species fertilization (Wassarman & Albertini 1994). ZP seems to contain specific sperm receptors

that prevent the recognizing of a sperm of different species (Epifano & Dean 1994, Wassarman 2005).

- Polyspermy prevention by the inhibition of the binding of additional spermatozoa to eggs. This processes is induced by ZP3 (Wassarman & Litscher 2008). More compactions of ZP inner layer glycoproteins by the increase of ZP1 cross-links prevent polyspermy (Sun *et al.* 2005). This physiological barrier is reinforced by further activation of the cortical granules release and ZP2 cleavage (Wassarman & Albertini 1994).
- Maintaining the initial early embryo development microenvironment in the perivitelline space. ZP is involved also in the early embryo exchanges with the oviduct fluids (Hunter 1994).
- Physical, chemical and immunological protection of the early blastomeres. ZP conferred a degree of cohesion to the blastomeres during the early embryo development where they still lack cell junctions (Edwards 1964, Kamo *et al.* 2004, Ueno *et al.* 2007).

Further investigations of the ZP selective permeability would be helpful to find additional potential roles mainly in signal transduction between the oocyte and its environment.

1.2.5. Communications within the cumulus-oocyte complex (COC)

Cell-cell communications between adjacent cells is an important way whereby key physiological processes in live tissues are regulated. Many types of junctions and communications gateways are found between the plasma membranes of CCs and oocyte, mainly the gap junctions (GJC). GJC are recognized as specialized junctions involved in small molecules exchange in a multicellular context. These structures increased in number and size in the rat preovulatory stages following FSH and/or estrogen actions (Burghardt & Matheson 1982). This intercellular coupling seems to be involved in the transmission of the FSH signal transduction of GCs cultured *in vitro*. In fact, LHR expression by GCs was reported to be induced within aggregated cells with presumptive intact and functional GJC (Amsterdam *et al.* 1981). The inhibition of GJC in bovine COCs during maturation decreases oocyte developmental competence with no blastocyst after IVF (Ali *et al.* 2005).

GJC intercellular channels are dimmers of connexons. Each connexon is an oligomer of six connexins. Their formation starts with the passage of connexins through the Golgi apparatus, their oligomerization into hexameric connexons and their insertion into the plasmic membrane.

These hemichannels are kept closed until their attachement to their complementary counterparts in the other side of the adjacent cell membrane. GJC are reported to cluster in particle-poor regions of the cell membrane called plaques (Szollosi 1978, Musil & Goodenough 1993). Connexin is the elementary unit of GJC channels that have a transmembrane domain and the ability to associate *in vitro* and form functional channels. Many connexin isoforms are reported to join together in diverse combinations, affecting the channel permeability and properties. Homotypic channel is composed of the association of the same connexin molecule in both hemichannels of each cell, even the connexon (hemichannel) are heteromeric (reviewed by (Bruzzone et al. 1996, Kumar & Gilula 1996)) (Figure 1.7). Although, it appears that theoretically many types of GJC could be formed, a selective association of connexins particular to each tissue was reported (Mese et al. 2007). This cell-type connexon association is thought to be related to functional specialization and particular conductive properties. For example, GJC are found at all the follicular stages in mammals (Anderson & Albertini 1976). They are believed to be involved in the synchronization of cytoplasmic and nuclear maturation of the oocyte (Carabatsos et al. 2000, Vozzi et al. 2001). The main connexins explored and reported in the follicle are Cx43 (GJA1), Cx 37 (GJA4) and Cx32 (GJB1) (Sutovsky et al. 1993, Simon et al. 1997, Juneja et al. 1999). GJA4-null mice are unable to overcome the preantral stage (Simon et al. 1997). Consequently, they were anovulatory and therefore infertile. Moreover, the oocyte is even unable to attain its meiotic competency (Carabatsos et al. 2000). Worse yet, the follicular development using an organ culture technique of GJA1 null-mice blocks at the primary follicle stage (Juneja et al. 1999).

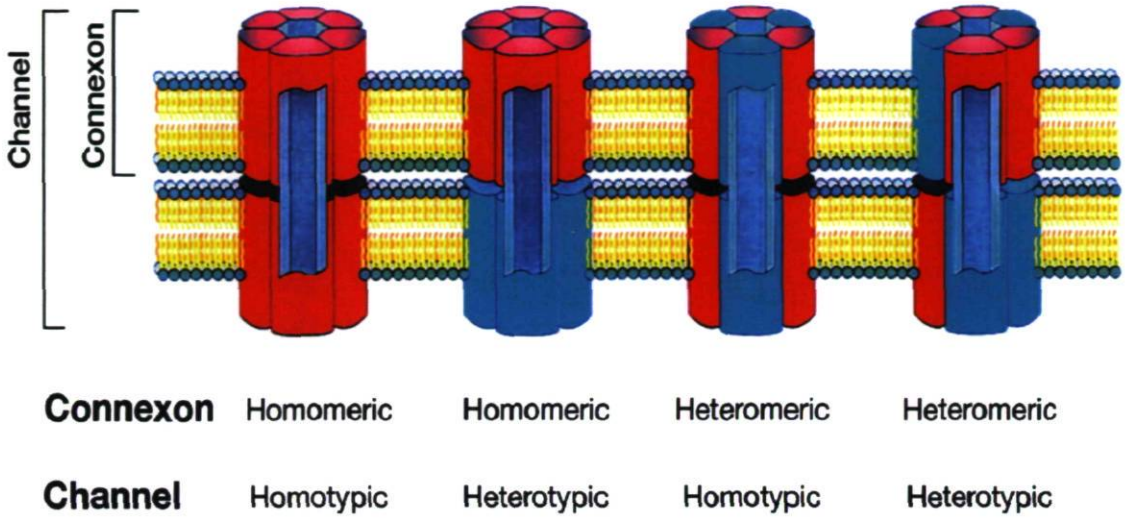

Figure 1.7: Potential arrangements of connexons within gap junctions: connexon hexamers (hemichannel) could be homomeric (identical connexion isoforms) or heteromeric. The extracellular association between correspondent connexins of the same connexon leads to homotypic (the association between the same connexins) or heterotypic (Kumar & Gilula 1996).

Concerning communications between cumulus and oocyte, Sutovsky has shown that cumulus-oocyte GJC are rich in GJA1. While GJA1-based GJC were ruptured or obstructed after 6h of culture in the bovine (at GVBD), interestingly the oocyte starts expressing GJB1 (Sutovsky *et al.* 1993). The same team using microinjection of fluorescent dyes and immunohistochemistry documented a slight and constant decrease in GJC number during 24h of culture despite the cumulus expansion process. GJC diameter could also be reduced due to long term incubation (Sutovsky *et al.* 1993, Sutovsky *et al.* 1994). Recently, GJA1 upregulation in the porcine CCs *in vitro* was transcriptionally upregulated, but its downregulation look to be due to its gonadotropins-dependent clustering in the lipid rafts around the GVBD (Sasseville *et al.* 2009b). Interestingly in the same study, the GJA1 increased activity since the beginning of culture was shown to be gonadotropins-independent, rising additional questions concerning the intricate regulation of CCs-oocyte exchanges. Therefore, measuring just one type connexin at one time point becomes not sufficient to assess GJC communication.

For GJC transfer potential, they allow direct passage of small molecules less than 1 KDa of size. It includes ions (Ca^{2+}, K^{+}), second messengers (cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), inositol 1,4,5-triphosphate (IP3)), nucleotides, amino acids, metabolites (puryvate, glucose) and small peptides. They are reported to be involved in many

signalling pathways and cellular functions like tissue-homeostasis maintenance, electrical (action potential) and biochemical coupling between adjacent cells (Schwarzmann *et al.* 1981, Wei *et al.* 2004, Mese *et al.* 2007). The under-1000-Da molecule passage is considered as the basic and not the selective permeability. The number of genes coding for connexins has increased the GJC potential sub-types (great diversity) and therefore their permeabilities and regulation (Kumar & Gilula 1996). Specific, selective and higher molecular molecules transfer through GJC could be therefore envisioned. In 2002, the hypothesis of possible effects of diverse connexins combinations per connexon on selective permeability of GJC was confirmed. Interestingly, the permeability of GJA1-based GJC was more permissive to high molecular weight and charged molecules than the GJB1-based one (Goldberg *et al.* 1999, Goldberg *et al.* 2002, Goldberg *et al.* 2004). Moreover, the GJA1 connexin increased in the cattle oocyte following the LH surge (Sutovsky *et al.* 1993). Moreover, LH was reported to phosphorylate GJB1 in the rat ovary which probably promote the specific closure of these junctions (Granot & Dekel 1997). In contrast, the FSH induced GJB1 overexpression in rat granulosa cell lines (Sommersberg *et al.* 2000). Intact CCs communication (i.e. functional GJC) was also reported to be crucial in the improvement of oocyte competence *in vitro*. CCs co-culture with denuded oocytes didn't restore their developmental potential (Luciano *et al.* 2005).

Recently, GJC were reported to allow the passage of small interfering RNA (siRNA) up to 24 oligonucleotides. This permeability was specific to the GJB1 (Valiunas *et al.* 2005). Other exchange alternatives between CCs and oocyte are therefore possible. Taken together, these findings highlighted the need of additional studies to identify the different connexins expressed both in CCs and oocyte during folliculogenesis, the possible combinations between them and their effects on the CCs-oocyte communications at each stage of follicle development. A careful interpretation of the connexins' patterns of expression is required for better assessment of the communication flow between CCs and the oocyte.

1.2.6. Additional potential means of the oocyte-cumulus cells dialog?

Despite recent developments in ovarian physiology and regulation, the real exchanges between the inner layer of CCs, the corona radiata, and the oocyte remain an enigma. Based on recent reports about the follicle physiology and gene expression, additional intercellular communications may occur.

1.2.6.1. Are the cytoskeleton and molecular motors involved in cumulus-oocyte interplay?

Optimal cumulus expansion is required for the oocyte final maturation, ovulation and fertilization (Tanghe *et al.* 2002, Yokoo & Sato 2004). This process remains not completely understood. While some studies considered CCs mucification as volumetric enlargement of the extracellular space and the rupture of cell-to-cell contact leading to oocyte meiosis resumption (Larsen *et al.* 1986, Eppig 1991), increasing arguments are supporting the maintain of CCs contacts within each other and with oocyte via GJC (even their number start decreasing since the GVBD) during the entire culture time (Sutovsky *et al.* 1993, Thomas *et al.* 2004).

In order to better understand the CCs behavior during the gonadotropins-induced expansion, it is crucial to know the main intracellular modifications occurring in this somatic compartment and their possible involvement in the cell-cell interactions. In this context, it was shown that the globular CCs became polarized with rapid development of their Golgi apparatus and the accumulation of lipid droplets following 6h of bovine IVM (GVBD)(Sutovsky *et al.* 1994). The same authors have described interesting cytoskeletal arrangements of CCs marked by the assembly of actin microfilaments, leading to the formation of subsequent cytoplasmic projections. These ultrastructural phenotypes as well as the cumulus expansion process were prevented by the cytochalasin B, a specific inhibitor of microfilaments assembly. Microtubules and intermediate filaments were mainly located in a radial position and were not detected in the cytoplasmic projections across the ZP (Sutovsky *et al.* 1993, Sutovsky *et al.* 1994). Although the Albertini's group reported similar observations in bovine CCs (polarization and expansion) following gonadotropins stimulation, they showed a progressive decrease in the microfilaments but an important rise in microtubules in the transzonal cytoplasmic projections close to the oocyte (Allworth & Albertini 1993). This study supports a continuous relationship between CCs microtubules and the oocyte's membrane. Therefore, CCs elongation and depolarization might be crucial to maintain intercellular communications and molecules' exchange in the COC despite the intensive mucification. In fact, cytoskeleton plays critical roles in cell structure and development in higher eukaryotes. Organelles and molecules' transport along microtubules and microfilaments to their cell-specific compartment maintains cell polarity. Both microtubules and microfilaments were reported to contribute in the molecular intracellular transport responsible of the asymmetric distribution of organelles and/or molecules within the cell. Kinesin and dynein are molecular motors that use microtubules as a track to transport molecules to specific destination. First

described in the nervous system, kinesin moves the cargo molecule/vesicle along microtubules to the cell periphery (the microtubule positive end), and dynein achieves the transport towards the microtubule minus end close to the nucleus. These motors were reported to contribute in many processes as mRNA transport, nuclear envelope breakdown and organelles' transport (Goldstein & Yang 2000, Hays & Karess 2000, Salina *et al.* 2002, Bullock *et al.* 2003). Similar processes were suggested in the mammalian oocyte (Albertini 1992). Similarly, the microfilament molecular motors include mainly myosins that hold the cargo to the plus end of actin filaments. They are involved in endocytosis, and RNA and organelles transport (Bhaskar *et al.* 2007).

Taken together, the CCs cytoskeleton of polarized CCs could be the site of action of several molecular motors involved in the molecular transport and distribution of key factors in time and space coordinated way, allowing quick and efficient physiologic responses. Such processes could be involved in the prerequisite CCs support to maturation, ECM formation and stabilization, as well as the maintenance of the TZP extensions and depolarization.

1.2.6.2. What about CCs exocytosis, endocytosis, phagocytosis and synapses?

The Golgi apparatus produced secretory granules in many cellular types. These vesicles are conducted to the cell periphery via the microtubule motors before been delivered by exocytosis (Rudolf *et al.* 2001). An exocytosis process of cortical granules was reported in the oocyte (Leguia *et al.* 2006). Additionally, the SNAP25-dependent exocytosis was also reported in mouse GCs following the LH peak (Shimada *et al.* 2007). Thus, together the events of the cytoskeleton rearrangements, the Golgi development and lipid-vesicle accumulation described above in CCs provide the needed ingredients for possible exocytosis process (Figure 1.8). The vesicles content may diffuse, bind to a specific receptor or a specific structures such as lipid rafts found recently in mouse oocytes and porcine CCs membranes where they play functional roles (Comiskey & Warner 2007, Sasseville *et al.* 2009b). These rafts structures are cell membrane microdomains made of structured association of glycosphingolipids, cholesterol and protein receptors. They are organizing scaffold for signal transduction molecules and receptor trafficking, characterized by their tight and movable properties (Simons & Toomre 2000).

Interestingly, the EGF /MAPK pathway is also reported to interact with the actin-microfilaments to transport vesicles from the cell surface to the cytoplasm where internalized particles are processed (Pol et al. 2000). The EGF pathway was also essential in the oocyte maturation, CCs expansion, and ovulation (Conti *et al.* 2006, Hsieh *et al.* 2007, Conti 2010). Given the importance

of the EGF pathway in CCs, their microfilaments arrangements as well as the cytoplasmic extensions (Sutovsky *et al.* 1995, Park *et al.* 2004, Panigone *et al.* 2008), EGF-dependent vesicle endocytosis could be envisioned within CCs or with the oocyte.

Similar interactions of the myosin family motors with the actin filaments were reported in phagocytosis. The phagosomes' transport in mouse may involve both microfilaments and/or microtubules molecular motors (Toyohara & Inaba 1989). Moreover, autophagy, which is a conserved degradation process of proteins and organelles involved in tissue homeostasis, acted concomitantly with apoptosis in removing atretic oocytes in immature rats (Escobar *et al.* 2008). Increased chances of phagocytosis are furthermore supported by the immune-like genes reported in CCs prior to ovulation (Hernandez-Gonzalez *et al.* 2006, Shimada *et al.* 2006a). Besides their involvement in the ovulation process, these immune factors in CCs may be involved in a vesicle phagocytosis role to be determined (Figure 1.8).

Several neuronal-like genes were also differentially expressed in mouse CCs prior to ovulation (Hernandez-Gonzalez *et al.* 2006, Richards 2007). It was also shown that synaptic vesicles used both microtubules and actin filaments during their axonal transport (Bhaskar *et al.* 2007). Moreover, some synaptic proteins, as synaptosomal-associated protein of 25 kDa (SNAP25; 25 KDa), were reported in the preovulatory and luteal GCs, CCs as well as the oocyte of mouse, rat and human species. SNAP25, which is a crucial element of the molecular machinery required for the neurotransmitter exocytosis and synapses, was transcriptionnally up-regulated by FSH and estradiol. These SNAP25 transcripts levels remained high following the LH action (Grosse *et al.* 2000, Shimada *et al.* 2007). Possibility of CCs-oocyte exchanges by a synapse-like process could be therefore assumed.

Based on these studies, some molecular events associated to exocytosis, synaptic transmission, endocytosis and/or phagocytosis could occur within CCs or with the oocyte before and following the LH surge (Figure 1.8). Assuming the occurrence of these processes, future CCs-oocyte dialog studies should focus on targeting secreted/released factors that carry the CCs induction of oocyte competence. The identification of this/these precious factors would revolutionize both the *in vitro* system and ovarian stimulation protocols already used for both human and livestock species.

1.2.6.3. Could CCs communicate via membrane nanotubes?

Membrane nanotubes are cell-cell long distance connections through membrane protrusions. They are involved in vesicle and signal traducers trafficking between joined animal cells. These

membrane tunnels were reported in many tissues mainly the neuronal and immune cells (Watkins & Salter 2005, Gerdes *et al.* 2007). These nanotubes could be formed by actin-filaments extensions or by cell-cell contacts followed by their draw out (Rustom *et al.* 2004, Pontes *et al.* 2008). Many functions were attributed to these structures in various cell types as intercellular cargo transport, electrostatic coupling, cell-surface protein transfer, endocytosis and signal transduction (Davis & Sowinski 2008, Gurke *et al.* 2008). A relationship was recently made between the exosome, the nanotubes and the RNA and DNA transport (Valadi *et al.* 2007, Belting & Wittrup 2008). This physiological transfer of nucleic acids between eukaryotic cells has opened a challenging field in physiological cell regulation to explore. The cytoplasmic projections of the corona radiata cells that cross the ZP and remain nearby the oocyte (Figure 1.6) rising therefore many questions concerning possible involvement of nanotubes connections in their particles transfer process (Figure 1.8).

Interestingly, the CCs polarization and their TZP look to assign them a neuronal- and/or immune-like morphology. Moreover, the CCs-gene expression induced by LH prior to ovulation have revealed an important number of neuronal, and immune cell-related genes (Hernandez-Gonzalez *et al.* 2006, Shimada *et al.* 2006a, Richards 2007, Koks *et al.* 2010). Taken together, these findings reinfore the possible occurrence of the CCs-oocyte communication processes suggested before and summarized in figure 1.8. Further studies are therefore needed to investigate these hypotheses.

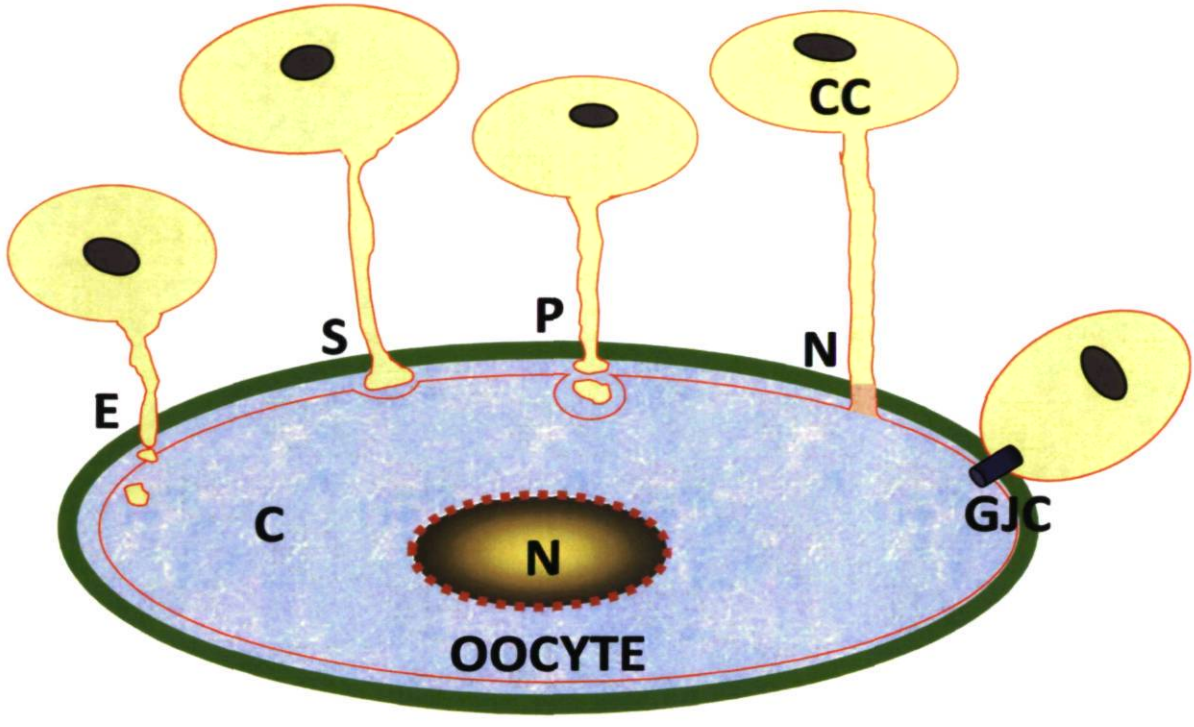

Figure 1.8: Schematic drawing of the potential means/ways of CCs-oocyte communication. CC= cumulus cell; C= cytoplasm, N= nucleus; GJC= gap junction; N: nanotubes;
P= phagocytosis; S=synapse; E= exocytosis

1.2.7. Cumulus cells metabolism

Being an interface between the oocyte and its environment, CCs are the site of well-orchestrated sequences that ensured the accomplishment of oocyte competence. The main visible transformation of CCs is the secretion and the arrangement of the ECM. This aforementioned process of mucification is the fruit of many coordinate cascades of gene expression, protein synthesis and macromolecules' deposition in the intercellular space (Zhuo & Kimata 2001, Hunter 2003, Feuerstein *et al.* 2007, Memili *et al.* 2007). These CCs activities are influenced by the oocyte and are required for its maturation and competence acquisition (Eppig 2001). Several already established metabolic processes take place in CCs. In this context, glycolysis is a main

metabolic CCs function that is shown to be promoted by the oocyte (Sugiura *et al.* 2007). Six key enzymes involved in the glycolysis pathway, including enolase (Eno1), pyruvate kinase (Pkm2) and Lactate Dehydrogenase (Ldh1) were reported as differentially expressed in mice CCs compared to MGCs. This glycolytic pathway in CCs is deeply affected by the oocyte removal (Sugiura *et al.* 2005). Moreover, the CCs glycolysis was reported as crucial nutritional need for the oocyte, known to metabolize glucose poorly. The CCs process therefore glucose to pyruvate (both *in vivo* & *in vitro*), which provides a glycolytic support for the oocyte during its growth and meiosis resumption in mice, porcine and bovine species (Biggers *et al.* 1967, Eppig 1976, Leese & Barton 1985, Mayes *et al.* 2007, Thompson *et al.* 2007).

Amino acids are also required for the oocyte to pursue its maturation. In fact, the oocyte is unable to use some amino acids even available in the culture media. This was probably due to poor transport capacity through its membrane (Colonna *et al.* 1983, Sugiura *et al.* 2005). Additionally, specific amino acid transporters were differentially overexpressed in CCs of antral follicles (Eppig *et al.* 2005). Theses specific transporters allowed the uptake of selected amino acids as L-alanine and L-histidine by CCs and their subsequent transfer to the oocyte through GJC (Colonna *et al.* 1983, Haghighat & Van Winkle 1990).

Cholesterol production is another metabolic process reported in CCs. It is reduced in denuded oocytes due to limitation in cholesterol biosynthesis (Su *et al.* 2008). Assuming reduced cholesterol concentrations in FF (Perret *et al.* 1985), CCs may biosynthesize cholesterol and transfer it to the oocyte through the raft structures. This metabolic activity is driven by oocyte through the FGF factors mainly BMP15 and GDF9 (Su *et al.* 2008). The oocyte cholesterol stored in the rafts was recently shown to be essential not only in maturation but during the early embryo development stages (Comiskey & Warner 2007).

Steroidogenesis is another metabolic function ensured by CCs. Steroid hormones mainly progesterone and estradiol were reported to be produced *in vitro* by bovine CCs (Mingoti *et al.* 2002). These steroids are beneficial to the porcine and bovine oocyte maturation *in vitro* (Ali & Sirard 2002a, Li *et al.* 2008) as well as on human oocyte competence (Tesarik & Mendoza 1995, Loutradis *et al.* 2008). Moreover, the oocyte looks to prevent the CCs luteinization, promoting therefore their steroids production (el-Fouly *et al.* 1970, Vanderhyden *et al.* 1993, Li *et al.* 2000, Lucidi *et al.* 2003). Interestingly, the association between successful oocyte maturation and the overexpression of many steroidogenesis-related genes in their CCs reported in microarray studies

(Wise *et al.* 1994, Kawashima *et al.* 2008) are a further confirmation of the importance of this metabolic process.

1.2.8. Main signalling pathways in cumulus cells

In order to support the oocyte maturation and fertilization, the CCs are the site of action of several signalling pathways. PKA was the first identified kinase downstream the gonadotropins stimulation in mammalian CCs. It is involved in ECM formation and stabilization as well as oocyte meiotic maturation (Bornslaeger *et al.* 1986, Downs & Hunzicker-Dunn 1995, Ali & Sirard 2005, Yamashita *et al.* 2009). It is a linear pathway (GPCR (G-protein coupled receptor)/AC/cAMP/PKA) which induces the phosphorylation of some key factors as p38MAPK, ERK1/2 and CREB (cAMP-regulatory element binding protein)) in follicular somatic cells (Richards 2001, Ning *et al.* 2008). Additionally, the catalytic subunits of PKA could also translocate to the CCs' nucleus and activate some transcription factors leading to the expression of several key genes including HAS2, TNFAIP6, CYP19A1 and EGF-like factors (Tirone *et al.* 1997, Gonzalez-Robayna *et al.* 1999, Ochsner *et al.* 2003a, Ning *et al.* 2008, Edson *et al.* 2009, Yamashita *et al.* 2009, Zhang *et al.* 2009). Cyclic AMP (cAMP) has an established role in the oocyte meiosis resumption (reviewed in (Conti 2002b, Zhang *et al.* 2009)). It can enter from CCs to the oocyte via gap junctions and blocks the spontaneous meiosis resumption (Webb *et al.* 2002). The phosphodiesterase 3A (PDE3A) inhibition in the oocyte was reported also to support the high endogenous levels of cAMP (Conti *et al.* 2002a). Following the gonadotropins surge, the meiosis resumption was associated to a dramatic decrease of the cAMP due to the disruption of gap junctions (Thomas *et al.* 2004, Sela-Abramovich *et al.* 2005). The induction (end of inhibition) of phosphodiesterases (PDE3A in oocyte and/or PDE4B, PDE8 in CCs) activity (Thomas *et al.* 2002, Conti *et al.* 2002a, Conti 2002b, Sasseville *et al.* 2009a, Zhang *et al.* 2009), and/or the blockade of cGMP entry (Norris *et al.* 2009, Vaccari *et al.* 2009) were also involved in this process.

Cyclic AMP was also suggested to act in a PKA-independent manner to phosphorylate PKB/Akt via the phosphatidylinositol-dependent kinase (PI3K) in rat granulosa (Gonzalez-Robayna *et al.* 2000). The phosphorylation of PKB/Akt in mouse CCs promoted the oocyte developmental potential *in vitro* (Zhang *et al.* 2010). This PKB action in the oocyte was shown to occur via the cyclin-dependent kinase 1 (CDK1) and PDE activation, and/or the PKA (via the

dephosphorylation of CDK1 by the CDC25 phosphatase) which leads to the maturation promoting factor (MPF= heterodimer of CDK1 and cyclin B1)) activation (reviewed by (Zhang *et al.* 2009, Tripathi *et al.* 2010)). Moreover, the gonadotropins stimulation of the PI3K/PKB pathway was shown to prevent apoptosis and induce progesterone production in porcine CCs (Shimada *et al.* 2003, Shimada *et al.* 2003a).

PKC and MAPK pathways are also involved in the CCs signalling pathways of oocyte maturation in many species (Su *et al.* 1999, Shimada *et al.* 2001, Fan *et al.* 2004, Sela-Abramovich *et al.* 2005). The addition of phorbol 12-myristate13-acetate (PMA), a PKC activator, to the culture media activates the MAPK in CCs (Fan *et al.* 2004), induces gene expression cascades (including the EGF-like factors) and enhances therefore the oocyte competence *in vitro*. The inhibition of this pathway reverses these effects (Fan *et al.* 2004, Ali & Sirard 2005, Assidi *et al.* 2008). The PKC pathway PLC/PIP2/DAG/PKC is suggested to act in CCs upstream the MAPK cascades which are necessary for the gonadotropin-induced meiotic resumption before the GVBD, and involved in microtubule organization and meiotic spindle assembly thereafter (Fan *et al.* 2004, Fan & Sun 2004, Fan *et al.* 2009). The EGF-like factors are driving the PKC induction of oocyte maturation (mainly through MAPK) in mice (Downs & Chen 2008) and porcine (Chen *et al.* 2008, Li *et al.* 2008a) species (reviewed in (Mehlmann 2005)). The MAPK induction of meiotic resumption in oocyte was suggested to be mainly through MOS/MEK/ERK1/2 in mammalian species (Liang *et al.* 2007).

Other oocyte-secreted factors, mainly the TGFβ family, are crucial in the CCs differentiation, support of oocyte maturation and CCs expansion (Pangas *et al.* 2004, Su *et al.* 2004, Pangas & Matzuk 2005, Hussein *et al.* 2006). These TGFβ effects on CCs occurred mainly but non-exclusively through Smad 2/3 pathway (Drummond 2005, Dragovic *et al.* 2007, Diaz *et al.* 2007a, Edson *et al.* 2009).

Keeping in mind all the aforesaid signaling pathways, it clearly appears that CCs responds to gonadotropins, oocyte and other intrafollicular factors (e.g. EGF-like factors) stimulations by the activation of several signalling pathways. Despite their complexity, these signalling cascades look to act harmoniously in order to support suitable oocyte maturation and subsequent fertilization.

1.2.9. Gene expression in cumulus cells

Gene expression is an important process triggered by some of the various signaling pathways described in CCs. The inhibition of the transcription in the cumulus-oocyte complex (COC) impaired both oocyte maturation and fertilization (Motlik *et al.* 1989, Tatemoto & Terada 1995). These findings suggested that CCs gene expression is essential to oocyte maturation and early development. Moreover, gene expression patterns in CCs were shown to be influenced by the oocyte-secreted paracrine factors mainly the TGF family (Eppig 2001, Sugiura *et al.* 2007, Gilchrist *et al.* 2008, Su *et al.* 2008). The CCs gene expression profiles were studied in many mammalian species including mice (Hernandez-Gonzalez *et al.* 2006), pig (Kawashima *et al.* 2008), cow (Assidi *et al.* 2008, Bettegowda *et al.* 2008, Tesfaye *et al.* 2009) and human (McKenzie *et al.* 2004, Zhang *et al.* 2005, Assou *et al.* 2008). The analysis of these genome-wide studies (achieved at different contexts *in vivo* versus *in vitro*; and at different time points GV, GVBD or the ovulatory stage) has provided interesting molecular functions occurring in CCs (Assou *et al.* 2006, Feuerstein *et al.* 2007, Russell & Robker 2007, Assidi *et al.* 2008). Despite the valuable contributions of these excellent studies, the CCs molecular dynamics and the precise molecular pathways needed to support oocyte competence are still poorly understood. In fact, gene expression patterns vary by switching from one follicular stage to the other, or between mammalian species. This may affect most of the CCs signalling and metabolic cascades and consequently their functions. Comparative biology studies are a prequisite to enlighten on the species similarities and differences in terms of follicular development and oocyte molecular maturation pathways. Animal models are available to understand human reproduction studies where many restrictions disallow the availability of tissues at different timepoints. In order to rebuild the whole signalling and gene expression events that occurred in CCs and efficiently optimize our *in vitro* culture systems, additional studies that focus on time- and space-dependent molecular events are required. Retrospective analysis that links the signalling pathways together and the gene expressed to their inducer and/or target will be helpful in our understanding of the molecular communication enigma between oocyte and its environment particularly CCs.

1.2.10. Cumulus cells contributions

1.2.10.1. In vitro & in vivo oocyte maturation

While the oocyte-secreted TGFβ factors were essential to the CCs expansion, oocytes failed to fulfill their growth and to reach competence when their exchanges with CCs are disrupted (by denuding oocytes or just blocking the junctions) (Matzuk *et al.* 2002, Sutton *et al.* 2003, Ali *et al.* 2005). During this process *in vivo*, the oocyte resumes meiosis following the LH surge and then arrests again in MII (Dieleman *et al.* 1983). Appropriate nuclear and cytoplasmic maturations are necessary for the oocyte developmental competence (Krisher 2004, Sirard *et al.* 2006). While the process of meiosis resumption looks complex and remains no fully understood, it was reported that CCs secreted a gonadotropin-dependent signal that promote meiosis resumption involving the phosphatidylinositol 3-kinase (PI 3-kinase) and MAPK pathways, as well as GJC (Byskov *et al.* 1997, Shimada & Terada 2001). The activation of the MAPK / ERK 1/2 pathway in CCs is necessary to the generation of a paracrine meiosis-inducing signal and CCs expansion (Su *et al.* 2003). Other authors suggested a capital role of cAMP high concentrations in maintaining the meiosis blockage. The cAMP degradation could be induced by phosphodiesterases (PDE) or gonadotropins-dependent pathways including the EGF-like factors (reviewed by (Richard 2007)). In fact, FSH was shown to increase of the cAMP concentrations within the oocyte (via GJC) (Webb *et al.* 2002) and to overexpress the EGF-like factors in CCs (Freimann *et al.* 2004, Park *et al.* 2004, Assidi *et al.* 2008). The GJC ensure continuous corona-radiata-oocyte exchanges during the whole maturation (Isobe & Terada 2001).

When removed from the follicle, the oocyte resumes meiosis spontaneously and passively. This is probably due to the absence of the follicular environment inhibitory effect as suggested by (Tanghe *et al.* 2002). Similar results were reported with denuded rabbit oocytes *in vitro* that resume meiosis even when cultured with alpha-amanitin (a transcription inhibitor) and/or cycloheximide (protein synthesis inhibitor)(Motlik *et al.* 1989) but are developmentally incompetent. Interestingly and in the same study, the oocytes were unable to resume meiosis when kept in the COC and cultured in the same inhibitory conditions. Taken together, these findings reveal the importance of the transcriptional and translational processes in the CCs to produce and/or release some key factors involved in the oocyte maturation. These factors could

be transferred thereafter to the oocyte via their cell-cell or other potential communications ways suggested before, triggering therefore key pathways of oocyte competence.

To achieve successful maturation, the oocyte needs to achieve properly both nuclear and cytoplasmic maturations. Since the oocyte nuclear status was more visible and easier to recognize, it has been extensively studied and used as a reference for the oocyte maturation progression (Su & Eppig 2002b, Luciano *et al.* 2005, Mehlmann 2005, Kalous *et al.* 2006, Richard 2007, Vaccari *et al.* 2009). It was suggested that the meiotic arrest serves mainly to the synchronization of cytoplasmic and nuclear maturation of the oocyte, as well as the estrus cycle events through the ovulation onset (Carabatsos *et al.* 2000, Vozzi *et al.* 2001). For the cytoplasmic maturation, it remains a complex process where few/no efficient criteria to assess. It requires the metabolic and nutritive support of CCs (Haghighat & Van Winkle 1990, Eppig *et al.* 2005, Su *et al.* 2008). CCs are involved in maintaining high levels of gluthatione (GSH) within the pig oocyte. GSH contributes in oxidative damages prevention, helps the male pronucleus decondensation and increased therefore the oocyte developmental competence (Yoshida 1993, Lim *et al.* 1996). CCs removal during bovine IVM, before and even 7h after IVF fertilization affected negatively fertilization as well as the blastocyst rates (Zhang *et al.* 1995). Similar conclusions were reported by Sasseville (Sasseville *et al.* 2009b) during porcine IVM. Since the nuclear progression looked normal, these effects were probably associated to an impaired cytoplasmic maturation or the lack of a fertilization promoting-factor produced by the CCs. Similar support of CCs to the cytoplasmic maturation have been reported in mouse oocytes (Yamazaki *et al.* 2001). Furthermore, other ultrastructural rearrangements related to the cytoplasmic maturation including mitochondrial polarity and cortical granules migration were shown to be induced by the CCs-produced nitric oxide (NO) in mouse, cattle and human oocyte (Van Blerkom *et al.* 2008, Matta *et al.* 2009). A recent report showed that mitochondria and lipid droplets distributions, GSH content and the intracellular calcium release are also improved by CCs in porcine IVM leading to higher developmental competence (Cui *et al.* 2009).

1.2.10.2. Contribution in the ovulation process

Ovulation is a complex mechanism that allows the COC ejection to the fallopian tube following the rupture of the follicle and the ovarian epithelium. It is a crucial step in the reproductive function that ensured two key roles: the release of the oocyte and the luteinisation of the remaining parts of the follicle. Whilst the former is essential to fertilization, the second is critical

for pregnancy maintenance (Hunter 2003). Following the LH surge, final and rapid changes occurred including FF volume increase, CCs mucification and ECM's water attraction. An augmentation of the antrum volume and therefore of the pressure within the Graafian follicle may occurred. However, this modest pressure was reported to be constant during ovulation but decrease dramatically after follicle rupture (Espey & Lipner 1963, Espey 1994). Following ECM expansion, the COC acquired viscoelastic properties that ease its release through the follicle rupture (Hunter 2003, Russell & Salustri 2006). Impaired ECM structure or the KO of some of its structural genes as TNFAIP6 and/or ADAMTS1 deeply affected the ovulation rates and therefore fertility (Hess *et al.* 1999, Fulop *et al.* 2003, Mittaz *et al.* 2004). The ovulation process involves different signals from the blood supply in the theca cells as well as steroidogenic and proteases factors produced within the follicle. These signals trigger CCs expression of many genes associated to an inflammatory-like response (Hernandez-Gonzalez *et al.* 2006, Russell & Robker 2007). These immune-related genes supported the CCs contribution in the ovulation and fertilization processes (Richards 2005). These findings suggested also that CCs and the ECM around ensure the protection of the oocyte in an inflammatory and proapoptotic environment during ovulation (Espey 1980, Espey 1994, Hunter 2003, Richards 2005).

Just after ovulation and rapid luteinization, an increase of progesterone production has been reported. Moreover, PGR induces the transcription of ADAMTS1 and Cathepsin L (Robker *et al.* 2000) which are key genes in the proteolytic events of follicle rupture. Prostaglandins (PGs) were also expressed in CCs and involved in the activation of the proteolytic process leading to the follicular wall rupture by the activation of collegenases (Robker *et al.* 2000, Hernandez-Gonzalez *et al.* 2006). The inhibition of this PGs local production in monkey follicles impaired ovulation but not luteinisation (Duffy & Stouffer 2002). Other cytokines and neuronal factors have been reported to be differentially expressed within human and mouse CCs, and therefore suggested as mediator of the ovulatory process (Machelon & Emilie 1997, Zhang *et al.* 2005, Feuerstein *et al.* 2007). Until few years ago, ovulation was considered as dependent only on blood flow, theca vascularisation and basal membrane disruption (Espey 1994). Recent gene expression analysis showed that CCs gained some immune and neuronal functions required during ovulation in many mammal species including human (McKenzie *et al.* 2004, Zhang *et al.* 2005, Feuerstein *et al.* 2007), mouse (Shimada *et al.* 2006a, Richards 2007), rat (Espey *et al.* 2000) and bovine (Assidi *et al.* 2008). Overall, these findings support a significant role of CCs in ovulation. Additional

work to elucidate the ovulation molecular pathways would yield supplementary alternatives to treat ovulatory defects both in livestock and IVF clinics.

1.2.10.3. Contribution in fertilization & early embryo development

Following maturation, the expanded CCs provide a suitable coat for the oocyte that facilitates its ovulation and its transport inside the infundibulum (Hunter 2003). Moreover, the ECM and particularly the HA was shown to prevent CCs apoptosis (Saito *et al.* 2000). Because CCs are maintained in the vicinity of the oocyte during fertilization, the spermatozoa must traverse in between CCs and their ECM before binding to the ZP. Intriguingly, CCs and ECM look to be more permissive to the spermatozoa with normal morphology, good motility and intact acrosome (Hong *et al.* 2009). This selection may be mediated via ECM molecules like HA (Bains *et al.* 2001, Chiu *et al.* 2007). CCs were also reported to secrete sperm attractants that contribute to enhance the fertilization rates (Sun *et al.* 2005b). This sperm chemotaxis may attract and concentrate the full-capacitated spermatozoa around the oocyte. This CCs attractive action is reinforced by another chemotaxis exerted by the mature oocyte (Sun *et al.* 2005b). The progesterone was suggested as the suitable CCs chemoattractant (Guidobaldi *et al.* 2008, Oren-Benaroya *et al.* 2008). These finding were supported by previous reports where the impairment of ECM expansion or the CCs removal lead to fertilization problems in most mammal species (Zhuo & Kimata 2001, Fulop *et al.* 2003, Tanghe *et al.* 2003). Following the CCs penetration, the sperm binding to the ZP is achieved through the ZP3 protein. This ZP-sperm recognition induces the acrosomal exocytosis and triggers the proteasome pathway (Sutovsky *et al.* 2004, Wassarman 2005, Ni *et al.* 2007, Bansal & Gupta 2009). The progesterone interactions with specific binding sites on the sperm membrane caused the intracellular Ca2+ increase and therefore the membranes' fusions and the release of the cortical granules avoiding polyspermy (reviewed by (Familiari *et al.* 2006)). The precise process of oocyte-sperm fusion remains unclear despite the ultrastructural changes of the mature oocyte zona and the key factors involved as integrins and sperm proteins reported in recent works (Schultz 2005, Familiari *et al.* 2006a, Wassarman & Litscher 2008). Preventing harmful changes in the oocyte and ZP biochemical properties (e.g. zona hardening) was also considered as one of the main functions of CCs during maturation and until fertilization (Tanghe *et al.* 2002).

Successful early embryo development is the consequence of suitable achievement of all steps of maturation, ovulation and fertilization. Consequently, CCs are indispensable to the successful

achievement of the early embryo development. Moreover, CCs expansion offers a suitable environment in the oocyte vicinity that is helpful for its cytoplasmic maturation which is correlated to the developmental competence. It was shown also that the degree of CCs expansion (expanded area) is correlated with the porcine developmental potential, and therefore suggested as predictor of early development competence (Tanghe *et al.* 2002, Qian *et al.* 2003). Interestingly, CCs co-culture with denuded bovine zygotes improved the cleavage, blastocyst and hatching rates (Zhang *et al.* 1995, Goovaerts *et al.* 2009). It is important to mention that CCs are found in the early embryo vicinity in the human oviduct at 80 hours following the LH surge. They maintain translational and steroidogenesis activities with first signs of luteinization both in human and bovine (Motta *et al.* 1995, Familiari *et al.* 1998); and are suggested to have beneficial effects on fertilization, cleavage, implantation and up to the first week of pregnancy (Yoshida 1993, Stanger *et al.* 2001, Hunter *et al.* 2005). Taken together, these findings support a helpful role of CCs during early embryo development.

1.2.10.4. Prediction of oocyte competence

To reach full competency, the oocyte must maintain optimal and mutual exchanges with CCs. These interactions are prerequisite during oocyte journey to achieve developmental competence since maturation and until pregnancy. As noted before, CCs contribute to oocyte quality by their physical presence, their morphology, their metabolic functions, their communications (signalling molecule exchange) as well as their gene expression patterns. Based on this, CCs is suggested as a suitable target of non invasive approaches of oocyte competence prediction. While some studies used the immature CCs morphology as an *in vitro* indicator of oocyte developmental potential (Blondin & Sirard 1995, Qian *et al.* 2003), others focused on its metabolic activities (Sugiura *et al.* 2005, Thompson *et al.* 2007). CCs have also gene expression patterns that intimately reflect the oocyte competence and may therefore reflect later embryo development success (Assou *et al.* 2006, Assou *et al.* 2008, van Montfoort *et al.* 2008, Assou *et al.* 2010). Therefore, additional genomic markers could be added to the morphological criteria to accurately and non-invasively predict the oocyte developmental competence. In this context, some gene candidates expressed in CCs were suggested as potential markers of oocyte competence mainly in bovine and human (McKenzie *et al.* 2004, Zhang *et al.* 2005, Assidi *et al.* 2008, Assou *et al.* 2008). Keeping in mind all the aforementioned CCs functions, confirmed genomic markers differentially expressed in

CCs of competent oocytes should offer powerful tools that contribute in more accurate oocyte selection and therefore improved pregnancy outcomes.

1.3. SECTION # 3 HUMAN ASSISTED REPRODUCTIVE TECHNOLOGIES

1.3.1. The infertility issue

According to the World Health Organization (WHO) and the International Committee for Monitoring Assisted Reproductive Technologies (ICMART), infertility is "a **disease** of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse"(Zegers-Hochschild *et al.* 2009). This recognition of infertility as a disease gives hope to those, who struggle against infertility and lack enough resources to release their suffering, to have government and insurance companies' adequate support to be treated as for other diseases. It reflects also an increasing of both the incidence and the scientific community interest toward this issue. In fact, more fact than 80 millions of citizens are experiencing infertility troubles worldwide. This number represents more than one couple out of ten (>10%). These infertility rates vary largely among countries (5% to 40%) or patient age, as well as between developed versus developing countries. Restricted access to ART and the lack of suitable treatments to dangerous diseases (Malaria, tuberculosis, postpartum or postabortal infections, sexually transmitted diseases...) are suggested as possible factors behind the high rates of infertility (Zegers-Hochschild *et al.* 2009). This limited accessibility to infertility treatments could be attributed to the ART procedure costs, its efficiency, the national policies and other socio-economic factors.

In Canada, the demographic situation is marked by a continuous decrease of the birth rate to reach its lowest level (10.29 per thousand citizens) in 2008. Simultaneously, infertility affects 10 to 20% of the Canadians. According to statistics Canada, this infertility affected as more than 1 out of 6 Canadian couples (Statistics Canada 2008). To achieve their parental project, an increase of couples seeking for ART support has therefore been recorded. It is expected that the decision of some Canadian provinces to cover some IVF trials fees will make ART more accessible to the whole population, raising therefore the RFR (replacement fertility rate) per couple.

1.3.2. Ovarian stimulation strategies

To satisfy the couple reproductive ambitions, clinicians perform an ovarian stimulation program to increase the number of produced oocytes through a multifollicular recrutement, which may increase the outcome of ready-to-transfer embryos. Moreover, the better understanding of

folliculogenesis regulation, the development of new preparations techniques of hormones and the availability of additional forms of agonists and antagonists have lead to constant improvement of superovulation strategies. A successful ovarian induction involves the administration of a hormone/drug in optimal timing and dose/concentration to efficiently stimulate the target receptor/organ leading to both successful pregnancy and no /the least side effects. In practice, the first goal of superovulation treatments is to make the ovarian hormones feedback on the pituitary gonadotropins ineffective, and therefore take the control of the spontaneous cycle. Since the ovulation disturbances are the most common causes of female infertility, several strategies/protocols are in use to achieve controlled ovarian stimulation:

1.3.2.1.Antiestrogen-based program:

The antiestrogens are the first-line therapy to treat anovulation disturbances. They include mainly the clomiphene citrate (CC) and the tamoxifen citrate (TMX).

The CC is an antiestrogenic (competitive antagonist of estrogen) that binds to the ER receptors reducing therefore their effect mainly in the hypothalamus. This increasing insensitivity to E2 removes/reduces its feed-back and improves consequently the GnRH and then the FSH & LH pulses' frequency. CC has a half life of 5 days and acts preferentially on LH secretion. It is not a direct inducer of ovulation but intensify the physiological events of the natural cycle. It is recommended in case of missing /irregular ovulation, insufficient FSH stimulation, polycystic ovary (PCO), corpus luteum (CL) deficiency, or to improve the timing of ovulation. It is used (at an average of 50 mg/day, 5 days) starting from the 5^{th} day of the cycle to support dominant follicle selection. To increase multiple ovulations, CC may be used earlier. By careful following the plasma E2 concentrations and/or using ultrasounds, ovulation ($\approx$ 38 h later) can be induced by hCG. Too early hCG treatment could give immature oocytes. However, the main CC limitations are its reduced estrogenic-like effect on the ovary and the endometrium, as well as possible complications like premature LH surge, poor cervical glands secretions, multiple pregnancies, high E2 and P4 concentrations, and dilation of blood vessels. Its combination with gonadotropins and GnRH agonists may offer effective alternative (Hunter 2003, Waldmann-Rex *et al.* 2005, Dickey 2010). The recent use of GnRH antagonists could improve better the CC-gonadotropins combination (Hugues 2002).

Similar to CC, Tamoxifen citrate (TMX) is an antiestrogenic non-steroidal compound but with weak estrogenic activity. It therefore blocks / modifies the ER action through a competitive

approach. It is administrated on the second day of the cycle (during menses) (at an average of 20 mg/day, 5 days). Intramuscularly injection of hCG is achieved at follicular diameter of 20-22 mm can improve the ovulation rate. TMX is recommended in the same cases as CC and gives similar success and probably more side effects. These effects include higher incidence of OHSS, reduced mucus secretion from cervical glands, uterine bleeding, and endometrium carcinoma, as well as the absence of endometrial cycle and abnormalities of physiological development. However, it represents an alternative in case of CC resistance, PCO patients with high LH levels or patient with poor cervical secretions (Patil 2005). Both CC and TMX lack a constant predictability of the individual responses and high risk of multiple pregnancies (Hunter 2003). Depending on the cause of infertility, they are recommended for 4 to 6 cycles of treatments combined with gonadotropins before moving to IVF (Hugues 2002, Dickey 2010).

1.3.2.2.Gonadotropins-based program:

The use of gonadotropins in ovarian stimulation have started with the human pituitary gonadotropins (hPG) (pituitary glands of cadavers) then moved to the human menauposal gonadotropins (hMG) due to the low availability of hPG and possibility of Creutzfeld-Jakob disease transmission. Both hPG and and hMG contains FSH and LH and were/are used during the follicular phase to induce the follicular growth (FSH-like effect). For the purified gonadotropins, they are also available with a predominant FSH effect. With the recent introduction of recombinant gonadotropins (rFSH and rLH), there are an increasing tendency to use them in order to make the ovarian response more predictable and more constant despite their cost (Howles 2000, Hunter 2003, Adams & Boime 2008, Franco *et al.* 2009, Ledger *et al.* 2009). Natural cycle is the suitable reference for ovarian induction strategies and is marked by specific oscillation of circulating levels of gonadotropins (Pelinck *et al.* 2002, Shahin 2007). Variation of these levels is function of their flow of release, their biological function (binding receptor/tissue) and their half-life/ turnover (degradation). The gonadotropins are secreted following ($\approx$ 20 min of delay) GnRH pulses. Although they improve the pregnancy rate/cycle, gonadotropins are not the first option in ovarian stimulation. The use of exogenous gonadotropins is indicated in cases of unexplained infertility or intolerance to the antiestrogen therapy. They may be also tested after failure of at least 4 cycles of tests with CC or TMX. Even when used, the starting dose should be very low (low-dose protocol) in order to have a close assessment of the ovarian response. This is

due their high incidence of both OHSS and multiple pregnancies (triplet and higher-order pregnancies) (Dickey & Brinsden 2010).

The gonadotropins treatment aims firstly at maintaining FSH over a threshold necessary to ensure proper recruitment of follicles. Increasing moderately its levels is favourable to induce multiple follicular developments. Low or very high FSH leads to respectively fewer follicles or lower pregnancy rates. In both cases, the pregnancy outcomes are weak synonym of an incomplete oocyte competence acquisition. For hCG, it may be used at follicular diameter around 18 mm. It may be injected before to reduce multiple pregnancies (Rosen *et al.* 2008). It is injected when a possible spontaneous LH surge is expected in order to ensure ovulation and high levels of P4 later.

In addition to the low-dose protocol, the gonadotropins could also be used in a high-dose protocol (called also controlled ovarian hyperstimulation) in order to induce multiple follicular ovulations especially for the IVF/ICSI procedures or for the low responders. Despite the absence of consensus about the number of cycles of gonadotropins stimulation, the number of developed follicles per patient per cycle may be a good criterion for the clinician to decide to pursue or not this dangerous stimulation or to switch to another approach (Blumenfeld 2005). It is to note that due to the gonadotropins side effects, their use in ovarian stimulation requires experience and dexterity of the clinician, whole examination of the patient, frequent monitoring of follicle development and endocrine levels measurements (E2, LH, FSH, and P4).

There are two approaches of gonadotropins-based ovarian stimulation: the step-up and step-down protocols. For the step-up protocols, they are recommended mainly in PCO patients who lack FSH compared to LH. The goal is to correct this hormonal imbalance and slowly stimulate the follicle growth (one or few) and emergence until the preovulatory stage. Once the dominant follicle(s) is observed (12-14 mm), a steady state of hormonal level is maintained with monitoring of hormonal levels (LH and E2) and follicular growth. Ovulation is induced by hCG as in the other protocols (Bremner & Benadiva 2005). Concerning the step-down programs, they are based on initial high dose of FSH to overcome the threshold needed to induce follicle growth and to ensure full recruitment of a growing follicles pool. Decreasing doses start at the beginning of follicle dominance (12-14 mm) marked by high sensitivity to FSH (FSHR) in order to prevent premature LH surge. Careful monitoring is required in these types of protocols. The goal is to activate the growth of a cohort of preovulatory follicles and then control their number using a

decremental hormonal support based on exogenous FSH or hMG. It is recommended in case of PCO patients who failed after some trials with CC; and is case of high responding women for IVF purposes (Scholtes 2005).

1.3.2.3. GnRH-agonists:

GnRH-agonists are characterized by longer half life and better affinity to GnRH receptor compared to the original hormone. They act by downregulating their receptor so they have an initial stimulating (rise in gonadotropins) effect named «flare-up» during the 24-48h before decreasing.

GrRH-agonists are used in ovulation induction with insemination/intercourse, superovulation followed by ART, or for patients with OHSS. For IVF and other ART purposes, the GnRH agonists prevent the surge of gonadotropins due to increasing E2 levels, allowing therefore large number of follicles to be activated and harvested. It is also a precious tool for oocyte collection timing/planning. The GnRH agonist is used first (even since the mid-luteal phase of the previous cycle in the long protocol) to ensure pituitary suppression, and then the gonadotropins are added to stimulate the follicular growth. The agonist is removed only on the day of hCG administration reducing therefore the monitoring tasks and costs (Allahbadia & Majmudar 2005, Buckett 2005). These agonists have some limitations like the initial surge of gonadotropins at the time of first administration, possible P4 rise before ovulation (premature luteinisation), slow recovery of the pituitary activity after cessation (refractory period) and therefore less chances of ovulation using the endogenous LH surge (Hugues 2002, de Jong *et al.* 2005).

1.3.2.4.GnRH-antagonists:

GnRH-antagonists are also synthetic analogues of GnRH. they bind specifically and competitively to the GnRH receptor inhibiting immediately the gonadotropins release, and allow a rapid recovery of endogenous FSH/LH secretion once its administration is stopped (possibility of endogenous LH surge induction). Therefore, some authors recommend their use at the late follicular stage. Their use is limited mainly for IVF purposes to avoid premature LH surge. Despite reduced outcome compared to the GnRH-agonists, optimal use of endogenous FSH followed by mid-cycle addition of both exogenous FSH and GnRH antagonist was shown to be less expensive and shorter for patients, and more efficient and softer for the oocyte final maturation. Possibility of triggering ovulation by arresting treatment (only endogenous LH) could

be envisioned with these antagonists, which may considerably reduce the OHSS risk (Hugues 2002, de Jong *et al.* 2005).

1.3.2.5.Combined programs and suitable ovulation induction protocol

There are several ways of ovulation induction by the aforementioned approaches alone or in combination. Despite the several combination documented as (CC + FSH + GnRH antagonist ± hCG/LH), (GnRH agonist + FSH + hCG/LH), (GnRH antagonist + FSH ± hCG/LH) (Allahbadia & Majmudar 2005, Buckett 2005, Silberstein & Lunenfeld 2005, Brinsden & Dickey 2010, Dickey 2010, Dickey & Brinsden 2010), the suitable protocol chosen by the clinician based on several parameters remains subjective since patient reaction differs with these ovulation inducers and their possible interactions are still poorly identified.

Therefore, the choice of the suitable program should be preceded by meticulous etiology of the anovulatory state of the patient and a complete assessment of the patient/couple health state. Several parameters including the medical history, the clinical examination for possible infertility's causes and risk of complications, the serum hormones levels determination (FSH, AMH (Anti-Müllerian hormone), P4), the body weight measurement as well as ultrasound examination of the ovarian status are required. Moreover, it is also necessary to keep couples informed of the costs and the success and risks probabilities. Suitable counselling and accompanying of the patients should be envisioned before the treatment starting (Hugues 2002, Hunter 2003, Brinsden & Dickey 2010).

1.3.3. Overview of Assisted Reproductive Technologies (ART)

According to the WHO, the assisted reproductive technologies (ART) cover « all treatments or procedures that include the *in vitro* handling of human oocytes and sperm or embryos for the purpose of establishing a pregnancy. This includes, but is not limited to, *in vitro* fertilization and transcervical embryo transfer, gamete intrafallopian transfer, zygote intrafallopian transfer, tubal embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation and gestational surrogacy» (Vayena *et al.* 2002).

Within the past decades, the increase of infertility incidence and the better understanding of the pharmaceutical control of folliculogenesis have opened new alternatives to achieve pregnancy. In addition to the widely use of ovarian stimulation, other ART have been used for the first time in human reproduction. It is estimated that more than one million babies worldwide have been born

using ART with more than 5% of births in Europe (Vayena *et al.* 2002). *In vitro* fertilization (IVF) is one of the most popular techniques used during the last decades. In fact, and since the birth of the first human following from IVF in 1978 (Steptoe & Edwards 1978), this technology has been widely used mainly to treat the infertility due to the blockage or damage of fallopian tubes. It necessitates sperm and oocyte collection, then their mixture to allow them to fertilize outside the body (*in vitro*). The oocyte retrieval is achieved following hCG stimulation and the fertilization occurs spontaneously in a dish in the laboratory setting. The IVF protocols are under constant review in order to increase their efficiency, and therefore pregnancy outcomes (Ahmad *et al.* 2009). Following early embryo development *in vitro*, named also *in vitro* culture (IVC), the cleaved embryo is transferred to the woman uterus for implantation and embryo development. In some cases of female infertility, immature oocytes can be harvested and matured *in vitro*. Some media are available for *in vitro* maturation (IVM) although the process is not well controlled. The studies on livestock species have offered available models where the media for the IVM, IVF and IVC technologies are optimized. Despite the advantages of human IVM to reduce the fertility drugs use, it is still a rudimentary strategy with poor clinical application (Trounson 2003, Lacham-Kaplan & Trounson 2008).

If the sperm analysis reveals low count and/or motility, the new micromanipulation technique named the intracytoplasmic sperm injection (ICSI) can be used. In fact, ICSI is based on the injection of a single sperm through the zona pellucidae into the oocyte avoiding therefore all the physiological steps of fertilization. The first case of birth due to ICSI was reported on 1992 (Palermo *et al.* 1992). While the sperm is obtained through standard preparation protocol (washing, dilution), the mature (MII) oocytes are retrieved following enzymatic denudation (hyaluronidase). The ICSI technique has high efficiency in achieving successful fertilization in wide cases of male factors where even few spermatozoa are available surgically or through ejaculation. Consequently, the ICSI has been so famous and ubiquitously used in most of IVF clinics worldwide (Malter & Cohen 2002, Nadalini *et al.* 2009).

The intrauterine insemination (IUI) is another technique based on direct introduction of the seminal fluid/sperm dilution into the uterus that could be to overcome male fertility difficulties. It could be also in some endometriosis, hostile or irregular cervical mucus (CFAS Report 2009). Gamete transfer could be also achieved via the gamete intrafallopian transfer (GIFT) technique

which is an intracorporeal fertilization where both spermatozoa and oocyte are transferred to the fallopian tubes. It may be also an alternative to cervix or immunological limitations (Yee 2006).

Instead of gametes, zygote could be transferred to the fallopian tubes by the technique named tubal embryo transfer or commonly the zygote intrafallopian transfer (ZIFT). The gamete transfer by ZIFT occurred normally at the pronuclear stage of development following IVF. Theoretically, the ZIFT success rate should be better than the IVF and GIFT ones since only fertilized oocytes are transferred. However and in addition to both surgical interventions (retrieve the oocyte transvaginally and zygote transfer by laparoscopy), no significant differences of ZIFT birth rate were reported compared to the GIFT (Vayena *et al.* 2002, Yee 2006, CFAS Report 2009).

Assisted Hatching (AH) is another ART procedure to induce the ZP rupture of the early embryo. It is achieved just before the embryo transfer in order to ease its hatching and therefore subsequent implantation.

Sperm selection has been also subject to recent ART advances including the collection procedure from the epididymis by surgical or mechanical (aspiration) techniques. This technology is called microscopic epididymal sperm aspiration (MESA) and is used mainly in cases of ejaculation defects or azoospermia, but their improvement of pregnancy rates following ICSI remains unconfirmed (Vayena *et al.* 2002, Van Peperstraten *et al.* 2008).

The sperm quality enhancement and selection have been the focus of new microscopy-based ART. In fact, new observation methods of motile spermatozoa to assess in real time and at high magnification (>x6000) its fine nuclear morphology have been recently suggested through the motile sperm organelle morphology examination (MSOME) technique. This has leaded to new microinjection/ICSI procedure called intracytoplasmic morphologically selected sperm injection (IMSI). However, the IMSI effectiveness was not higher than conventional ICSI based on cleavage rate and pregnancy outcomes (Nadalini *et al.* 2009, Mauri *et al.* 2010).

Cryopreservation is another strategy of human tissues preservation for infertility treatment or fertility preservation. It has been an efficient, safe and beneficial approach for sperm over many years (Pyrzak 2010). Encouraging results were also reported for embryo (Trounson & Dawson 1996) which may be an efficient approach to increase the cumulative pregnancy outcome. Unfortunately, the oocyte cryopreservation is still challenging. Many studies using MII oocytes suggest that oocyte survival and future developmental potential may be affected by the freezing/thawing processes. Cytoplasmic organelle distribution, reduced number of cortical

granules, premature zona hardening, and meiotic spindle disruption are possible complications associated to cryopreservation (Pyrzak 2010). Therefore, Oocyte cryopreservation strategies remain to be optimized.

1.3.4. The bovine as model for human reproduction

Since human tissues are not easily obtained and preclude exploratory experimentation, many ovarian research programs have focused on animal models such as the bovine. In fact, several ethical and logistical restrictions ban repeated or prolonged examinations, invasive trial, frequent tissue collection and doses optimization. The use of animal model should therefore be a prerequisite to test and confirm new approaches or techniques, before their preclinical trial in human. Because the mouse model is easier and less expensive to achieve gene KO, it is more popular and largely used in several contexts. The specie prolificacy, pubertal age, short follicular cycle and pregnancy duration have also contributed to this choice. However, the mouse model shows some major physiological differences compared to both bovine and human. In fact, it is a polyovulatory specie with «accelerated» physiological processes (Menezo & Herubel 2002), reflecting probably different signalling pathways and molecular regulation. The use of the bovine model to study fundamental mechanism and new technologies before clinical investigation has offered a better and valuable solution. Actually, the choice of this model has solid arguments mainly interesting similarities with human. This includes the common monovulatory characteristic, the similar ovarian size, mature follicle diameter and CL dimension. The ovarian anatomy (superficial cortex, central stroma) and even the reproductive pathology (polycystic ovaries) were also comparable (Adams & Pierson 1995, Sirard & Trounson 2003). Interestingly, the follicular dynamics (waves), the physiology of ovulation and lactation as well as the early embryo chronology are analogous and are well studied and documented (Adams & Pierson 1995, Sarty *et al.* 2000). Additionally, the bovine has been a precious model to study the physiological and molecular events during the maternal-Embryonic transition (MET), which occurred in both human and bovine around the 8-cell stage. Moreover, it was shown that oocyte mRNA polyadelation is finely regulated and prolonged in both human and bovine (compared to the mouse) (Menezo & Herubel 2002, Sirard & Trounson 2003, Tremblay *et al.* 2005, Massicotte *et al.* 2006). Furthermore, common molecular markers of oocyte quality were reported in both bovine and human species (McKenzie *et al.* 2004, Feuerstein *et al.* 2007, Assou *et al.* 2008, Bettegowda *et al.* 2008).

Several experiments using the bovine model including ovulation induction, IVF optimization, endocrine mechanism exploration, monovulatory follicular development kinetics, ovarian aging, oocyte/embryo cryopreservation, assisted hatching and early embryo development (Adams & Pierson 1995, Menezo & Herubel 2002, Baerwald *et al.* 2003b, Campbell *et al.* 2003, Malhi *et al.* 2006, Malhi *et al.* 2007, Malhi *et al.* 2008) have permitted a better understanding of ART process and consequences.

1.3.5. Markers of oocyte and embryo quality

Despite the important development in ART and the improvement of our understanding of the maturation events, accurate selection of good quality oocytes is still challenging. Therefore, several studies have focused on the research of some parameters that could be helpful to predict the oocyte developmental potential. In the absence of efficient methods of competence measurement, successful fertilization and early embryo development have been used. In both human and livestock species, some relationships/correlations were made between the oocyte and its somatic environment and the pregnancy or IVF outcome to find early morphological parameters that best reflect the oocyte quality both *in vivo* and *in vitro*.

In human IVF, it is reported that the oocyte diameter should be 110-120 µm to acquire the ability to resume meiosis *in vitro* (Durinzi *et al.* 1995). Only follicles having more than 6-mm diameter (better more than 12 mm) at the moment of hCG injection are able to produce blastocysts, and CCs presence in culture is prerequisite in gonadotrophin-mediated IVM (Trounson *et al.* 2001). In addition to keeping oocyte-corona radiata cells contacts, Smitz et al. (2001) recommend the following of the meiotic maturation state (e.g. first polar body extrusion) as a marker of oocyte maturation progression. Using microscopic tools, the meiotic spindle and cytoplasm granulation, have been also used as morphological markers of oocyte developmental competence (Trounson 2003, Rama Raju *et al.* 2007, Van Blerkom 2009, Yu *et al.* 2009).

For the ZP, the thicker and uniform the zona is, the higher the oocyte developmental competence is (Montag *et al.* 2000b, Host *et al.* 2002, Rama Raju *et al.* 2007, Ueno *et al.* 2007). Recent approaches that automatically (user-independent) measure the ZP birefringence have reported positive correlation with human pregnancy outcome (Montag *et al.* 2008, Ebner *et al.* 2009, Madaschi *et al.* 2009). The degree of CCs expansion was also used to have an idea about the COC response to gonadotropins (Ebner *et al.* 2003). Additionally, the CCs presence, their

apoptosis and expansion were matched with the human oocyte subsequent embryo development (Host *et al.* 2000, Lee *et al.* 2001, Yamazaki *et al.* 2001, Host *et al.* 2002, Assou *et al.* 2010).

Other late parameters that link the oocyte developmental competence to the embryo morphology were also reported. Among the early embryo morphological markers, the pronuclei appearance, cleavage rate and speed, multinucleation, blastomere number, ovarian/follicular vascularity, metabolic products during development were the mostly used (Montag *et al.* 2000b, Jones *et al.* 2002, Ebner *et al.* 2003, Borini *et al.* 2005, Van Blerkom 2009).

Although there is evidence of improvement of pregnancy outcomes, these morphological criteria remain subjective, in some cases invasive and/or poorly correlated to the oocyte competence. Finding relevant biomarkers to predict the oocyte quality is therefore of primary interest to offer efficient approaches to improve the pregnancy outcome especially for infertile couple. The transfer of multiple embryos and its heavy consequences on both woman and babies health has made this biomarkers research more urgent (Felberbaum 2007, Gelbaya *et al.* 2009). In fact, the emphasis on increasing the pregnancy outcomes by multiple embryo transfer have raised several complications for the mother (miscarriage, preterm labour and delivery, anaemia, hypertension, operative delivery) (Ozturk & Templeton 2002) and the babies (preterm birth, low birth weight, perinatal mortality, neonatal care) (Finnstroem 2002), associated to additional social and economic consequences.

In order to reduce the incidence of these high-order pregnancies, recent approaches aiming at finding better non-invasive markers of oocyte developmental potential that could accurately and repeatability predict the oocyte quality are necessary (Guerif *et al.* 2007, Assou *et al.* 2010). Since the CCs gene expression and mucification are influenced by the oocyte, CCs gene expression studies have been suggested as quantitative tool that may strengthen the morphological aforesaid criteria and improve the oocyte selection. In fact, and based on the CCs-oocyte cross-talk, successful embryonic development looks to be the result of meticulous and time-and-site-specific gene expression program in CCs to support proper oocyte competence acquisition. Interestingly, the level of expression of some genes in CCs was reported to be correlated with human oocyte ability to achieve successful embryo development (McKenzie *et al.* 2004, Assou *et al.* 2008, Hamel *et al.* 2008b). Therefore, the identification of the gene markers that are expressed in CCs and prognostic of oocyte developmental potential will offer precious

contributions in both oocyte selection efficiency and in the understanding of the pathways that underlies the molecular mechanisms of oocyte competence.

RATIONALE, HYPOTHESIS & OBJECTIVES

Rationale and Hypothesis:

Several ART studies achieved in the last decades on both human and livestock species have offered worthy information regarding key molecular processes associated to the oocyte progression since the preovulatory stage to the early embryo development stages. The identification of some oocyte-derived factors has improved our understanding of the extend of oocyte-associated follicular cells communications (Eppig 2001). Thought until a near past as a passive gamete that receives the gonadotropins stimulation to fulfill maturation, the oocyte was shown to drive complex signalling pathways and interplay with its immediate environment. In this context, the CCs differentiation, metabolism, signalling and gene expression were shown to be influenced by the oocyte (Matzuk *et al.* 2002, Sugiura *et al.* 2007, Su *et al.* 2008). Therefore, one could assume that CCs are the site of expression of interesting genes that may reflect the oocyte maturity or quality.

The *in vitro* embryo production system has also been subject to exciting progress mainly in livestock species by mimicking the *in vivo* environment of oocyte maturation, fertilization and early embryo development. This system was also reinforced by the extending use of the recombinant gonadotropins and the recent metabolic studies that are helpful to optimize the used media (Sutton *et al.* 2003). Consequently, such system represents a valuable framework to achieve several tests and assays that are difficult, expensive or impossible to test *in vivo* in livestock species and human due to several limitations. In fact, the molecular mechanism of oocyte competence remains still an enigma. Moreover, the bidirectional communications between oocyte and CCs, the intricate signalling pathways and the gene expression patterns required for oocyte developmental competence are poorly understood and require further exploration. The comprehension of these molecular processes will represent a valuable contribution in the improvement of the *in vitro* system and the optimization of the superovulation schedules in livestock species. Benefiting of less legal, technical and ethical restrictions, these studies both *in vitro* or using an animal model offer powerful tools to clinicians to enhance the infertile couples' chances to achieve successful pregnancy and bring a healthy baby.

Keeping in mind the importance of CCs, the advantages of the *in vitro* system and the magnitude of using an animal model *in vivo*, we consider that the exploration of the signalling pathways and gene expression events in bovine and human CCs involved in proper final maturation of the oocyte are crucial steps before establishing possible strategies of improvement in the future. To do this, we assume that:

- Besides being essential to the oocyte developmental competence, CCs are also a mirror that reflects its quality. Their contribution is achieved mainly through well-established sequence of both signalling pathways and gene expression events during both maturation and fertilization.
- The *in vitro* system is a precious support on which we can investigate and reproduce at least part of the signalling and transcriptional events of the oocyte maturation events. The study of these events and their validation *in vivo* markedly improves our understanding of the molecular pathway of oocyte competence.
- Just before the GVBD where the oocyte transcriptional activity is markedly reduced (Hyttel *et al.* 2001), the CCs have already produced some factors that are transmitted to the oocyte to achieve its final maturation and prepare its final RNA stockpile. The identification of these factors is main step toward the comprehension of the molecular pathway of CCs action both *in vivo* and *in vitro*.
- The identification of the LH-induced genes expressed in CCs *in vivo* allows a better understanding of the oocyte final maturation physiology and the CCs contribution in the ovulatory process.
- The identification of genes differentially expressed in CCs of oocytes with successful developmental competence should provide quantitative and non-invasive tools for accurate oocyte selection.

Specific objectives:

The general goal of this thesis is to explore the CCs gene expression under different contexts (*in vitro* versus *in vivo*) and different species (bovine versus human) in order to:

- Find relevant signalling pathways and gene biomarkers induced by FSH and/or PKC around the GVBD in CCs and involved in bovine oocyte competence *in vitro*
- Explore the LH genomic effect in CCs *in vivo*. Early molecular biomarkers of oocyte developmental competence in bovine CCs around the GVBD *in vivo* could be suggested.

- Use the bovine model to compare the genomic effect of FSH *in vitro* versus LH *in vivo* on CCs
- Identify gene biomarkers differentially expressed in human CCs prior to ICSI and associated to successful pregnancy. These biomarkers are quantitative and non-invasive tools that improve the oocyte selection in IVF clinics, increasing therefore the pregnancy outcome.
- Evaluate possible correlation between two markers of human oocyte competence: ZP birefringence versus prognostic gene biomarkers expressed in CCs

CHAPTER 2:

Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with FSH and/or PMA *in vitro*

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2.1. RÉSUMÉ

La compétence au développement de l'ovocyte consiste en sa capacité à réussir les étapes successives de maturation, de fécondation pour atteindre le stade blastocyste et donner une progéniture en santé. Étant indispensables pour ce processus, la suppression des cellules cumulus réduit significativement le taux de blastocystes. De même, les propriétés et les fonctions des cellules du cumulus sont influencées par l'ovocyte et peuvent refléter ainsi son degré de maturation. Notre étude visait à identifier des marqueurs de compétence ovocytaires qui sont exprimés dans les cellules du cumulus bovin. Selon une étude précédente de notre laboratoire, l'ajout de la FSH ou le phorbol myristate acétate (PMA) au milieu de maturation *in vitro* a donné un rendement en blastocystes de 45%. Par conséquent, nous avons étudié ici quatre séries de traitements pendant les 6 premières heures de la maturation *in vitro* (MIV): FSH (0,1 mg / ml), PMA (0,1 uM), FSH + PMA ainsi que le contrôle négatif. Chacun des 3 traitements de MIV a été hybridé contre le même contrôle négatif sur une biopuce contenant une librairie partielle de gènes différentiellement exprimés dans le cumulus d'ovocytes compétents prélevés à 6 heures post-LH *in vivo*. Les clones surexprimés dans les CCs et communs entre les 3 traitements ont été sélectionnés et 15 candidats ont été validés par PCR en temps réel. Ainsi, les principaux candidats surexprimés dans les cellules du cumulus et qui pourraient être des marqueurs potentiels de la compétence ovocytaire sont hyaluronane synthase 2 (HAS2), inhibine βA (INHBA), epidermal growth factor (EGFR), gremlin (GREM), betacellulin (BTC), CD44, tumor necrosis factor-induced protein-6 (TNFAIP6) et prostaglandin-endoperoxide synthase 2 (PTGS2). Ces biomarqueurs peuvent être des candidats potentiels pour prédire la compétence des ovocytes et ainsi sélectionner des embryons de meilleure qualité pour le transfert. De plus, ces biomarqueurs non-invasifs de la compétence d'ovocytes et de la santé folliculaire pourraient améliorer notre connaissance des profils d'expression génique dans les CCs ainsi que les voies moléculaires qui régissent la compétence ovocytaire.

2.2. ABSTRACT

Oocyte competence is the ability of the oocyte to complete maturation, undergo successful fertilization and reach the blastocyst stage. Cumulus cells are indispensable for this process. Their removal significantly affects the blastocyst rates. Moreover, the properties and functions of cumulus cells are regulated by the oocyte. They also reflect the oocyte's degree of maturation. Our study was aimed at identifying markers of oocyte competence that are expressed in bovine cumulus cells. In a previous study in our laboratory, the blastocyst yield following FSH or phorbol myristate acetate (PMA) treatment was 45%. Therefore, we tested four sets of conditions during the first 6 hrs of *in vitro* maturation (IVM): FSH (0.1 μg/ml), PMA (0.1 μM), FSH+PMA and negative control. Extracts from each IVM treatment were hybridized against the same negative control on a microarray containing partial library of differentially expressed transcripts in the cumulus of competent oocytes collected at 6 hrs post-LH *in vivo*. Common positive clones between diffentially-treated cells were selected and 15 candidates were validated by real time PCR. Based on this, the main candidates expressed in cumulus cells and that could be valuable and indirect markers of oocyte competence are hyaluronan synthase 2 (*HAS2)*, inhibin βA (*INHBA)*, epidermal growth factor receptor (*EGFR*), gremlin (*GREM*), betacellulin (*BTC*), *CD44*, tumor necrosis factor-induced protein-6 (*TNFAIP6*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*). These biomarkers could be potential candidates to predict oocyte competence and to select higher quality embryos for transfer. Additionally, these indirect predictors of oocyte competence and follicular health could improve our knowledge of gene expression patterns in the cumulus and yield insights into the molecular pathways involved in oocyte competence.

2.3. INTRODUCTION

As our understanding of factors regulating folliculogenesis increases, it appears the relationship between the oocyte and the surrounding somatic cells is more complex than previously thought and represents a determining factor of later developmental competence. It is currently established that communication between cumulus cells and their oocyte is essential for the competence acquisition process. *In vitro* culture (IVC) of denuded bovine oocytes considerably decreases their competence (Tanghe *et al.* 2002). Oocytes clearly depend on the presence of follicle cells to generate specific cellular signals that coordinate their growth and maturation. The cumulus cells were thought to express some of the signals that are crucial to the oocyte maturation fulfilment (Taft *et al.* 2002, Gilchrist *et al.* 2004). The study of the gene expression in follicles, including cumulus and oocyte, may contribute to a better understanding of the maturation and the successful fertilization processes (Lonergan *et al.* 2003).

Despite successful results of the *in vitro* maturation (IVM), the *in vitro* competence is still reduced compared to *in vivo-derived* oocytes. Moreover, and because of the absence of appropriate analysis tools, the maturation completion is still assessed through successful fertilization and blastocyst yield. Bovine embryos produced *in vitro* show some differences with *in vivo-derived* embryos in terms of morphology, timing of development, resistance to low temperature, embryonic metabolism, and especially gene expression (Niemann & Wrenzycki 2000, Lazzari *et al.* 2002). Given that the morphological appearance of the oocyte and embryo does not accurately predict the health of the embryo (Munne *et al.* 1994), studies of the gene expression in both *in vivo* and *in vitro* maturing cumulus-oocyte complexes (COCs) could potentially lead to the elucidation of signaling pathways involved in the intricate crosstalk between the oocyte and its somatic compartment during the maturation and the development processes, and to a better understanding of the origin of disturbances in oocyte maturation possibly involved in reduced fertility. Successful embryonic development is dependent on a rigorous, time- and site-specific gene expression program of appropriate genes. Identification of these differentially expressed genes and the analysis of their pattern of expression are powerful tools to gain informations about functions relevant to processes such as the oocyte competence. Such further competence is mainly due to the molecular memory acquired during maturation by the oocyte and the surrounding somatic cells (Sirard *et al.* 2003). Thus, it is valuable to examine the gene transcription in both the oocyte and the cumulus cells when investigating developmental

competence. Communication between the oocyte and cumulus cells is accomplished mainly through the gap junction type of intercellular communication. These communications are considered to play important roles in supporting oocyte maturation (Bruzzone *et al.* 1996). Previous studies have shown that the removal of the cumulus cells before IVM or the blockade of the gap junction were associated with an inhibition of oocyte maturation (Vozzi *et al.* 2001). Moreover, cumulus cell expansion, gene expression as well as other essential properties are governed by the oocyte and therefore reflect the follicle growth stage (Luvoni *et al.* 2001, Sugiura *et al.* 2007). Given the absence of FSH receptor (FSHR) on the oocyte, the study of cumulus cell responses to FSH stimulation, especially their gene expression patterns, is therefore valuable. In addition, the presence of cumulus cells and intact gap junctions were reported to support oocyte competence *in vitro* (Hashimoto *et al.* 1998). Inhibition of the functional coupling between the oocyte and its cumulus using gap junction inhibitors significantly reduced the oocyte competence expressed in terms of blastocyst rate (Atef *et al.* 2005). These findings support a key role for the cumulus and its communication with the oocyte in the competence acquisition process. Consequently, competent oocytes influence the pattern of expression of a set of biochemical markers in the cumulus that might be crucial to achieve maturation and successful subsequent development (Lucidi *et al.* 2003).

Many studies have focused on gene expression in oocytes in order to find specific molecular markers to characterize successful oocyte maturation. Many genes were identified as potential predictors of oocyte competence (Robert *et al.* 2000, Sirard *et al.* 2003, Krisher 2004). Unfortunately, data on cumulus gene expression in the cow is lacking despite its established support to oocyte competence, a process that seems to be mediated by programs of differential gene expression. It is expected that some specific metabolites in the cumulus are indicators of the oocyte maturation state.

It was also previously demonstrated in our laboratory that during the first 6 hours of IVM, FSH or phorbol myristate acetate (PMA) treatment led to improved oocyte competence and yielded more than 45% blastocysts (Ali & Sirard 2005). Based on these results, the improvement in oocyte competence was thought to be mediated via gene expression in cumulus cells and then in oocyte. Hence, the present study aims to explore the transcriptional effect of FSH and/or PMA after 6 hours of IVM on oocyte competence. Differentially expressed genes, especially those common to FSH- and PMA-treated extracts, were identified and could represent valuable

molecular markers to predict oocyte competence through cumulus gene expression analysis. The identification of relevant subsets of these molecular cascades can provide powerful knowledge and new tools to individually assess oocyte competence by analyzing its cumulus. Moreover, these biochemical markers for embryo development would improve pregnancy outcomes by optimizing oocyte and embryo selection especially in human IVF, and allow therefore fewer embryos to be transferred.

2.4. MATERIALS AND METHODS

2.4.1. COC recovery and cumulus collection

Two groups of COCs were collected. In the first (*in vivo*) group, cows were synchronized and treated with FSH to induce superovulation. Briefly, the ovaries were collected 6 hrs after the GnRH-induced LH surge. The cows were presynchronized using an ear implant (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, Netherlands) for 9 days. Two days before implant removal, prostaglandin (22.5 mg, Intervet Internatinal, BV, Boxmeer) was administered to induce a new oestrus cycle. From day 9 to 14, cows received another Crestar implant, and FSH was administered twice a day in decreasing doses from day 10 to 13. In addition to FSH, cows received prostaglandin 48 hrs before removal of the Crestar implant (Figure 2.1). Cows received an injection of GnRH (1 mg) at the time of implant removal and were ovariectomized 8 hrs after GnRH administration. Ovaries were collected in 0.9% (w/v) NaCl at 37 °C and were immediately transported to the laboratory (Knijn *et al.* 2002). Next, an individual preovulatory follicle (POF) selection was performed. POFs have decreased estradiol (E2) and low progesterone (P4) and testosterone levels in their follicle fluid (FF). *In vivo*-derived cumulus was separated through gentle pipetting.

In the second (*in vitro*) group, bovine ovaries were collected at a slaughterhouse and divided immediately into two subgroups before being transported to the laboratory: 5 ovaries were immediately put on ice, whereas the remaining ovaries were kept at 37 °C in a 0.9% (w/v) saline solution containing 100,000 IU/L penicillin, 100 mg/L streptomycin and 250 µg/L amphotericin B (Sigma-Aldrich, Oakville, ON, Canada) and were used for an *in vitro* culture protocol. All further steps of cumulus collection in this group were performed at 4 °C. The COCs from 3- to 8-mm follicles were collected by aspiration. 1, 2 and 3 Classes of COCs were selected according to morphological classification proposed by Blondin and Sirard (Blondin & Sirard 1995). Cumulus

cells on ice were physically separated from immature COCs (GV stage) by gentle pipetting and washed several times in phosphate buffered saline (PBS) to prevent any contamination with oocyte content. Isolated and washed cumulus cells of this iced subgroup and from the *in vivo* group were then immediately frozen at -80 °C for subsequent RNA extraction and suppressive subtractive hybridization (SSH). Each IVM treatment and tissue collection for further molecular analysis were repeated four times.

2.4.2. In vitro maturation and cumulus cell collection at 6 hrs of IVM

Ovaries transported in saline solution were punctured and only COCs with more than three layers of compact cumulus cells were selected by using a stereomicroscope (Ali & Sirard 2002). Selected COCs were washed three times in Hepes-buffered Tyrode medium (TLH) supplemented with 0.3% (w/v) BSA (fraction V), pooled into groups of 10 COCs and then put into 50-µl droplets of maturation medium (SOF) with 0.8% BSA-FAF (BSA fatty acid free; Sigma-Aldrich, Oakville, ON, Canada) under 9 ml mineral oil (Sigma-Aldrich, Oakville, ON, Canada). To study the effects of recombinant human FSH (r-hFSH) (Gonal F, Serono, ON, Canada) and PMA (Sigma-Aldrich, Oakville, ON, Canada), four treatments were applied during the first 6 hrs of IVM. For each treatment, 40 to 50 COCs were used. Maturation occurred in saturated-atmosphere humidity (5% CO_2 in air) at 38.5 °C for 22 to 24 hours. The four treatments were: 1) SOF without hormone, 2) SOF + r-hFSH (0.1 µg/ml), 3) SOF + PMA (0.1µM), and 4) SOF + FSH (0.1µg/ml) + PMA (0.1µM). After 6 hrs of IVM, COCs from each treatment group were collected and cumulus cells physically separated from the oocyte (GVBD stage) by gentle pipetting, washed by PBS and then immediately frozen at -80 °C for further RNA extraction.

2.4.3. Total RNA extraction

Total RNA of cumulus cells for *in vitro-derived* samples was isolated by Trizol extraction (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol. The total RNA pellet was then resuspended in 30 µl of nuclease-free water and stored at -80 °C. For *in vivo-derived* samples, RNA was extracted using Stratagene's RNA isolation kit (Stratagene, San Diego, CA, USA) according to their user manual recommendations. For both *in vivo* and *in vitro* replicates, total RNA quantification and quality assessment were achieved using Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's protocol.

2.4.4. Construction of CCs SSH cDNA library of the from in vivo competent oocytes

2.4.4.1. PCR-based RNA amplification

Because of the small amounts of starting material from bovine COCs, the mRNA of the first (*in vivo*) and second (*in vitro*) groups of collected cumulus were reverse transcribed and the full-length cDNA was subjected to PCR using the Super SMART PCR cDNA synthesis kit (Clontech, Mountain View, CA, USA).

Protocols were performed according to the manufacturer's instructions. The reaction was stopped at 17 cycles. The amplified cDNA was then used for SSH.

2.4.4.2. Suppressive subtractive cDNA hybridization (SSH)

The tester was the cDNA of *in vivo-derived* cumulus cells at 6 hrs post-LH (oocyte at GVBD stage), whereas the driver cDNA came from *in vitro* cumulus cells that were collected at the slaughterhouse and kept on ice. These driver transcripts came from 1, 2 and 3 classes of immature COCs (GV) from 3- to 8-mm follicles. The subtracted cDNAs were ligated into the pGEM-T plasmid vector (Promega, Madison, WI, USA). The ligated product was then transformed into *Escherichia coli* DH5α-T1 cells (Invitrogen, Burlington, ON, Canada), plated on LB agar plates containing ampicillin (50 mg/ml) and X-gal (40 mg/ml), and incubated overnight at 37 °C. Recombinant white colonies were randomly selected and cultured in 96-well plates containing 150 µl of LB broth with 50 mg/ml ampicillin for 6 hrs at 37 °C with agitation. The clones were amplified in a 50-µl reaction using 1Uof HotMaster Taq DNA polymerase (Eppendorf, ON, Canada), 10 mM dNTPs, in1X buffer, and 30.8 µl of nuclease-free water, as well as 0.25 µM of nested primers 1 and 2R.

2.4.4.3. Sequencing and BLAST homology search

The PCR products were purified using the unifilter 384-well purification plates (Whatman, Clifton, NJ, USA) and then sequenced by using nested primer 1 with the ABI 3730 DNA sequencer and the ABI prism BigDye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Foster City, CA, USA). The resulting sequences were analyzed by using the Chromas software (Chromas), then blasted using cDNA Library Manager, a server of Genome Canada Bioinformatics that allows clone identification using the Genbank database as reference (Vallee *et al.* 2006). The BLAST results including Genbank accession number, Unigene number, score, e-value, homologous nucleotide alignment and position of alignment were finally compiled in a report chart.

2.4.5. Microarray printing

A home-made microarray containing the PCR products of our partial library composed of randomly selected clones (n=405) was performed by using the VersArray ChipWriter Pro (BioRad, Mississauga, ON, Canada). Each selected clone was spotted in two replicates on GAPS II glass slides (Corning, Corning, NY, USA). Various controls were printed on the slide: SpotReport Alien cDNA Array Validation System (Stratagene) as negative controls, the *GFP* (green fluorescent protein) gene as an exogenous positive control, and housekeeping genes (*ACTB, GAPDH*) as internal positive controls. Slides were then cross-linked with UV rays according to the manufacturer's instructions. Quality-control printing was achieved with Terminal Transferase dye (Roche Diagnostics, Laval, QC, Canada) according to manufacturers' instructions.

2.4.6. Hybridization of cumulus cells collected at 6 hrs in vitro

2.4.6.1. Linear amplification of RNA

For the cumulus cells collected after 6 hrs of IVM, RNA amplification was achieved by using 2-round IVT (*in vitro* transcription) following the instructions of the RiboAmp RNA Amplification kit (Arcturus LCM Instruments, Molecular Devices, Sunnyvale, CA, USA). The amplification product was eluted in 30 µl of RNA elution buffer (RE) and 1 µl was used to quantify the aRNA amplification yield by using the NanoDrop (Wilmington, DE, USA).

2.4.6.2. Reverse transcription and cDNA precipitation labelling

Briefly, 3 µg of aRNA from each IVM treatment was reverse-transcribed by using 50 µM of random primers (Ambion), dNTP mix (10 mM dATP, dCTP, and dGTP, 3 mM TTP, 6 mM dUTP), according to the Superscript III RT kit (Invitrogen, Carisbad, CA, USA) instructions. An alkaline hydrolysis of the RT product with 1 N NaOH for 10 min at 70 °C was then performed, followed by neutralization with 1 N HCl. Twenty microliters of sodium acetate (3 M, pH 5.2) was then added to each tube, and the final volume was adjusted to 100 µl with nuclease-free water. Purification was performed by using the Qiaquick PCR purification kit (Qiagen, Maryland, USA) according to the manufacturers' instructions, except that the washing step was repeated three times, followed by isopropyl alcohol precipitation. Briefly, 3 µl of sodium acetate (3 M, pH 5.2) and 150 µl of isopropyl alcohol (100%) were added to each treatment before incubation for 30 min at -80 °C. The pellet was washed with 500 µl of 70% ethanol and resuspended in 5 µl of nuclease-free water.

Since the SOF without hormone treatment served as a hybridization reference, 9 µg of aRNA was reverse-transcribed and labelled.

2.4.6.3. Complementary DNA indirect labelling, purification and precipitation

The cDNA labelling was performed by using a two-step method with fluorescent Alexa Fluor 647 and 555 dyes (Molecular Probes, Eugene, OR, USA). Sodium bicarbonate (0.1 M, pH 9) was added to each treatment's amino allyl cDNA as a labelling buffer, and the mix (8 µl) was added to the Alexa Fluor dyes already dissolved in 2ul of dimethylsulfoxide (DMSO), and incubated in the dark for 1 hr. The 3 IVM treatments (PMA, FSH, and FSH+PMA) were labelled with the 647 dye whereas the control treatment (SOF without hormones) with 555 dye. Probes were purified by using a PCR purification kit (Qiagen, Maryland, USA) according to the instructions, washed three times and then eluted in 50 µl of nuclease-free water. Isopropyl alcohol precipitation was carried out as described above. The precipitated probes were resuspended in 8 µl of 10 mM EDTA for the 3 treatments and 24 µl for the control.

2.4.6.4. Quality and quantity of labeling assessment

A 1-µl aliquot of the final eluate of the purified labelled cDNA of each treatment was assessed on the NanoDrop. Each two differentially labelled probes (using Alexa Fluor 555 and 647 reactive dyes (Molecular Probes, Eugene, OR, USA)) used for hybridization on the same microarray slide were then mixed together in equimolar proportions.

2.4.6.5. Probe hybridization and microarray analysis

Three hybridizations were performed on the custom-made array containing the PCR-amplified cDNA inserts in order to select potential candidates for quantitative PCR validation:

- FSH (647) versus Control (SOF without hormones) (555)
- PMA (647) versus Control (555)
- FSH + PMA (647) versus Control (555)

Hybridizations were performed in the ArrayBooster by using the Advacard AC3C (The Gel Company, San Francisco, CA, USA) for 18 hrs at 55 °C by using Slide Hyb#1 (Ambion, Austin, TX, USA). The slides were then washed twice in 2X SSC/0.5% SDS buffer for 15 min at 55 °C and twice in 0.5X SSC/0.5% SDS buffer. Two quick final washes at room temperature in 1X SSC and water were achieved before a spin dry for each slide in a centrifuge at 900g for 5 min. The arrays were then scanned and analyzed by using the ChipReader and ArrayPro Analyzer software (Media Cybernetics, San Diego, CA, USA).

Microarray data was normalized (Loess normalization) by using the ArrayPro and background subtracted to eliminate uninformative data and to define a calculated threshold (*t*). This value was calculated as $t = m + 2$ x SD, where *m* is the mean of the negative-control raw data and SD is the standard deviation, and then used to select clones that were subsequently used in data analysis. Finally, 647/555 ratios were used to obtain a list of candidates of each hybridization, and common positive candidates between treatments (i.e., FSH vs PMA, FSH vs FSH+PMA, and PMA vs FSH+PMA) were selected for further analysis and quantitative real-time PCR validation.

2.4.7. Quantification by real time PCR

Non-amplified aliquots of our four biological replicates that were used for hybridization were analyzed by real time PCR. This quantification analysis was performed by using a two-step RT-PCR. The single-stranded cDNA templates for real-time PCR analysis were generated from 50 ng of non-amplified total RNA samples by reverse transcription using Sensiscript RT (Qiagen, Maryland, USA) and an oligo dT according to the manufacturer's instructions. Samples were diluted to 80 µl for subsequent real-time PCR in a LightCycler instrument (Roche, Germany) using the LightCycler FastStart DNA Master SYBR Green I (Roche), according to the manufacturer's instructions. Each gene was amplified with specific primers. Primer sets were designed using Primerquest software (Integrated DNA Technologies, Coralville, IA) (Primerquest). Primer sets are summarized in Table 2.1. Quantification analysis was based on a standard curve produced using series of 4 dilutions of a standard purification for each studied gene ranging from 2×10^{-4} to 2×10^{-7} ng/reaction. The following settings in the LightCycler PCR were used: a preincubation step (10 min at 95 °C) and a 3-step cycling amplification (50 cycles): denaturation (5 sec at 95 °C), annealing (5 sec at Tm of each primer sets), extension (20 sec at 72 °C). Melting curves were performed by cooling the samples down to 72 °C and then increasing the temperature to 95 °C (with a slope of 2 °C/sec). Results were quantified by using the relative quantification approach with the software provided by the manufacturer (Roche, Germany). *GAPDH*, β-actin (*ACTB*) and polyubiquitin were used as internal controls.

2.4.8. Statistical analysis

Each real-time PCR experiment was performed using 4 biological replicates and data were normalized using the GeNorm software (PRimerDesign Ltd., Southampton, UK) (GeNorm) as described by Vandesompele and al. (Vandesompele *et al.* 2002). Briefly, we used a set of three

candidates composed of *GAPDH, β-actin (*ACTB*) and polyubiquitin* to choose our reference genes. Using the GeNorm software, we obtained the most stable reference genes, i.e. *GAPDH* and *ACTB*, as well as the appropriate normalization factors using the the $2^{(-\text{Delta Delta C(T)})}$ method (Livak & Schmittgen 2001). The mRNA quantities of analyzed candidates were then divided by preceding normalization factor, transformed (log base 10), and a one-way ANOVA using GraphPad PRism4 software (GraphPad Software, San Diego, CA, USA) was performed. Multiple comparisons were performed with Tukey's test ($P < 0.05$).

2.5. RESULTS

2.5.1. Identification of differentially expressed clones using SSH

SSH between the tester transcripts (cDNA of *in vivo-derived* cumulus cells 6 hrs post-LH) and the driver cDNA (*in vitro* cumulus collected on ice) provided us with valuable differentially expressed genes that covered a wide range of maturation steps from medium-size growing follicles to 6 hrs post-LH stage. A total of 419 clones were sequenced and blasted against the known sequences in the GenBank database. After identification step based on nucleotide homologies, 300 different clones were selected and printed on slides. For slide description and clones printing, they were described elsewhere by Hamel and al.,(Hamel *et al.* 2008a).

2.5.2. Microarray analysis of in vitro maturation treatments

Each of the three treatments (FSH, PMA and (FSH+PMA)) was hybridized against the control treatment. Microarray normalized data were analyzed and candidates with the highest hybridization ratio were selected. Candidates common to two treatments or to all three treatments taken together were selected (Figure 2.2). Because of the poor RNA quality of the FSH+PMA IVM replicate when assessed with the bioanalyzer before amplification, another series of IVM replicates for the fourth IVM treatments had to be performed. To prevent bias, the control sample of the second series of IVM treatment replicates (named CTRL2) was added to the FSH+PMA sample in the real-time PCR validation step. Real-time PCR analysis was therefore based on CTRLI, FSH and PMA from the first series of replicates, and CTRL2 and FSH+PMA from the second series.

Microarray analysis provided us with a non exhaustive list of differentially expressed genes per treatment. Twenty-four candidates were common to the three IVM treatments (Table 2.2). These common genes represented 48%, 30% and 15.5% of the differentially expressed candidates in the

FSH, PMA and FSH+PMA treatments, respectively. Common candidates were chosen based on the ability of FSH, PMA, and FSH+PMA to increase oocyte competence and hence blastocyst yield. The three treatments seemed to induce the oocyte competence molecular process through the expression of a set of genes in cumulus cells. Therefore, suitable potential markers associated with oocyte competence should be differentially expressed in all three IVM treatments (Table 2.2). Some of these 24 common candidates were subjected to further RT-PCR validation.

2.5.3. Analysis of mRNA expression by real-time PCR

Real-time PCR quantification was used to validate differentially expressed genes in cumulus cells. Three main criteria were used to select clones to be confirmed by quantitative PCR in this study: 1) the hybridization ratio and the redundancy of each clone in our SSH library, 2) its function as a potential target of the FSH and/or the PKC pathways, and 3) a gene-candidate approach, i.e. certain candidates that were not in our library but were reported by others to be expressed in the cumulus of competent COCs. A total of 15 candidates were selected for real-time PCR validation: 7 candidates from the overexpressed genes that are common between our three IVF treatments (*HAS2, INHBA, TNFAIP6, HSP90AA1, THBS, EREG* and *HSP90B1*). The 8 other candidates were chosen according to the 2) and 3) criteria (*CD44, EGFR, BTC, PTGS2, PGR, SERPINE2, GREM* and *PTX3*) (figure 2.3). To reveal actual differences in gene expression, we used multiple comparisons statistical analysis (Tukey's test, α=0.05) to classify gene level expression among the four IVM treatments.

Analysis of the expression profiles of cumulus cells after 6 hrs of IVM revealed six major patterns of gene expression: (a) FSH-specific without additive effect with PMA; (b) PMA-specific without additive effect with FSH; (c) PMA-specific with additive effect with FSH; (d) both FSH- and PMA-specific with additive effect, (e) both FSH- and PMA-specific without additive effect, and (f) neither FSH- nor PMA-specific. The latter pattern contained two subgroups depending on whether or not the additive effect was significant.

Compared to control (hormone-free) treatment after 6 hrs of IVM, 43% of candidates (7 of 16 genes) were differentially expressed in the FSH treatment, including *HAS2* (α<0.05), *INHBA* (α<0.001), *TNFAIP6* (α<0.05), the hyaluronan receptor *CD44* (α<0.01), *GREM* (α<0.05), *EGFR* (α<0.01) and *BTC* (α<0.05). For PMA treatment, *PGR* (α<0.01), *HAS2* (α<0.01), *INHBA* (α<0.001), *GREM* (α<0.05), *EGFR* (α<0.01), the protein chaperone *HSP90AA1* (α<0.05), *THBS* (α<0.01) and *HSP90B1* (α<0.05) were the major differentially expressed genes. The additive

effect between FSH and PMA was studied via the IVM treatment PMA+FSH. Differentially expressed genes were significant compared to either FSH and/or PMA treatment and to the control treatment. This was the case of more than 45% of the candidates (Figure 2.3). Four candidates were not differentially expressed among the four treatments: *EREG* (p= 0.54), *PTX3* (p= 0.16), and *PTGS2* and *SERPINE2,* which were only differentially expressed in the FSH+PMA treatment compared to PMA and FSH, respectively (Figure 2.5).

2.6. DISCUSSION

2.6.1. SSH and library construction

Suppressive subtractive cDNA hybridization (SSH) has been used as a powerful tool to both isolate and identify cDNAs of differentially expressed genes. It has the advantage of enriching low abundance transcripts which are differentially expressed in the tester population (Diatchenko *et al.* 1996). For these reasons, we opted for SSH as a suitable tool for the realisation of our experience.

The role of cumulus cells in supporting oocyte maturation and competence acquisition is well established (Tatemoto & Terada 1995, Vozzi *et al.* 2001). Furthermore, the major transcriptional activity of the oocyte occurs during meiotic arrest before ovulation (GV) (Hyttel *et al.* 2001). In order to better understand the cumulus functions related to oocyte competence improvement, we looked for differentially expressed genes between cumulus cells collected *in vivo* at 6 hrs post-LH (tester) and those collected on ice (driver) at the slaughterhouse (cumulus of immature oocytes at GV stage) using SSH technology. The aim was to keep the driver's COCs at their minimal level of maturation and to avoid the beneficial effect of transportation in saline solution at 30 °C on oocyte competence (Blondin *et al.* 1997). On the other hand, cumulus cells collected following the LH surge (tester) are believed to contain the whole set of transcripts and proteins required to allow the final maturation of the oocyte. All these facts supported the idea that the 6-hr post-LH peak is the optimal moment to study gene expression in cumulus cells to find potential markers associated with oocyte competence.

2.6.2. Microarray analysis

Using the LH surge as reference, the corresponding time after aspiration to look for cumulus response was set at 6 hrs (Sirard *et al.* 1989). Four treatments were performed in order to explore, using microarrays, the genomic effect of FSH, PMA and their eventual additive effect

(FSH+PMA) during the first 6 hrs of IVM that enhances oocyte competence. The choice of these treatments was made according to previous findings in that shown that both rFSH (0.5 μg/ml) and PMA (0.1 μM) added at 6 hrs of IVM improved oocyte competence and yielded more than 45% of blastocysts (Ali & Sirard 2005). Since cumulus cells are essential for normal development *in vivo* and *in vitro* through continuous association with the oocyte, their pattern of mRNA expression might represent an efficient and indirect alternative to assess oocyte competence (Raman *et al.* 2001). Moreover, a positive correlation between specific gene expression in human cumulus and subsequent embryo development was reported (McKenzie *et al.* 2004). In addition to their usefulness in improving COC selection, these differentially expressed molecular signals may constitute a sign of oocyte competence or fulfilment of maturation; and their expression pattern is reported to be regulated by the oocyte itself in many species (Gilchrist *et al.* 2004, Feuerstein *et al.* 2006, Sugiura *et al.* 2007, Su *et al.* 2008). Because of this cross-dialog between cumulus and oocyte as well as the fact that FSH and PMA led to the same improvement in blastocyst yield, it was expected that common candidates revealed by these hybridizations would likely be associated with the molecular process of acquisition of competence.

The analysis of the physiological processes covered by common hybridization candidates revealed at least 7 major functions. Cell defense and regulation of apoptosis was one of the main cellular processes in which hybridization candidates were involved. This process includes anti-oxidative stress response, DNA repair, heat shock protection, and immune and anti-inflammatory responses (e.g., *GSTA1, UTMP, HSP90AA1, HIGD1A*). The other major cellular processes were signal transduction (*AKAP7, EREG*) and cell structure and communication, including extracellular matrix formation (*TNFAIP6, ADAMTS1*), cell adhesion and molecular export. In contrast, cell cycle was limited to only 4% of the common candidates (Figure 2.4). This cellular process distribution appears to make sense in light of cumulus cell differentiation and functions. In fact, apoptosis incidence in the outer layers of cumulus cells was reported as a morphological criterion correlated with oocyte competence enhancement (Blondin & Sirard 1995). Cumulus cells were also found to protect porcine oocytes against oxidative stress-induced apoptosis by enhancing glutathione (GSH) content in oocytes and to express many immune cell-related genes that are indispensable to the inflammatory-like response preceding ovulation (Tatemoto *et al.* 2000, Hernandez-Gonzalez *et al.* 2006). Thus, a cumulus cell expression pattern related to defense and apoptotic response may be considered as a sign of oocyte final maturation

progression just prior to ovulation (Shimada *et al.* 2006a, Stouffer *et al.* 2007). In the case of signal transduction, it has been demonstrated that cumulus cells represent a signalling interface between the oocyte and its surrounding environment. This cellular process is believed to play a key role in the oocyte competence acquisition through a functional signaling cascade in both the somatic and germinal compartments of the follicle (Sirard *et al.* 2007). Among the main documented functions attributed to cumulus cells were extracellular matrix expansion and "ovulation processes" (Richards 2005). This is supported by the fact that many candidates related to the cumulus expansion process were differentially expressed after 6 hrs of IVM. For cell cycle, cumulus cells are differentiated and specialized cells that divide in a very coordinated way, related to maturation progression. Their mitotic index is reported to decrease with maturation progression in mouse and to stop at around 8 hrs of culture (Hernandez-Gonzalez *et al.* 2006). The main common biological function in which these candidates are involved is reproduction or fertility in general, including oestrus cycle, cumulus expansion, ovulation, embryo nidation, development of early pregnancy and embryogenesis.

2.6.3. Real-time PCR analysis

The relative expression level of each candidate in each IVM treatment is summarized in Figure 2.5. Interestingly, we note the absence of a FSH-specific pattern with additive effect with PMA. This is a strong indication that the effect of FSH probably involves most of the pathways induced by PMA, while the converse is false. Under this assumption, the response to FSH appears to be more general and complete than the response to PMA. The PMA effect may be considered as a principal pathway included in FSH action on cumulus cells to reach oocyte competence.

The first pattern observed following FSH treatment and that included an FSH effect without a PMA's additive effect (Figure 2.3A) is illustrated mainly by *TNFAIP6*, *CD44*, *BTC* and *PTGS2*. *TNFAIP6* expression was detected under inflammatory conditions *in vivo*. It is induced by pro-inflammatory cytokines in a variety of cell types *in vitro* and has anti-inflammatory and chondroprotective effects (Wisniewski *et al.* 1993). The TNFAIP6 protein can also interact with molecules that form the backbone of the COC matrix such as hyaluronan (HA) through its Link module and inter-α-trypsin inhibitor (IαI) (Parkar & Day 1997, Carrette *et al.* 2001). Its expression was reported to occur upstream of PGE2, especially PTGS2 and/or PTGER2 (prostaglandin E receptor 2), in mice, and was induced by FSH through the cAMP/PKA/ERK (Ochsner *et al.* 2003a, Ochsner *et al.* 2003b) pathway, supporting our current findings. Moreover,

TNFAIP6-deficient mice (TNFAIP6$^{-/-}$) failed to assemble the extracellular matrix of COCs *in vivo* due to the lack of HA incorporation, and were infertile (Fulop *et al.* 2003). These characteristics suggest a key role of TNFAIP6 in the organization of the extracellular matrix and ovulation.

CD44 is a cell adhesion receptor with HA as one of its main ligands in the viscoelastic extracellular matrix. It is a normal component of extracellular matrix and fluids and is mainly involved in cell adhesion and/or migration, inflammatory responses and apoptosis inhibition (Kaneko *et al.* 2000, Lesley *et al.* 2004, Eshkar Sebban *et al.* 2007). Expression of the CD44 mRNA increased during bovine IVM of COCs under either LH or FSH+LH treatments (Schoenfelder & Einspanier 2003), possibly through the PKA/Raf/MEK1,2/ERK1,2 pathway, as proposed for the salivary gland (Yeh *et al.* 2005). Possible transactivation of receptor tyrosine kinases (mainly *EGFR*) after G-protein stimulation may also induce CD44 expression through Gαi- and/or Gβγ-mediated signalling pathway (Kraus *et al.* 2003). CD44 was suggested as a candidate related to bovine oocyte maturation *in vitro* (Furnus *et al.* 2003) and is differentially expressed in human cumulus of mature COCs compared to their immature counterparts (Ohta *et al.* 1999).

PTGS2 is an enzyme that catalyzes the conversion of arachidonic acid (AA) into PGH_2 and various specific prostaglandins (PG). It is involved in several female reproductive functions such as ovulation, fertilization, pregnancy, and parturition (Arosh *et al.* 2004). Its expression is induced by gonadotropins during the preovulatory stage. Furthermore, the interval from expression induction to follicular rupture was around 10 hrs in rat, cow and horse (Sirois & Dore 1997). *PTGS2-/-* mice exhibited impaired cumulus expansion and had severely reduced ovulation rates. *PTGS2* is essential to induce *TNFAIP6* expression in cumulus cells. In fact, *PTGS2* or *PTGER2* null mice exhibit an important decrease in *TNFAIP6* mRNA which leads to an impaired cumulus matrix and infertility (Ochsner *et al.* 2003b). In human cumulus cells, a 6-fold increase in *PTGS2* expression in competent COCs was reported, hence its proposed role as a marker of human oocyte competence (McKenzie *et al.* 2004). This increased expression was induced by either the PKA pathway or EGF, and was reported to be synchronized with cumulus expansion progression in bovine COCs (Nuttinck *et al.* 2002, Chen *et al.* 2007). Taken together, these data support the FSH-stimulated expression of *PTGS2* in bovine cumulus cells *in vitro* through PKA. *PTGS2* expression may be also amplified through EGF-like factors (*EREG, AREG, BTC*).

BTC belongs to the EGF family, as does *EREG*. Although there was an additive effect of FSH and PMA on EREG expression, it was not enough to be significant (data not shown). *BTC* expression was significantly higher in FSH than PMA without any additive effect. The expression of these EGF-like factors was triggered by gonadotropins, in particular LH through the activation of the cAMP cascade (Freimann *et al.* 2004). These growth factors induced the main morphological and biochemical events initiated by pituitary hormones, including cumulus expansion, oocyte maturation and luteinization, upon incubation with follicles (Park *et al.* 2004). These factors are therefore paracrine mediators that propagate the FSH and LH signals throughout the mammalian ovulatory follicles. Their expression in granulosa cells was associated with dominant follicles in rat and cattle (Robert *et al.* 2001, Conti *et al.* 2006). In mouse cumulus cells, their expression increased dramatically after 4 hrs of culture in FSH-enriched media, which required EGFR expression and was downstream of the p38MAPK pathway (Shimada *et al.* 2006a). *BTC* and *EREG* are thereafter synthesized as integral membrane precursors with a single transmembrane domain and, in order to be biologically active, they must be cleaved by members of the A Disintegrin And Metalloproteinase (ADAMs) family. The mature proteins are subsequently able to bind to EGFR and activate downstream pathways, mainly ERK1/2 (Ben-Ami *et al.* 2006a). They were also reported to act through the cAMP/PKA pathway (Johansson *et al.* 2004). Our data confirms the expression of these factors in cumulus cells and reveals for the first time their differential expression following treatment with FSH. Their role in cumulus expansion, steroidogenesis, and ovulation (Sekiguchi *et al.* 2004) indicates *BTC* and *EREG* are putative indicators of further development competence. Based on our results, the FSH-specific expression of *BTC* in cumulus *in vitro* suggests it is a more likely marker of oocyte maturation progression than *EREG*.

The second pattern (Figure 2.3B) we observed included genes induced specifically by PMA without additive effect with FSH. Representative genes include *THBS* (thrombospondin) and *HSP90B1* (heat shock protein 90kDa beta, member 1*)*. *THBS* belongs to a family of five multidomain glycoproteins and is involved in cell-cell and cell-matrix interactions through a calcium-dependent pathway, and in angiogenesis inhibition (Adams 2001). In the rat ovary, *THBS* expression was induced by FSH in cultured granulosa cells compared to control (Petrik *et al.* 2002) and was suggested to maintain avascular granulosa in mammal preantral follicles (Greenaway *et al.* 2005). The *THBS* expression pathway was reported to be induced by PMA

and/or PKC pathway in hepatocarcinoma cell lines (Kim *et al.* 2001). In fact, PMA is an inducer of the PKC pathway through a DAG-like process which leads to IP3-calcium release from the endoplasmic reticulum (ER) or the extracellular media. These data, taken together with our results, suggest important roles for *THBS* in cumulus cells such as angiogenesis control and extracellular matrix stabilization. PMA and more generally the PKC pathway appear to be the appropriate inducer of THBS expression in bovine cumulus cells *in vitro*.

HSP90B1 (also known as *TRA1)* and HSP90AA1 are two chaperones that belong to the HSP90 family and are involved in many cellular functions such as protein folding, cell signaling, transcription, kinase regulation, and DNA replication and repair (Richter *et al.* 2007). Our results showed that these two chaperones share similar expression patterns, with a significant effect of PMA and not of FSH. The additive effect of FSH and PMA was significant only for *HSP90AA1*. This supports a slight improvement of their expression under FSH treatment. Given that PMA is a potent activator of PKC and may activate inflammation-related events *in vitro*, its action in the cumulus may be the result of increased HSP90AA1 expression and mRNA stability, as reported in human monocytes (Jacquier-Sarlin *et al.* 1995). *HSP90B1* has also some specific functions such as folding and assembly of proteins in the ER, monitoring of protein transport through the cell and protection against cell death (Yang & Li 2005). In bovine dominant follicles, *HSP90AA1* was proposed as a regulator of follicular maturation through its action on aromatase (Driancourt *et al.* 1999). *HSP90B* was also found to be expressed on the sperm surface, which may mediate its interactions with cumulus cells, the extracellular matrix, and the oocyte plasma membrane during fertilization (Stein *et al.* 2006). The association between the oocyte maturation process and chaperone expression seems to be necessary to protect the oocyte, prevent cumulus cell apoptosis, support signal transduction and prepare the inflammatory-like ovulation process.

In addition to *HSP90AA1*, PGR is the other candidate from the pattern (Figure 2.3C). *PGR* expression is induced by gonadotropins, mainly LH, hCG or PMSG. It was reported to be essential in reproduction and particularly in the ovulatory process through stimulation of the expression of enzymes crucial to ovulation such as ADAMTS1 and CTSL (cathepsine L), and inhibition of cell apoptosis (Robker *et al.* 2000). *PGR* is differentially expressed in the granulosa of periovulatory follicles (Shao *et al.* 2003). Despite previous findings indicating the absence of *PGR* expression in the cumulus before the LH surge (Teilmann *et al.* 2006), our results indicate it is expressed in cumulus cells *in vitro*, especially following PMA treatment. This effect was

additive when PMA was combined with FSH. The detailed expression pathway of *PGR* is still unknown.

SERPINE2 is a proteinase inhibitor regulated mainly during the transcriptional process. Its expression increased with follicle growth progression and was maintained in the corpus luteum. *SERPINE2* mRNA is regulated in a spatio-temporal pattern with highest levels in granulosa cells of growing dominant bovine follicles and follicle fluid (FF) (Bedard *et al.* 2003). This supports the hypothesis that high expression of *SERPINE2* may contribute to final follicular growth and therefore increased oocyte competence. It is also involved in the remodeling of the extracellular matrix (ECM) of bovine granulosa cells during antral follicle growth and may have an anti-apoptotic role (Cao *et al.* 2004). This chronology of expression suggests an important role of SERPINE2 in cumulus ECM organization. Our data confirm the expression of *SERPINE2* in bovine cumulus cells *in vitro* downstream of the PMA pathway.

The next pattern (Figure 2.3D) is represented by *HAS2* which showed both FSH and PMA effects that were additive. In fact, HA is a glycosaminoglycan (GAG) with antioxidative activity. Its synthesis by mouse cumulus cells was first detected 2-3 hrs after gonadotropin stimulation, reached and maintained a maximum rate at 4-10 hrs, and declined and ceased by about 18 hrs (Tirone *et al.* 1997). The main enzyme responsible for HA production by cumulus cells is HAS2 (Schoenfelder & Einspanier 2003). The timing of *HAS2* mRNA expression in cumulus cells correlates well with HA synthesis (Fulop *et al.* 1997). *HAS2* expression was also proposed to be under the control of GDF9 (Growth and differentiation Factor 9) (Gui & Joyce 2005) and considered as an important prerequisite for initiating HA-mediated effects during final oocyte maturation, as well as sperm-egg interactions (Schoenfelder & Einspanier 2003). *HAS2* is differentially expressed in the cumulus of human competent COCs (McKenzie *et al.* 2004). All these arguments suggest *HAS2* is a potential marker of oocyte competence. Concerning the expression pathway, our data confirmed the FSH stimulation of *HAS2* expression through cAMP/PKA (Tirone *et al.* 1997, Nagyova *et al.* 2004). We reported also an additional pathway via PKC that is probably mediated through EGFR tyrosine kinase activity. In fact, Tirone et al. (Tirone *et al.* 1997) reported the absence of any effect of EGF stimulation on cAMP levels in mouse cumulus cells, which supports the idea of two complementary pathways.

The pattern (Figure 2.3E), where we observed non-additive effects of FSH and PMA, contains three candidates: *INHBA, EGFR* and *GREM. INHBA* is one of two α:β heterodimers that are

members of the TGFβ (transforming growth factor β) superfamily. Measurement of *INHBA* serum levels during human follicle growth showed a basal level secretion that increased for 1-2 days throughout the FSH treatment during maturation progression. It was suggested that the serum levels of *INHBA* have a predictive value in monitoring ovarian stimulation treatment for IVF and hence to assess the rate of healthy oocytes for selection for IVF (Eldar-Geva *et al.* 2002). *INHBA* expression is differentially upregulated in granulosa cells of sheep and cattle competent follicles (Campbell & Baird 2001, Fayad *et al.* 2004). *INHBA* mode of action is not yet fully understood, although some models have been proposed.

EGFR is the receptor for EGFs (Epidermal Growth Factors). Its expression increased with progression of folliculogenesis due to gonadotropin stimulation, reached its maximum at the preovulatory stage and was modulated through the MAPK and PI3K pathways (Choi *et al.* 2005). It was also reported in rat granulosa cells and bovine cumulus (Park *et al.* 2004) which is consistent with our findings. Seven members including EGF, TGF-α (transforming growth factor-α), HB-EGF (heparin-binding epidermal growth factor-like growth factor), AREG, EREG and BTC were reported to be EGFR ligands (Harris *et al.* 2003). The PLC/IP2/PKC pathway is one of the main EGFR signaling pathways (Sun *et al.* 2005). Its intracytoplasmic domain has tyrosine kinase activity that induces PLC and therefore intracellular calcium, PI3 kinase and MAPK. EGF's anti-apoptotic effect and activation of the PI3K pathway through RAS-RAF-MEK-ERK were reported in pig cumulus cells (Yuan-Chen Wu *et al.* 2007). This MAPK/ERK signaling pathway, that is independent of PKA and downstream of EGFR, was also reported in chicken granulosa cells and in murine cumulus cells (Shimada *et al.* 2006a, Wang *et al.* 2007). Interestingly, EGFR amplifies both the FSH and the PMA gene expression pathways. Once expressed, EGFR can induce other pathways (whether by its autophosphorylation or when activated by cleaved EGF-like factors), mainly through ERK1/2 (Lee & Juliano 2002, Harris *et al.* 2003).

GREM is an antagonist of bone morphogenic protein (BMP) signalling, but not of the GDF9 pathway. Its expression was associated with competent oocytes in preovulatory human cumulus cells and reported to be downstream of the GDF9 pathway (McKenzie *et al.* 2004). The differential regulation of BMP through GREM is thought to contribute simultaneously to granulosa cell luteinization and cumulus cell expansion, and therefore to the final maturation of oocytes (Pangas *et al.* 2004). Considering that GREM inhibits BMP15, an oocyte-derived factor,

the oocyte exerts a meticulous control on gene expression in the somatic compartment, especially in cumulus cells (Pangas *et al.* 2004). GREM therefore reflects the BMP15 activity and was suggested to be a predictor of oocyte competence.

The last pattern (data not shown) includes candidates with neither FSH- nor PMA-specific effect. This pattern contains two subgroups according to whether there was a significant additive effect, such as *SERPINE2*, or no effect for all four IVM treatments, which was the case for *PTX3* and *EREG*. Both *SERPINE2* and *EREG* were discussed before. *PTX3* encodes an acute phase protein of 45 kDa that acts downstream of GDF9 (Bottazzi *et al.* 1997) and is expressed in anti-inflammatory responses (Garlanda *et al.* 2005). *PTX3* null mice were subfertile despite their ability to ovulate with impaired cumulus cell mucification (Varani *et al.* 2002). Its expression pattern was similar to *TNFAIP6*, and their co-expression seems to be crucial for matrix stability and mouse fertility *in vivo*. It was induced by primary inflammatory signals, hCG, FSH, cAMP and EGF depending on the cellular type, and was reported in mouse and human cumulus cells during the preovulatory phase *in vivo* (Salustri *et al.* 2004, Garlanda *et al.* 2005, Bottazzi *et al.* 2006). Recently, a direct interaction between PTX3 and IαI was reported (Scarchilli *et al.* 2007). This provided further confirmation of its essential role in cumulus viscoelastic matrix stabilization prior to ovulation. While Zhang et al. (Zhang *et al.* 2005) suggested PTX3 was a marker of oocyte quality and hence of its competence, our data reported a similar expression of *PTX3* in the cumulus following the four IVM treatments. This may be due to the difference in collection time of cumulus cells. While our real time PCR analysis was at 6 hrs of IVM, Zhang et al. used human cumulus cells matured *in vivo*. It is possible that *PTX3* expression continues to increase until ovulation in order to ensure maximal cumulus expansion and contribute to fertilization (Wisniewski & Vilcek 2004). In addition to the possible differences between species, there may be differences between *in vitro* and *in vivo* conditions. Thus, we speculate that PTX3 expression is basal or ubiquitous at 6 hrs *in vitro*, independently of IVM treatment. Under these conditions, PTX3 appears to be an inadequate marker of oocyte competence.

Candidates analyzed in the present study have provided us with the expression pattern of 15 genes. At least 8 of them could be potential candidates to predict oocyte competence (Figure 2.5). To our knowledge, these candidates are reported for the first time together in bovine cumulus *in vitro*. As mentioned before, the most interesting candidates are those with FSH-specific, PMA-specific and FSH+PMA additive effects. This conclusion emerges from the IVF results where the

three treatments gave statistically similar blastocyst levels. Thus, the different effects induced by FSH, PMA or both (FSH+PMA) are very important to better understand the contribution of these pathways, in particular PKC, to the process of oocyte maturation and subsequent fertilization. Based on our results, the major candidates expressed in cumulus cells *in vitro* at 6 hrs of IVM and that could constitute a valuable set of oocyte competence markers are *HAS2, INHBA, EGFR, GREM, BTC, CD44, TNFAIP6* and *PTGS2*.

EGFR is therefore a major contributing factor to PMA-induced transcription (Lee *et al.* 2002). STAT3 and SP1 are the main transcription factors activated downstream of PMA action (Hwang *et al.* 2007, Yuan-Chen Wu *et al.* 2007). Transcriptional activation by PMA should be achieved through two pathways, PKC/Ras/Raf/MEK/ERK or PKC/MEK/ERK. The latter pathway (Fan *et al.* 2004) may represent the vectors of PMA-specific, FSH-independent transcription. Additive effects between FSH and PMA could be modulated through the PI3K/Akt pathway (Figure 2.6).

Extending these studies to identify differentially expressed genes *in vivo* will be a valuable tool to compare with candidate genes identified *in vitro*. Together, these findings will contribute to a better understanding of the cumulus contribution in the molecular mechanisms that lead to oocyte competence acquisition. The differentially expressed genes may be important markers of the oocyte's ability to reach the blastocyst stage and allow direct assessment of the fertility potential of an individual oocyte without compromising its integrity.

2.7. ACKNOWLEDGMENTS

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2.8. REFERENCES

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2.9. FIGURES

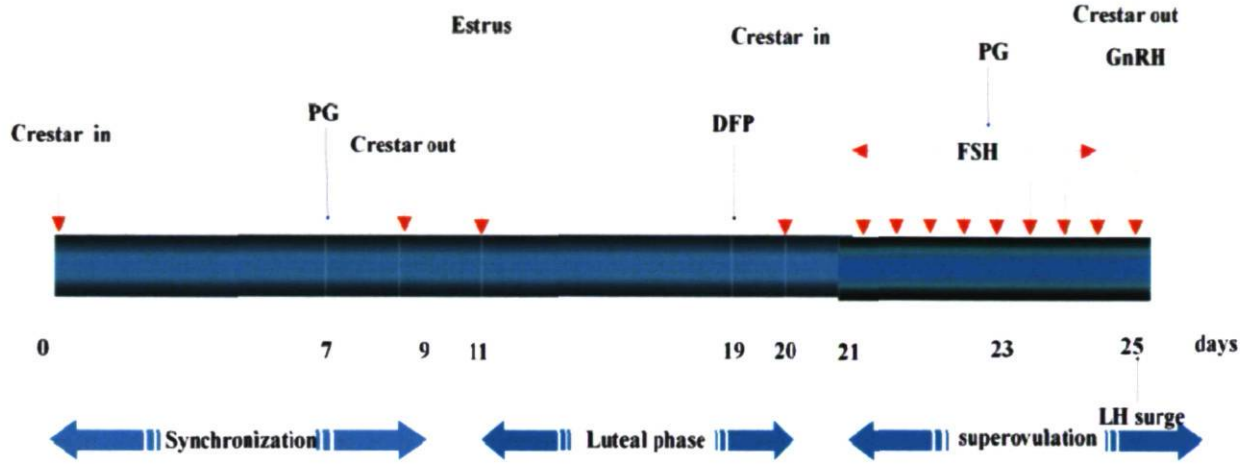

Figure 2.1: Schedule of treatments for superovulation in cows with a fixed LH surge to collect in vivo *matured COCs (6 hrs post-LH). FSH: administration of eight consecutive, decreasing doses of FSH; PG: administration of prostaglandin; GnRH: administration of GnRH; LH surge: the time at which peak GnRH-induced LH surge occurs; DFP: dominant follicle punction.*

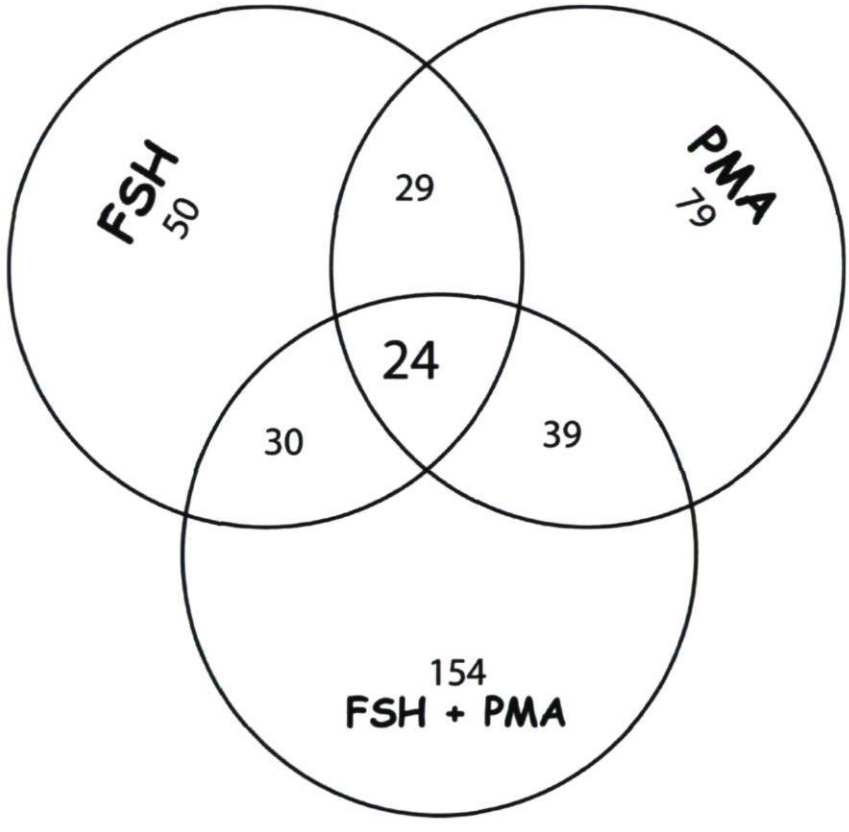

Figure 2.2: Summary of common hybridization candidates between the three 6-hr IVM treatments FSH, PMA and (FSH+PMA).

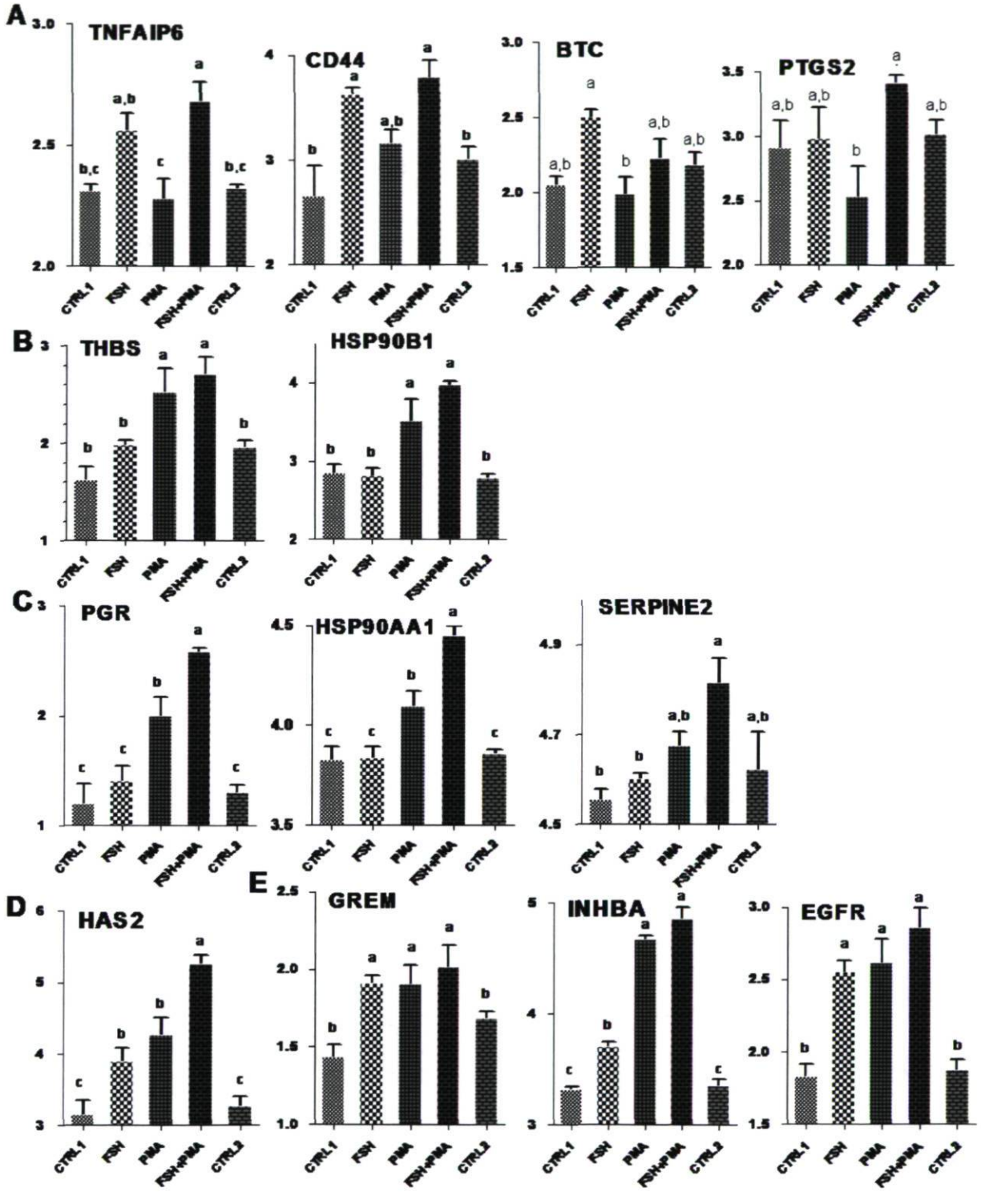

Figure 2.3: Cumulus expression patterns at 6 hours of IVM of HAS2, INHBA, EGFR, CD44, TNFAIP6, GREM, BTC, PTGS2, THBS, PGR, HSP90AA1, HSP90B1 and SERPINE2. Means' multiple comparisons to classify gene level expression of each gene among the four IVM treatments was achieved using Tukey's test at $\alpha=0.05$. $^{a-c}$values with different superscripts are significantly different ($P< 0.05$).

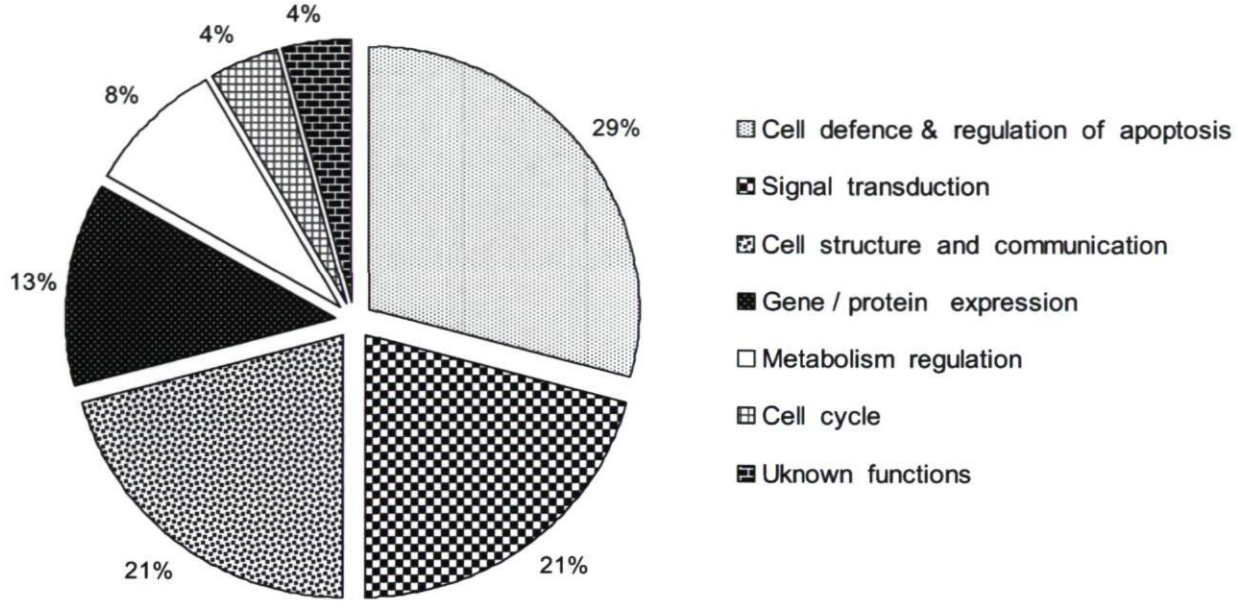

Figure 2.4: Main cellular processes of differentially expressed candidates common to the three IVM treatments FSH, PMA and (FSH+PMA) at 6 hr revealed by hybridization

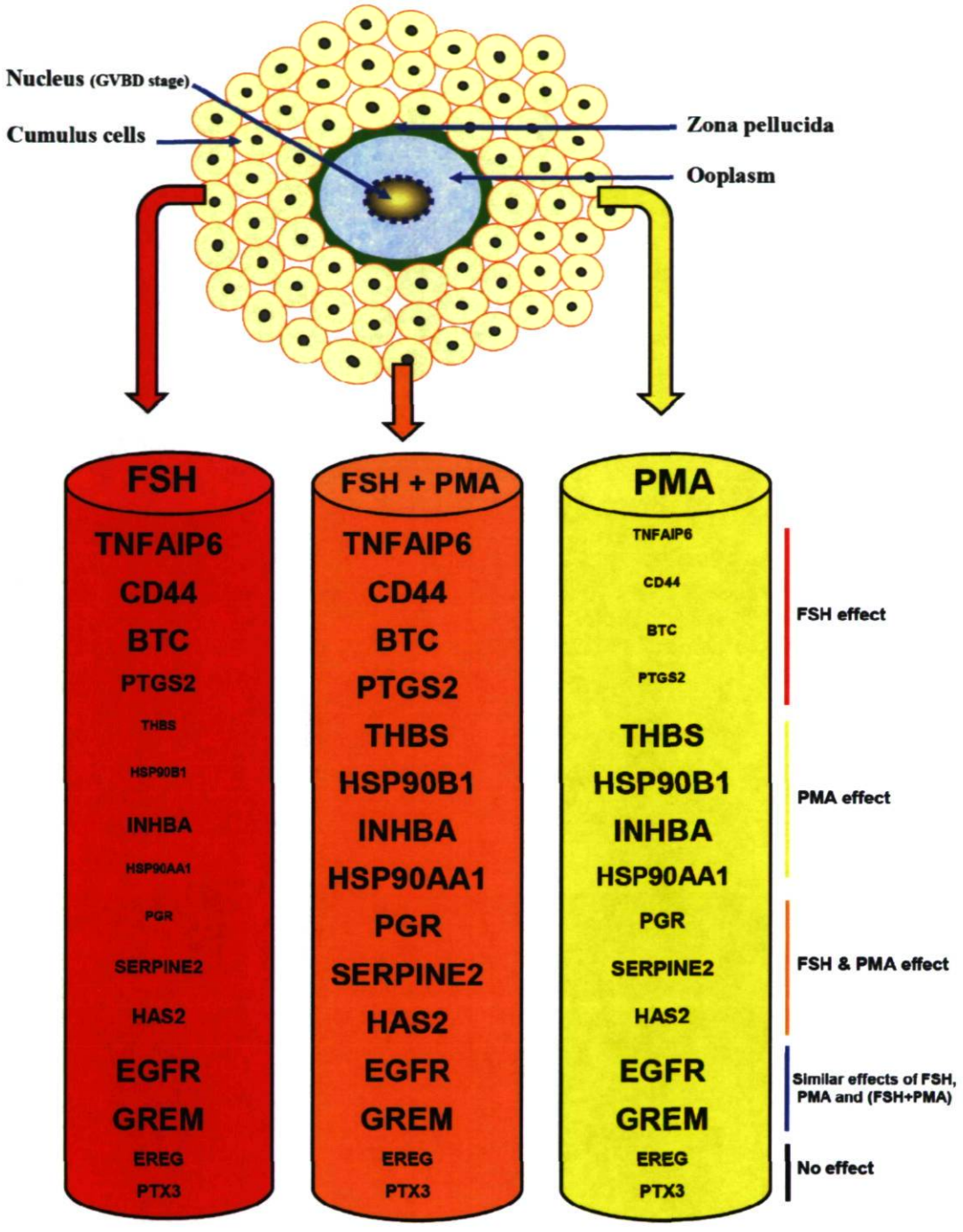

Figure 2.5: Differentially expressed candidates in cumulus cells at 6 hrs of IVM with three treatments: FSH, PMA and (FSH+PMA) revealed by RT-PCR. Bold type and font size are correlated with the expression level of each candidate in the corresponding treatment.

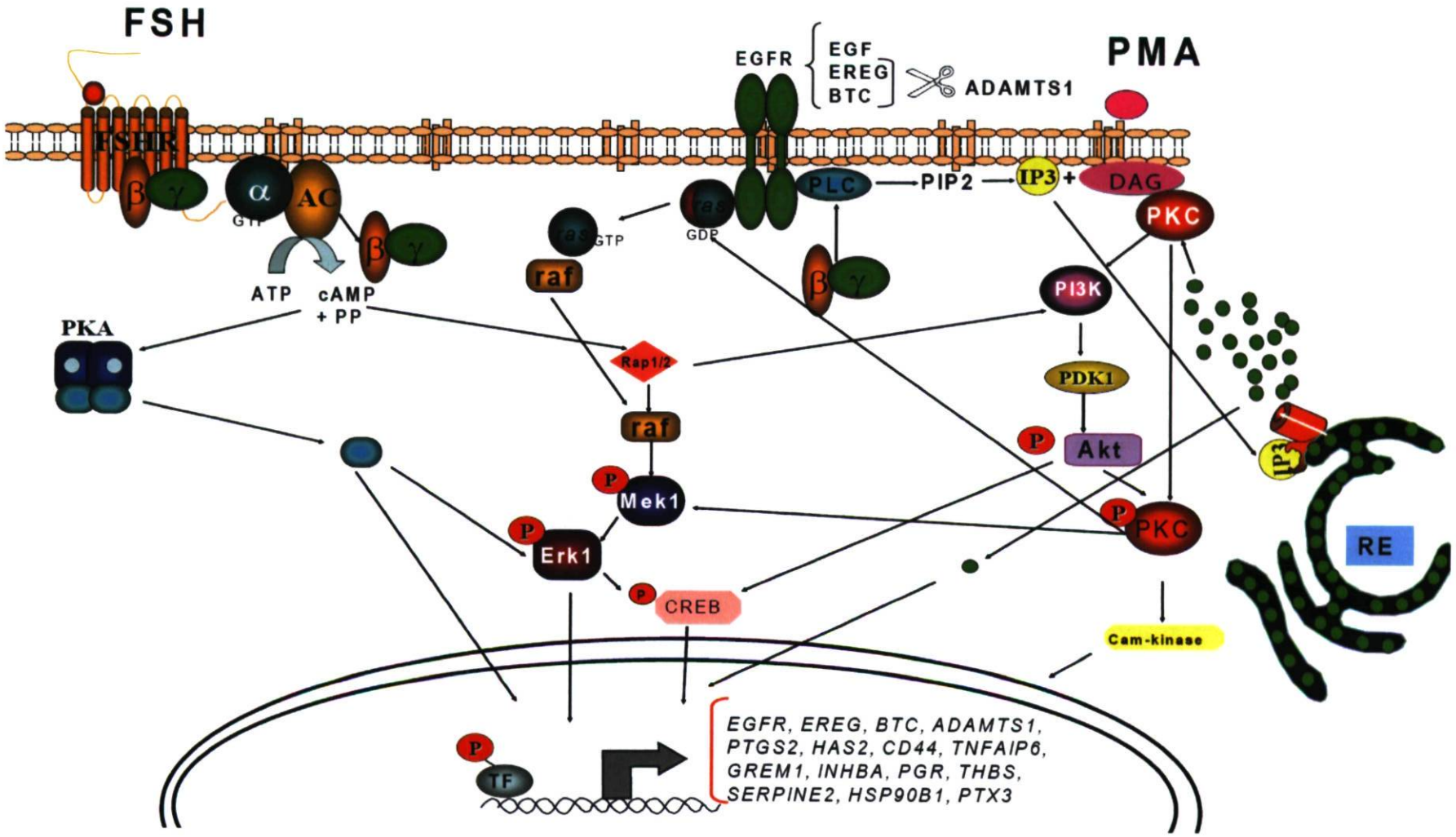

Figure 2.6: Potential pathways of differentially expressed genes in bovine cumulus cells after FSH and/or PMA IVM treatments

2.10. TABLES

Table 2.1: Sequences of specific primers of candidates used in real time PCR quantification

Gene name	Full name	Unigene accession	Primer set (5'-3')	Annealing temperature (°C)	Reference (if applicable)
PTGS2	prostaglandin-endoperoxide synthase 2	Bt.15758	Up 5'-CATGGGTGTGAAAGGGAGGAAAGA-3' low 5'-CCTTAGTGAAAGCTGGTCCTCGTT-3'	58	
EREG	epiregulin	Bt.63437	up 5'-GTGTGGCTCAAGTGTCAATAAC-3' low 5'-TCGATTTCTGTACCATCTGC-3'	53	
GREM	gremlin	Bt.41128	up 5'-AACAGCCGTACCATCATCAAC-3' low 5'-TTCAGGACAGTTGAGAGTGACC -3'	55	
PGR	progesterone receptor	Bt.13036	Up 5'-GTCGCCTTAGAAAGTGCTGT-3' low 5'-CACATCTGGTTCAATGCTCA-3'	53	
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	Bt.91085	up 5'-GTCTGGCAAATACAAGCTCACCT-3' low 5'-GGATCTGTAAACACACCACCACA-3'	57	
ACTB	actin, beta	Bt.14186	up 5'-CGTGACATTAAGGAGAAGCTGTGC-3' low 5'-CTCAGGAGGAGCAATGATCTTGAT-3'	57	
BTC	betacellulin	Bt.236	Up 5'- TACCACCACACAACCAAAGCGAAG- 3' low 5'- CGTTTCCGAAGAGGATGACAGCAT- 3'	59	
CD44	CD44	Bt.5494	Up 5'- GATGGTGCATTTGGTGAACAAGG-3' Low 5'- TGAAGTGTCCCAGCTCCCTGTAAT- 3'	58	
EGFR	epidermal growth factor receptor	Bt.6422	Up 5'- ACCACCCATCCTGCCTGTATCAAT-3' Low 3'- TGCCCAAACGGACAACATTCTTCC- 3'	60	
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Bt.87389	Up 5'-CCAACGTGTCTGTTGTGGATCTGA-3' Low 5'- GAGCTTGACAAAGTGGTCGTTGAG-3'	57	
HAS2	hyaluronan synthase 2	Bt.63577	Up 5'- ATAAATGTGGCAGGCGGAAGAAGG-3' Low 5'- GTCTTTGTTCAAGTCCCAGCAGCA – 3'	60	
HSP90AA1	heat shock protein 90kDa alpha, class A member 1	Bt.61915	up 5'-GTATGGACAATGACTCCAATCAAGT-3' Low 5'-CCGTTTGTTGTAAGGTGTGTATGTA-3'	55	(Dode *et al.* 2006)
INHBA	inhibin, beta A	Bt.12760	Up 5'-TCCTTTCACTCGACGGTCATCAAC-3' Low 5'-TGTCTTCGTGTCACCACTGTCTTC-3'	58	
PTX3	pentraxin-related gene 3	Bt.7541	Up 5'- GGCAGACTCACAGGCTTCAATATC- 3' Low 5'- CCTTCTCCAGTCTCCCTTTCAACT -3'	57	
SERPINE2	serpin peptidase inhibitor, clade E	Bt.13676	Up 5'-TCCGTGACGTTGCCCTCTGTG-3' Low 5'-CCGTGATCTCCACAAACCCTT-3',	57	(Bedard *et al.* 2003)
THBS	thrombospondin	Bt.5301	Up 5'- CTACATTGGCCACAAGACAA- 3' Low 5' – AGCAAGAGGTCCACTCAGAC- 3'	53	(Weiss *et al.* 2004)
HSP90B1 (TRA1)	heat shock protein 90kDa beta, member 1	Bt.8686	Up 5'-TGGCAGAGACCATCGAAAG-3' Low 5'-GGTAACTTCCCCTTCAGCAG-3'	55	(Rottmayer *et al.* 2006)
GSTA1	glutathione S-transferase A1	Bt.227	Up 5'- TCCAGCAAGTGCCAATGGTTGA -3' Low 5'- ATTTCACCCAAATCTGCCACACCC -3'	57	

Table 2.2: Common differentially expressed candidates in the cumulus cells of the three 6-hr IVM treatments FSH, PMA and (FSH+PMA) revealed by microarray hybridizations.

	Gene name	Full name of Gene / Protein	Accesion No.
1	*ADFP*	*Bos taurus* adipose differentiation-related protein	NM_173980
2	*ATP6V1C1*	*Bos taurus* ATPase, H+ transporting, lysosomal 42kDa, V1 subunit	NM_176676
3	*GSTA1*	*Bos taurus* glutathione S-transferase subunit isoform I	U49179
4	*HSPA8*	*Bos taurus* heat shock 70 kDa protein 8	NM_174345
5	*HNRPF*	*Bos taurus* heterogeneous nuclear ribonucleoprotein F	NM_001014860
6	*HSP90AA1*	*Bos taurus* hsp90alpha mRNA, class A member 1	AB072368
7	*HAS2*	*Bos taurus* hyaluronan synthase 2	NM_174079
8	*INHBA*	*Bos taurus* inhibin, beta A (activin A, activin AB alpha polypeptide)	NM_174363
9	*SELK*	*Bos taurus* similar to selenoprotein K	NM_001037489
10	*TNFAIP6*	*Bos taurus* tumor necrosis factor, alpha-induced protein 6	NM_001007813
11	*HSP90B1*	*Bos taurus* tumor rejection antigen 1,	NM_174700
12	*UTMP*	*Bos taurus* uterine milk protein precursor	NM_174797
13	*AKAP 7*	*Bos taurus* similar to A-kinase (PRKA) anchor protein 7	XM_864553.2
14	*EREG*	*Bos taurus* epiregulin (EREG),	XM_596732
15	*HIGD1A*	*Homo sapiens* HIG1 domain family, member 1A,	BC070277
16	*THBS*	*Bos taurus* thrombospondin (THBS)	NM_174196
17	*HSPA5*	*Bos taurus* 78 kDa glucose-regulated protein	GeneID: 281829
18	*YWHAZ*	*Bos taurus* tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_174814 .2
19	*ADAMTS1*	PREDICTED: *Bos taurus* similar to ADAMTS-1 precursor	XM_589626
20	*BTG2*	PREDICTED: *Bos taurus* similar to BTG2 protein	XM_586687
21	*LOC519739*	PREDICTED: *Bos taurus* hypothetical LOC519739	XM_597964
22	*SLC18A2*	*Bos taurus* solute carrier family 18 (vesicular monoamine), member 2	NM_174653
23	*SLC39A10*	PREDICTED: *Bos taurus* similar to solute carrier family 39 (zinc transporter), member 10	XM_599261
24	*ZNF643*	PREDICTED: *Bos taurus* similar to zinc finger protein 643 (LOC529132),	XM_607572

CHAPTER 3

Cumulus cells gene expression following the LH surge in bovine preovulatory follicles: potential early markers of oocyte competence

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3.1. RÉSUMÉ

Les cellules du cumulus sont nécessaires pour permettre à l'ovocyte d'acquérir une compétence optimale au développement. Il est aussi établi que beaucoup de grandes propriétés fonctionnelles des CCs sont induites par les gonadotrophines et régies par l'ovocyte. Par conséquent, le cumulus est donc capable de refléter la qualité des ovocytes et il est d'ailleurs souvent utilisé dans leur sélection. La fonction la plus visible des CCs est leur capacité d'expansion suite au de pic de LH. Encore partiellement inexpliquée, la LH induit la maturation finale et améliore la qualité des ovocytes. Pour étudier les voies de signalisation de la LH ainsi que les cascades d'expression génique au moment de la GVBD, les CCs bovins furent collectées à 2 heures avant et 6 heures après le pic de LH puis hybridées sur une biopuce-maison afin de mieux comprendre l'action génomique de LH et de trouver des gènes différentiellement exprimés associés à l'induction par la LH de la maturation finale des ovocytes. Une analyse génomique fonctionnelle des 141 clones surexprimés et les 161 sous-exprimés a été effectuée en fonction de leurs fonctions moléculaires, des réseaux de gènes (gene networks) et des compartiments cellulaires dans lesquels ils agissent. Après la validation par PCR quantitative de nos gènes candidats, leurs rôles potentiels dans l'action génomique de la LH sur les CCs, la maturation finale des ovocytes, l'ovulation et la fécondation sont discutés. Une liste de marqueurs précoces de la compétence ovocytaire *in vivo* et *in vitro* a été ensuite suggérée. De tels biomarqueurs sont une ébauche intéressante pour comprendre les voies moléculaires LH qui déclenchent le processus final acquisition de la compétence ovocytaire chez le bovin.

3.2. ABSTRACT

Cumulus cells are essential for oocytes to reach full development competency and become fertilized. Many major functional properties of cumulus cells are triggered by gonadotropins and governed by the oocyte. Consequently, cumulus may reflect oocyte quality and is often used for oocyte selection. The most visible function of cumulus cells is their ability for rapid extracellular matrix expansion after the LH surge. Although unexplained, LH induces the final maturation and improves oocyte quality. To study the LH signaling and gene expression cascade patterns close to the GVBD, bovine cumulus cells collected at 2 hours before and 6 hours after the LH surge were hybridized to a custom-made microarray to better understand the LH genomic action and find differentially expressed genes associated with the LH-induced oocyte final maturation. Functional genomic analysis of the 141 overexpressed and 161 underexpressed clones was performed according to their molecular functions, gene networks and cell compartments. Following real time PCR validation of our gene lists, some interesting pathways associated to the LH genomic action on CCs and their possible roles in oocyte final maturation, ovulation and fertilization are discussed. A list of early potential markers of oocyte competency *in vivo* and *in vitro* is thereafter suggested. These early biomarkers are a preamble to understand the LH molecular pathways that trigger the final oocyte competence acquisition process in bovine.

Additional Key-words: ovulation, oocyte quality, gene expression, cumulus, luteinizing hormone

3.3. INTRODUCTION

In large mammals, oocyte maturation is a prerequisite to fulfill the subsequent steps of embryo development (Krisher 2004). The ability to achieve such development, known as oocyte competence, is acquired mainly during the antral phase of folliculogenesis through well-coordinated molecular processes, including proper nuclear maturation and final cytoplasmic maturation (Sirard *et al.* 2003, Krisher 2004, Gilchrist & Thompson 2007, Kimura *et al.* 2007a). Successful oocyte final maturation is therefore a complex process that implies many factors such as intrinsic oocyte quality (Lonergan *et al.* 2003, Wang & Sun 2007), dialog with neighboring somatic compartment (Matzuk *et al.* 2002, Tanghe *et al.* 2002, Yokoo & Sato 2004, Gilchrist *et al.* 2008, Li *et al.* 2008), and functional gap junctions (Thomas *et al.* 2004, Ali *et al.* 2005, Lodde *et al.* 2007). It has been established that inside the follicle, the oocyte is surrounded by granulosa cell populations that, through folliculogenesis, differentiate into both mural granulosa cells (GCs) and cumulus cells (CCs). Cumulus cells are closer to the oocyte and were shown to maintain a proximity relationship with the oocyte, providing nutrients, maturation-enabling factors, and an optimal microenvironment to ensure successful maturation and further developmental competence (Eppig 1991, Pangas & Matzuk 2005, Gilchrist *et al.* 2008). The layers of differentiated granulosa cells that surround the oocyte are essential to oocyte maturation, ovulation as well as fertilization (Tanghe *et al.* 2002). Premature rupture of the communication between cumulus cells and oocyte affected the competence level (Modina *et al.* 2001). Such communication is ensured via cytoplasmic extensions of corona radiata cells that pass through the zona pellucida and exchange with oocyte during the whole maturation process (Allworth & Albertini 1993). The inhibition of these communicative junctions considerably affects bovine oocyte cytoplasmic maturation and hence the blastocyst rate *in vitro* (Ali *et al.* 2005). This is likely due to the lack of transfer of specific molecular signals that coordinate oocyte final maturation (Gilchrist *et al.* 2004, Lodde *et al.* 2007). Moreover, extracellular matrix (ECM) formation, cumulus cells differentiation, gene expression, metabolic activity and steroidogenesis in the preovulatory stage were reported to be governed by the oocyte in many mammalian species like mouse and pig (Salustri 2000, Lucidi *et al.* 2003, Su *et al.* 2008, Paradis *et al.* 2010). Conversely, inhibition of transcription and/or translation in the cumulus-oocyte complex (COC) impaired oocyte maturation and fertilization (Motlik *et al.* 1989, Sirard *et al.* 1989, Kastrop *et al.* 1991b, Tatemoto & Terada 1995). Optimal and reciprocal exchanges between oocyte and its

cumulus cells (CCs) are therefore a key factor to successful maturation, fertilization, and early embryo development. Based on this, it is thought that cumulus cells could be considered as a mirror that reflects the oocyte's level of competence and could thus be used as one of the main criteria for COC selection (Tanghe *et al.* 2002, McKenzie *et al.* 2004). This led us to focus on cumulus cells in order to increase our knowledge concerning the gene expression patterns in cumulus cells and contribute to the establishment of a non-invasive approach for oocyte quality prediction.

Other parameters, in particular the number of cumulus layers, their degree of expansion and/or apoptosis, oocyte diameter and ooplasm homogeneity, are also used in COC selection (Blondin & Sirard 1995, Patel *et al.* 2007). However, these criteria are subjective and lack the required precision to select highly competent oocytes that allow successful embryo development. Finding reliable tools that efficiently assess the oocyte quality is a prerequisite to predict its developmental potential. Alternative approaches were suggested to define molecular markers of oocyte competence that are expressed in the oocyte itself (Lonergan *et al.* 2003, Paradis *et al.* 2005) or in the surrounding somatic compartments (Robker *et al.* 2000, Ochsner *et al.* 2003, McKenzie *et al.* 2004, Assidi *et al.* 2008, Hamel *et al.* 2008b). Investigation of the molecular pathways of competence through the identification of potential candidates expressed in cumulus cells and associated with the oocyte quality is a key step to demystify the complex pathway of oocyte final maturation and competence acquisition. CCs gene expression patterns have the advantage of being a specific (for each oocyte) and non-invasive method that preserves oocyte integrity and allows its fertilization, early developmental and even subsequent transfer.

The capacity of the cumulus to support oocyte maturation during antral follicle growth requires two types of stimulation: oocyte factors GDF9 and BMP-15 (Lucidi *et al.* 2003, Pangas & Matzuk 2005), and gonadotropin stimulation with follicle stimulating hormone (FSH) and/or luteinizing hormone (LH) (Adriaens *et al.* 2004, Ali & Sirard 2005, Sirard *et al.* 2007). Additionally, gonadotropin signal transduction by the somatic compartment requires de novo mRNA synthesis within cumulus cells (Meinecke & Meinecke-Tillmann 1993). Most assisted reproductive technologies, including ovarian stimulation and *in vitro* maturation (IVM), are based on gonadotropins actions. The investigation of LH differential gene expression *in vivo* and its correlation with oocyte competence is the focus of our present study. In fact, LH was reported to be important to oocyte final maturation, ovulation as well as optimal fertilization and early

embryo development (Dieleman *et al.* 2002). This is achieved through activation of intrafollicular signaling and gene expression pathways leading to cumulus cells expansion, meiosis resumption, oocyte final maturation and ovulation in most mammalian species including mouse (Panigone *et al.* 2008), pig (Kawashima *et al.* 2008), cow (Hyttel *et al.* 1997, Dieleman *et al.* 2002), and human (Filicori 1999, Feuerstein *et al.* 2007). Although it has been established that LH triggers key functions in both oocyte and cumulus, there are questions about the presence of LH receptors (LHR) proteins in cumulus cells membrane, suggesting an indirect induction pathway (Peng *et al.* 1991) probably through the EGFR pathway (Panigone *et al.* 2008, Reizel *et al.* 2010).

In order to accurately explore the effect of LH, we used suppressive subtractive cDNA hybridization (SSH) as a powerful strategy to both isolate and identify cDNAs of differentially expressed genes. SSH also allows the enrichment of low-abundance transcripts that are differentially expressed in the tester population (Diatchenko *et al.* 1996). A custom-made library of differentially expressed genes revealed by SSH and associated to confirmed competence contexts was made (Assidi *et al.* 2008, Hamel *et al.* 2008b) and used in our microarray study.

The process of competence, which includes well space and time-coordinated sequences of molecular events required for the oocyte to fulfill maturation and gain the ability to pass through the stages of early embryo to blastocyst, is poorly understood. Its proper achievement requires the contribution of cumulus cells (Dieleman *et al.* 2002, Tanghe *et al.* 2002, Kimura *et al.* 2007a). Herein, we investigated the genomic patterns of two cumulus treatments collected *in vivo* from superstimulated cows at 2 hours before and at 6 hours after the LH surge. At 2 h pre-LH, the oocyte is still in prophase I and is, with its somatic environment, ready to start the final maturation step induced by the LH peak. It corresponds to the *in vivo* prematuration stage (Dieleman *et al.* 2002). At this moment, we assume that the cumulus cells gene expression machinery is set to receive the last major induction of oocyte final maturation (the LH surge). The 6 h post-LH time point corresponds to the beginning of the germinal vesicle breakdown (GVBD) of the oocyte and the transcriptional arrest (Dieleman *et al.* 1983, Sirard *et al.* 1989, Hyttel *et al.* 1997). From the LH surge until the GVBD, we believe that cumulus cells express key factors that are inducers or consistent markers of oocyte final maturation and therefore competency. The genomic study of gene expression pattern evolution from 2 h before to 6 h after the LH surge should yield insights about cumulus cells contribution to the final maturation process and provide powerful markers of oocyte quality. These cumulus cells at 6h post LH are

considered to be associated to high competent oocytes. In fact, our control treatment in Dr Dieleman laboratory using the same superovulation protocol yielded more than 60% of expanded blastocyst as reported previously (Dieleman *et al.* 2002). This high competence level could indicate that our *in vivo* cumulus cells are the site of expression of final markers of oocyte quality. The identification of these candidates may be a valuable contribution in our understanding of the molecular events of oocyte competence fulfillment and may serve as predictors of high quality oocytes.

3.4. RESULTS

3.4.1. Microarray data analysis

Ovarian stimulation was achieved in order to increase the number of follicles that reach maturity. After COC aspiration and cumulus cells isolation, total RNA of cumulus cells was extracted, amplified and hybridizations were performed. Our hybridization design consisted of twelve replicates (3 x 2 x 2) to be analyzed: 3 biological replicates, 2 technical replicates (dye-swap design) and 2 technical sub-replicates or blocks (SSH clones were printed twice on our custom-made microarray). Hybridization analysis of overexpressed and underexpressed clones at 6 h post-LH peak compared to 2 h pre-LH peak was therefore based on these 12 replicates. Correlation values between the log-intensities of the 12 replicates for each group were very high (all above 0.85). These findings were also confirmed by hierarchical clustering of replications (data not shown). A minimum cutoff limit of 2.25 allowed us to filter data, lower the error variance, and select candidates with higher probabilities to be true positives (fold change > 1.5). Using the National Institute of Aging (NIA) array analysis tool at 5% false discovery rate (FDR), we obtained two lists of 141 overexpressed and 161 underexpressed clones that were affected by the LH surge. These clones represent respectively 63 upregulated and 112 downregulated separate genes whose expression level vary significantly at 6h post LH compared to the 2h preLH. The complete gene lists are provided (Tables 3.3 and 3.4, supplemental data).

3.4.2. Functional genomic analysis

Using the IPA software, functional genomic analysis was performed to investigate of the main molecular functions (Figure 3.2) related to our gene lists. Among the molecular pathways triggered by our differentially expressed genes, protein biosynthesis and maturation (gene expression, protein synthesis, protein folding, post-translational modification), cell differentiation (cell morphology, cell function and maintenance, cell signaling), molecular transport, and cell-to-

cell interactions (cellular assembly & organization, cell-to-cell signaling and interaction, cell-mediated immune response, nervous system function, antigen presentation) were the main general functions (Figure 3.2).

Using the IPA software, three gene pathway networks, with the highest scores, were selected for further analysis. The first network includes mainly candidate genes overexpressed (red colour) following LH/hCG action (Figure 3.3). On the other hand, the second network is composed of candidate genes (green colour) that were downregulated following the LH surge (figure 3.4). Concerning the third gene network selected, it included both overexpressed and downexpressed genes (Figures 3.5).

3.4.3. Real-time PCR validation

To validate both our positive and negative genes lists, 7 candidate genes (4 overexpressed and 3 underexpressed) were selected for real-time PCR validation. This QPCR validation was achieved on the initial biological replicates (pools of CCs) of the two (2 h before) and (6h after) the LH surge treatments groups (Figure 3.1). Following QPCR analysis, all the 4 overexpressed candidates assessed were statistically very significant ($p< 0.01$) (Figure 3.6A). For the 3 underexpressed candidates tested, two were statistically significant ($P<0.05$) (Figure 3.6B). The significant candidates are *THBS1* ($p<0.0001$), *EREG* ($p=0.0002$), *UBE2N* ($p=0.0043$), *TNFAIP6* ($p=0.0068$), *TRIB2* ($p=0.008$) and *ERRFI1* ($p=0.050$) (Figure 3.6).

3.5. DISCUSSION

3.5.1. Study approach

The choice of the 2 h pre- and 6 h post-LH peak time points was based on previous studies where oocytes exposed to LH *in vivo* had better chances of becoming viable embryos after fertilization (Blondin *et al.* 2002, Dieleman *et al.* 2002). The gonadotropins signal transduction, mainly LH, through the somatic compartment contributes to provide the oocyte with the required molecular tools and ingredients reserve to attain competence and undergo fertilization (Dieleman *et al.* 2002, Sirard *et al.* 2003, Sirard *et al.* 2007).

It is to note also that the ovarian stimulation used herein to collect our samples is an established stimulation protocol previously used in Dr Dieleman Laboratory. This protocol always yields around 60% of expanded blastocysts (Dieleman *et al.* 2002). This high rate of blastocyst with best grade allowed us to assume that our collected *in vivo* tissues are associated to high competent oocytes.

Additionally, finding efficient tools to measure oocyte competence and successful embryo development necessitates a better understanding of the *in vivo* molecular pathways induced following the LH surge. It is established that CCs are beneficial for the oocyte maturation, fertilization and later embryo development in many mammalian species including mouse (Zhou *et al.* 2010), bovine (Assidi *et al.* 2008) and human (Russell & Robker 2007, Anderson *et al.* 2009). Keeping in mind that most of the transcriptional activity of the oocyte occurred during meiotic arrest before the GVBD (Hyttel *et al.* 2001), the study of the CCs gene expression patterns at the GVBD stage (6h post LH) could reflect the oocyte quality. In fact, the oocyte at this stage has acquired most if not all the transcript stockpile needed for its final maturation and could therefore, through its continuous and intimate communication with its neighbouring cells, trigger the expression of some potential and indirect biomarkers in CCs. In addition to their usefulness in improving COC selection, the study of these potential biomarkers' pathways is in the scope of this study in order to yield insights into the complex molecular of competence that remains still ambiguous.

Following a solid statistic design based on 12-highly-correlated replicates, the microarray data analysis yielded 141 overexpressed and 161 underexpressed clones (Tables 3.3 and 3.4, supplemental data). RNA profiles from 2 h pre- versus 6 h post-LH peak were analysed using a functional approach according to their cellular and molecular functions, as well as the corresponding cell compartments.

3.5.2. Real time PCR analysis

In order to strengthen our gene lists provided by microarray analysis, seven candidates were chosen for real-time PCR validation. The relative expression level of each candidate in each treatment (2h before versus 6h after LH) is summarized in figure 3.6. Among the candidates selected, over 85.7% of them were statistically significant. This high level of fidelity between microarray and QPCR data could be explained by our hybridization design based on twelve replicates (3 biological replicates X 2 technical replicates (dye-swap) X 2 technical sub-replicates (2 blocks/chip)). Correlation values between the log-intensities of microarrays replicates were very high ($r^2 > 0.95$). Our results are in line with what is recommended in microarray analysis to get relevant data that highly correlates with further real-time PCR validation, and therefore true biological differences (Gupta *et al.* 2008).

3.5.3. Cellular and molecular functions analysis

Many cellular and molecular functions were statistically significant (over the threshold) following the LH surge. As expected, cumulus cells start showing high degree of cell differentiation accompanied by slight apoptosis. In fact, the cumulus cells are very specialized cells that undertake final differentiation after the LH peak. This differentiation phenotype included cell morphology, cellular maintenance and development (Figure 3.2); and was required to CCs contribution at oocyte final maturation and successful ovulation (Tanghe *et al.* 2002). In this context, the cell morphology pathway at the GVBD was associated to a change in CCs from round to polarized shape. Cytoskeleton arrangements were marked by the assembly of actin microfilaments leading to cytoplasmic projections' formation that may serve to cell-cell communications despite intensive mucification (Allworth & Albertini 1993, Sutovsky *et al.* 1994).

Protein translation and maturation is another interesting phenotype exposed by the CCs. It reinforces our knowledge of the CCs aforementioned differentiation status. It includes protein synthesis, nucleic and amino acids' metabolism, as well as post-translational modifications (Hernandez-Gonzalez *et al.* 2006). These findings are supported by the overexpression of genes associated to the transcription machinery (gene expression) (*BAMBI, PGR*...) and the cell cycle (*FOXO3, FGF2*...) (Figure 3.2). Moreover, expression of apoptosis-related genes in CCs reported herein was also associated to final oocyte maturation progression in several previous studies in many mammalian species (Host *et al.* 2002, van Montfoort *et al.* 2008); and were even suggested as an indicator to predict human oocyte quality (Lee *et al.* 2001). However, at this stage (GVBD) the CCs tissue maintains a proliferative phenotype (cell cycle and cellular growth functions) that prepares the prerequisite structure for rapid expansion and oocyte competence acquisition support. These findings do not match with the conclusions of Okazaki et al.,(2003) during their *in vitro* assays in the porcine species where the LH was reported to decrease the proliferative activity of CCs in culture. This may be due to timing of tissue analysis, the *in vitro* bias and species differences. Interestingly, the expression of some proliferative genes was reported to be induced by the oocyte (Hussein *et al.* 2005) and we can assume that may compensate/counteract the early apoptosis signs. This survival behaviour was also reinforced by the activation of cell morphology and cellular assembly functions. These functions involved the expression of the

extracellular matrix (ECM) genes that prevent or delay the follicular cells apoptosis (Kaneko *et al.* 2000).

This «mucification» process that distinguishes CCs is induced by the LH surge. Our differentially expressed genes at 6h post-LH triggered many molecular and cellular functions associated to this mechanism including cell morphology, cellular assembly and organization, connective tissue development, as well as cell-to cell signalling and interaction (Figure 3.2). They include crucial genes reported to be essential to ECM formation and oocyte competence like *TNFAIP6, EREG, SPRY2, PGR* and *HAS2* (Chen *et al.* 1993, Fulop *et al.* 2003, Ochsner *et al.* 2003a, Cillo *et al.* 2007).

Another remarkable pathway induced by LH is the metabolic activity. This activity includes the metabolism of amino acids, lipids, nucleic acids as well as carbohydrates. It appears to be crucial to CCs activity and their dialog with the oocyte in order to reach its full developmental potential (Thompson *et al.* 2007, Su *et al.* 2008). This metabolic pathway includes mainly glycolysis, steroidogenesis and Carbone metabolism (Krisher & Bavister 1999, Lucidi *et al.* 2003, Kwong *et al.* 2010, Sutton-McDowall *et al.* 2010).

Concurrently with their active differentiation, CCs are the site of intensive signalling pathways (both intra- and intercellular) (Figure 3.2). These signalling pathways, including many protein kinases as PKC, PKB, PKA, PI3K and ERK1/2, were activated in CCs *in vitro* or following the LH surge and are required to the oocyte final maturation as well as ovulation (Mattioli & Barboni 2000, Shimada & Terada 2001, Fan *et al.* 2004, Russell & Robker 2007).

Molecular transport is another important function that is overrepresented by our LH-induced genes in CCs. This pathway includes mainly genes involved in intracellular organelles' motility and molecular transport (*TUBA1B, TMED5, TSNAX, LMAN1, BCAP29, and NRP1*) (supplemental data). The expression of these factors looks to be necessary for CCs membrane and cellular organelles to maintain their morphology, their content and ensure their functions regardless of the rapid molecular events occurring following the LH surge. The transported cargo may be proteins, anions and cations. Interestingly, this cytoplasmic streaming may drive CCs polarization required during mucification and thereafter as reported by Sutovsky et al. (1994). In fact, authors have shown that following the gonadotropins stimulation, bovine CCs microtubules were associated to the Golgi, whereas the intermediate filaments combine with the lipid droplets

close to the nucleus. Similar findings were also reported elsewhere (Hernandez-Gonzalez *et al.* 2006, Assou *et al.* 2008, van Montfoort *et al.* 2008, Racedo *et al.* 2009, Tesfaye *et al.* 2009).

Finally, we should highlight that in addition to the LH stimulation, these changes in transcriptome patterns of CCs are also influenced by the oocyte-cumulus reciprocal dialog documented by other previous studies (Eppig *et al.* 1997a, Russell & Salustri 2006, Kimura *et al.* 2007a, Su *et al.* 2008)

3.5.4. Gene network analysis

To investigate CCs genomic behaviour following the LH surge, four important IPA gene networks of both overexpressed and underexpressed genes are discussed (Figures 3.3, 3.4 and 3.5). We aim at finding relationships between identified gene candidates, the cumulus functions and the established pathways in the cumulus-oocyte complex. The analysis of such pathways reveals interesting pathways in CCs that are crucial to the oocyte final maturation process. Moreover, the analysis of these networks has allowed the identification of several key functions accomplished by CCs according to their gene expression profile at the GVBD stage.

3.5.4.1. ECM formation and stabilization:

Following the LH surge, several genes involved in ECM formation including *EREG, THBS1, TNFAIP6, HAS2, PLOD2, PTX3* and other cell-adhesion molecules (CD9, integrins, laminin, and fibrinogen) were overexpressed. These candidate genes, illustrated mainly in figures 3.3 and 3.5 pathways, are good illustration of the signalling pathways that occur in CCs following the gonadotropin (LH/hCG, FSH) stimulation. In fact, LH induced some key genes known to be involved in CCs proper expansion mainly *TNFAIP6, EREG* and *HAS2*. It has been established that *TNFAIP6* is essential to the ECM stabilization through its covalent links between hyaluronan (HA) (backbone of the ECM) and inter-α-trypsin inhibitor (IαI)(Carrette *et al.* 2001). Its expression downstream the LH/hCG preovulatory surge has been confirmed in most mammals including mice (Hernandez-Gonzalez *et al.* 2006), bovine (Tesfaye *et al.* 2009) and human (Haouzi *et al.* 2009). Moreover, *TNFAIP6*-deficient mice (*TNFAIP6*$^{-/-}$) failed to form a stable ECM and are infertile (Fulop *et al.* 2003).

HAS2 is also a key enzyme for ECM structure through its induction of HA production. Its expression correlates with the HA production and the ECM kinetic formation and was differentially expressed in human competent CC (McKenzie *et al.* 2004). Our network also shows that *HAS2* and *TNFAIP6* are activated by the EGF-like factors pathway (mainly EREG in our

case, Figure 3.3) during final oocyte maturation. This LH stimulation of the EGF pathway is established in mammals (Park *et al.* 2004) and considered as main player in the gonadotropins signal transduction and amplification in CCs (Edson *et al.* 2009). The proper ECM construction requires additional immune-like factors as pentraxin (*PTX3*) (Bottazzi *et al.* 2006) and glucoproteins such as laminin, fibronectin and integrins that are involved in cell differentiation and migration, as well as cell-to-cell adhesion (Sutovsky *et al.* 1995).

CD9 is also another member of the tetraspanins family. It is known to be an important protein in the fertilization process through its involvement in egg-sperm fusion (Ito *et al.* 2010, Lefevre *et al.* 2010) and sperm-CCs interactions (Mattioli *et al.* 2009, Sutovsky 2009). Taken together, these constituents provide ECM viscoelastic properties crucial to oocyte ovulation and further fertilization (reviewed by (Zhuo & Kimata 2001, Tanghe *et al.* 2002, Russell & Salustri 2006)).

3.5.4.2. Ovulation preparation and inflammatory-like response:

Ovulation is one of the main functions associated to the LH preovulatory surge. It is a complex process marked by the rupture of the ovarian epithelium and the release of the COC into the fallopian tube (Hunter 2003). In fact, the LH surge induces rapid changes, mainly the CCs expansion, the ECM's water attraction, and hence the raise of antrum volume (Hunter 2003, Russell & Salustri 2006). Many crucial genes involved in this process are overexpressed in our data sets like *TNFAIP6, HAS2* and *PGR* (Figures 3.3 and 3.5). These candidates are involved in both CC expansion and proteolysis, required for ovulation (Tsafriri 1995, Tsafriri & Reich 1999, Espey & Richards 2002). The KO of these genes in mice resulted in impaired mucification and the inhibition/reduction of ovulation rates and therefore fertility (Lydon *et al.* 1995, Hess *et al.* 1999, Fulop *et al.* 2003, Mittaz *et al.* 2004, Sugiura *et al.* 2009). Moreover, *PGR* was even reported to be induced by LH (through MAPK) and suggested as crucial playmaker (hormone receptor and transcription factor) upstream the ovulatory process pathway (Robker *et al.* 2000, Richards 2005, Richards 2007, Kawashima *et al.* 2008, Kim *et al.* 2009).

Additionally, we report herein the expression of several inflammatory-like factors as *CD58, IL1, FTH1, THBS1, DNAJB6, IFN-alpha, TGF-beta* and *TNF* (Figures 3.4 and 3.5). These results are in agreement with previous studies where ovulation was considered as an inflammatory-like process finely regulated through CCs (Hernandez-Gonzalez *et al.* 2006, Richards 2007, Russell & Robker 2007). Recently, it was reported that IFN-alpha, which expression in follicular cells is

induced by LH, is involved in the rat preovulatory follicular differentiation (through PI3-K/ERK pathway) (Lee da *et al.* 2009).

Additionally, our gene networks showed that FGF2 which contributes in both the expression of these immune-like factors and CCs expansion (Figure 3.3) was recently suggested as biomarker of high quality human oocytes that leads to successful pregnancy (van Montfoort *et al.* 2008) and therefore involved in the developmental competence acquisition process.

Uterine milk protein (*UTM*P) is an interesting candidate that is reported for the first time to be upregulated following the LH surge (fold change > 7). It is a protein known to be expressed in the endometrium downstream the progesterone pathway. It is associated with important protease inhibition functions (protease functions similar to SERPIN's superfamily) and is believed to support early pregnancy through nutritional supply and auto-immune protection of the embryo (Moffatt *et al.* 1987, Ing & Roberts 1989, Khatib *et al.* 2007). It also seems to be involved in protein metabolism (Figure 3.2) by reducing the negative impact of the inflammatory-like environment (required for ovulation) in the immediate vicinity of the oocyte and therefore preserving its quality.

3.5.4.3. Steroid biosynthesis:

The analysis of our gene networks at 6h following the LH peak revealed many steroidogenesis-related genes including *HSD3B2, INHBA, PGR, HPGD* and *DHCR24* (Figures 3.3, 3.4 and 3.5). These patterns are indicative of increased steroïdogenic activity within CCs at the GVBD stage, and may reflect an oocyte control of this process (Vanderhyden *et al.* 1993). The steroids synthesis was significantly higher in the late preovulatory stage COC (Vanderhyden & Macdonald 1998). . The presence / absence of steroids in IVM –IVF protocols may affect the expected oocyte quality and therefore its developmental competence both *in vivo* and *in vitro* (Dieleman *et al.* 2002, Ali & Sirard 2002a). *PGR* (Figure 3.3) could play an essential role especially in CCs (Li *et al.* 2004) through an increased sensitivity to progesterone induced genes. The *PGR* is expressed in CCs and associated to the preovulatory stage in many mammals including mice (Hernandez-Gonzalez *et al.* 2006), bovine (Mingoti *et al.* 2002, Schoenfelder *et al.* 2003), pig (Lucidi *et al.* 2003) and human (Chian *et al.* 1999). The changes in steroid output and sensitivity, especially progesterone could be required not only for mammalian oocyte final maturation, but also for ovulation and subsequent embryo survival (Wise *et al.* 1994, Richards 2005, Christian & Moenter 2010, Lynch *et al.* 2010). Interestingly progesterone is also reported

to be useful to the human sperm acrosome reaction (Teves *et al.* 2009) and therefore could be useful for cumulus-oocytes complexes in the fallopian tube. The fine regulation of steroid profile is suggested to influence the granulosa differentiation and to increase IVF yields (Lucidi *et al.* 2003).

3.5.4.4. Cell signalling:

Cell signalling is one of the main functions governed by the LH-activated genes as shown by figure 3.2. The cellular signalling pathway included mainly signal transduction and intracellular signaling. *TNFAIP6, EREG, INHBA, PGR* (Figure 3.3) and uterine milk protein (*UTMP*) (supplemental data) were the major redundant candidates in our gene networks. While *TNFAIP6, INHBA, EREG* and *TNF* were reported previously as potential candidates induced by LH in CC in association with competence (Ben-Ami *et al.* 2006b, Feuerstein *et al.* 2007), we reported herein an interesting gene network (Figure 3.5) where the *TNF* seems to play a crucial and central role in CC expansion and steroidogenesis via its interactions with several known genes as *PTX3, TNFAIP6, GJA1* and progesterone. Moreover, the *TNF* alpha and its receptor type II were previously reported to be present in both human CCs and oocyte (Naz *et al.* 1997). More studies on this candidate are needed to better understand its contribution in the follicle differentiation.

It is widely known that cumulus cells-oocyte communications occurred mainly through the gap-junctions (Szollosi 1978, Carabatsos *et al.* 2000). These communications played important roles in the oocyte-somatic compartment cell signalling and decreased at the GVBD (Sutovsky *et al.* 1993, Mattioli & Barboni 2000). Such connections breakdown was suggested to be gonadotropin-dependent in pig COCs (Sasseville *et al.* 2009b). Our data confirmed these findings in the bovine at 6h post LH by the downregulation of a fundamental protein of these cell-cell connections: the gap junction protein alpha 1 (*GJA1*).

Progesterone receptor (PGR) is probably involved as a coordinator in the CCs intracellular signalling. It is an essential factor that is expressed through the LH/MAPK pathway and required for the oocyte final maturation and subsequent ovulation. It acts as a hormone-receptor as well as a transcription factor (Richards 2007, Kawashima *et al.* 2008). PGR knockout mice have impaired reproductive functions as an anovolatroy phenotype, troubles in sexual behavior and even inflammatory symptoms (Lydon *et al.* 1995). It is suggested to control the expression and/or the activity of key genes needed in the ovulatory process like key transcription factors,

proteases, cell-adhesion molecules and inflammation factors (Robker *et al.* 2000, Li *et al.* 2004, Kim *et al.* 2009).

The LH surge is believed to induce multiple intracellular signaling and second messengers in the follicle, including the protein kinase A (PKA), the phospholipase C (PLC) and inisitol triphosphate 3 (IP3) pathways. It is also known to increase the intracellular calcium (reviewed in (Russell & Robker 2007)). The Akt (Figure 3.3) is becoming phosphorylated in the follicular cells respectively in rat and bovine species following LH treatment (Carvalho *et al.* 2003, Fukuda *et al.* 2009).

Additionally, the ERK 1/2 pathway (Figure 3.3) is involved in the response to LH by the maturational events in mouse COC including cumulus expansion and oocyte meiosis resumption (GVBD) (Su *et al.* 2003). The ERK ½ is also suggested to phosphorylate several transcriptional factors implicated in the final maturation and the ovulation processes, including Fos, Myc, Stat3 and AP1 (Sharma & Richards 2000, Roux & Blenis 2004, Russell & Robker 2007). The AP1 for example (Figure 3.4) was confirmed as an essential transcription factor for the expression of the *TNFAIP6* gene in bovine granulosa cells just prior to ovulation (Sayasith *et al.* 2008).

EREG gene which is an EGF-like factor (Figure 3.3) is considered as major factors in competence acquisition via the amplification of the gonadotropin signal in cumulus cells (particularly LH *in vivo* and FSH *in vitro*) that leads to the oocyte final maturation and ovulation (Park *et al.* 2004, Ashkenazi *et al.* 2005, Assidi *et al.* 2008, Conti 2010, Su *et al.* 2010). Following the LH surge, it was demonstrated that EREG activates the EGFR in order to spread the ovulation signal (Park *et al.* 2004).

The *TGFB1* gene seems to triggered interesting pathways that should be explored further (Figure 3.5). This gene is member of the TGFB superfamily and was not present in our gene list. This gene candidate may therefore represent the oocyte control of the major molecular processes in CCs through some oocyte-derived paracrine factors mainly the TGFB members (BMP15, GDF9, FGF8, TGFB1…) (Galloway *et al.* 2000, Pangas & Matzuk 2005, Sugiura *et al.* 2007, Su *et al.* 2008). Recently, it was reported that the *TGFB -/-* mice suffered from severe perturbation including the LH synthesis, oocyte incompetence and early embryo arrest (Ingman & Robertson 2009), which is a further confirmation of its importance in the oocyte developmental competence acquisition.

3.5.4.5. Cumulus cells neuronal-like functions:

Following the LH surge, we showed up the overexpression of several neuronal-related genes as *THBS1* (Liauw *et al.* 2008), *FGF2* (Chen *et al.* 2010), *MYO1D* (Brown & Bridgman 2004), *SYNPO* (Vlachos *et al.* 2009) and *CD9* (Ishibashi *et al.* 2004) (Figures 3.3 and 3.5). Strikingly, the common functions ensured by these genes were mainly neuron plasticity, the neurogenesis (including the dendrogenesis and the axogenesis) and the synaptogenesis. These pathways were also reported to be finely controlled by steroids and particularly the estradiol (Fester *et al.* 2009). Some synaptic proteins, as SNAP25 (synaptosomal-associated protein; 25 KDa), were previously reported in the preovulatory and luteal GCs as well as the oocyte. This protein, which is a crucial element of the molecular machinery required for the neurotransmitter exocytosis, was overexpressed by FSH and estradiol, and maintained by the LH (Grosse *et al.* 2000). Moreover, it was demonstrated that some of the ECM matrix proteins of CC swere also involved in neuronal development (Moore *et al.* 2009).

In addition to their immune-like phenotype discussed before, CCs were also reported herein to have some neuronal-like functions confirming the previous study of Hernandez-Gonzalez *et* al. (2006). Other cytokines and neuronal factors were also shown to be differentially expressed within CCs, and therefore suggested as mediator of the ovulatory process in many mammal species including human (McKenzie *et al.* 2004, Zhang *et al.* 2005, Feuerstein *et al.* 2007), mouse (Shimada *et al.* 2006a, Richards 2007), rat (Espey *et al.* 2000) and bovine (Assidi *et al.* 2008).

Based on these gene expression reports, the CC plasticity could be clearly highlighted leading to crucial questions concerning the real pathways of communications between the oocyte and the surrounding CCs restricted until now to the gap junctions. Considering both the cytoplasmic extensions that meet the oocyte, and these immune- and neuronal-like factors, we can assume the possibility of signalling vesicles exchange through phagocytosis and/or synapses between CCs and oocyte. CCs might need an active transport process to carry specific molecules to their polarization site of action to ensure efficient and rapid biological effect. This hypothesis needs more investigation in future studies to enlighten the intricate and mysterious cumulus-oocyte communication ways.

3.5.5. Potential in vivo markers of oocyte competence

In previous work in our laboratory, we reported a set of potential markers of oocyte competence expressed in bovine cumulus cells *in vitro* with 3 different IVM treatments (FSH; PMA [phorbol myristate acetate]; FSH+PMA) (Assidi *et al.* 2008). Using the oocyte meiotic state (GVBD) as a reference, we compared herein the reported potential genes associated with oocyte competence and regulated by all three *in vitro* treatments (Assidi *et al.* 2008), with the genes overexpressed herein *in vivo* at 6 h after the LH surge. Potential candidates associated with oocyte competence that were upregulated in three IVM treatments and confirmed *in vivo* should offer more efficient markers to assess oocyte quality and maturation fulfillment. Table 3.2 summarizes the common candidates between the two contexts.

Interestingly, among the 25 candidates that were overexpressed in competence contexts *in vitro*, 16 were also overexpressed *in vivo* (64%). Some of these candidates were reported in other species and/or contexts as potential markers of competence, *e.g.*, *TNFAIP6* in mouse, porcine cumulus cells (Fulop *et al.* 2003, Nagyova *et al.* 2009), *HAS2* in human cumulus granulosa cells (McKenzie *et al.* 2004), *PGR* in mouse granulosa cells (Shao *et al.* 2003) and *INHBA* in bovine granulosa cells (Fayad *et al.* 2004).

SPRY 2 (sprouty homolog 2) is another interesting gene candidate that is overexpressed following the LH surge (Figure 3.3). Its overexpression in follicular cells was differentially associated to developmentally competent oocytes in mammals mainly the bovine (Robert *et al.* 2001) and human (Hamel *et al.* 2008b) species. This gene was furthermore shown to be induced by the FGF pathway and acts to reduce cell proliferation (Edwin *et al.* 2006) and to amplify the EGFR pathway (through MAPK/ERK 1/2; Figure 3.3) (Egan *et al.* 2002). It may be assumed that *SPRY2* could be a biomarker of oocyte quality that contributes to CCs final differentiation and is involved in the EGF-like factors amplification of the gonadotropins' signals.

Taken together, these results reinforce our findings and yield insights concerning the main factors involved in cumulus cells contribution to the oocyte competence acquisition. These biomarkers will serve as a precious tool for a non-invasive assessment of oocyte quality and therefore competence. The interactions of these factors and other yet unidentified candidates will be a precious itinerary to explore in future studies in order to complete the oocyte competence puzzle.

This non-exhaustive inventory of the main up- and down-regulated molecular functions and gene networks involved in the CCs is a preamble to enrich our comprehension of molecular

contribution of CCs in oocyte final maturation, ovulation and subsequent fertilization. To our knowledge, this is first study that analyzed these molecular events around the LH surge (GVBD) in the bovine cumulus *in vivo*. Many potential candidates (see supplemental data) expressed hours prior to ovulation are reported for the first time. More exploratory studies are required to draw the chronology of the whole molecular events in CCs starting before the LH surge and ending at ovulation, and leading to oocyte final maturation.

3.6. MATERIALS AND METHODS

3.6.1. Animals

Cyclic, non-lactating and clinically healthy Holstein-Friesian cows were selected as described previously (Knijn *et al.* 2002). Animals were synchronized using a 9-day ear implant (3mg, Norgestomet, Crestar, Intervet International BV, Boxmeer, The Netherlands) followed by prostaglandin (PG) (Prosolvin, Intervet International BV). The detailed protocol was approved by the Ethical Committee of the Veterinary Faculty of Utrecht University.

3.6.2. Superovulation schedule

Selected cows (n=20) were approximately 4-year old and 185 days post-partum. They were randomized on ten (10) cows per treatment and then selected for regular superovulatory response and synchronous cycle using progesterone measurement three times a week. The aim was to avoid possible effects of asynchronous cycles on the LH surge (Atkins et al. 2008). A crestar/GnRH-controlled LH surge was used in our superovulation protocol (Knijn et al. 2002).The LH concentration was monitored to determine the time of maximum LH and ovariectomy. The few cows with deviating LH profiles were excluded. Following estrus synchronization, cows were kept in similar conditions of housing, food (silage and concentrate) as well as ad libitum water. Superstimulatory treatments were initiated on the 9th day of the new estrus cycle (day 0 = estrus) by implanting another ear Norgestomet. Cows then received a total dose of ovine FSH (oFSH) (Ovagen ICP, Auckland, New Zealand) equivalent to 299 IU NIH-FSH-S1 units in eight decreasing doses administered at 12-h intervals from day 10 to day 13 (3.5, 2.5, 1.5 and 1 ml) (Knijn et al. 2002). PG (22.5 mg) was given simultaneously with the fifth dose of FSH. Fifty hours after PG treatment, the ear implant was removed and GnRH (0.021 mg, Receptal im, Intervet International BV) was administered to induce the LH peak. Ovaries were collected immediately after cow ovariectomy (by laparotomy through flank incision and under

local anesthesia) at 50 and 58 hours following PG administration, which respectively corresponded to 2 h before and 6 h after the highest level of LH. They were immediately transported to the laboratory in saline solution at 37°C.

3.6.3. Collection of cumulus cells

Two experimental groups were set up: 1) 2 h before LH peak, and 2) 6 h after LH peak, to explore the genomic events of the final maturation steps of COC *in vivo*, using the LH surge as reference. For each treatment, follicular contents were aspirated from ovaries and stored at 4°C. Collected follicle fluid was used to predict follicular diameter. COC were then retrieved under a stereomicroscope and washed twice in PBS-5% PVA (Sigma-Aldrich, St-Louis, MO). COC were individually incubated with 5% (w/v) hyaluronidase (Sigma-Aldrich) for 1 min, and cumulus cells were denuded by gentle pipetting, washed and classified on the basis of steroid concentrations of the follicle fluid. Using steroid concentration patterns, and follicle fluid volume as reference (Dieleman *et al.* 1983, van de Leemput *et al.* 1999, Dieleman *et al.* 2002), 10 and 8 cows had regular superovulatory response respectively at 2h before and 6h after the LH surge. Cumulus of selected follicles were pooled together to get four biological replicates, and stored at -80°C for total RNA extraction. Each replicate corresponded to the cumulus of 10 healthy follicles collected from 5 of 10 cows at 2h before LH and from 4 of 8 cows at 6h after LH.

3.6.4. Total RNA extraction

Cumulus cells were subjected to total RNA extraction using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Briefly, total RNA was protected from RNases using a mixture 0.7 % (v/v) beta-mercaptoethanol (β-ME) added to 100 µl of lysis buffer. After homogenization and addition of an equal volume of 70% ethanol, the mix was transferred to a column microcentrifuge and spun for 30 sec. Column product was washed with 600 µl of 1x low-salt wash buffer for 30 sec, then immediately for 2 min to air-dry the spin cup. To prevent contamination, DNase digestion of the retained RNA was then performed for 15 min at 37°C according to the kit recommendations. Extracted RNA was washed once in 1x high-salt and twice in 1x low-salt wash buffer, and resuspended in 30 µl of Elution buffer provided in the kit. The concentration and quality of the RNA were assessed using Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's protocol.

3.6.5. Messenger RNA linear amplification

Based on RNA quality, the three best biological replicates of cumulus cells collected both at 2 h before and 6 h after the LH surge were amplified using 2-round *in vitro* transcription (IVT) following the instructions of the RiboAmp RNA Amplification kit (Arcturus, Mountain View, CA). Briefly, 10 ng total RNA of each biological replicate of each treatment were annealed to 1 µl of RiboAmp primer A for 5 min at 65°C, reverse-transcribed in a total volume of 20 µl containing 7µl of first strand master mix and 2 µl of first strand enzyme mix at 42°C for 2 h, and then put on ice. Two microliters of first strand nuclease mix was added to the reaction for 30 min at 37°C, followed by 5 min at 95°C. After annealing with 1 µl of RiboAmp primer B for 2 min at 95°C, second strand synthesis was achieved using 29 µl of second strand master mix, and 1 µl of second strand enzyme following this thermocycling program (5 min at 25°C, 10 min at 37°C, 5 min at 70°C), hold at 4°C (on ice), subsequently purified with the columns provided, and eluted with 11 µl of DNA elution buffer (DE). The IVT of round one was carried out by successive addition of 8 µl of IVT buffer, 12 µl of IVT master mix and 4 µl of IVT enzyme mix for 6 h at 37°C. The IVT product was DNase-treated, purified and eluted in 12 µl of RNA elution buffer. One microliter of this elution was used for NanoDrop (Wilmington, DE) quantification of the first round yield, whereas the rest served as a template for the second round.

As for the first round, the second round started with an annealing step with 1 µl of RiboAmp primer B at 65°C for 5 min, then the complete first strand was synthesised using the same mix as the first round for 10 min at 25°C, followed by 45 min at 37°C. Second strand synthesis was performed using the RiboAmp primer A and the same mix as the second strand synthesis of the first round using this program (30 min for 37°C, 5 min for 70°C), purified, and the amplification product was eluted in 30 µl of RNA eluted buffer (RE) and 1 µl was used to quantify the aRNA amplification yield using NanoDrop as before.

3.6.6. Messenger RNA direct labelling and hybridization

Amplified Messenger RNA of each replicate was divided into 2 sub-replicates to get dye-swap and labelled using the Universal Linkage System (ULS™) aRNA Fluorescent Labelling Kit (KREATECH Biotechnology, Amsterdam, The Netherlands) according to the manufacturer's instructions. Briefly, for each sub-replicate, 2.5 µg of amplified RNA was labelled by incubation with 2.5 µl of Cy5/DY647-ULS or Cy3/DY547-ULS, and 2 µl of 10x Labeling solution in a 20-µl total volume at 85°C for 15 min. Unbound dye was then removed, as recommended, using the

KREApure columns provided in the kit. Labelled RNA was quantified on the NanoDrop. Each of the two differentially labelled sub-replicates (Cy5/DY647-ULS vs Cy3/DY547-ULS) used for hybridization on the same microarray slide (2 h before LH vs. 6 h after LH) were then mixed together in equimolar proportions. Six hybridizations were performed in a dye-swap design (Figure 3.1) on our custom-made array of differentially expressed transcripts described elsewhere (Hamel *et al.* 2008b). Hybridizations were performed in the ArrayBooster using the Advacard AC3C (The Gel Company, San Francisco, CA) for 18 h at 50°C using Slide Hyb#1 (Ambion, Austin, TX). The slides were then washed twice in 2X SSC/0.5% SDS buffer, and twice in 0.5X SSC/0.5% SDS buffer. After two quick final washes at room temperature in 1X SSC and water, slides were spin-dried, scanned and analyzed using the ChipReader and ArrayPro Analyzer software (Media Cybernetics, San Diego, CA).

3.6.7. Microarray data analysis

Microarray data were subjected to Loess normalization using the ArrayPro software and uninformative data that were below background were then removed. Data analysis of our dye-swap experiments was subsequently achieved through the free-software National Institute on Aging (NIA) Array Analysis Tool (Baltimore, MD) (NIA 2010) developed at NIA. The analysis was performed with our three biological replicates and the two technical replicates generated by the dye-swap using the (2 h before LH) as a reference and, at FDR=5% and a minimum cutoff limit of 2.25. Since each clone was printed twice on our slide (Hamel *et al.* 2008b), two additional technical sub-replicates that emerged from this design were taken into account. Two lists of over-expressed and under-expressed clones in the (6 h after LH) treatment compared to (2 h before LH) were generated for further functional genomic analysis using mainly the Ingenuity Pathway Analysis (IPA) software (IPA 2010). Briefly, the candidate gene official names and fold change were uploaded into the IPA. Using its web database based on previous studies, IPA is able to automatically find the potential connections between the uploaded candidates and to classify them into scored networks according to the molecular pathways. A score and a p-value were therefore associated to each network, which is composed of selected genes from the uploaded list linked together and with other molecules (suggested by the software), and mapped in a whole signaling pathway. Each molecular relationship among the network members is represented in a conventional mapping that allows identification and therefore interpretation of the type of interactions. This genomic analysis aims at discovering some gene networks and

pathways in CCs associated to the LH genomic effect and therefore to the oocyte final maturation.

3.6.8. Real-time PCR validation

Real time PCR validation of some candidates was achieved on the three initial biological replicates (non-pooled) (Figure 3.1). Equal amounts of total RNA were taken from each CC pool of both 2 h before and 6h after the LH surge treatments. To denature the RNA and remove secondary structures, the RNAs were heated at 65°C for 5 min and then quenched rapidly on ice for at least 2 minutes. Samples were then reversed transcribed using the SensiScript reverse transcriptase kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's recommendations. Real-time PCR was performed on the 7 selected candidates (4 overexpressed and 3 underexpressed) in LightCycler capillaries (Roche Applied Science, Mannheim, Germany) using the LightCycler FastStart DNA Master SYBR Green I (Roche) as detailed elsewhere (Assidi *et al.* 2008). For each candidate, a specific set of primers was designed using the NCBI's primer-blast software and the candidates' specific sequences (NCBI) (Table 3.1). Normalization was achieved using three housekeeping genes, *ACTB* (β-actin), *GAPDH*, and *SDHA* (succinate dehydrogenase complex, subunit A). Following the GeNorm software analysis, *SDHA* and *GAPDH* were successively the most stable control genes (M value= 0.121; $P > 0.05$). These two housekeeping genes were subsequently used in for QPCR data normalization. The real-time PCR product specificity of each candidate was confirmed by sequencing as well as through the analysis of the Lightcycler melting curve (Roche). Each gene mRNA expression level was then divided by its normalization factor and log-transformed (Vandesompele *et al.* 2002). A t-test to compare gene expression levels between both treatments was then performed using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA) at $\alpha=0.05$.

3.7. DECLARATIONS OF INTEREST

The authors declare free of any conflict of interest that would prejudice the impartiality of the present research.

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3.11. FIGURES

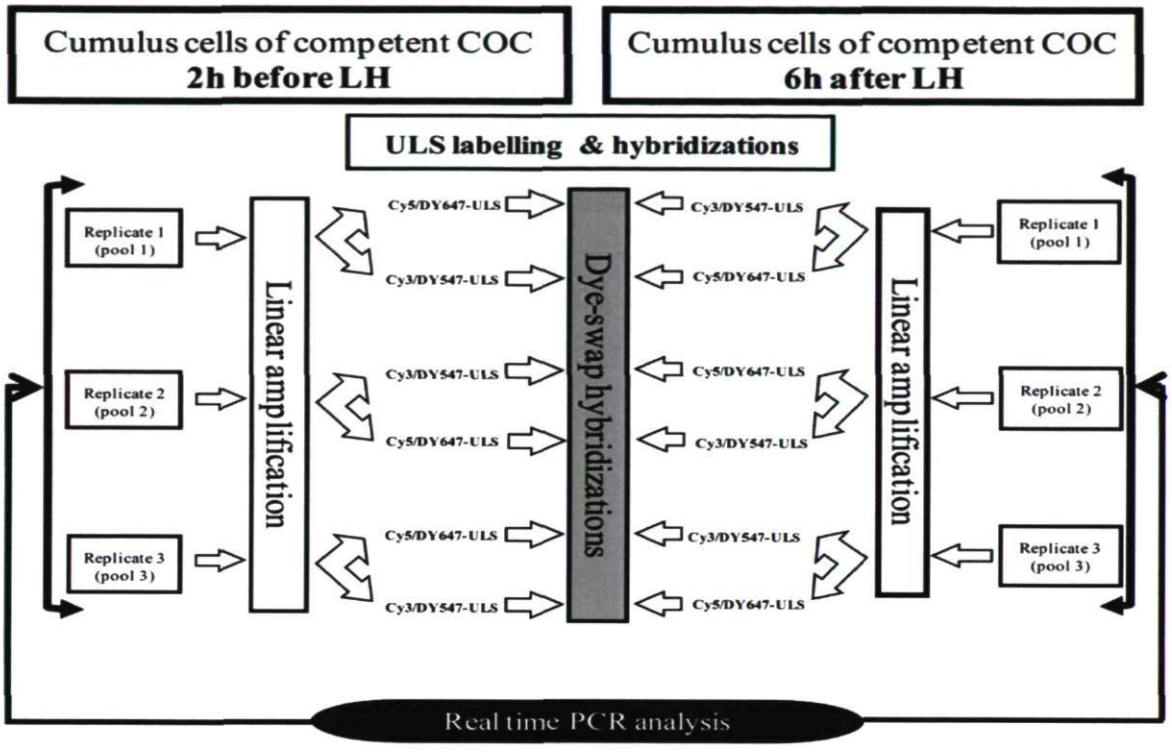

Figure 3.1. Experimental design of linear amplifications, dye-swap hybridizations and real-time PCR validation. Each pool corresponded to the cumulus cells of 10 healthy follicles collected from cows with regular superovulatory response.

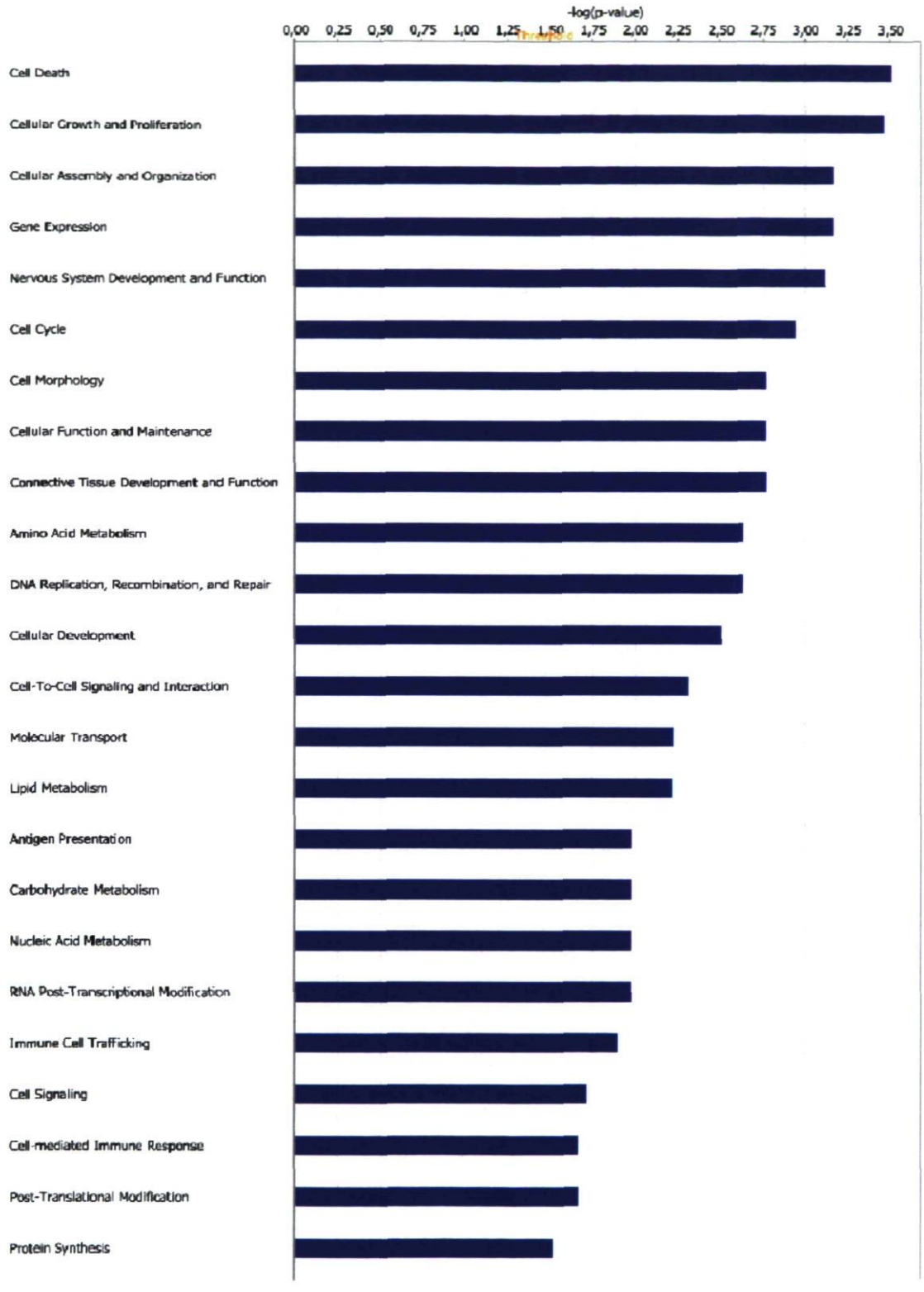

Figure 3.2. Summary of the main molecular and cellular functions affected by the LH surge around the GVBD. Only significant functions (over the thresold, $p<0.05$) were shown.

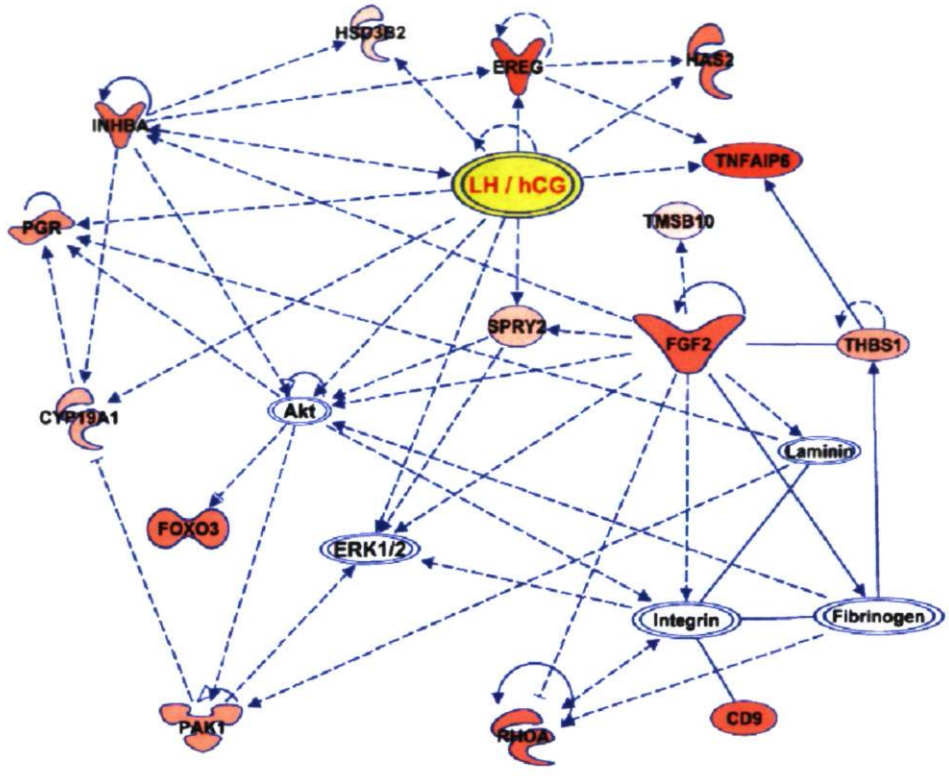

Figure 3.3. Ingenuity network 1 generated from the overexpressed genes (red) 6h after the LH surge in bovine CCs. The genes in this first network are EREG (epiregulin), TNFAIP6 (tumor necrosis factor, alpha-induced protein 6), HAS2 (hyaluronan synthase 2), LH/hCG (luteinizing hormone/ human chorionic gonadotropin), HSD3B2 (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2), INHBA (inhibin, beta A), BAMBI (BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)), PGR (progesterone receptor), CYP19A1 (cytochrome P450, family 19, subfamily A, polypeptide 1), Akt (protein kinase B), SPRY2 (sprouty homolog 2 (drosophila)), TMSB10 (thymosin beta 10), FGF2 (fibroblast growth factor 2), THBS1 (thrombospondin 1), ERK1/2 (extracellular signal-regulated kinase 1/2), FOXO3 (forkhead box O3), CD9 (CD9 molecule), RHOA (ras homolog gene family, member A) and PAK1 (p21 protein (Cdc42/Rac)-activated kinase 1)

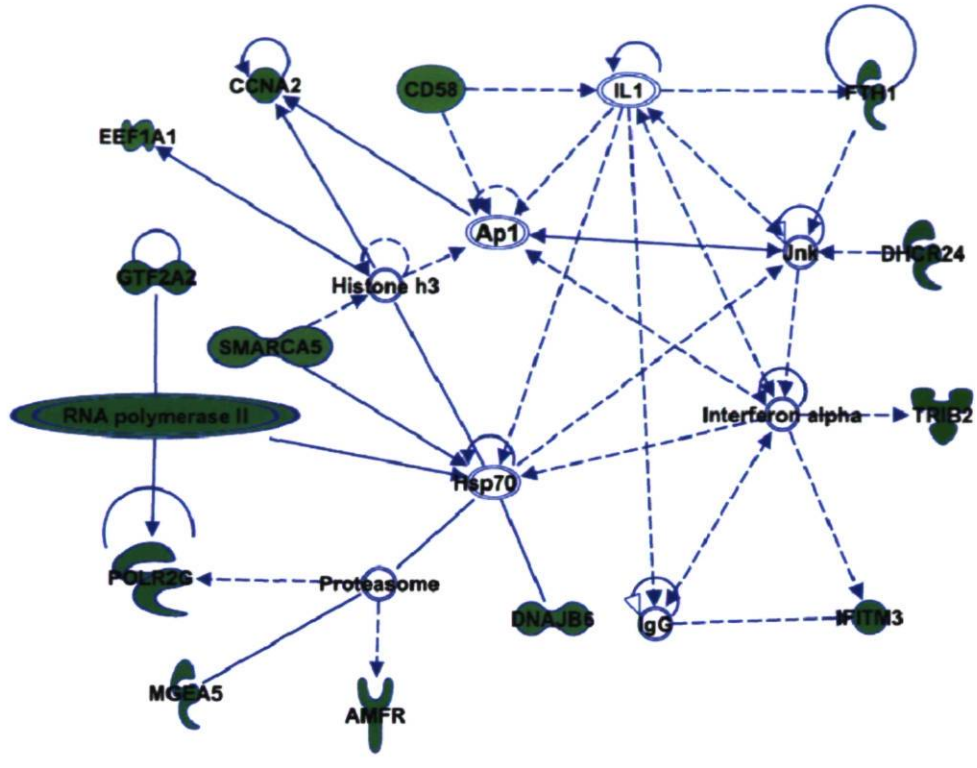

Figure 3.4. Ingenuity network 2 generated from the underexpressed genes (green) following the LH surge in bovine CCs. The genes in this second network are IL1 (interleukin 1), FTH1 (ferritin, heavy polypeptide 1), JNK (c-Jun N-terminal kinase), DHCR24 (24-dehydrocholesterol reductase), TRIB2 (tribbles homolog 2 (drosophila)), IFITM3 (interferon induced transmembrane protein 3 (1-8U)), IgG (Immunoglobulin G), DNAJB6 (DnaJ (Hsp40) homolog, subfamily B, member 6), AP1 (transcription factor AP-1), CD58 (CD58 molecule), CCNA2 (cyclin A2), EEF1A1 (eukaryotic translation elongation factor 1 alpha 1), SMARCA5 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5), GTF2A2 (general transcription factor IIA, 2, 12kD), POLR2G (polymerase (RNA) II (DNA directed) polypeptide G), MGEA5 (meningioma expressed antigen 5 (hyaluronidase)) and AMFR (autocrine motility factor receptor)

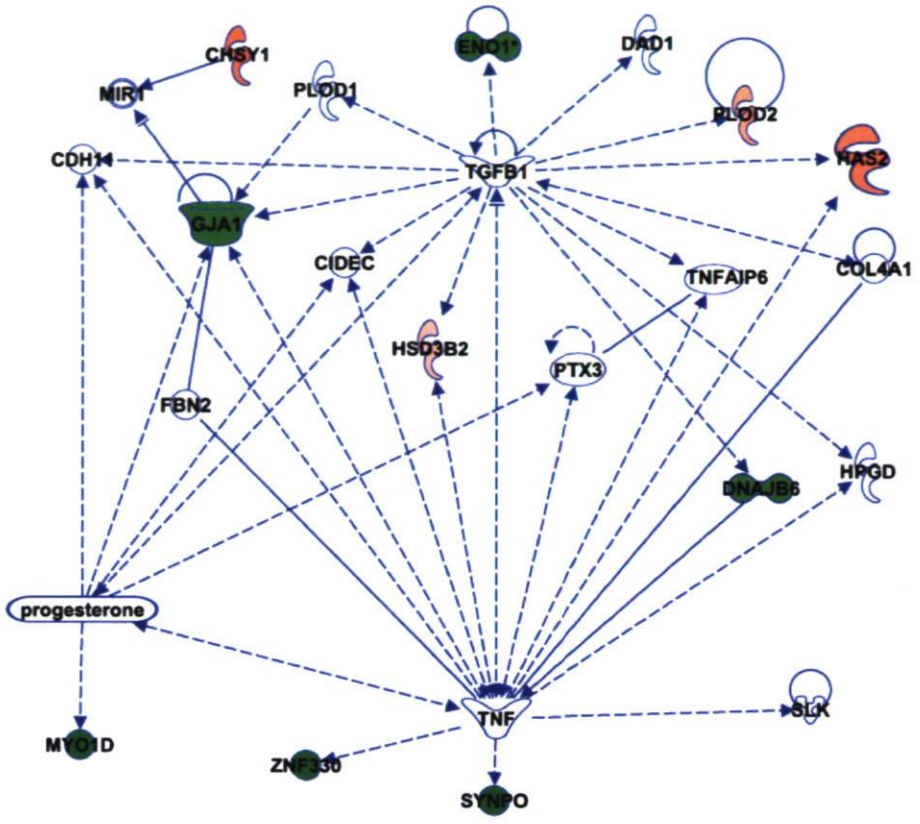

Figure 3.5. Ingenuity network 3 generated from both the over - and underexpressed gene candidates 6h after the LH surge in bovine CCs. Overexpressed genes (red) are TNFAIP6 (tumor necrosis factor, alpha-induced protein 6), HAS2 (hyaluronan synthase 2), HSD3B2 (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2), PLOD2 (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2) and CHSY1 (chondroitin sulfate synthase 1). Underexpressed genes (green) are ENO1 (enolase 1, (alpha)), DNAJB6 (DnaJ (Hsp40) homolog, subfamily B, member 6), GJA1 (gap junction protein, alpha 1, 43kDa), SYNPO (synaptopodin), ZNF330 (zinc finger protein 330) and MYO1D (myosin ID). The other genes (uncoloured), recommended by the IPA software to achieve the network, are TGFB1 (transforming growth factor, beta 1), TNF (tumor necrosis factor (TNF superfamily, member 2)), PTX3 (pentraxin-related gene, rapidly induced by IL-1 beta), DAD1 (defender against cell death 1), PLOD1 (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1), COL4A1 (collagen, type IV, alpha 1), CIDEC (cell death-inducing DFFA-like effector c), CDH11 (cadherin 11, type 2, OB-cadherin (osteoblast)), FBN2 (fibrillin 2), HPGD (hydroxyprostaglandin dehydrogenase 15-(NAD)) and SLK (STE20-like kinase (yeast)).

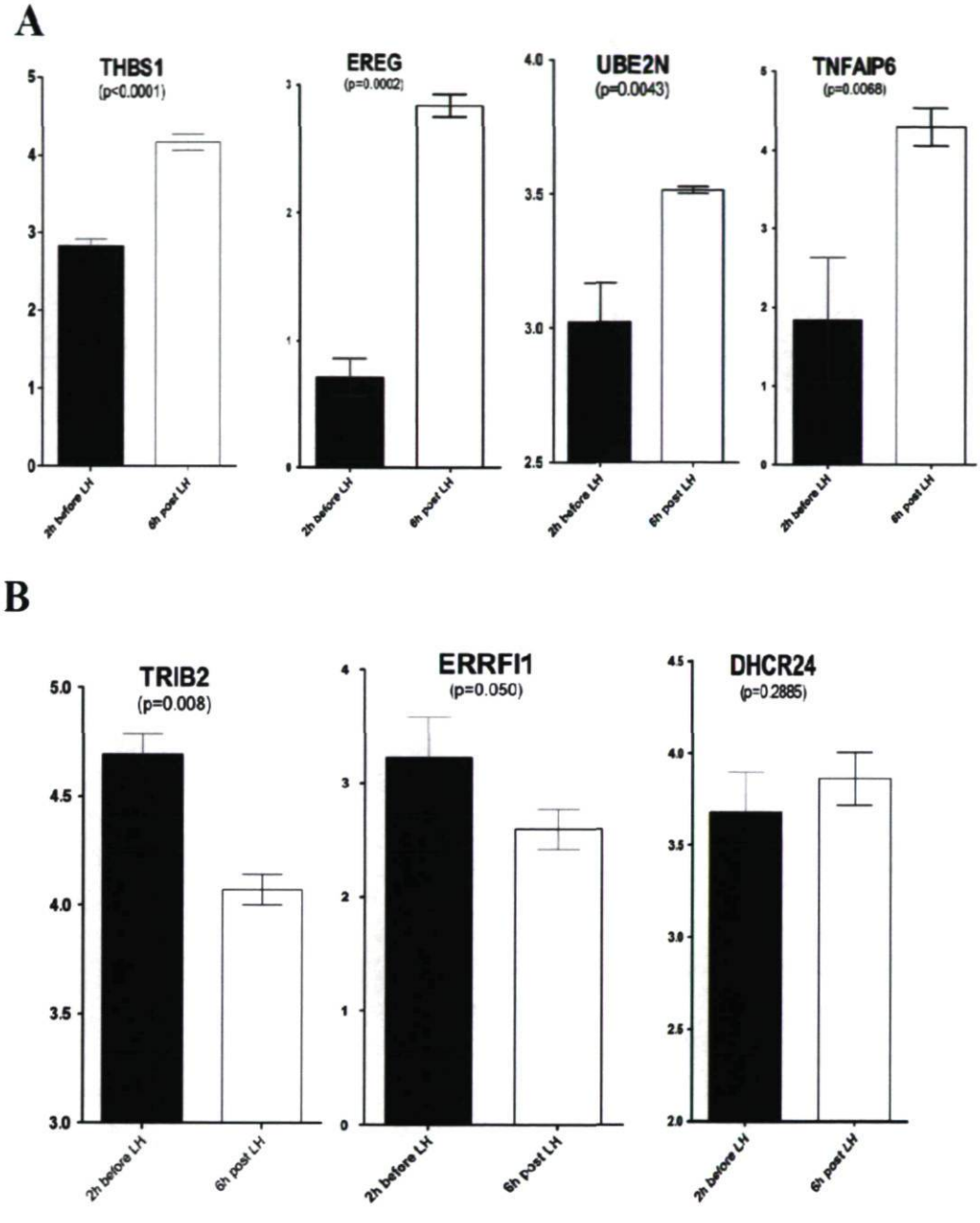

Figure 3.6. Real-time PCR analysis of some differentially expressed genes in original and non-pooled bovine CCs samples at 6h after versus 2h before the LH surge. (A), gene candidates overexpressed at 6h after the LH surge. (B), Underexpressed genes at 6h after compared to 2h before the LH surge. The analyzed genes are THBS1 (thrombospondin 1), EREG (epiregulin), UBE2N (ubiquitin-conjugating enzyme E2N), TNFAIP6 (tumor necrosis factor, alpha-induced protein 6), TRIB2 (tribbles homolog 2 (drosophila)), ERRFI1 (ERBB receptor feedback inhibitor 1) and DHCR24 (24-dehydrocholesterol reductase). Candidates were ranked according to their p-values, which were determined following a T-test analysis achieved on normalized data at $\alpha=0.05$

3.12. TABLES

Table 3.1: Sequences of real-time PCR specific primers of gene candidates expressed in bovine CCs 6h following the LH surge

Gene Name	Primer set (5'-3')	Genbank	Annealing temperature	Fluorescence acquisition temperature
UBE2N	up 5'- AAGACCAATGAAGCCCAAGCCA -3' low 5'- GCAGCTAACCCTGACAACTACC -3'	NM_001076258.1	60	81
TRIB2	Up 5'- CACACATCTTGGCATCGCACTGTT -3' low 5'- AGCACCCAGGTTTCACATCAGTCT -3'	NM_178317.3	61	79
DHCR24	up 5'- ACAAACCTGAGTCCAGTTCCCAGT -3' low 5'- AAGTGTCTTCTCCAAGCACACGGT -3'	NM_001103276.1	62	89
ERRFI1	up 5'- TGCCTGCTTTAAGTCGTCCTGAGA -3' low 5'- ACCCACAACACACATCTCCACACA -3'	NM_001077930.1	61	83
EREG	up 5'-GTGTGGCTCAAGTGTCAATAAC-3' low 5'-TCGATTTCTGTACCATCTGC-3'	XM_596732	53	81
TNFAIP6	up 5'-GTCTGGCAAATACAAGCTCACCT-3' low 5'-GGATCTGTAAACACACCACCACA-3'	NM_001007813.1	57	84
THBS1	up 5'- CTACATTGGCCACAAGACAA-3' low 5'– AGCAAGAGGTCCACTCAGAC-3'	NM_174196.1	53	89
GAPDH	up 5'-CCAACGTGTCTGTTGTGGATCTGA-3' low 5'- GAGCTTGACAAAGTGGTCGTTGAG-3'	NM_001034034.1	57	86
ACTB	up 5'-CGTGACATTAAGGAGAAGCTGTGC-3' low 5'-CTCAGGAGGAGCAATGATCTTGAT-3'	NM_173979.3	57	87
SDHA	up 5'- GCAGAACCTGATGCTTTGTG -3' low 5'- CGTAGGAGAGCGTGTGCTT -3'	NM_174178.2	57	88

Table 3.2. Common differentially expressed genes in bovine CCs between upregulated candidates in three IVM treatments (FSH; PMA; FSH+PMA), and the overexpressed candidates *in vivo* at 6 h after the LH surge: possible biomarkers of oocyte developmental competence

	Gene Name	Full Name of Gene / Protein	Accesion No.
1	*ADFP*	*Bos taurus* adipose differentiation-related protein	NM_173980
2	*ATP6V1C1*	*Bos taurus* ATPase, H+ transporting, lysosomal 42kDa, V1 subunit	NM_176676
3	*HSPA8*	*Bos taurus* heat shock 70 kDa protein 8	NM_174345
4	*HAS2*	*Bos taurus* hyaluronan synthase 2	NM_174079
5	*INHBA*	*Bos taurus* inhibin, beta A (activin A, activin AB alpha polypeptide)	NM_174363
6	*SELK*	*Bos taurus* selenoprotein K	NM_001037489.2
7	*TNFAIP6*	*Bos taurus* tumor necrosis factor, alpha-induced protein 6	NM_001007813
8	*UTMP*	*Bos taurus* uterine milk protein precursor	NM_174797
9	*AKAP 7*	Bos taurus A-kinase (PRKA) anchor protein 7	NM_001102266.1
10	*EREG*	*Bos taurus* epiregulin (EREG),	XM_596732
11	*HIGD1A*	*Homo sapiens* HIG1 domain family, member 1A,	BC070277
12	*THBS1*	*Bos taurus* thrombospondin 1	NM_174196.1
13	*HSPA5*	*Bos taurus* 78 kDa glucose-regulated protein	NM_001075148
14	*SLC18A2*	*Bos taurus* solute carrier family 18 (vesicular monoamine), member 2	NM_174653
15	*SLC39A10*	PREDICTED: *Bos taurus* similar to solute carrier family 39 (zinc transporter), member 10	XM_599261
16	*PGR*	*Bos taurus* progesterone receptor	XM_583951.4

Table 3.3 (supplemental data): List of overexpressed genes in bovine cumulus cells *in vivo* at 6h following the LH surge

No.	Gene name	*Gene/protein full name (if available)*	Accession no.	Redundancy	Fold Change
1	*THBS1*	Bos taurus thrombospondin 1	NM_174196.1	**2**	**9- 10.4**
2	*UBE2N*	*Bos taurus ubiquitin-conjugating enzyme E2N*	NM_001076258.1	**1**	**7.778**
3	*LOC286871*	*Bos taurus uterine milk protein precursor (LOC286871),(UTMP)*	NM_174797	**13**	**7- 10.2**
4	*SLC39A8*	*PREDICTED: Bos taurus similar to Solute carrier family 39 (zinc transporter), member 8*	XM_584935	**2**	**7,1**
5	*AKAP7*	*Bos taurus A kinase (PRKA) anchor protein 7*	NM_001102266	**1**	**6.36**
6	*PAPD1*	*Bos taurus PAP associated domain containing 1,*	BC104501	**1**	**6.258**
7	*NEAT1*	*Homo sapiens nuclear enriched abundant transcript 1*	EF177379	**1**	**6.046**
8	*TNFAIP6*	*Bos taurus tumor necrosis factor, alpha-induced protein 6*	NM_001007813	**16**	**5.2 - 10**
9	*CHSY1*	*Homo sapiens carbohydrate (chondroitin) synthase 1*	NM_014918	**1**	**5.134**
10	*ATP1B4*	*Bos taurus ATPase, (Na+)/K+ transporting, beta 4 polypeptide*	NM_001101919	**1**	**5.117**
11	*LRRN3*	*PREDICTED: Bos taurus similar to leucine rich repeat neuronal 3*	XM_588627	**1**	**5.097**
12	*EREG*	*Homo sapiens epiregulin*	NM_001432	**10**	**5 - 9.8**
13	*UCHL1*	*Bos taurus ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)*	NM_001046172	**1**	**4.698**
14	*RBMX*	*PREDICTED: Bos taurus similar to Heterogeneous nuclear ribonucleoprotein G (RNA-binding motif protein, X linked)*	XM_875611	**1**	**4.572**
15	*HSPA8*	*Bos taurus heat shock 70 kDa protein 8*	NM_174345	**1**	**4.544**
16	*HSPA5*	*Bos taurus heat shock 70kDa protein 5*	BT030726	**2**	**4.441**
17	*CD9*	*Bos taurus CD9 antigen (p24)*	NM_173900	**2**	**4.4**
18	*FGF2*	*Bos taurus fibroblast growth factor 2 (basic)*	NM_174056	**2**	**4.391**
19	*AGPAT9*	*PREDICTED: Bos taurus 1-acyl-sn-glycerol-3-phosphate O-acyltransferase 9*	XM_597964	**5**	**4 - 6.9**
20	*SGMS2*	*Homo sapiens sphingomyelin synthase 2*	NM_001136258	**3**	**4 - 4.87**
21	*RHOA*	*Bos taurus ras homolog gene family, member A*	NM_176645	**3**	**4,2**
22	*PSMA2*	*Bos taurus proteasome (prosome, macropain) subunit, alpha type, 2*	BC102206	**1**	**3.998**
23	*HAS2*	*Bos taurus hyaluronan synthase 2*	NM_174079	**4**	**3.9 - 5.2**
24	*FOXO3A*	*Homo sapiens forkhead box O3 (FOXO3), transcript variant 2,*	NM_201559	**1**	**3,9**
25	*ADFP*	*Bos taurus adipose differentiation-related protein*	NM_173980	**2**	**3.7**
26	*NONO*	*Bos taurus non-POU domain containing, octamer-binding ,*	NM_001046554	**1**	**3.584**
27	*BCO2*	*Bos taurus beta-carotene oxygenase 2*	NM_001101987	**1**	**3.491**

No.	Gene name	Gene/protein full name (if available)	Accession no.	Redundancy	Fold Change
28	*HNRPAB*	*Homo sapiens heterogeneous nuclear ribonucleoprotein A/B*	BC002625	1	3.462
29	*PLOD2*	*Bos taurus procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2*	NM_001101149	1	3.305
30	*SLAIN1*	*PREDICTED: Bos taurus similar to SLAIN motif family, member 1*	XM_583891	1	3.24
31	*PGR*	*Homo sapiens progesterone receptor (PGR) gene,*	AY525610	4	3.1- 5.3
32	*SLC25A5*	*Bos taurus solute carrier family 25 member 5,*	BC102950	1	3.069
33	*ATP6V1C1*	*Bos taurus ATPase, H+ transporting, lysosomal 42kDa, V1 subunit*	NM_176676	4	3.064
34	*SELK*	*Bos taurus similar to selenoprotein K,*	BC108150	1	3.006
35	*SC4MOL*	*Bos taurus sterol-C4-methyl oxidase-like,*	NM_001098863.	1	2.935
36	*THBS1*	*Homo sapiens thrombospondin 1*	NM_003246	2	2,6
37	*UBA6*	*Bos taurus ubiquitin-like modifier activating enzyme 6*	NM_001083438	2	2.516
38	*SMC4*	*Homo sapiens structural maintenance of chromosomes 4*	NM_005496	1	2.436
39	*PPM1G*	*Homo sapiens protein phosphatase 1G (formerly 2C), magnesium-dependent,gamma isoform*	BC007531	1	2.405
40	*CYP19A1*	*Homo sapiens cytochrome P450, family 19, subfamily A, polypeptide*	NM_000103	1	2.402
41	*FIBIN*	*Bos taurus fin bud initiation factor homolog (zebrafish) (FIBIN),*	NM_001015541	1	2.314
42	*ACTG1*	*Bos taurus actin, gamma 1*	NM_001033618	4	2.246
43	*MPHOSPH6*	*Bos taurus M-phase phosphoprotein 6*	NM_001075564	1	2.23
44	*HIGD1A*	*Homo sapiens hypoxia inducible domain family, member 1A*	BC000601	1	2.158
45	*TTC3*	*Homo sapiens tetratricopeptide repeat domain 3*	NM_001001894.1	1	2.121
46	*SPRY2*	*Bos taurus sprouty homolog 2 (Drosophila) (SPRY2),*	NM_001076147	4	2.1- 3.2
47	*INHBA*	*Bos taurus inhibin, beta A*	NM_174363	5	2.1 - 3.7
48	*RIMKLB*	*Homo sapiens ribosomal modification protein rimK-like family*	NM_020734	1	2.074
49	*MRO*	*Bos taurus maestro, mRNA (cDNA clone MGC:165702 IMAGE:8839632),*	BC146146	1	2.051
50	*PAK1*	*Homo sapiens p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)*	NM_002576	3	2- 3.1
51	*HSD3B2*	*Homo sapiens hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2*	NM_000198	1	1.979
52	*RWDD4A*	*Bos taurus RWD domain containing 4A*	NM_001076824	1	1.759
53	*MYL6*	*Bos taurus myosin, light chain 6, alkali*	BC103428	1	1.701
54	*TMSB10*	*Bos taurus thymosin beta-10*	AF294616	1	1.695
55	*BCAP29*	*Homo sapiens B-cell receptor-associated protein 29*	NM_018844	1	1.678
56	*EIF1AX*	*Homo sapiens eukaryotic translation initiation factor 1A, X-linked,*	BC047573	1	1.635

No.	*Gene name*	*Gene/protein full name (if available)*	Accession no.	Redundancy	Fold Change
57	*ENO1*	*Homo sapiens enolase 1, (alpha)*	AK315417	**1**	**1.634**
58	*KCIP-1*	*PREDICTED: Bos taurus similar to 14-3-3 protein zeta/delta (Protein kinase C inhibitor protein 1)*	XM_001788370	**1**	**1.627**
59	*NDUFB5*	*Bos taurus NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5*	NM_176656	**1**	**1.627**
60	*EIF4B*	*Homo sapiens eukaryotic translation initiation factor 4B,*	BC001097	**1**	**1.611**
61	*BAMBI*	*Bos taurus BMP and activin membrane-bound inhibitor homolog*	NM_001046309	**1**	**1.587**
62	*RNF13*	*Bos taurus ring finger protein 13*	NM_001076142	**1**	**1.579**
63	*RPL3*	*Bos taurus ribosomal protein L3*	BT021012	**1**	**1.532**
64	###	*Unknown genes*	###	**5 clones**	**7 – 3.9**

Table 3.4 (supplemental data): List of underexpressed genes in bovine cumulus cells in vivo at 6h following the LH surge

No.	Gene name	Gene/protein full name (if available)	Accession no.	Redundancy	Fold Change
1	*DPYSL2*	*Bos taurus dihydropyrimidinase-like 2*	NM_001076000	**1**	**3.069**
2	*MT-COX2*	*Homo sapiens mitochondrial coxII mRNA for cytochrome C oxidase II subunit*	X55654	**1**	**2.995**
3	*TRIB2*	*Homo sapiens tribbles homolog 2*	NM_021643	**1**	**2.988**
4	*CCBL2*	*Homo sapiens cysteine conjugate-beta lyase 2*	NM_019610	**4**	**2.741**
5	*DHCR24*	*Homo sapiens 24-dehydrocholesterol reductase*	NM_014762	**1**	**2.603**
6	*RGS3*	*Homo sapiens regulator of G-protein signalling 3*	BC019039	**1**	**2.525**
7	*TMPO*	*Homo sapiens mRNA for Lamina-associated polypeptide 2,*	AB209297	**1**	**2.445**
8	*C7H19orf10*	*Bos taurus chromosome 19 open reading frame 10*	NM_001001164	**1**	**2.332**
9	*ST3GAL3*	*Homo sapiens ST3 beta-galactoside alpha-2,3-sialyltransferase 3*	NM_006279	**1**	**2.304**
10	*RHOC*	*Homo sapiens ras homolog gene family, member C,*	BC052808	**1**	**2.302**
11	*STMN1*	*Bos taurus stathmin 1/oncoprotein 18*	NM_001034790	**2**	**2.301**
12	*SYNPO*	*Homo sapiens synaptopodin*	NM_007286	**1**	**2.291**
13	*ZNF330*	*Bos taurus zinc finger protein 330*	NM_001038157	**1**	**2.278**
14	*LMAN1*	*Homo sapiens lectin, mannose-binding, 1*	NM_005570	**2**	**2.277**
15	*PDE4DIP*	*Homo sapiens phosphodiesterase 4D interacting protein*	BC025406	**1**	**2.277**
16	*ERRFI1*	*Bos taurus ERBB receptor feedback inhibitor 1*	NM_001077930	**1**	**2.261**
17	*YWHAE*	*Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide*	BC001440	**1**	**2.234**
18	*LOC782502*	*PREDICTED: Bos taurus misc_RNA (LOC782502),*	XR_027712	**1**	**2.207**
19	*RPL3*	*B.taurus mRNA for ribosomal protein L3*	Z29555	**4**	**2.204**
20	*MYO1D*	*Homo sapiens myosin ID*	NM_015194	**2**	**2.203**
21	*SELPLG*	*Bos taurus P-selectin glycoprotein ligand 1*	AY929857	**1**	**2.202**
22	*NFXL1*	*Homo sapiens nuclear transcription factor, X-box binding-like 1*	NM_152995.	**1**	**2.194**
23	*STT3B*	*Homo sapiens highly similar to Source of immunodominant MHC-associated peptides.*	AK027789	**1**	**2.188**
24	*TOB2*	*Homo sapiens transducer of ERBB2, 2*	NM_016272	**1**	**2.186**
25	*RPL8*	*Homo sapiens similar to ribosomal protein L8*	AK289459	**1**	**2.161**
26	*POLR2G*	*Homo sapiens polymerase (RNA) II (DNA directed) polypeptide G*	NM_002696	**1**	**2.146**
27	*PXMP4*	*Homo sapiens peroxisomal membrane protein 4,*	BC001147	**1**	**2.143**

No.	Gene name	Gene/protein full name (if available)	Accession no.	Redundancy	Fold Change
28	*OPHN1*	*Homo sapiens cDNA, FLJ99737 highly similar to Oligophrenin 1*	AK309696	1	2.139
29	*VEZF1*	*Bos taurus vascular endothelial zinc finger 1*	NM_001102227	1	2.139
30	*TSNAX*	*Homo sapiens translin-associated factor X*	NM_005999	1	2.137
31	*MGEA5*	*Homo sapiens meningioma expressed antigen 5 (hyaluronidase)*	NM_001142434	1	2.136
32	*CCDC53*	*Homo sapiens coiled-coil domain containing 53*	NM_016053	1	2.12
33	*OGDH*	*Homo sapiens oxoglutarate (alpha-ketoglutarate) dehydrogenase*	NM_002541	1	2.11
34	*CSTB*	*Homo sapiens cystatin B (stefin B)*	NM_000100	1	2.105
35	*IFITM3*	*Homo sapiens interferon induced transmembrane protein 3*	NM_021034	3	2.097
36	*AMFR*	*Bos taurus autocrine motility factor receptor*	NM_001045974	1	2.095
37	*ANKRD40*	*Bos taurus ankyrin repeat domain 40*	NM_001075586	2	2.093
38	*GTF2A2*	*Bos taurus general transcription factor IIA, 2,*	NM_001037619	2	2.072
39	*BDKRB2*	*Homo sapiens bradykinin receptor B2*	NM_000623	1	2.065
40	*FTH1*	*Homo sapiens proliferation-inducing protein 15 / ferritin, heavy polypeptide 1*	AY258285	1	2.058
41	*RPL27A*	*Bos taurus ribosomal protein L27a*	NM_001024471	4	2.057
42	*DOM3Z*	*Bos taurus DOM-3 homolog Z,*	BC120425	1	2.055
43	*ENY2*	*Homo sapiens e(y)2 homolog*	AF173296	1	2.033
44	*RPS27*	*Homo sapiens ribosomal protein S27*	NM_001030	1	2.028
45	*CTNS*	*Homo sapiens cystinosis, nephropathic*	NM_001031681	1	2.023
46	*RPS12*	*Homo sapiens ribosomal protein S12*	NM_001016	1	2.015
47	*RPS6*	*Bos taurus ribosomal protein S6,*	BC102493	2	1.997
48	*ENO1*	*Homo sapiens enolase 1, (alpha),*	BC050642	1	1.995
49	*EDA*	*Bos taurus partial ectodysplasin A, exons 4-9*	AJ278907	1	1.992
50	*DMN*	*PREDICTED: Bos taurus similar to desmuslin*	XM_870453	1	1.989
51	*TPI1*	*Homo sapiens triosephosphate isomerase 1,*	BC009329	2	1.984
52	*CLK1*	*Bos taurus CDC-like kinase 1*	NM_001102271	1	1.952
53	*GJA1*	*Bos taurus gap junction protein, alpha 1,*	NM_174068	2	1.926
54	*CCNA2*	*Homo sapiens cyclin A2*	BC021067	4	1.925
55	*ND4*	*Homo sapiens, Similar to NADH dehydrogenase 4,*	BC014376	1	1.915
56	*PHF8*	*Homo sapiens PHD finger protein 8,*	BC053861	1	1.907

No.	Gene name	Gene/protein full name (if available)	Accession no.	Redundancy	Fold Change
57	*RPLP0*	*Bos taurus acidic ribosomal phosphoprotein PO*	AF013214	2	1.904
58	*MALAT1*	*Homo sapiens metastasis associated lung adenocarcinoma transcript 1*	BC025986	1	1.901
59	*AK7*	*Bos taurus adenylate kinase 7*	NM_001104972	1	1.9
60	*NRP1*	*Homo sapiens neuropilin 1*	NM_003873	1	1.898
61	*HBB*	*Homo sapiens hemoglobin, beta*	NM_000518	2	1.897
62	*TMED5*	*Homo sapiens transmembrane emp24 protein transport domain containing 5*	BC070051	1	1.884
63	*MTND5*	*Homo sapiens NADH dehydrogenase subunit 5*	AF339086	2	1.877
64	*DNAJB6*	*Bos taurus DnaJ (Hsp40) homolog, subfamily B, member 6,*	NM_174532	1	1.876
65	*ARL6IP5*	*Bos taurus ADP-ribosylation-like factor 6 interacting protein 5*	NM_001014891	1	1.865
66	*SMARCD3*	*SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 3.*	BX648385	1	1.859
67	*SARS*	*Homo sapiens seryl-tRNA synthetase*	NM_006513	1	1.846
68	*EIF4G3*	*Homo sapiens eukaryotic translation initiation factor 4 gamma, 3,*	BC072413	1	1.832
69	*ATP6V1D*	*Homo sapiens ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D*	NM_015994	1	1.816
70	*EFCAB8*	*Homo sapiens EF-hand calcium binding domain 8,*	NM_001143967	1	1.793
71	*LASS5*	*Homo sapiens LAG1 homolog, ceramide synthase 5*	NM_147190	1	1.785
72	*EXPH5*	*PREDICTED: Bos taurus similar to Slp homolog lacking C2 domains b (Exophilin-5)*	XM_605435	1	1.779
73	*HNRPDL*	*Bos taurus heterogeneous nuclear ribonucleoprotein D-like*	NM_001083725	1	1.771
74	*PTDSS1*	*Homo sapiens phosphatidylserine synthase 1,*	BC002376	1	1.766
75	*TUBA1B*	*Bos taurus similar to tubulin, alpha 1,*	BC146060	1	1.766
76	*GAPDH*	*Homo sapiens glyceraldehyde-3-phosphate dehydrogenase,*	BC083511	1	1.764
77	*SMARCA5*	*Bos taurus SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5*	NM_001105416	1	1.764
78	*OS9*	*Homo sapiens amplified in osteosarcoma*	NM_006812	1	1.761
79	*HSP90AB1*	*Bos taurus 90-kDa heat shock protein beta,*	AB072369	2	1.749
80	*EEF1G*	*Bos taurus eukaryotic translation elongation factor 1 gamma*	BT025451	1	1.742
81	*SDC4*	*Homo sapiens syndecan 4*	NM_002999	1	1.732
82	*EMX2OS*	*Homo sapiens empty spiracles homolog 2 (EMX2) opposite strand*	AY117413	1	1.721
83	*TM9SF2*	*Homo sapiens highly similar to Transmembrane 9 superfamily protein member 2 precursor*	AK129798	1	1.721
84	*GSTP1*	*Homo sapiens mutant glutathione transferase GSTP1*	AY887902	1	1.704
85	*EEF1A1*	*PREDICTED: Bos taurus similar to elongation factor 1 alpha*	XM_001251644	1	1.681

No.	Gene name	Gene/protein full name (if available)	Accession no.	Redundancy	Fold Change
86	*SLC25A3*	*Homo sapiens solute carrier family 25, member 3*	BC015379	**1**	**1.679**
87	*BCAP29*	*Homo sapiens B-cell receptor-associated protein 29*	NM_018844	**1**	**1.678**
88	*RPL23A*	*Homo sapiens ribosomal protein L23a*	NM_000984	**1**	**1.67**
89	*RPL19*	*Homo sapiens ribosomal protein L19*	NM_000981	**1**	**1.665**
90	*CD58*	*Bos taurus CD58 molecule*	BC134443	**1**	**1.652**
91	*RPS25*	*Bos taurus ribosomal protein S25*	BC102560	**1**	**1.639**
92	*RPL35A*	*Homo sapiens ribosomal protein L35a*	NM_000996	**1**	**1.637**
93	*SCOC*	*Homo sapiens short coiled coil protein*	AF448857	**1**	**1.634**
94	*CMAS*	*Homo sapiens cytidine monophosphate N-acetylneuraminic acid synthetase*	BC016609	**1**	**1.631**
95	*TMEM181*	*Homo sapiens transmembrane protein 181*	NM_020823	**1**	**1.63**
96	*WDR61*	*Bos taurus WD repeat domain 61*	NM_001038097	**1**	**1.626**
97	*HNRPF*	*Bos taurus heterogeneous nuclear ribonucleoprotein F*	BT020929	**1**	**1.625**
98	*SNRPB2*	*PREDICTED: Bos taurus small nuclear ribonucleoprotein polypeptide B*	XM_600315	**1**	**1.619**
99	*EDIL3*	*PREDICTED: Bos taurus similar to EGF-like repeats and discoidin I-like domains 3*	XM_618255	**1**	**1.618**
100	*TMEM45A*	*Bos taurus similar to Transmembrane protein 45a*	NM_001075405	**1**	**1.607**
101	*SMS*	*Bos taurus spermine synthase*	BT029883	**1**	**1.6**
102	*STAR*	*Homo sapiens steroidogenic acute regulator*	NM_001007243	**1**	**1.599**
103	*GNAS*	*Homo sapiens highly similar to Guanine nucleotide-binding protein G(s)subunit alpha ,*	NM_001077490	**1**	**1.594**
104	*COX7C*	*Homo sapiens cytochrome c oxidase subunit VIIc, nuclear*	NM_001867	**1**	**1.555**
105	*PPIA*	*Homo sapiens peptidylprolyl isomerase A (cyclophilin A)*	NM_021130	**1**	**1.55**
106	*SSLP-1*	*Bos taurus secreted seminal vesicle Ly-6 protein 1*	NM_001105478	**1**	**1.545**
107	*HNRNPA0*	*Homo sapiens heterogeneous nuclear ribonucleoprotein A0*	NM_006805	**1**	**1.544**
108	*RPS3A*	*Homo sapiens ribosomal protein S3A,*	BC018954	**1**	**1.543**
109	*HIGD1A*	*Bos taurus HIG1 domain family, member 1A*	NM_001077114	**1**	**1.537**
110	*CNN3*	*Homo sapiens calponin 3, acidic*	BC025372	**1**	**1.529**
111	*THOC2*	*Homo sapiens THO complex 2*	NM_001081550	**1**	**1.511**
112	*PKIA*	*Homo sapiens protein kinase (cAMP-dependent, catalytic) inhibitor alpha*	NM_006823	**1**	**1.501**
113	###	*Unknown genes*	###	**23 clones**	**1.8 - 3**

CHAPTER 4

FSH *in vitro* versus LH *in vivo*: complementary or substitutive genomic effects?

Mourad Assidi, François J. Richard and Marc-André Sirard

This chapter is a mini-review supported by some data. It will be submitted in the near future to « **Reproduction**»

4.1. RÉSUMÉ

L'utilisation des gonadotrophines pour stimuler la maturation ovocytaire aussi bien *in vivo* qu'*in vitro* a permis une meilleure compréhension de la fonction ovarienne. De nos jours, la plupart des techniques de reproduction assistée (ART) aussi bien chez l'homme ainsi que chez les espèces animales ont recours aux gonadotrophines principalement la FSH et la LH. Il est également établi que la réussite de la maturation ovocytaire et l'acquisition de la compétence au développement nécessite la synthèse de novo ARNm. Cette activité transcriptionnelle vise à équiper l'ovocyte et son environnement somatique avec des facteurs essentiels au bon déroulement de la maturation, l'ovulation et le développement embryonnaire. Par conséquent, une attention particulière est de plus en plus accordée aux effets transcriptionnels des gonadotrophines afin de mieux comprendre le mécanisme moléculaire de la compétence et d'améliorer les systèmes de culture *in vitro* et les protocoles de stimulation ovarienne présentement utilisés aussi bien chez les animaux ainsi qu'en milieu clinique.

Malgré les nombreuses études effectuées, les mécanismes moléculaires d'action de la FSH et de la LH ne sont pas encore entièrement définis, et sont même sujets à des variations en passant du contexte *in vivo* à celui *in vitro*. Cette compréhension approximative de la synergie FSH/LH est d'ailleurs la cause de l'absence d'un consensus sur leur utilisation essentiellement *in vitro* ou dans les protocoles d'induction de l'ovulation. Afin d'optimiser leur utilisation, des travaux supplémentaires focalisant surtout sur la comparaison des effets *in vitro* versus *in vivo* s'avèrent donc nécessaires. Dans ce contexte, cette revue vise à adresser brièvement les voies de d'expression géniques induites par la FSH *in vitro* versus celles de la LH *in vivo*. En utilisant des données de biopuces récentes obtenues dans notre laboratoire, on rapporte pour la première fois que la FSH pourrait accomplir *in vitro* au moins une partie de l'activité transcriptionnelle assurée normalement par la LH *in vivo*. Cette compensation/substitution de l'effet transcriptionnel de LH par la FSH dans les cellules du cumulus *in vitro* permet de mieux interpréter des résultats précédents où l'addition de LH à la FSH en milieu de MIV n'améliore pas significativement la compétence ovocytaire. Toutefois, des études complémentaires sont nécessaires pour reconfirmer ces résultats et mieux explorer la synergie FSH / LH. L'étude des profils d'expression génique induite par la FSH, LH et (FSH + LH) dans un système de culture séquentiel pourrait être un moyen intéressant pour valider ces résultats.

4.2. ABSTRACT

The use of gonadotropins to trigger the oocyte maturation both *in vivo* and *in vitro* has provided precious and powerful knowledge that have significantly increased our understanding of the ovarian function. Moreover, the efficiency of most of the assisted reproductive technologies (ART) used in both human and livestock species rely on gonadotropins' input mainly FSH and LH. It is also established that the induction of oocyte maturation and subsequent developmental competence acquisition require de novo mRNA synthesis. This gene expression activity aims at supplying the oocyte and its surrounding somatic compartment with crucial factors to achieve subsequent events of maturation, ovulation and embryo developmental. Therefore, an increasing importance has been given to the gonadotropins-transcriptional effects to increase our understanding of the molecular mechanism of oocyte competence and to improve both the *in vitro* culture systems and the ovarian stimulation protocols already in use (in both livestock and clinic).

Despite numerous works achieved and the relevant findings reported, the exact molecular pathways of action of the two pituitary hormones FSH and LH remain not fully understood. Moreover, these pathways may not be the same when moving from the *in vivo* to the *in vitro* contexts. This misunderstanding of the intricate synergy between these two hormones engenders a lack of consensus about their use mainly *in vitro* or in ovulation induction schedules *in vivo*. In order to optimize their use, additional works are hence required with a special focus on the *in vitro* versus the *in vivo* effects. In this context, this overview tries to briefly summarize the downstream gene expression pathways induced by both FSH *in vitro* and LH *in vivo*. Based on recent data in our laboratory, we report for the first time that FSH *in vitro* looks to be able to achieve at least part of the LH gene expression activity *in vivo*. It means that FSH may reproduce/substitute at least partially the *in vivo* activity of LH. This transcriptomic compensation of the LH action by FSH *in vitro* in cumulus cells make sense to previous results where the addition of LH to FSH-based IVM media doesn't provide significant increase in oocyte competence measured by the blastocyst rate. However, additional studies are required to confirm these findings and investigate the FSH/LH synergy. Studying the gene expression patterns induced by FSH, LH and (FSH+LH) in sequential culture system could be an interesting way that may validate these findings.

4.3. INTRODUCTION

Mammalian female reproductive function is finely regulated by a set of coordinated endocrine signals that allowed successful events of oocyte developmental competence, granulosa cells differentiation, extracellular matrix (ECM) production, ovulation, fertilization and even early embryo development. Gonadotropins (mainly FSH and LH) are the main extraovarian endocrine factors involved in the control of these ovarian functions (Dekel *et al.* 1979, Eppig 1980, Hillier 1994, Richards 1994, Espey & Richards 2002, Sha *et al.* 2010). The release of these two anterior-pituitary hormones is governed by the hypothalamus via the GnRH (gonadotropins releasing hormone) and modulated by other ovarian factors as activin and inhibin (Gregory & Kaiser 2004). Following a gonadotropin-independent phase, the mammalian follicular growth becomes first FSH-dependent since the secondary stage and thereafter LH-dependent prior to ovulation (Hillier *et al.* 1994, Kumar *et al.* 1997, Ginther *et al.* 2000, Hunter 2003, Zeleznik 2004). While FSH is involved mainly in the follicular growth, cellular proliferation and oestrogens' production (aromatase activity), LH induces the androgens biosynthesis, the oocyte final maturation and ovulation (Wise *et al.* 1994, Howles 2000, Mattioli & Barboni 2000, Shimada & Terada 2002, Accardo *et al.* 2004, Sirard *et al.* 2007, Panigone *et al.* 2008). To achieve their functions, FSH and LH trigger multiple downstream cascades of intraovarian pathways that are necessary for proper female fertility (Howles 2000, Mattioli & Barboni 2000, Accardo *et al.* 2004, Assidi *et al.* 2008, Edson *et al.* 2009, Hillier 2009, Zhang *et al.* 2009). Besides, it is established that the efficiency of most of the assisted reproductive technologies (ART) used in both human and livestock including ovarian stimulation and oocyte *in vitro* maturation (IVM) rely on gonadotropins' input (Zuelke & Brackett 1990, Blondin *et al.* 2002, Dieleman *et al.* 2002, Ali & Sirard 2002a, Shimada *et al.* 2003, Weghofer *et al.* 2007, Baerwald *et al.* 2009, Adriaenssens *et al.* 2010).

Interestingly, successful gonadotropins-induced maturation of oocyte was shown to necessitate de novo mRNA synthesis. This gene expression activity aims at supplying the oocyte and follicular cells with crucial factors to achieve subsequent events of maturation and ovulation (Meinecke & Meinecke-Tillmann 1993, Farin & Yang 1994, Tatemoto & Terada 1995, Rodriguez & Farin 2004a).

In vitro, it is established that FSH improves oocyte maturation (both nuclear and cytoplasmic), cumulus cells (CCs) expansion, *in vitro* fertilization (IVF) and early embryo development in

several mammalian species. Its receptor FSHR is expressed in mural granulosa cells (MGCs) and CCs since the secondary follicular stage in most mammals including murine, porcine, ovine, bovine and human species (Salustri *et al.* 1989, Merriman *et al.* 1998, Anderiesz *et al.* 2000, Accardo *et al.* 2004, Ali & Sirard 2005, Ye *et al.* 2005). FSH stimulation is also a key step in the superovulation protocols in livestock animals and human (Zafeiriou *et al.* 2000, Blondin *et al.* 2002, Dieleman *et al.* 2002, Baerwald *et al.* 2009). Considering the hypothesis of absence of functional LH receptors in CCs in the *in vitro* context (Peng *et al.* 1991), FSH has been the major gonadotropin used in IVM to trigger the oocyte maturation fulfillment (Ali & Sirard 2002a, Calder *et al.* 2003, Sirard *et al.* 2007).

To be effective, the LH action depends hence on the expression of functional LHCGR in the follicle. LHCGR expression was reported in theca and granulosa cells (Camp *et al.* 1991, Robert *et al.* 2003, Park *et al.* 2004) but was absent in both oocytes and CCs (Amsterdam *et al.* 1975, van Tol *et al.* 1996, Nuttinck *et al.* 2004). Therefore, the LH action on CCs was assumed to be indirect mainly through the EGF-like factors (Park *et al.* 2004, Panigone *et al.* 2008, Zhang *et al.* 2009, Reizel *et al.* 2010). The addition of LH into the IVM media might be hence not needed *in vitro* (Anderiesz *et al.* 2000, Choi *et al.* 2001, Ali & Sirard 2002a). However, Peng et al. (1991) have reported the expression of LHCGR in rat CCs, raising therefore a controversy that needs further exploration.

In this review, we attempt to briefly addresse the general pathways of FSH and LH in follicular cells (mainly CCs) that result in downstream transcriptional activity. A special focus will be made on the common features between the FSH-transcriptionnally upregulated genes *in vitro* versus their LH counterparts *in vivo*. Based on common structural and functional features between FSH and LH, the hypothesis of partial replacement or "compensation" of LH action by FSH *in vitro* is explored. Using several findings reported in previous works and microarray data in our laboratory, we propose herein an interesting aspect of the gonadotropins actions that may increase our understanding of their molecular pathways as well as their intricate synergy. This aspect, once confirmed, should be considered in the future.

4.4. GONADOTROPINS-MEDIATED GENE EXPRESSION AND OOCYTE DEVELOPMENTAL COMPETENCE

In view of the gonadotropins' beneficial effects, they are used both *in vitro* and *in vivo* to improve oocyte developmental competence. Although the molecular mechanism of their action remains not fully defined, we supposed that their genomic effects *in vitro* could be different from those *in vivo*, where they act in synergy. This hypothesis emerged from the difference in blastocyst rates between *in vivo* and *in vitro* oocyte maturation. For the bovine specie for example, the proportion of oocytes with successful developmental competence *in vivo* is between 60 to 80% (Blondin *et al.* 2002, Dieleman *et al.* 2002). This percentage drops off *in vitro* up to 45% at best (van de Leemput *et al.* 1999, Ali & Sirard 2002a). It is clear that the *in vivo* context including the gonadotropins (FSH & LH) synergetic actions and other intrafollicular players is far suitable to the oocyte maturation and competence acquisition. Interestingly, data in our laboratory and elsewhere showed that an FSH-based IVM media allowed *in vitro* at least half of the *in vivo* developmental competence outcome (Rizos *et al.* 2002, Ali & Sirard 2005). To explain these findings in LH-free media (use of recombinant human FSH (rhFSH) without contamination risk), we assume that FSH is able to accomplish both its own biological function as well as at least part of the LH one, leading therefore to full developmental competence of some oocytes (with still undetermined properties). In a LH-lacking context, FSH looks to be able to exert key functions normally achieved by LH reflecting probably a resistance mechanism (i.e. adaptation reaction) that ensures the specie fertility and therefore perpetuation (Hunter, 2003). This compensation process could be an adaptation process in case of particular/stressful situations (as the *in vitro* context).

In order to explore possible compensation / substitution of LH *in vivo* action by the FSH *in vitro*, many levels could be studied (metabolic, physiological, morphological, transcriptomic, etc.). This work is an overview of possible transcriptomic compensation of LH by FSH *in vitro* by briefly reviewing their respective signalling pathways that may induce gene expression, followed by a case report of genomic effect comparison of FSH *in vitro* versus LH *in vivo* in bovine CCs.

4.4.1. Main signaling pathways of FSH in vitro

It is established that FSH is a key regulator of ovarian function particularly follicular growth and granulosa cell differentiation (Kumar *et al.* 1997, Robker & Richards 1998). FSHβ deficient mice were unable to move over the preantral stage (Kumar *et al.* 1997). These observations confirm

that folliculogenesis is gonadotropins-dependent since the antral stage. The main functions triggered by FSH in the mammalian ovary are cell proliferation, apoptosis prevention, estradiol production and gene expression (Richards 1994, Kaipia & Hsueh 1997, Okazaki *et al.* 2003, Slomczynska *et al.* 2003, Adriaens *et al.* 2004, Sirard *et al.* 2007, Kawashima *et al.* 2008). Additionally, FSH is the main ingredient in IVM media and was shown to efficiently promote oocyte maturation in several mammals *in vitro* including bovine (Farin & Yang 1994, Ali & Sirard 2002a), murine (Schultz *et al.* 1983) and porcine (Jin *et al.* 2006, Sha *et al.* 2010) species. This FSH *in vitro* action is initiated in cumulus-oocyte complex (COCs) via its receptor (FSHR) on cumulus cells (CCs). The FSHR is a GPCR (G-protein-coupled receptor) with a specific seven-transmembrane domain that was shown to activate the linear FSHR/AC/cAMP/PKA pathway. Among the two activated isoforms of PKA, only the PKAII was shown to be involved in the transcriptional events in CCs required to meiosis resumption (GVBD) (Rodriguez *et al.* 2002, Ning *et al.* 2008). This de novo gene expression is indispensable for gonadotropins-induced oocyte maturation in murine and feline species (Farin *et al.* 2007, Ning *et al.* 2008) and was shown to involve the MAPK downstream the cAMP dependent-PKA pathway in most mammals including mice (Su *et al.* 2001, Su *et al.* 2002a), rat (Maizels *et al.* 1998) and bovine (Fissore *et al.* 1996). The inhibition of the MAPK pathway in CCs (or COCs) impaired gonadotropins-induced oocyte maturation and prevent the overexpression of crucial genes as PTSG2 and HAS2 required for oocyte maturation fulfillment, CCs expansion and steroidogenesis. Interestingly, this MAPK effect downstream the PKA pathway was oocyte-paracrine-factors dependent (Su *et al.* 2003). Additional PKA gene expression activity was associated to its two catalytic subunits that were able to traverse to the CCs' nucleus. Several key genes were reported to be expressed downstream this pathway including HAS2, TNFAIP6, PTGS2, CYP19A1 and EGF-like factors ((Tirone *et al.* 1997, Gonzalez-Robayna *et al.* 1999, Nuttinck *et al.* 2002, Ochsner *et al.* 2003a, Ning *et al.* 2008, Yamashita *et al.* 2009), reviewed recently in (Edson *et al.* 2009, Zhang *et al.* 2009)). This transcriptional activity is mediated mainly – but not exclusively- through the phosphorylation of the CREB (CRE-binding protein) and therefore its binding to the CRE (cAMP-responsive-element) region in the promoter (Johannessen *et al.* 2004a).

FSH-mediated gene expression activity occurs also in a PKA-independent manner. In fact, it was demonstrated that FSH phosphorylates PKB/Akt and SGK1via the PI3K (phosphatidylinositol-

dependent kinase)/PDK1 (phosphoinositide-dependent kinase1) pathway in rat granulosa cells (Gonzalez-Robayna *et al.* 2000) mouse CCs (Kalous *et al.* 2006, Zhang *et al.* 2010) and porcine CCs (Shimada *et al.* 2003a) to support the oocyte maturation *in vitro*. Interestingly, the PI3K/PKB pathway downstream FSH was shown to induce cell survival and progesterone production in porcine CCs (Shimada *et al.* 2003, Shimada *et al.* 2003a).

PKC was also reported to mediate the FSH action in CCs by the activation of the MAPK. This PKC action upstream the MAPK pathway (and possibly through other pathways) induced the expression of key factors (de novo proteins) required for the oocyte meiotic maturation including the EGF-like factors in most mammalian species (Fan *et al.* 2004, Freimann *et al.* 2004, Ali & Sirard 2005, Chen *et al.* 2008). Similar effects induced by PMA (phorbol 12-myristate13-acetate), which is a PKC activator, were shown in CCs. Moreover, the induction of PKC pathway by FSH was associated with the mobilization of intracellular calcium that is assumed to be favorable to oocyte maturation and subsequent fertilization (Su *et al.* 1999, Fan *et al.* 2004, Assidi *et al.* 2008, Baldi *et al.* 2009, Teves *et al.* 2009).

For the EGF-like factors overexpressed by FSH in CCs, they play crucial role in gonadotropins-induced maturation in mammalian COCs (Mehlmann 2005, Chen *et al.* 2008, Downs & Chen 2008, Li *et al.* 2008) and probably further fertilization (Halet 2004). Once expressed, these EGF-like peptides triggered gene expression in CCs through the extracellular signal-regulated kinases (ERK1/2) (Shimada *et al.* 2006b).

FSH was also able to rapidly (within 1hour) activate the MEK/MAPK pathway in mouse CCs allowing the oocyte maturation (Su *et al.* 2001). The most studied MAPK are the ERK1/2, the JNK/SAPK *(c-jun*terminal kinase/stress-activated protein kinases) and p38MAPK. Several transcription factors were reported to act downstream the MAPK including AP1, ATF2 and CMYC (reviewed in (Choi *et al.* 2003)). In this context, P38MAPK was also phosphorylated by FSH through the cAMP/Epac (exchange protein activated by cAMP)/Rap (Ras-like related proteins)/Raf pathway, which is PKA-independent (Gonzalez-Robayna *et al.* 2000). The ERK1/2 was also involved in MGCs and CCs steroidogenesis (progesterone and estradiol) induced by FSH (Hillensjo *et al.* 1981, Moore *et al.* 2001). Once produced, these steroids mainly the progesterone was shown to promote gene expression and contribute in oocyte competence and CCs expansion (Robker *et al.* 2000, Richards *et al.* 2002a, Kim *et al.* 2009).

Several studies were performed to assess the gene expression patterns in follicular cells induced by FSH *in vitro*. These sets of genes induce numerous biological and molecular functions associated to cell signaling, CCs expansion, steroidogenesis, gene expression, etc. (Salustri 2000, Rodriguez & Farin 2004a, Perlman *et al.* 2006, Assidi *et al.* 2008, Kawashima *et al.* 2008, Caixeta *et al.* 2009, Yamashita *et al.* 2009, Paradis *et al.* 2010, Richards & Pangas 2010). The analysis of these gene expression patterns has yield insights concerning the molecular involvement of FSH in CCs function leading to oocyte developmental competence acquisition.

4.4.2. **Do cumulus cells express the LHCGR?**

Before reviewing the LH pathways mainly those leading to gene expression in CCs, it is important to discuss available data about the possible expression of LHCGR in this compartment. Some studies have reported the absence of LHCGR in CCs (Amsterdam *et al.* 1975, van Tol *et al.* 1996, Nuttinck *et al.* 2004). The addition of LH in FSH-based media for COCs maturation *in vitro* does not therefore improve the oocyte developmental competence (Izadyar *et al.* 1996, van Tol *et al.* 1996, Ali & Sirard 2002a). In contrast, others works documented the LHCGR expression in CCs and therefore possible direct action of LH (Peng *et al.* 1991, Chen *et al.* 1994). Additional evidences look to confirm these latter findings in CCs of several mammal species including pig (Shimada *et al.* 2003, Kawashima *et al.* 2008), mouse (Fu *et al.* 2007), rat (Bukovsky *et al.* 1993), bovine (isoform E)(Robert *et al.* 2003) and human (Yang *et al.* 2005). Beneficial effects of LH on *in vitro* embryo yields were even shown (Younis *et al.* 1989, Zuelke & Brackett 1993). These opposite findings may be due to differences in several parameters as the COCs' follicular stage, the tissue type (granulosa or cumulus), the gonadotropins origin (recombinant versus purified) and the detection technique and its sensitivity. But with the production of recombinant pure gonadotropins and the use of accurate detection techniques as the real-time PCR, one could assume that the heterogeneity of the selected pool of COCs used for IVM (for each study) may contain variable proportion of follicles that have already express the LHCGR in CCs, yielding therefore unpredictable and inconstant response to LH *in vitro*. In the same way, the expression variation of particular LHCGR isoforms in CCs according to the follicular stage could also be behind this discrepancy. The analysis of only some isoforms may be insufficient to confirm the absence of these receptors in CCs. Sufficient data about the differential expression of LHCGR according to both the cell subtype (theca, granulosa or cumulus) and the follicular stage are still lacking. Possible reconciliation that reinforces our hypothesis was

reported recently by the sequential culture system (FSH followed by LH) suggested by Kawashima et al. (2008). In their study, the FSH was shown to trigger the expression of functional LHCGR able to respond to the subsequent LH action, yielding therefore greater developmental competence until the blastocyst stage both *in vivo* and in culture.

4.4.3. **Main in vivo pathways of LH**

Similarly to FSH, the LH contribution in follicle dominant selection, oocyte final maturation, ovulation and subsequent fertilization was studied. In fact, the LH was necessary in the selection of the dominant follicle in cattle and horse ((Ginther 2000) for review). This dominance is marked in cattle by an increasing dependence of the follicle on LH mainly its signaling and transcriptional activities (Fayad *et al.* 2004, Mihm *et al.* 2006). While only FSH was able to induce CCs expansion *in vitro*, the LH and hCG were able to promote this mucification *in vivo* through the Ras/Raf/MAPK pathway (downstream the cAMP) as well as oocyte maturation (Eppig 1980, Su *et al.* 2002a, Shimada *et al.* 2003, Liang *et al.* 2005, Sela-Abramovich *et al.* 2005). Dr Richards group have recently shown *in vitro* that the LH-induced transcriptional events are required for oocyte maturation, CCs mucification, ovulation and luteinization through the activation of some downstream transcription effectors as the C/EBPβ (CCAAT/Enhancer-binding protein-beta) via the ERK1/2 pathway ((Fan *et al.* 2009), reviewed in (Duggavathi & Murphy 2009, Edson *et al.* 2009, Sun *et al.* 2009, Richards & Pangas 2010)).

As FSH *in vitro*, LH was also shown to activate the PKAII isoform which triggers gene expression events required for oocyte maturation fulfilment (Downs & Hunzicker-Dunn 1995, Rodriguez & Farin 2004b, Newhall *et al.* 2006). Additionally, LH mediates the cAPM-dependent expression of the EGF-like factors (EREG, AREG, BTC) through the p38 MAPK. These growth factors propagate and amplify the LH signal in CCs as suggested elsewhere ((Park *et al.* 2004, Shimada *et al.* 2006b), reviewed in (Liang *et al.* 2007)). Other key genes were also induced by LH mainly those involved in CCs expansion and prostaglandin synthesis as HAS2, TNFAIP6, PTX3, CSPG2 and PTSG2 (Hernandez-Gonzalez *et al.* 2006). The knockout of these crucial genes in mouse causes severe defects in the animal reproductive phenotype and subsequent fertility (reviewed in (Edson *et al.* 2009)).

LH was also shown to induce steroidogenesis in porcine and bovine CCs mainly progesterone and estradiol (Dieleman *et al.* 1983, Shimada & Terada 2002). LH receptor null mice were infertile with defected steroid production (Lei *et al.* 2001, Zhang *et al.* 2001). Moreover, the gene

expression patterns in CCs were deeply affected in PGR null mice supporting a key transcriptional role of progesterone required for oocyte maturation, and subsequent ovulation and fertilization. This nuclear receptor acts as a transcription factor nuclear receptor to mediate the LH ovulatory response by the expression of key genes as ADAMTS1 and Catepsin L ((Lydon *et al.* 1995) for reviews (Richards *et al.* 2002, Richards 2005, Richards 2007, Richards & Pangas 2010)).

In addition to the PGR, LH surge induces also other various transcription factors leading to diverse transcriptional effects and consequently physiological responses (Richards *et al.* 2002). The PKC pathway was suggested as a possible transduction mode of this LH stimulation (Park *et al.* 2007). PKC epsilon was furthermore shown to induce a survival (anti-apoptotic) effect on human CCs downstream the PI3K/Akt pathway (Maraldi *et al.* 2009). This PKC action is possibly associated to the reported intracellular LH-induced rise of calcium in follicular cells (Davis *et al.* 1987, Mattioli *et al.* 1998).

Human Chorionic Gonadotropin (hCG) is another gonadotropin that have a high affinity to the LH receptor, named for this reason the LHCGR. In addition to the same α-subunit shared between LH and hCG, this affinity is due first to high similarities between the two β-subunits. Interestingly, the hCG is able to trigger most of the LH effects for longer periods due to its greater half-life. This property is desired in the ovarian stimulation drugs since it allows more time, flexibility and management possibilities during the ovarian stimulation programs mainly in human IVF. For these reasons, hCG have often been used instead of LH due to its LH-like effect (reviewed in (De Rensis *et al.* 2010))

Analogous to the FSH effect, the LH activation of several signal transduction pathways in CCs leads to diverse but well organised *in vivo* transcriptional responses that contribute into suitable oocyte acquisition of competence, subsequent ovulation fertilization and early embryo development. These beneficial effects were confirmed both *in vivo* and *in vitro* ((Dieleman *et al.* 2002, Park *et al.* 2004, Panigone *et al.* 2008, Su *et al.* 2010) reviewed by (Mattioli & Barboni 2000, Weghofer *et al.* 2007)).

4.4.4. **Comparative analysis of FSH and LH pathways**

Despite their specific biological functions, FSH and LH share some exciting similarities. In fact, both of them is pituitary-derived glycoprotein composed of a heterodimer of two subunits (α and β). These two subunits are linked by non-covalent bonds. While the α-subunit is common

between all the pituitary gonadotrophins, the β-subunit is specific and therefore responsible of the biological effect (Howles 2000). The expression of these subunits is differentially induced by the pulsatile gonadotropin-releasing hormone (GnRH) via the PKC/MAPK signaling pathway (Ciccone & Kaiser 2009). Interestingly, both FSH and LH exert their stimulatory effects through a seven transmembrane receptor (7TMR). These receptors are members of G protein-coupled receptors (GPCR) that stimulate several signaling pathways mainly through the G protein family (Gilchrist *et al.* 1996, Simoni *et al.* 1997, Pierce *et al.* 2002). Moreover and as discussed before, the two gonadotropins are able to induce gene expression events by targeting numerous transcription factors downstream key signaling pathways as PKA, PKC, PKB/Akt, MAPK and PI3K. These transcriptional activities of FSH *in vitro* or LH *in vivo* were essential to successful oocyte developmental competence attainment. These similarities in molecular structure, the specific receptor, and the transcriptional pathways and gene targets led support to the hypothesis of possible overlapping genomic roles between these two gonadotropins.

4.5. EVIDENCES OF COMPENSATION OF LH GENOMIC EFFECT IN VITRO BY FSH

In addition to their structural and functional (receptor and downstream pathways) resemblances, the goal here is to look for common genes transcriptionnally upregulated (directly or indirectly) by the two gonadotropins that could support our hypothesis of a partial replacement or "compensation" of LH action by FSH *in vitro*. To do this, we compared herein the FSH-induced genes in CCs and associated with oocyte competence *in vitro* (Assidi *et al.* 2008) with the *in vivo* context 6h following the LH surge. Because finding a timeline to compare cumulus cells status and gene expression patterns *in vivo* versus *in vitro* can be difficult, we used the meiotic status of the oocyte as a suitable reference. Thus, our analysis focused on the comparison of gene expression patterns *in vitro* (6 h of IVM, oocyte entering GVBD stage) (Assidi *et al.* 2008) with the overexpressed genes *in vivo* at 6 h after the LH surge (when the oocyte is again entering the GVBD stage) (Hyttel *et al.* 1986).

For the *in vitro* study, the focus was on the genomic effect of FSH in bovine CCs (around GVBD) associated with oocyte competence *in vitro* (Assidi *et al.* 2008). Although the whole molecular pathway of FSH action in CCs is not fully defined, we supposed that this genomic effect *in vitro* (Figure 4.2 B) could be different from its counterpart *in vivo*, where it acts in synergy with LH (Figure 4.2 A). The *in vitro* versus *in vivo* differences in blastocyst outcome

support this assumption. Concerning the subsequent *in vivo* study, we have analyzed the LH genomic effect *in vivo* (close to the GVBD) again on bovine CCs (unpublished/submitted data). This latter context should better reflect the real mechanism of CCs contribution into the oocyte competence acquisition. In fact, LH was reported to induce oocyte *in vivo* final maturation by action on CCs which express and deliver competence inducers to the oocyte (Peng *et al.* 1991, Dieleman *et al.* 2002). Keeping all these considerations in mind, our analysis focused on the comparison of the genomic action of FSH *in vitro* versus LH *in vivo*. A non-exhaustive list of common molecular genes between LH and FSH, expressed in CCs, and associated with oocyte final maturation is provided (Table 4.1). Among the 133 significant induced candidates by FSH *in vitro*, 22 genes were also induced by LH *in vivo*. This means that LH *in vivo* is able to induce the transcription of around 16.5% (22/133) of all the genes overexpressed by FSH *in vitro*. Strikingly, these common candidates correspond to almost 32 % (22/69) of the clones overexpressed via LH *in vivo* (Figure 4.1).

These findings mean that around one third of the genes induced by LH *in vivo* could be induced by FSH *in vitro*. FSH action *in vitro* seems therefore to compensate for both its own and LH's *in vivo* functions (Sirard *et al.* 2007). Using its common downstream pathways of gene expression with LH, FSH *in vitro* looks to reproduce its *in vivo* function and substitute at least partially the *in vivo* activity of LH (Figure 4.2 B).

The analysis of the gene networks of the common 22 genes using the gene Ingenuity Pathway Analysis (IPA) software (IPA 2010) confirm the high overlap at the transcriptional level between FSH and LH. The IPA analysis of the 22 common candidates reveals a gene network with the highest score (Figure 4.3). It is obvious the number of common candidates potentially targeted by both FSH and LH/hCG supporting therefore our initial hypothesis. Interstingly, a TGFB factor looks to share several downstream targets with the two gonadotropins, reminding therefore the oocyte-derived effects (via TGFβ family) on CCs (Figure 4.3). To our knowledge, this is the first time that compensation of LH action by FSH *in vitro* is highlighted at the transcriptomic level. It is to note that this analysis was made using a partial custom-made microarray (Assidi *et al.* 2008). It is expected that the number of common candidates between the two gonadotropins may increase if commercial whole genome microarray were used.

4.6. CONCLUDING REMARKS

Despite the LH-induced genes *in vivo* (6h post LH) were significantly overexpressed compared to the reference time point (2 hour before the LH surge), these data deserve attentive interpretation. In fact, our experimental design was performed to assess the LH additonnal transcriptomic effect compared to the residual effect (FSH effect). But, one must be careful in the interpretation of these data since these gene expression effects measured at 6h post LH *in vivo* may include a remaining / residual FSH effect. Therefore, additional studies in others species and other contexts both *in vivo* and *in vitro* are required to confirm these findings. Studying the gene expression patterns induced by recombinant FSH, LH and (FSH+LH) in sequential culture system could be an interesting way to explore.

These conclusions, even still preliminary, support our hypothesis of potential functional substitution between FSH and LH. It also makes sense to previous results where the addition of LH to FSH-based IVM media doesn't provide significant increase in oocyte competence measured by the blastocyst yield. Moreover, this probable high functional substitution of LH function by FSH *in vitro*, once confirmed by further studies in other species and contexts, should be helpful in improving *in vitro* culture systems and ovulation induction programmes through a better comprehension of the FSH/LH synergy *in vivo*. Furthermore, these common candidates will serve as a precious preamble tool that advances our understanding of the molecular pathways leading to successful oocyte maturation, ovulation and subsequent fertilization.

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4.8. **FIGURES**

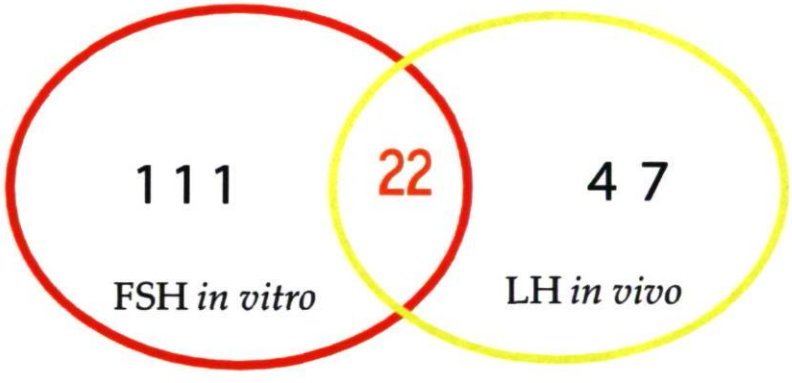

Figure 4.1: Common genes overexpressed by FSH in vitro versus LH in vivo in bovine CCs around the GVBD as revealed by microarray

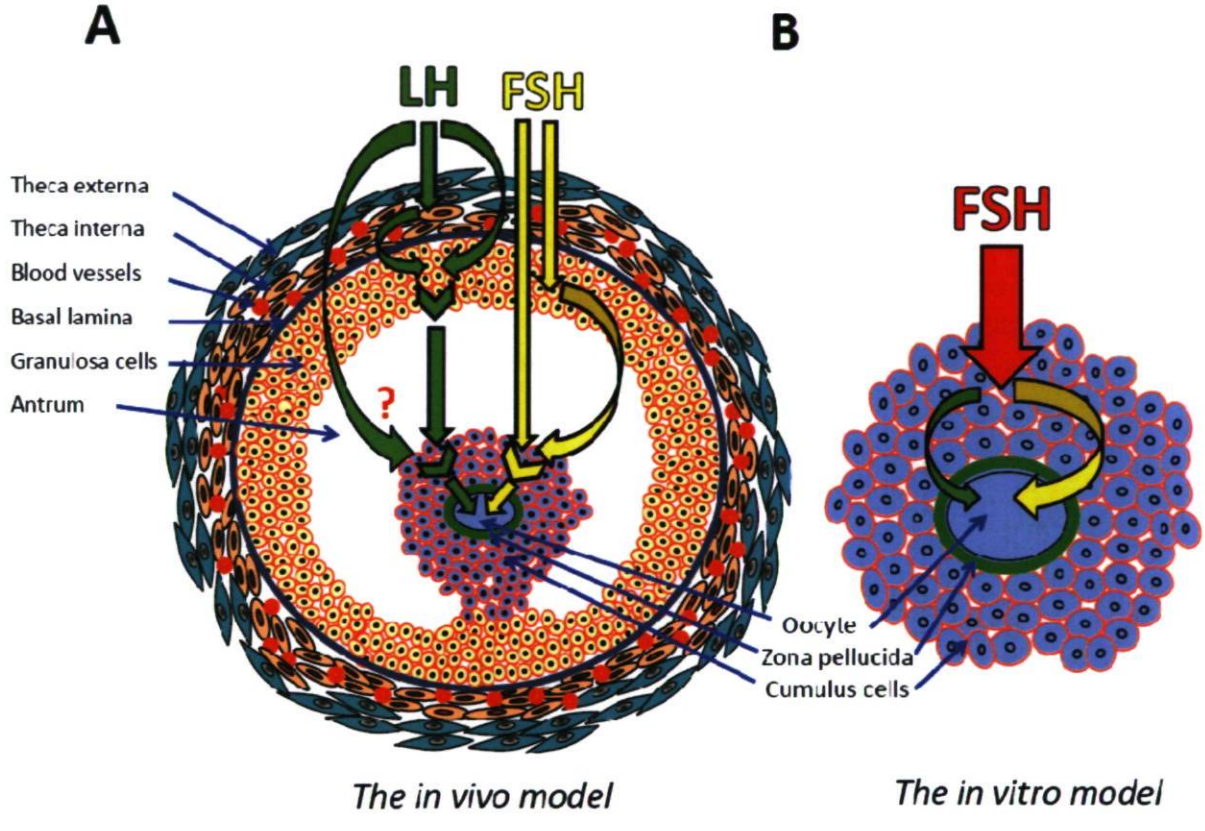

Figure 4.2: Model of gonadotropins induction of oocyte competence in vivo versus in vitro: Could FSH in vitro, and in addition to its own effects, achieve a part of those of LH?

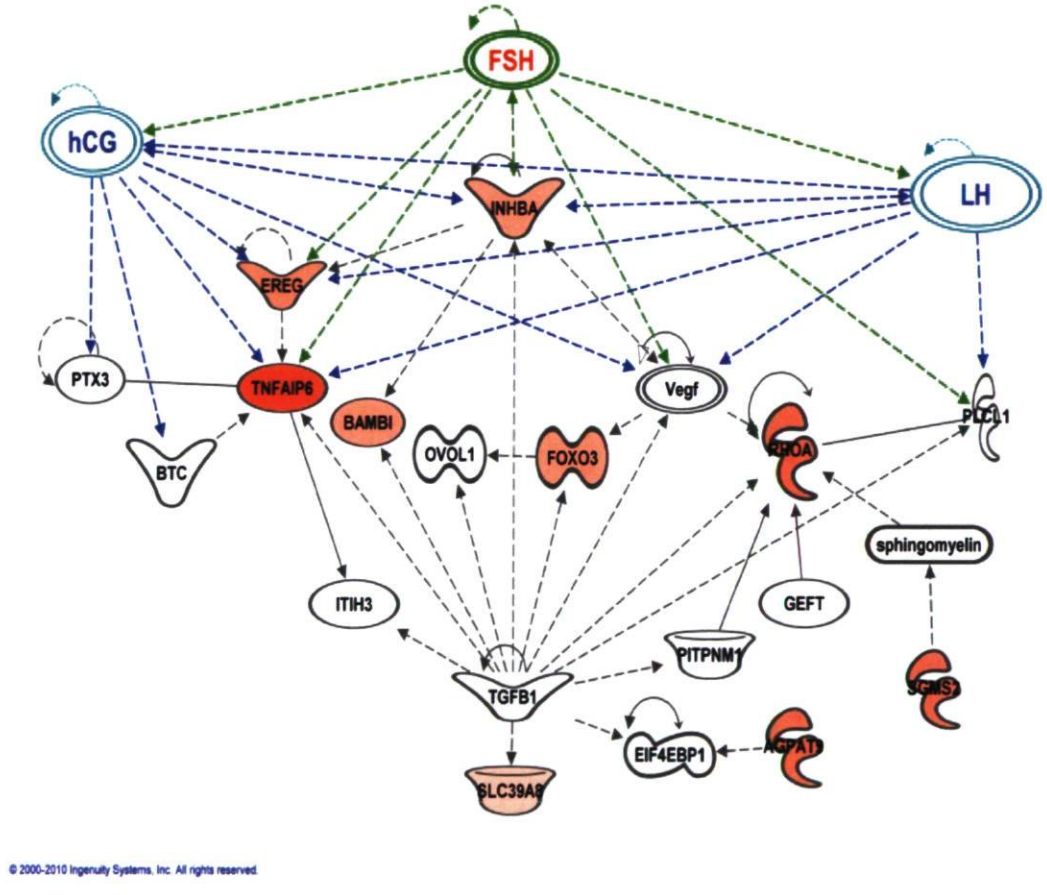

Figure 4.3: Common gene candidates induced by FSH in vitro versus LH/hCG in vivo as revealed by the IPA software.

4.9. TABLE

Table 4.1: Common overexpressed genes in bovine CCs around the GVBD between FSH *in vitro* versus LH *in vivo*

No.	Gene name	Gene/protein full name (if available)	Accession no.
1	ATP1B4	Bos taurus ATPase, (Na+)/K+ transporting, beta 4 polypeptide	NM_001101919
2	ATP6V1C1	Bos taurus ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C1	NM_176676
3	BAMBI	Bos taurus BMP and activin membrane-bound inhibitor homolog	NM_001046309
4	HSPA8	Bos taurus heat shock 70 kDa protein 8	NM_174345
5	INHBA*	Bos taurus inhibin, beta A	NM_174363
6	PAPD1	Bos taurus PAP associated domain containing 1,	BC104501
7	PSMA2	Bos taurus proteasome (prosome, macropain) subunit, alpha type, 2	BC102206
8	RHOA	Bos taurus ras homolog gene family, member A	NM_176645
9	RPL3	Bos taurus ribosomal protein L3	BT021012
10	SELK	Bos taurus similar to selenoprotein K	BC108150
11	SLC25A5	Bos taurus solute carrier family 25 member 5,	BC102950
12	TNFAIP6*Δ	Bos taurus tumor necrosis factor, alpha-induced protein 6	NM_001007813
13	UBA6	Bos taurus ubiquitin-like modifier activating enzyme 6	NM_001083438
14	CHSY1	Homo sapiens carbohydrate (chondroitin) synthase 1	NM_014918
15	EREGΔ	Homo sapiens epiregulin	NM_001432
16	FOXO3A	Homo sapiens forkhead box O3 (FOXO3), transcript variant 2, mRNA	NM_201559
17	-	Homo sapiens mRNA differentially expressed in malignant melanoma,	AJ293391
18	NEAT1	Homo sapiens nuclear enriched abundant transcript 1	EF177379
19	SGMS2	Homo sapiens sphingomyelin synthase 2 (SGMS2), transcript variant	NM_001136258
20	AGPAT9	PREDICTED: Bos taurus similar to 1-acyl-sn-glycerol-3-phosphate O-acyltransferase 9	XM_597964
21	RBMX	PREDICTED: Bos taurus similar to Heterogeneous nuclear ribonucleoprotein G	XM_875611
22	SLC39A8	PREDICTED: Bos taurus similar to Solute carrier family 39 (zinc transporter), member 8	XM_584935

*: overexpression validated *in vitro* by real time PCR ; Δ: overexpression validated *in vivo* by real time PCR; α=0.05%

CHAPTER 5

Biomarkers of human oocyte developmental competence expressed in cumulus cells before ICSI: A preliminary study

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5.1. RÉSUMÉ

Objectif: identifier des biomarqueurs génomiques fiables exprimés dans les cellules du cumulus qui peuvent prédire de façon précise et non invasive la compétence au développement des ovocytes et de renforcer les critères morphologiques déjà utilisés en cliniques de FIV.

Méthodes: Huit patientes consentantes ont été sélectionnées pour la stimulation ovarienne et les procédures de l'ICSI. Les complexes ovocyte-cumulus ont été collectés par voie transvaginale et individuellement choisis en fonction de deux critères : des propriétés morphologiques satisfaisantes et une haute biréfringence de la zone pellucide. À la suite de l'ICSI, deux embryons âgés de 3 jours ont été transférés à chaque patiente. Les résultats du test de grossesse et de la confirmation ultérieure de l'implantation ont été enregistrés et confirmés. L'expression différentielle de gènes a été évaluée au moyen de deux plateformes de biopuces. Outre la validation par PCR en temps réel, l'analyse des réseaux de gènes (Ingenuity Pathways Analysis) et l'analyse intra-patientes ont été effectuées sur les 17 gènes candidats sélectionnés.

Résultats: Sept gènes étaient différentiellement exprimés ($p \leq 0,05$) dans les cellules de cumulus associées à des ovules de patientes ayant réussi une grossesse. Ces biomarqueurs peuvent être utilisés pour prédire la compétence des ovocytes de développement.

Conclusions: Ces marqueurs génomiques sont un renfort puissant des approches morphologiques de sélection des ovocytes. Leur validation en utilisant un nombre plus grand de patientes pourrait augmenter le taux de grossesses réussies ainsi que l'efficacité du transfert monoembryonaire.

5.2. CAPSULE

Cumulus cells expressed some molecular biomarkers that discriminate the developmental competence of oocytes having similar and satisfactory morphological and ZP birefringence properties in human IVF.

5.3. ABSTRACT

Purpose: To identify reliable genomic biomarkers expressed in cumulus cells that accurately and non-invasively predict the oocyte developmental competence and reinforce the already used morphological criteria.

Methods: Eight consenting patients were selected for ovarian stimulation and ICSI procedures. Cumulus-oocyte complexes were transvaginally punctured and individually selected based on both good morphological criteria and high zona pellucida birefringence. Following ICSI, two 3-day embryos per patient were transferred. Pregnancy outcome was recorded and proven implantation was thereafter confirmed. Differential gene expression was assessed using two microarray platforms. Further real-time PCR validation, Ingenuity pathways analysis and intra-patient analysis were performed on 17 selected candidates.

Results: Seven genes were differentially ($p \leq 0.05$) associated to successful pregnancy and implantation. These biomarkers could be used to predict the oocyte developmental competence.

Conclusions: These genomic markers are a powerful reinforcement of morphological approaches of oocyte selection. Their large-scale validation could increase pregnancy outcome and single embryo transfer efficiency.

KEY WORDS

Cumulus cells; genomic biomarkers; infertility; oocyte developmental competence; pregnancy; zona pellucida birefringence;

5.4. INTRODUCTION

Infertility is the biological inability of a couple to conceive a baby after one year of unprotected sexual intercourse. Its incidence is rising mainly in the developed countries. Assisted reproductive technologies (ART) offer interesting treatment possibilities to infertile couples by means of procedures that include the selection of the suitable gametes to fertilize and thereafter of the appropriate embryos to transfer (Balaban & Urman 2006). This selection of high-quality embryos to transfer is still challenging in human ART. It was reported to be dependent largely on the oocyte quality acquired during the maturation process (Krisher 2004).

To clinically predict the oocyte developmental potential, several non-invasive parameters including early embryo (number and appearance of blastomeres, multinucleation, etc.) and oocyte morphology (oocyte diameter, an integral first polar body and a less granulated cytoplasm) were used (Ebner *et al.* 2003, Yu *et al.* 2009). Additional criteria related to the oocyte and its neighboring cells were also used (Coticchio *et al.* 2004). In this context, cumulus cells (CCs) appearance and number of layers were used (Chan 1987, Tanghe *et al.* 2002, Borini *et al.* 2005). Unfortunately, these morphological and microscopic criteria are subjective and lack enough accuracy and therefore weakly correlated with successful pregnancy (Guerif *et al.* 2007). To balance the weakness of these morphological criteria and increase the pregnancy outcome, clinicians have resorted to transferring more than one embryo. Consequently, the incidence of multiple pregnancies, perinatal mortality as well as health problems for both the mother and the babies increased considerably (Adashi *et al.* 2003, Templeton 2004, Bromer & Seli 2008).

In order to avoid these drawbacks on both the mother and the children, and reduce health care costs, elective single embryo transfer (eSET) is therefore a necessary approach (Gerris 2005, Pinborg 2005). In addition to the reduction of multiple pregnancy rates, eSET is reported to provide several benefits not only associated to the safety of the mother and the offspring, but also at the socio-economic level. Indeed, a successful eSET requires the accurate selection of high-quality embryo to transfer (Hamamah *et al.* 2007, Sunde 2007, Gerris 2009).

It is established in most mammal species including human that embryo quality depends mainly on the competence of the oocyte selected for fertilization. This competence is the result of mutual interactions of the oocyte with its follicular environment (Blondin & Sirard 1995, Mikkelsen & Lindenberg 2001, Krisher 2004, Balaban & Urman 2006). CCs are the somatic cells that occupy the immediate vicinity of the oocyte and represent the first interface of the oocyte with its

environment (Hussein *et al.* 2006). CCs maintain a continuous and reciprocal relationship with the oocyte and are involved in the support and the chronology of oocyte maturation and competence acquisition (Hashimoto *et al.* 1998, Eppig *et al.* 2002, Sirard & Trounson 2003, Hussein *et al.* 2006). They are also reported to be involved in the oocyte maturation, ovulation as well as the fertilization process (Tanghe *et al.* 2002, Thompson *et al.* 2007). Given their importance in oocyte maturation and therefore embryo quality, CCs are usually used both in animal IVF and clinics as one of the main morphological criteria to assess the oocyte developmental potential (Blondin & Sirard 1995, Gardner & Sakkas 2003, Wang & Sun 2007). Despite the interesting morphological criteria used including CCs morphology, there is still little correlation between these parameters and the pregnancy final outcome (Borini *et al.* 2005, Guerif *et al.* 2007). Additional early approaches to reinforce these previous criteria were explored. Genomic biomarkers expressed in CCs are an interesting approach to non-invasively and quantitatively predict the oocyte developmental competence (Assou *et al.* 2008, Anderson *et al.* 2009).

Based on the previous reports, we used herein the morphological properties of CCs to reflect the oocyte quality. These morphologically good cumulus-oocyte complexes (COCs) were associated with their pregnancy outcome. We sought to identify quantitative biomarkers expressed in CCs that could accurately reflect the oocyte developmental potential. Finding differentially expressed genes associated with oocytes that lead to successful pregnancy would offer concise predictors to select high-quality oocytes and improve pregnancy rates in human ART.

Besides the oocyte and CCs morphology, zona pellucida (ZP) birefringence is another morphological criterion that was also used in our study to reinforce our selection of high-quality oocytes. In fact, the inner immediate layers of CCs are connected with the ZP, which is a thick protective layer that surrounds the oocyte. Many studies have demonstrated a positive correlation between ZP thickness and oocyte developmental competence, successful embryo implantation and subsequent pregnancy rates (Montag *et al.* 2008, Montag & van der Ven 2008, Madaschi *et al.* 2009). To prevent any user bias and to increase objectivity and repeatability, ZP thickness and homogeneity measurements in Dr. Montag's lab were automated (Montag *et al.* 2008, Ebner *et al.* 2009).

The goal was therefore to define a preliminary list of genomic markers expressed differentially in the CCs of oocytes with high ZP birefringence (HZB) and associated with successful pregnancy.

These molecular biomarkers should offer a powerful tool to considerably improve oocyte selection, and therefore increase the pregnancy outcomes and diminish multiple pregnancy incidences. Ultimately, once the large-scale validation of these biomarkers is achieved, efficient single embryo transfer could be envisioned.

5.5. MATERIALS AND METHODS

5.5.1. Patient selection

Eight consenting patients (**n=8**) were selected for this study at the IVF clinic of the University of Bonn Medical School. ICSI (intracytoplasmic sperm injection) was the recommended procedure for all the patients.

5.5.2. Ovarian stimulation and cumulus-oocyte complex (COC) retrieval

Ovarian stimulation with the administration of the gonadotrophin releasing hormone agonist (GnRHa) triptorelin acetate (Decapeptyl (0.1 mg/day), Ferring, Germany) was started on the 22nd day of the preceding oestral cycle. Daily administration of the human menopausal gonadotrophin (HMG; Menogon, Organon) and/or follicular stimulating hormone (FSH; Gonal-F, Serono) was carried out 12 to 15 days later. The HMG/FSH (225 IU) dose was adjusted through transvaginal ultrasound monitoring of the patient's individual response, mainly follicular size and oestradiol levels. When some follicles of the ovulatory wave were larger than 18 mm in diameter, human chorionic gonadotrophin (HCG; 10,000 IU) was administered and 36 to 38 h later, COCs were transvaginally punctured. All the protocols used herein were approved by the institutional review board of the University of Bonn Medical School.

5.5.3. Cumulus cells collection and zona birefringence analysis

Following follicular aspiration, collected COCs were immediately washed in HEPES-buffered medium (Cook, Brisbane, Australia) and individually cultured in fertilization media (Cook) for 2 hours. Incubation was performed in a mini-incubator (Minc, Cook) using pre-mixed gas with low oxygen (6% CO_2, 5% O_2, 89% N_2) at 37°C. Each COC was put in a dish containing HEPES-buffered medium under oil. CCs were dissected using a sterile scalpel and transferred immediately into a sterile tube and stored at -80°C for further analysis.

Next, a hyaluronidase treatment to remove the remaining cumuli was performed and denuded oocytes were individually incubated at 37°C in 5-µL droplets of fertilization medium covered

with mineral oil in a glass-bottom dish (Willco, Wells BV dish, MTG, Altdorf, Germany) for 1 to 2 h. Oocytes with vacuolization were excluded and neither used for zona imaging nor for ICSI. Prior to switching to the birefringence analysis mode to assess the zona score, immature oocytes (absence of the first polar body when scanned by conventional light microscopy) were also removed. As described previously by Dr. Montag's team (Montag *et al.* 2008), unfertilized MII oocytes were classified based on their inner zona layer birefringence measurement using an automatic module Octax polairAide™ (Octax ICSI Guard™, OCTAX Microscience GmbH, Altdorf, Germany) connected to a polarization imaging software (OCTAX Eyeware™) that recorded images combining bright field (green) and birefringence (red). Zona score was therefore automatically and non-invasively measured in real time based on the intensity and the uniformity of the birefringence at 180 measuring points of the inner zona layer. The temperature of the heated plate was linked to a calibrated sensor to maintain 37.0 ± 0.5°C in the medium droplet during microscopic observation. A micromanipulation system (Eppendorf, Hamburg, Germany) adapted to the microscope allowed rotation of oocytes to optimize zona visualization and scoring. MII oocytes with an irregular and/or low birefringence distribution in the inner zona layer were classified as low zona birefringence (LZB). However, those with a high intensity and uniform birefringent inner zona layer were classified as high zona birefringence (HZB) (Montag *et al.* 2008).

5.5.4. Intracytoplasmic sperm injection (ICSI)

All media used for oocyte retrieval, denuding, ICSI treatment and subsequent culture were of pharmaceutical grade, free of phenol red and provided by Cook company (Fertilization, Cleavage, Gamete, PVP, Hyaluronidase, Culture oil). The selection of patients for ICSI treatment was based on the diagnosis of male factor infertility due to reduced sperm quality (mainly morphological abnormalities). ICSI was the recommended and the most efficient approach in this case to prevent male factor effect (Mangoli *et al.* 2008). In a collaborative approach, all patients underwent an extensive andrological, gynaecological and cytogenetic examination prior to ICSI to avoid any other bias.

ICSI was performed within 1 h after zona imaging. Oocytes were kept in the same order as during zona imaging and subsequently cultured individually in 30-µL medium droplets under oil. The spermatozoa ejaculate was first diluted by a mini-swim-up technique, then washed first with Gamete-100 buffer and finally with 1 mL of fertilization medium. After each wash step, a

centrifugation step in a microfuge (Biofuge 13, Heraeus, Osterode, Germany) was achieved. The final sperm pellet was resuspended in 20–50 mL of fertilization medium and stored in a CO_2 incubator. A few microliters of the motile sperm suspension were placed into a central polyvinylpyrrolidone (PVP) droplet in the injection dish where their morphological criteria were quickly checked. ICSI was carried out on the heated stage of an inverted microscope (DMIRB; Leica, Bensheim, Germany) equipped with microinjection devices for holding the oocyte and sperm injection (Narishige, Tokyo, Japan). All MII oocytes were fertilized by ICSI. Following injection, oocytes were cultured in cleavage medium up to the time of transfer.

5.5.5. Embryo culture and transfer

Eighteen (18) hours following ICSI, oocytes with two pronuclei (2PN) of equal size in close proximity and centrally located within the ooplasm were considered as successfully fertilized. Among them and due to legal restrictions, only two fertilized oocytes were chosen for transfer. The principal criterion for selection was the intensity of zona birefringence (the two top zone scorer were taken). Two oocytes with initially HZB were chosen for further embryo culture and transfer; whereas the supernumerary oocytes were cryopreserved. The selected 2 x 2PN were further individually cultured until transfer on day 3 using the Cook culture system (COOK, Brisbane, Australia). Incubation was done in a Minc benchtop incubator at 5% O_2, 6% CO_2, 98% N_2. Transvaginal intrauterine embryo transfer was performed in 30 µL of culture medium using a Sydney IVF catheter (COOK, Brisbane, Australia) as described previously (Montag *et al.* 2008). Progesterone vaginal suppositories (200 mg/day) were used twice a day to support the luteal phase support. This treatment began on the day following the HCG administration. Pregnancy was assessed first through a positive HCG test at day 14 after transfer and then a higher value 2 days later. Proven implantation and pregnancy were thereafter confirmed by ultrasonic detection of gestational sacs and a positive heart beat (viable embryo) 3 weeks later.

5.5.6. Patient groups

Based on the pregnancy results, individual cumulus cells from the eight (n=8) patients were divided into two main groups to explore *in vivo* genomic markers expressed in CCs and associated with oocyte competence, embryo quality as well as pregnancy. The CCs of the zona good oocytes with successful pregnancy (ZGP) was the first (positive) group. It included 8 cumuli of individual oocytes (from 4 patients) that led to pregnancy. Only one patient gave

heterozygous twins while the other three got a single embryo after transferring two HZB fertilized oocytes. On the other hand, the second (negative) group contained 6 individual cumuli of individual oocytes (again from 4 different patients) with zona good score but an unsuccessful pregnancy (ZGNP). While ZGP represented the positive group, ZGNP was the negative one.

5.5.7. Custom-made cDNA microarray preparation

Four suppressive subtractive cDNA hybridizations (SSH) previously achieved in our lab were printed on our custom-made microarray. Differentially expressed cDNAs in both cumulus and granulosa cells associated to *in vivo* competent oocytes (Robert *et al.* 2001, Assidi *et al.* 2008, Hamel *et al.* 2008b) were amplified, purified, sequenced, and identified by BLAST analysis against the cDNA Library Manager Program (Genome Canada bioinformatics, Quebec, Canada). SSH products and negative and positive controls were dissolved in equal volumes of dimethyl sulfoxide (DMSO) and H_2O and spotted in duplicate on different locations on GAPSII glass slides (Corning, Corning, NY) using VersArray Chip WriterPro robot (Bio-Rad, Mississauga, Canada) as detailed elsewhere (Assidi *et al.* 2008). UV rays served to cross-link the oligonucleotides before the Terminal Deoxynucleotidyl Transferase quality control Assay (GE healthcare, Quebec, Canada).

5.5.8. Total RNA extraction

The CCs samples of each oocyte in both experimental groups were subjected to total RNA extraction using the PicoPure RNA Isolation Kit (Arcturus, Molecular Devices Analytical Technologies, Sunnyvale, CA) according to the manufacturer's instructions. Briefly, CCs were extracted in 100 µL of Extraction Buffer (XB), incubated for 30 min at 42°C and centrifuged 2 min at 3000g. The supernatant containing the RNA was collected, mixed with an equal volume of 70% ethanol, transferred to a previously conditioned purification column and spun for 1 min. To prevent contamination and immediately after a first wash with 100 µL of w1 wash buffer, an on-column DNase digestion for 15 min on benchtop with the RNase-Free DNase Set (Qiagen, Maryland, USA) was performed according to the manufacturers' instructions. Following the two washing steps respectively with buffers w1 and w2 provided, the column product was resuspended in 30 µL of Elution buffer (EB) provided in the kit. The concentration and quality of the RNA were assessed using the Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's protocol.

5.5.9. Messenger RNA linear amplification

Based on RNA concentrations of each individual CC (biological replicate), 10 ng of total RNA were pooled for each experimental group (pool of 8 and 6 replicates respectively for the pregnant and the non-pregnant groups) for amplification using 2-round *in vitro* transcription (IVT) following the instructions of the RiboAmp^{plus} RNA Amplification kit (Arcturus, Molecular Devices Analytical Technologies). Briefly, RNA was first reversed transcribed with the incorporation of a primer containing a T7 RNA polymerase promoter sequence (RiboAmp primer A). Double-stranded cDNA was then synthesized, column-purified and used as a template to drive the first 6-h round of the T7-polymerase IVT. One microliter of this elution was used for NanoDrop (NanoDrop Technologies, Wilmington, DE) quantification of the first round yield, whereas the rest served as a template for the second round. Similarly to round 1, the second linear amplification round was carried out according to the kit recommendations and the resulting RNA was column-purified and eluted in 30 µL of RNA eluted buffer (RE). The final RNA amplification yield was quantified by spectrophotometry at 260 nm using the NanoDrop ND-1000 (NanoDrop Technologies) as before.

5.5.10. Messenger RNA indirect labelling

Amplified messenger RNA of each group (ZGP vs. ZGNP) was divided into 2 sub-replicates per chip type (Figure 5.1) to get a dye-swap design and labelled using the Universal Linkage System (ULS™) aRNA Fluorescent Labelling Kit (KREATECH Biotechnology, Amsterdam, The Netherlands) according to the manufacturer's instructions. Briefly, for each sub-replicate, 2.5 µg of amplified RNA was labelled by incubation with 2.5 µL of Cy5/DY647-ULS or Cy3/DY547-ULS dyes, and 2 µL of 10x labelling solution in a 20-µL total volume at 85°C for 15 min. Unbound dye was then removed, as recommended, using the KREApure columns provided in the kit. Labelled RNA was quantified on the NanoDrop ND-1000 (NanoDrop Technologies). Each probe of the ZGP group was mixed with its corresponding one in the ZGNP group in equimolar proportions before hybridization.

5.5.11. Hybridization design

5.5.11.1. Custom-made cDNA microarray hybridizations

Two hybridizations were performed in a dye-swap design (Figure 5.1) on our custom-made array. Hybridizations were performed in the ArrayBooster using the Advacard AC3C (The Gel

Company, San Francisco, CA) for 18 h at 50°C using Slide Hyb#1 (Ambion, Austin, TX). The slides were then washed twice in 2X SSC/0.5% SDS buffer, and twice in 0.5X SSC/0.5% SDS buffer. After two quick final washes at room temperature in 1X SSC and water, slides were spin-dried, scanned and analyzed using the ChipReader and ArrayPro Analyzer software (Media Cybernetics, San Diego, CA).

5.5.11.2. Hybridizations using the OneArray microarray

In order to achieve an extensive candidate search, two additional hybridizations in a dye-swap design (Figure 5.1) were performed using two One Array chips (Phalanx Biotech, Palo Alto, CA). It consisted of a 60-mer sense-strand polynucleotide microarray that contains 30,968 of highly sensitive and specific human probes and 1,082 experimental control probes. After a prehybridization step of 10 min at 60°C, the hybridization protocol was performed according the DNA microarray user guide available at http://www.phalanxbiotech.com/Support/Usr_Gd_combo.pdf and using the recommended hybridization and washing buffers.

5.5.12. Hybridization data analysis

Following hybridization, both microarray slides were scanned using the VersArray ChipReader 3.1 System (Bio-Rad, Mississauga, Canada) and analyzed using the ArrayPro Analyzer software (Media Cybernetics, Bethesda, MD). Raw microarray data were first Loess-normalized and corrected for background as described elsewhere (Assidi *et al.* 2008). Ratio of net fluorescence intensities of our dye-swap experiments between positive (pregnant) and negative (non-pregnant) group was analysed using the free-software National Institute on Aging (NIA) Array Analysis Tool (Baltimore, MD) developed at NIA (NIA 2010) at FDR=5% and a minimum cut-off limit of 2.25. Since each clone was printed twice on our slide (Hamel *et al.* 2008b), two additional technical sub-replicates that emerged from this design were taken into account during statistical analysis. Two lists of more than two-fold change in both over-expressed and under-expressed clones in the ZGP group compared to ZGNP were generated for subsequent analysis to define suitable markers expressed in CCs and associated with good-quality oocytes.

Functional genomic analysis including network mapping and molecular pathways of both overexpressed and underexpressed genes within the two patient groups (ZGP vs. ZGNP) was achieved using the Ingenuity Pathways Analysis software (IPA) (IPA 2010). Briefly, the candidate genes were uploaded with their official name and fold change into the IPA. Using its web database based on previous studies, IPA is able to automatically find the potential

connections between the uploaded genes and to classify them into networks (using numerical scores) according to the molecular pathways involved and the quality of the gene relationships already established. Each network is therefore composed of selected genes from the uploaded list linked together and with other molecules (suggested by the software), and mapped in a whole signalling pathway. Each molecular relationship among the network members is represented in a conventional mapping that allows identification and therefore interpretation of the type of interactions.

The use of IPA analysis in our study aims at discovering some gene networks and exploring the possible interactions between the genomic biomarkers differentially expressed in CCs and associated to oocytes with successful pregnancy.

5.5.13. Real-Time PCR validation

Equal amounts of total RNA were taken from each replicate (non-amplified original material) of individual CCs of each patient group. To denature the RNA and remove secondary structures, the RNAs were heated at 65°C for 5 min and then quenched rapidly on ice for at least 2 minutes. Samples were then reversed transcribed using the SensiScript reverse transcriptase kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's recommendations. Real-time PCR was performed on the **17** selected candidates from both hybridizations of our custom-made cDNA array and the 60-mer oligonucleotide OneArray chip in LightCycler capillaries (Roche Applied Science, Mannheim, Germany) using the LightCycler FastStart DNA Master SYBR Green I (Roche) as detailed elsewhere (Assidi *et al.* 2008). Candidate selection for QPCR was based on their fold change and their redundancy within our libraries. For each candidate, a specific set of primers was designed using the NCBI's primer-blast software and the candidates' specific sequences (NCBI) (Table 5.1). Additionally, three housekeeping genes, ACTB (β-actin), GAPDH, and PPIA, were quantified and used in GeNorm normalization. The two most stable housekeeping genes (ACTB and PPIA; $P > 0.05$) had an M value=1.17 which is less than the 1.5 value recommended by the GeNorm software. These two housekeeping genes were therefore used in both groups as the suitable control genes for quantitative real-time PCR (QPCR) data normalization. The real-time PCR product specificity of each candidate was confirmed by sequencing to validate the amplification of the appropriate product as well as by analysis of the Lightcycler melting curve (Roche). Each gene mRNA expression level was then divided by its

normalization factor and log-transformed. A t-test to compare gene expression levels between both groups was then performed using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA) at α=0.05.

5.5.14. Intra-patient profound analysis

In order to discriminate between the positive samples (associated to successful pregnancy) and the negative ones within each patient (two embryos were transferred per patient), we used the principal component analysis (PCA) on normalized gene expression levels of the 7 significant genes revealed by QPCR. PCA is a powerful method that allows the accurate projection of a high-dimensional data set onto a new space with reduced dimensions for easy visualization and interpretation. Mathematically, the new space axes, called principal components (PC), are determined successively by order of importance according to the amount of variance they represent in the original data. Each PC is therefore a linear combination of the levels of expression of the measured genes. Based on the maximal Euclidean distance between the positive and negative samples, PCA was used to determine the true positive sample associated with successful pregnancy among the two transferred embryos as described elsewhere (Hamel *et al.* 2010).

Using PCA, the true positive sample (associated with successful pregnancy) in each patient was determined. Based on these results, binary logistic regression was used to establish a preliminary predictive model that links the pregnancy probability (successful pregnancy=1 vs. failure pregnancy =0) to the expression level of each biomarker gene measured in CCs. This preliminary model allows the determination of the relative importance of our biomarker genes in the pregnancy success probability at α=0.05

5.6. RESULTS

5.6.1. Microarray data analysis

CCs biomarker candidates were selected using two different platforms. The first was a custom-made microarray platform obtained by suppressive subtractive hybridizations of cDNA sequences, whereas the second was the OneArray commercial arrays. Following microarray

experiment analysis, candidate gene selection was achieved based on the microarray results from the dye-swap of both our custom-made cDNA array and the OneArray slides. By comparing the positive clone lists from the two different groups, two main categories of candidates were selected based on their fold change (fold > 2, FDR=5%). The first category corresponds to the competence markers and includes **260** candidate genes (69 from our library and 191 from OneArray) that were differentially expressed in the CCs of pregnant patients compared to the non-pregnant group. Conversely, the second group contains **231** potential incompetence markers (29 in our library and 202 from OneArray) that were underexpressed in the CCs of pregnant patients compared to the non-pregnant. These candidates are potential negative indicators of oocyte quality.

The selected differentially expressed candidates revealed by our analysis were then ordered according to their redundancy in different libraries, their signal intensities (Figure 5.2) and their recurrence inside the same library. Given the importance of the cross-dialog between CCs and oocyte to fulfil maturation, it was expected that these candidates would likely be potential indicators associated with the molecular process of oocyte competence.

5.6.2. Real-time PCR analysis and biomarker determination

In order to validate both our positive and negative genes lists, 17 candidates (14 overexpressed genes and 3 underexpressed genes) were chosen for subsequent validation by quantitative real-time PCR. The QPCR validation was achieved on the CCs tissues of the two ZGP and ZGNP groups (positive and negative groups). Seven (7) candidates among the 17 assessed were statistically significant following QPCR analysis, which represents a proportion of more than 41%. In fact, among the 14 positive candidates selected, 6 positive markers of oocyte quality and successful pregnancy were statistically significant between pregnant and non-pregnant patient groups. These candidates are DPP8 (p=0.0441), HIST1H4C (p=0.0482), UBQLN1 (p=0.0236), CALM1 (p=0.05), NRP1 (p=0.0107) and PSMD6 (p=0.0412) (Figure 5.3A).

As for the 3 underexpressed markers assessed, only TOM1 (p=0.0126) was confirmed as a negative marker differentially expressed in the CCs of the non-pregnant patient group. TOM1 is therefore an interesting incompetence marker that was highly significant following the QPCR validation (Figure 5.3B).

5.6.3. Intra-patient analysis and positive embryo determination

This profound statistical analysis was achieved only on CCs from patients who got a single embryo after transferring two HZB fertilized oocytes. The objective was to distinguish the embryo that led to successful pregnancy among the two HZB transferred embryo. Principal component analysis (PCA) is a multivariate procedure able to transform a great number of correlated variables into a reduced and independent number of variables named the principal components (PC). We applied PCA to normalized gene expression levels of the 7 significant candidates to maximize the variance (Euclidian distance) between the negative and the positive embryos. Following PCA analysis and eigenvalues determination, five uncorrelated PCs were reported. The first principal component F1 accounted for 78.15% of the data total variability, while the F2 accounted for 17.95%. The representation of our six CCs zona good (ZG) samples (two samples for each patient) in the new space including just F1 and F2 represented 96.10% of the total variability (Figure 5.4). This representation allowed us to discriminate the true positive embryo associated with successful pregnancy from the two embryos transferred.

5.6.4. Pregnancy prediction model

The logistic regression was used to explore mathematical relationships between diverse input variables and an output explained variable that takes values between 0 and 1. If the output variable has just two potential outcome ("successful pregnancy" or "failure", *i.e.*, 1 or 0), the appropriate model is the binary logistic regression (BLR). Therfore, we used BLR to predict the probability of occurrence of pregnancy using the normalized expression levels of the significant genomic biomarkers and their pregnancy outcome according to the PCA analysis described before. Given the high correlation between most of our gene candidates (r^2>0.95 between 4 genes), only two biomarker genes (HIST1H4C and NRP1) were maintained in the predictive model whose determination coefficient (R^2= 0.75) was very satisfactory. The two genes' coefficients (slope) as well as the constant term were very significant ($P < 0.0001$) following the Khi² test at the 95% confidence level. The preliminary model of pregnancy probability prediction is therefore simply written as shown by figure 5.5. This simplified model was established using the minimum number of gene markers that were significant. It is to note that this model required further validation step (with a large group of patients receiving SET) in order to be useful in clinical applications.

5.6.5. Gene network analysis

Genomic analysis of network mapping and molecular pathways of the differentially expressed genes using the Ingenuity Pathways Analysis (IPA) software was performed to investigate potential signalling pathways and biological processes driven by our gene predictors of pregnancy. Two networks were selected for deep analysis and exploration of gene candidates' interactions. The first network included in particular the candidates validated by QPCR (Figure 5.6). Most of the 17 modulated candidates were involved in diverse molecular and cellular functions including intercellular signalling and cell cycle (AR, UBQLN1, PKN2), cell assembly and interactions (NRP1, TOM1, PKN2), molecular and vesicle trafficking (CHGB, TOM1, SYT11), protein folding and/or apoptosis (CALU, PSMD6, DPP8), calcium-dependent pathways (CALM1, CALU), gene expression and cell differentiation (HIST1H4C, RPL9, THOC2).

The second pathway shows a custom-made network that reflects the complexity of the signal transduction pathways in CCs (P38 MAPK, PI3K and PLC pathways). At this physiological period just prior to ovulation, CCs have reached full expansion marked by cell distension and selective gap-junction down-regulation (AR, NRP1, CAV1, PTX3, and GJA1). In order to prepare ovulation as an inflammatory-like process, many candidates were reported herein to be regulated including caspases, and chaperone proteins (HSPD1). Most of these pathways occurred downstream the preovulatory gonadotropin stimulations (LH/hCG) (Figure 5.7).

5.7. DISCUSSION

Despite several morphological criteria used, prediction of oocyte developmental competence is still challenging. Finding quantitative biomarkers that could accurately predict oocyte quality and subsequent pregnancy would be a valuable tool to improve the clinical outcomes of IVF and ICSI procedures. CCs are the site of expression of gene sets that are correlated to the oocyte maturation status and could serve to predict the pregnancy outcome (McKenzie *et al.* 2004, van Montfoort *et al.* 2008). Moreover, CCs maintain a close relationship with the oocyte during ovulation, fertilization and even until early embryo development (Motta *et al.* 1995, Hunter 2003, Hernandez-Gonzalez *et al.* 2006). In addition to the effect of gonadotropins on CCs, it was also established that their functions are under the control of oocyte-secreted factors (Lucidi *et al.* 2003, Sugiura *et al.* 2007, Gilchrist *et al.* 2008). These properties offer CCs key roles in the competence acquisition process and could therefore be the site of expression of valuable markers

that reflect oocyte competence. The main goal of this work was the identification of genomic markers expressed in CCs and associated with high developmental potential oocytes. CCs were harvested just prior to ICSI to ensure that the CCs at this stage are the most reflective of oocyte quality, and therefore express suitable markers to investigate.

To find valuable biomarkers that provide added value to the selection criteria already used, all the selected COCs had good morphological properties as well as a high ZP score. Although morphological criteria are widely used in IVF clinics, several studies have supported a significant improvement of pregnancy outcome in oocytes with high and uniform ZP birefringence (Host *et al.* 2002, Montag *et al.* 2008, Ebner *et al.* 2009, Madaschi *et al.* 2009). Using similar and good morphological and ZP criteria, the individual CCs were classified based on the pregnancy outcome of their oocytes. Two groups of pooled CCs samples were analysed: ZGP (8 CCs of the pregnant group) and ZGNP (6CCs of the non-pregnant group).

Hybridizations were performed using two different microarray platforms. The first was a custom-made microarray platform obtained by suppressive subtractive hybridizations of cDNA sequences (200 to 1000 bp) differentially expressed in follicular cells (cumulus and granulosa cells) of oocytes that successfully achieved embryo development (Hamel *et al.* 2008b). The second microarray platform was the OneArray commercial arrays, which contains more than 32,000 60-mer polynucleotide probes (OneArray 2010). While the whole genome OneArray microarray was expected to yield more gene candidates, our custom-made library should reveal more specific candidates (Figure 5.2); and thus the two platforms are thought to be complementary. In fact, the OneArray candidates represented respectively 73.4% (191/260) and 87.4% of the overexpressed and the underexpressed candidates with more than 2-fold change that were kept in our microarray data analysis. We believe that these *in vivo* candidates expressed just prior to ICSI could reflect the normal physiological and genomic contexts needed for good oocyte production and successful pregnancy. The proximity of the oocyte would confer CCs a high potential to notify its developmental potential both in ICSI programs or IVF cycles (Feuerstein *et al.* 2007, Assou *et al.* 2008). Due to the complexity of the signalling pathways in CCs, the study of both over- and underexpressed genes seems to be important in order to understand the molecular events in this cell compartment and identify potential pathways that might yield insights about the oocyte competence acquisition process.

The global analysis of both up- and down-regulated candidates in CCs of ZGP versus ZGNP by the IPA software revealed the main molecular and cellular functions triggered by each gene expression pattern (Table 5.2). Just prior to ovulation, CCs are in full expansion which may explain that around 18% of the overexpressed genes are involved in cellular assembly and organization. At this stage, CCs were reported to be less homogenous and therefore of various shapes (Motta *et al.* 1995, Familiari *et al.* 2006) which may explain the down-regulation of cell morphology genes.

The underexpression of main functions such as cell development and protein folding and/or post-translational modifications in CCs (Table 5.2) is consistent with an apoptotic phenotype shown by 40 % of the overexpression genes that are related to the cell death pathways and confirmed by previous studies (Lee *et al.* 2001). It is expected that CCs show signs of apoptosis at the final maturation stage. Some authors have even suggested to use these apoptotic genes during folliculogenesis as potential markers of oocyte development potential (Haouzi & Hamamah 2009). Surprisingly and in parallel to these apoptotic events, the expression of some cell death genes in CCs was markedly reduced. Furthermore, many genes associated with cell cycle, cell growth and proliferation, as well as gene expression were activated (Table 5.2). These gene patterns show a proliferative phenotype that may reduce or delay the impact of the apoptotic events discussed earlier. This CCs genomic behaviour supports a fine gene regulation within CCs to ensure their functions (reviewed by (Tanghe *et al.* 2002)) and highlight their roles as an interface between the oocyte and its environment. It was also established in most mammals that in addition to environmental stimulations, the CCs are governed by the oocyte at many molecular levels including steroidogenesis, gene expression, apoptosis prevention, metabolic activity and inflammatory-like response production (Lucidi *et al.* 2003, Hussein *et al.* 2005, Hernandez-Gonzalez *et al.* 2006, Gilchrist *et al.* 2008, Su *et al.* 2008, Paradis *et al.* 2010). The presence of several genes associated with most of these reported molecular functions in our overexpressed gene list is a further confirmation of our findings. CCs are therefore a suitable target to look for efficient biomarkers associated with oocyte developmental potential (Assou *et al.* 2010). Among the IPA provided pathways, two networks will be discussed later.

Based on microarray data, 17 gene candidates (14 overexpressed and 3 underexpressed) were chosen for further validation by real-time PCR. This validation was achieved on the 14 individual CCs samples (8CCs for ZGP versus 6 for ZGNP). Following normalization and statistical

analysis, 6 competence biomarkers among the 14 overexpressed genes (≈ 43 %) analyzed were found to be statistically significant ($P \leq 0.05$). On the other hand, one of the three incompetence markers assessed was highly significant (TOM1 ($p=0.0126$)). It should be noted that the number of significant markers could have been higher without the technical limitation due to the reduced number of available patients and therefore of the CCs samples used during the study.

To our knowledge, the idea of looking for incompetence markers using high- throughput tools such as microarray is still emerging. We assume / believe that CCs are the site of a two-way dialog between the oocyte and the follicular environment, and could therefore respond by the expression of several useful markers (Feuerstein *et al.* 2007, Hasegawa *et al.* 2007, Assou *et al.* 2008). Biomarkers of oocyte incompetence could therefore constitute additional and important indicators of oocyte quality. Taken together, our preliminary positive and negative markers, once validated on a large number of patients, could be efficient tools to discriminate the competent oocytes to select for IVF / ICSI procedures among a set of high-grade morphological oocytes. The confirmed biomarkers are useful tools that strengthen the morphological selection procedures already used.

In order to increase the chances of pregnancy in our study, two embryos with high morphological grade and ZP birefringence were transferred to each one of the 8 patients. Among them, three women were pregnant with singleton. PCA analysis was the suitable tool to overcome the challenge of determining which embryo from the two transferred induced the pregnancy. In fact, PCA allows an exact projection (based on Euclidian distance) of a high-dimensional data set onto a new reduced space to enable data visualization and interpretation. The new space is composed of few independent axes (independent PC) that represent accurately the original data and therefore maximizing the power to detect true positives. This mathematical tool was previously used in similar context to successfully identify the embryo that led to pregnancy among the two transferred (Hamel *et al.* 2010). Gene expression variation of normalized real-time PCR data of the three patients was used to identify the true positive versus the false positive embryo. The 2D representation of the six CCs samples allowed the identification of the positive embryo among the two embryos transferred for each patient (Figure 5.4). This intra-patient analysis was very helpful to establish a preliminary mathematical model that predicts the oocyte's pregnancy probability based on the significant biomarkers reported herein. While previous oocyte quality predictive approaches were based on morphological and subjective criteria (Hunault *et al.* 2002,

Verberg *et al.* 2008), our preliminary pregnancy model might be more efficient since it relies on quantitative and significant biomarkers associated with successful pregnancy. These markers were measured under similar and satisfactory morphological criteria, and are therefore more discriminative. Keeping in mind the dichotomous criteria of our explained variable (pregnancy= 0 or 1), binary logistic regression was the appropriate model to predict the pregnancy success probability. The simplified model described before (Figure 5.5) was established following many iterative steps to keep the lowest number of gene markers that accurately predict the pregnancy outcome. This model was made using the normalized (but not $\log_{10}$-transformed) gene expression levels. Odds ratio (OR) analysis was also achieved to make this preliminary model more useful to predict the pregnancy chances of an oocyte once the levels of expression of HIST1H4C and NRP1 of its CCs are known. As it is statistically recommended, the relative OR analysis of each gene in the model was measured by fixing the other candidate to its average normalized value from our data set. An increase of one log-expression unit of HIST1H4C or NRP1 provides an increase in pregnancy chances of 59% or 39% respectively. Given the experimental limitations in our study due to the reduced number of samples, this preliminary model needs an additional validation step with large number of samples and patients. Once this validation is achieved, it will be a valuable and quantitative selection tool that could be added to the morphological criteria already in use in clinics. A large-scale study using SET is recommended to validate both the gene biomarkers list and the pregnancy prediction model independently of the possible bias associated to the stimulation regimen. Additional genomic predictors could be added to the present list. The cognate protein levels of the confirmed candidates should be thereafter checked as protein measurements may be seen as an easier way to assess candidate levels in a clinical setting. This validation would offer in the few next years a valuable set of efficient biomarkers that may be helpful to increase both the pregnancy outcome and the SET efficiency.

The use of genomic approaches to study gene expression patterns in CCs should contribute to shed light on the molecular pathways leading to oocyte competence. This is why two IPA networks were selected for analysis. The first network (Figure 5.6) included most of the 17 candidates selected for real-time PCR validation. AR is one of the main genes involved in this pathway. It is a steroid hormone receptor that, once activated, acts as an intracellular transducer signal and/or a transcription factor that interacts with transcription regulators such as TMF1. AR is also involved in CCs steroidogenesis which is under both the oocyte and gonadotropins control

(Lucidi *et al.* 2003). It is expressed in the cumulus granulosa cells at the pre- and post-ovulatory stages in many mammalian species including human and rat (Kimura *et al.* 2007b, Catteau-Jonard *et al.* 2008, Szoltys *et al.* 2009). AR also interacts with the Ca^{2+} pathway via CALU, a calcium-binding protein localized in the endoplasmic reticulum, and CALM1. The calcium pathway in CCs was recently reported to interact with progesterone, PKA and PI3K to induce a chemical attraction of the spermatozoa to the oocyte surface during human fertilization (Teves *et al.* 2009). The presence of PI3K, Ca, PLCB2 and p38 MAPK in both the first and especially the second network 1 downstream of the gonadotropins (LH/hCG) (Haouzi *et al.* 2009) (Figure 5.7) supports these findings.

CCs also exhibited a differentiation status with a fine regulation of some genes associated with the catabolic process, including the ubiquitination machinery (UBQLN1), caspases (CASP9) and the proteasome activity (TOM1). In fact, selective proteolysis was shown to be required for ovulation (Tsafriri 1995, Russell *et al.* 2003). Moreover, this proteolytic activity was shown to involve a specific ubiquitin-proteasome pathway during porcine oocyte maturation, CCs expansion and fertilization (Huo *et al.* 2004, Assidi *et al.* 2008). This selective proteolysis appears to occur simultaneously with an increase in gene expression and translational activities in CCs (RPL9, HIST1H4C) at the preovulatory stage. The upregulated genes at this stage are a bank of potential candidates that may reflect the oocyte quality (Feuerstein *et al.* 2007, Assou *et al.* 2008, van Montfoort *et al.* 2008, Huang & Wells 2010).

We reported also in figure 5.6 the expression of some neuronal-like factors (NRP1, CHGB, and SYT11) involved in vesicle guidance and transport following E2 induction. SYT11 is a Calcium sensor that triggered the membranes' fusion in the intracellular vesicle trafficking, cell exocytosis and the neuronal synaptic vesicle secretion in eukaryotes (Gustavsson & Han 2009, Colvin *et al.* 2010, Lee *et al.* 2010). For CHGB, it is an ubiquitous component of the secretory vesicles in eukaryote cells. This protein was also shown to be co-released with the hormone or the neurotransmitter following the appropriate stimuli. NRP 1, which is the most studied candidate, is a tyrosine kinase coreceptor that can bind to the membrane and is involved in angiogenic and neuronal traffic through the cytoskeleton (Fantin *et al.* 2009). Its expression was also reported in granulosa cells of rat and bovine species (Miyabayashi *et al.* 2005, Shimizu *et al.* 2006). Keeping in mind the fact that synaptic vesicles transport was also achieved via microfilament and microtubule networks (Bhaskar *et al.* 2007) and that similar cytoskeleton structures were

reported in the transzonal cytoplasmic projections between CCs and the oocyte (Albertini 1992, Allworth & Albertini 1993, Sutovsky *et al.* 1994, Barrett & Albertini 2010), one can assume the involvement of neuronal genes and molecules/vesicles exchange in CCs-oocyte dialogue.

Other immunity-associated genes that reflect the inflammatory-like context in CCs just prior to ovulation were also found (AP1, PTX3, HSPD1) (Figure 5.7). These molecules interact with other apoptotic and chaperone molecules (PSMD6, CASP9, TOM1, CAV1, heat shock proteins) to respond adequately to environmental signals and protect the oocyte. The expression of some neuronal and immune-like genes in CCs was previously associated with ovulation (Bottazzi *et al.* 2006, Hernandez-Gonzalez *et al.* 2006, Richards 2007). In the same way, the preovulatory hCG was reported to activate the AP1 transcription factors (through PKA/ERK pathway) (Sharma & Richards 2000); and to increase the CAV1 expression (Diouf *et al.* 2006) in granulosa cells of respectively rat and bovine species. Additionally, the CAV1expression was proportional to the degree of tissue vascularisation (Bullejos *et al.* 2002). Its downregulated in CCs is in agreement with our understanding of the CCs physiology at this stage.

PTX3 is another gene candidate that is involved in CCs expansion and extracellular matrix formation (Salustri *et al.* 2004, Scarchilli *et al.* 2007). Although it was previously suggested as a positive oocyte competence marker (Zhang *et al.* 2005), our microarray data shows its underexpression in the CCs of oocytes that led to pregnancy(Figure 5.7). Other similar reports were unable to find positive correlation between this candidate gene and the oocyte competency (McKenzie *et al.* 2004, Cillo *et al.* 2007). Experimental conditions related to ovarian stimulation, sample collection and processing could be factors that may explain this discrepancy.

GJA1 (Cx43) is one of the proteins involved in cumulus-oocyte gap junctions (GJC) which play a pivotal role in this intercellular communication (Cieniewicz & Woodruff 2010). Its expression is regulated by gonadotropins (LH/hCG) through PKC and PI3K (Figure 5.7) (Shimada *et al.* 2001).These connexin-43-rich GJC were ruptured or obstructed after 6 h of culture in the bovine (GVBD) (Atlas 2010), and substituted thereafter by GJB1 (Sutovsky *et al.* 1993, Sutovsky *et al.* 1994). This GJA1 breakdown was attributed to its integration into the lipid raft microdomains (Sasseville *et al.* 2009b). Consistent with our results which showed a significant decrease of its expression, the GJA1 underexpression was suggested as a good marker of oocyte quality and successful pregnancy (Hasegawa *et al.* 2007). Interestingly, the CCs transzonal projections (TZP) structures based on actin filaments were recently shown to be intact in mouse CCs *in vivo*

prior to ovulation (Barrett & Albertini 2010). Based on these findings, selective regulation of cumulus-oocyte exchanges looks to occur through membrane cytoplasmic extensions mainly the transzonal projections (TZP) until ovulation. These communications are prerequisite for oocyte developmental competence acquisition.

The Calcium pathway is also markedly overrepresented in our data including $Ca^{2+,}$ CALU (calcium-binding protein in the ER), Calmodulin, PLCB2, CALM1, and PI3K (Figure 5.7). This pathway is involved in several signalling pathways and secretory vesicle trafficking (reviewed in (Atlas 2010)). It is established that the LH/hCG stimulation prior to ovulation triggers a very quick rise of intracellular Calcium in CCs. This Calcium was shown to move to the oocyte within few minutes especially when the TZP are not disrupted (Davis *et al.* 1987, Mattioli *et al.* 1998). It is mainly in the *in vivo* context where this CC-oocyte coupling is maintained during the whole maturation phase (Barrett & Albertini 2010). In addition to its established roles in oocyte maturation and meiosis resumption (Homa 1991, Homa 1995, Mattioli *et al.* 1998, Tosti 2006), the calcium pathways is essential to the spermatozoa capacitation, acrosome reaction and subsequent fertilization (Fukami *et al.* 2001, Jose *et al.* 2010)

TOM1 is a gene candidate with still poorly known functions. It was reported to bind to endosomes where it regulated the ubiquitinated protein sorting and the intracellular vesicles trafficking (Katoh *et al.* 2004, Seet & Hong 2005). Moreover, it inhibits some immune-like factors pathways including interleukins and the tumor necrosis factor-induced proteins. Other transcription factors like AP1 were also downregulated by TOM1(Yamakami & Yokosawa 2004). This transcription factor (AP1) is involved in the LH pathway of TNFAIP6 expression in the preovulatory bovine granulosa cells (Sayasith *et al.* 2008). Taken together, TOM1 looks to prevent some molecular events associated to the ovulation process. As a negative biomarker of oocyte competence, its inhibition in the CCs at this stage prior to ICSI becomes therefore understandable.

We should highlight that the two networks studied herein may be only the tip of the iceberg of the whole molecular pathway of oocyte developmental competence acquisition. The protein kinases' interactions (PI3K, AKT, PKA, MAPK, ERK, PKC, PKN2, and PLCB2) are very common pathways and involve several cross-talks which emphasizes the complexity of the molecular events in CCs prior to ovulation. Unfortunately, the whole molecular network of these candidates is still poorly understood despite recognized studies conducted in rodents and

mammalian species ((Assidi *et al.* 2008, Yamashita *et al.* 2009) and reviewed by (Russell & Robker 2007)). Further studies are required to improve our understanding of these pathways (disruption in animal models) and to facilitate oocyte developmental potential prediction.

5.8. CONCLUSION

We reported herein a list of preliminary *in vivo* markers that may reflect the normal physiological and genomic context needed for optimal oocyte maturation and successful pregnancy. The oocyte, through its proximity impact, confers to CCs a high capacity to notify its developmental potential both in ICSI programs and IVF cycles. These CCs genomic biomarkers once validated will represent a valuable tool in the clinical setting not only in the selection of good quality oocytes that lead to successful pregnancy and healthy embryo, but also to optimize the ovulation induction protocols reported to influence the oocyte quality (Hillier 2009). Consequently, the elective SET outcome could be improved, and thus reducing the risks associated with multiple pregnancies (Hamamah *et al.* 2007, Gerris 2009). These biomarkers could also serve to optimize the culture media used in IVM protocols. The levels of expression of these positive and negative markers in CCs collected following IVM or ovarian stimulation should correlate with those found in the *in vivo* context that has led to successful pregnancy.

Our study has the advantage of taking into account most of the COC morphological criteria, the ZP birefringence, the early embryo morphology and the CCs gene expression patterns associated to later pregnancy outcome. It therefore provides a preliminary list of 7 non-invasive biomarkers with high discriminative potential of the oocyte developmental competence that could increase the pregnancy outcome, reduce multiple pregnancy incidences and improve the efficiency of the elective SET procedure.

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5.11. FIGURES

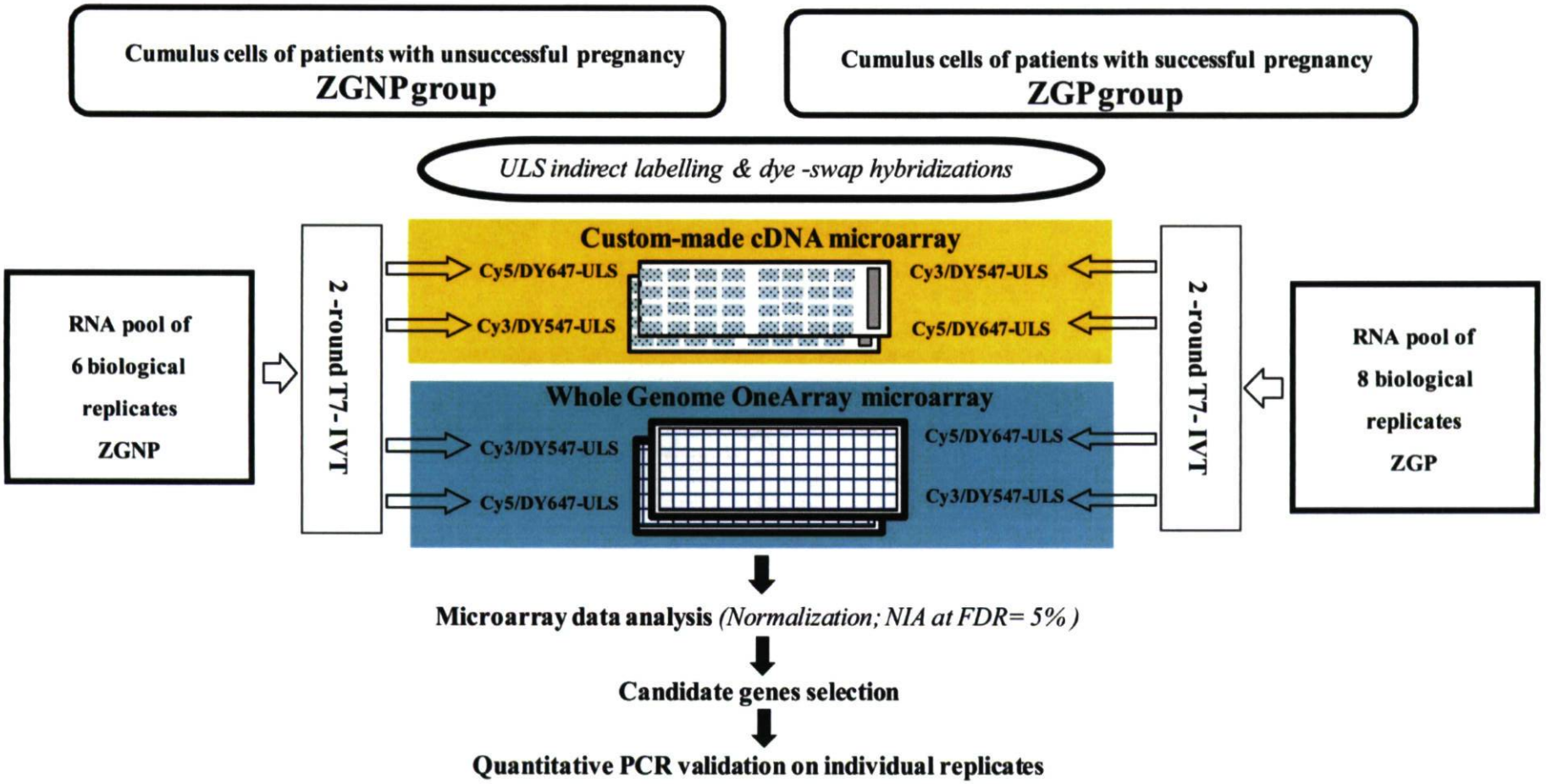

Figure 5.1. Schematic representation of the experimental design

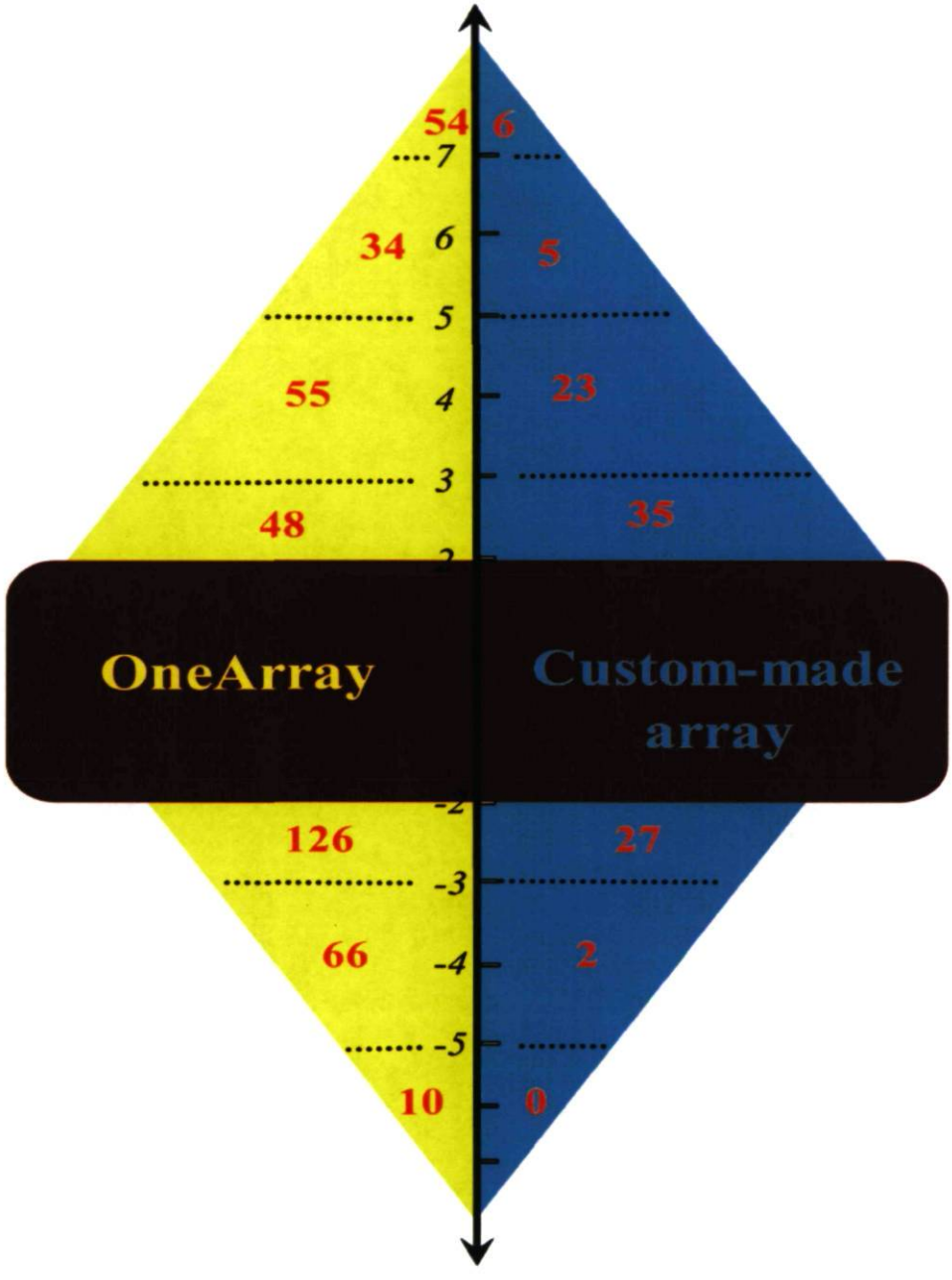

Figure 5.2. Schematic representation of the differentially expressed genes in CCs of ZGP oocytes compared to the ZGNP group according to both their fold change and the microarray platform

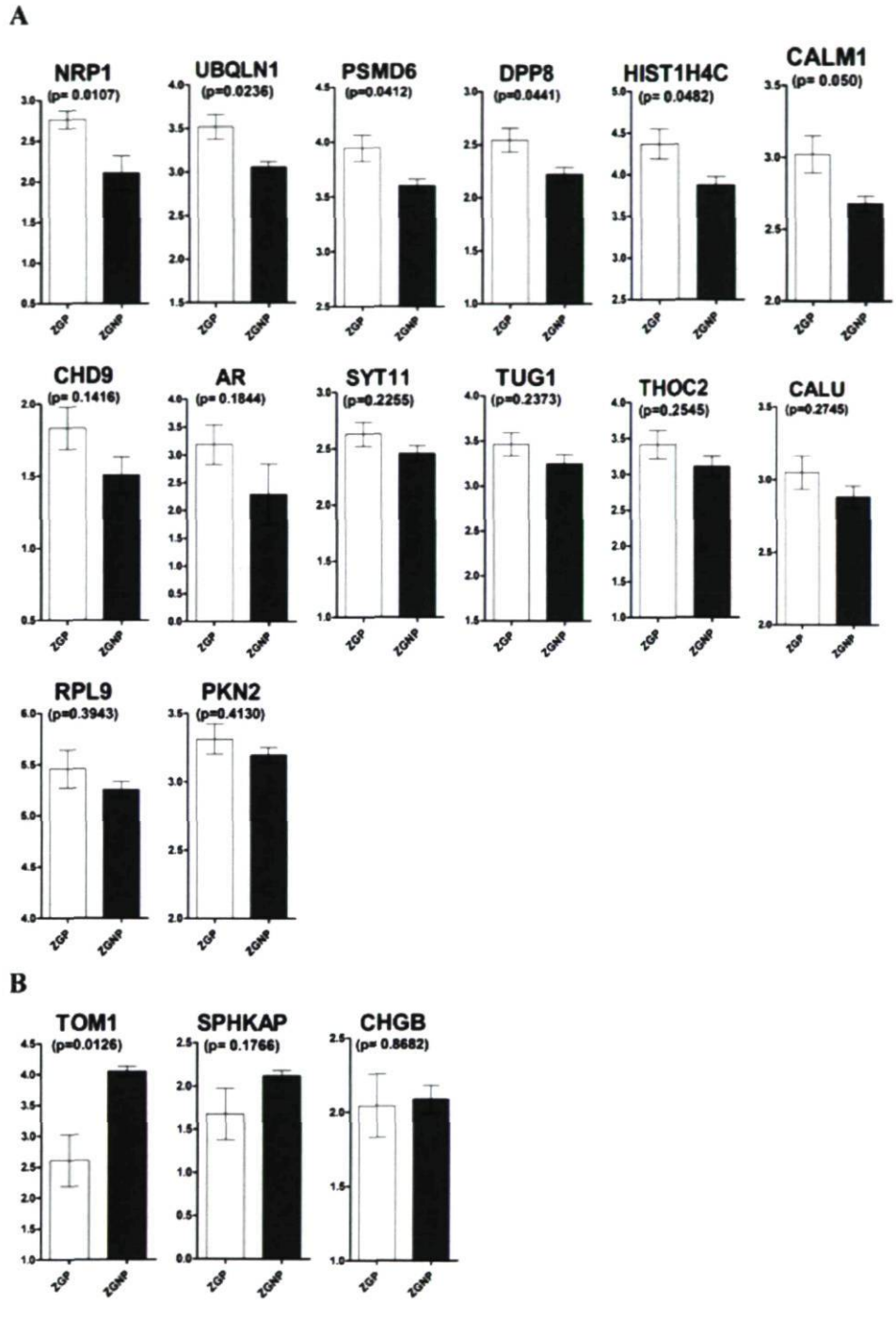

Figure 5.3. Real-time PCR analysis of selected genes in individual CCs from ZGP and ZGNP groups. (A), positive gene markers associated with pregnancy. (B), negative markers associated with pregnancy failure. Candidates were ranked according to their p-values, which were determined following a t-test analysis achieved on normalized data at $\alpha=0.05$

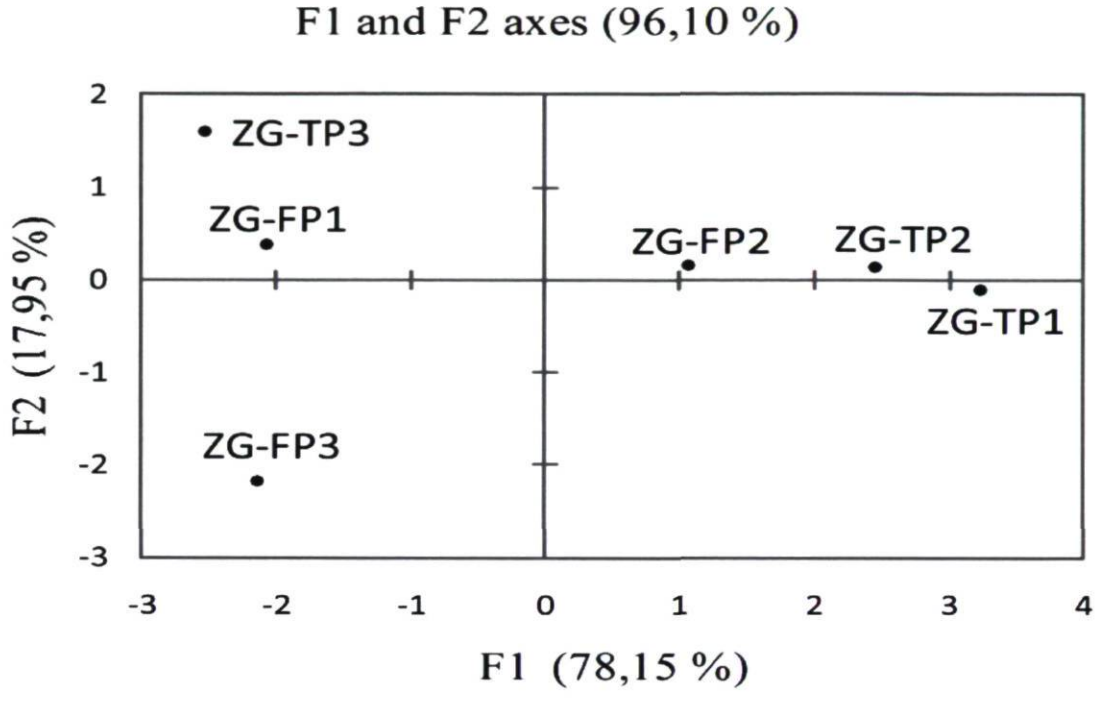

Figure 5.4. Bi-dimensional PCA representation of the six CCs samples to distinguish the positive embryo that led to successful pregnancy among the two transferred for each patient. For each patient and using the Euclidian distance and the origin (negative control), the nearer embryo is the false positive (FP, associated with pregnancy failure), while the farther is the true positive (TP, associated with successful pregnancy). If the distances have opposite signs, the embryo with positive Euclidian distance is automatically the true positive. (F1, F2), the two main PCA axes. (ZG), cumulus cells of oocytes with high zona score; (TP), true positive; (FP), false positive; (1, 2, 3), patient number.

$$Pregnancy = \begin{cases} 1\ (i.e. pregnant)\ with\ a\ probability\ P = \dfrac{1}{\left[1 + \exp\left(-(12347{,}135 + 46417186{,}605 * \mathrm{HIST1H4C} - 3397039537{,}268 * \mathrm{NRP1})\right)\right]} \\ 0\ (i.e. pregnancy\ failure)\ with\ a\ probability\ (1 - P) \end{cases}$$

Figure 5.5. Equation of the pregnancy prediction model. (P), probability of successful pregnancy

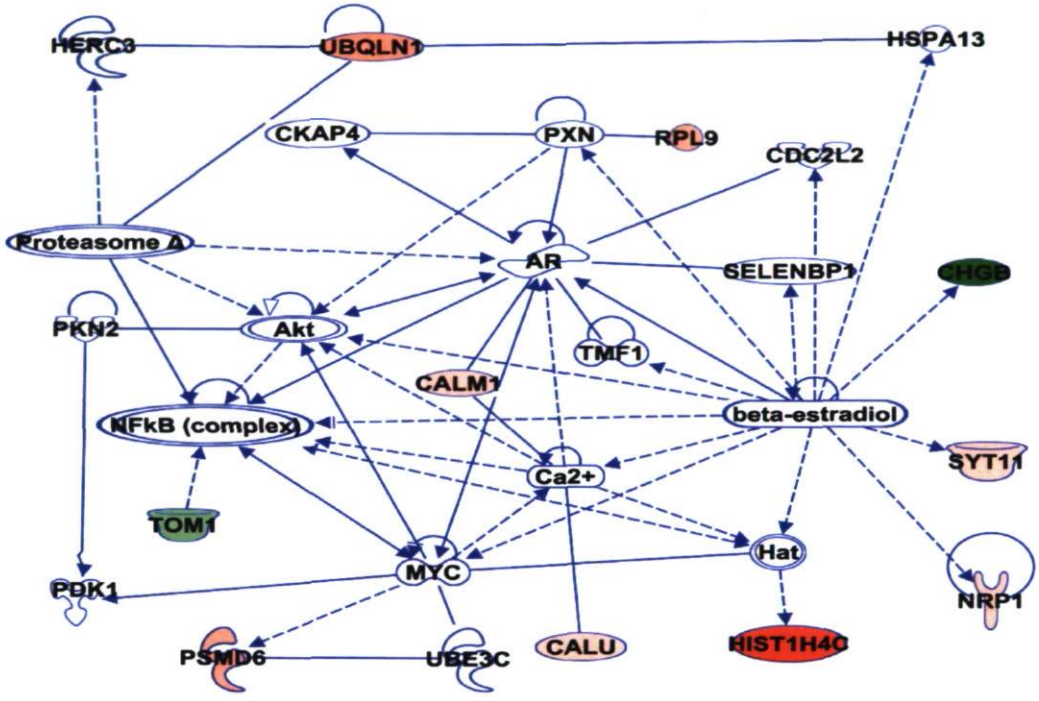

Figure 5.6. Ingenuity pathways network generated from the QPCR validated candidates in human CCs prior to ICSI. Overexpressed genes (red) are RPL9 (ribosomal protein L9), UBQLN1 (ubiquilin 1), CALM1 (calmodulin 1), AR (androgen receptor), NRP1 (neuropilin 1), PKN2 (protein kinase N2), SYT11 (synaptotagmin XI), HIST1H4C (histone cluster 1, H4c), CALU (calumenin) and PSMD6 (proteasome (prosome, macropain) 26S subunit, non-ATPase, 6). Underexpressed genes (green) are TOM1 (target of myb1 (chicken)) and CHGB (chromogranin B). Other genes: TMF1 (TATA element modulatory factor 1), CDC2L1 and CDC2L2 (cell division cycle 2-like 1 and 2), PXN (paxillin), CKAP4 (cytoskeleton-associated protein 4), HSPA13 (heat shock protein 70kDa family, member 13), HERC3 (hect domain and RLD 3), Akt (protein kinase B), Hat (histone acetylase or acetyltransferase), PDK1 (pyruvate dehydrogenase kinase, isozyme 1), UBE3C (ubiquitin protein ligase E3C), and SELENBP1 (selenium binding protein 1)

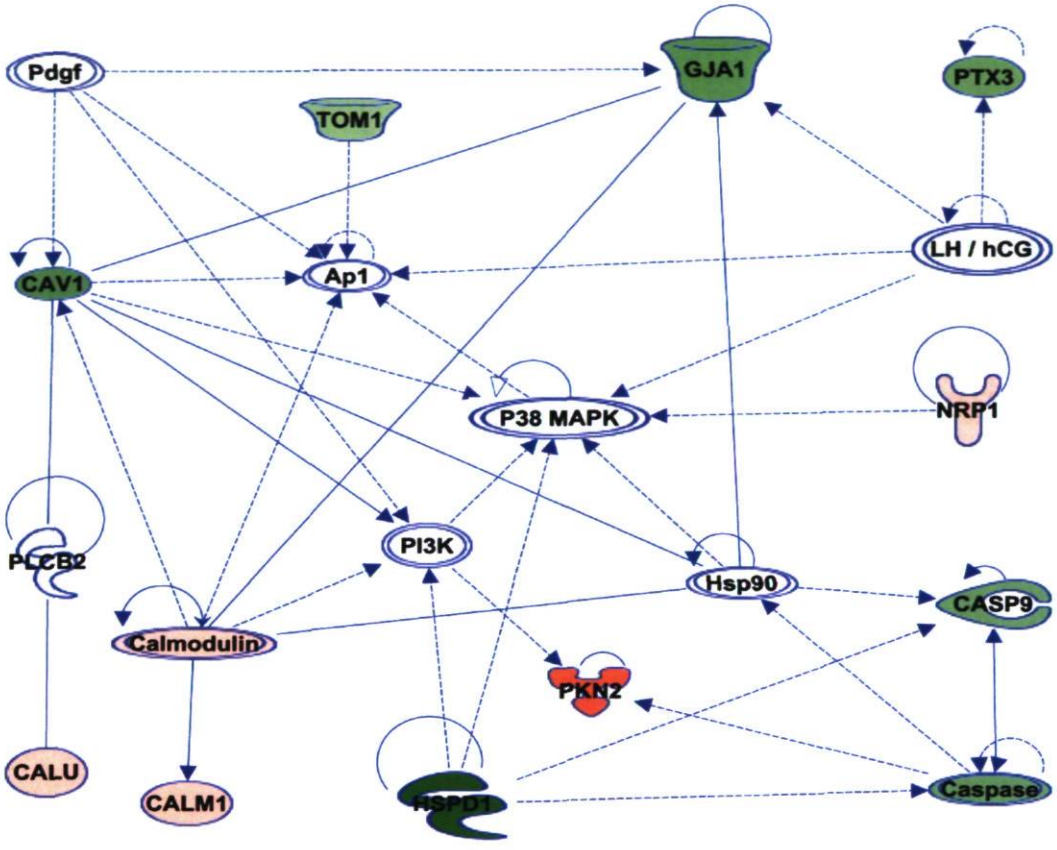

Figure 5.7. A custom-made IPA network made of selected list of microarray differentially expressed genes of human CCs between the ZGP and ZGNP groups. Overexpressed genes (red) are CALM1 (calmodulin 1), AR (androgen receptor), NRP1 (neuropilin 1), PKN2 (protein kinase N2), CALU (calumenin), and PLCB2 (phospholipase C, beta 2). Underexpressed genes (green) are TOM1 (target of myb1 (chicken)), GJA1 (gap junction protein, alpha 1, 43kDa), PTX3 (pentraxin-related gene, rapidly induced by IL-1 beta), PGRMC1 (progesterone receptor membrane component 1), IL8 (interleukin 8), CASP9 (caspase 9, apoptosis-related cysteine peptidase), CAV1 (caveolin 1, caveolae protein, 22kDa) and HSPD1 (heat shock 60kDa protein 1 (chaperonin)). Other genes: hCG (human chorionic gonadotropin), FSH (follicle stimulating hormone), LH (luteinizing hormone), Akt (protein kinase B), AP1(JUN, enhancer-binding protein AP1), PDGF family (platelet-derived growth factor), VEGF family (vascular endothelial growth factor), PKC (protein kinase C), ERK1/2 and p38 MAPK (mitogen-activated protein kinase family), PI3K family (phosphoinositide-3-kinase), and HSP70 & 90 (heat shock protein 70 and 90 kDa family).

5.12. TABLES

Table 5.1: Sequences of specific primers of candidates used in real time PCR quantification

Gene Name	Primer set (5'-3')	Genbank	Unigene	Annealing temperature	Fluorescence acquisition temperature
CALU	Up 5'- ACAAGGATGGAGACCTCATTGCC -3' low 5'- TGCTCTCGCTCTGTCTTTACCC -3'	AF013759	Hs.7753	62	80
DPP8	Up 5'- GCTGCCTGCTCCAAGTGATTTCAA -3' low 5'- GCAAGAATGTGAGTAGCCACGGT -3'	NM_197960.2	Hs.591106	61	83
HIST1H4C	Up 5'- CCATCGTAAGGTGCTCCG -3' low 5'- TTGGCGTGCTCCGTATAGGT -3'	NM_003542	Hs.46423	60	81
PKN2	Up 5'- ACAAGCCTGATACTCCTCAGTCAG -3' low 5'- GCAACCCAAGAACTACACAAGCAG -3'	NM_006256.2	Hs.440833	61	81
PSMD6	Up 5'- TTCCAGCAGTTCGGCAGTATCTGT -3' low 5'- TTCCACACCAACACCAAACGCTTC -3'	BC000630	Hs.152536	61	81
RPL9	Up 5'- TGAAGGGACGCACAGTTATCGTGA -3' low 5'- AAGCAACACCTGGTCTCATCCGAA -3'	BC066318	Hs.719072	60	83
SYT11	Up 5'- ACCCTGTGTTTGACGAGACCTTCA -3' low 5'- CATCTTCGGCAAGTGTCTGGCTTT -3'	BC039205	Hs.32984	63	87
THOC2	Up 5'- GGTAATCTTTCAGGAAGGTGGAGA -3' low 5'- GCTGATGTCATCCCAGACTTTG -3'	NM_001081550.1	Hs.592243	60	81
TOM1	Up 5'- CAATCTCAACAATGTGTTCCTGCG -3' low 5'- TACCTCTTTCCGTTGGTCAGCC -3'	NM_001135732	Hs.474705	60	77
TUG1	Up 5'- CTTCAGATCAGCAGGACAGTTGG -3' low 5'- GGGAGTTGTTACAAGATGGAACGG -3'	NR_002323.1	Hs.554829	62	80
AR	Up 5'- AACCCTATTTCCCCACCCCAG -3' low 5'- GCTCTCTAAACTTCCCGTGGCA -3'	NM_000044.2	Hs.496240	59	83
CALM1	Up 5'- TACTTCGTGTGCTCCGACCCAT -3' low 5'- AGTCCACAGCCACAGCCTACTC -3'	BC007965	Hs.282410	60	84
NRP1	Up 5'- ACCTGAAACCCAGTGCCCAGAA -3' low 5'- TGTTGTTGCGGTTGTCAGCAGT -3'	NM_003873.5	Hs.131704	60	84
CHD9	Up 5'- ACCAGCCTCGTCAATTTCCCAA -3' low 5'- CATCTCCTGCAAGTCTCGTTCCA -3'	NM_025134.4	Hs.622347	59	80
SPHKAP	Up 5'- GCAGCGATTTGCCTTGACAAC -3' low 5'- TTTAAGCTCAGGGTGCTCGTCC -3'	NM_001142644.1	Hs.436306	58	85
CHGB	Up 5'- CAACTGGACCAGCTCCTTCAC -3' low 5'- GCACAGTCATTGTCATAAGCATGT -3'	NM_001819.2	Hs.516874	58	85
UBQLN1	Up 5'- CAGTGATACATTTGGCTGACTCTGG -3' low 5'- GCCTCCACCGTAACCTTTGTACTT -3'	BC017289	Hs.9589	62	82
PPIA	Up 5'- TGCTGGACCCAACACAAATGGTTC -3' low 5'- TGGTGATCTTCTTGCTGGTCTTGC -3'	NM_021130.3	Hs.356331	60	84
ACTB	Up 5'- CGTGACATTAAGGAGAAGCTGTGC -3' low 5'- CTCAGGAGGAGCAATGATCTTGAT -3'	NM_001101	Hs.520640	59	89

Table 5.2. The main molecular and cellular functions governed by human CCs harvested before ICSI. These functions are ranked by p-value according to the IPA software analysis

Underexpressed genes			Overexpressed genes		
Function	p-value	% of genes	Function	p-value	% of genes
Cell morphology	**1,24E-04 - 3,45E-02**	**23,6**	**Cell death**	**3,13E-04 - 3,21E-02**	**40,2**
Cellular development	**1,32E-04 - 3,45E-02**	**11,3**	**Cellular growth & proliferation**	**3,46E-04 - 3,21E-02**	**21,4**
Cell death	**1,85E-04 - 3,45E-02**	**28,1**	**Cellular assembly & organization**	**6,88E-04 - 3,21E-02**	**17,9**
Post-translational modification	**2,73E-04 - 2,61E-02**	**30,3**	**Gene expression**	**6,88E-04 - 3,21E-02**	**12**
Protein folding	**2,73E-04 - 2,73E-04**	**6,7**	**Cell cycle**	**1,14E-03 - 3,21E-02**	**8,5**

CHAPTER 6

Analysis of correlations between zona pellucida birefringence and molecular markers of oocyte competence

6.1. CONTEXT

This chapter focuses on the analysis of possible correlations between ZP birefringence and gene biomarkers of oocyte competence. When Dr. Montag had provided us with human CCs, he wanted to check whether the ZP properties variation is associated to a differential CCs gene expression deviation. Therefore, he provide us with 3 types of CCs ZGP (zona good pregnant), ZGNP (zona good non pregnant) and ZBNP (zona bad non pregnant). Whilst the two first groups served to find gene biomarkers associated to good ZP and successful pregnancy (chapter 5), the ZBNP group was hybridized against the first group and transcriptional patterns were compared. This work is a preliminary analysis that raises more questions than it answers. The presentation of these data is a follow up of our commitment with Dr. Montag group.

6.2. ZONAE PELLUCIDAE AS MORPHOLOGICAL CRITERIA OF OOCYTE COMPETENCE

ZP is filamentous matrix of glycosylated glycoproteins that appears around the oocyte at the secondary follicle stage (Wassarman & Albertini 1994).This ZP acts as an oocyte's coat since the oocyte growth and maturation, remains during the early embryo development until the blastocyst hatching (Dunbar *et al.* 1994, Lunn & Wright 2006). Three different genes (ZP1, ZP2 and ZP3) were shown to govern the expression of mammalian ZP proteins (Wassarman 1988, Gook et al. 2008). The structure of ZP is based on filaments of ZP2-ZP3 heterodimers proteins, linked in some binding sites by ZP1 (Figure 6.1)(Green 1997).

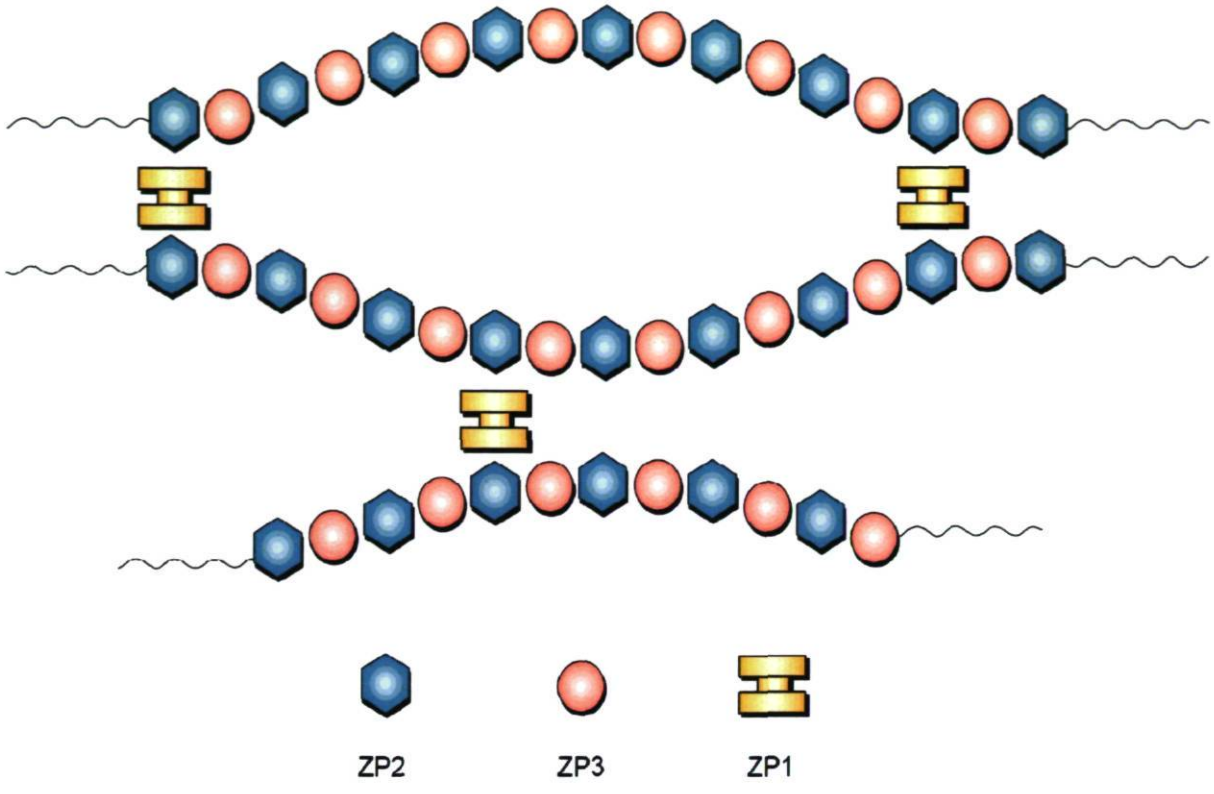

Figure 6.1: Schematic representation of the three-dimensional structure of the zona pellucida. ZP filaments of repeating ZP2–ZP3 units (ZP2:ZP3 ratio= 1) are cross-linked by ZP1 (Green 1997).

As discussed before in chapter 1 (section #2), the structure and thickness of ZP vary during oocyte maturation, ovulation and early cleavage (Familiari et al. 2006a).

The ZP achieves several crucial roles including CCs-oocyte exchanges control/support events such as, sperm recognition, acrosome reaction, prevention of polyspermy, and support of early embryo development (physical and immunological protection) (Epifano & Dean 1994, Hunter 2003, Kamo et al. 2004, Sun et al. 2005, Wassarman 2005, Ni et al. 2007, Ueno et al. 2007, Wassarman & Litscher 2008). Interestingly, ZP biochemical properties were affected by the oocyte aging, which decreases the oocyte fertilizability (Longo 1981). Moreover, the thickness of human oocyte ZP was positively correlated to the successful early embryo development (Chan 1987, Gabrielsen *et al.* 2000). Additionally, the number and size of ZP pores were shown to be negatively correlated with the bovine oocyte developmental competence *in vitro* (Santos *et al.* 2008), supporting the correlation between the ZP structure and oocyte quality.

Despite discrepancy about the ZP origin, it is sure that the oocyte is the main producer of the ZP proteins (Epifano *et al.* 1995, Hunter 2003). Therefore, harmful/stressful intrafollicular conditions could affect the oocyte and consequently the ZP structure and thickness. The ZP structure is hence used as a morphological parameter that may reflect the oocyte health/quality in many human IVF clinics. Positive correlation between the ZP thickness and oocyte early developmental potential was confirmed in number of IVF clinics (Chan 1987, Montag *et al.* 2000b, Montag *et al.* 2008, Ebner *et al.* 2009, Madaschi *et al.* 2009).

6.3. ZONAE PELLUCIDAE THICKNESS ASSESSMENT

The first attempts of measuring the ZP thickness were based on solubilization techniques (Inoue & Wolf 1974). Laser-based techniques were thereafter used to assess the ZP hardness, a problem that may cause improper embryo hatching and subsequent implantation failure (Montag et al. 2000a). The introduction of polarization microscopy has offer the possibility of non-invasive live zona imaging of individual oocytes. This polarizing microscope (Polscope) is based on the principle of alteration and retardance of polarized light when it gets through an object. The polscope was first used to non-invasively analyze the meiotic spindle of human oocytes regardless of their orientation (Oldenbourg 1996, Moon *et al.* 2003). Next, it has been used to assess qualitatively (thickness) and quantitatively (heterogeneity) the hamster ZP structure (Keefe *et al.* 1997).

Unfortunately and despite the improvement in ZP evaluation, the investigator-related bias became the new challenge to overcome (Pelletier *et al.* 2004). The development of suitable softwares able to be combined to the polscope has allowed objective measurements of the ZP birefringence. The ZP scoring has become an automatic (user-independent) and non-invasive technique that is able to measure the ZP density and uniformity (Pelletier *et al.* 2004, Rama Raju *et al.* 2007, Montag *et al.* 2008, Madaschi *et al.* 2009). The addition of robot-like micromanipulation system adapted to the microscope has improved the zona 3D visualisation and scoring. Using around 180 measurements, Dr Montag group classified the MII oocytes into two main classes. While the low zona birefringence (LZB) oocytes have an irregular and/or low birefringence distribution, those with a high and uniform ZP birefringence were designed as high zona birefringence (HZB) (Figure 6.2). In IVF clinics using the ZB assessment criterion, the

priority in ICSI and subsequent embryo transfer is given to the second HZB group (Montag et al. 2008).

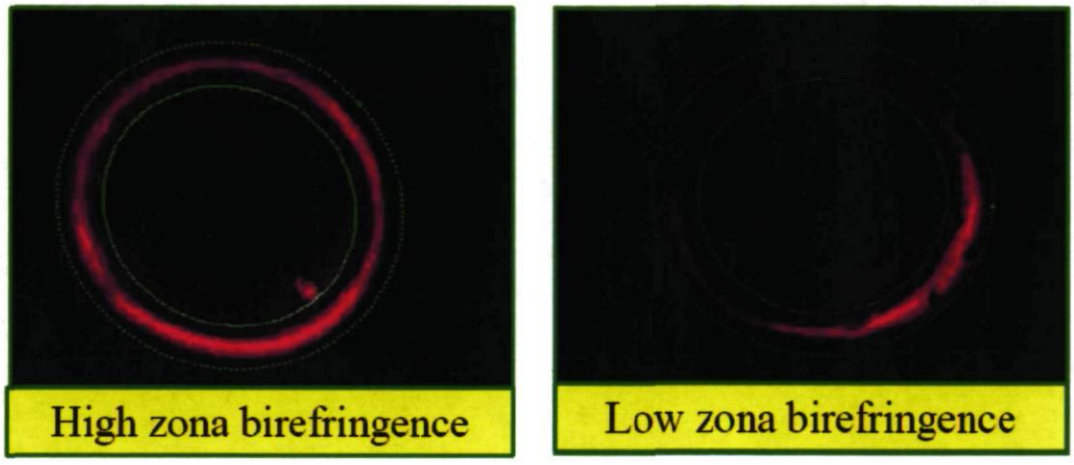

Figure 6.2: Automatic scoring at 180 points of ZP of MII oocytes using the Octax PolarAIDE polarized microscopy. Left: HZB oocyte with +4.6 score; Right: LZB with score= -6.2 (MTG 2010)

6.4. ZP BIREFRINGENCE AND MOLECULAR BIOMARKERS CORRELATIONS

6.4.1. Rationale

Oocyte selection for subsequent steps of fertilization (including ICSI) and embryo transfer is based on several morphological criteria. These parameters have been used in animal and human assisted reproduction (Blondin & Sirard 1995, Ebner *et al.* 2003, Balaban & Urman 2006, Wang & Sun 2007). In order to increase the morphological criteria accuracy, the ZP birefringence of MII oocytes was shown to be positively correlated to their developmental potential, supporting its use as a predictive parameter of oocyte competence in several ART clinics (Pelletier et al. 2004, Montag et al. 2008, Ebner et al. 2009, Madaschi et al. 2009).

Being subjective and still unable to accurately predict the oocyte developmental competence, the perspective to reinforce these morphological criteria by each other or using molecular markers differentially expressed in the competence-associated context is promising. Several studies were performed to look for non-invasive biomarkers that are highly correlated to the embryo

development outcome (McKenzie *et al.* 2004, Wang & Sun 2007, Bettegowda *et al.* 2008, Hamel *et al.* 2008b, Li *et al.* 2008, Verberg *et al.* 2008, Assou *et al.* 2010). As discussed before, the CCs are the target of large part of these genomic-markers-research studies due to their physical, physiological and molecular relationships with the oocyte ((McKenzie *et al.* 2004, Feuerstein *et al.* 2007, Anderson *et al.* 2009, Adriaenssens *et al.* 2010, Ferrari *et al.* 2010), reviewed in (Assou *et al.* 2010)).

Since we have already defined a preliminary list of genomic markers expressed differentially in the CCs of oocytes with high ZP birefringence (HZB) and associated with successful pregnancy, our goal is to investigate possible correlation between these biomarkers and the ZP birefringence. This correlation, if confirmed, could offer an interesting combination of morphological (ZP) and molecular (biomarkers) criteria allowing the selection of high competent oocytes.

6.4.2. Experimental design

In the previous chapter, we have defined a list of differentially expressed biomarkers within CCs of oocytes with HZB (ZGP vs ZGNP) (n=8 patients) and associated to successful pregnancy. We attempt herein to check whether these biomarkers are also suitable for the (ZGP vs ZBNP) comparison. We hypothesized that positive correlation between the ZP birefringence and molecular predictor of oocyte competence should lead to a powerful combined approach (combination of ZP birefringence and gene biomarkers) that may precisely estimate oocyte developmental potential, allowing therefore an efficient single embryo transfer.

In order to check our hypothesis, we have performed:

- Microarray comparison of CCs pools of ZGP vs ZBNP
- Real time PCR quantification of the 7 significant biomarkers between the ZGP and the ZBNP groups.

For the detailed material and methods, they are the same as those of the chapter 5.

6.4.3. Microarray hybridization

Following hybridization, 32 and 50 candidate genes were respectively underexpressed and overexpressed in the ZGP compared to the ZBNP (Table 6.1 & 6.2). Interestingly, three (3) positive biomarkers from the (ZGP vs ZGNP) comparison appear in the overexpressed gene list of the (ZGP vs ZBNP). These biomarkers are PSMD6, CALM1 and NRP1.

Table 6.1: Underexpressed genes in CCs of ZGP vs ZBNP

	Gene	GenBank name	Link to hit record	Fold
1	SLC25A3	Homo sapiens solute carrier family 25 (mitochondrial carrier;	NM_213611	3.907
2	HNRNPM	heterogeneous nuclear ribonucleoprotein M	Hs.465808	3.206
3	CCNA2	Homo sapiens cyclin A2, mRNA	BC021067	3.087
4	ARFGAP3	Homo sapiens ADP-ribosylation factor GTPase activating protein	NM_014570	3.018
5	RPL10	Homo sapiens ribosomal protein L10 (RPL10), mRNA	NM_006013.3	2.843
6	MT-CYB	Homo sapiens mitochondrially encoded cytochrome b	NP_536855.1	2.747
7	TAX1BP1	Homo sapiens Tax1 (human T-cell leukemia virus type I) binding protein 1	BC001764	2.712
8	HSPA8	Homo sapiens heat shock 70kDa protein 8 (HSPA8), transcript variant	NM_006597	2.684
9	CYP11A1	Homo sapiens cytochrome P450, family 11, subfamily A, polypeptide	NM_000781	2.613
10	ENO1	Homo sapiens mRNA for enolase 1 variant, clone: adSE00169	AK222517	2.585
11	FDPS	Human farnesyl pyrophosphate synthetase mRNA, complete cds	J05262	2.567
12	-	Homo sapiens mRNA; cDNA DKFZp586B0922 (from clone DKFZp586B0922)	AL110200	2.548
13	FOSB	Homo sapiens FBJ murine osteosarcoma viral oncogene homolog B	NM_001114171	2.407
14	RPL35A	Homo sapiens ribosomal protein L35a, mRNA (cDNA clone MGC:9770	BC017093	2.357
15	LAMB1	Homo sapiens laminin, beta 1 (LAMB1), mRNA	NM_002291	2.351
16	DPH1	Homo sapiens DPH1 homolog (S. cerevisiae) / candidate tumor suppressor in ovarian cancer 2 (OVCA2)	NM_001383	2.333
17	HBB	Homo sapiens hemoglobin, beta (HBB), mRNA	NM_000518	2.323
18	MT-ATP6	Homo sapiens ATP synthase 6 mRNA, complete cds; mitochondrial	AF368271	2.258
19	TNFAIP1	Homo sapiens tumor necrosis factor, alpha-induced protein 1 (endothelial),	BC006208	2.238
20	Unkown	full-length cDNA clone CS0DK008YI09 of HeLa cells Cot 25-normalized of Homo sapiens (human)	CR622106	2.228
21	IQWD1	Homo sapiens IQ motif and WD repeats 1, mRNA (cDNA clone IMAGE:3929120),	BC015957	2.214
22	TMED5	Homo sapiens transmembrane emp24 protein transport domain containing	NM_016040	2.209
23	HLA-DRA	Homo sapiens major histocompatibility complex, class II, DR alpha	NM_019111	2.187
24	Unknown	Bovine lysozyme c isozyme 3a mRNA, complete cds	M26242	2.181
25	RPL14	Homo sapiens ribosomal protein L14, mRNA (cDNA clone MGC:88594	BC071913	2.14
26	TTC15	Homo spaiens tetratricopeptide repeat domain 15	NM_016030.5	2.139
27	ZNF232	Homo sapiens mRNA similar to zinc finger protein 232 (cDNA clone	BC002852	2.083
28	RPS13	Homo sapiens ribosomal protein S13 (RPS13), mRNA	NM_001017	2.054
29	IFITM3	Homo sapiens interferon induced transmembrane protein 3 (1-8U)	NM_021034	2.031
30	RLIM	Homo sapiens ring finger protein 12, mRNA (cDNA clone IMAGE:3343080),	BC002451	2.03
31	C20orf4	Homo sapiens chromosome 20 open reading frame 4 (C20orf4), mRNA	NM_015511	2.019
32	DOM3Z	Homo sapiens dom-3 homolog Z (C. elegans), mRNA (cDNA clone MGC:29502	BC019083	2.006

Table 6.2: Overexpressed genes in CCs of ZGP vs ZBNP

	Gene	GenBank name	Link to hit record	Fold
1	MOBKL1B	Homo sapiens MOB1, Mps One Binder kinase activator-like 1B (yeast)	NP_060691.2	6.443
2	ZNF793	Homo sapiens zinc finger protein 793 (ZNF793), mRNA	NM_001013659	5.44
3	RPL9	Homo sapiens ribosomal protein L9, mRNA (cDNA clone MGC:87207	BC066318	5.267
4	GGNBP2	Bos taurus similar to zinc finger protein 403,transcript variant 2	XM_863956	5.207
5	PSMD6	Bos taurus proteasome (prosome, macropain) 26S subunit, non-ATPase,	NM_001034649	5.162
6	RBMS2	Homo sapiens RNA binding motif, single stranded interacting protein 2 (RBMS2), mRNA	NM_002898	4.542
7	RAN	Bos taurus RAN, member RAS oncogene family, mRNA (cDNA clone	BC151426	4.535
8	KIF13B	kinesin family member 13B	NT_023666.17	4.38
9	-	PREDICTED: Bos taurus similar to endonuclease reverse transcriptase	XM_001790089	4.302
10	CCAR1	Bos taurus cell division cycle and apoptosis regulator 1 (CCAR1),	NM_001076532	4.278
11	CALM1	Homo sapiens calmodulin 1 (phosphorylase kinase, delta) (CALM1	NM_006888.3	4.136
12	PPM1G	Homo sapiens protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	BC007531	4.113
13	CCNA2	Homo sapiens cyclin A2, mRNA (cDNA clone IMAGE:3510175), ****	BC021067	4.069
14	THOC2	PREDICTED: Bos taurus similar to Tho2 (LOC507738), partial mRNA	XM_584407	3.804
15	HBA2	Bos taurus hemoglobin alpha chain, mRNA (cDNA clone MGC:157272	BC133477	3.688
16	EIF4GI	PREDICTED: Bos taurus eukaryotic translation initiation factor eIF4GI protein (EIF4GI), mRNA	XM_585239	3.453
17	FAM73A	Homo sapiens family with sequence similarity 73, member A (FAM73A),	NM_198549	3.429
18	TIE1	Homo sapiens tyrosine kinase with immunoglobulin-like and EGF-like domains 1	AL833389	3.393
19	SYT11	Bos taurus synaptotagmin XI (SYT11), mRNA	NM_001099171	3.271
20	SGCB	Bos taurus sarcoglycan, beta (43kDa dystrophin-associated glycoprotein) (SGCB), mRNA.	NM_001102188	3.141
21	DDX51	Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 51 (DDX51),	NM_175066	3.116
22	PRRT2	Homo sapiens proline-rich transmembrane protein 2 (PRRT2), mRNA	NM_145239.2	3.109
23	PSIP1	Homo sapiens PC4 and SFRS1 interacting protein 1 (PSIP1),	NM_021144.2	3.093
24	GABARAP	Homo sapiens GABA(A) receptor-associated protein (GABARAP), mRNA	NM_007278	3.042
25	RDH11	PREDICTED: Bos taurus similar to retinol dehydrogenase 11, transcript variant 1 (RDH11), mRNA	XM_582373.	2.833
26	MRPS18B	PREDICTED: Bos taurus similar to Mitochondrial 28S ribosomal protein S18-2 (MRPS18B),	XM_001250819	2.832
27	SCARA5	Homo sapiens scavenger receptor class A, member 5 (putative) (SCARA5), mRNA	NP_776194.2	2.817
28	RLIM	Homo sapiens ring finger protein, LIM domain interacting	BC002451	2.784
29	CASP9	Homo sapiens caspase 9 splice variant (CASP9) mRNA, complete	AY732490	2.776
30	CDKN1A	Homo sapiens cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389	2.744
31	VPS16	Homo sapiens vacuolar protein sorting 16 homolog (S. cerevisiae),	BC073959	2.732
32	HSD11B2	Bos taurus 11-beta-hydroxysteroid dehydrogenase type 2 mRNA,	AF074706	2.722
33	CALM2	Bos taurus similar to calmodulin 2 (LOC514457)	XM_001250668	2.681
34	HBB	Homo sapiens hemoglobin, beta (HBB), mRNA	NM_000518.4	2.604
35	GLB1	Bos taurus GLB1 mRNA for galactosidase, beta 1, complete cds	AB325580	2.546
36	RPS4X	Homo sapiens ribosomal protein S4, X-linked (RPS4X), mRNA	NM_001007	2.519
37	PANX1	Homo sapiens mRNA for pannexin 1 (PANX1 gene),	NM_015368	2.379
38	PRDX2	Bos taurus peroxiredoxin 2 (PRDX2), mRNA	NM_174763	2.378
39	RNF121	Homo sapiens ring finger protein 121 (RNF121), transcript variant	NM_018320	2.36
40	HSP90B1	Bos taurus tumor rejection antigen (gp96) 1 / heat shock protein 90kDa beta (Grp94), member 1	BC104549	2.322
41	POMP	Bos taurus proteasome maturation protein (POMP), mRNA	NM_001034376	2.294
42	CALU	Homo sapiens calumein (Calu) mRNA, complete cds	AF013759	2.249
43	VPS33A	Homo sapiens vacuolar protein sorting 33 homolog A (S. cerevisiae)	NM_022916	2.231
44	ZFYVE20	Homo sapiens zinc finger, FYVE domain containing 20, mRNA (cDNA	BC021246	2.159
45	RGS3	Bos taurus regulator of G-protein signaling 3 (RGS3), mRNA	NM_001077973	2.15
46	RPL37A	Bos taurus ribosomal protein L37a (RPL37A), mRNA	NM_001035008	2.123
47	HDAC2	Bos taurus histone deacetylase 2 (HDAC2), mRNA	NM_001075146	2.102
48	NRP1	Homo sapiens neuropilin 1 (NRP1)	Hs.131704	2.102
49	EMP2	Bos taurus epithelial membrane protein 2 (EMP2), mRNA	NM_001075324	2.091
50	MALAT1	Homo sapiens MALAT1 mRNA, complete sequence	FJ209305	2.046

6.4.4. Real time PCR analysis

In addition to the 7 biomarkers associated to both HZB and successful pregnancy, we have analysed by qRT-PCR the gene TUG1 which was not significantly different between the ZGP versus ZGNP groups. Among the 7 biomarkers, 5 genes (out of 7) were also significantly different between the ZGP versus the ZBNP (i.e. 71.4 % of biomarkers confirmed), which is a further quantitative confirmation of the validity of our biomarkers on separate biological samples. These candidates are NRP1 ($p = 0.003$), CALM1 ($p = 0.005$), DPP8 ($p = 0.006$), UBQLN1 ($p = 0.025$) and PSMD6 ($p = 0.048$) (Figure 6.3).

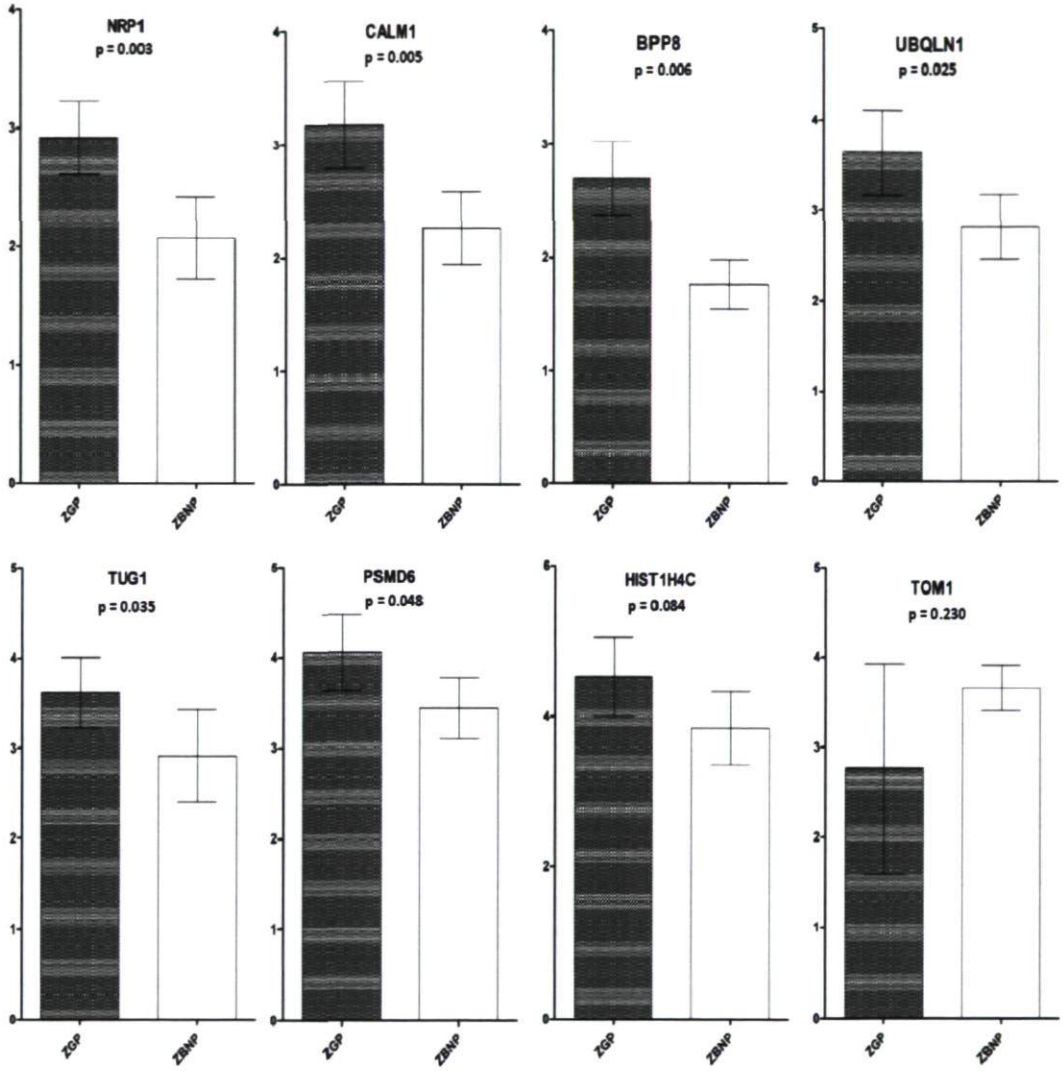

Figure 6.3. Real-time PCR analysis of differentially expressed genes in individual CCs of ZGP group versus ZBNP. Candidates were ranked according to their p-values, which were determined following a T-test analysis achieved on normalized data at $\alpha=0.05$

The combination of the real time PCR analysis in the 3 ZP quality groups (ZGP; ZGNP; ZBNP) is also summarized in Table 6.3.

Table 6.3: Real-time PCR comparative analysis of differentially expressed genes in individual CCs among the three ZP quality groups (ZGP versus ZGNP versus ZBNP). Candidates with different letters are significantly different following a t-test analysis at α=0.05

Gene		CALM1	DPP8	HIST1H4C	NRP1	PSMD6	TOM1	UBQLN1	TUG1
ZP quality groups	ZGP	a	a	a	a	a	b	a	a
	ZGNP	b	b	b	b	b	a	b	a,b
	ZBNP	b	b	a	b	b	a,b	b	b

6.5. DISCUSSION AND CONCLUDING REMARKS

In addition to the confirmation of most significant candidates of the (ZGP vs ZGNP) in the ZGP vs ZBNP shown by figure 6.3, we documented herein that most of the analyzed candidates were not significantly between the two groups with failed pregnancy. In fact and except the HIST1H4C gene, no statistical differences were found in the ZGNP vs ZBNP contrast ($p > 0.05$). Therefore and despite having two opposite ZP birefringences, few or no transcriptional differences were found between ZGNP versus ZBNP (Figure 6.4). These findings incited us to be careful in the interpretation of the ZP birefringence. According to these data, it looks that the ZP morphological phenotype is associated to a transcriptomic gene pattern not related to competence. Therefore, it is difficult under this assumption to establish a positive correlation between these two criteria (morphological vs molecular). Using the case of TUG1 gene and despite being non significant biomarker of competence, it was significantly associated to the ZP morphology. It may be therefore assumed that other genes that are not in our confirmed markers list may reflect a transcriptomic difference due to the ZP morphology.

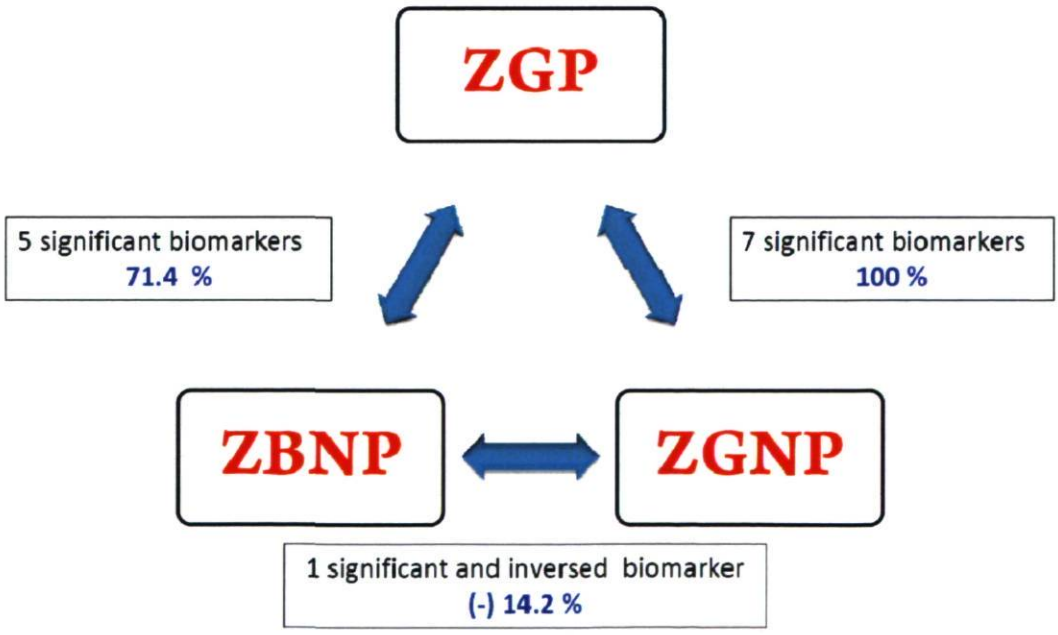

Figure 6.4. Schematic representation of the correlation (expressed in %) of significant gene biomarkers between the three morphological groups of ZB

Although these data are preliminary and require more investigations, several reasons could be associated to the lack of correlation between these two parameters of oocyte competence selection:

- The first reason is that the ZP birefringence could be an independent parameter that may not correlate with the genomic predictors of oocyte competence. It means that the ZP pellucida phenotype may not necessarily imply a gene expression difference at least at this stage. Therefore, these parameters could be joined together in an additive and independent manner. In fact and as mentioned before, the ZP formation started at the secondary follicular stage and is achieved at the preovulatory stage. At the ICSI moment, low correlation between the ZP birefringence and molecular markers may be expected. This independency is supported by the TUG1 gene which was not a significant marker of oocyte competence, but was differentially expressed between the ZGNP versus ZBNP.
- Given that the oocyte is the main producer of the ZP, CCs may weakly affect the ZP morphology which may be another reason of this absence of correlation.
- A differential gene expression between CCs and corona radiata cells (CRCs) was previously reported by Dr. Montag group (Van Der Ven *et al.* 2009). Therefore and due to the high similarities of the compared tissues, only corona CRCs may show a transcriptomic difference associated to ZP birefringence. This assumption is further supported by possible involvement of the CRCs in ZP protein biosynthesis. Therefore, the analysis of the whole CCs in our case may dilute possible transcriptional difference in CRCs with opposite ZP birefringences.
- It is also possible that despite the reported correlations, the ZP birefringence could be a non suitable parameter that may accurately reflect the oocyte quality and subsequent developmental competence.
- It is to note that the gene markers reported in the chapter 5 remain preliminary and require further confirmation in separate and larger group of patients. Despite the PCA input in the gene markers analysis, the transfer of two embryos that lead to singular pregnancy is an important bias that may affect the effectiveness of the molecular biomarkers and therefore their correlation with the ZP birefringence criterion.

Ultimately, the idea of finding related biomarkers of oocyte quality would reinforce the accuracy of oocyte selection. Potential correlates criteria should strengthen each other offering a precious prognostic tool of the pregnancy outcome. The improvement of oocyte selection procedures would increase the pregnancy outcomes and diminish multiple pregnancy incidences. Therefore, efficient single embryo transfer could be envisioned.

DISCUSSIONS AND GENERAL CONCLUSION

As more additional players involved in the ovarian function regulation are revealed, the molecular bidirectional interplay between the oocyte and its somatic environment becomes more complex and crucial to unravel the mystery of developmental competence. The CCs importance in this molecular mechanism doesn't raise any doubt in view of the fact that their removal and/or the inhibition of their exchanges with oocyte affect significantly the blastocyst rates (Ali & Sirard 2005, Luciano *et al.* 2005). Moreover, CCs main properties including expansion, steroidogenesis and gene expression are influenced by the oocyte and hence reflect its degree of maturation (As discussed in chapter 1(section #2)). Therefore, the CCs ensure pivotal roles in oocyte competence and are thought to express gene markers that reflect the oocyte developmental potential (Assou *et al.* 2010). However, this competence decreases considerably when moving from the *in vivo* context to the *in vitro* system (Rizos *et al.* 2002). As a consequence and because of its importance in both commercial and research purposes, the *in vitro* embryo production has been the focus of several studies to improve the blastocyst yield mainly through the optimization of the IVM media (Rodriguez & Farin 2004a). Using a completely defined IVM media in our laboratory, the FSH or PMA during just the first 6h of IVM were able to induce an interesting blastocyst yield close to 50 % (Ali & Sirard 2005). Therefore, our first objective was to look for differentially expressed genes in CCs between three IVM treatments (FSH, PMA, FSH+PMA) collected at 6h *in vitro* (GVBD). In fact, the GVBD stage is a crucial step that is marked by the achievement of oocyte of all (most) the transcriptional events needed to reach the MZT (Hyttel *et al.* 2001). We believe that CCs contribution in triggering oocyte competence is clearly present at this moment. Our experiments have shown similar blastocyst rate between the three IVM treatments (without additive effect). Besides the confirmation of the PKC pathway as main target of the FSH-induced oocyte competence *in vitro* (Downs *et al.* 2001, Chen *et al.* 2008), these findings report a strong evidence of the FSH-action amplification loop driven by the EGF-like factors and the EGFR (Conti *et al.* 2006, Downs & Chen 2008). The presence of additive effects in the gene expression of some candidates between FSH and PMA confirm that the PKC is one of other targets of this pituitary hormone action. Based on these data, it was expected that common overexpressed candidates between the three treatments have serious chances to be associated with the molecular

process of acquisition of competence. Among 16 candidates analysed by QPCR, a list of 8 potential biomarkers of oocyte competence was confirmed, inluding HAS2 (Hyaluronan synthase 2), Inhibin βA (INHBA), EGFR (Epidermal Growth Factor Receptor), Gremlin (GREM), BTC (Betacellulin), CD44, TNFAIP6 (Tumor necrosis factor, alpha-induced protein 6) and PTSG2 (prostaglandin-endoperoxide synthase 2). These early biomarkers could serve to explore the quality of oocytes and offer an idea about interesting pathways in a crucial time point in the oocyte itinerary toward the competence acquisition.

In order to strengthen our *in vitro* findings and explore the CCs response to LH surge, we have analyzed a parallel study in the bovine CCs *in vivo*. To date, the suitable reference available for CCs differentiation and function is the meiotic stage of their oocyte. At the GVBD stage, CCs are believed to have the entire package of signals required to induce the last step of oocyte *in vivo* final maturation. Moreover, it is established that major functional properties of CCs are triggered by gonadotropins and governed by the oocyte in mammalian species (Eppig 2001, Matzuk *et al.* 2002, Kawashima *et al.* 2008, Richards & Pangas 2010, Sha *et al.* 2010). Although the process is still unexplained, LH improves the oocyte quality by inducing some signaling and gene expression pathways able to trigger its final maturation (Dieleman *et al.* 2002, Richards 2005, Richards 2007). To explore the LH effect close to the GVBD, gene expression in bovine CCs collected 2 h prior to and 6 h past the LH surge were analyzed according to their molecular functions, gene networks and cell compartments. In line with our expectation, CCs have shown a very particular phenotype marked mainly by increasing cell organization and differentiation, slight apoptosis, as well as intensive metabolic, signalling and gene expression activities. Interestingly, both LH-overexpressed and -underexpressed genes corroborate to carry out crucial biological functions related to CCs as mucification, oocyte competence induction, ovulation preparation and steroidogenesis (Tanghe *et al.* 2002). Although cell-cell distensions, the CCs morphology looks to shift to a polarized shape with cytoplasmic projections' formation that are suggested to play a crucial role in cell-cell communications (Allworth & Albertini 1993, Sutovsky *et al.* 1994, Hunter 2003). To our knowledge, this is the first time that such findings are documented and discussed in the bovine CCs at this stage. Keeping in mind the necessity of transcriptional activity in CCs to support successful gonadotropins-induced oocyte maturation (Meinecke & Meinecke-Tillmann 1993, Rodriguez & Farin 2004a), the analysis of the LH gene expression response in this compartment should yield insights about this gonadotropins

involvement in the molecular events of the final maturation process around the GVBD, a contribution until now poorly understood. Given that the PMA induces reduced CCs expansion at 6h of IVM, common overexpressed candidates in CCs between the *in vitro* (for the three IVM treatments) and the *in vivo* contexts are suggested as powerful and non-invasive early biomarkers expressed in CCs and associated to high competent oocytes. In fact, the PMA treatment could be considered as a filter that reduces the chances of mucification-related genes and increases those of real biomarkers involved more directly in the pathway of competence. Deep investigation of the protein levels and the molecular pathways of these common pathways (chapter 4) would reveal crucial molecular pathways occurring in CCs and involved in the molecular process of oocyte competence acquisition. These biomarkers may be used as relevant tool to optimize the IVM media, offering therefore the possibility to increase the livestock *in vitro* system productivity and serve as a framework to efficient human IVM.

Interestingly, the analysis of FSH *in vitro* versus LH *in vivo* activities has revealed for the first time a possible substitution/compensation of the *in vivo* LH transcriptional effect by FSH *in vitro* (discussed chapter 4). These data support previous reports where the addition of LH to FSH-based IVM media doesn't provide significant increase in oocyte competence measured by the blastocyst rate (Anderiesz et al. 2000, Choi et al. 2001, Ali & Sirard 2002a). Further studies are needed to validate these findings and provide better understanding of the real FSH/LH synergy.

The last part of my thesis deals with a worldwide concern, the human infertility. Fortunately, several ART are used to lessen the incidence of this disease and help afflicted couples achieving their parenthood dream. To clinically select good-quality oocyte with high developmental potential, several morphological parameters related to the oocyte, ZP, CCs and/or the early embryo were used (Ebner *et al.* 2003, Coticchio *et al.* 2004, Montag *et al.* 2008, Madaschi *et al.* 2009, Yu *et al.* 2009). Despite little improvement recorded through the use of these morphological criteria, the pregnancy outcomes remains reduced (Guerif *et al.* 2007). Neither the ovarian induction programmes nor the multi-embryo transfer adopted by clinicians have offered a suitable solution. Worst yet, severe medical, perinatal and neonatal complications that endanger both the mothers and the babies have increased considerably (Adashi *et al.* 2003, Templeton 2004, Bromer & Seli 2008). Therefore, the selection of the best embryo to transfer remains challenging. Moreover, single embryo transfer (SET) option is gaining popularity but requires the availability of accurate tools of oocyte and/or embryo selection (Gerris 2009). Since the oocyte

developmental competence is largely dependent on its maturation process fulfilment (Krisher 2004) where CCs are a crucial contributor, we aim at finding quantitative and non-invasive markers associated to the oocyte quality and produced by CCs. Using powerful molecular and mathematical tools, we have explored relationships between CCs gene expression and both morphological parameters and pregnancy outcome just prior to ICSI. Under good morphological parameters, seven (7) gene candidates were differentially associated to successful pregnancy and implantation and suggested as prognostic tools of oocyte developmental competence. These candidates are composed of six positive biomarkers (DPP8 ($p=0.0441$), HIST1H4C ($p=0.0482$), UBQLN1 ($p=0.0236$), CALM1 ($p=0.05$), NRP1 ($p=0.0107$), PSMD6 ($p=0.0412$)), and a negative biomarker TOM1 ($p=0.0126$). Our study has the advantage of taking into account most of the COC morphological criteria, the ZP birefringence, the early embryo morphology and the CCs gene expression patterns associated to successful pregnancy and confirmed implantation. Therefore, the biomarkers (once validated) could be of high discriminative potential to predict the oocyte developmental competence, which may increase the pregnancy outcome, reduce multiple pregnancy incidences and improve the efficiency of the elective SET procedure.

We should highlight that the suggested biomarkers lack a final validation step. We suggest herein the validation of these significant biomarkers and even those non significant in larger number of patients using SET approach. An efficient SET will therefore make the dream of many infertile couples a reality.

Recently, several «omics» approaches have been undertaken to target good and non-invasive biomarkers of oocyte quality (Seli *et al.* 2010). In this context, it becomes important to focus also on a useful form/kit of the biomarker that eases its use in IVF clinics. We should not expect that IVF clinics can begin measuring RNA levels in cumulus samples overnight or even one day. Therefore, it would preferable to use biomarkers either secreted or proteins that could be measured and quantified in CCs, FF or the culture media. According to Van Blerkom (2009), studying correlation of these biomarkers with the already used morphological criteria is necessary. In fact, the clinicians experience over the years has provided them enough skills to achieve several successful pregnancies using the sole morphological parameters. Furthermore, it is not expected that biomarkers-selected oocytes will have entirely opposite morphological parameters. Based on this, the decision-making of selection of the high-quality embryo should be staggered along all the steps of the ART procedure (Borini et al. 2005) and may require

successive use of different types of prognostic parameters. The marker selective tools would be used only to discriminate one good morphology embryos amongst others at day 3 or 5 before SET.

In the perspective of using additional non-invasive indicators, we have checked possible positive correlation between the ZP birefringence and our molecular predictors of oocyte competence to find powerful combined approach (combination of ZP birefringence and gene biomarkers) that may increase the accuracy of oocyte developmental potential estimation. Interestingly, our analysis showed that ZP morphological phenotype is independent or at best weakly correlated with our predictors list. This absence of correlation may be due to the independence of these parameters especially that the ZP proteins are produced mainly by oocyte at the early stages of folliculogenesis. It is also suggested that this correlation could have been positive if we had used only the corona radiata cells (CRCs). Under this assumption, the use of the whole CCs would dilute possible transcriptional difference in CRCs with opposite ZP birefringences. Despite the lack of final validation of our gene biomarkers, it is also possible that the ZP birefringence is not a strong parameter that may accurately reflect the oocyte developmental competence. The fact that around half of the ZGP embryos transferred in our study failed to achieve successful pregnancy may support this latter hypothesis.

An important care should also be addressed to the gene transcription levels. The analysis of possible pathways where these candidates could be involved should not mask the fact that their protein level may be different or even nil. Not only protein validation is useful to assess function but it could become an easier way to measure these markers compared to RNA levels.

Ultimately, the study of gene expression using microarrays has the advantage of providing a macro-view about the molecular events occurring at a given physiological state. By using both human and bovine tissues during this thesis, we have investigated the CCs genomic behaviour at three key time points (2h before LH, 6h after LH and 36h post hCG/LH) which correspond respectively to the oocyte GV, GVBD and MII meiotic stages. We believe that the effectiveness of oocyte selection, the *in vitro* media optimization, the suitable superovulation program depend on a profound comprehension of the molecular players and the time-and space-sequence of events from the follicular recruitment to ovulation. Therefore, these 3 time points offer precious meaningful-points to launch a retrospective approach to link players together (using the cause-effect and/or inducer-target and/or dose-response relationships) and to try to progressively rebuild

the molecular pathway of oocyte competence acquisition. The addition of other complementary time-points could be also envisioned.

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