

## TABLE DES MATIÈRES

RESUME.....	III
ABSTRACT.....	V
TABLE DES MATIERES.....	VII
LISTE DES TABLES .....	X
LISTE DES FIGURES.....	XI
LISTE DES ABREVIATIONS ET DES SIGLES.....	XII
REMERCIEMENTS.....	XVI
AVANT-PROPOS.....	XVIII
<b>1. INTRODUCTION .....</b>	<b>1</b>
<b>1.1. Le follicule ovarien .....</b>	<b>1</b>
1.1.1. Ovogenèse .....	1
1.1.2. Folliculogenèse.....	2
1.1.3. Ovulation .....	10
<b>1.2. Le <i>cumulus oophorus</i>.....</b>	<b>11</b>
1.2.1. Différenciation des cellules du cumulus, rôle de l'ovocyte .....	11
1.2.2. Communications bidirectionnelles .....	13
1.2.3. Métabolisme : missions du cumulus .....	14
1.2.4. Réponse du COC au pic préovulatoire de LH.....	19
1.2.5. Cumulus post-ovulatoire .....	21
<b>1.3. La compétence ovocytaire au développement.....</b>	<b>22</b>
1.3.1. Définition.....	22
1.3.2. Évaluation de la compétence ovocytaire .....	23
1.3.3. Mesure de la compétence ovocytaire .....	23
1.3.4. Optimisation de la stimulation ovarienne .....	29
<b>1.4. Hypothèse et objectifs.....</b>	<b>30</b>
<b>2. CUMULUS CELL GENE EXPRESSION ASSOCIATED WITH PRE-OVULATORY ACQUISITION OF DEVELOPMENTAL COMPETENCE IN BOVINE OOCYTES .....</b>	<b>32</b>
<b>2.1. Résumé.....</b>	<b>33</b>
<b>2.2. Abstract .....</b>	<b>34</b>
<b>2.3. Introduction .....</b>	<b>35</b>
<b>2.4. Results.....</b>	<b>37</b>
2.4.1. COC morphology.....	37
2.4.2. Genes inventory.....	37
2.4.3. Hierarchical cluster analysis .....	37
2.4.4. Variation of gene expression across time.....	38
2.4.5. Ingenuity Pathway Analysis (IPA).....	38
2.4.6. qRT-PCR results.....	38
<b>2.5. Discussion.....</b>	<b>40</b>

<b>2.6. Conclusion .....</b>	<b>46</b>
<b>2.7. Materials and methods.....</b>	<b>47</b>
2.7.1. Ovarian stimulation treatment, <i>in vitro</i> production and cumulus–oocyte complex (COC) recovery from super-stimulated animals.....	47
2.7.2. Cumulus cell retrieval .....	47
2.7.3. RNA extraction.....	47
2.7.4. RNA processing for microarray analysis.....	48
2.7.5. Microarray data normalization and statistical analysis .....	48
2.7.6. RNA processing for qRT-PCR and statistical analysis .....	49
2.7.7. Statistical analysis of COC morphology .....	49
<b>2.8. References.....</b>	<b>50</b>
<b>2.9. Tables.....</b>	<b>53</b>
<b>2.10. Figures .....</b>	<b>54</b>
<b>3. THE EFFECTS OF BASAL LH INHIBITION WITH CETRORELIX ON CUMULUS CELL GENE EXPRESSION DURING THE LUTEAL PHASE UNDER OVARIAN COASTING STIMULATION IN CATTLE .....</b>	<b>61</b>
<b>3.1. Résumé.....</b>	<b>62</b>
<b>3.2. Abstract .....</b>	<b>63</b>
<b>3.3. Introduction .....</b>	<b>64</b>
<b>3.4. Results.....</b>	<b>66</b>
3.4.1. COC Morphology .....	66
3.4.2. Genes Inventory and Clustering.....	66
3.4.3. IPA analysis .....	67
3.4.4. qRT-PCR results.....	67
<b>3.5. Discussion.....</b>	<b>68</b>
<b>3.6. Conclusion .....</b>	<b>71</b>
<b>3.7. Materials and methods.....</b>	<b>72</b>
3.7.1. Ovarian stimulation treatment, <i>in vitro</i> production, and recovery of cumulus-oocyte complexes (COC) from super-ovulated animals .....	72
3.7.2. Cumulus cell retrieval .....	72
3.7.3. RNA extraction.....	72
3.7.4. RNA processing for microarray analysis .....	73
3.7.5. Microarray data normalization and statistical analysis .....	73
3.7.6. RNA processing for qRT-PCR and statistical analysis .....	73
3.7.7. Statistical Analysis of COC Morphology .....	74
<b>3.8. References.....</b>	<b>75</b>
<b>3.9. Tables.....</b>	<b>78</b>
<b>3.10. Figures .....</b>	<b>79</b>
<b>4. ANALYSIS OF LHCGR AND SELECTED STEROID ENZYME mRNA EXPRESSION IN BOVINE CUMULUS CELLS DURING <i>IN VITRO</i> MATURATION.....</b>	<b>82</b>
<b>4.1. Résumé.....</b>	<b>83</b>
<b>4.2. Abstract .....</b>	<b>84</b>
<b>4.3. Introduction .....</b>	<b>85</b>
<b>4.4. Results.....</b>	<b>86</b>
4.4.1. LHCGR and MVK mRNA expression during <i>in vitro</i> maturation .....	86

4.4.2. Steroidogenic enzyme and progesterone receptor mRNA expression during <i>in vitro</i> maturation .....	86
<b>4.5. Discussion.....</b>	<b>87</b>
<b>4.6. Conclusion .....</b>	<b>89</b>
<b>4.7. Materials and methods.....</b>	<b>90</b>
4.7.1. Cumulus-Oocyte Complex <i>in vitro</i> maturation and retrieval.....	90
4.7.2. RNA processing and quantification .....	90
<b>4.8. References.....</b>	<b>92</b>
<b>4.9. Tables.....</b>	<b>94</b>
<b>4.10. Figures.....</b>	<b>95</b>
<b>5. INDIVIDUAL BOVINE <i>IN VITRO</i> EMBRYO PRODUCTION AND CUMULUS CELL TRANSCRIPTOMIC ANALYSIS TO DISTINGUISH CUMULUS-OOCYTE COMPLEXES WITH HIGH OR LOW DEVELOPMENTAL POTENTIAL.....</b>	<b>97</b>
<b>5.1. Résumé.....</b>	<b>98</b>
<b>5.2. Abstract .....</b>	<b>99</b>
<b>5.3. Introduction .....</b>	<b>100</b>
<b>5.4. Results.....</b>	<b>102</b>
5.4.1. <i>In vitro</i> embryo production and quality assessment .....	102
5.4.2. Gene inventory .....	102
5.4.3. Ingenuity Pathway Analysis .....	102
5.4.4. Quantitative reverse transcription-polymerase chain reaction results .....	103
<b>5.5. Discussion.....</b>	<b>104</b>
<b>5.6. Conclusion .....</b>	<b>108</b>
<b>5.7. Materials and methods.....</b>	<b>109</b>
5.7.1. <i>In vitro</i> culture and CC recovery .....	109
5.7.2. Analysis of RNA.....	111
<b>5.8. Acknowledgments.....</b>	<b>114</b>
<b>5.9. References.....</b>	<b>115</b>
<b>5.10. Tables .....</b>	<b>118</b>
<b>5.11. Figures .....</b>	<b>119</b>
<b>6. CONCLUSION ET DISCUSSION GENERALES .....</b>	<b>124</b>
<b>BIBLIOGRAPHIE.....</b>	<b>128</b>

## **LISTE DES TABLES**

Table 2-1. List and characteristics of primer pairs used in qRT-PCR for housekeeping and candidate genes in bovine cumulus cells.....	53
Table 3-1. Cumulus cell portrait of the main cellular functions affected by the absence of LH using functional categories and annotations of IPA software.....	78
Table 4-1. List and characteristics of primer pairs used in qRT-PCR for investigated genes in bovine cumulus cells .....	94
Table 5-1. Cleavage and Blastocyst rates. ....	118

## **LISTE DES FIGURES**

Figure 1-1. Coupe immunohistologique d'un follicule antral bovin.....	4
Figure 1-2. Représentation schématique des vagues folliculaires et des variations hormonales au cours d'un cycle œstral à trois vagues chez la vache. ....	5
Figure 1-3. Ultrastructure de l'extrémité d'une projection transzonale (TZP) .....	14
Figure 2-1. Morphological evolution of the recovered COC during FSH coasting. ....	54
Figure 2-2. Venn diagrams representing the number of expressed genes in bovine cumulus cells for the different FSH withdrawal times. ....	55
Figure 2-3. Dendrogrammatic representation of the distance between the four different FSH withdrawal treatments. ....	56
Figure 2-4. Proportion of probes for which expression in bovine cumulus cells.....	57
Figure 2-5. Expression network from 10 selected genes of bovine cumulus.....	58
Figure 2-6. Quantification by qRT-PCR of mRNA profiles in cumulus cells for each FSH withdrawal time.....	59
Figure 2-7. Top 14 IPA biofunctions and their significance at 20, 44, 68 and 92h of FSH. (originally published as Figure S1, Supplementary Material) .....	60
Figure 3-1. Ovarian stimulation protocol used to assess the effects of LH support during the final follicle growth and differentiation in bovine.....	79
Figure 3-2. Morphological annotation of the recovered COC at the different FSH withdrawal durations with or without Cetorelix.....	80
Figure 3-3. Cumulus cell mRNA quantification by qRT-PCR at 68 h of FSH withdrawal with or without Cetorelix.....	81
Figure 4-1. Bovine cumulus cell mRNA expression during IVM. ....	95
Figure 4-2. Bovine cumulus cell mRNA expression of STAR, CYP11A1, HSD3b, and PGR during IVM. ....	96
Figure 5-1. Venn diagram representing the number of expressed genes in bovine CCs depending on the fate of their oocyte, i.e. 2- to 8-cells arrested embryos or Day 8 blastocysts.....	119
Figure 5-2. Top 12 Ingenuity Pathway Analysis biofunctions in bovine cumulus cells.....	120
Figure 5-3. Quantification of selected genes by qRT-PCR in CCs from each COC fate category.....	121
Figure 5-4. Quantification of selected genes by qRT-PCR in CCs for each COC fate category. ....	122
Figure 5-5. Bovine cumulus cell gene expression fold change comparison.....	123

## LISTE DES ABRÉVIATIONS ET DES SIGLES

%	pourcent
°C	degrés Celsius
µL	microlitre(s)
µm	micromètre(s)
3'UTR	3' Untranscribed Regions
AA	Acide(s) Aminé(s)
ACTNB	Actin Beta
ADAMTS	ADAM metallopeptidase with ThomboSpondin
ADN	Acide DésoxyRibonucléique
AG	Acide(s) Gras
AGPAT9	1-AcylGlycerol-3-Phosphate O-acylTransferase 9
AH	Acide Hyaluronique
AMPc	Adénosine MonoPhosphate cyclique
ANK3	Ankyrin 3, node of Ranvier (ankyrin G)
ANOVA	Analysis Of Variance
AR	Aldose Reductase
AREG	Amphireguline
ARN	Acide RiboNucléique
aRNA	Antisense RNA
ART	Assisted Reproductive Techniques
ATP	Adénosine Triphosphate
Bax	BCL2-Associated X protein
Bcl-2	B-cell CLL/lymphoma 2
BMP	Bone Morphogenic Protein
bp	Base Pair(s)
BSA	Bovine Serum Albumin
BTC	Betacelluline
CARTR	Canadian Assisted Reproductive Technologies Register
CC\CCs	Cellule(s) du Cumulus
CEEF	Cumulus-Expansion Enabling Factors
CG\CGM	Cellule(s) de la Granulosa \ Murales
CGP	Cellules Germinales Primordiales
CKB	Creatine Kinase B
CLIC3	Chloride Intracellular Channel 3
COC	Complexe Ovocyte-Cumulus
CSF	Cytostatic Factor
Cx	Connexine
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1
DEG	Differentially Expressed Genes
DNA	DeoxyriboNucleic Acid
e.g.	<i>exempli gratia</i> (par exemple)

E2	Estradiol
EGA	Embryonic Genome Activation
EGF	Epidermal Growth Factor
EGR1	Early Growth Response 1
EREG	Epiregulin
FADS2	Fatty Acid Desaturase 2
FAM105A	Family with sequence similarity 105, member A
FC	Fold Change
FD	Follicule Dominant
FDR	False Discovery Rate
FSH	Folliculo-Stimulating Hormone
G6PDH	Glucose-6-Phosphate Dehydrogenase
GADD45	Growth Arrest and DNA Damage 45
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GATM	Glycine Amidinotransferase (L-arginine : glycine amidinotransferase)
GDF9	Growth and Differentiation Factor 9
GMPc	Guanosine Monophosphate cyclique
GnRH	GonadoReleasing Hormone
GV	Germinal Vesicle
GVBD	Germinal Vesicle BreakDown
h	heure(s)
HAS2	Hyaluronic Acid Synthase 2)
HCA	Hierarchical Cluster Analysis
HDL	High-Density Lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSD3b /HSD3 $\beta$	Hydroxysteroid Dehydrogenase 3 beta
HSP	Heat-Shock Protein
<i>i.e.</i>	<i>id est</i> (c'est-à-dire)
IFI27	Interferon $\alpha$ -Inducible protein 27
IGF-I	Insulin Growth Factor I
INSIG1	Insulin Induced Gene 1
IPA	Ingenuity Pathway Analysis
IVF	<i>in vitro</i> Fertilization
IVM	<i>in vitro</i> Maturation
IVP	<i>in vitro</i> Production
kDa	kiloDalton
KRT8	Keratine 8
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LH	Luteinizing Hormone
LHCGR	Luteinizing Hormone/ChorioGonadotropin Receptor
LIMMA	Linear Models for Microarray
lncRNA	long non-coding RNA

LUM	Lumican
MAN1A1	Mannosidase $\alpha$ , class 1A, member 1
MEC	Matrice Extracellulaire
mg	milligramme(s)
MII	Métaphase II
min	minute(s)
miRNA	microRNA
MIV	Maturation <i>in vitro</i>
mL	millilitre(s)
mm	millimètre(s)
mM	milliMolaire(s)
MPF	M-phase-Promoting Factor
mRNA	messenger RiboNucleic Acid
MTCH1	Mitochondrial carrier 1
MVK	Mevalonate Kinase
NADPH	Nicotinamide Adénine Dinucléotide Phosphate
ng	nanogramme(s)
NPPC	Natriuretic Peptitde type C
NPR2	Natriuretic Peptide Receptor 2
NRP1	Neuropilin 1
NSDHL	NAD(P)-dependent Steroid Dehydrogenase-Like
ODPF	Oocyte-Derived Paracrine Factor(s)
OHSS	Ovarian HyperStimulation Syndrome
OSF	Oocyte-Secreted Factor(s)
P4	Progéstérone
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDE3	Phosphodiesterase 3A
PKF	Phosphofructokinase
PGE2	Prostaglandine type E 2
PGR	Progesterone Receptor
PKA	Proteine Kinase A
PMA	Procréation Médicalement Assistée
PO	Ponction(s) Ovocytaire(s)
PRPP	Phosphoribosylpyrophosphate
PTGS2	Prostaglandine Synthase 2
PTX3	Pentraxin 3
PTZ	Projection(s) Transzonale(s)
PVA	Polyvinyl Alcohol
qRT-PCR	quantitative Reverse-Transcription Polymerase Chain Reaction
RE	Reticulum Endoplasmique
RELN	Reelin
R-FSH	Recepteur Folliculo-Stimulating Hormone

R-LH	Recepteur Luteinizing Hormone
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
RT	Reverse Transcription
s	seconde(s)
S100A4	S100 calcium binding protein A4
SCARB1	Scavenger Receptor class B member 1
siRNA	small interfering RNA
Slc38a3	Solute Carrier family 38 member 3
SOF	Synthetic Oviductal Fluid
STAR	Steroidogenic Acute Regulatory Protein
TGF- $\beta$	Transforming Growth Factor $\beta$
TLH	Tyrode lactate solution
TZP	Transzonal Projection(s)
VG	Vésicule Germinale
VNN1	Vanin 1
YBX1	Y box binding protein 1

## **REMERCIEMENTS**

C'est non sans larmes que je m'attaque à cette section... Le chemin a été long et éprouvant comme on dit, et aussi semé d'embuches personnelles qui vous gâchent le quotidien jusque sous la hotte de PCR... (Non mes échantillons n'ont pas été contaminés par mes larmes, j'ai fait attention!) Pourtant, tout est bien qui finit bien puisque j'en suis à rédiger ces lignes !!! Et d'ailleurs, il y a des personnes à qui je dois et je veux dire merci pour leur contribution plus ou moins réelle et/ou consciente à ce projet. Certaines ont pris d'autres directions, d'autres, à ma grande satisfaction, sont restées proches!

Marc-André tout d'abord. En tant que mon directeur de recherche, merci de m'avoir accueillie dans ton laboratoire et de m'avoir ainsi donné la chance de vivre la formidable expérience de l'expatriation. Merci de m'avoir conseillée et fait confiance envers et contre toutes mes phases de profond découragement. Merci de ta bienveillance, de ton humanité, de m'avoir soutenue et aidée à surmonter ces phases difficiles. Et, même si les mots me paraissent insuffisants : Merci de m'avoir offert l'exceptionnelle opportunité de revenir finir mon doctorat, même après 3 ans d'absence! La gratitude que j'ai à ton égard est grande.

Merci aussi à François Richard, mon co-directeur, Claude Robert, François Castonguay et Catherine Combelles d'avoir accepté de constituer mon jury d'évaluation.

Merci à l'équipe de Boviteq dont Patrick Blondin et Christian Vigneault.

Merci à Ellen Jorssen, Jo Leroy et Peter Bols d'avoir embarqué dans le projet de collaboration.

Un chaleureux merci à Isabelle Dufort, la « Maman du labo », pour son soutien technique mais aussi pour son naturel, les longues discussions sur tout et rien, les rigolades et les éclaboussures en tous genres ;) ...

Merci à Isabelle Laflamme de m'avoir formée à faire des bébés *in vitro*!

Julie, Dominic, Isabelle G, merci pour les dépannages, la bonne ambiance, encore des discussions et des rires... Un merci spécial à Dominic pour le prêt de Lapinou!

Merci à tous les étudiants et post-docs pour les bons moments : Béatrice ma « grande-sœur de labo », Anne-Laure, Maëlla ma voisine bretonne, Gaël le « grand-frère » toulousain, Florence, Angus, Gab, Rémi, Éric, Élise, Ernesto, Luis, Annie Ga., Nico, Eve-Lyne, ...

Merci à Sara Scantland d'être elle-même! Merci pour ta présence encore aujourd'hui! Merci de me laisser venir te prêter main-forte de temps à autre, ça fait du bien!

Merci à Meg et Jason pour votre présence et votre soutien du côté plus personnel!

Merci à Annie Girard pour les discussions et les moments salvateurs à l'écurie!

Merci à Rim, Libou et Marie!

Merci au soutien et à l'amour de la famille : Papa Bernard, Maman Jocelyne, mes grands-frères Olivier et Jérôme (le fou des vaches!) ainsi que leurs douces moitiés Sandrine et Claire, ma grande-sœur Bénédicte (la seule qui comprenne la folie des études!) et son mari Ghislain. Merci à mes neveux et nièces : Elsa, Maïa, Clément, Colin, Malo, Tristan, Alexandre, Capucine et Oscar pour leur joie, leur amour et leurs câlins à chacun de mes retours au pays!

Merci aussi aux grands-parents, Mamie Geneviève et Père Noël, pour leurs cadeaux de départ!

Merci aux Tontons et Tatas et Cousins et Cousines pour leurs encouragements!

Merci à Mithra et Magnificat, vos ronrons pour l'un et cabrioles pour l'autre, ont été important pendant la rédaction!

Et finalement, merci à mon amour, François, pour ton soutien, tes encouragements, tes « coups de pieds au c.. » et ta confiance.

## **AVANT-PROPOS**

Les chapitres 2 et 5 sont des articles publiés. Les chapitres 3 et 4 seront prochainement soumis pour publication.

### **Chapitre 2 :**

Bunel A., Nivet A. L., Blondin P., Vigneault C., Richard F. J., Sirard M. A. (2013)

**Cumulus cell gene expression associated with pre-ovulatory acquisition of developmental competence in bovine oocytes.**

*Reproduction, Fertility and Development* **26**, 855-865.

### **Chapitre 3 :**

Bunel A., Nivet A. L., Blondin P., Vigneault C., Richard F. J., Sirard M. A.

**The effects of basal LH inhibition with Cetrorelix on cumulus cell gene expression during the luteal phase under ovarian coothing stimulation in cattle.**

Dans ces chapitres, Audrey Bunel était responsable de la réalisation de toutes les expériences, de l'analyse et l'interprétation des données et de l'écriture du manuscrit. Anne-Laure Nivet et Christian Vigneault ont pris part à la mise en place du projet et se sont impliqués dans la récolte des échantillons biologiques. Patrick Blondin et François Richard ont participé à la conception du projet. Marc-André Sirard a contribué à la création du projet, l'analyse et l'interprétation des données et également la révision du manuscrit. Les coauteurs ont tous lu et approuvé le manuscrit du chapitre 2 dans sa version finale.

### **Chapitre 4 :**

Bunel A., Girard A., Dufort I., Sirard M. A.

**Analysis of LHCGR and selected steroid enzyme's expression in bovine cumulus cells during *in vitro* maturation.**

Dans ce chapitre, Audrey Bunel a pris part à l'élaboration du projet et était responsable de la récolte des échantillons biologiques, de la réalisation des expériences finales, de l'analyse et l'interprétation des données et de l'écriture du manuscrit. Annie Girard et Isabelle Dufort ont réalisé les expériences préliminaires et participé à l'analyse des séquences nucléotidiques. Marc-André Sirard a contribué à la conception du projet, l'analyse et l'interprétation des données ainsi qu'à la révision du manuscrit.

**Chapitre 5 :**

Bunel A., Jorssen E., Merckx E., Leroy J. L., Bols P. E., Sirard M.-A. (2015)

**Individual bovine *in vitro* embryo production and cumulus cell transcriptomic analysis  
to distinguish cumulus-oocyte complexes with high or low developmental potential.**

*Theriogenology , Volume 83 , Issue 2 , 228 - 237*

Dans ce chapitre, Audrey Bunel et Ellen Jorssen ont collaboré à la conception du projet. Audrey Bunel était responsable de la réalisation des expériences de transcriptomique, de l'analyse et l'interprétation des données et de l'écriture du manuscrit. Ellen Jorssen et Els Merckx étaient responsables des expériences de culture *in vitro* et d'évaluation de l'apoptose. Marc-André Sirard a participé à l'élaboration du projet, l'analyse et l'interprétation des données et aussi la révision du manuscrit. Les coauteurs ont tous lu et approuvé la version finale du manuscrit.

## 1. INTRODUCTION

### 1.1. Le follicule ovarien

La formation et le développement d'un follicule ovarien complet impliquent deux processus morphogènes spécifiques : l'ovogenèse et la folliculogenèse. Ces deux processus démarrent au stade embryonnaire, l'ovogenèse en premier, et finissent à l'âge adulte avec l'ovulation. Ovogenèse et folliculogenèse sont interdépendantes. L'ovocyte est en effet considéré comme le chef d'orchestre de la folliculogenèse tandis que les cellules somatiques folliculaires vont le soutenir et l'influencer dans son développement.

#### 1.1.1. Ovogenèse

L'ovogenèse correspond à la formation et au développement de la cellule germinale femelle. Elle débute avec l'induction de cellules germinales primordiales et s'achève avec l'obtention d'un ovocyte fécondable.

##### 1.1.1.1. Cellules germinales primordiales

C'est au cours de la gastrulation embryonnaire que la lignée cellulaire germinale s'établie, par l'induction de cellules germinales primordiales (CGP) ([Biason-Lauber 2010](#)). Alors que les crêtes génitales se développent, les CGP entreprennent une longue migration au travers de différents tissus de l'embryon afin de les rejoindre. Chez le bovin, la formation des crêtes génitales à lieu entre le 28<sup>e</sup> et 32<sup>e</sup> jours et la migration se déroule du 30<sup>e</sup> au 64<sup>e</sup> jour de gestation ([Rüsse and Sinowitz 1991](#)).

À leur arrivée dans les crêtes génitales, les CGP intensifient leur activité mitotique et proliférative entreprise pendant la migration ([Erickson 1966](#)). Chez la femelle, une fois la gonade primordiale formée, les cellules germinales primordiales installées vont se différencier en ovogonies ([Oktem and Oktay 2008](#)).

##### 1.1.1.2. Ovogonies

Après avoir abondamment proliféré au sein des gonades, les ovogonies s'engagent dans une première phase de méiose qui, pendant la gestation, s'arrêtera en prophase I et le noyau cellulaire portera dorénavant le nom de vésicule germinale (VG). Cette différenciation par méiose des ovogonies en ovocytes primaires intervient alors que la folliculogenèse débute ([Erickson 1966](#)).

### **1.1.2. Folliculogenèse**

La formation du follicule ovarien s'organise autour de l'ovocyte. Par la suite, le développement du follicule se divise en deux phases : une première phase appelée *croissance folliculaire basale* qui est indépendante des hormones gonadotropes, et une deuxième phase appelée *croissance folliculaire terminale* qui elle dépend des hormones gonadotropes.

#### **1.1.2.1. Mise en place du follicule primordial**

Encore au stade d'ovogonies, les cellules germinales s'entourent d'une monocouche de cellules somatiques aplatis qui seront à l'origine des cellules de la granulosa. Cet assemblage constitue une ébauche de follicule qui va progressivement s'envelopper d'une lame basale et ainsi s'isoler du stroma ovarien. Ces follicules, désormais individualisés et contenant un ovocyte arrivé au stade primaire, sont appelés les *follicules primordiaux* ([Merchant-Larios and Chimal-Monroy 1989](#)).

Peu avant la naissance, cette population de follicules subit une importante phase d'apoptose entraînant une chute sévère de ses effectifs ([De Felici et al. 2005](#)). Les follicules primordiaux restants constituent alors la réserve ovarienne dans laquelle des follicules seront régulièrement recrutés tout au long de la vie de l'individu.

Chez la vache, les follicules primordiaux sont observables dès le 90<sup>e</sup> jour de gestation ([Yang and Fortune 2008](#)) et leur nombre maximal est d'environ 2,1 millions puis chute avant la naissance à environ 130 000 ([Erickson 1966](#)). Chez la femme, au 5<sup>e</sup> mois de grossesse, on compte environ 7 millions de follicules primordiaux alors qu'après la naissance, la réserve ovarienne n'en contient plus qu'environ 2 millions ([Baker 1963](#)).

#### **1.1.2.2. Croissance folliculaire basale**

L'entrée en croissance des follicules primordiaux survient lorsque le noyau de l'ovocyte atteint environ 0,015 mm chez la vache et 0,019 mm chez la femme ([Driancourt 2001](#)). Dès lors le diamètre de l'ovocyte va grandement s'accroître tandis que le follicule se développe et grandit d'autant plus.

##### **1.1.2.2.1. Follicule primaire**

L'initiation de la croissance folliculaire basale se caractérise par le passage à la forme cubique de la couche de cellules somatiques entourant l'ovocyte primaire. Cette transformation des premières *cellules de la granulosa* (CG) reflète le passage du follicule primordial au stade de *follicule primaire*. C'est aussi

à ce stade que l'ovocyte initie sa croissance et que des cellules dérivées du stroma ovarien viennent entourer le follicule et commencent à constituer la *thèque* ([Hirshfield 1991](#)).

#### **1.1.2.2.2. Follicule secondaire et pré-antral**

La croissance folliculaire basale se poursuit avec la prolifération des CG en plusieurs couches et l'apparition de la *zone pellucide* autour de l'ovocyte primaire. Cette nouvelle structuration folliculaire est celle du follicule secondaire ([Fair 2003](#)).

Une communication directe entre les cellules du follicule est rendue possible par la mise en place de jonctions communicantes – ou *gap junctions* ([Senbon et al. 2003](#)). Plus particulièrement pour les échanges entre les CG et l'ovocyte, les jonctions communicantes se trouvent au bout de projections cellulaires envoyées au travers de la zone pellucide par les CG et entrant en contact avec l'ovocyte ([de Loos et al. 1991](#)). Cette communication bidirectionnelle permet la régulation du développement folliculaire, notamment par un contrôle ovocytaire de la prolifération et de la différenciation des cellules de la granulosa et de la thèque ([Oktem and Oktay 2008](#)).

À ce stade, l'ovocyte continue sa croissance jusqu'à environ 50 µm ([Braw-Tal and Yossefi 1997](#)) et la thèque s'étend avec l'apparition de la thèque externe. C'est dans ce compartiment que la vascularisation du follicule s'installe puis se densifie conjointement au développement du follicule. La granulosa, quant à elle, restera dépourvue de vascularisation ([Oktem and Oktay 2008](#)).

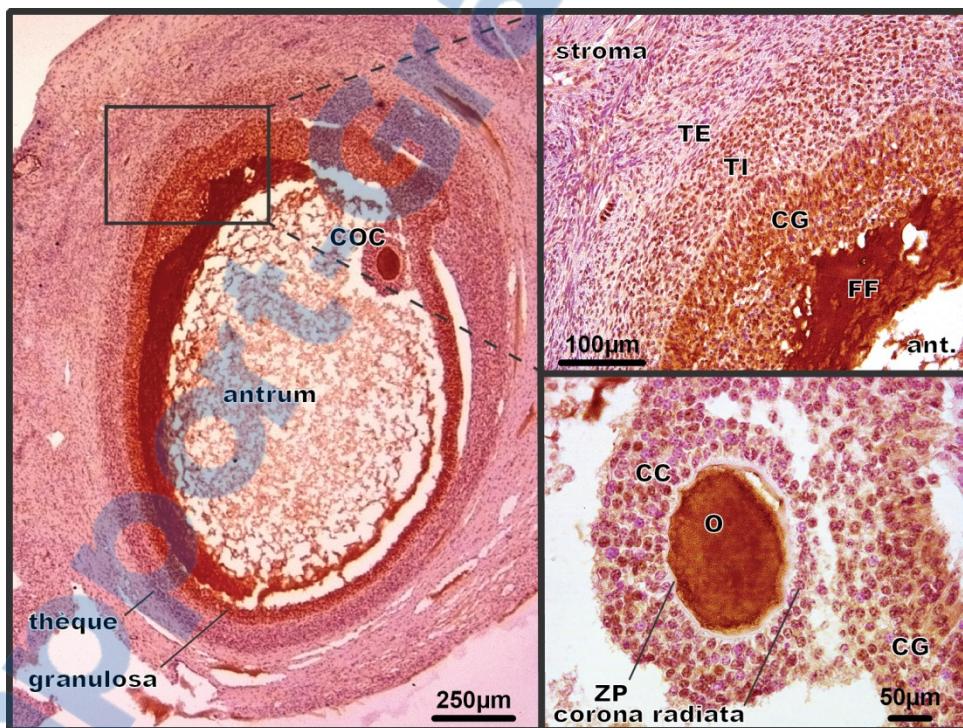
L'apparition de cellules épithélioïdes dans la thèque interne du follicule secondaire caractérise le *stade pré-antral* du follicule. L'expression de récepteurs à l'hormone luténisante (LH) dans la thèque et à l'hormone folliculo-stimulante (FSH) dans la granulosa ([Findlay and Drummond 1999](#)), potentialise dès lors une réponse du follicule à une stimulation gonadotrope.

#### **1.1.2.3. Croissance folliculaire terminale**

Le début de la croissance folliculaire terminale se manifeste par l'installation d'une dépendance aux gonadotropines des follicules dont le diamètre a atteint, chez la vache, 3 mm ([Driancourt 2001](#)). Cette phase d'accroissement d'un groupe de follicules, qui a lieu 2 ou 3 fois par cycle oestral, prend 4 à 5 jours, et s'achèvera par l'ovulation d'un seul follicule lors de la 2<sup>e</sup> ou de la 3<sup>e</sup> vague de croissance, tandis que les autres seront éliminés par atrésie. Le follicule préovulatoire bovin mesurera finalement entre 15 et 20 mm et contiendra un ovocyte mesurant lui environ 135 µm de diamètre ([Driancourt 2001](#)).

#### 1.1.2.3.1. Follicule tertiaire ou le follicule antral

La transition des follicules secondaires au *stade tertiaire* – ou *stade antral* – est figurée par la formation d'une cavité appelée *antrum* ([Smitz and Cortvriendt 2002](#)). Ce nouveau compartiment contient le liquide folliculaire composé de molécules dérivées du sang et de molécules sécrétées par les cellules somatiques : entre autres, des radicaux libres et des antioxydants, des hormones et des métabolites (revue par ([Hennet and Combelles 2012](#)). La formation de l'*antrum* engendre deux nouveaux types de cellules somatiques dérivées de la granulosa : les *cellules du cumulus* (CC) et les *cellules de la granulosa murale* (CGM). Plusieurs couches de ces CC enveloppent l'ovocyte et composent avec lui le *complexe ovocyte-cumulus* (COC), aussi appelé *cumulus oophorus*. Les CC situées à la périphérie immédiate de l'ovocyte forment la *corona radiata*, et elles sont arrimées à celui-ci par des projections cellulaires traversant la zone pellucide ([Rüsse 1983](#)), ou projections transzonales (PTZ). Le COC quant à lui reste partiellement rattaché à la granulosa désormais répartie à la périphérie de l'*antrum*, le long de la lame basale ([Figure 1-1](#)).



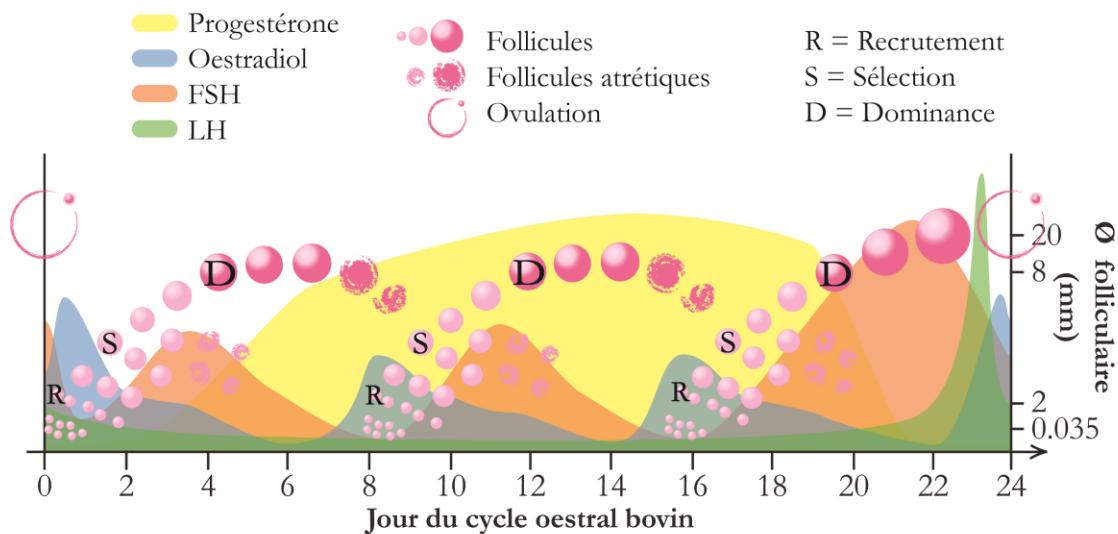
*Figure 1-1. Coupe immunohistologique d'un follicule antral bovin.*

COC : Complexe ovocyte-cumulus; TE : thèque externe; TI : thèque interne; CG : cellules de la granulosa; FF : fluide folliculaire; ant. : antrum; CC : cellules du cumulus; O : ovocyte; ZP : zone pellucide (Photos utilisées en accord avec la Dre Joëlle Dupont, Institut National de Recherche Agronomique, Nouzilly, France).

### 1.1.2.3.2. Les trois phases de la croissance folliculaire terminale

L'acquisition de la sensibilité aux gonadotropines de certains follicules secondaires va leur permettre d'entrer en croissance folliculaire terminale. Ce processus relativement bref et hautement dynamique se divise en trois phases : le *recrutement* d'une cohorte de follicules secondaires destinés à croître, la *sélection* – ou bien l'atrésie – et enfin la *dominance* du follicule sélectionné sur le reste de la cohorte. Ces trois phases constituent une vague folliculaire ([Evans et al. 1994](#)).

Les vagues folliculaires sont au nombre de 2 ou 3 par cycle oestral. Chez la vache, un cycle à 2 vagues dure environ 21 jours, et plutôt 23 jours pour un cycle à 3 vagues ([Sirois and Fortune 1988](#)). Seul le follicule dominant (FD) de la dernière vague du cycle achèvera son développement avec l'ovulation. Le FD de la ou des vagues précédentes devra quant à lui régresser et disparaître ([Figure 1-2](#)).



**Figure 1-2. Représentation schématique des vagues folliculaires et des variations hormonales au cours d'un cycle oestral à trois vagues chez la vache.**

(figure adaptée de ([Aerts and Bols 2010b](#); [Martin et al. 2013](#))

- *Le Recrutement*

Suite à la dégénérescence du follicule dominant de la vague précédente, ou bien à l'ovulation du cycle précédent, une forte augmentation de FSH circulante permet le *recrutement* de la cohorte des follicules qui formera une nouvelle vague folliculaire. Cette cohorte est généralement constituée de 5 à 10 follicules ([Driancourt 2001](#)) de 2 à 5 mm pourvus de différentes sensibilités aux gonadotropines. La croissance de ces follicules est dépendante de la sécrétion hypophysaire de FSH et est amplifiée par l'augmentation du nombre de leurs récepteurs à cette hormone.

- *La Sélection ou l'Attrésie?*

Lorsqu'au sein de la cohorte, un des follicules atteint 8,5 mm de diamètre pour la vache, 12 mm pour la femme, une *déviation* ([Ginther et al. 1997](#); [Ginther et al. 1998](#); [Ginther et al. 1999](#); [Beg and Ginther 2006](#)) se met en place entre le plus gros follicule qui va croître rapidement, et les plus petits dont la croissance va s'interrompre. Cette déviation est imposée par la différence d'expression des récepteurs aux gonadotropines. En effet, le plus gros follicule commence à exprimer le récepteur à la LH (R-LH) au niveau de la granulosa ([Ginther et al. 2001](#)) tandis que les autres ne possèdent que celui pour la FSH (R-FSH). La croissance des follicules ayant entraîné une augmentation de la production d'inhibine et d'œstradiol (E2), la production de FSH se voit inhibée. La croissance des plus petits follicules n'est alors plus stimulée par la FSH et seul le follicule exprimant R-LH peut poursuivre son développement, c'est la *sélection*. On parle alors du follicule dominant et des follicules subordonnés. Ces derniers ne pouvant continuer leur croissance de par l'insuffisance de FSH et l'absence de R-LH, ils vont régresser par *attrésie folliculaire* ([Ginther et al. 2000](#)) en utilisant les mécanismes de mort cellulaire programmée ou apoptose. Au sein du follicule, l'atrésie progresse de la périphérie vers le centre, affectant d'abord la granulosa, puis le cumulus et *in fine* l'ovocyte ([Irving-Rodgers et al. 2001](#)).

- *La Dominance*

La croissance du follicule dominant se poursuit dorénavant sous la dépendance à la LH ([Mihm et al. 2006](#)) et sa forte production d'E2 et d'inhibine maintient la FSH à des concentrations basales, lui assurant ainsi sa *dominance* sur les follicules subordonnés ([Ginther et al. 2000](#); [Ginther et al. 2001](#)). Pendant la phase lutéale du cycle œstral, et donc en présence de fortes concentrations de progestérone (P4) produite par le corps jaune, la fréquence et l'amplitude de la sécrétion pulsatile de LH ne permettent pas la maturation finale et l'ovulation du follicule dominant qui régressera ([Rahe et al. 1980](#); [Mihm and Austin 2002](#)). Une nouvelle vague folliculaire émergera alors. En phase folliculaire cependant, le follicule peut continuer son développement et devenir un follicule préovulatoire.

#### **1.1.2.3. Follicule préovulatoire, pic de LH et maturation ovocytaire**

Ayant la possibilité de se développer en *follicule préovulatoire* lors de la phase folliculaire, le diamètre du follicule dominant atteindra de 16 à 22 mm chez la vache, et environ 20 mm chez la femme. Il se sera écoulé 180 jours pour que le follicule primordial bovin atteigne le stade préovulatoire ([Lussier et al. 1987](#)).

Pendant cette phase, la présence de P4 à des concentrations basales – suite à la régression du corps jaune ou *lutéolyse* –, et la forte production d'E2 par le follicule dominant ([Ireland and Roche 1982](#);

[Ireland and Roche 1983](#)) entraînent une augmentation de GnRH (Gonadotropin-Releasing Hormone) permettant d'induire le pic préovulatoire de LH ([Sunderland et al. 1994](#)). Ce pic de LH conduit à la maturation finale du follicule dominant et mènera à son ovulation. Notons que les fortes concentrations d'E2 sont également responsables du comportement reproductif nécessaire à l'accouplement ([Ireland 1987](#)).

La maturation finale du follicule inclut des changements au niveau somatique ainsi qu'au niveau ovocytaire et comprend des modifications nucléaires et cytoplasmiques (revue par [Ferreira et al. 2009](#)).

- *Impacts du pic de LH sur la thèque et la granulosa*

Le pic préovulatoire de LH est perçu par le follicule au niveau des cellules de la thèque et de la granulosa, là où se trouvent les R-LH. Le pic de LH induit alors une diminution d'expression de l'aromatase dans la granulosa (conversion des androgènes en œstrogènes) ainsi qu'une augmentation d'expression de STAR, CYP11A1 et HSD3 $\beta$  (conversion du cholestérol en progestagènes, [Espey and Richards 2002](#)). Ceci aura pour effet de diminuer la production d'E2 au profit de la production de P4. Ces changements préparent le follicule à la *lutéinisation*, soit la reconversion du follicule en corps jaune suite à l'ovulation ([Smith et al. 1994](#)). Le corps jaune, *via* sa production de P4 comme signal pour l'endomètre, permettra le maintien d'une éventuelle gestation.

Par ailleurs, dans la granulosa, la synthèse de prostaglandines, notamment la PGE2, est initiée par l'activité croissante de la prostaglandine synthase 2 (PTGS2) induite par la LH ([Sirois and Dore 1997](#)). Ces prostaglandines seront nécessaires à la rupture du follicule pour l'ovulation ([Filion et al. 2001](#)).

- *Maturation nucléaire de l'ovocyte*

La méiose qui était restée bloquée au stade pachytène de prophase I au sein de la vésicule germinale (GV) depuis le stade de follicule primaire, reprend sous l'effet du pic de LH. Environ 4 à 8 h après ce pic ([Kruip et al. 1983](#)), la rupture de la vésicule germinale – ou GVBD, *germinal vesicle breakdown*, est le premier événement observable de la reprise de la méiose. Dès 1935, Pincus et Enzmann ([1935](#)) remarquent que la reprise de la méiose peut également avoir lieu spontanément lorsque l'ovocyte de mammifère ([Edwards 1965](#)) est ôté de son follicule. Une inhibition folliculaire de la méiose est alors suggérée. L'AMPc (adénosine monophosphate cyclique), un second messager, doit effectivement être retrouvée à de fortes concentrations au sein de l'ovocyte afin de bloquer la méiose en prophase I dans un premier temps, puis de maintenir ce blocage ([Bilodeau et al. 1993](#)). L'AMPc est synthétisée par l'ovocyte mais aussi par le cumulus, lequel transmet une partie de sa production à l'ovocyte par les PTZ

([Bornslaeger and Schultz 1985](#)). Toutefois, chez le bovin, le maintien de concentrations ovocytaires élevées d'AMPc requiert l'intervention de GMPc (guanosine monophosphate cyclique, ([Norris et al. 2009](#)). En effet, la GMPc, passant par les jonctions communicantes, permet d'inhiber une protéine responsable de la dégradation de l'AMPc dans l'ovocyte, la PDE3A (phosphodiesterase 3A, ([Shitsukawa et al. 2001](#)).

La régulation – arrêt comme reprise – de la méiose de l'ovocyte fait appel au facteur de promotion de la maturation ou MPF (M-phase-promoting factor, revue par ([Han and Conti 2006](#)) ainsi qu'au facteur cytostatique ou CSF (cytostatic factor, ([Masui and Markert 1971](#)), le dernier participant à la stabilisation du premier. Lors de la chute des concentrations d'AMPc, l'activité PKA (proteine kinase A) diminue permettant l'activation du MPF ([Han and Conti 2006](#)) et ainsi la maturation ovocytaire peut débuter.

L'ovocyte passe alors en métaphase, anaphase et enfin télophase de première division de méiose – ou *division réductionnelle* –, lors de laquelle le premier globule polaire est expulsé. L'ovocyte, dorénavant désigné comme ovocyte II, débute la deuxième division de méiose – ou *division équationnelle* –, puis subit un second arrêt méiotique, en MII cette fois. Cet arrêt, dû au MPF et survenant environ 24 h après le début de la maturation ([Hashimoto and Kishimoto 1988](#)), perdurera jusqu'à fécondation.

Par ailleurs, la compétence méiotique de l'ovocyte est aussi en lien avec sa propre taille. Ainsi, les ovocytes de diamètre inférieur à 100 µm ont moins de chance d'atteindre le stade de GVBD que les ovocytes de diamètre compris entre 100 et 110 µm qui eux peuvent progresser jusqu'en métaphase I. Finalement, c'est à partir de 110 µm que la capacité à atteindre le stade de métaphase II s'installe ([Fair et al. 1995](#)).

- *Maturation cytoplasmique et moléculaire de l'ovocyte (passée en revue par Ferreira 2009)*

Au cours de la maturation ovocytaire, une réorganisation des organelles est observable. Celle-ci s'appuie sur les filaments du cytosquelette de l'ovocyte. En effet, ces structures adaptables et dynamiques permettent notamment la ségrégation des chromosomes pendant la méiose ainsi que le déplacement des molécules et des organelles au sein de la cellule ([Alberts et al. 2004](#)). Ces filaments ont la capacité de s'associer et de se dissocier rapidement et trois types de filaments entrent en jeu : les microtubules, les filaments d'actine et les filaments intermédiaires. Au fur et à mesure de la progression de l'ovocyte vers la MII, le réseau de filaments d'actine va se densifier à la périphérie de l'ovocyte ([Schatten et al. 1986](#)).

Initialement en position plutôt périphérique, les mitochondries, fournissant l'énergie nécessaire à l'ensemble des processus de la maturation ([Stojkovic et al. 2001](#)), se dispersent dans tout l'ooplasme suite à l'expulsion du premier globule polaire, environ 19 h après le pic de LH chez le bovin. Une fois la métaphase II atteinte, les mitochondries sont retrouvées en position centrale ([Kruip et al. 1983](#)).

L'intense activité mitochondriale déployée pour la maturation, puis le développement embryonnaire précoce, produit de grandes quantités de dérivés réactifs de l'oxygène (ou ROS, *reactive oxygen species*) induisant un fort stress oxydatif ([Taramona et al. 2006](#)). En éliminant les ROS, les enzymes antioxydantes comme par exemple la glutathione, la catalase ou encore la superoxyde dismutase, protègent l'ovocyte puis l'embryon contre les dommages de l'oxydation. Ainsi, une augmentation de la glutathione intervient au cours de la progression de l'ovocyte du stade de GV au stade de MII ([Luciano et al. 2005](#)).

Le réticulum endoplasmique (RE) qui est responsable de la régulation intracellulaire du calcium essentiel à l'activation de l'ovocyte lors de la fécondation ([Machaca 2007](#)) est, au stade de GV, uniformément réparti dans l'ooplasme. Le RE se retrouve ensuite en position corticale et finalement, en approchant de la MII, dispersé dans l'ooplasme et arrangé en grappes. Outre la régulation des gradients de calcium, le RE prend également part au repli des protéines et à leur dégradation, au métabolisme lipidique ainsi qu'à la synthèse des membranes ([Lippincott-Schwartz et al. 2000](#)).

L'appareil de Golgi quant à lui apparaît fragmenté au stade ovocytaire de GV puis sous forme vésiculaire au moment de la GVBD ([Moreno et al. 2002](#)).

Dérivés de l'appareil de Golgi, les granules corticaux sont d'abord organisés en paquets répartis dans l'ooplasme du stade GV ([Hosoe and Shioya 1997](#)), ils vont ensuite se disposer le long de la membrane plasmique au stade de MII ([Thibault et al. 1987](#)). Cette organisation permet, lors de la fécondation, d'empêcher la polyspermie par une modification rapide de la matrice extracellulaire de l'ovocyte *via* l'exocytose des granules corticaux et la libération de leur contenu ([Hosoe and Shioya 1997](#)). Ce processus de *réaction corticale* fait suite à la décharge de calcium résultant de l'entrée du spermatozoïde dans l'ovocyte ([Lawrence et al. 1997](#)) dont la zone pellucide devient alors impénétrable pour d'autres spermatozoïdes ([Ozil 1998](#)).

Afin d'assurer le développement de l'ovocyte pendant sa maturation, et les premiers clivages embryonnaires jusqu'à l'activation du génome de l'embryon – ou EGA, *embryonic genome activation* ; au stade de 8-16 cellules chez le bovin et 4-8 cellules chez l'humain –, l'ovocyte doit également accumuler un grand nombre d'ARN messagers (ARNm) et de protéines. Ces molécules seront soit utilisées directement, soit stockées sous forme de ribonucleoprotéines jusqu'au moment opportun de leur

utilisation ([Sirard 2001](#)). Une fois la méiose redémarrée, l'expression génique, ou transcription, est minimale voire nulle. Le stockage approprié des ARNm est donc crucial de façon à les protéger de la dégradation ([Fulka et al. 1998](#)). De plus, afin d'assurer l'élévation de la synthèse protéique nécessaire à la maturation ovocytaire, tout particulièrement pendant la métaphase I, une augmentation de la production de ribosomes est requise ([Van Blerkom et al. 2000; Ferreira et al. 2009](#)). Finalement, la traduction des ARNm redescendra à un niveau moindre lors de l'arrivée en MII ([Tomek et al. 2002](#)).

*En 1935, Ethel Browne Harvey a montré que chez l'oursin de mer, les premières divisions embryonnaires n'ont même pas besoin de la présence d'un noyau au sein de l'embryon ! En effet, elle a retiré le noyau de l'œuf, a activé son développement en l'immergeant dans l'eau de mer, et les divisions se sont effectuées jusqu'à atteindre environ 500 cellules avant de s'arrêter ([Harvey 1935](#)) !!!*

### 1.1.3. Ovulation

L'ovulation fait suite au pic de LH et à la maturation finale du follicule. Elle correspond à la rupture dudit follicule qui libère alors le complexe ovocyte-cumulus mature en direction du site de fécondation. Chez la vache, l'ovulation a lieu entre 29 et 31 heures après le pic de LH et chez la femme, environ 35 heures après ([Driancourt 2001](#)).

L'action des prostaglandines et du stress oxydatif en réponse au pic de LH, ainsi que la présence de cellules du système immunitaire suggèrent une ressemblance entre l'ovulation et une réaction de type inflammatoire ([Espey 1980](#)). Ultimement, la combinaison d'une élévation de la pression intra-folliculaire ([Matousek et al. 2001](#)) et de l'activité de type protéase permettrait la rupture du follicule ([Curry et al. 1992](#)).

## 1.2. Le *cumulus oophorus*

Dans le follicule antral, les cellules du cumulus se distinguent des cellules de la granulosa non seulement d'un point de vue anatomique, mais aussi du point de vue de leurs fonctions auprès de l'ovocyte. En effet, les CG assument un rôle préférentiellement endocrine – FSH, LH, stéroïdogenèse – alors que les CC assurent plutôt les fonctions « nourricières » – métabolites, énergie, réserves ([Diaz et al. 2007](#)).

Le *cumulus oophorus* ou complexe ovocyte-cumulus (COC) qui apparaît avec la formation de l'*antrum* au sein du follicule tertiaire, est une structure tridimensionnelle très spécialisée, dynamique et où la communication réciproque – ou bidirectionnelle – est essentielle.

L'ablation des CC, la rupture de leurs communications avec l'ovocyte, la perturbation de leur métabolisme ou bien de leurs activités transcriptionnelle ou traductionnelle ont démontré le rôle crucial des CC auprès de l'ovocyte. Ainsi les CC interviennent tant dans la maturation finale de l'ovocyte que dans sa capacité à assurer les premières divisions embryonnaires – ou compétence ovocytaire au développement, et ce dans plusieurs espèces ([Sirard and First 1988; Isobe and Terada 2001; Modina et al. 2001; Matzuk et al. 2002; Sutton et al. 2003b](#)).

Quant à l'ovocyte, il permet le maintien de l'organisation et de l'intégrité du follicule antral et régule les fonctions somatiques. Ainsi, l'ovocyte assure par exemple la survie des CC en empêchant leur apoptose ([Hussein et al. 2005](#)).

### 1.2.1. Différenciation des cellules du cumulus, rôle de l'ovocyte

Cellules murales de la granulosa et cellules du cumulus sont toutes deux issues des cellules de la granulosa pré-antrale, de se fait elles partagent certaines potentialités d'expression génique. Cependant, lors du développement du follicule antral, l'ovocyte induit leur différenciation spécifique en CC et CGM. Ainsi, les CC et les CGM accompagnant un ovocyte mature pourraient différemment exprimer jusqu'à un peu plus de 3150 gènes ([Burnik Papler et al. 2015](#)).

La différenciation CC/CGM nécessite que l'ovocyte sécrète des facteurs spécifiques appelés OSF (oocyte-secreted factors) ou ODPF (oocyte-derived paracrine factors) dont la majeure partie appartient à la super-famille des protéines TGF- $\beta$  (*transforming growth factor- $\beta$* ) ([Erickson and Shimasaki 2000](#)). La gonadotropine FSH reste toutefois nécessaire à la différenciation CC/CGM et au développement du

follicule antral comme le démontrent les expériences avec des souris *knockout* pour la FSH ([Kumar et al. 1997](#)), ou bien son récepteur ([Dierich et al. 1998](#)), dont les follicules ne parviennent pas à se développer au-delà du stade antral précoce.

Parmi les OSF, GDF9 (growth and differentiation factor 9) intervient très tôt dans la folliculogenèse puisqu'en son absence – souris *knockout* pour GDF9 – la transition de follicule primaire à secondaire n'a pas lieu ([Dong et al. 1996](#)). Il en va de même avec BMP15 (bone morphogenetic protein 15) chez les brebis ayant une mutation homozygote pour ce gène ([Galloway et al. 2000](#)). Chez le bovin, l'immunisation active contre GDF9 et/ou BMP15 entraîne également un développement folliculaire anormal, compromettant alors la fertilité ([Juengel et al. 2009](#)). Plus spécifiquement vis-à-vis du cumulus, chez la souris, l'absence de BMP15 entrave sa différenciation ([Su et al. 2004; Yoshino et al. 2006](#)). Outre son rôle de différenciateur, l'ovocyte assure aussi la survie des CC notamment *via* les OSF que sont BMP15 et BMP6, la promotion de Bcl-2 (protéine anti-apoptotique) et la suppression Bax (pro-apoptotique), ce qui permet de prévenir l'apoptose des CC chez le bovin ([Hussein et al. 2005](#)).

Au sein du follicule antral, le gradient d'efficacité des OSF s'oppose à celui des gonadotropines : les OSF étant plus efficaces à proximité de l'ovocyte, ils ont donc plus d'impact sur les CC que sur les CGM ; tandis que les gonadotropines exercent plus fortement leur influence sur la thèque et les CGM que sur les CC (revue par ([Gilchrist et al. 2008](#))). Si bien que, par exemple, un gradient d'expression du transcrit du R-LH est observé chez la souris, où plus les cellules sont éloignées de l'ovocyte, plus le transcrit du R-LH est exprimé et, à l'inverse, dans les cellules les plus proches de l'ovocyte, cette expression est totalement supprimée ([Eppig et al. 2002](#)). L'absence caractéristique de R-LH au sein du cumulus est effectivement due à la suppression de son expression par l'ovocyte au stade de GV ([Eppig et al. 1997](#)). Ainsi, alors que cumulus et granulosa murale expriment tous deux le récepteur à la FSH ([Richards and Midgley 1976](#)), seule la granulosa murale exprime significativement le R-LH. Ceci sert d'ailleurs de marqueur de la différenciation des CGM ([Lei et al. 2001](#)).

Également, les CC affichent une stéroïdogenèse nettement moins active que les CGM, particulièrement concernant la production de progestérone ([Li et al. 2000](#)). En effet, l'ovocyte empêche la lutéinisation des CC en inhibant notamment l'expression de l'enzyme stéroïdogène P450 *side chain cleavage* codée par CYP11A1 ([Diaz et al. 2007](#)), ainsi que l'expression du récepteur à la LH ([Eppig et al. 1997](#)). Aussi, l'ovocyte, incapable d'utiliser directement le glucose comme source d'énergie ([Rieger and Loskutoff 1994](#)), stimule l'activité glycolytique du cumulus ([Sutton-McDowall et al. 2010](#)) ou encore le transport d'acides aminés ([Eppig et al. 2005](#)).

### 1.2.2. Communications bidirectionnelles

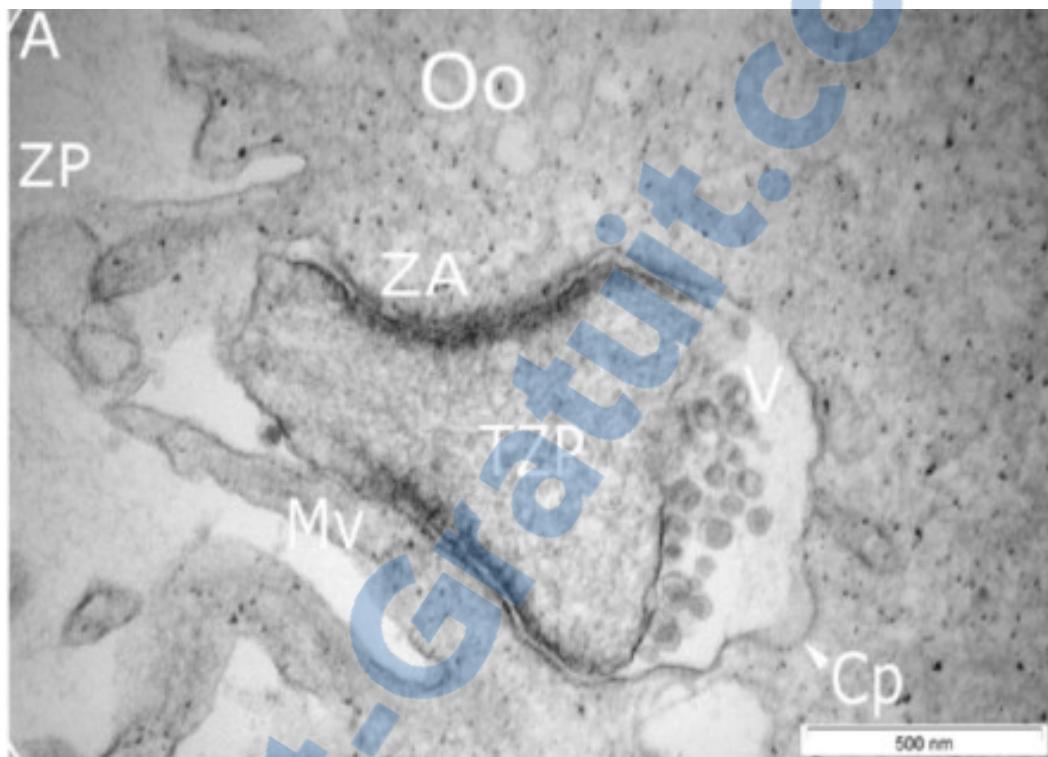
Outre les communications de nature paracrine abordées précédemment, l'ovocyte et les cellules du cumulus entretiennent une communication plus directe par le transfert de molécules.

En effet, la communication entre l'ovocyte et les CC *via* les jonctions communicantes (JC) – ou *gap junctions* – permet le transfert de molécules hydrophiles de poids moléculaire inférieur à 1 kDa, telles que des ions, des nucléotides, des acides aminés ou encore des glucides simples ([Winterhager and Kidder 2015](#)). La communication par le biais de ces jonctions au sein du COC a d'ailleurs été largement démontrée pour des molécules de petit poids moléculaire ([Moor et al. 1980; Racowsky and Satterlie 1985; Schultz 1985; Webb et al. 2002; Thomas et al. 2004; Li et al. 2012](#)). Les JC sont présentes entre les CC d'une part, mais aussi entre les CC de la *corona radiata* et l'ovocyte d'autre part. En raison de la présence de la zone pellucide entourant l'ovocyte, les jonctions entre les CC et l'ovocyte se font à l'extrémité des projections transzonales (PTZ) émises par la *corona radiata* ([Anderson and Albertini 1976](#)). Une JC est formée de deux connexons, un pour chaque cellule en interaction, eux-mêmes constitués de six connexines. Les principales connexines retrouvées dans le COC sont les Cx37 et Cx43 ([Nuttinck et al. 2000](#)).

Par ailleurs, les Cx43 semblent autoriser le passage de siRNA (small interfering RNA, 24 oligonucléotides maximum) ([Valiunas et al. 2005](#)). Le liquide folliculaire, lui, contient des microARN (miRNA, 20-24 oligonucléotides) qui sont fréquemment retrouvés dans les exosomes, et ces derniers peuvent être récupérés par les CG ([Sohel et al. 2013](#)) et les CC ([Hung et al. 2015](#)). De plus, chez l'humain, les miRNA exprimés par la *corona radiata* sont associés au métabolisme des acides aminés et à la glycolyse, et diffèrent de ceux exprimés par les CC plus éloignées de l'ovocyte ([Tong et al. 2014](#)). En outre, chez le bovin, ovocyte et CC semblent avoir une influence réciproque sur leurs populations de miRNA ([Abd El Naby et al. 2013](#)). Des transferts de miRNA entre les cellules du follicule *via* les JC sont ainsi imaginables.

Un autre type d'ARN, les longs ARN non-codants (lncRNA), a été relié au potentiel développemental dans les COC humains ([Yerushalmi et al. 2014; Li et al. 2015](#)). Des lncRNA, dont trois associés à la qualité embryonnaire, ont également été retrouvés dans les PTZ de cumulus bovins ([Caballero et al. 2014](#)). Le transfert de mRNA et de lncRNA depuis les CC vers l'ovocyte a d'ailleurs été montré chez le bovin, ainsi qu'une structure de type synapse, avec la présence de vésicules à l'extrémité des projections transzonales ([Figure 1-3 \(Macaulay et al. 2014\)](#)). Le déplacement de transcrits a également été suivi dans les PTZ jusqu'à l'ovocyte avec une accumulation progressive d'ARN dans ces PTZ suggérant un

transfert possible, et ce avant la reprise de méiose puisque les PTZ se rétractent au cours de la maturation ovocytaire ([Macaulay et al. 2016](#)). Ce transfert pourrait ainsi participer à l'accumulation d'ARNm par l'ovocyte en vue des premiers clivages et de l'activation du génome embryonnaire.



**Figure 1-3. Ultrastructure de l'extrémité d'une projection transzonale (TZP)**

De nombreuses vésicules (V) sont présentes à la rencontre entre la projection et l'ovocyte (Oo). Une fois la zone pellucide (ZP) traversée, la projection entre en contact avec des microvillosités (Mv) et forme une jonction de type *zonula adherens* (ZA) avec la membrane plasmique de l'ovocyte ([Macaulay et al. 2014](#)). Biol Reprod | © 2014 by the Society for the Study of Reproduction, Inc.

### 1.2.3. Métabolisme : missions du cumulus

Une véritable coopération existe entre l'ovocyte et les CC. En effet, ce que l'ovocyte ne fait pas lui-même, il le fait « sous-traiter » par le cumulus. Particulièrement lors de la maturation finale de l'ovocyte, le COC doit fournir différents métabolites, électrolytes, purines et pyrimidines, ou encore acides gras, acides aminés et glucides.

- *Le glucose*

Le glucose est la source d'énergie de processus nécessaires à la maturation du COC et il est utilisé par le biais de différentes voies de métabolisation. En effet, un COC bovin mature consomme deux fois plus d'oxygène, de pyruvate et de glucose qu'un COC immature ([Sutton et al. 2003a](#)). En outre, lors de maturation *in vitro* de COC, des concentrations inadéquates de glucose – trop basses ou trop élevées – provoquent des perturbations de la maturation nucléaire et cytoplasmique de l'ovocyte ainsi que de l'expansion du cumulus, et par-là même une compétence au développement réduite (revue par ([Sutton-McDowall et al. 2010](#))). Ces effets semblent être dus, dans le cas des concentrations en glucose trop faibles chez la souris, à un manque d'approvisionnement des voies de la glycolyse et des pentoses phosphates, limitant alors la synthèse d'acides nucléiques et la production d'énergie ([Downs et al. 1998](#)). Dans le cas de concentrations en glucose trop élevées chez le bovin, c'est l'augmentation de la production de ROS et le déclin de la glutathione réduite – enzyme anti-oxydante – qui sont mises en cause ([Hashimoto et al. 2000](#)).

Afin de servir la cause ovocytaire, le glucose doit d'abord passer par le cumulus car, bien que chez le bovin ou l'humain, l'ovocyte exprime certains transporteurs de glucose ([Dan-Goor et al. 1997; Augustin et al. 2001](#)), il n'en absorbe que très peu. En revanche, le cumulus, lui, possède un transporteur supplémentaire qui lui permet d'incorporer efficacement ce sucre ([Nishimoto et al. 2006](#)). Thompson et ses collègues ([Thompson et al. 2007](#)) ont ainsi calculé que les CC de COC bovins immatures consomment 23 fois plus de glucose que les ovocytes.

La voie majeure de métabolisation du glucose par le COC est celle de la glycolyse. Cependant, l'ovocyte bovin affiche une activité phosphofructokinase (PFK) faible ([Cetica et al. 2002](#)) – enzyme-clé de la glycolyse transformant le glucose en fructose – de ce fait c'est le cumulus qui prend en charge cette voie. Le cumulus, afin de fournir le COC en énergie, convertit ainsi le glucose en pyruvate et lactate ([Harris et al. 2009](#)). L'ovocyte bovin peut alors utiliser ces substrats dans le cycle de Krebs, puis la phosphorylation oxydative lui permet de produire de l'ATP ([Steeves and Gardner 1999](#)). Chez le bovin, la consommation de glucose par le COC au cours de la maturation *in vitro* augmente, toutefois, la production de lactate reste constante, suggérant alors une stabilité de l'activité glycolytique au cours de ce processus ([Sutton et al. 2003a](#)).

Dans le COC, le glucose entre aussi dans la voie des pentoses phosphates. D'ailleurs, lors de la maturation *in vitro* de COC porcins, l'inhibition de cette voie modifie l'activité mitochondriale et oxydative de l'ovocyte, faisant alors obstacle à la progression de la méiose ([Alvarez et al. 2016](#)). Chez le

bovin, l'ovocyte révèle une activité G6PDH (glucose-6-phosphate dehydrogenase, enzyme-clé de cette voie) plus élevée que le cumulus ([Cetica et al. 2002](#)). La voie des pentoses phosphates permet : d'une part, la production de NADPH – *via* la G6PDH – pour la régulation des ROS par réduction de la glutathione ; et d'autre part, la production de PRPP (phosphoribosylpyrophosphate) servant à la synthèse des purines nécessaires à la régulation de la méiose ([Sutton-McDowall et al. 2010](#)). La voie des pentoses phosphates est donc impliquée dans la maturation cytoplasmique et nucléaire de l'ovocyte.

Une autre voie de métabolisation du glucose est mise en œuvre par le COC, celle de la biosynthèse des hexosamines. Dans les CC, la conversion du produit final de cette voie, la UDP-N-acetyl glucosamine, en acide hyaluronique par HAS2 (hyaluronic acid synthase 2) est requise pour la formation de la matrice extracellulaire nécessaire à l'expansion du cumulus ([Sutton-McDowall et al. 2004](#); [Gutnisky et al. 2007](#)). La UDP-N-acetyl glucosamine participe également à la régulation post-traductionnelle en intervenant dans la O-glycolysation des protéines. La voie des hexosamines servirait en outre de sonde métabolique permettant une réponse cellulaire adaptée au statut énergétique de la cellule en question (revue par [\(Wells et al. 2003\)](#). Chez le xénope, la O-glycosylation semble varier au cours de l'ovogenèse et du développement précoce ([Dehennaut et al. 2009](#)). Dans les COC murins, la O-glycosylation de la HSP90 (heat-shock protein 90) due à une hyperglycémie mimée par l'ajout de glucosamine, réduit la compétence au développement de l'ovocyte ([Frank et al. 2014](#)).

Pour ce qui est de l'utilisation du glucose par la voie des polyols, peu d'information est disponible concernant le COC. Dans ce cas, le glucose est converti en sorbitol par l'aldose réductase (AR) puis en fructose par la sorbitol déshydrogénase. Chez la souris, l'ajout d'activateurs de l'AR ou l'augmentation des concentrations en sorbitol réduit la communication entre l'ovocyte et les CC ainsi que la synthèse de purines, ou encore la production d'AMPc et finalement, supprime la maturation ovocytaire induite par la FSH ([Colton and Downs 2004](#)).

### Régulation par l'ovocyte?

L'influence ovocytaire dans la régulation de la consommation de glucose du COC pourrait être spécifique de l'espèce puisque chez la souris, sans ovocyte, l'activité glycolytique des CC est fortement réduite ([Sugiura et al. 2005](#)) tandis que chez le bovin, l'absence de l'ovocyte n'aurait pas d'impact sur la consommation de glucose par les CC ([Sutton et al. 2003a](#)). Toutefois, une autre étude chez le bovin a montré que l'activité glycolytique des CC est réduite lorsque l'ovocyte est retiré du COC ([Zuelke and Brackett 1992](#)).

- *Les lipides*

Les cellules du cumulus détiennent également un rôle dans le transport et le métabolisme des lipides. En effet, lors de la maturation *in vitro*, l'absence des CC entraîne une diminution des réserves lipidiques de l'ovocyte bovin ([Auclair et al. 2013](#)).

Les acides gras (AG) interviennent dans la constitution des membranes et l'ancrage de protéines dans celles-ci. Les AG servent aussi de précurseurs aux prostaglandines et de source potentielle d'énergie (revue par ([Dunning et al. 2014b](#)). Chez le bovin, les ovocytes ([Wonnacott et al. 2010](#)) et les COC ([Adamiak et al. 2006](#)) contiennent plus d'AG saturés que les cellules de la granulosa. Le déroulement de la méiose ainsi que l'expansion du cumulus et son apoptose sont différemment influencés selon le type et les concentrations d'AG utilisés ([Leroy et al. 2005](#)). De plus, le traitement en maturation *in vitro* de COC bovins avec un mélange spécifique d'acides gras stimule l'expression de gènes du métabolisme énergétique et du stress oxydatif dans l'ovocyte alors qu'il inhibe ces mêmes gènes dans le cumulus ([Van Hoeck et al. 2013](#)). Par ailleurs, les blastocystes issus de la fécondation de ces ovocytes comptent moins de cellules, montrent d'avantage d'apoptose ainsi qu'une altération de l'expression de gènes reliés au métabolisme ([Van Hoeck et al. 2011; Van Hoeck et al. 2013](#)).

La β-oxydation des AG dans la mitochondrie permettant la synthèse d'ATP augmente drastiquement au cours de la maturation *in vitro* de COC murins, et bien qu'une partie de cette oxydation ait lieu dans l'ovocyte, la majeure partie se déroule dans le cumulus ([Dunning et al. 2010](#)). La même étude indique qu'*in vivo*, en période de maturation péri-ovulatoire, l'expression de *Cpt1b*, relié à l'entrée d'AG à longue chaîne dans la mitochondrie, augmente significativement dans le COC. La comparaison entre maturation *in vitro* et *in vivo* a montré qu'*in vitro*, la β-oxydation est moins active pour l'ovocyte félin ([Spindler et al. 2000](#)) et l'expression de gènes de cette voie est dérégulée dans les CC simiesques ([Lee et al. 2011](#)) et murines ([Dunning et al. 2014a](#)). Également, chez le bovin, durant la maturation *in vitro*, l'activité catalytique des lipases pour l'utilisation des acides gras comme source d'énergie, est plus élevée dans l'ovocyte que dans les CC où en plus elle décline avec le temps ([Cetica et al. 2002](#)). Finalement, la β-oxydation apparaît indispensable à la reprise de la méiose et à la maturation nucléaire ([Paczkowski et al. 2013](#)) ainsi qu'au développement embryonnaire, notamment chez le bovin ([Ferguson and Leese 2006](#)), le porc ([Sturmy et al. 2006](#)) ou encore la souris ([Dunning et al. 2010](#)).

En plus de prendre part au métabolisme des acides gras, le cumulus a une importance capitale dans le métabolisme du cholestérol au sein du COC. En effet, chez la souris, le cumulus exprime beaucoup plus que l'ovocyte les transcrits de certaines enzymes de la voie de biosynthèse du cholestérol, tels que *Mvk*, *Pmvk*, *Fdps*, *Sqle*, *Cyp51*, *Sc4mol* et *Ebp* ([Su et al. 2008](#)). Dans la même étude, les auteurs ont montré

que l'expression de ces transcrits dans les CC se trouve réduite lorsque l'ovocyte est excisé du COC, et qu'il en va de même pour la production *de novo* de cholestérol. Également, en l'absence des CC, l'ovocyte ne synthétise quasiment pas de cholestérol. D'autre part, l'ovocyte semble incapable d'incorporer le cholestérol lié aux lipoprotéines sous forme de HDL ou de LDL (high-density ou low-density lipoprotein) car il ne possède pas les récepteurs adéquats SCARB1 ([Trigatti et al. 1999](#)) et LDLR ([Sato et al. 2003](#)) respectivement. Le cumulus quant à lui exprime ces deux récepteurs, cependant le liquide folliculaire ne contient que peu de LDL-cholestérol ([Perret et al. 1985](#)) et l'étude de Trigatti *et al.* ([1999](#)) suggère que le HDL-cholestérol a peu d'influence dans l'apport folliculaire en cholestérol. Ainsi la source principale de cholestérol pour l'ovocyte viendrait du cumulus.

- *Les acides aminés*

Au début de la maturation, l'ovocyte bovin manifeste une synthèse protéique particulièrement active ([Tomek et al. 2002](#)) et a donc besoin d'un apport en acides aminés (AA) suffisant. Par ailleurs, les acides aminés tels que la cystine et la glycine ont un intérêt particulier dans la synthèse de glutathione pendant la maturation ovocytaire ([Luberda 2005](#)). De surcroît, l'ajout d'AA essentiels et non-essentiels en maturation *in vitro* a montré ses effets positifs sur la transcription des ARNm maternels ainsi que sur le développement embryonnaire ([Watson et al. 2000](#)). Par exemple, chez la souris, l'addition de carnitine dans le milieu de culture augmente les taux de maturation, de fécondation et de blastocystes ([Dunning et al. 2011](#)).

Comme pour le glucose ou les lipides, l'ovocyte requiert l'assistance du cumulus concernant certains de ses besoins en AA. Chez la souris, l'ovocyte ne peut en effet pas se fournir lui-même en alanine et c'est donc le cumulus qui le récupère et lui transmet *via* les jonctions communicantes ([Colonna and Mangia 1983](#)). Également, le transcrit d'un transporteur, *Slc38a3*, ayant une haute affinité pour l'alanine, le glutamate et l'histidine, est fortement exprimé dans le cumulus mais n'est exprimé ni dans les CG, ni dans l'ovocyte ([Eppig et al. 2005](#)). Les CC améliorent ainsi l'absorption de l'alanine, la glycine, la lysine ou encore l'histidine pour les transférer à l'ovocyte ([Eppig et al. 2005](#)). Chez le bovin, les ovocytes possèdent six différents transporteurs d'AA, toutefois, la présence de cellules somatiques améliore la capture de glycine, alanine, lysine et taurine par l'ovocyte ([Pelland et al. 2009](#)). Les CC bovines affichent en outre une plus grande activité des aspartate- et alanine-aminotransférases ainsi que de la malate déshydrogénase ([Cetica et al. 2003](#)). Finalement, la LH induit une augmentation du métabolisme oxydatif de la glutamine des ovocytes et des COC bovins ([Zuelke and Brackett 1993](#)).

#### 1.2.4. Réponse du COC au pic préovulatoire de LH

Le pic préovulatoire de LH induit de profonds remaniements au sein du COC, allant de variations d'expression génique jusqu'à des modifications structurelles et morphologiques, sans oublier la reprise de méiose par l'ovocyte. Les changements, du cumulus notamment, sont essentiels à l'ovulation, à la capture et au transport du COC par l'oviducte ainsi qu'à la fécondation (revue par [\(Russell and Salustri 2006\)](#)).

Le cumulus jouait jusque là un rôle dans le maintien de l'arrêt méiotique de l'ovocyte, mais ceci va prendre fin. Ce rôle lui était assuré par l'ovocyte lui-même *via*, entre autres, une coopération entre les OSF et l'E2 permettant au cumulus d'exprimer le récepteur NPR2 (natriuretic peptide receptor 2) qui fixe NPPC (natriuretic peptide type C) en provenance des CG. L'implication du système NPPC/NPR2 dans le maintien de l'arrêt méiotique a été démontré chez plusieurs mammifères (revue par [\(Emori and Sugiura 2014\)](#)).

La modification la plus évidente engendrée par le pic de LH intervient au niveau de la matrice extracellulaire (MEC) du cumulus, et est appelée *mucification* ou *expansion du cumulus*. Plus précisément, l'expansion du cumulus débute avec l'augmentation drastique et rapide de la synthèse d'acide hyaluronique (AH) par la HAS2 (hyaluronic acid synthase 2) en réponse au pic de LH *in vivo* ([\(Salustri et al. 1992\)](#), ou en réponse à la FSH *in vitro* ([\(Salustri et al. 1989\)](#)). Ce glycosaminoglycane composé d'acide glucuronique et de N-acétyl-glucosamine – issue de la voie de synthèse des hexosamines abordée précédemment – constitue l'ossature de la MEC. L'analyse ultra-structurale de la matrice du COC évoque un filet dont les propriétés visco-élastiques permettent au COC de se déformer et ainsi passer sans rompre dans la brèche du follicule ovulatoire lors de sa rupture ([\(Yudin et al. 1988\)](#)).

L'étude fonctionnelle des propriétés de l'acide hyaluronique dans d'autres tissus a montré son implication dans des processus tels que la morphogenèse, le remodelage tissulaire ou encore l'inflammation ([\(Toole 2004\)](#). De surcroît, dans les cumuli porcins, humains et bovins ([\(Ohta et al. 1999; Yokoo et al. 2002; Schoenfelder and Einspanier 2003\)](#)), le récepteur CD44 est synthétisé en parallèle de l'AH et leur interaction améliore la survie des CC en abaissant leur taux d'apoptose ([\(Kaneko et al. 2000; Saito et al. 2000\)](#)). RHAMM (receptor for HA-mediated motility) est un autre récepteur de l'AH capable de promouvoir la prolifération et la mobilité des cellules ([\(Turley et al. 2002\)](#)), et son expression est retrouvée dans les CC bovins ([\(Schoenfelder and Einspanier 2003\)](#)). En plus de la fabrication de la matrice extracellulaire, l'expansion du cumulus implique une mobilité des CC qui passerait en partie par l'activation de la calpaine suite à la stimulation du EGFR ([\(Kawashima et al. 2012\)](#)). En période

préovulatoire, suite à l'augmentation de GnRH, les concentrations d'I $\alpha$ I (inter- $\alpha$  trypsin inhibitor) augmentent dans le fluide folliculaire et leurs chaînes lourdes se lient avec l'AH assurant la stabilité de la MEC du cumulus. Cette liaison est catalysée par Tnfaip-6 (tumor necrosis factor alpha-induced protein-6) qui est aussi capable de se lier à l'AH et est synthétisé par les CC et les CG au cours de l'expansion. Parallèlement à la synthèse de Tnfaip-6, la MEC s'équipe de complexes de PTX3 (pentraxine 3) capables de se lier à Tnfaip-6 et qui assurent la rétention de l'AH dans la matrice participant alors à sa stabilisation (revue par [\(Russell and Salustri 2006\)](#)). Versican, un protéoglycane agrégatif produit initialement par les CG, se lie aussi à l'AH et à CD44, s'incorporant ainsi à la matrice du cumulus suite au pic de LH ([\(Russell et al. 2003\)](#)). Dans les follicules ovulatoires, la transcription de la protéase ADAMTS1 (*a disintegrin and metalloproteinase with thrombospondin-like motifs type 1*) est induite dans les CGM et détient un rôle dans la formation de la MEC du COC *via* le clivage sélectif de versican requis pour l'ovulation (revue par [\(Russell and Salustri 2006\)](#)). Enfin, la MEC du cumulus contient également d'autres protéines de structure telles que la *cartilage link protein* (Crtl1) ([\(Sun et al. 2003\)](#)), la fibronectine, la tenacine-C ou encore la laminine (revue par [\(Russell and Salustri 2006\)](#)).

*In vivo*, l'expansion du cumulus est induite par le pic de LH, toutefois, comme indiqué précédemment, les CC expriment faiblement le R-LH. Par conséquent, l'action de la LH passe initialement par les CGM où elle déclenche une cascade de signalisation entraînant une augmentation des concentrations d'AMPc qui pourrait être transmise au cumulus ([\(Russell and Salustri 2006\)](#)). Simultanément, la LH induit l'expression de facteurs appartenant à la famille de l'EGF (epidermal growth factor) par la granulosa ([\(Park et al. 2004\)](#)). Ces facteurs que sont l'amphiréguline (AREG), la bétacelluline (BTC) et l'épiréguline (EREG) sont appelés des EGF-*like* et se lient au récepteur de l'EGF (EGFR) au niveau du cumulus ([\(Su et al. 2010\)](#)). Chacun de ces facteurs est capable de provoquer la synthèse de PTGS2, HAS2 et Tnfaip-6 (revue par [\(Russell and Salustri 2006\)](#)). La PTGS2 permet la production de prostaglandines, PGE2 particulièrement, importantes dans l'expansion du cumulus et également dans l'ovulation ([\(Davis et al. 1999\)](#)).

De plus, *in vitro*, la production d'AH, ainsi que l'expansion du cumulus sont inductibles *via* l'ajout d'EGF et/ou de FSH (revue par [\(Russell and Salustri 2006\)](#)). Plus récemment, l'ajout de versican recombinante dans le milieu de maturation de COC murins a démontré une activité EGF-*like* partielle de versican. En effet, l'ajout de cette protéine accroît l'expansion du cumulus de manière dose-dépendante et stimule également l'expression de gènes de la MEC, avec toutefois quelques différences comparativement à EGF ([\(Dunning et al. 2015\)](#)).

Le passage du signal LH depuis les CG jusqu'au COC n'est pas encore tout à fait clairement défini, conséquemment, la contribution des vésicules extracellulaires (VE) contenues dans le liquide folliculaire a été explorée ([Hung et al. 2015](#)). Ces VE sont effectivement capables de transporter différentes protéines ainsi que des ARN tels que les miARN et peuvent être incorporées par les CC. De ce fait, l'ajout de VE en maturation *in vitro* permet l'expansion du cumulus et modifie l'expression de gènes reliés, *in vivo*, à cette expansion ([Hung et al. 2015](#)). De plus, EGFR a été détecté dans les VE de cellules cancéreuses ([Sanderson et al. 2008](#)).

Finalement, l'expansion du cumulus est dépendante de l'ovocyte, en effet celui-ci sécrète des facteurs régulant cette transformation ([Buccione et al. 1990](#)) ou CEEF (cumulus-expansion enabling factors) ([Prochazka et al. 1998](#)). Par exemple, une coopération entre GDF9 et BMP15, sécrétés par l'ovocyte et activant les facteurs de transcriptions SMAD ([Kaivo-Oja et al. 2005](#)), est nécessaire à l'expansion correcte du cumulus ([Sugiura et al. 2010](#)).

### 1.2.5. Cumulus post-ovulatoire

Suite à l'ovulation, les CG restent dans le follicule ovulé et lutéinisent afin de former, avec la thèque, le corps jaune. Les CC quant à elles restent attachées à l'ovocyte et le suivent dans l'oviducte à la rencontre éventuelle de spermatozoïdes.

La matrice extracellulaire du cumulus occupe un rôle essentiel au processus ovulatoire, toutefois, son rôle ne s'arrête pas là. D'autres étapes subséquentes de la reproduction dépendent de la formation et de l'expansion en bonne et due forme de la matrice du COC (revue par ([Russell and Salustri 2006](#))).

Ainsi la matrice du cumulus faciliterait la capture du COC par l'*infundibulum* ([Mahi-Brown and Yanagimachi 1983](#)) et son transport dans l'oviducte ([Lam et al. 2000](#)). Elle protégerait également l'ovocyte du stress oxydatif par le biais de l'acide hyaluronique et de versican ([Wu et al. 2005](#)).

Par ailleurs, le cumulus aurait des propriétés chimio-attractives sur les spermatozoïdes – *via* la sécrétion de P4 notamment ([Guidobaldi et al. 2008](#)) –, un rôle dans la capacitation de ceux-ci, ou encore pourrait agir tel un filtre afin de ne laisser passer que les spermatozoïdes ayant un haut potentiel fécondant. Chez l'humain par exemple, le COC contient un protéoglycan suspecté d'être versican qui accélère la réaction acrosomique ([Drahorad et al. 1991](#)). D'autre part, la protéine PTX3, présente dans la MEC du cumulus, est capable de fixer les spermatozoïdes ([Salustri et al. 2004](#)).

## 1.3. La compétence ovocytaire au développement

### 1.3.1. Définition

La compétence ovocytaire au développement a été établie selon cinq critères représentant les étapes charnières impliquant la cellule germinale femelle ([Sirard et al. 2006](#)) :

- Reprise de la méiose ;
- Clivage post-fécondation ;
- Développement jusqu'au stade de blastocyste ;
- Induction d'une gestation allant jusqu'à son terme ;
- Développement à terme d'une descendance en bonne santé.

Toutefois, certaines des étapes présentées dans cette définition ne dépendent pas uniquement de l'ovocyte. Par exemple, une fois le génome embryonnaire activé, le développement subséquent de l'embryon dépend certes des réserves et de l'ADN d'origine maternelle, mais aussi de l'ADN d'origine paternelle. En effet, la capacité de l'embryon à poursuivre son développement complet jusqu'au terme de la gestation repose aussi sur la réorganisation appropriée des marques épigéniques, notamment pour les gènes soumis à l'empreinte parentale ([Ogawa et al. 2006](#)). Également, l'étape d'implantation de l'embryon nécessaire à la poursuite du développement dépend à la fois de la fonctionnalité du corps jaune et de celle de l'utérus...

La définition de *compétence ovocytaire au développement* pourrait alors être limitée à la possibilité d'obtenir un embryon génomiquement autonome à partir d'un ovocyte, indépendamment du spermatozoïde fécondant.

Par ailleurs, l'ovocyte n'est pas seul dans l'acquisition de sa compétence. En effet, chaque compartiment du follicule joue un rôle précis dans la réussite des étapes préparatoires de l'ovocyte (revue par [\(Sirard et al. 2006\)](#). Ainsi, avant même l'ovulation, le follicule va collaborer à :

- ✓ Reprendre la méiose et atteindre la métaphase II ;
- ✓ Réguler les fonctions du cumulus quant à l'expansion, l'ovulation et l'attraction des spermatozoïdes ;
- ✓ Constituer des réserves d'énergie, de métabolites, de transcrits...

Une fois ces étapes réussies, l'ovocyte pourra alors :

- ✓ Assurer la fécondation et fusionner avec le noyau mâle ;
- ✓ Réaliser les premiers clivages ;
- ✓ Permettre l'activation du génome embryonnaire.

### **1.3.2. Évaluation de la compétence ovocytaire**

L'évaluation de la compétence ovocytaire correspond globalement à la réussite des étapes évoquées dans la section précédente. Ces critères sont le plus souvent évalués pour une population d'ovocytes et ainsi exprimés sous forme de taux de réussite :

- Reprise et complétion de la méiose : taux d'ovocytes au stade de GVBD, de métaphase I, expulsion du 1<sup>er</sup> globule polaire et finalement de métaphase II ;
- Clivage post-fécondation : taux de formation de zygotes, de formation de morulas ;
- Développement au stade de blastocyste : taux de blastocyste et/ou de blastocystes éclos ;
- Taux de gestation ;
- Taux de naissance.

En outre, le temps passé pour effectuer chaque étape est un autre des critères d'évaluation de la compétence ovocytaire. Ainsi, la vitesse de réalisation du premier clivage est corrélée avec le taux de blastocystes ([Fenwick et al. 2002](#)) ou encore avec les taux d'implantation et de gestation ([Lundin et al. 2001](#)).

Dans le cadre de la recherche en biologie de la reproduction, surtout chez l'animal, l'évaluation de la compétence ovocytaire est souvent limitée au taux de formation de blastocystes. En effet, pour des raisons techniques et économiques, il n'est pas toujours possible de pousser le développement des embryons au-delà de ce stade.

Que ce soit chez l'animal ou chez l'humain, l'évaluation de la compétence ovocytaire vise notamment à : une meilleure compréhension du fonctionnement folliculaire ; l'amélioration des conditions de stimulation ovarienne ; l'optimisation des conditions de maturation *in vitro*.

### **1.3.3. Mesure de la compétence ovocytaire**

#### ***1.3.3.1. Mise en contexte***

Parce que chez l'humain, les taux de naissances vivantes restent relativement bas et que le recours à la procréation médicalement assistée (PMA) n'est pas sans « effets secondaires », l'amélioration des conditions de stimulation ovarienne et de production *in vitro*, ainsi que la capacité à prédire le destin d'un ovocyte (et également d'un embryon) de manière non invasive sont nécessaires et requièrent une meilleure compréhension des mécanismes cellulaires et moléculaires menant à un ovocyte compétent et de bonne qualité.

En effet, les traitements hormonaux de stimulation ovarienne peuvent avoir pour conséquence une hyperstimulation (OHSS, *ovarian hyperstimulation syndrome*) potentiellement fatale pour les cas dits critiques ; les cas dits modérés et sévères concerneraient 2 à 3 % des patientes et les cas dits légers représenteraient 20 à 30 % des cycles de fécondation *in vitro* (pour revue ([Nelson 2017](#)). Une autre conséquence problématique de la PMA est l'occurrence de grossesses multiples dont les risques concernent la santé tant des mères que des enfants, mais également des troubles psychologiques et financiers pour le couple ([Little 2010](#)). Cependant, grâce à l'accroissement des connaissances en biologie reproductive, les méthodes de stimulation ovarienne ainsi que les conditions *in vitro* pour les gamètes et les embryons ont été grandement améliorées même si des progrès sont encore à faire. Ainsi au Canada, le taux de grossesses multiples suite à une procédure de PMA est passé de 29.4 % en 2005 à 10.1 % en 2015 ([Canadian ART Register, CARTR](#)), et a légèrement diminué aussi en France en passant de 14.7 % en 2012 à 12.3 % en 2015 ([Agence de la Biomédecine, Rapport d'activité d'Assistance Médicale à la Procréation 2015](#)).

En ce qui concerne la maturation *in vitro* (MIV) chez l'humain, cette méthode n'est que très peu utilisée, essentiellement en raison d'une maîtrise encore insuffisante de la technique. Par exemple, en 2015 en France, cette méthode a été utilisée pour 76 tentatives de PMA et 10 enfants en sont nés soit 13 % de réussite, alors que 61 341 cycles de prélèvement d'ovocytes ont été réalisés au total avec un taux d'accouchement entre 14 et 22 % selon les méthodes employées (hors MIV), tous âges confondus ([Agence de la Biomédecine, Rapport d'activité d'Assistance Médicale à la Procréation 2015](#)).

Par ailleurs, des risques à plus ou moins long terme pour la santé de la progéniture née de PMA sont crants. En effet, d'une part, la plupart des étapes ne sont pas physiologiques et d'autre part, la manipulation des gamètes et de l'embryon à une période critique quant à la reprogrammation épigénétique, notamment avec la mise en place des empreintes parentales, est particulièrement sensible (revue par ([Jiang et al. 2017](#)). Les mêmes questions se posent évidemment chez l'animal ([Urrego et al. 2014](#)) avec l'exemple du syndrome du gros veau (*large offspring syndrome*, ([Young et al. 1998](#)). Bien que ce syndrome ait presque disparu avec l'optimisation des milieux de culture ([Galli and Lazzari 2008](#)), il démontre bien l'importance de l'environnement dans lequel sont produits les embryons ([McEvoy et al. 2000](#)). Par ailleurs, l'évaluation de paramètres cardiovasculaires et métaboliques d'enfants et de jeunes adultes issus de procédures de PMA a montré que par exemple, comparativement à une progéniture conçue naturellement, leur pression sanguine était plus élevée ([Guo et al. 2017](#)).

Alors que chez l'humain, la PMA est utilisée pour circonvenir à différentes sources de trouble de la fertilité (mâle ou femelle, âge...), ou encore pour de la préservation de fertilité (cas de traitements en chimiothérapie par exemple), chez l'animal, l'utilisation de procédures de reproduction assistée (ART, *assisted reproductive techniques*) est essentiellement à visée économique et plus rarement pour la préservation d'espèces menacées ou en captivité ([Jewgenow et al. 2017](#)).

Chez la vache, la recherche fondamentale sur le fonctionnement folliculaire, l'acquisition de la compétence ovocytaire au développement et la qualité embryonnaire, a un rôle important à jouer afin de mieux comprendre et aider à trouver une solution au déclin de fertilité constaté depuis cinq décennies dans les troupeaux, laitiers notamment.

En effet, parallèlement à la sélection génétique pour l'amélioration de la production laitière, une baisse de fertilité (baisse du taux de conception et augmentation de la mortalité embryonnaire précoce) est survenue et représente une importante perte économique pour les élevages (revu par ([Perkel et al. 2015](#)). Cette corrélation négative entre production laitière et reproduction est attribuée aux perturbations métaboliques (déclin des taux de glucose, insuline, IGF-I et élévation des acides gras non-estérifiés et des corps cétoniques) induites par une balance énergétique négative en début de lactation (période où la production de lait est maximale) ([Lonergan et al. 2016](#)). D'ordinaire, les taux de fécondation pour les troupeaux de bovins laitiers sont d'environ 80 %, tandis que les taux de vêlages sont de 55-60 % pour les génisses et seulement de 35-40 % pour les vaches en lactation ([Lonergan et al. 2016](#)). Comme le suggèrent les auteurs de cette étude, la baisse de fertilité observée ne paraît donc pas être liée à la fécondation mais serait plutôt associée aux importantes pertes embryonnaires survenant dans les 2 semaines suivant cette fécondation, mettant alors en cause la qualité des ovocytes qui ne se seraient pas développés sous les meilleures auspices (revu par ([Lonergan et al. 2016](#)). Les auteurs suggèrent également une implication de la fonction utérine qui pourrait, elle aussi, être affectée par les perturbation métaboliques de début de lactation.

En outre, une fertilité réduite a également été observée en lien avec la saison de reproduction (printemps/été vs. automne/hiver), les principales raisons invoquées étant : un débalancement du métabolisme énergétique accompagné d'une augmentation du stress oxydatif et/ou un débalancement de l'axe hypothalamo-hypophysaire-gonadique dus à la baisse d'appétit des animaux en période chaude ; mais aussi une influence de la photopériode sur le follicule par le biais de la mélatonine (revu par ([De Rensis et al. 2017](#)).

Chez les bovins, les ART sont très utilisées et sont vues comme un moyen d'optimiser le rendement des élevages par la génétique ([Baruselli et al. 2011](#)). L'insémination artificielle diffuse la génétique mâle, la stimulation ovarienne associée au transfert embryonnaire permet de diffuser également la génétique femelle ([Pontes et al. 2010](#)). La production des embryons pour le transfert, peut se faire *in vivo* mais aussi *in vitro*. L'utilisation des techniques de production d'embryons *in vitro*, en plus de participer à la diffusion de la génétique femelle, offre notamment la possibilité de réduire les temps de générations. Ceci pourrait d'ailleurs aider à optimiser la sélection génétique sur les critères de fertilité en plus des critères de production et de morphologie habituels ([Berry et al. 2016](#)).

#### **1.3.3.2. Méthodes de prédiction de la compétence ovocytaire**

En matière de prédiction du destin d'un ovocyte, de nombreuses méthodes ont vu le jour, toutefois aucune n'est fiable à 100 % et la littérature suggère plutôt une association de différents critères afin d'obtenir des prédictions plus justes. Par ailleurs, certaines méthodes permettent plutôt d'exclure les moins bons candidats que de sélectionner les meilleurs, ce qui peut compléter le processus de tri. Parmi ces critères, les plus usités, certainement de par leur accessibilité, sont morphologiques. Toutefois, la part de subjectivité et d'expérience de l'évaluateur est souvent remise en cause. La recherche de méthodes non-invasives pour l'obtention de critères objectifs tels que l'expression de gènes, la présence de protéines ou encore le bilan métabolique des milieux de cultures, ne cesse donc de s'intensifier. L'utilisation des « *omics* » (transcriptomique, protéomique, métabolomique) sur l'ovocyte lui-même n'est possible que dans le cadre de la recherche car ces méthodes sont généralement destructives, et donc très restreintes chez l'humain par exemple. La situation est similaire concernant le globule polaire dont la récupération est invasive ([Campbell et al. 2013](#)). Au contraire, les « *omics* » utilisées sur les cellules somatiques ou le liquide folliculaire, s'avèrent être des techniques puissantes en recherche pour la compréhension des mécanismes et l'amélioration des conditions de production *in vitro* d'une part, et prometteuses pour l'établissement de critères de discernement des ovocytes (et embryons) de potentiel développemental différents d'autre part.

La présente thèse se concentrant sur les cellules du cumulus, l'emphase sera mise sur les critères reliés à ce type cellulaire.

### **1.3.3.2.1. Morphologie**

- *Taille du follicule*

Un lien existe entre la taille du follicule de provenance de l'ovocyte et sa capacité à reprendre la méiose, et donc sa compétence ([Lonergan et al. 1994](#); [Blondin and Sirard 1995](#)). En effet, chez le bovin, si les follicules d'origine ont un diamètre inférieur à 2 mm, leurs ovocytes auront une capacité de reprise de la méiose plus faible que ceux provenant de follicules de taille supérieure ([Pavlok et al. 1992](#); [Bergh et al. 1998](#)). Nonobstant, certaines études montrent que la corrélation entre compétence ovocytaire et dimensions folliculaires ne serait pas linéaire que ce soit avec ([Salha et al. 1998](#); [Nivet et al. 2012](#)) ou sans stimulation ovarienne ([Trounson et al. 2001](#)). Par ailleurs, certains follicules de grande taille échouent à former un embryon tandis que d'autres de taille moyenne y parviennent ([Lee et al. 2010](#)).

- *Degré d'atrésie folliculaire et de compaction du cumulus*

Chez la vache, les follicules légèrement atrétiques contenant un ovocyte entouré d'un cumulus non totalement compact sont les plus compétents ([Blondin et al. 1996b](#)). Ces COC présentent effectivement des taux de formation d'embryon  $\geq 16$  cellules de 43 %, tandis que les COC au cumulus très compact n'atteignent que 31%, finalement, les COC au cumulus complètement expansé ainsi que les COC n'ayant que la *corona radiata* et les ovocytes sans cumulus ne dépassent pas 11 % d'embryons ([Blondin and Sirard 1995](#)).

- *Observation de l'ovocyte*

En prêtant attention à la granulosité de l'ooplasmme et à l'épaisseur de la zone pellucide ([Gerris 2005](#)), ou encore au diamètre de l'ovocyte ([Anguita et al. 2007](#)), on peut avoir une indication sur sa compétence. La configuration de la chromatine ovocytaire peut également s'ajouter aux autres critères car le potentiel développemental lui est corrélé ([Luciano et al. 2012](#)). Pour une revue plus complète des critères de morphologie ovocytaire voir ([Rienzi et al. 2011](#)).

- *Cinétique développementale*

La vitesse d'expulsion du premier globule polaire par l'ovocyte ([van der Westerlaken et al. 1994](#)), la rapidité avec laquelle survient la première division embryonnaire ([Van Soom et al. 1992](#); [Lonergan et al. 1999](#)), ainsi que les divisions subséquentes ([Grisart et al. 1994](#); [Holm et al. 1998](#)) ont été associées à un plus grand potentiel de développement ([Hlinka et al. 2012](#); [Sugimura et al. 2012](#)).

### **1.3.3.2.2. Transcriptomique**

La transcriptomique correspond à l'étude de l'ensemble des transcrits (ARN) d'une ou plusieurs cellules. Ainsi lorsqu'on parle de transcriptome, on se réfère à l'expression génique d'une cellule ou d'un ensemble de cellules. Les transcrits de type ARN messager (ARNm) dont le destin, pour la plupart d'entre eux, est d'être traduits en protéines, sont nécessaires au fonctionnement cellulaire. L'étude de l'abondance des transcrits ainsi que leur variation peut donc donner des informations intéressantes quant à l'activité en cours et à venir d'une cellule.

Toutefois, lorsqu'on entreprend d'étudier le transcriptome du cumulus, il faut garder à l'esprit la possibilité qu'une partie de la production d'ARN puisse être vouée à être transférée à l'ovocyte pour y être stockée ([Macaulay et al. 2016](#)). Par ailleurs, les miRNA ([Moussaddykine et al. 2012](#); [Maalouf et al. 2016](#)) ou encore les lncRNA ([Caballero et al. 2014](#); [Xu et al. 2015](#)) évoqués précédemment font bien sûr partie du transcriptome du cumulus mais leur usage dans la prédiction de la compétence ovocytaire ne sera pas abordé ici.

Parmi les différentes techniques utilisées pour l'étude des ARNs, la qRT-PCR (quantitative Reverse Transcription - Polymerase Chain Reaction) permet de mesurer précisément l'abondance d'un ou plusieurs transcrits. Cette technique est utilisée dans la comparaison de l'abondance de transcrits de gènes dits candidats : c'est-à-dire que leur implication dans un processus est déjà suspectée et, en utilisant la qRT-PCR, on cherche à confirmer ou infirmer cette implication. La puissance d'exploration de cette technique est toutefois limitée. Cependant, la qRT-PCR permet de valider les résultats obtenus avec une autre méthode : l'hybridation sur puces à ADN ou *microarray*. Avec cette technique, l'exploration du transcriptome dans sa quasi-totalité est rendue possible sans être contraint d'identifier préalablement, et un par un, les gènes d'intérêt.

Ainsi, suite à l'annotation complète du génome bovin, notre laboratoire a développé une puce à ADN spécialement éditée pour l'étude du transcriptome de l'ovocyte et de l'embryon ([Robert et al. 2011](#)). En outre, cette puce, comportant des sondes pour tous les gènes de référence, ainsi que des sondes pour des variants d'épissage, pour des régions 3'UTR alternatives, ou encore pour des régions non traduites, permet aussi l'étude du transcriptome des cellules folliculaires.

Récemment, des cumuli censés être associés à des ovocytes hautement compétents ont été comparés à des cumuli associés à des ovocytes à faible compétence en utilisant, respectivement, des cumuli issus de vaches prétraitées avec de la FSH et des cumuli issus de vaches non traitées. L'acquisition de la compétence ovocytaire au développement serait ainsi associée à l'intégrité des communications intracellulaires du cumulus et à la survie de celui ([Sugimura et al. 2017](#)).

#### **1.3.4. Optimisation de la stimulation ovarienne**

Lors d'un cycle naturel chez la vache, les follicules en phase de croissance montrent un meilleur potentiel au développement que les follicules récupérés en phase de dominance ([Hagemann 1999](#)). En outre, si les follicules sont obtenus en phase de sélection, donc entre les deux phases évoquées juste avant, les follicules ont un potentiel encore plus grand ([Vassena et al. 2003](#)).

Dans le cas d'un cycle stimulé chez la vache, l'emploi de multiples injections de FSH afin de récupérer un grand nombre de follicules en croissance ([Blondin et al. 1996a](#)) suivies de l'application d'une période de sevrage de 48 h en FSH – ou *coasting*, afin de mimer la phase de dominance permettant ainsi la différenciation finale des follicules – a donné lieu à des taux de blastocystes de 80 % suite à la maturation et la fécondation *in vitro* des ovocytes ponctionnés ([Blondin et al. 2002](#)). Ce protocole a encore été optimisé, permettant de maximiser les taux de blastocystes et de déterminer qu'une période de sevrage en FSH de 54 h serait idéale quant à la différenciation folliculaire ([Nivet et al. 2012](#)). Une coordination chrono-physiologique serait donc une bonne voie d'optimisation des protocoles de stimulation ovarienne.

## 1.4. Hypothèse et objectifs

L'ovocyte est la cellule germinale femelle qui permettra, en association avec un spermatozoïde, de générer une progéniture et ainsi perpétuer une espèce. Toutefois, la réussite de cette mission n'est pas une tâche aisée. En effet, il peut arriver que la mission échoue et que ce soit, parfois, la faute de l'ovocyte. Mais pourquoi? L'ovocyte, niché au creux de son follicule, doit acquérir, tout particulièrement avec l'aide des cellules folliculaires, différentes capacités qui le rendront compétent pour cette mission mais quelque fois... cela ne se passe pas selon les plans prévus. Et quels sont ces plans? Quoique déjà bien ébauchés et étayés par les plus de 90 années de recherche dans le domaine, les plans des mécanismes moléculaires et cellulaires menant à la construction de la compétence ovocytaire ne sont pas encore assez précis pour être fidèlement reproduits, dans le cas de la production d'embryon *in vitro* chez le bovin par exemple, ou même parfaitement réparés, comme dans les cas d'infertilité humaine. Enfin, l'ovocyte doit acquérir sa compétence graduellement et avec l'aide du follicule, tout particulièrement avec celle des cellules du cumulus qui le suivront jusqu'à la rencontre avec les spermatozoïdes. L'hypothèse de travail adoptée ici est donc que la population d'ARN messagers du cumulus reflète la compétence de l'ovocyte.

L'objectif principal de cette thèse a été d'identifier les variations de l'expression génique globale – ou transcriptome – de cellules du cumulus associées à des ovocytes de compétence au développement différente.

Dans un premier temps, l'utilisation d'un protocole de stimulation ovarienne basé sur la différenciation des follicules en croissance terminale ([Nivet et al. 2012](#)) a permis l'obtention de trois stades distincts de compétence ovocytaire : compétence en cours d'acquisition ; compétence acquise ; et compétence en déclin. Le transcriptome des cumuli associés aux différents ovocytes de ce protocole a été alors été examiné *via* l'utilisation de puces à ADN.

Par la suite, de manière à étudier l'influence des concentrations basales de LH précédant l'ovulation lors de cycles naturels, un antagoniste de GnRH a été intégré au protocole ci-dessus afin de faire cesser la sécrétion de LH pendant la différenciation folliculaire. Le transcriptome des cellules du cumulus issues de ce protocole a été comparé au transcriptome des cumuli associés à une compétence ovocytaire acquise lors du protocole présenté au paragraphe précédent.

Puisque l'étude ci-dessus a montré une influence de la LH sur le transcriptome des cellules du cumulus, l'expression du récepteur à la LH a été examinée dans des cumuli bovins en cours de maturation *in vitro*.

Finalement, dans le but d'obtenir une image transcriptomique plus contrastée, une collaboration avec le centre de recherche sur les gamètes de l'université d'Anvers (ou *Antwerp*) en Belgique, a permis de réaliser des biopsies de cumuli bovins dont les COC correspondants ont pu être suivis individuellement lors de la culture *in vitro*. Les cumuli ont alors été associés *a posteriori* soit : à un ovocyte qui aura été capable de former un blastocyste, *i.e.* un ovocyte compétent ; ou bien à un ovocyte dont le développement embryonnaire se sera arrêté aux préminces de l'activation du génome embryonnaire, à 4-8 cellules, *i.e.* un ovocyte incompétent.

## **2. CUMULUS CELL GENE EXPRESSION ASSOCIATED WITH PRE-OVULATORY ACQUISITION OF DEVELOPMENTAL COMPETENCE IN BOVINE OOCYTES**

Audrey Bunel<sup>1</sup>, Anne-Laure Nivet<sup>1</sup>, Patrick Blondin<sup>2</sup>, Christian Vigneault<sup>2</sup>, François J. Richard<sup>1</sup> and Marc-André Sirard<sup>1</sup>

<sup>1</sup> Centre de recherche en biologie de la reproduction, Faculté des sciences de l'agriculture et de l'alimentation, Département des sciences animales, Université Laval, Québec, Canada.

<sup>2</sup> L'Alliance Boviteq, Saint-Hyacinthe, Québec, Canada.

Cet article a été publié dans la revue « Reproduction, Fertility and Development » sous la référence suivante :

**Bunel A., Nivet A. L., Blondin P., Vigneault C., Richard F. J., Sirard M. A. (2013)** Cumulus cell gene expression associated with pre-ovulatory acquisition of developmental competence in bovine oocytes. *Reproduction, Fertility and Development* **26**, 855-865.

## 2.1. Résumé

Les derniers jours avant l'ovulation affectent grandement la fonctionnalité du follicule ainsi que la qualité de l'ovocyte. Dans cette étude, les changements transcriptomiques des cellules du cumulus (CC) pendant la période d'acquisition de la compétence au développement de l'ovocyte ont été examinés. Des injections de FSH deux fois par jour pendant 3 jours, suivies d'un sevrage de FSH ( $-FSH$ ) de 20, 44, 68 et 92 heures, ont été réalisées sur 6 vaches laitières en 4 cycles œstraux différents, menant à 24 ponctions ovocytaires (PO). Afin d'évaluer les taux de blastocystes, la moitié des complexes ovocyte-cumulus ont subi la maturation, fécondation et culture *in vitro*. Les CC de l'autre moitié des COC ont été analysées par puces à ADN ( $n = 3$  vaches, 12 PO) et par qRT-PCR ( $n = 3$  autres vaches, 12 PO). D'après les taux de blastocystes,  $-FSH$  20h amènerait à des follicules sous-différenciés (49 %),  $-FSH$  44 et 68h donneraient les follicules les plus compétents (71 % et 61 %) et  $-FSH$  92h formerait des follicules sur-différenciés (51 %). Les expériences de qRT-PCR ont été réalisées sur dix gènes issus des listes associées aux trois statuts folliculaires. Les profils d'expression de *CYP11A1* et *NSDHL* reflètent celui des taux de blastocystes. Toutefois, la majorité des gènes tels que *GATM*, *MAN1A1*, *VNN1* et *NRP1*, sont associés au statut de sur-différenciation. La plus courte période de sevrage de FSH n'a eu que peu d'effet sur l'expression génique du cumulus, tandis que la période la plus longue en a un beaucoup plus significatif et indique un début d'atrésie.

## 2.2. Abstract

The final days before ovulation impact significantly on follicular function and oocyte quality. This study investigated the cumulus cell (CC) transcriptomic changes during the oocyte developmental competence acquisition period. Six dairy cows were used for 24 oocyte collections and received FSH twice daily over 3 days, followed by FSH withdrawal for 20, 44, 68 and 92 h in four different estrous cycles for each of the six cows. Half of the cumulus–oocyte complexes were subjected to *in vitro* maturation, fertilization and culture to assess blastocyst rate. The other half of the CC underwent microarray analysis ( $n=3$  cows, 12 oocyte collections) and qRT-PCR ( $n=3$  other cows, 12 oocyte collections). According to blastocyst rates, 20 h of FSH withdrawal led to under-differentiated follicles (49%), 44 and 68 h to the most competent follicles (71% and 61%) and 92 h to over-differentiated ones (51%). Ten genes, from the gene lists corresponding to the three different follicular states, were subjected to qRT-PCR. Interestingly, *CYP11A1* and *NSDHL* gene expression profiles reflected the blastocyst rate. However most genes were associated with the over-differentiated status: *GATM*, *MAN1A1*, *VNN1* and *NRP1*. The early period of FSH withdrawal has a minimal effect on cumulus gene expression, whereas the longest period has a very significant one and indicates the beginning of the atresia process.

### **2.3. Introduction**

Fertility has significantly decreased over the last twenty years in dairy cows. This phenomenon is most likely related to the increase in individual milk production; however, the relationship between these two parameters remains unclear (reviewed in Walsh *et al.* 2011). This situation, combined with the desire to disseminate genetics, has driven the dairy-cow industry to further develop assisted reproductive technologies (ART). The process by which ART can improve fertility in cattle implies a better understanding of how oocytes acquire the ability to produce an embryo and, ultimately, offspring. In fact, since its beginning, bovine ART has been confronted with the dilemma of recovering matured oocytes from a cow (as in human IVF) which gives very high development rates, and the poor success of using *in vitro* matured oocytes (Sirard and Coenen 2006). Using slaughterhouse ovaries as a source of immature bovine oocytes, it has been almost impossible to obtain more than 30–35% blastocyst rate with suboptimal pregnancy rates. Using *in vivo*-collected immature oocytes, the success rates are not better unless some hormonal pre-treatment is used (Blondin *et al.* 2002). Recently, our laboratory has developed an ovarian stimulation protocol that better mimics the natural estrous cycle. This protocol uses a follicle-stimulating hormone (FSH) withdrawal period between the last FSH injection and oocyte retrieval, also known as the coasting period. Four withdrawal times (20, 44, 68 or 92h) were tested in lactating dairy cows. The best blastocyst rates were reached at 44 and 68 h with a mean of 71% and 61%, respectively and with some animals reaching 100% for both times (Nivet *et al.* 2012). The 20h condition, with a 49% blastocyst rate, was considered to be too early or to lead to under-differentiated follicles, while the 92h condition, with a 51% blastocyst rate, was considered to be too late or to generate over-differentiated follicles. This time-course assay was designed to distinguish oocyte developmental competence acquisition and loss. During this protocol, companion cumulus cells (CC) were retrieved to perform global transcriptome analysis.

CC are known to be crucial for oocyte development and maturation (Eppig 2001; Gilchrist *et al.* 2004). These cells are differentiated granulosa cells that emerge during formation of the secondary follicle. CC have an intimate connection with the oocyte by having cytoplasmic projections passing through the zona pellucida and forming gap junctions with the ooplasmic membrane. Because of this particular connection between CC and the oocyte, CC play an important role in supporting oocyte growth and maturation. Indeed, *in vitro*, denuded oocytes can mature and be fertilized but do not continue their development past the 8-cell stage (First *et al.* 1988). It is therefore believed that the cumulus can influence oocyte competence until ovulation and consequently that the signaling process involved might be revealed by a transcriptome analysis performed at the right time. In order to improve ART outcomes, a method to anticipate oocyte fate would be useful. The particular relationship that

exists between the oocyte and its CC makes the analysis of CC transcriptomic profiles a quick, non-invasive method of assessing oocyte quality. This hypothesis has been explored mainly by the quest for biomarkers of oocyte quality in humans using, for example, cumulus cells recovered post-LH (Assou *et al.* 2008; Assidi *et al.* 2010). In human CC, expression of many genes, such as PTGS2 (McKenzie *et al.* 2004), HAS2, GREM1 (McKenzie *et al.* 2004; Cillo *et al.* 2007), PTX3 (Zhang *et al.* 2005), CKB, PRDX2 (Lee *et al.* 2010) or RGS2 (Feuerstein *et al.* 2012) has previously been positively associated with embryo quality and blastocyst stage achieved. RGS2 has also been associated with follicles containing oocytes leading to pregnancy in humans (Hamel *et al.* 2010). CC gene expression has also been directly associated with pregnancy, with genes like BCL2 L11, PCK1 (Assou *et al.* 2008) or VCAN, PTGS2, GREM1 and PFKP (Gebhardt *et al.* 2011). The cumulus can also indicate a negative embryo formation outcome from the expression of genes like PR (Hasegawa *et al.* 2005), GJA1 (Feuerstein *et al.* 2007; Hasegawa *et al.* 2007), STAR, AREG, COX2, SCD1, SCD5 (Feuerstein *et al.* 2007) and CCND2, CTNND1, CXCR4, DHCR7, DVL3, GPX3, HSPB1, TRIM28 (van Montfoort *et al.* 2008) or even a negative pregnancy outcome with genes such as NFIB (Assou *et al.* 2008). However, in cattle, very few large-scale investigations have been performed. The most relevant study is from Bettegowda *et al.* (2008) using juvenile versus adult oocytes as a competence contrast, which has shown that CTSB, CTSS and CTSZ mRNAs are negative markers of blastocyst stage formation in CC.

The aim of this study was to analyze the gene expression profile of CC during the acquisition, the maximal and the ensuing loss of oocyte developmental competence to uncover signaling pathways and genes associated with such different competence states.

## 2.4. Results

### 2.4.1. COC morphology

The first observations made on the cumulus complexes in this study were morphological. COC were observed and categorized and then split into two groups for *in vitro* culture and RNA analysis. Four categories were established depending on the approximate number of cumulus cell layers around the oocyte (4–5, 2–3, 0–1 layers) and whether or not the COC was already expanded ('Exp'; [Figure 2-1](#)). At 20h, significant differences were observed between 4–5 layers and 0–1 layer ( $P<0.05$ ) and between 4–5 layers and 'Exp' ( $P<0.01$ ). At 44h, significant differences were observed between 2–3 layers and 0–1 layer ( $P<0.001$ ) and between 2–3 layers and 'Exp' ( $P<0.001$ ). At 68h, significant differences were observed between 2–3 layers and 0–1 layer ( $P<0.05$ ) and between 2–3 layers and 'Exp' ( $P<0.01$ ). At 92h, significant differences were observed between 2–3 layers and 0–1 layer ( $P<0.001$ ) and between 2–3 layers and 'Exp' ( $P<0.001$ ). Looking at all conditions, significant differences exist between 20h and 44h and between 20h and 92h, at the 2–3 layers level ( $P<0.05$ ). Clearly, cumulus layers are being lost during the FSH withdrawal period.

### 2.4.2. Genes inventory

To better understand what happens during oocyte developmental competence acquisition in CC, we performed a large scale transcriptomic analysis using a new microarray tool made from RNAseq analysis of bovine oocytes and embryos. The microarray slide includes 42 242 probes for 21 139 known reference genes, 7 230 splice and 3'UTR variants in addition to 9 322 novel transcribed regions (Robert *et al.* 2011). The CC samples came from follicles of different differentiation status, *i.e.* under-differentiated (20 h), competent (44–68 h) and over-differentiated (92 h). For hybridization, each FSH withdrawal duration was compared on a dye-swap design with the other three time samples from the same animal to minimize individual variation effects. Out of those 21 139 target genes, 12 048, 10 179, 8 771 and 8 804 genes were considered to be expressed at 20, 44, 68 and 92h of coasting respectively, with signal above a threshold defined as the mean background plus two times the standard deviation ([Figure 2-2](#)). This observation indicates that around 10% of genes are shut down after FSH withdrawal.

### 2.4.3. Hierarchical cluster analysis

A hierarchical cluster analysis (HCA) was performed with Pirouette 4.0 software (Informetrics, Bothell, WA, USA) and showed on the one hand that the data can be divided into two groups, *i.e.* the 20 and 92h conditions together and the 44 and 68h conditions together, and on the other hand that the

data can also be divided into three groups, *i.e.* the 20h condition alone, the 44 and 68h conditions together and the 92h condition alone ([Figure 2-3](#)). This segregation corresponds to the blastocyst rate observations where the 20 and 92h conditions were lowest and the 44 and 68h the highest.

#### 2.4.4. Variation of gene expression across time

Microarray experiments showed that as time passed, variation of gene expression increased ([Figure 2-4](#)). In fact, considering a 2-fold change and a FDR $\leq$ 0.05, 0.48%, 1.94% and 3.18% of the expressed genes varied between 20 and 44h, between 44 and 68h and between 68 and 92h, respectively ([Figure 2-4 a](#)). Considering a 1.5-fold change and a FDR $\leq$ 0.05, 4.96%, 6% and 13.31% of the expressed genes varied between 20 and 44h, between 44 and 68h and between 68 and 92h, respectively ([Figure 2-4 b](#)).

The microarray slide also included probes for 9 322 novel regions, 3 677 alternatively spliced exons and 3 353 3'UTR tiling. Taking into account those probes, with a 2-fold change and a FDR $\leq$ 0.05 cut-off, 96, 183 and 135 probes showed a positive change and 16, 143 and 246 probes showed a negative change between 20 and 44h, between 44 and 68h and between 68 and 92h, respectively.

#### 2.4.5. Ingenuity Pathway Analysis (IPA)

Data were also analyzed with IPA (Ingenuity Systems, [www.ingenuity.com](http://www.ingenuity.com)). Using the 10 selected genes for qRT-PCR validation, IPA allows the building of a network to see if links exist between those genes ([Figure 2-5](#)). Note that on this network, FSH, TGFB1 or INHBA act through their receptors and their own pathways; the direct link is shown to simplify the scheme. The selection of the 10 genes was based on the statistical data from the microarray analysis (fold change, *P* value and false discovery rate). The datasets of positive probes were also used for an IPA analysis to find any functional clue related to the HCA analysis. This analysis did not reveal clear differences between biofunctions significantly represented in the four FSH withdrawal times (see [Figure 2-7](#) available as Supplementary Material to this paper).

#### 2.4.6. qRT-PCR results

Ten genes selected from microarray and functional analysis (*IFI27*, *FADS2*, *CYP11A1*, *NSDHL*, *ANK3*, *RELN*, *NRP1*, *VNN1*, *MAN1A1*, *GATM*) were quantified by qRT-PCR using three independent biological replicates (three other cows than the ones used for microarrays; [Figure 2-6](#)). Results showed six of 10 genes displaying significantly different expression between at least two conditions. Significant differences were found for *CYP11A1* between 20h and 68h, *P*=0.0115; *NSDHL*

between 68h and 92h,  $P=0.0473$ ; *NRP1* between 20h and 92h,  $P=0.0386$ ; *VNN1* between 20–44–68h and 92h,  $P<0.0001$ ; *MAN1A1* between 20–44–68h and 92h,  $P=0.0026$  and *GATM* between 20–44–68h and 92h,  $P<0.0001$ . Among all the candidates, gene expression was significantly correlated with coasting duration in four instances: *NRP1* ( $R^2=0.6104$ ,  $P=0.0076$ ); *VNN1* ( $R^2=0.6903$ ,  $P=0.0015$ ); *MAN1A1* ( $R^2=0.5926$ ,  $P=0.0056$ ) and *GATM* ( $R^2=0.7630$ ,  $P=0.0004$ ). For *IFI27*, this correlation tended to be significant with  $R^2=0.3323$  and  $P=0.0634$ .

## 2.5. Discussion

This is the first study exploring the potential relationship of cumulus cell gene expression with oocyte competence using the same animals over several precisely timed conditions. This design represents 24 different oocyte collections with minimal genomic variations associated with individual animals. Also, in this study, high competence was not compared with zero competence (very immature follicles) but to suboptimal conditions as they may arise in the clinical setting. Nonetheless, microarray experiments have allowed us to distinguish the three major follicular state categories, *i.e.* under-differentiated, competent and over-differentiated.

Indeed, when HCA analysis was performed on the four datasets, three main groups became apparent: on one side 20h and 92h segregated separately, while on the other side, 44h and 68h were grouped together. The comparison of these groups with the corresponding blastocyst rates shows some possible similarities between 20h and 92h and between 44h and 68h. The higher blastocyst rates and the clustering of 44h and 68h together suggest similar beneficial physiological conditions associated with gene expression in both conditions that ensure oocyte developmental competence acquisition from the cumulus cells. When the HCA dendrogram is more closely observed, 20h is separated from 92h in contrast to 44h and 68h, which are fused. The under- and over-differentiated conditions were both different from the competence condition but also different from each other. Indeed, at 20h, a fraction of the follicles were not yet ready to generate a competent oocyte, whilst at 92h some follicles were already too advanced in differentiation and the enclosed oocyte may already have been compromised. The two time points, 20 and 92h, are not more similar but equally different from 44 and 68h. IPA analysis, performed with positive probes data sets of the four conditions, showed few differences between the FSH withdrawal durations. In fact, the first 10 biological functions were almost similar in all conditions (see [Figure 2-7](#)). This indicates that in the cumulus cells, the different competence states of the oocyte are reflected by limited differences in gene expression. In the granulosa cells coming from the same experiments (Nivet *et al.* 2013), the datasets gave indications of a preparation process leading to ovulation for the first half of the coasting period, which resembled indications of a preparation process leading to atresia for the second half of the coasting period; this was not as clear in cumulus cells. It is possible the same phenomenon occurred in cumulus cells but the analysis did not show it.

Because blastocyst rates vary and gene expression was not exactly the same in cumulus cells at the four FSH withdrawal durations, cumulus cells may affect oocyte phenotype. However, cumulus

cells do not have to change as rapidly and as much as the whole follicle, which responds more directly to environmental and systemic changes to synchronize ovulation. Therefore, the fact that just a modest proportion of the genes expressed fluctuates is not that surprising. Cumulus cells could act as some kind of check-point to filter and smoothly mediate influences from the follicle. This buffering role could protect and sustain the oocyte in its acquisition of competence. It is likely that the cumulus cells from stimulated and coaxed follicles are already differentiated enough to support the major roles of cumulus in ovulation such as triggering oocyte maturation and cumulus matrix expansion. The cumulus may begin to react to FSH starvation at the third day of coaxing as the follicles move into early atresia, which may explain the increased gene expression modifications observed between 68 and 92h. It is important to note that these animals are in the luteal phase and cannot ovulate due to progesterone inhibition (Nivet *et al.* 2012). Before associating atresia in the cumulus and atresia in the follicle, it is important to state that *in vivo*, the cumulus is affected later than the mural granulosa by the atresia process. In fact, the cumulus in general shows a slower or inhibited differentiation compared with granulosa, and the oocyte would be the source of this inhibition (Eppig 2001; Gilchrist *et al.* 2008). Therefore, some of the changes associated with the last FSH coaxing period may still be indicative of a differentiation associated with a positive outcome while some others may indicate the beginning of the cumulus demise. It is not known where and how the program deviates from a pre-ovulatory function to an atretic function. Furthermore, the cumulus is bound to self-destruct very soon after ovulation either by self-generated apoptosis or by being targeted by the immune system once in the oviduct. The following examples illustrate the different options the cumulus may have at the end of its journey.

The investigation of COC morphology tends to show a loss of cumulus cell layers as time passes, from 4–5 layers of CC to 2–3 layers from 44h to 92h. This phenomenon could be due to some apoptosis in the first layers of cumulus while FSH is decreasing. However, no significant increase in apoptosis-related genes was highlighted during this study, maybe because the cells undergoing those changes were detached from the COC due to apoptosis.

The first gene tested by quantitative PCR was *IFI27*. Its function has been characterized mainly in cancer cells where it promotes apoptosis *via* mitochondrial perturbations (Cheriyath *et al.* 2011). The role of this gene in cumulus is not known and would require further investigation as we believe that apoptosis could decrease as the oocyte approaches ovulation to ensure cumulus–oocyte survival in the dominant follicle. However, it should then increase at the end of the FSH withdrawal according to the preceding analysis; our results show a constant decrease throughout the coaxing period. The second gene that decreased throughout FSH coaxing in our arrays was *FADS2*, a delta-6 fatty-acid-desaturase

(D6D), which catalyses the first step in the synthesis of highly unsaturated fatty acids (HUFA) such as arachidonic acid. Prostaglandins, which are synthesized from arachidonic acid, are known to be important for cumulus expansion (Nuttinck *et al.* 2008). The decrease of an enzyme, FADS2, that provides substrate for prostaglandin-endoperoxide synthase 2 (PTGS2, also known as COX2) is probably associated with the accumulation of enough fatty acids to meet cumulus–oocyte requirements. Note that PTGS2 has been associated with high developmental potential oocytes in human cumulus cells (Gebhardt *et al.* 2011).

The next gene with an interesting profile is *CYP11A1* (cytochrome P450, family 11, subfamily A, polypeptide 1, also known as P450scc), as it mimicked developmental competence in terms of blastocyst rates. The encoded enzyme is involved in steroidogenesis by converting cholesterol to pregnenolone. In dominant follicles, the expression of *CYP11A1* is upregulated, independent of size (Irving-Rodgers *et al.* 2009). Moreover, in a study comparing the transcriptome of CC and their corresponding oocytes at germinal vesicle stage, *CYP11A1* has been found to be cumulus-cell specific (Regassa *et al.* 2011). In bovine cumulus obtained from the slaughterhouse, *CYP11A1* expression increases with maturation like *PTGS2* and is likely required for progesterone production. The fact that expression of this gene, which is normally upregulated by the LH surge, increased with 44 and 68 h of FSH starvation probably indicates that the cumulus is getting ready to move to progesterone production as reported in cattle (Mingoti *et al.* 2002, Aparicio *et al.* 2011) and pigs (Yamashita *et al.* 2003). The ability of maturing bovine COC to secrete progesterone in culture systems has been reported and like PGE2, progesterone is thought to be required for successful terminal differentiation of the COC (Nuttinck *et al.* 2008). Furthermore, Grupen and Armstrong (2010) have shown that, in porcine IVM, the more CC produce progesterone, the less apoptosis occurs within them. CC also secrete progesterone after ovulation, which is thought to be involved in sperm chemotaxis to the *cumulus oophorus* in both human (Teves *et al.* 2006) and rabbit (Guidobaldi *et al.* 2008).

Nsdhl protein is an enzyme involved in cholesterol synthesis. In steroidogenic cells, Nsdhl is suspected to have a role in regulating cholesterol release or storage and biosynthesis (Ohashi *et al.* 2003). In our data, *NSDHL* gene expression was upregulated at 68h and downregulated at 92h and showed a profile comparable with *CYP11A1*. As those two genes may act synergistically to generate progesterone, it seems plausible that this change is important for oocyte competence either directly or through progesterone signaling.

The next series of genes involve the ones progressively increasing during coasting. Six genes were measured by qPCR and although all six showed the same pattern as the microarrays, only four had a significant linear increase.

*NRP1* encodes neuropilin-1, a member of the NRP family, which are Type I transmembrane receptors. It binds vascular endothelial growth factor (VEGF) family members and semaphorin-3 family members. Through these interactions, neuropilin-1 is implicated in angiogenesis and in neuronal guidance, respectively (Rossignol *et al.* 2003). VEGF, which is known to have a role in ovarian follicles, is able to upregulate *NRP1* expression (Kurschat *et al.* 2006). *NRP1* was found in the cytoplasm of *Xenopus* oocytes, and maternal *NRP1* mRNA has been shown to accumulate in the egg (Good *et al.* 1993; Valdembri *et al.* 2009). In cattle, *NRP1* expression was also found in granulosa and theca cells of follicles of 7mm or larger (Shimizu *et al.* 2006). In the same study, the authors have shown in granulosa cell cultures that *NRP1* mRNA is increased when the estradiol : progesterone (E2 : P4) ratio is high. At the same time, they showed no effect of FSH on *NRP1* expression. Because a high ratio of E2 : P4 can stimulate *NRP1* expression, the authors suggest a role of *NRP1* in the final growth of follicles. Furthermore, in granulosa cells coming from the same follicles as the present study, the expression pattern of *NRP1* is the same (Nivet *et al.* 2013). Those data indicate a role for *NRP1* in the follicle where granulosa and cumulus cells act the same way. Still another function suggested for *NRP1* is the regulation of RNA metabolism such as localization of specific mRNAs within the cytoplasm, transport of RNAs, polyadenylation and regulation of translation (Good *et al.* 1993) either in itself (trans-zonal projections) or in the nearby oocyte.

Vanin-1 is a glycosylphosphatidylinositol-anchored surface molecule with pantetheinase activity. By hydrolyzing pantetheine, vanin-1 generates an antioxidant, cysteamine, which plays an important role in oxidative stress response in the oocyte (Pitari *et al.* 2000). Vanin-1 is therefore a key molecule to regulate the glutathione-dependent response to oxidative injury (Berruyer *et al.* 2004). In our study, *VNN1* expression increased at 92 h of FSH withdrawal when blastocyst rate decreased. The decline of oocyte competence and the beginning of follicular atresia could be associated with the release of free radicals. This release could in turn trigger the expression of oxidative-stress sensors like *VNN1* in cumulus cells. Moreover, in granulosa cells coming from the same follicles as the present study, *VNN1* showed the same expression pattern (Nivet *et al.* 2013). This suggests a role for *VNN1* in the global follicle oxidative state, in the granulosa and in cumulus cells to protect the whole follicle.

*MAN1A1* codes for mannosidase a, class 1A, member 1, a transmembrane protein that hydrolyses terminal mannose residues. The major neutral carbohydrate chain of bovine zona pellucida glycoproteins is a high-mannose-type chain (Katsumata *et al.* 1996). Furthermore, mannose-binding sites are present on the bovine sperm surface (Revah *et al.* 2000). Tanghe *et al.* (2004) showed that, in cattle, removing D-mannosyl residues from the zona pellucida by a-D-mannosidase decreases oocyte–sperm binding. In our results, the expression of *MAN1A1* increased at 92h while the blastocyst rate decreased. If the protein activity follows the same pattern, it could be possible that mannose residues present on the oocyte surface are removed. This absence of mannose residues could prevent spermatozoa from binding the oocyte and fertilizing it.

*GATM*, an L-arginine : glycine amidinotransferase, encodes a mitochondrial enzyme and is a member of the amidinotransferase family. This enzyme participates in creatine biosynthesis, which occurs in tissues deprived of oxygen. Fischer *et al.* (1992) showed a decrease in oxygen tension correlated with increase in follicular size. In a previous study from our laboratory (Nivet *et al.* 2012), the follicular size increases as time passes from 20 to 92h of FSH coacting. In the ovary, oxygen is provided to the follicle by blood vessels around it. VEGF is known to stimulate neovascularisation in response to an FSH stimulus in mouse (Shin *et al.* 2005) and rat follicles (Kuo *et al.* 2011). Furthermore, in primate follicles, VEGF inhibition has been shown to upregulate hypoxia-inducible factor 1a (HIF1a; Duncan *et al.* 2008). In our experiment, FSH decreased over time so VEGF should not be stimulated anymore (VEGF expression, in fact, decreased in our study; data not shown) and this may increase the expression of HIF1a (HIF1a was actually highly expressed in our experiment; data not shown). This could explain why a hypoxic-related gene like *GATM* increases with FSH withdrawal.

When the differently expressed genes selected on the microarray for qRT-PCR analysis are imported into a program like Ingenuity Pathway Analysis, it becomes possible to investigate the links between them based on a large source of literature. The program generates networks of interactions (Figure 2-5) that may be relevant for a particular condition, even though the interactions may have been observed in different tissues and different species. In the presented network, *TGFB1* could be responsible for the effects observed with *RELN*, *GATM*, *CYP11A1* and *IF127*. The TGF-b super-family is, in fact, known to play an important role in ovarian follicular functions (reviewed in Knight and Glister 2003). In this network, *INHBA*, which is also part of the TGF-b super-family, is shown to influence *CYP11A1* and *NR5A1*. It is important to note that *INHBA* is upregulated in bovine *in vivo*-matured cumulus cells (Tesfaye *et al.* 2009). More importantly, transcription factors SREBF2 and NR5A2 are involved in the response to gonadotrophin and steroid production in granulosa cells. In

fact, in cultured granulosa cells, *SREBF1*–2 and *NR5A1*–2 were identified as targets induced by FSH (Liu *et al.* 2009). In our study, their level did not change significantly across the 4 days but they were present and, according to IPA analysis, they could influence *IFI27*, *FADS2*, *CYP11A1*, *NSDHL*, *VNN1*, *MAN1A1* and *GATM* gene expression. Because *SREBF1*–2 and *NR5A1*–2 are transcription factors, their impact could be more or less extended in time, suggesting that their expression may be more important than their variation.

## 2.6. Conclusion

This study provides for the first time an image of the evolution of cumulus cell gene expression during the final period of oocyte developmental competence acquisition in cattle. As cumulus are losing external layers of cells, the transcriptomic profiles show significant changes in genes involved in progesterone production, which may be potentially associated with a better outcome. Several genes appear progressively and may play roles in signaling to the oocyte. The detrimental effect on blastocyst rate of an excessive waiting time after FSH withdrawal is also reflected in the cumulus cells' transcriptome. The biomarkers identified here could indicate potential treatment of cumulus cells *in vitro* to enhance oocyte quality during IVM.

## **2.7. Materials and methods**

All reagents and media supplements used in these experiments were of tissue-culture grade and were obtained from Sigma Chemicals Co. (St. Louis, MO, USA) unless otherwise indicated.

### **2.7.1. Ovarian stimulation treatment, *in vitro* production and cumulus-oocyte complex (COC) recovery from super-stimulated animals**

Ovarian stimulation, *in vitro* production and COC recovery were performed as described in Nivet *et al.* (2012). The four different FSH withdrawals were performed in four different estrous cycles for each of the six dairy cows. Follicles from 5mm were aspirated. Blastocyst rates were determined at Day 8 after fertilization. For each sampling session, one half of the recovered COC were used for *in vitro* production until Day 8, and the other half of the COC were pooled for each animal and kept for RNA analysis and processed as described in the ‘Cumulus cell retrieval’ section. COC numbers have been computed at each coasting time for each cow. At 20h, 15, 6, 20, 20, 15 and 24 COC were recovered for a total of 100 COC. At 44h, 12, missing value, 20, 36, 11 and 18 COC were recovered for a total of 97 COC. At 68h, missing value, 17, 15, 14, 16 and 19 COC were recovered for a total of 81 COC. At 92h, 15, missing value, 15, 27, 9 and 18 COC were recovered for a total of 84 COC. Total number of COC for each cow was 42 (one missing value), 23 (two missing values), 70, 97, 51 and 79. Considering the four coasting times and the six cows, a total of 362 COC were used in this experiment.

### **2.7.2. Cumulus cell retrieval**

After three washes in modified Tyrode’s medium with HEPES (TLH) solution, COC were collected in Petri dishes containing phosphate-buffered saline (PBS) and polyvinyl alcohol (PVA). COC were washed three times in different dishes with PBS–PVA. They were then transferred into 1.5mL conical tubes and vortexed for 2min in 150mL PBS. Cells were placed into a new dish and oocytes and CC were collected separately into new 1.5mL conical tubes. CC were washed by centrifugation three times in 150mL PBS. Cells were then snap-frozen in nitrogen in a minimum volume and conserved at -80°C.

### **2.7.3. RNA extraction**

Total RNA of CC was extracted using silica-based ion exchange columns (PicoPure RNA Isolation kit; Life Technologies, Foster City, CA, USA) following the manufacturer’s protocol. Contaminating genomic DNA was removed using an on-column DNase treatment (Qiagen,

Mississauga, ON, Canada) while nucleic acids were bound to the column. RNA elution was done with the minimum recommended volume of 11mL. RNA concentration and quality were assessed using a 2100 Bioanalyzer apparatus (Agilent Technologies, Santa Clara, CA, USA) and samples were run on RNA Pico Chips (Agilent Technologies). All samples used showed an RNA Integrity Number  $\geq 7$ .

#### **2.7.4. RNA processing for microarray analysis**

Three of the six biological replicates were used for microarray analysis and 5ng of their total RNA were amplified using T7 RNA polymerase (RiboAmp HS RNA Amplification kit; Life Science, Foster City, CA, USA) according to the manufacturer's indications. A fixed amount of 2mg antisenseRNA (aRNA) per array was labeled indirectly using ULS aRNA Fluorescent Labelling (ULS Fluorescent Labelling Kit for Agilent arrays; according to the manufacturer's protocol. To purify aRNA samples, the PicoPure RNA Isolation kit (Life Science) was used. Amplification and labeling efficiency were measured using the Nano Drop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). All possible comparisons were made using a two color dye swap on Agilent-manufactured EmbryoGENE slides (Robert *et al.* 2011). These comparisons gave four results per condition. Hybridizations were performed according to the manufacturer's protocol (Agilent) using 825ng of labeled aRNA per replicate. After the washing steps, slides were scanned with a PowerScanner (Tecan, Männedorf, Switzerland) and images were analyzed with the ArrayPro 6.3 software (MediaCybernetics, Bethesda, MD, USA).

#### **2.7.5. Microarray data normalization and statistical analysis**

Signal intensity data files were normalized and analyzed using the FlexArray 1.6.1 software (Genome Quebec, URL: <http://genomequebec.mcgill.ca/FlexArray>). The first step in the data processing was to remove the background of intensity files using a simple background subtraction. Data were then normalized for dye bias using a within-array loess, and a between-array 'quantile' normalization was then performed in order to minimise array effects. To calculate fold changes of probe intensity, normalized data were assessed using e-Bayes moderated *t*-test (LIMMA) included in the FlexArray software. A false discovery rate (FDR) algorithm (Benjamini–Hochberg) was also applied. Positive probe signals were determined on normalized data by establishing a significant threshold of cut-off based on a degree of confidence associated with the variability of the negative controls. This cut-off threshold was calculated as the mean background plus two times the standard deviation. All the data equal to or lower than the cut-off threshold determined previously were not considered in the inventory analysis.

### **2.7.6. RNA processing for qRT-PCR and statistical analysis**

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed with the three other biological replicates. Total extracted RNA was reverse transcribed using the qScript Flex cDNA Synthesis Kit with a mix of oligo-dT (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's instructions. Each sample was processed with 1 ng of RNA. At the end of the reverse transcription (RT), 40mL of nuclease-free water was added to the final 20mL RT reaction. Primers used for qRT-PCR were designed from sequences based on the UMD3.1/bosTau5 assembled version of the bovine genome using the IDT PrimerQuest web interface (<http://www.idtdna.com/Scitools/Applications/Primerquest/>, accessed November 2011) and are listed in **Table 2-1**. For each sample and each primer pair, 2mL of cDNA were used to perform qPCR on a LightCycler 2.0 apparatus with the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Applied Science, Laval, QC, Canada). Reactions were performed in glass capillaries in a final volume of 20mL (Roche Applied Science). The reaction mix contained 13.9mL PCR-grade water, 2mL SYBR Green mix, 3mM MgCl<sub>2</sub> and 0.125mM of each primer. For each candidate gene tested, PCR products were purified on 2% agarose gel and extracted with the QIAquick Gel Extraction kit (Qiagen) and quantified with a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies) to perform sequencing in order to confirm the specificity of each primer pair. The standard curves for quantification experiments consisted of five standards of the purified and confirmed PCR products diluted from 2.10<sup>-4</sup> to 2.10<sup>-8</sup>ng mL<sup>-1</sup>. The PCR conditions used for all genes were as follows: denaturing cycle for 10min at 95°C, 50 PCR cycles (denaturing at 95°C for 5s, annealing at the adapted temperature for 5s, elongation at 72°C for 30s, acquisition at the adapted temperature for 1s), a melting curve at 95°C for 1s, 65°C for 5s and a step cycle starting at 65°C up to 95°C at 0.1°C s<sup>-1</sup>) and a final cooling step at 40°C. Data normalization was performed with GeNORM normalization factor (Vandesompele *et al.* 2002) from expression values of three reference genes (ACTB, GAPDH, PPHLN1) and after GeNORM processing only ACTB and GAPDH were kept. To determine the statistically significant differences in mRNA content between each coasting time in cumulus cells, a one-way ANOVA and Tukey's multiple comparison tests with a *P* value<0.5 were applied using Prism 5.02 (GraphPad software, La Jolla, CA, USA). Linear regression was also performed on data.

### **2.7.7. Statistical analysis of COC morphology**

To assess the statistically significant differences concerning COC morphology between each coasting time and each COC category, a two-way ANOVA and Bonferroni post-tests with a *P* value<0.5 were performed using Prism 5.02 (GraphPad software).

## 2.8. References

- Aparicio, I.M., Garcia-Herreros, M., O'Shea, L.C., Hensey, C., Lonergan, P., and Fair, T. (2011) Expression, regulation, and function of progesterone receptors in bovine cumulus oocyte complexes during *in vitro* maturation. *Biol Reprod* **84**(5), 910-21
- Assidi, M., Dieleman, S.J., and Sirard, M.A. (2010) Cumulus cell gene expression following the LH surge in bovine preovulatory follicles: potential early markers of oocyte competence. *Reproduction* **140**(6), 835-52
- Assou, S., Haouzi, D., Mahmoud, K., Aouacheria, A., Guillemin, Y., Pantesco, V., Reme, T., Dechaud, H., De Vos, J., and Hamamah, S. (2008) A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. *Mol Hum Reprod* **14**(12), 711-9
- Berruyer, C., Martin, F.M., Castellano, R., Macone, A., Malergue, F., Garrido-Urbani, S., Millet, V., Imbert, J., Dupre, S., Pitari, G., Naquet, P., and Galland, F. (2004) Vanin-1/- mice exhibit a glutathione-mediated tissue resistance to oxidative stress. *Mol Cell Biol* **24**(16), 7214-24
- Bettegowda, A., Patel, O.V., Lee, K.B., Park, K.E., Salem, M., Yao, J., Ireland, J.J., and Smith, G.W. (2008) Identification of novel bovine cumulus cell molecular markers predictive of oocyte competence: functional and diagnostic implications. *Biol Reprod* **79**(2), 301-9
- Blondin, P., Bousquet, D., Twagiramungu, H., Barnes, F., and Sirard, M.A. (2002) Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biol Reprod* **66**(1), 38-43
- Cheriyath, V., Leaman, D.W., and Borden, E.C. (2011) Emerging roles of FAM14 family members (G1P3/ISG 6-16 and ISG12/IFI27) in innate immunity and cancer. *J Interferon Cytokine Res* **31**(1), 173-81
- Cillo, F., Brevini, T.A., Antonini, S., Paffoni, A., Ragni, G., and Gandolfi, F. (2007) Association between human oocyte developmental competence and expression levels of some cumulus genes. *Reproduction* **134**(5), 645-50
- Duncan, W.C., van den Driesche, S., and Fraser, H.M. (2008) Inhibition of vascular endothelial growth factor in the primate ovary up-regulates hypoxia-inducible factor-1alpha in the follicle and corpus luteum. *Endocrinology* **149**(7), 3313-20
- Eppig, J.J. (2001) Oocyte control of ovarian follicular development and function in mammals. *Reproduction* **122**(6), 829-38
- Feuerstein, P., Cadoret, V., Dalbies-Tran, R., Guerif, F., Bidault, R., and Royere, D. (2007) Gene expression in human cumulus cells: one approach to oocyte competence. *Hum Reprod* **22**(12), 3069-77
- Feuerstein, P., Puard, V., Chevalier, C., Teusan, R., Cadoret, V., Guerif, F., Houlgate, R., and Royere, D. (2012) Genomic assessment of human cumulus cell marker genes as predictors of oocyte developmental competence: impact of various experimental factors. *PLoS One* **7**(7), e40449
- First, N.L., Leibfried-Rutledge, M.L., and Sirard, M.A. (1988) Cytoplasmic control of oocyte maturation and species differences in the development of maturation competence. *Prog Clin Biol Res* **267**, 1-46
- Fischer, B., Kunzel, W., Kleinsteiner, J., and Gips, H. (1992) Oxygen tension in follicular fluid falls with follicle maturation. *Eur J Obstet Gynecol Reprod Biol* **43**(1), 39-43
- Gebhardt, K.M., Feil, D.K., Dunning, K.R., Lane, M., and Russell, D.L. (2011) Human cumulus cell gene expression as a biomarker of pregnancy outcome after single embryo transfer. *Fertil Steril* **96**(1), 47-52 e2
- Gilchrist, R.B., Lane, M., and Thompson, J.G. (2008) Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod Update* **14**(2), 159-77
- Gilchrist, R.B., Ritter, L.J., and Armstrong, D.T. (2004) Oocyte-somatic cell interactions during follicle development in mammals. *Anim Reprod Sci* **82-83**, 431-46
- Good, P.J., Rebbert, M.L., and Dawid, I.B. (1993) Three new members of the RNP protein family in Xenopus. *Nucleic Acids Res* **21**(4), 999-1006
- Grupen, C.G., and Armstrong, D.T. (2010) Relationship between cumulus cell apoptosis, progesterone production and porcine oocyte developmental competence: temporal effects of follicular fluid during IVM. *Reprod Fertil Dev* **22**(7), 1100-9
- Guidobaldi, H.A., Teves, M.E., Unates, D.R., Anastasia, A., and Giojalas, L.C. (2008) Progesterone from the cumulus cells is the sperm chemoattractant secreted by the rabbit oocyte cumulus complex. *PLoS One* **3**(8), e3040
- Hamel, M., Dufort, I., Robert, C., Leveille, M.C., Leader, A., and Sirard, M.A. (2010) Genomic assessment of follicular marker genes as pregnancy predictors for human IVF. *Mol Hum Reprod* **16**(2), 87-96
- Hasegawa, J., Yanaihara, A., Iwasaki, S., Mitsukawa, K., Negishi, M., and Okai, T. (2007) Reduction of connexin 43 in human cumulus cells yields good embryo competence during ICSI. *J Assist Reprod Genet* **24**(10), 463-6

- Hasegawa, J., Yanaihara, A., Iwasaki, S., Otsuka, Y., Negishi, M., Akahane, T., and Okai, T. (2005) Reduction of progesterone receptor expression in human cumulus cells at the time of oocyte collection during IVF is associated with good embryo quality. *Hum Reprod* **20**(8), 2194-200
- Irving-Rodgers, H.F., Harland, M.L., Sullivan, T.R., and Rodgers, R.J. (2009) Studies of granulosa cell maturation in dominant and subordinate bovine follicles: novel extracellular matrix focimatrix is co-ordinately regulated with cholesterol side-chain cleavage CYP11A1. *Reproduction* **137**(5), 825-34
- Katsumata, T., Noguchi, S., Yonezawa, N., Tanokura, M., and Nakano, M. (1996) Structural characterization of the N-linked carbohydrate chains of the zona pellucida glycoproteins from bovine ovarian and fertilized eggs. *Eur J Biochem* **240**(2), 448-53
- Knight, P.G., and Glister, C. (2003) Local roles of TGF-beta superfamily members in the control of ovarian follicle development. *Anim Reprod Sci* **78**(3-4), 165-83
- Kuo, S.W., Ke, F.C., Chang, G.D., Lee, M.T., and Hwang, J.J. (2011) Potential role of follicle-stimulating hormone (FSH) and transforming growth factor (TGFbeta1) in the regulation of ovarian angiogenesis. *J Cell Physiol* **226**(6), 1608-19
- Kurschat, P., Bielenberg, D., Rossignol-Tallandier, M., Stahl, A., and Klagsbrun, M. (2006) Neuron restrictive silencer factor NRSF/REST is a transcriptional repressor of neuropilin-1 and diminishes the ability of semaphorin 3A to inhibit keratinocyte migration. *J Biol Chem* **281**(5), 2721-9
- Lee, M.S., Liu, C.H., Lee, T.H., Wu, H.M., Huang, C.C., Huang, L.S., Chen, C.M., and Cheng, E.H. (2010) Association of creatin kinase B and peroxiredoxin 2 expression with age and embryo quality in cumulus cells. *J Assist Reprod Genet* **27**(11), 629-39
- Liu, Z., Rudd, M.D., Hernandez-Gonzalez, I., Gonzalez-Robayna, I., Fan, H.Y., Zeleznik, A.J., and Richards, J.S. (2009) FSH and FOXO1 regulate genes in the sterol/steroid and lipid biosynthetic pathways in granulosa cells. *Mol Endocrinol* **23**(5), 649-61
- McKenzie, L.J., Pangas, S.A., Carson, S.A., Kovanci, E., Cisneros, P., Buster, J.E., Amato, P., and Matzuk, M.M. (2004) Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum Reprod* **19**(12), 2869-74
- Mingoti, G.Z., Garcia, J.M., and Rosa-e-Silva, A.A. (2002) Steroidogenesis in cumulus cells of bovine cumulus-oocyte-complexes matured *in vitro* with BSA and different concentrations of steroids. *Anim Reprod Sci* **69**(3-4), 175-86
- Nivet, A.L., Bunel, A., Labrecque, R., Belanger, J., Vigneault, C., Blondin, P., and Sirard, M.A. (2012) FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction* **143**(2), 165-71
- Nivet, A.L., Vigneault, C., Blondin, P., and Sirard, M.A. (2013) Changes in granulosa cells gene expression associated with increased oocyte competence in bovine. *Reproduction*
- Nuttinck, F., Marquant-Le Guenne, B., Clement, L., Reinaud, P., Charpigny, G., and Grimard, B. (2008) Expression of genes involved in prostaglandin E2 and progesterone production in bovine cumulus-oocyte complexes during *in vitro* maturation and fertilization. *Reproduction* **135**(5), 593-603
- Ohashi, M., Mizushima, N., Kabeya, Y., and Yoshimori, T. (2003) Localization of mammalian NAD(P)H steroid dehydrogenase-like protein on lipid droplets. *J Biol Chem* **278**(38), 36819-29
- Pitari, G., Malergue, F., Martin, F., Philippe, J.M., Massucci, M.T., Chabret, C., Maras, B., Dupre, S., Naquet, P., and Galland, F. (2000) Pantetheinase activity of membrane-bound Vanin-1: lack of free cysteamine in tissues of Vanin-1 deficient mice. *FEBS Lett* **483**(2-3), 149-54
- Regassa, A., Rings, F., Hoelker, M., Cinar, U., Tholen, E., Looft, C., Schellander, K., and Tesfaye, D. (2011) Transcriptome dynamics and molecular cross-talk between bovine oocyte and its companion cumulus cells. *BMC Genomics* **12**, 57
- Revah, I., Gadella, B.M., Flesch, F.M., Colenbrander, B., and Suarez, S.S. (2000) Physiological state of bull sperm affects fucose- and mannose-binding properties. *Biol Reprod* **62**(4), 1010-5
- Robert, C., Nieminen, J., Dufort, I., Gagne, D., Grant, J.R., Cagnone, G., Plourde, D., Nivet, A.L., Fournier, E., Paquet, E., Blazejczyk, M., Rigault, P., Juge, N., and Sirard, M.A. (2011) Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays. *Mol Reprod Dev* **78**(9), 651-64
- Rossignol, M., Pouyssegur, J., and Klagsbrun, M. (2003) Characterization of the neuropilin-1 promoter; gene expression is mediated by the transcription factor Sp1. *J Cell Biochem* **88**(4), 744-57
- Shimizu, T., Jayawardhana, B.C., Nishimoto, H., Kaneko, E., Tetsuka, M., and Miyamoto, A. (2006) Hormonal regulation and differential expression of neuropilin (NRP)-1 and NRP-2 genes in bovine granulosa cells. *Reproduction* **131**(3), 555-9
- Shin, S.Y., Lee, H.J., Ko, D.S., Lee, H.C., and Park, W.I. (2005) The regulators of VEGF expression in mouse ovaries. *Yonsei Med J* **46**(5), 679-86

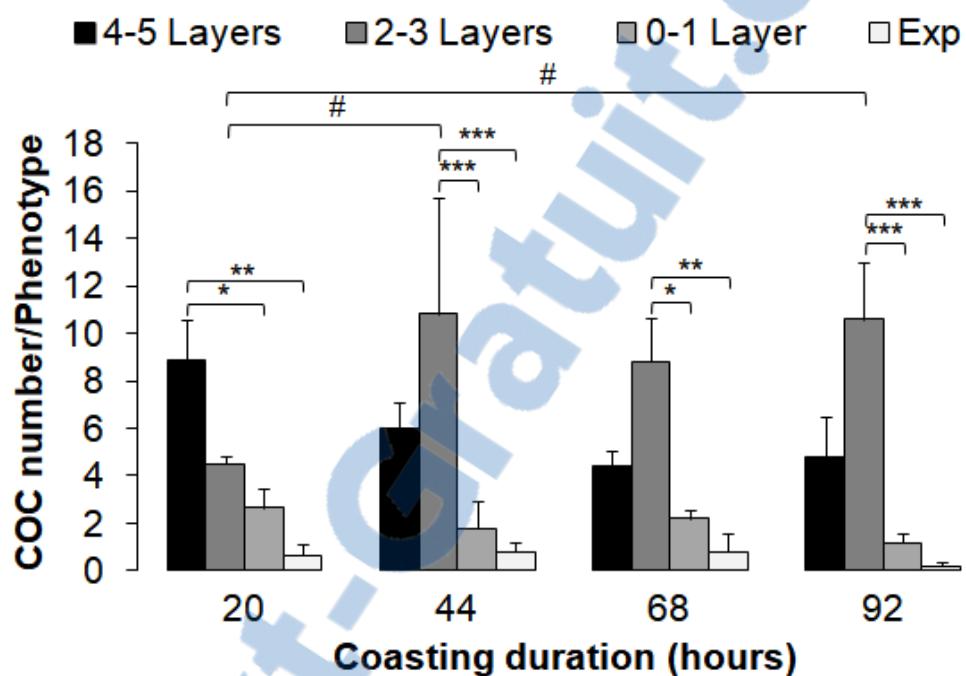
- Sirard, M.A., and Coenen, K. (2006) In vitro maturation and embryo production in cattle. *Methods Mol Biol* **348**, 35-42
- Tanghe, S., Van Soom, A., Duchateau, L., and De Kruif, A. (2004) Inhibition of bovine sperm-oocyte fusion by the p-aminophenyl derivative of D-mannose. *Mol Reprod Dev* **67**(2), 224-32
- Tesfaye, D., Ghanem, N., Carter, F., Fair, T., Sirard, M.A., Hoelker, M., Schellander, K., and Lonergan, P. (2009) Gene expression profile of cumulus cells derived from cumulus-oocyte complexes matured either *in vivo* or *in vitro*. *Reprod Fertil Dev* **21**(3), 451-61
- Teves, M.E., Barbano, F., Guidobaldi, H.A., Sanchez, R., Miska, W., and Giojalas, L.C. (2006) Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. *Fertil Steril* **86**(3), 745-9
- Valdembri, D., Caswell, P.T., Anderson, K.I., Schwarz, J.P., Konig, I., Astanina, E., Caccavari, F., Norman, J.C., Humphries, M.J., Bussolino, F., and Serini, G. (2009) Neuropilin-1/GIPC1 signaling regulates alpha5beta1 integrin traffic and function in endothelial cells. *PLoS Biol* **7**(1), e25
- van Montfoort, A.P., Geraedts, J.P., Dumoulin, J.C., Stassen, A.P., Evers, J.L., and Ayoubi, T.A. (2008) Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. *Mol Hum Reprod* **14**(3), 157-68
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**(7), RESEARCH0034
- Walsh, S.W., Williams, E.J., and Evans, A.C. (2011) A review of the causes of poor fertility in high milk producing dairy cows. *Anim Reprod Sci* **123**(3-4), 127-38
- Yamashita, Y., Shimada, M., Okazaki, T., Maeda, T., and Terada, T. (2003) Production of progesterone from de novo-synthesized cholesterol in cumulus cells and its physiological role during meiotic resumption of porcine oocytes. *Biol Reprod* **68**(4), 1193-8
- Zhang, X., Jafari, N., Barnes, R.B., Confino, E., Milad, M., and Kazer, R.R. (2005) Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. *Fertil Steril* **83 Suppl 1**, 1169-79

## 2.9. Tables

**Table 2-1. List and characteristics of primer pairs used in qRT-PCR for housekeeping and candidate genes in bovine cumulus cells.**

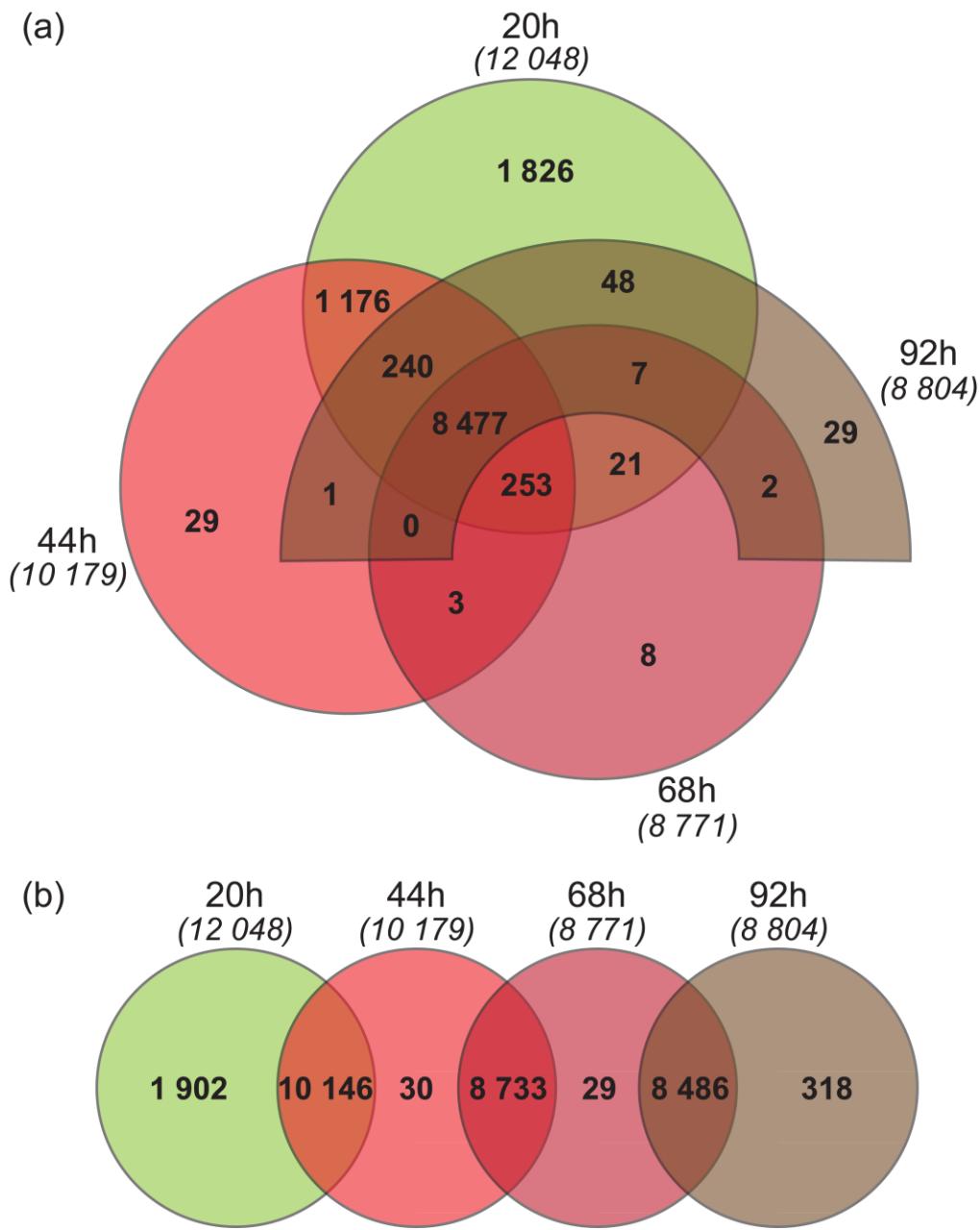
Gene	Forward primer (sense) Reverse primer (anti-sense)	Annealing temperature (°C)	Product length (bp)	Accession number
<b>ANK3</b>	CCCAAATGAGGAATTCTGTAGTTCTG CCTAGTATGACATGGCGAGAATGG	60	484	NM_001105620
<b>CYP11A1</b>	TAGCATCAAGGAGACGCTGAGA TAGCTGGATTGGTGGAAAGGG	57	470	NM_176644
<b>FADS2</b>	AGCTGAGGGGAGAGGAAAGTGAGA TTCCAAAAGTGTAGAGGTGGGAAGC	60	310	NM_001083444
<b>GATM</b>	GGCAGCTTGAATGTTGGTCCT GGAACTGTAACITATCGTTATATCCTATTGCAC	59	392	NM_001045878
<b>IFI27</b>	GC GGCCAAGATGATGTCAATATCTGC ACTGGGTCCCCTCACTGCATC	58	269	NM_001038050
<b>MAN1A1</b>	ACTGAAGTCCTTAGGTAGAGGCTG TGTGCCAATCTATTATGTTCCCATAAC	55	319	XM_002690078
<b>NRP1</b>	AAGGCAAGGGCTCCGAAGATT TTGTTTGCCATTCCCAGCAGGT	59	252	XM_002692084
<b>NSDHL</b>	TTATCATCCACAGAGCCCTTTGTCC TATTCCAATCTTCCCAGGTGCCTC	59	337	NM_001035482
<b>RELN</b>	CTGCGGGTCATATTCACACCTT AGCTTGTTCACCCCTAAAGTGC	59	123	XM_002686760
<b>VNN1</b>	TGGCACGTTGGAACCCAGTAT CTTTGGATTGAGCCCTAGCGCTTG	58	223	NM_001024556
<b>ACTNB</b>	ATCGTCCACCGCAAATGCTTCT GCCATGCCAATCTCATCTCGTT	59	101	NM_173979
<b>GAPDH</b>	CCAACGTGTCTGTGGATCTGA GAGCTTGACAAAGTGGTCGTTGAG	58	217	NM_001034034

## 2.10. Figures



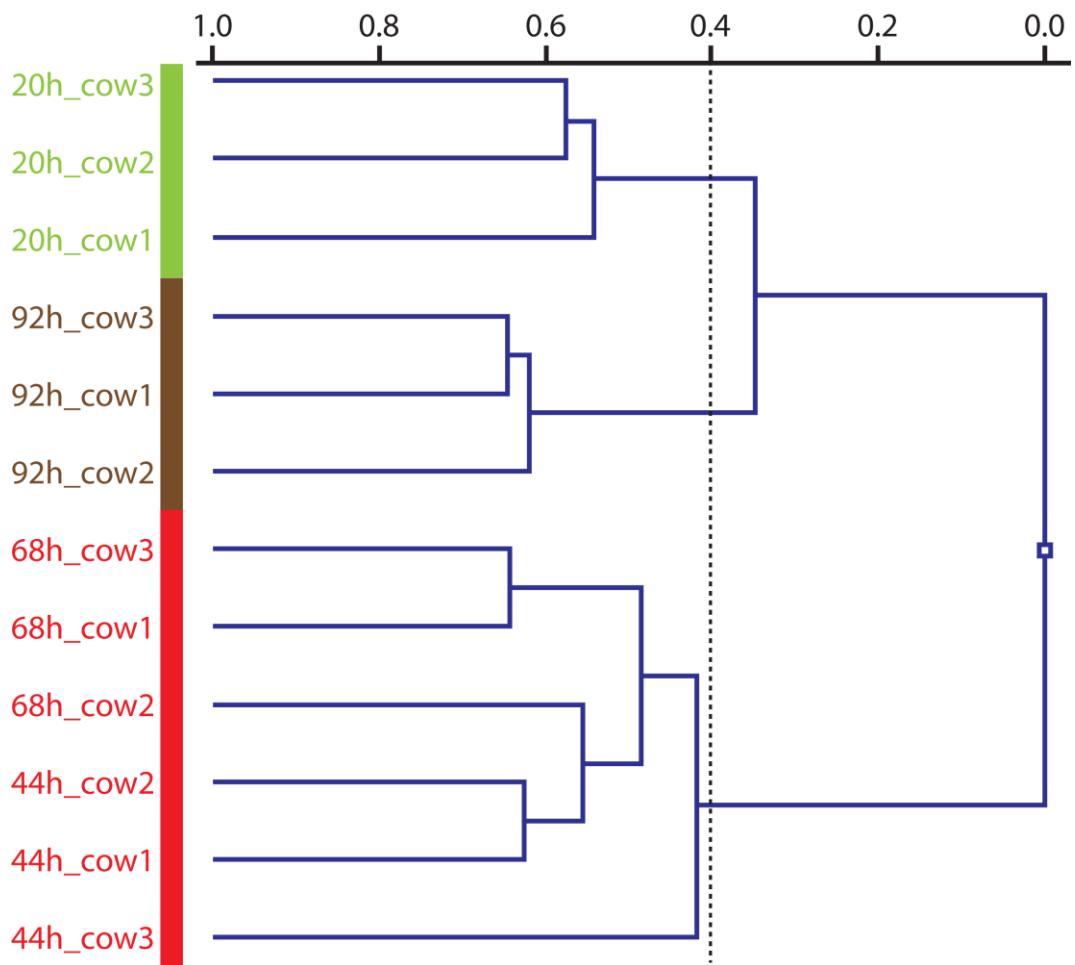
*Figure 2-1. Morphological evolution of the recovered COC during FSH coasting.*

COC were categorized into four classes depending on the approximate number of cumulus cell layers around the oocyte (4–5, 2–3, 0–1 layers) and whether or not there was an already expanded cumulus (Exp).  $n = 6$  and data are mean  $\pm$  s.e.m. \*, #  $P < 0.05$ .



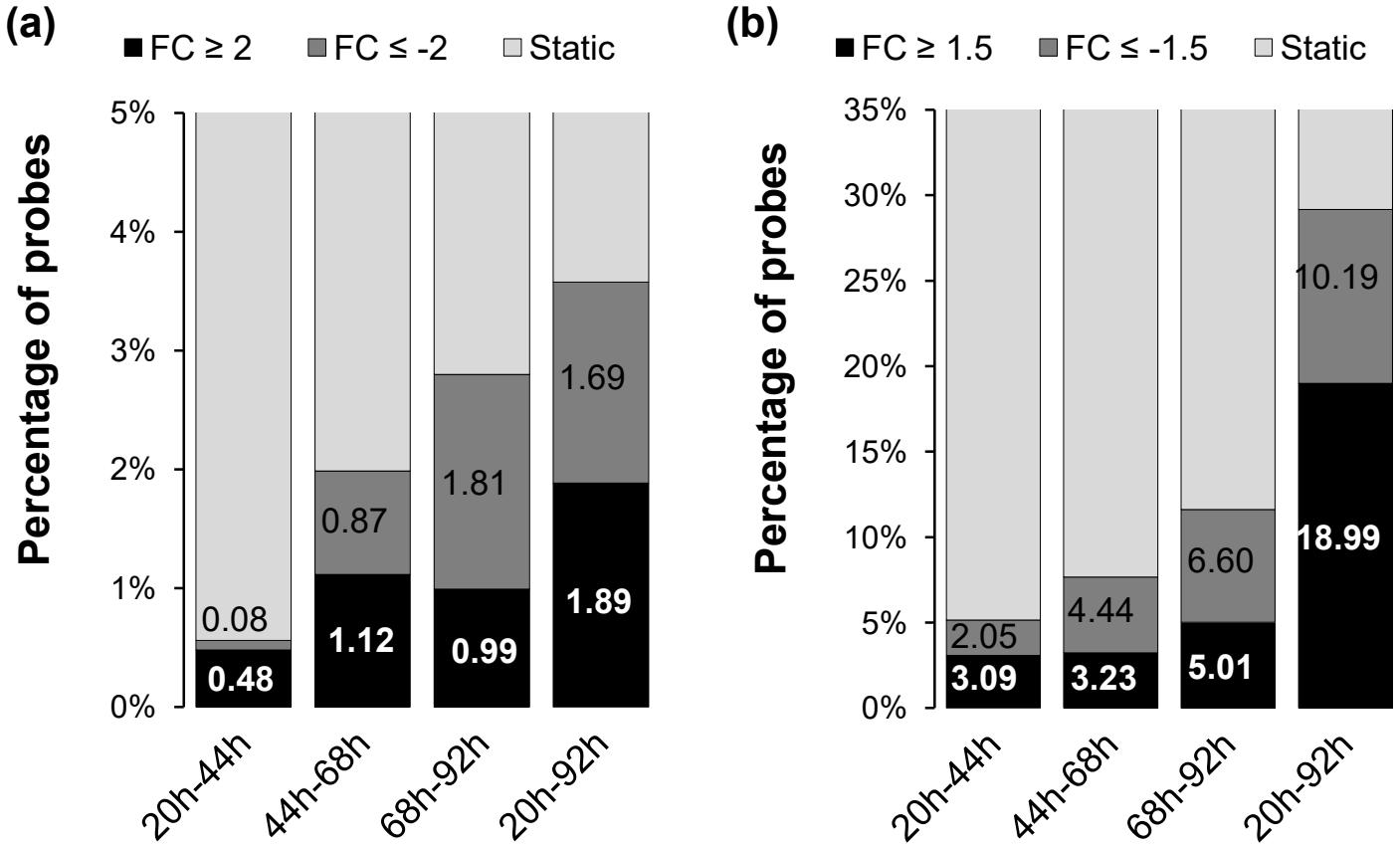
**Figure 2-2.** Venn diagrams representing the number of expressed genes in bovine cumulus cells for the different FSH withdrawal times.

Diagrams indicate the number of probes corresponding to unique gene symbols with at least one probe having an intensity signal higher than the defined threshold (background intensity plus 2 times its standard deviation for each condition). Numbers between brackets indicate the total gene number for each corresponding condition. (a) Exclusive comparisons of each gene list. (b) Chronological but non-exclusive comparisons of gene lists.  $n = 3$ .



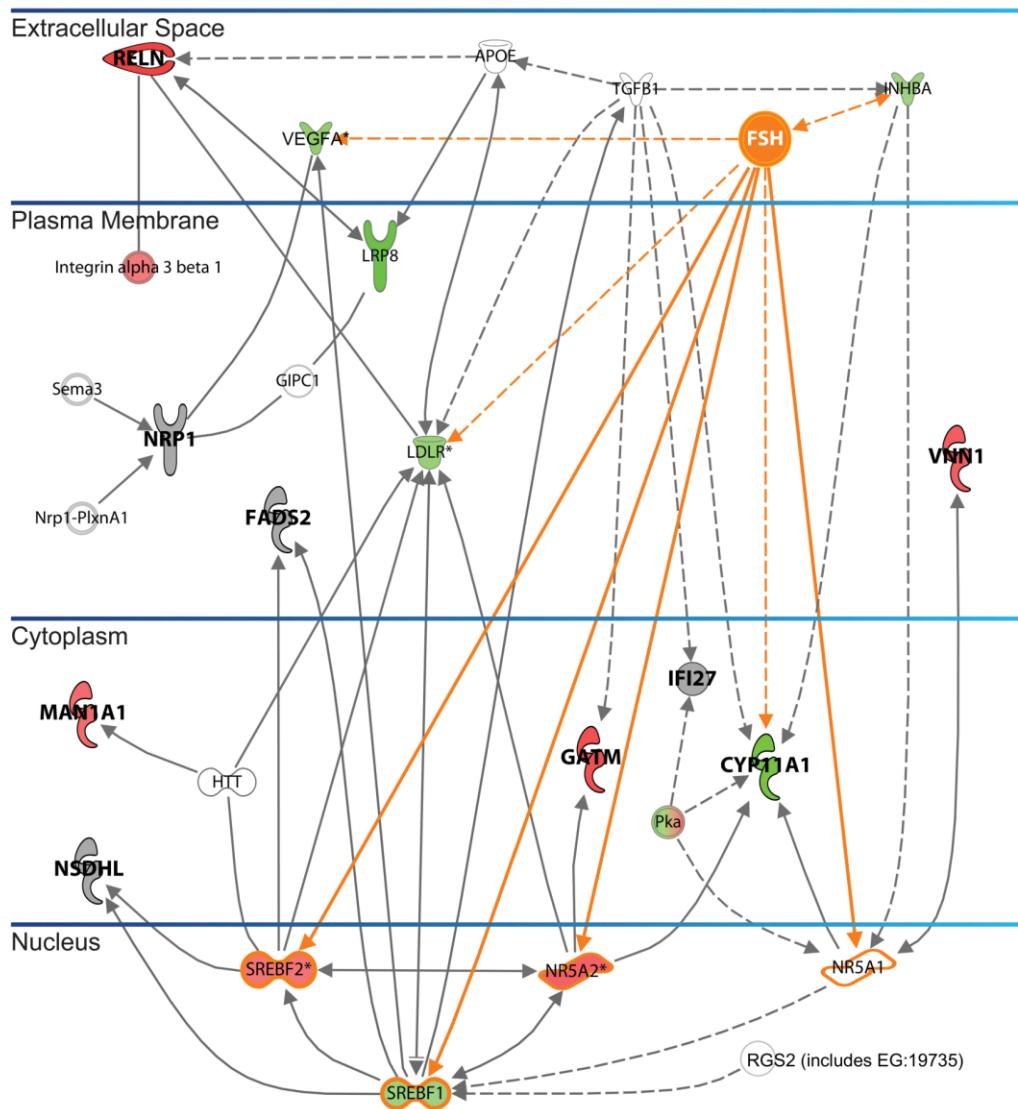
**Figure 2-3. Dendrogrammatic representation of the distance between the four different FSH withdrawal treatments.**

Includes the three biological replicates using positive probes in the bovine cumulus cells (Pirouette 4.0; Infometrics, Bothell, WA, USA).  $n = 3$ .



*Figure 2-4. Proportion of probes for which expression in bovine cumulus cells*

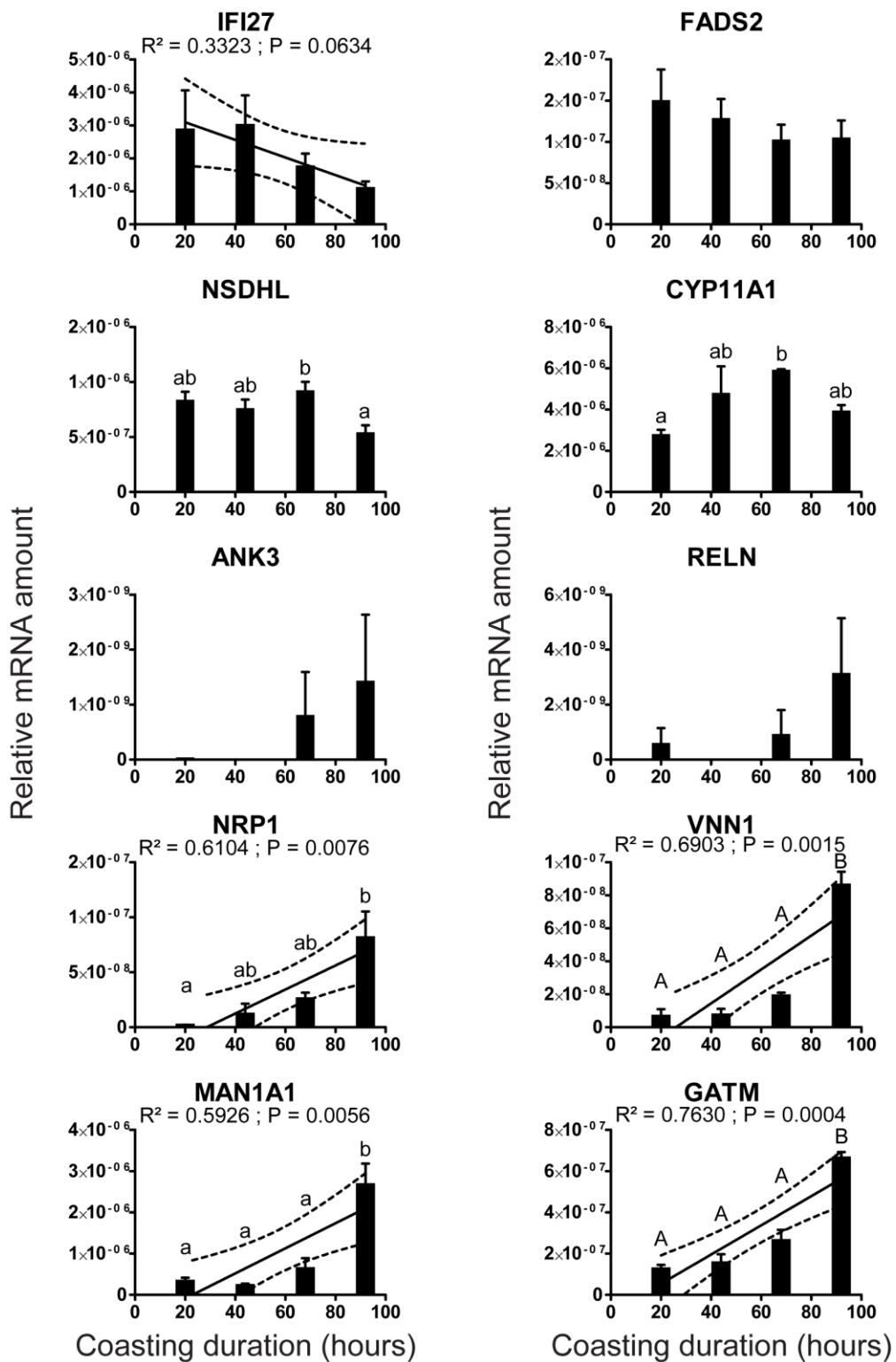
varies considering (a) a 2-fold change or (b) a 1.5-fold change, and for both a false discovery rate (FDR)  $\leq 0.05$ , between the different FSH withdrawal times.  $n = 3$ .



© 2000-2012 Ingenuity Systems, Inc. All rights reserved.

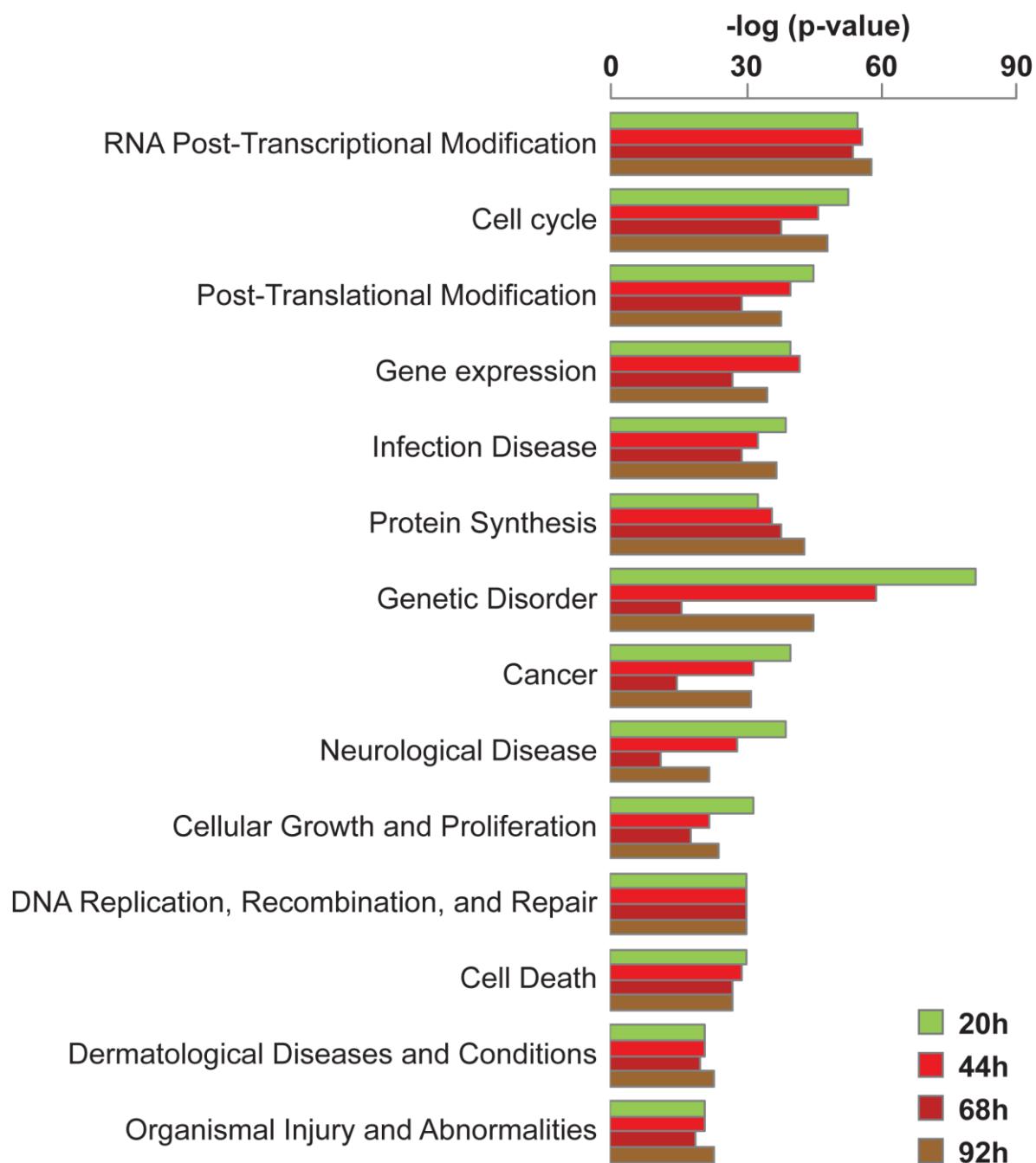
**Figure 2-5. Expression network from 10 selected genes of bovine cumulus.**

IPA software was used to construct a network based on 10 genes of the bovine cumulus cells processed in qRT-PCR validation. Colors indicate positive (red) or negative (green) gene expression changes between 20 h and 44 h as example. *RELN*, reelin; *ANK3*, ankyrin 3, node of Ranvier (ankyrin G); *NRP1*, neuropilin 1; *FADS2*, fatty acid desaturase 2; *VNN1*, vanin 1; *MAN1A1*, mannosidase  $\alpha$ , class 1A, member 1; *GATM*, glycine amidinotransferase (L-arginine : glycine amidinotransferase); *CYP11A1*, cytochrome P450, family 11, subfamily A, polypeptide 1; *IFI27*, interferon  $\alpha$ -inducible protein 27; *NSDHL*, NAD(P)-dependent steroid dehydrogenase-like. (—) direct interaction; (---) indirect interaction.



**Figure 2-6. Quantification by qRT-PCR of mRNA profiles in cumulus cells for each FSH withdrawal time.**

$n = 3$  and data are mean  $\pm$  s.e.m. Different letters indicate significant difference: a, b or c,  $P < 0.05$ ; A or B,  $P < 0.0001$ . Solid lines represent linear regressions and dashed lines show the 95% confidence intervals of the best-fit line.



*Figure 2-7. Top 14 IPA biofunctions and their significance at 20, 44, 68 and 92h of FSH. (originally published as Figure S1, Supplementary Material)*

**3. THE EFFECTS OF BASAL LH INHIBITION WITH CETRORELIX ON CUMULUS CELL  
GENE EXPRESSION DURING THE LUTEAL PHASE UNDER OVARIAN COASTING  
STIMULATION IN CATTLE.**

Audrey Bunel<sup>1</sup>, Anne-Laure Nivet<sup>1</sup>, Patrick Blondin<sup>2</sup>, Christian Vigneault<sup>2</sup>, François J. Richard<sup>1</sup>  
and Marc-André Sirard<sup>1</sup>

<sup>1</sup> Centre de recherche en reproduction, développement et santé inter-générationnelle, Faculté des sciences de l'agriculture et de l'alimentation, Département des sciences animales, Université Laval, Québec, Canada.

<sup>2</sup> L'Alliance Boviteq, Saint-Hyacinthe, Québec, Canada.

### 3.1. Résumé

Les cellules du cumulus jouent un rôle important dans la préparation finale de l'ovocyte avant l'ovulation. Pendant la phase de différenciation folliculaire finale, alors que les taux de FSH sont bas, c'est la LH qui maintient la croissance folliculaire ; l'influence éventuelle de la LH sur les cellules du cumulus (CC) au sein du follicule pendant cette période n'est toutefois pas connue. Chez l'humain, la LH est souvent inhibée afin d'éviter un pic préovulatoire de LH prématûre lors de cycles de stimulation ovarienne. Cette procédure permet d'examiner le rôle de la LH dans le développement folliculaire. Dans cette étude, nous avons analysé l'impact de la suppression de LH avec un antagoniste de GnRH, le Cetorelix, au cours d'un protocole de stimulation ovarienne avec sevrage en FSH, sur le transcriptome de CC bovins. Les ovocytes ont été collectés en deux fois sur six vaches laitières. Pour la première collection, les vaches ont reçu de la FSH deux fois par jour pendant trois jours, suivis d'un sevrage en FSH de 68 h comme condition contrôle. Pour la seconde collection ovocytaire, les vaches ont reçu le même protocole avec toutefois l'injection de l'antagoniste de GnRH au deuxième jour de stimulation avec la FSH jusqu'au jour de la récupération des complexes ovocyte-cumulus (COC). La moitié des COC a été mise en maturation, fécondation et culture *in vitro* afin d'évaluer les taux de blastocystes associés aux deux traitements. L'autre moitié des CC a été soumise à une analyse par puces à ADN ( $n = 3$  vaches, 2 traitements, 6 ponctions ovocytaires) et par qRT-PCR ( $n = 6$  vaches : 3 vaches des puces à ADN + 3 autres vaches, 2 traitements, 12 ponctions ovocytaires). L'expression différentielle de gènes spécifiques a été confirmée par qRT-PCR : ATP6AP2, SC4MOL, OSTC et PTGDS. L'analyse transcriptomique globale des cellules du cumulus a démontré que l'absence de LH en circulation diminuerait notamment la survie et la croissance du follicule. De plus, les résultats suggèrent qu'au niveau du cumulus la LH interviendrait dans le maintien de mécanismes cellulaires tels que l'expression globale d'ARN, le métabolisme des protéines et des acides nucléiques ainsi que la production énergétique. Ces résultats supportent l'hypothèse de l'importance du soutien de la LH dans la maturation finale du follicule par son influence sur les cellules du cumulus.

### **3.2. Abstract**

Cumulus cells have an important role to play in the final preparation of the oocyte before ovulation. During the final phase of follicular differentiation, FSH levels are low and LH maintains follicular growth; however, it is not known if at that time LH has an influence on cumulus cells inside the follicle. In humans, LH is often inhibited in order to avoid a premature ovulatory LH surge. This procedure provides a tool to investigate the role of LH in follicular development. In this study, we investigated the impact of suppressing LH using the GnRH antagonist Cetrorelix during an ovarian coothing stimulation protocol on the transcriptome of bovine cumulus cells (CC). Oocytes were collected twice from six dairy cows. For the first collection, the cows received FSH twice daily for three days, followed by FSH withdrawal for 68 hours as control protocol. For the second collection, the same stimulation protocol was used, but the cows also received, starting on day 2 of FSH stimulation, a GnRH antagonist once a day until recovery of the cumulus-oocyte complexes (COC). Half of the COC were subjected to *in vitro* maturation, fertilization and culture to assess blastocyst rates. The other half of the CC underwent microarray analysis (n=3 cows, 2 treatments, 6 oocyte collections) and qRT-PCR (n=6 cows: 3 microarray cows + 3 other cows, 2 treatments, 12 oocyte collections). The differential expression of specific genes was confirmed by RT-qPCR: ATP6AP2, SC4MOL, OSTC and PTGDS. The global transcriptomic analysis of cumulus cells demonstrated that the inhibition of LH secretion may decrease survival, and growth of the follicle. Moreover, the results suggested that LH may be important to cumulus for the maintenance of cellular mechanisms such as global RNA expression, protein and nucleic acid metabolism and energy production. These results support the hypothesis that LH support is important during the final part of follicle maturation through its influence on the cumulus cells.

### 3.3. Introduction

A functional synchronization of all the follicular components is required to ensure the optimal timing for the preparation and release of a mature and competent oocyte. In large mammals, the last stage of follicular maturation is characterized by a dominant follicle which suppresses FSH secretion. In these conditions, only follicles with LH receptors (functional dominants) can continue to grow. In bovine, as the dominant follicle grows while FSH levels are low, it moves from a state of FSH dependence to a state of LH dependence (Mihm *et al.* 2006). In a previous study, we analyzed the impact of FSH decrease during super-ovulation as a way to mimic a natural cycle with several dominant follicles instead of only one. These conditions were associated with a very significant improvement of oocyte quality (Blondin *et al.* 2002, Nivet *et al.* 2012). More recently, we explored the consequences of removing LH support using GnRH antagonist during the low FSH period both in granulosa cells (GC) and oocytes from stimulated cows (Labrecque *et al.* 2014, Nivet *et al.* 2017). In these studies, a decrease (although not significant) in oocyte quality and an arrest in follicular growth were associated with a shift in the transcriptome of GC towards atresia (Nivet *et al.* 2017). In addition, the comparison of the transcriptome of GnRH antagonist-treated GC to LH-treated GC revealed a list of 64 common basal LH associated genes, which are related to functions such as cell cycle, angiogenesis, Wnt signaling and reproduction (Sirard 2016). The role of cumulus cells in this context is unknown as they may be non-responsive to LH (Robert *et al.* 2003). However, they are probably sensitive to follicular signals.

The use of GnRH analogues is widely spread in human artificial reproduction technologies (ARTs) and their impact on the outcomes has been analysed in several studies in different species (antagonists: Bittner *et al.* 2011, Madill *et al.* 1994, Oussaid *et al.* 2000, Oussaid *et al.* 1999, Ulker *et al.* 2001; agonists: Bergfeld *et al.* 1996; both: Al-Inany *et al.* 2007, Maclellan *et al.* 1997). Agonists and antagonists are both used to avoid a premature LH surge which can arise before the leading follicle is ready and/or can compromise the induction of maturation of multiple follicles. However, while agonists down-regulate and desensitize GnRH receptors on gonadotrophic cells, antagonists bind to GnRH receptors competitively, preventing the stimulatory effect of endogenous GnRH and inhibiting production and release of LH by pituitary cells. Therefore in humans, the ovarian stimulation and the growth of follicles happen with minimal (agonist) or no endogenous LH support (antagonist). Several studies compared the effects of agonists and antagonists (Devjak *et al.* 2012). The two analogue types seem to be equivalent in term of ART outcomes even if some meta-analysis were contradictory. However, the use of agonists triggers cysts formation and more cases of ovarian hyper-stimulation syndrome than the use of antagonists (Chang *et al.* 2013).

As mentioned above, we previously demonstrated the beneficial value of removing FSH support at the end of stimulation to allow the influence of basal LH to maintain growth and prepare the follicle for ovulation (Nivet *et al.* 2012). These conditions, which increased oocyte competence, also had an influence on the cumulus transcriptome (Bunel *et al.* 2013) and such transformations may play a crucial role in preparing the oocyte for embryonic development. To assess if the changes occurring during coasting are due to the absence of FSH support, or alternatively, to the presence of basal LH support, a simple GnRH antagonist treatment was applied on the same animals used in the first study and the consequences on the cumulus transcriptome and developmental competence were explored. We therefore compared the presence or absence of the GnRH antagonist during the optimal coasting procedure in order to better understand how the cumulus may translate the LH support to the oocyte. Our results indicated that the presence of basal LH is important and maintains the cumulus cells in a pro-survival profile.

### 3.4. Results

#### 3.4.1. COC Morphology

The COC (81 and 111 COCs for the 68h and the 68h+Cetro conditions respectively) were observed and categorized before being split into two groups: one half for *in vitro* culture and one half for RNA analysis. Four morphological categories were established depending on the approximate number of cumulus cell layers surrounding the oocyte (4-5, 2-3, 0-1 layers), or whether the cumulus was already expanded (« Exp ») (Figure 3-2). For the 68h condition, significant differences were observed between 2-3 layers and 0-1 layer ( $P < 0.05$ ). For the 68h+Cetro condition, significant differences were highlighted between 4-5 layers and 2-3 layers ( $P < 0.01$ ), between 2-3 layers and « Exp » ( $P < 0.001$ ) and between 0-1 layers and « Exp » ( $P < 0.05$ ). We also compared these results to those of the 92h of coasting from our previous study using the same protocol on the same cows (Bunel *et al.* 2013) where significant differences were observed between 2-3 layers and 0-1 layer ( $P < 0.001$ ) and between 2-3 layers and « Exp » ( $P < 0.001$ ). No significant difference was observed between the three coasting conditions.

#### 3.4.2. Genes Inventory and Clustering

To get an overview of the transcriptomic differences between CC recovered following 68h of FSH withdrawal and CC recovered following 68 h of FSH withdrawal in the presence of the LH-inhibitor Cetrorelix, a large-scale transcriptomic analysis was performed using the EmbryoGENE microarray slide. This bovine specific slide includes 42,242 probes for 21,139 known reference genes, 7,230 splice and 3'UTR variants, and 9,322 novel transcribed regions (Robert *et al.* 2011). The hybridization was set to compare the two conditions from the same animal in a dye-swap design, resulting in two technical replicates for each of the three biological replicates with a minimum individual variation effect. Out of the 21,139 genes represented on the slide, 20,191 genes were considered to be expressed in the 68h condition and 20,295 genes were considered to be expressed in the 68h+Cetro condition with signals above a threshold defined as the mean background plus two times the standard deviation. Furthermore, considering a 2-fold change (FC) cut-off, 140 genes were differentially expressed between the two conditions (26 genes down-regulated and 114 up-regulated after LH inhibition). When a False Discovery Rate (FC 2; FDR < 0.05) test was apply, a total of 76 genes were differentially expressed (12 for 68h condition and 64 for 68h+Cetro condition).

### **3.4.3. IPA analysis**

The analysis of the transcriptomic data with the IPA software (20805 mapped IDs and 3426 analysis-ready molecules included by IPA), revealed that the five main functions impacted by the absence of LH were: Cell death and survival, Cellular growth and proliferation, Gene expression, Cell cycle, and Protein synthesis. [Table 3-1](#) shows a general cumulus cell portrait of the main cellular functions affected by the absence of LH.

### **3.4.4. qRT-PCR results**

Four genes were submitted to qRT-PCR: ATP6AP2, OSTC, SC4MOL and PTGDS. These genes were quantified using six replicates (the three used for microarray plus three independent replicates) ([Figure 3-3](#)). Significant differences were found for ATP6AP2, SC4MOL and OSTC between 68 h and 68 h +Cetro with a higher expression in the 68h condition ( $P < 0.01$ ,  $P < 0.001$  and  $P < 0.05$  respectively). Whereas, a significantly higher expression of PTGDS was found in the 68h +Cetro compared to the 68h condition,  $P < 0.05$ .

### 3.5. Discussion

In this study, we first described the transcriptomic variations in bovine cumulus cells induced by basal LH suppression during an optimal ovarian coothing stimulation protocol (the protocol was previously developed and optimised by our team: (Blondin *et al.* 2002, Nivet *et al.* 2012). To do so, we used a GnRH antagonist (Cetrorelix) with a 68h of FSH withdrawal stimulation protocol and compared it to 68h of FSH withdrawal without the GnRH antagonist. In a previous study, suppressing LH during an FSH-based ovarian stimulation led to a slight but not significant decrease in the developmental potential of the COC ( $52.4 \pm 5.6\%$  vs.  $67.1 \pm 13.9\%$ , for 68h +Cetro and 68h alone respectively, (Labrecque *et al.* 2014). Because of the importance of cumulus cells for the proper developmental competence acquisition by the oocyte (Gilchrist *et al.* 2004), the loss of developmental competence observed could be reflected by modifications of the morphological quality of the COC. In fact, even if it was not statistically significant, the morphology of the COC also tended to deteriorate in the presence of Cetrorelix. Thus, a greater proportion of COC with 0-1 layer of cumulus cells surrounding the oocyte and a lesser proportion of COC with 4-5 layers were observed when LH was inhibited. As shown in Table 3-1, an IPA functional category called '*Cellular movement*' was predicted to be activated in the absence of LH. One may infer that if cellular movement is enhanced, cell adhesion or cell-to-cell interactions would be looser and explain, at least in part, the decrease in the number of layers of cumulus cells. However, the microarray data did not confirm a link between expression of genes related to cell adhesion or extracellular matrix and the absence of LH.

At the end of the ovarian stimulation cycle, basal LH is presumed to support follicular growth after FSH withdrawal. Another study from our team (Nivet *et al.* 2017) regarding mural granulosa cells and the morphological aspect of the follicular growth on the same cows, revealed a drop in the number of greater than 10 mm follicles in the presence of Cetrorelix, confirming the importance of basal LH in the support of follicular growth. Furthermore, LH has already been shown to sustain cellular growth and proliferation of goat granulosa cells in culture (Gupta *et al.* 2012). Also, cell proliferation was enhanced when the LH receptor was ligand-activated in human granulosa cells (Casarini *et al.* 2016). According to transcriptomic IPA analysis, LH inhibition resulted in the biofunction '*Cell cycle*' with the annotation for '*duplication of centrosome*' to be predicted as inhibited in cumulus cells. Moreover, the '*Cellular growth and proliferation*' biofunction was predicted to be inhibited for '*proliferation of cells*' and activated for '*cytostasis*'. Inhibiting basal LH while FSH levels were at their lowest seemed to have hindered proliferation in bovine cumulus cells. Likewise, according to our microarray data, the anti-proliferative gene GADD45a (Perugini *et al.* 2009) was up-regulated (FC= 2.83, p= 0.001) in the

absence of basal LH. Note that FSH is able to inhibit GADD45a at the mRNA and protein levels in human cumulus cells (Stocco *et al.* 2017).

In the present study, the ATP6AP2 (also named (P)RR) and SC4MOL genes were down-regulated by the GnRH antagonist treatment. The gene ATP6AP2 is pro-proliferative (Wanka *et al.* 2017) and knocking it down led to deformations of spindle assembly in agreement with the predicted inhibition of '*duplication of centrosome*' annotation. Otherwise, deficiency in SC4MOL, which expression was diminished here, promoted proliferation of human fibroblasts (He *et al.* 2014). Moreover, deficiency of SC4MOL led to FF-MAS accumulation (He *et al.* 2011) which is known to engage meiosis resumption (reviewed in Grondahl 2008). Finding down-regulated pro-proliferative genes as well as up- and down-regulated anti-proliferative genes supports the prediction by the IPA computing of activated cytostasis and inhibited proliferation in cumulus cells in the absence of basal LH. Luteinizing hormone is believed to maintain the differentiation of the follicle when FSH is low. However, the positive z-score of the biofunction '*Cellular development*', illustrated by the '*differentiation of cells*' annotation, indicates that LH is not working alone to insure the progressive differentiation of the follicle. Effectively, the oocyte drives the differentiation of the somatic follicular cells (Eppig *et al.* 2002) and is responsible for maintaining the cumulus cell phenotype (Diaz *et al.* 2007) through the secretion of oocyte-derived factors such as GDF9 or BMP15 (Sugiura *et al.* 2010). Otherwise, ATP6AP2 has also been shown to prevent differentiation (Wanka *et al.* 2017) and is here decreased by the inhibition of basal LH.

Luteinizing hormone is also assumed to take part in the avoidance of atresia in follicles. In the present study, this assumption was confirmed as the biofunction '*Cell death and survival*' exhibited activation of the '*Cell death*' process and inhibition of '*Cell survival*' in the absence of LH. When comparing our differentially expressed genes (DEG;  $p < 0.05$ ) to the list of those differentially expressed between healthy and atretic bovine follicles published by Hatzirodos *et al.* (2014), we found 282 common DEG. Out of these 282 DEG, the majority were regulated in the same direction in atretic follicles as in our basal LH inhibition treatment. The down-regulated DEG list included FSHR, MAD2, and PTTG1. In pig follicular cells, FSHR was down-regulated in the early stage of atresia (Pan *et al.* 2012) and MAD2 deregulation impaired proper meiosis I in mouse oocytes (Niault *et al.* 2007). The down-regulation of PTTG1 was associated with the failure of pregnancy after embryo transfer in bovine (El-Sayed *et al.* 2006). The up-regulated DEG list included CTGF, EGR1, and GADD45A. The growth factor CTGF is essential to normal follicular development and ovulation in the mouse

(Nagashima *et al.* 2011), EGR1 has been associated with follicular atresia (Yuan *et al.* 2016), and GADD45A has been identified as a potential marker of atresia in the pig (Terenina *et al.* 2017).

The transcriptomic analysis of CC revealed a very global role for LH at the cellular level. In fact, the absence of LH may result in energy deprivation in cumulus cells as shown by the predicted inhibited functions '*Synthesis of ATP*', '*Beta-oxidation of fatty acids*', and '*Degradation of mitochondria*'. Furthermore, the '*Nucleic acid metabolism*' functional category was also affected by the lack of LH as suggested by the inhibited state of '*Synthesis of purine nucleotide*' and '*Biosynthesis of nucleoside triphosphate*'. Cumulus cells are in fact of great importance for supplying energy to the oocyte. As reviewed by Dunning *et al.* (2014), fatty acid  $\beta$ -oxidation in cumulus cells and subsequent ATP production are crucial for optimal oocyte and embryo development. The RT-qPCR validation of the down-regulation of SC4MOL, in addition to the IPA analysis of our data suggest an interruption of the processing of cholesterol as SC4MOL (named also MSMO1) encodes for a key enzyme in the cholesterol synthesis pathway and is highly expressed in cumulus cells (Su *et al.* 2008). Furthermore, SC4MOL deficiency is associated with a decrease in total cholesterol in tumor cells (Gabitova *et al.* 2015).

Biofunctions concerning '*Gene expression*' such as '*Initiation of expression of RNA*' and '*Transcription of RNA*' were also repressed by the inhibition of LH secretion. Likewise, the '*Protein synthesis*' biofunction was negatively impacted as shown by an inhibited state for '*Synthesis of protein*' and an activated state for '*Catabolism of protein*'. Besides, the '*Cellular assembly and organisation*' category highlighted a specific function that was inhibited in absence of LH: '*Formation of vesicles*'. As shown by Macaulay *et al.* (2014), the trans-zonal projections of the cumulus cells present synapse-like endings with vesicular structures which may be responsible for the transport of large molecules such as mRNA. If this exchanging function between cumulus cells and the oocyte is impaired, the further development of the embryo could be affected.

### **3.6. Conclusion**

Even if the blastocyst rates of oocytes recovered following the optimized ovarian stimulation protocol with and without the Cetrorelix LH-inhibitor were not significantly different (Labrecque *et al.* 2014), consequences of the absence of LH during the last days of follicular maturation could appear later in embryo or fetus development. In fact, in human patients with more than two episodes of low LH, there was no differences in fertilization, implantation, clinical pregnancy, and live-birth rates but there was a significant increase in early pregnancy loss rates compared to patients with normal LH levels (Chen *et al.* 2016). In the mouse, the use of Cetrorelix to delay ovulation during stimulation cycles induced more resorption sites and lowered embryonic weight (Bittner *et al.* 2011). In this study, we showed that the use of a GnRH antagonist was not without impact on follicular cells, on cumulus cells in particular, and this impact should not be underestimated. Indeed, suppressing LH during one of the crucial steps of the final maturation of the ovarian follicle may have serious consequences on the full and correct molecular preparation of the oocyte. Finally, the molecular mechanism(s) by which LH influences the CC transcriptome is not yet fully understand and requires more investigations especially since it is not clear if LH receptors are expressed and functional in the bovine cumulus.

### **3.7. Materials and methods**

All reagents and media supplements used in these experiments were of tissue-culture grade and were obtained from Sigma Chemicals Co. (St. Louis, MO) unless otherwise indicated.

#### **3.7.1. Ovarian stimulation treatment, *in vitro* production, and recovery of cumulus-oocyte complexes (COC) from super-ovulated animals**

Ovarian stimulation, *in vitro* embryo production, and COC recovery were done as described in Nivet *et al.* 2012. Briefly, six dairy cows were stimulated with FSH given twice daily for three days followed by an FSH withdrawal period of 68h « 68h condition ». For the « 68h +Cetro » condition, the GnRH agonist Cetorelix (Cetrotide, Merckx-Serono), which inhibits LH secretion, was given from day 2 of FSH stimulation until COC recovery 68h following the last FSH injection (12mg per day for 5 days, [Figure 3-1](#)). For each sampling session, half of the recovered COC were used for *in vitro* embryo production, and the remaining COC were kept for RNA analysis. Blastocyst rates were determined at day 8 post-fertilization.

#### **3.7.2. Cumulus cell retrieval**

The recovered COC were washed thrice in TLH solution and were then collected in Petri dishes containing PBS and polyvinyl alcohol (PVA). The COC were washed three times with PBS-PVA in different dishes, transferred into 1.5-ml conical tubes, and vortexed in 150µl PBS for 2min. Cells were placed into a new dish to collect oocytes and cumulus cells (CC) separately into new 1.5-ml conical tubes. The CC were washed by centrifugation three times in 150µl PBS. Cells were then snap frozen in liquid nitrogen and conserved at -80°C.

#### **3.7.3. RNA extraction**

Total cumulus cell RNA was extracted using silica-based ion exchange columns (PicoPure RNA Isolation kit, Life Technologies, Foster City, CA, USA) following the manufacturer's protocol. An on-column DNase treatment was used to remove contaminating genomic DNA (Qiagen, Mississauga, ON, Canada) while nucleic acids were bound to the column. The RNA was eluted with the minimum recommended volume of 11µl.

The concentration and quality of RNA were assessed using a 2100 Bioanalyzer apparatus (Agilent Technologies, Santa Clara, CA, USA) and samples were run on RNA Pico Chips (Agilent Technologies). All samples used had an RNA Integrity Number  $\geq 7.8$ .

#### **3.7.4. RNA processing for microarray analysis**

Three of the six biological replicates were used for microarray analysis and 5 ng of their total RNA were amplified using T7 RNA polymerase (RiboAmp HS RNA Amplification kit, Life Science) according to the manufacturer's indications. A fixed amount of 2 $\mu$ g aRNA per array was labeled indirectly using ULS aRNA Fluorescent Labeling (ULS Fluorescent Labeling Kit for Agilent arrays, Kreatech Biotechnology, Amsterdam, the Netherlands) according to the manufacturer's protocol. To purify aRNA samples, the PicoPure RNA Isolation kit (Life Science) was used. Amplification and labelling efficiency were measured using the Nano Drop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The two treatments were compared using a 2-color dye swap on Agilent-manufactured EmbryoGENE slides (Robert *et al.* 2011). Hybridizations were performed according to the manufacturer's protocol (Agilent) using 825ng of labeled aRNA per replicate. After the washing steps, slides were scanned with a PowerScanner (Tecan, Männedorf, Switzerland) and images were analysed with the ArrayPro 6.3 software (MediaCybernetics, Bethesda, MD, USA).

#### **3.7.5. Microarray data normalization and statistical analysis**

Signal intensity data files were normalized and analyzed using the FlexArray 1.6.1 software (Genome Quebec, URL: <http://genomequebec.mcgill.ca/FlexArray>). The first step in the data processing was to remove the background of intensity files using a simple background subtraction. Data were then normalized for dye bias using a within-array loess and a between-array « quantile » normalization to minimize array effects. To calculate fold changes of probe intensity, normalized data were assessed using the e-Bayes moderated t-test (LIMMA) included in the FlexArray software. A False Discovery Rate algorithm (Benjamini-Hochberg) was also applied.

#### **3.7.6. RNA processing for qRT-PCR and statistical analysis**

Quantitative RT-PCR was performed on the six biological replicates. Total RNA (1 ng) was reverse transcribed using the qScript Flex cDNA Synthesis Kit with a mix of oligo-dT (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's instructions. At the end of the reverse transcription, 40 $\mu$ l of nuclease-free water were added to the final 20 $\mu$ l reaction.

Primers used for qRT-PCR were designed from sequences based on the UMD3.1/bosTau5 assembled version of the bovine genome using the IDT PrimerQuest web interface (<http://www.idtdna.com/Scitools/Applications/Primerquest/>).

For each sample and each primer pair, 2 $\mu$ l of cDNA were used to perform quantitative polymerase chain reaction on a LightCycler 480 apparatus with LightCycler 480 SYBR Green I Master (Roche Applied Science, Laval, QC, Canada). Reactions were performed in 96-well plastic plates in a final volume of 20 $\mu$ l (Roche Applied Science). The reaction mix contained 7 $\mu$ l of PCR grade water, 10 $\mu$ l of SYBR Green I Master, and 0.25mM of each primer. For each candidate gene tested, PCR products were observed on 2% agarose gels, extracted with the QIAquick PCR Purification kit (Qiagen), quantified with a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies), and sequenced to confirm the specificity of each primer pair. For quantification experiments, standard curves were built using the purified and confirmed PCR products diluted from  $2 \times 10^{-4}$  to  $2 \times 10^{-8}$ ng/ $\mu$ l. The PCR conditions applied for all genes were as follows: a denaturing cycle of 10min at 95°C; 50 PCR cycles (denaturing, 95°C for 5s; annealing at the adapted temperature for 5s; elongation, 72°C for 30s; acquisition at the adapted temperature for 1s), a melting curve at 95°C for 1s, 65°C for 5s and a step cycle starting at 65°C up to 95°C at 0.1°C/s, and a final cooling step at 40°C. Data normalization was performed with GeNORM normalization factor ([Vandesompele et al. 2002](#)) from the expression values of two reference genes (ACTB, GAPDH).

To determine whether the mRNA content in cumulus cells was significantly different between the coasting conditions, one-way ANOVA and Newman-Keuls multiple comparison tests with a  $p$ -value < 0.5 were performed using Prism 5.02 (GraphPad software, La Jolla, CA).

### 3.7.7. Statistical Analysis of COC Morphology

To assess the statistically significant differences in COC morphology between each coasting conditions and each COC categories, two-way ANOVA and Bonferroni post-tests with a  $p$ -value < 0.5 were performed using Prism 5.02 (GraphPad software).

### 3.8. References

- Al-Inany HG, Abou-Setta AM, Aboulghar M. 2007. Gonadotrophin-releasing hormone antagonists for assisted conception: a Cochrane review. *Reproductive biomedicine online* 14(5):640-649.
- Bergfeld EG, D'Occio MJ, Kinder JE. 1996. Pituitary function, ovarian follicular growth, and plasma concentrations of 17 beta-estradiol and progesterone in prepubertal heifers during and after treatment with the luteinizing hormone-releasing hormone agonist deslorelin. *Biology of reproduction* 54(4):776-782.
- Bittner AK, Horsthemke B, Winterhager E, Grummer R. 2011. Hormone-induced delayed ovulation affects early embryonic development. *Fertility and sterility* 95(7):2390-2394.
- Blondin P, Bousquet D, Twagiramungu H, Barnes F, Sirard MA. 2002. Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biology of reproduction* 66(1):38-43.
- Bunel A, Nivet AL, Blondin P, Vigneault C, Richard FJ, Sirard MA. 2013. Cumulus cell gene expression associated with pre-ovulatory acquisition of developmental competence in bovine oocytes. *Reproduction, fertility, and development* 26(6):855-865.
- Casarini L, Reiter E, Simoni M. 2016. beta-arrestins regulate gonadotropin receptor-mediated cell proliferation and apoptosis by controlling different FSHR or LHCGR intracellular signaling in the hGL5 cell line. *Molecular and cellular endocrinology* 437:11-21.
- Chang HJ, Lee JR, Jee BC, Suh CS, Lee WD, Kim SH. 2013. Cessation of gonadotropin-releasing hormone antagonist on triggering day in flexible multiple-dose protocol: A randomized controlled study. *Clinical and experimental reproductive medicine* 40(2):83-89.
- Chen CD, Chiang YT, Yang PK, Chen MJ, Chang CH, Yang YS, Chen SU. 2016. Frequency of low serum LH is associated with increased early pregnancy loss in IVF/ICSI cycles. *Reproductive biomedicine online* 33(4):449-457.
- Devjak R, Fon Tacer K, Juvan P, Virant Klun I, Rozman D, Vrtacnik Bokal E. 2012. Cumulus cells gene expression profiling in terms of oocyte maturity in controlled ovarian hyperstimulation using GnRH agonist or GnRH antagonist. *PloS one* 7(10):e47106.
- Diaz FJ, Wigglesworth K, Eppig JJ. 2007. Oocytes determine cumulus cell lineage in mouse ovarian follicles. *Journal of cell science* 120(Pt 8):1330-1340.
- Dunning KR, Russell DL, Robker RL. 2014. Lipids and oocyte developmental competence: the role of fatty acids and beta-oxidation. *Reproduction* 148(1):R15-27.
- El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, Sirard MA, Schellander K, Tesfaye D. 2006. Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiological genomics* 28(1):84-96.
- Eppig JJ, Wigglesworth K, Pendola FL. 2002. The mammalian oocyte orchestrates the rate of ovarian follicular development. *Proceedings of the National Academy of Sciences of the United States of America* 99(5):2890-2894.
- Gabitova L, Restifo D, Gorin A, Manocha K, Handorf E, Yang DH, Cai KQ, Klein-Szanto AJ, Cunningham D, Kratz LE, Herman GE, Golemis EA, Astsaturov I. 2015. Endogenous Sterol Metabolites Regulate Growth of EGFR/KRAS-Dependent Tumors via LXR. *Cell reports* 12(11):1927-1938.
- Gilchrist RB, Ritter LJ, Armstrong DT. 2004. Oocyte-somatic cell interactions during follicle development in mammals. *Animal reproduction science* 82-83:431-446.
- Grondahl C. 2008. Oocyte maturation. Basic and clinical aspects of *in vitro* maturation (IVM) with special emphasis of the role of FF-MAS. *Danish medical bulletin* 55(1):1-16.
- Gupta C, Chapekar T, Chhabra Y, Singh P, Sinha S, Luthra K. 2012. Differential response to sustained stimulation by hCG & LH on goat ovarian granulosa cells. *The Indian journal of medical research* 135:331-340.
- Hatzirodos N, Hummitzschi K, Irving-Rodgers HF, Harland ML, Morris SE, Rodgers RJ. 2014. Transcriptome profiling of granulosa cells from bovine ovarian follicles during atresia. *BMC genomics* 15:40.
- He M, Kratz LE, Michel JJ, Vallejo AN, Ferris L, Kelley RI, Hoover JJ, Jukic D, Gibson KM, Wolfe LA, Ramachandran D, Zwick ME, Vockley J. 2011. Mutations in the human SC4MOL gene encoding a methyl sterol oxidase cause psoriasisform dermatitis, microcephaly, and developmental delay. *The Journal of clinical investigation* 121(3):976-984.
- He M, Smith LD, Chang R, Li X, Vockley J. 2014. The role of sterol-C4-methyl oxidase in epidermal biology. *Biochimica et biophysica acta* 1841(3):331-335.
- Labrecque R, Vigneault C, Blondin P, Sirard MA. 2014. Gene expression analysis of bovine oocytes at optimal cooasting time combined with GnRH antagonist during the no-FSH period. *Theriogenology* 81(8):1092-1100.

- Macaulay AD, Gilbert I, Caballero J, Barreto R, Fournier E, Tossou P, Sirard MA, Clarke HJ, Khandjian EW, Richard FJ, Hyttel P, Robert C. 2014. The gametic synapse: RNA transfer to the bovine oocyte. *Biology of reproduction* 91(4):90.
- Maclellan LJ, Bergfeld EG, Earl CR, Fitzpatrick LA, Aspden WJ, Kinder JE, Walsh J, Trigg TE, D'Occhio MJ. 1997. Influence of the luteinizing hormone-releasing hormone agonist, deslorelin, on patterns of estradiol-17 beta and luteinizing hormone secretion, ovarian follicular responses to superstimulation with follicle-stimulating hormone, and recovery and *in vitro* development of oocytes in heifer calves. *Biology of reproduction* 56(4):878-884.
- Madill S, Rieger D, Johnson WH, Walton JS, Coy DH, Rawlings NC. 1994. Effects of an LHRH antagonist on the time of occurrence and amplitude of the preovulatory LH surge, progesterone and estradiol secretion, and ovulation in superovulated Holstein heifers. *Theriogenology* 41(4):951-960.
- Mihm M, Baker PJ, Ireland JL, Smith GW, Coussens PM, Evans AC, Ireland JJ. 2006. Molecular evidence that growth of dominant follicles involves a reduction in follicle-stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle. *Biology of reproduction* 74(6):1051-1059.
- Nagashima T, Kim J, Li Q, Lydon JP, DeMayo FJ, Lyons KM, Matzuk MM. 2011. Connective tissue growth factor is required for normal follicle development and ovulation. *Mol Endocrinol* 25(10):1740-1759.
- Niault T, Hached K, Sotillo R, Sorger PK, Maro B, Benezra R, Wassmann K. 2007. Changing Mad2 levels affects chromosome segregation and spindle assembly checkpoint control in female mouse meiosis I. *PloS one* 2(11):e1165.
- Nivet AL, Vigneault C, Blondin P, Sirard MA. 2017. Influence of luteinizing hormone support on granulosa cells transcriptome in cattle. *Anim Sci J* doi: 10.1111/asj.12856.
- Nivet AL, Bunel A, Labrecque R, Belanger J, Vigneault C, Blondin P, Sirard MA. 2012. FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction* 143(2):165-171.
- Oussaid B, Lonergan P, Khatir H, Guler A, Monniaux D, Touze JL, Beckers JF, Cognie Y, Mermilliod P. 2000. Effect of GnRH antagonist-induced prolonged follicular phase on follicular atresia and oocyte developmental competence *in vitro* in superovulated heifers. *Journal of reproduction and fertility* 118(1):137-144.
- Oussaid B, Mariana JC, Poulin N, Fontaine J, Lonergan P, Beckers JF, Cognie Y. 1999. Reduction of the developmental competence of sheep oocytes by inhibition of LH pulses during the follicular phase with a GnRH antagonist. *Journal of reproduction and fertility* 117(1):71-77.
- Pan Z, Zhang J, Lin F, Ma X, Wang X, Liu H. 2012. Expression profiles of key candidate genes involved in steroidogenesis during follicular atresia in the pig ovary. *Molecular biology reports* 39(12):10823-10832.
- Perugini M, Kok CH, Brown AL, Wilkinson CR, Salerno DG, Young SM, Diakiw SM, Lewis ID, Gonda TJ, D'Andrea RJ. 2009. Repression of Gadd45alpha by activated FLT3 and GM-CSF receptor mutants contributes to growth, survival and blocked differentiation. *Leukemia* 23(4):729-738.
- Robert C, Gagne D, Lussier JG, Bousquet D, Barnes FL, Sirard MA. 2003. Presence of LH receptor mRNA in granulosa cells as a potential marker of oocyte developmental competence and characterization of the bovine splicing isoforms. *Reproduction* 125(3):437-446.
- Robert C, Nieminen J, Dufort I, Gagne D, Grant JR, Cagnone G, Plourde D, Nivet AL, Fournier E, Paquet E, Blazejczyk M, Rigault P, Juge N, Sirard MA. 2011. Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays. *Molecular reproduction and development* 78(9):651-664.
- Sirard MA. 2016. Somatic environment and germinal differentiation in antral follicle: The effect of FSH withdrawal and basal LH on oocyte competence acquisition in cattle. *Theriogenology* 86(1):54-61.
- Stocco C, Baumgarten SC, Armouti M, Fierro MA, Winston NJ, Scoccia B, Zamah AM. 2017. Genome-wide interactions between FSH and insulin-like growth factors in the regulation of human granulosa cell differentiation. *Hum Reprod* 32(4):905-914.
- Su YQ, Sugiura K, Wigglesworth K, O'Brien MJ, Affourtit JP, Pangas SA, Matzuk MM, Eppig JJ. 2008. Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. *Development* 135(1):111-121.
- Sugiura K, Su YQ, Li Q, Wigglesworth K, Matzuk MM, Eppig JJ. 2010. Estrogen promotes the development of mouse cumulus cells in coordination with oocyte-derived GDF9 and BMP15. *Mol Endocrinol* 24(12):2303-2314.
- Terenina E, Fabre S, Bonnet A, Monniaux D, Robert-Granie C, SanCristobal M, Sarry J, Vignoles F, Gondret F, Monget P, Tosser-Klopp G. 2017. Differentially expressed genes and gene networks involved in pig ovarian follicular atresia. *Physiological genomics* 49(2):67-80.
- Ulker H, Gant BT, de Avila DM, Reeves JJ. 2001. LHRH antagonist decreases LH and progesterone secretion but does not alter length of estrous cycle in heifers. *Journal of animal science* 79(11):2902-2907.

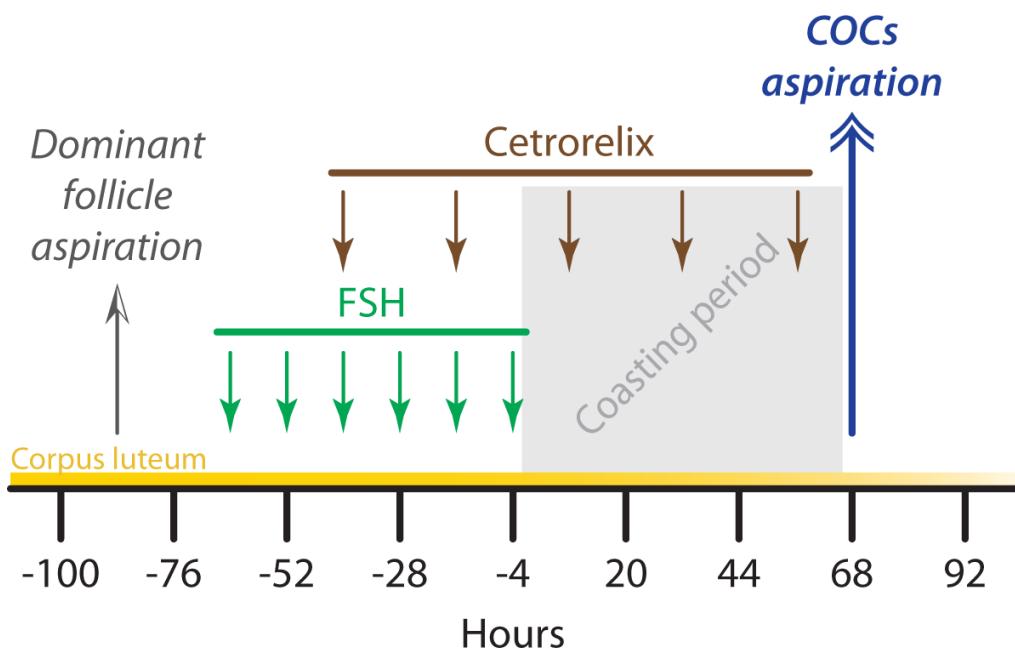
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* 3(7):RESEARCH0034.
- Wanka H, Lutze P, Staar D, Peters B, Morch A, Vogel L, Chilukoti RK, Homuth G, Sczodrok J, Baumgen I, Peters J. 2017. (Pro)renin receptor (ATP6AP2) depletion arrests As4.1 cells in the G0/G1 phase thereby increasing formation of primary cilia. *Journal of cellular and molecular medicine*.
- Yuan S, Wen J, Cheng J, Shen W, Zhou S, Yan W, Shen L, Luo A, Wang S. 2016. Age-associated up-regulation of EGR1 promotes granulosa cell apoptosis during follicle atresia in mice through the NF-kappaB pathway. *Cell Cycle* 15(21):2895-2905.

### 3.9. Tables

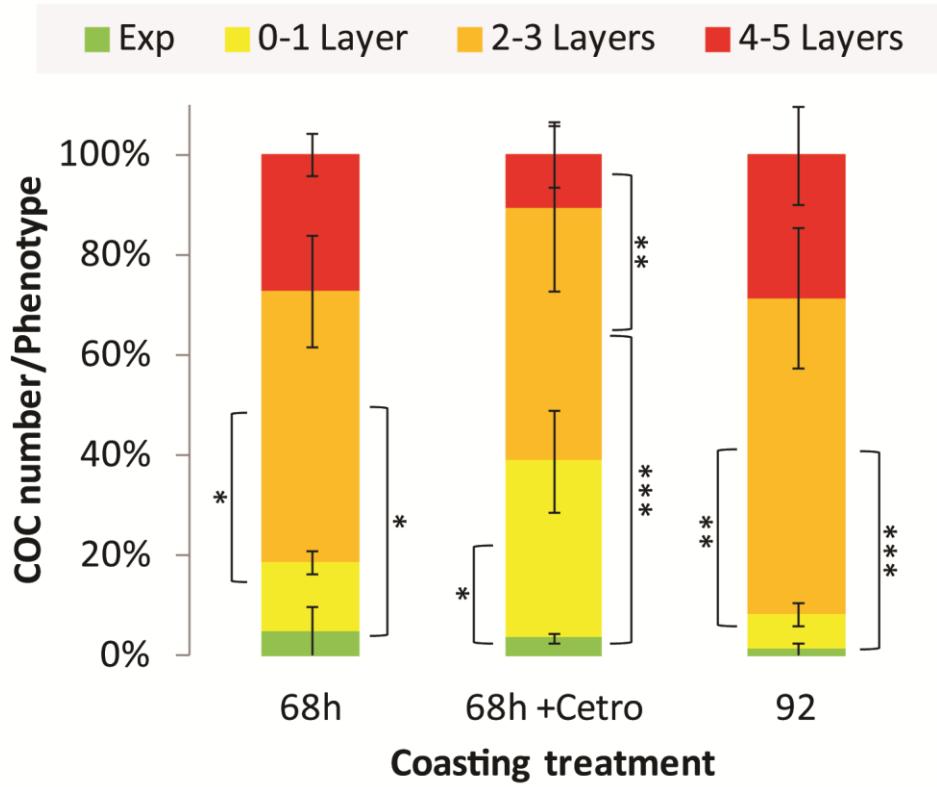
**Table 3-1. Cumulus cell portrait of the main cellular functions affected by the absence of LH using functional categories and annotations of IPA software.**

IPA Functional categories and sub-Annotations	Activation z-score	Molecules included	P-value	Predicted activation status
<b><i>Cell Cycle</i></b>				
Duplication of centrosome	-2.224	18	1.42 E-3	Inhibited
<b><i>Cell Death and Survival</i></b>				
Cell death	2.640	1027	3.62E-31	Activated
Cell survival	-1.503	398	2.42E-09	
<b><i>Cellular Growth and Proliferation</i></b>				
Proliferation of cells	-3.608	1040	5.74E-20	Inhibited
Cytostasis	0.932	78	2.24E-04	
<b><i>Cellular Development</i></b>				
Differentiation of cells	0.990	562	1.67E-03	
<b><i>Cellular Assembly and Organization</i></b>				
Formation of vesicles	-2.107	50	8.05E-08	Inhibited
<b><i>Cellular Movement</i></b>				
Cellular movement of fibroblast cell lines	2.159	59	2.63E-04	Activated
<b><i>Energy production</i></b>				
Synthesis of ATP	-1.662	28	1.35E-03	
Beta-oxidation of fatty acids	-0.328	23	1.66E-03	
Degradation of mitochondria	-1.569	17	1.52E-03	
<b><i>Gene expression</i></b>				
Initiation of expression of RNA	-1.819	32	5.15E-05	
Transcription of RNA	-0.975	561	2.20E-17	
<b><i>Protein Synthesis</i></b>				
Synthesis of protein	-1.731	139	1.95E-08	
Catabolism of protein	1.119	175	2.98E-07	
<b><i>Nucleic Acid Metabolism</i></b>				
Synthesis of purine nucleotide	-2.253	53	1.21E-04	Inhibited
Metabolism of purine nucleotide	-2.186	31	1.30E-04	Inhibited
Biosynthesis of nucleoside triphosphate	-1.836	38	5.59E-06	
<b><i>Molecular transport</i></b>				
Transport of molecule	0.339	411	1.12E-03	
Transport of RNA	-0.277	22	1.02E-03	
Transport of protein	1.054	103	3.51E-10	

### 3.10. Figures

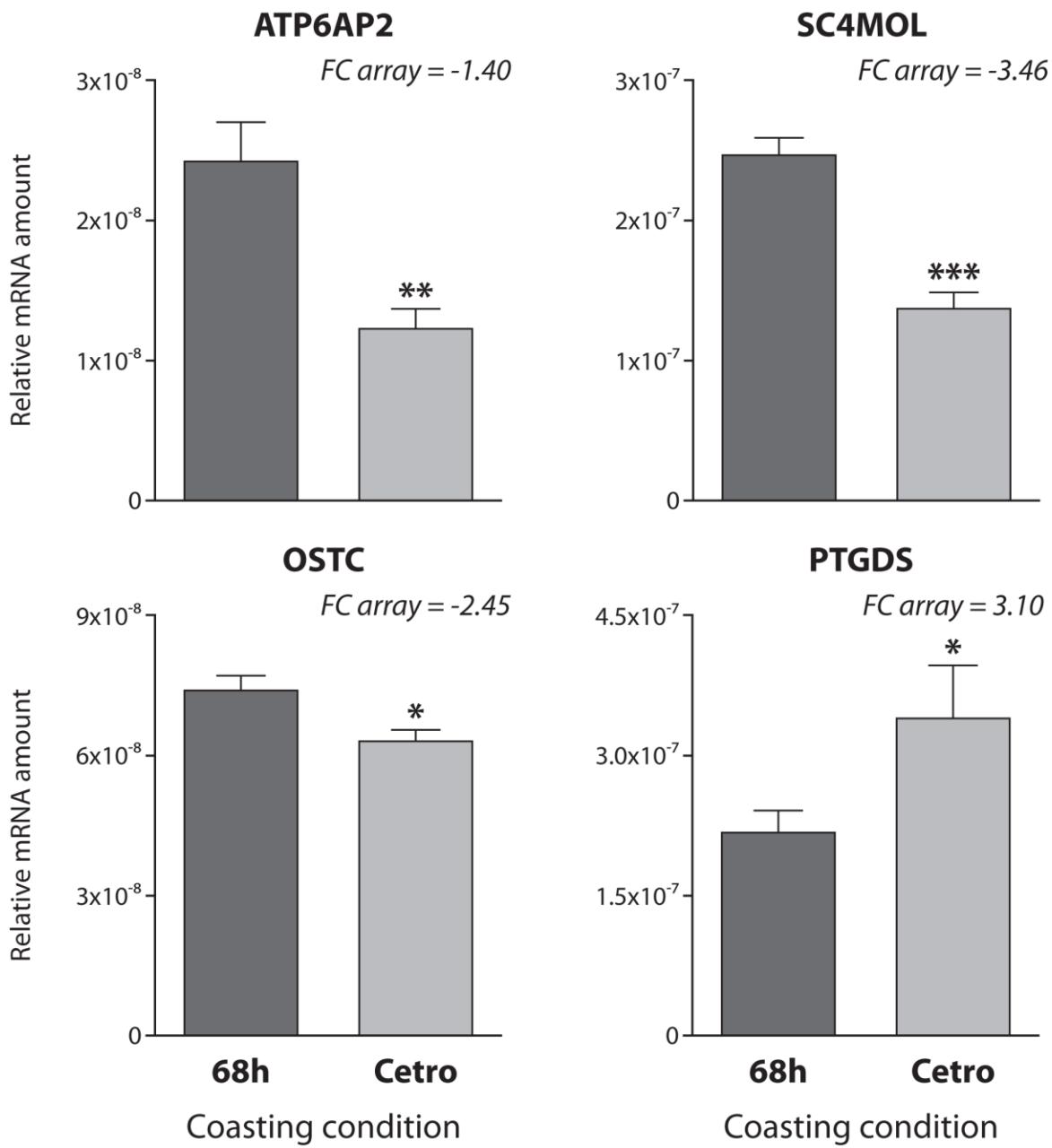


*Figure 3-1. Ovarian stimulation protocol used to assess the effects of LH support during the final follicle growth and differentiation in bovine.*



**Figure 3-2. Morphological annotation of the recovered COC at the different FSH withdrawal durations with or without Cetrorelix.**

COC were categorized in 4 classes depending on the approximate number of cumulus cell layers around the oocyte (4-5, 2-3, 0-1 layers), and whether the cumulus was already expanded (Exp). The 92 h condition results come from our previous study on the same cows, using the same protocol (Bunel *et al.* 2013). No significant difference was observed between the three coasting conditions.  $n = 5$  and data are mean  $\pm$  SEM, \* and #  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



**Figure 3-3.** Cumulus cell mRNA quantification by qRT-PCR at 68 h of FSH withdrawal with or without Cetrorelix.

$n = 6$  and data are mean  $\pm$  SEM, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . FC: Fold change from the microarray assay.

**4. ANALYSIS OF LHCGR AND SELECTED STEROID ENZYME mRNA EXPRESSION IN  
BOVINE CUMULUS CELLS DURING *IN VITRO* MATURATION.**

Audrey Bunel<sup>1</sup>, Annie Girard<sup>1</sup>, Isabelle Dufort<sup>1</sup> and Marc-André Sirard<sup>1</sup>

<sup>1</sup> Centre de recherche en reproduction, développement et santé inter-générationnelle,, Faculté des sciences de l'agriculture et de l'alimentation, Département des sciences animales, Université Laval, Québec, Canada.

#### **4.1. Résumé**

*In vivo*, lors de la phase folliculaire, les concentrations basales de LH ont une influence sur le transcriptome de l'ensemble du follicule en différentiation finale. Toutefois, dans le cadre de la maturation *in vitro* (MIV) d'ovocytes de mammifères, les complexes ovocyte-cumulus ne sont récupérés qu'à partir de follicules immatures, de taille inférieure à celle de l'établissement de la dominance. Les milieux de culture utilisés pour cette maturation contiennent habituellement de la FSH dont l'utilité est bien connue, tandis que l'impact bénéfique de la LH est controversé en partie parce que la présence de son récepteur reste incertaine. Nous avons ainsi exploré l'expression du transcrit du Luteinizing Hormone/ Choriogonadotropin Receptor (LHCGR) et de ses isoformes, ainsi que l'un de ses régulateurs, la mévalonate kinase (MVK), toutes les trois heures pendant la MIV, de zéro à 24 heures. Parallèlement, l'expression génique d'enzymes clés de la stéroïdogenèse influencées par la présence de LH (STAR, CYP11A1, HSD3 $\beta$ ) pour la production de progestérone (P4) et l'expression de son récepteur (PGR) ont été évaluées à chaque temps. Aucun transcrit LHCGR n'a été détecté dans les CC à aucun temps de la MIV, alors que l'expression de MVK a augmenté significativement à 9, 12 et 15 heures de maturation. Les ARNm de STAR et HSD3 $\beta$  étaient faiblement exprimés tandis que les ARNm de CYP11A1 et PGR ont significativement augmenté à partir de 9 heures de MIV. Par conséquent, en absence d'expression de LHCGR, la supplémentation des milieux de maturation avec de la LH ne paraît pas pertinente. De plus, même sans ajout de LH dans le milieu de MIV, les cellules du cumulus semblent capables d'utiliser le cholestérol pour la stéroïdogenèse, particulièrement pour la production de prégnénolone, et pourraient répondre à une stimulation par la P4 car elles expriment son récepteur.

## 4.2. Abstract

*In vivo*, during follicular phase, at the final differentiation time, basal LH concentrations are influencing the whole follicle transcriptome. However, for the purpose of *in vitro* maturation (IVM) of mammalian oocytes, cumulus-oocyte complexes are recovered only from immature follicles, at diameters before dominance establishment. Media used for maturation normally contain FSH for which the usefulness is well established, whereas any beneficial impact of LH is controversial partly because the presence of its receptor on cumulus cells (CC) remains uncertain. Then, we assessed the mRNA expression of the Luteinizing Hormone/Choriogonadotropin Receptor (LHCGR) and its isoforms, as well as one of its regulators, the mevalonate kinase (MVK), every three hours during IVM, from zero to 24 hours. In parallel, the expression of genes for key steroidogenic enzymes for progesterone (P4) production (STAR, CYP11A1, HSD3 $\beta$ ) and the expression of the P4 receptor (PGR) were assessed at each of those time points. No LHCGR mRNA was detected in CC at any time during maturation, while the expression of MVK was significantly increased at 9, 12, and 15 hours of maturation. The STAR and HSD3 $\beta$  mRNAs were weakly expressed while CYP11A1 and PGR mRNAs were significantly increased from 9 hours of IVM. Therefore, because of the lack of LHCGR expression, maturation media supplementation with LH does not seem to be relevant. Furthermore, even without LH addition, in IVM media, cumulus cells appear to be able to use cholesterol for steroidogenesis, especially for pregnenolone production, and could respond to P4 stimulation as they express its receptor.

### 4.3. Introduction

The luteinizing hormone (LH) plays a crucial role in reproduction in most animal species as it triggers the final maturation of oocytes and ovulation. For *in vitro* maturation (IVM) of bovine oocytes, cumulus-oocyte complexes (COCs) are aspirated from small and medium non-preovulatory follicles and placed into culture. In this context, resumption of meiosis is triggered automatically by the removal of the follicular inhibition (Pincus and Enzmann 1935).

In some species, LH receptors are present on cumulus cells (CC) at the beginning or acquired during maturation. Recently, transcriptomes of *in vivo* GnRH antagonist-treated granulosa cells (GC) and *in vivo* 6 hours post-LH-GC have been compared. It revealed 64 common basal LH associated genes, which were related to functions such as cell cycle, Wnt signaling, angiogenesis and reproduction (Sirard 2016). In a following study, we equally found that the transcriptome of bovine CC is influenced *in vivo* when basal LH secretion is inhibited during FSH based ovarian stimulation (Bunel *et al.* in preparation). Also, the capacity of CC to synthesize progesterone post-ovulation (Chian *et al.* 1999) was proposed to be linked to the presence of LH receptors on them (Rispoli *et al.* 2013). Nevertheless, even if LH has an impact on CC, it is not clear whether LH receptors are expressed by these bovine cells.

In early studies, bovine oocyte maturation was improved when LH was added to IVM media (Brackett *et al.* 1989; Zuelke and Brackett 1990). However, at that time, only affinity purified LH contaminated with FSH was available (reviewed in Lunenfeld 2004). The increase of *in vitro* meiotic resumption rates and the improved competence of bovine oocytes observed with very high amounts of purified LH (100 $\mu$ g/ml) could have been caused by the presence of FSH since the FSH receptor mRNA is present while the LH receptor mRNA was undetected in cumulus cells (van Tol *et al.* 1996). The developmental competence of yak (*Bos grunniens*) oocytes following IVM was also improved when using FSH compared to LH, and this could be explained by higher expression of the FSH receptor compared to the LH receptor mRNA (Xiao *et al.* 2014).

The LH surge results in a change in steroid secretion from estradiol to progesterone in granulosa cells from pre-ovulatory follicles (Dieleman *et al.* 1983). Does this change happen also in cumulus cells and would it be mediated by LH? To clarify this issue, we studied the kinetics of the Luteinizing Hormone/Choriogonadotropin Receptor (LHCGR) gene expression in bovine cumulus cells at different times during *in vitro* maturation of COCs. We also studied the expression of key steroidogenic enzymes to determine if cumulus cells could be able to produce progesterone.

## 4.4. Results

### 4.4.1. LHCGR and MVK mRNA expression during *in vitro* maturation

Constitutive LHCGR mRNA (NM\_174381.1, named « 5' ») and the isoforms X3, X5 and X7 were found in *corpus luteum* but were not found in cumulus cells throughout the maturation period ([Figure 4-1 a](#)). Mevalonate kinase (MVK) expression was assessed due to its ability to target and promote LHCGR mRNA degradation (Menon *et al.* 2006). The MVK mRNA was found in cumulus cells and showed a bell-shaped pattern of expression during the 24h of IVM. Significant differences in MVK expression appeared between the 4°C control condition and the 9, 12, and 15h IVM time points ( $p<0.01$ ,  $p<0.05$ , and  $p<0.05$ , respectively). The same significant differences took place between the 0h IVM time point and the 9, 12, and 15h IVM time points ([Figure 4-1 b](#)).

### 4.4.2. Steroidogenic enzyme and progesterone receptor mRNA expression during *in vitro* maturation

The STAR mRNA was expressed in bovine cumulus cells during IVM but there was no significant variation ([Figure 4-2](#)). The CYP11A1 mRNA was expressed with a significant increase starting at 9h of IVM ( $p<0.01$  compared to the 4°C condition and to the 0h time point) and continuing until 18h of IVM ([Figure 4-2](#)). The expression of HSD3b mRNA was significantly higher in the 4°C control condition, *i.e.* at slaughter time, compared to all the IVM time points ( $p<0.001$ , [Figure 4-2](#)). The PGR mRNA expression was significantly higher at 9 h ( $p<0.01$ ) and 18 h of IVM ( $p<0.5$  compared to 4°C,  $p<0.01$  compared to 0h, [Figure 4-2](#)).

#### 4.5. Discussion

This study described the expression of LH receptor variants in bovine cumulus cells during *in vitro* maturation as a possible cause of specific steroidogenic enzyme regulation. Indeed, the changes observed in cumulus cell gene expression during IVM could be due to the appearance of the LH receptor in the wake of the spontaneous changes associated with the release of follicular inhibition or oocyte meiotic maturation. A previous study (Robert *et al.* 2003) explored the presence of LHCGR as a marker of follicular competence but failed to observe any of the isoforms of the LHCGR in cumulus cells of GV stage oocytes (immature). The LHCGR mRNA was not detected in bovine cumulus cells by *in situ* hybridization (Peng *et al.* 1991); it was detected by RT-PCR in one study (Calder *et al.* 2003) but not in other studies (van Tol *et al.* 1996, Robert *et al.* 2003). Additionally, radio-labeled LH binding was observed in bovine cumulus cells with a negative correlation to follicular size (Baltar *et al.* 2000); however, the presence of the receptor itself was not determined. The LH receptor protein was detected on immature bovine COCs by immunohistochemistry (Silvestre *et al.* 2012); but a human polyclonal antibody was used, so its specificity can be questioned. Surprisingly, without precise knowledge of its receptor expression in COCs, LH or CG (chorio-gonadotrophin, LH-like activity) is often used in IVM culture media (Brackett *et al.* 1989, Jang *et al.* 2010, Takada *et al.* 2012, Wang *et al.* 2013, Franciosi *et al.* 2014, Chasombat *et al.* 2015, Boruszewska *et al.* 2015, Bernal-Ulloa *et al.* 2016).

In addition to the constitutive form, the NCBI database predicts 7 isoforms of the LHCGR mRNA; however, isoforms X4 and X6 are not distinguishable from the other isoforms by conventional qRT-PCR. Using cumulus cell test samples and a positive control (*i.e. corpus luteum*), we were not able to find efficient primers for isoforms X1 and X2. Nevertheless, we were able to design suitable primers for isoforms X3, X5, and X7. These isoforms were found in the *corpus luteum*; however, they were not expressed in bovine cumulus cells during IVM, ruling out a contribution of this signaling pathway in the activation of the selected steroidogenic enzymes.

The regulation of the LHCGR is not simple even if this gene is highly conserved (Tu *et al.* 2012). The oocyte can inhibit the LHCGR expression in cumulus cells while it is expressed in the nearby and connected granulosa cells (mouse, Eppig *et al.* 1997). In 1998, Kash *et al.* (1998) discovered a protein that specifically binds to four different rat LHCGR transcripts. This linkage leads to post-transcriptional regulation of LHCGR expression by mRNA degradation. The authors later identified this binding protein as the mevalonate kinase (MVK) (Nair and Menon 2004), a key enzyme of cholesterol synthesis. The same mechanism was found in human granulosa cells (Wang *et al.* 2007). In the present study, the expression of the MVK mRNA increased at the beginning of maturation and

then declined while STAR and HSD3b expression started to rise (no significant correlation). The probability of the involvement of the MVK in the absence of LHCGR expression is low since the MVK bell-shape expression profile was not associated with an inverse distribution of LHCGR expression in cumulus cells.

The lack of LHCGR expression and the key role of MVK in cholesterol synthesis associated to the variations in its expression raised questions about its role in the steroidogenic activity of cumulus cells. Therefore, we looked at the expression of the first steroidogenic key enzymes: STAR and CYP11A1. Cholesterol requires the expression of STAR to enter mitochondria where it is converted to pregnenolone by CYP11A1. STAR was slightly expressed without major variation during IVM while CYP11A1 mRNA increased significantly starting at 9h of IVM. The presence of STAR mRNA and the increase of CYP11A1 expression suggest a possible use of cholesterol for steroidogenesis by cumulus cells. Bovine cumulus cells were previously shown to produce progesterone (P4) and blocking its synthesis by inhibition of HSD3b activity was related to poor blastocyst formation rates, without affecting fertilization nor cleavage rates (Aparicio *et al.* 2011). In the same study, blastocyst rates were rescued by the addition of exogenous P4. In the present study, HSD3b expression was weak but present. Progesterone receptors (PGR) are required for the P4 intracellular signaling to be effective (Aparicio *et al.* 2011). In our study, PGR mRNA started to be expressed at 6h of IVM and was maintained until the end. The production of progesterone by cumulus cells could have a beneficial effect on the oocyte directly (see Aparicio *et al.* 2011). Progesterone production could also be a post-ovulatory asset of the cumulus cells to signal the ovulation process to the oviduct. Indeed, in bovine embryos, progesterone supplementation up-regulated the non-classical-major histocompatibility complex class I (Al Naib *et al.* 2011) which is associated with endometrial re-modeling, angiogenesis, and maternal tolerance of the conceptus. In addition to signal ovulation, post-ovulatory progesterone from cumulus cells could help the release of sperm from the isthmus as observed in pigs (Bureau *et al.* 2002).

#### **4.6. Conclusion**

In conclusion, under the present experimental conditions of IVM, cumulus cells did not express the LH receptor. This brings interrogations about the utility of LH in bovine IVM media, and indicates that steroidogenic enzyme expression is not under the influence of the LH receptor. Also, albeit the uncertainty of P4 production by bovine CCs here, they are probably able to respond to P4 stimulation since they express its receptor. Finally, as LH was shown to have a transcriptomic influence on GC (Nivet *et al.* 2017; Gilbert *et al.* 2011) and on CC (Bunel *et al.* in preparation) *in vivo*; and as its receptor seems to not be expressed in these cells, at least *in vitro*, more research is needed to better understand how the LH signal would be transmitted from the GC to the CC in order to improve conditions used for bovine COC IVM.

## **4.7. Materials and methods**

All reagents and media supplements used in these experiments were of tissue-culture grade and were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise stated.

### **4.7.1. Cumulus-Oocyte Complex *in vitro* maturation and retrieval**

Cumulus-oocyte complexes (COCs) were aspirated from ovarian follicles of slaughtered bovines. After four washes in HEPES-buffered Tyrode lactate solution (TLH), groups of 10 selected COCs were placed in 50 $\mu$ l drops of maturation medium under mineral oil in petri dishes (Nunc, Denmark) and matured for 24h at 39°C under 5% CO<sub>2</sub> in air with maximum humidity. The maturation medium was TCM-199 supplemented with 10% fetal calf serum, 0.1 $\mu$ g/ml of follicle-stimulating hormone (Folltropin V; Bioniche), 1 $\mu$ g/ml of estradiol, 0.33mM of pyruvic acid, and 50 $\mu$ g/ml of gentamycin.

For each sampling session, 10 COCs were recovered from ovaries conserved at 4°C (representing slaughter time) and 10 COCs were recovered from ovaries conserved at body temperature as a control condition (0h). Then 10 COCs were retrieved every 3 hours during the 24h of IVM (at 3, 6, 9, 12, 15, 18, 21, and 24h). From this point, only RNase-free material and products were used. The COCs were washed three times in different dishes with PBS (phosphate-buffered saline) and the cumulus cells (CCs) were collected by mechanically denuding the oocytes by agitation for about 5min in PBS. The COCs were then transferred into 1.5-ml conical tubes and vortexed for 2min in 150 $\mu$ l PBS. Cells were placed into a new dish and oocytes and CCs were collected separately into new 1.5-ml conical tubes. Cumulus cells were washed by centrifugation three times in 150 $\mu$ l PBS. Cells were then frozen in liquid nitrogen in a minimum volume and conserved at -80°C.

### **4.7.2. RNA processing and quantification**

*RNA Extraction.* Cumulus cell total RNA was extracted using silica-based ion exchange columns (PicoPure RNA Isolation kit; Life Technologies, CA, USA) following the manufacturer's instructions. Contaminating genomic DNA was eliminated using an on-column DNase treatment (Qiagen, ON, Canada) while nucleic acids were bound to the column. Elution of RNA was done with 11 $\mu$ l, the minimum recommended volume. The concentration and quality of the RNA samples were assessed utilizing a 2100 Bioanalyzer apparatus (Agilent Technologies, CA, USA) and samples were loaded on RNA Pico Chips (Agilent Technologies). All samples used were of good quality with RNA Integrity Numbers over 7.5.

The total RNA of bovine *corpus luteum* was extracted using trizol extraction procedure and used as a positive control.

*RNA processing for qRT-PCR.* Extracted total RNA was reverse transcribed using the qScript Flex cDNA Synthesis Kit with an oligo-dT mix (Quanta Biosciences, MD, USA) according to the manufacturer's protocol. Each sample contained 1ng of RNA. At the end of the reverse transcription (RT), 20 $\mu$ l of nuclease-free water were added to the final 20 $\mu$ l RT reaction.

Primers used for qRT-PCR were designed from sequences based on the UMD3.1/bosTau5 assembled version of the bovine genome using the IDT PrimerQuest web interface (<http://www.idtdna.com/Scitools/Applications/Primerquest/>) and are listed in [Table 4-1](#).

For each sample and each primer pair, 2  $\mu$ l of complementary DNA were used to perform quantitative PCR on a LightCycler 480 apparatus with LightCycler 480 SYBR Green I Master (Roche Applied Science, Laval, QC, Canada). Reactions were performed in 96-well plastic plates in a final volume of 20 $\mu$ l (Roche Applied Science). The reaction mix contained 7 $\mu$ l of PCR grade water, 10 $\mu$ l of SYBR Green I Master, and 0.25mM of each primer. For each candidate gene tested, PCR products were observed on 2% agarose gels, extracted with the QIAquick PCR Purification kit (Qiagen), quantified with a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies), and sequenced to confirm the specificity of each primer pair. For quantification experiments, the standard curves consisted of five standards of the purified and confirmed PCR products diluted from  $2 \times 10^{-4}$  to  $2 \times 10^{-8}$ ng/ $\mu$ l. The PCR conditions applied to all genes were as follows: denaturing cycle for 10min at 95°C; 50 PCR cycles (denaturing, 95°C for 5s; annealing at the adapted temperature for 5s; elongation, 72°C for 30s, acquisition at the adapted temperature for 1s), a melting curve 95°C for 1s, 65°C for 5s and a step cycle starting at 65°C up to 95°C at 0.1°C/s and a final cooling step at 40°C. Data normalization was performed with GeNORM normalization factor (Vandesompele *et al.* 2002) from expression values of two reference genes (ACTB, GAPDH).

To determine the statistically significant differences in mRNA contents between each condition in cumulus cells, a one-way ANOVA and Newman-Keuls multiple comparison tests with a p-value<0.5 were applied using Prism 5.02 (GraphPad software, CA, USA).

## 4.8. References

- Al Naib A, Mamo S, O'Gorman GM, Lonergan P, Swales A, Fair T. 2011. Regulation of non-classical major histocompatibility complex class I mRNA expression in bovine embryos. *Journal of reproductive immunology* 91(1-2):31-40.
- Aparicio IM, Garcia-Herreros M, O'Shea LC, Hensey C, Lonergan P, Fair T. 2011. Expression, regulation, and function of progesterone receptors in bovine cumulus oocyte complexes during *in vitro* maturation. *Biology of reproduction* 84(5):910-921.
- Baltar AE, Oliveira MA, Catano MT. 2000. Bovine cumulus/oocyte complex: quantification of LH/hCG receptors. *Molecular reproduction and development* 55(4):433-437.
- Bernal-Ulloa SM, Lucas-Hahn A, Herrmann D, Hadeler KG, Aldag P, Baulain U, Niemann H. 2016. Oocyte pre-IVM with caffeine improves bovine embryo survival after vitrification. *Theriogenology* 86(5):1222-1230.
- Boruszewska D, Sinderewicz E, Kowalczyk-Zieba I, Grycmacher K, Woclawek-Potocka I. 2015. The effect of lysophosphatidic acid during *in vitro* maturation of bovine cumulus-oocyte complexes: cumulus expansion, glucose metabolism and expression of genes involved in the ovulatory cascade, oocyte and blastocyst competence. *Reproductive biology and endocrinology : RB&E* 13:44.
- Brackett BG, Younis AI, Fayerer-Hosken RA. 1989. Enhanced viability after *in vitro* fertilization of bovine oocytes matured *in vitro* with high concentrations of luteinizing hormone. *Fertility and sterility* 52(2):319-324.
- Bureau M, Bailey JL, Sirard MA. 2002. Binding regulation of porcine spermatozoa to oviductal vesicles *in vitro*. *Journal of andrology* 23(2):188-193.
- Calder MD, Caveney AN, Smith LC, Watson AJ. 2003. Responsiveness of bovine cumulus-oocyte-complexes (COC) to porcine and recombinant human FSH, and the effect of COC quality on gonadotropin receptor and Cx43 marker gene mRNAs during maturation *in vitro*. *Reproductive biology and endocrinology : RB&E* 1:14.
- Chasombat J, Nagai T, Parnpai R, Vongpralub T. 2015. Pretreatment of *in vitro* matured bovine oocytes with docetaxel before vitrification: Effects on cytoskeleton integrity and developmental ability after warming. *Cryobiology* 71(2):216-223.
- Chian RC, Ao A, Clarke HJ, Tulandi T, Tan SL. 1999. Production of steroids from human cumulus cells treated with different concentrations of gonadotropins during culture *in vitro*. *Fertility and sterility* 71(1):61-66.
- Dieleman SJ, Kruip TA, Fontijnne P, de Jong WH, van der Weyden GC. 1983. Changes in oestradiol, progesterone and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory bovine follicles relative to the peak of luteinizing hormone. *The Journal of endocrinology* 97(1):31-42.
- Eppig JJ, Wigglesworth K, Pendola F, Hirao Y. 1997. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biology of reproduction* 56(4):976-984.
- Franciosi F, Coticchio G, Lodde V, Tessaro I, Modina SC, Fadini R, Dal Canto M, Renzini MM, Albertini DF, Luciano AM. 2014. Natriuretic peptide precursor C delays meiotic resumption and sustains gap junction-mediated communication in bovine cumulus-enclosed oocytes. *Biology of reproduction* 91(3):61.
- Jang HY, Ji SJ, Kim YH, Lee HY, Shin JS, Cheong HT, Kim JT, Park IC, Kong HS, Park CK, Yang BK. 2010. Antioxidative effects of astaxanthin against nitric oxide-induced oxidative stress on cell viability and gene expression in bovine oviduct epithelial cell and the developmental competence of bovine IVM/IVF embryos. *Reproduction in domestic animals = Zuchthygiene* 45(6):967-974.
- Kash JC, Menon KM. 1998. Identification of a hormonally regulated luteinizing hormone/human chorionic gonadotropin receptor mRNA binding protein. Increased mRNA binding during receptor down-regulation. *The Journal of biological chemistry* 273(17):10658-10664.
- Lunenfeld B. 2004. Historical perspectives in gonadotrophin therapy. *Human reproduction update* 10(6):453-467.
- Menon KM, Nair AK, Wang L. 2006. A novel post-transcriptional mechanism of regulation of luteinizing hormone receptor expression by an RNA binding protein from the ovary. *Molecular and cellular endocrinology* 246(1-2):135-141.
- Nair AK, Menon KM. 2004. Isolation and characterization of a novel trans-factor for luteinizing hormone receptor mRNA from ovary. *The Journal of biological chemistry* 279(15):14937-14944.
- Peng XR, Hsueh AJ, LaPolt PS, Bjersing L, Ny T. 1991. Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. *Endocrinology* 129(6):3200-3207.
- Pincus G, Enzmann EV. 1935. The comparative behavior of mammalian eggs *in vivo* and *in vitro* : i. the activation of ovarian eggs. *The Journal of experimental medicine* 62(5):665-675.

- Rispoli LA, Payton RR, Gondro C, Saxton AM, Nagle KA, Jenkins BW, Schrick FN, Edwards JL. 2013. Heat stress effects on the cumulus cells surrounding the bovine oocyte during maturation: altered matrix metalloproteinase 9 and progesterone production. *Reproduction* 146(2):193-207.
- Robert C, Gagne D, Lussier JG, Bousquet D, Barnes FL, Sirard MA. 2003. Presence of LH receptor mRNA in granulosa cells as a potential marker of oocyte developmental competence and characterization of the bovine splicing isoforms. *Reproduction* 125(3):437-446.
- Silvestre F, Fissore RA, Tosti E, Boni R. 2012.  $[Ca^{2+}]_i$  rise at *in vitro* maturation in bovine cumulus-oocyte complexes. *Molecular reproduction and development* 79(6):369-379.
- Takada I, Junior AM, Mingoti GZ, Balieiro JC, Cipolla-Neto J, Coelho LA. 2012. Effect of melatonin on DNA damage of bovine cumulus cells during *in vitro* maturation (IVM) and on *in vitro* embryo development. *Research in veterinary science* 92(1):124-127.
- Tu Q, Cameron RA, Worley KC, Gibbs RA, Davidson EH. 2012. Gene structure in the sea urchin *Strongylocentrotus purpuratus* based on transcriptome analysis. *Genome research* 22(10):2079-2087.
- van Tol HT, van Eijk MJ, Mummery CL, van den Hurk R, Bevers MM. 1996. Influence of FSH and hCG on the resumption of meiosis of bovine oocytes surrounded by cumulus cells connected to membrana granulosa. *Molecular reproduction and development* 45(2):218-224.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* 3(7):RESEARCH0034.
- Wang L, Nair AK, Menon KM. 2007. Ribonucleic acid binding protein-mediated regulation of luteinizing hormone receptor expression in granulosa cells: relationship to sterol metabolism. *Mol Endocrinol* 21(9):2233-2241.
- Wang Z, Fu C, Yu S. 2013. Green tea polyphenols added to IVM and IVC media affect transcript abundance, apoptosis, and pregnancy rates in bovine embryos. *Theriogenology* 79(1):186-192.
- Xiao X, Zi XD, Niu HR, Xiong XR, Zhong JC, Li J, Wang L, Wang Y. 2014. Effect of addition of FSH, LH and proteasome inhibitor MG132 to *in vitro* maturation medium on the developmental competence of yak (*Bos grunniens*) oocytes. *Reproductive biology and endocrinology : RB&E* 12:30.
- Zuelke KA, Brackett BG. 1990. Luteinizing hormone-enhanced *in vitro* maturation of bovine oocytes with and without protein supplementation. *Biology of reproduction* 43(5):784-787.

## 4.9. Tables

**Table 4-1. List and characteristics of primer pairs used in qRT-PCR for investigated genes in bovine cumulus cells.**

Gene	Forward primer (sens) Reverse primer (anti-sens)	Annealing temperature (°C)	Product length (bp)	Accession number
<b>LHCGR</b> « 5' »	TCAGCCGACTATCACTCACCTA AGCCTCAATGTGCACCAGTTT	59	197	NM_174381.1
<b>LHCGR</b> <i>isoform X3</i>	AACGCTGATTTC CCTGGAGCTGAA GGCAGAATAATCGTTGTTGG	59	286	XM_005212546.3
<b>LHCGR</b> <i>isoform X5</i>	AACGCTGATTTC CCTGGAGCTGAA CCATGCAGAAGTAGTGTTTCAT	59	369	XM_015473420.1
<b>LHCGR</b> <i>isoform X7</i>	AACGCTGATTTC CCTGGAGCTGAA CCATGCAGAAGTCGTTGTT	59	288	XM_015473422.1
<b>MVK</b>	CCGAGGAGAAC TTGGAGTTAAT CGATCTCAGGGAACTTCAGTAG	53	249	NM_001015528.1
<b>PGR</b>	CCTTAGAAAGT GCTGTCAGG GAAGTTGTCTCTCACCAAGC	61	311	NM_001205356.1
<b>STAR</b>	GAATGCGGACAAGGCTCTTCTAAC GAECTCTGTCATGGAAGAAGTACG	58	408	NM_174189.2
<b>CYP11A1</b>	TAGCATCAAGGAGACGCTGAGA TAGCTGGATTGGTGGAAAGGG	57	470	NM_176644.2
<b>HSD3B</b>	GGATGAGCCTCCCTATTCTCTGC CTGCTAACTAATGTCCACGTTCCC	60	317	NM_174343.3
<b>ACTB</b>	ATCGTCCACCGCAAATGCTCT GCCATGCCAATCTCATCTCGTT	59	101	NM_173979.3
<b>GAPDH</b>	CCAACGTGTCTGTTGTGGATCTGA GAGCTTGACAAAGTGGTCGTTGAG	58	217	NM_001034034.2

#### 4.10. Figures

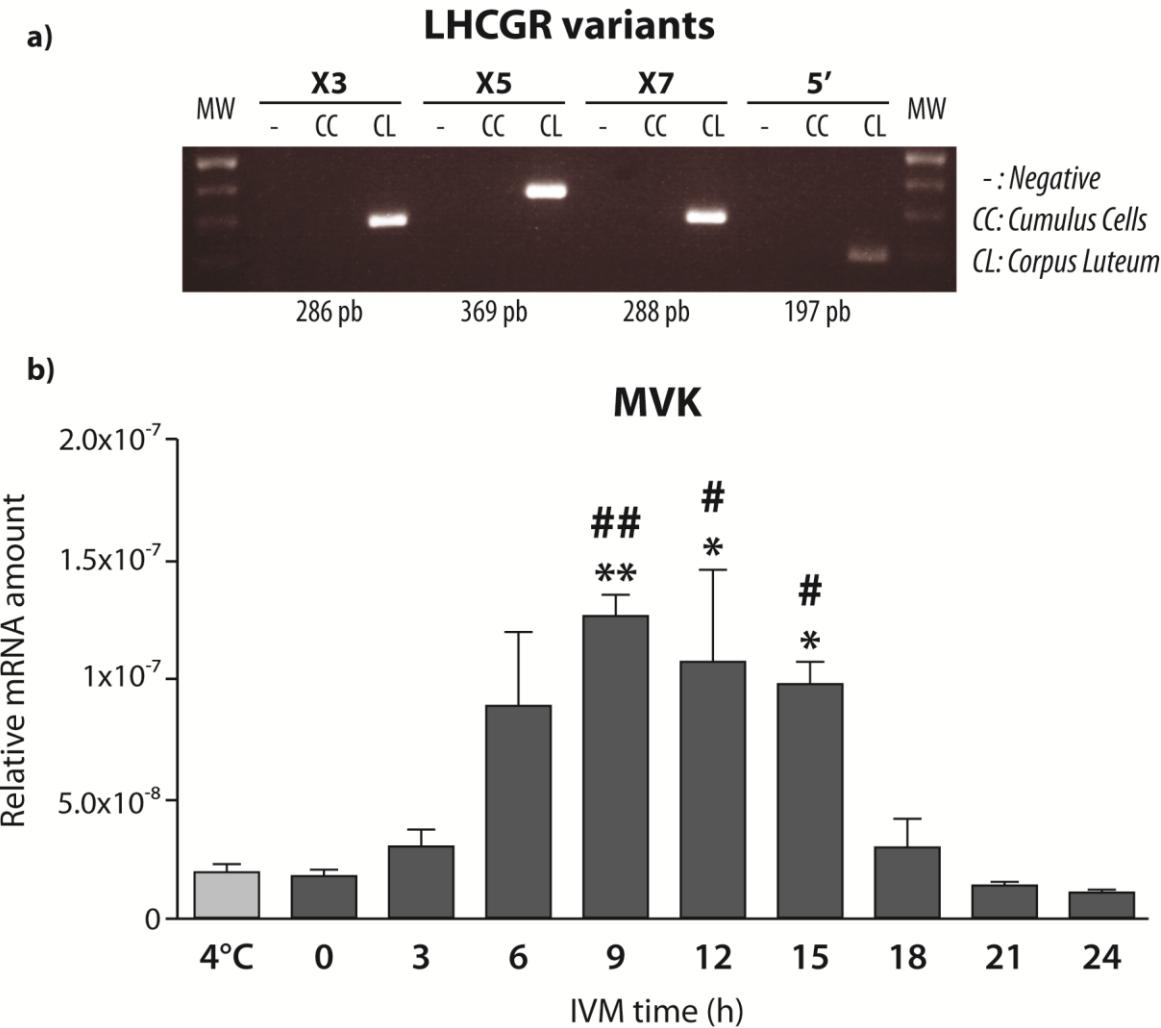
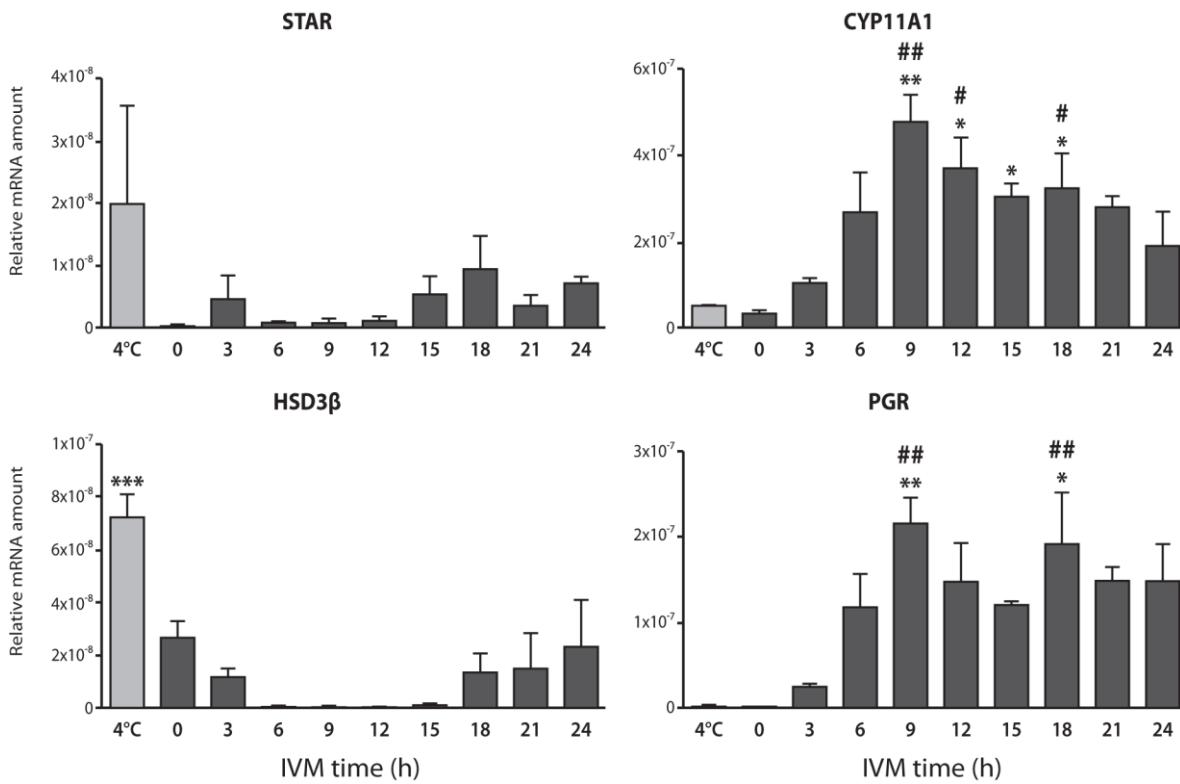


Figure 4-1. Bovine cumulus cell mRNA expression during IVM.

a) LHCGR variants X3, X5, X7 and constitutive isoform ('5'); b) one of its regulator, the mevalonate kinase (MVK).  $n = 3$ , \* depicts differences with 4°C condition and # differences with the 0 h IVM time point. \*,#  $p < 0.05$ , \*\*,##  $p < 0.01$ .



**Figure 4-2. Bovine cumulus cell mRNA expression of STAR, CYP11A1, HSD3b, and PGR during IVM.**

$n = 3$ , \* depicts differences with 4°C condition and # differences with the 0 h IVM time point. \*,# p<0.05, \*\*,## p<0.01.

## **5. INDIVIDUAL BOVINE *IN VITRO* EMBRYO PRODUCTION AND CUMULUS CELL TRANSCRIPTOMIC ANALYSIS TO DISTINGUISH CUMULUS-OOCYTE COMPLEXES WITH HIGH OR LOW DEVELOPMENTAL POTENTIAL**

Audrey Bunel<sup>1</sup>, Ellen Jorssen<sup>2</sup>, Els Merckx<sup>2</sup>, Jo L. Leroy<sup>2</sup>, Peter E. Bols<sup>2</sup> and Marc-André Sirard<sup>1</sup>

<sup>1</sup> Centre de Recherche en Biologie de la Reproduction, Faculté des Sciences de l’Agriculture et de l’Alimentation, Département des Sciences Animales, Université Laval, Québec, Canada.

<sup>2</sup> Gamete Research Center, Laboratory for Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Universiteitsplein 1, Wilrijk, Belgium

Cet article a été publié dans la revue « *Theriogenology* » sous la référence suivante :

Individual bovine *in vitro* embryo production and cumulus cell transcriptomic analysis to distinguish cumulus-oocyte complexes with high or low developmental potential

**Bunel, A. et al. 2015**

*Theriogenology*, Volume 83, Issue 2, 228 - 237

## 5.1. Résumé

Un des grands intérêts de l'étude du transcriptome des cellules du cumulus (CC) réside dans la possibilité d'évaluation non invasive de la qualité de l'ovocyte. Chez le bovin, la recherche de marqueurs de qualité ovocytaire n'a pas encore été réalisée avec des complexes ovocyte-cumulus (COC) cultivés de manière individuelle depuis la maturation jusqu'au stade de blastocyste. Ici, des biopsies de CC ont été réalisées à partir de COC à faible potentiel et de COC à haut potentiel développemental, à partir de follicules de 2-6 mm ( $n = 249$ ; 8 réplicats), afin d'examiner leurs différences au niveau transcriptomique. Chaque COC a été suivi individuellement puis catégorisé selon son destin : soit embryon au stade de blastocyste (CC-Blast), soit embryon arrêté au stade de 2 à 8 cellules (CC-2-8-cells). Les taux moyens de blastocystes ont été de 27.7 % pour la culture individuelle et de 31.2 % pour le groupe contrôle (non significativement différents). En vue de l'analyse transcriptomique, 5 biopsies de CC par réplicat ont été regroupées pour chaque phénotype observé. Trois réplicats ont été utilisés pour l'analyse par puces à ADN. De nettes différences d'expression génique ont été révélées entre les groupes de CC-Blast et de CC-2-8-cells. Considérant une variation de  $1.5 \times$  minimum ( $P < 0.05$ ), 68 gènes étaient différentiellement exprimés entre les CC-Blast et les CC-2-8-cells. Des validations par qRT-PCR ont été réalisées pour 12 gènes : 6 sur-exprimés pour chaque phénotype associé au COC. Une sur-expression de *AGPAT9* (métabolisme lipidique), *CLIC3*, *KRT8* et *LUM* (transport moléculaire) a été observée pour les CC-2-8-cells ( $P < 0.05$ ). L'analyse des CC-Blast a révélé une expression significativement plus élevée de *GATM* (modification post-transcriptionnelle, métabolisme des acides aminés et élimination des radicaux libres). Ce nouvel ensemble de gènes pourrait servir à distinguer les COC associés à des embryons de bonne qualité des COC au potentiel développemental limité.

## 5.2. Abstract

Studying cumulus cell (CC) transcriptome is of high interest as it could provide a non-invasive method to assess oocyte quality. In cattle, the search for quality markers has not been done with cumulus-oocyte-complexes (COCs) cultured individually from maturation to blastocyst stage. Here, differences between high- and low-potential COCs were examined by transcriptomic analysis of CC biopsies obtained from COCs of 2-6mm follicles ( $n = 249$ ; eight replicates) before individual *in vitro* maturation, fertilization and culture until Day 8 after fertilization. Each COC was individually tracked and categorized based on his fate: embryo at blastocyst stage (CC-Blast) or embryo arrested at 2- to 8-cell stage (CC-2-8-cells). Average blastocyst rates were 27.7% for individual culture and 31.2% for group control (not significantly different). For transcriptomic analysis, five cumulus biopsies per replicate were pooled for each fate. Three CC replicates underwent transcriptomic analysis using RNA microarray assay. Some clear differences in gene expression between the CC-Blast and the CC-2-8-cells groups were identified. Considering a 1.5-fold change ( $P < 0.05$ ), 68 genes were differentially expressed between CC-Blast and CC-2-8-cells. Quantitative reverse transcription-polymerase chain reaction validations were performed for 12 selected genes: six upregulated genes for each COC fate. Higher expression of 1-acylglycerol-3-phosphate O-acyltransferase 9 (*AGPAT9*) (lipid metabolism), Chloride intracellular channel 3 (*CLIC3*), Keratine 8 (*KRT8*), and Lumican (*LUM*) (molecular transport) was observed in CC-2-8-cells ( $P < 0.05$ ). The CC-Blast fate analysis revealed a significantly higher expression of Glycine amidinotransferase (L-arginine:glycine aminidotransferase) (*GATM*) (post-translational modification, amino acid metabolism, and free radical scavenging). This newly identified set of genes could provide new markers to distinguish COCs associated with good quality embryos from COCs with limited developmental potential.

### 5.3. Introduction

Cumulus cells (CCs) contribute to oocyte growth, maturation, and acquisition of developmental competence (Gilchrist *et al.* 2004, Eppig *et al.* 2001, Matzuk *et al.* 2002). Cytoplasmic projections embedded in the zona pellucida connect CCs intimately to the oocyte cytoplasm by the presence of gap junctions (Albertini *et al.* 2001). These connections allow the transfer of molecules essential for oocyte metabolism, for the support of oocyte development (Sutton-McDowall *et al.* 2010, Su *et al.* 2009), and for the regulation of meiotic arrest or resumption (Park *et al.* 2004). In 1988, it was reported that CCs are essential for cytoplasmic maturation because oocytes deprived of their companion CCs could be *in vitro* matured and fertilized, but the resulting embryos stopped developing before reaching the 8-cell stage (First *et al.* 1988). The absence of CC also affected lipid metabolism in bovine oocytes (Auclair *et al.* 2013). However, the precise mechanisms regulating the support provided by the CC to oocyte developmental competence acquisition remain elusive. Because of their functions, CCs represent a very promising tool to identify key factors that would allow researchers to distinguish high-potential cumulus-oocyte complexes (COCs) from low-potential COCs in a noninvasive way (Assou *et al.* 2008). This could potentially result in the improvement of embryo yields after *in vitro* production. One method to find such markers for oocyte potential is to analyze the transcriptome of their surrounding CCs.

The expression of potential marker genes in CCs has previously been associated with good embryo quality, developmental potential, and even pregnancy outcome in human (Assou *et al.* 2008, McKenzie *et al.* 2014, Zhang *et al.* 2005, Lee *et al.* 2010, Feuerstein *et al.* 2012, Assidi *et al.* 2010, Gebhardt *et al.* 2011, Iager *et al.* 2013). Similarly, negative outcomes have also been associated with the expression of specific genes in a CC (Hasegawa *et al.* 2005, Hasegawa *et al.* 2007, Feuerstein *et al.* 2007, van Montfoort *et al.* 2008).

Few large-scale investigations of CC gene expression have been conducted in cows. In a recent study of RNA transcript abundance in CCs harvested from oocytes of adult versus prepubertal animals (a model of poor oocyte quality), the genes Cathepsin B, Cathepsin S, and Cathepsin Z (cathepsins genes) were negatively correlated with blastocyst formation when overexpressed in a CC (Bettegowda *et al.* 2008). Our laboratory has developed a time course assay to characterize the oocyte developmental competence acquisition and loss during the coasting period (Nivet *et al.* 2012). The CC transcriptome was then explored in relation to developmental competence and time of coasting (Bunel *et al.* 2013).

Most studies in cows were conducted under routine group culture conditions in which the highly variable blastocyst developmental rates highlight the heterogeneity of the oocyte population and do not allow tracking of individual COCs. The « well of the well » (Vajta *et al.* 2000) and nylon mesh (Matoba *et al.* 2010) systems allow individual following but do not eliminate the influence of paracrine factors from other embryos.

To overcome these drawbacks, a fully individual production method has been developed for bovine oocytes, which results in similar blastocyst rates compared with group culture with (Goovaerts *et al.* 2010) or without (Goovaerts *et al.* 2012) serum. In this study, we hypothesized that by applying a fully individual *in vitro* production (IVP) system, new potential CC gene expression markers to distinguish COCs with high and low developmental potential could be identified in a noninvasive manner. Therefore, the aim of this study was to analyze the gene expression profile of CCs before maturation according to COCs' fate at Day 8 after fertilization, *e.g.* blastocyst or 2- to 8-cells, using a fully individual *in vitro* embryo production system.

## 5.4. Results

### 5.4.1. *In vitro* embryo production and quality assessment

No significant differences in cleavage or blastocyst rates were found between the individually produced embryos (SSS) from which cumulus biopsies were taken before *in vitro* maturation and the control group-produced embryos (GGG) (Table 5-1). In addition, there were no significant differences in the quality of expanded blastocysts from the control GGG and the SSS as assessed by mean total cell count ( $141.7 \pm 15.0$  and  $150.5 \pm 10.4$ ) and apoptotic cell ratio ( $3.2 \pm 2.2$  and  $2.8 \pm 1.4$ ), respectively.

### 5.4.2. Gene inventory

To obtain the transcriptomic differences between CC from COCs that developed to an 8-day blastocyst and CC from COCs that only reached the 2- to 8-cell stage, a large-scale transcriptomic analysis was performed using the EmbryoGENE microarray slide. This slide includes 42,242 probes for 21,139 known reference genes and 7230 splice and 3' untranslated region variants in addition to 9,322 novel transcribed regions (Robert *et al.* 2011). Two conditions were compared in a dye-swap design, providing two technical replicates for each of the three biological replicates. Out of the 21,139 genes represented on the slide, 20,547 genes were considered to be expressed in the CC-2-8-cells and 20,142 genes were considered expressed in the CC-Blast as indicated by signals above a threshold defined as the mean background plus two times the standard deviation. Considering a 1.5-fold change, 70 probes were differentially expressed between the two COC fates (Figure 5-1).

### 5.4.3. Ingenuity Pathway Analysis

An IPA (Ingenuity Systems; <http://www.ingenuity.com/>) was performed on the microarray data using the 1.5-fold change cutoff and  $P < 0.05$  data set. As shown in Figure 5-2, the main biofunctions revealed by the analysis are for the blastocyst fate: gene expression, posttranslational modification, protein folding, amino acid metabolism, free radical scavenging, and protein degradation; and for the 2- to 8-cell fate: cellular assembly and organization, cell death and survival, lipid metabolism, molecular transport, vitamin and mineral metabolism, and cellular growth and proliferation (Figure 5-2).

#### **5.4.4. Quantitative reverse transcription-polymerase chain reaction results**

Twelve genes selected from microarray analysis (1-acylglycerol-3-phosphate O-acyltransferase 9 [AGPAT9], Chloride intracellular channel 3 [CLIC3], early growth response 1, Family with sequence similarity 105, member A, Glycine amidinotransferase (L-arginine:glycine amidinotransferase) [GATM], Insulin induced gene 1[INSIG1], Keratin 8 [KRT8], Lumican [LUM], Mannosidase alpha, class 1A, member 1 [MAN1A1], Mitochondrial carrier 1 [MTCH1], S100A4, Y box binding protein 1 [YBX1]) were quantified by qRT-PCR using five independent biological replicates in addition to the three replicates used for microarray analysis ([Figure 5-3](#) and [Figure 5-4](#)). The differential expression of five of these genes between the two populations of CCs was confirmed. The expression of GATM was significantly higher in the blastocyst fate group ( $P = 0.02$ ) ([Figure 5-3](#)), whereas the expression of AGPAT9, CLIC3, KRT8, and LUM was significantly higher in the 2- to 8-cell fate group with  $P < 0.01$ ,  $P < 0.01$ ,  $P < 0.02$ , and  $P < 0.05$ , respectively ([Figure 5-4](#)). Nevertheless, the expression fold changes follow those of the microarray experiments ([Figure 5-5 A](#)) with  $r^2 = 0.59$  and  $P = 0.003$  ([Figure 5-5 B](#)).

## 5.5. Discussion

In the present study, a serum-free fully individual embryo production system was adopted to identify new noninvasive markers for embryonic developmental potential by gene expression analysis of CC biopsies obtained before *in vitro* maturation. To our knowledge, this is the first report, in cow, of transcriptomic analysis of CC biopsies from COCs of which the exact embryonic developmental outcome is known. Moreover, the developmental potential of these COCs was not influenced by paracrine factors produced by neighboring developing embryos or other unidentified molecules such as those included in serum.

Most of the assessment techniques routinely used to evaluate COC quality are still based on morphologic parameters, which are clearly not accurate enough, as *in vitro* blastocyst rates rarely exceed 40% (Goovaerts *et al.* 2009). Molecular analysis, *e.g.*, the omics (Hamamah *et al.* 2011, Seli *et al.* 2010), conceivably represents more reliable ways to objectively evaluate the COC potential. Even if the quality of the oocyte is the center of the debate, it is impossible to analyze its content without destroying it. Thus, oocyte quality assessment requires a noninvasive technique, and some researchers have used floating granulosa cells (Hamel *et al.* 2008, Hamel *et al.* 2010, Nivet *et al.* 2013) or follicular fluid to assess oocyte quality or outcome (Bayasula *et al.* 2013, Bertoldo *et al.* 2013, Li *et al.* 2011). However, this involves the individual collection of follicles, which is a task that is too tedious to be routinely performed. This context renders the CCs of great interest (Assou *et al.* 2010); in fact, they are attached to their own oocyte and can easily be sampled before the *in vitro* culture process. That is why, in this study, we cultured the COCs and further embryos in an individual manner. This system allowed us to follow each oocyte to the end of the culture period and to link each CC sample to the fate of his COC, *i.e.*, blastocyst or 2- to 8-cell embryo.

The microarray analysis revealed few differences between CC from COCs, which developed into Day 8 blastocysts, and CC from COCs, which arrested at 2- to 8-cell embryos. In fact, considering a 1.5-fold change cutoff, only 70 probes representing 65 unique known genes and three novel genes were differentially expressed in bovine CCs between the two COC fate categories. The low number of differentially expressed genes combined with the knowledge that follicular development and oocyte maturation require very fine regulation suggests that only small differences can determine the fate of a COC (Nivet *et al.* 2012, Hamel *et al.* 2008, Sirard *et al.* 2006).

The functional analysis brings to light a higher implication of different steps of protein formation in the CC-Blast. Indeed, five of the top six IPA biofunctions for the blastocyst fate are part of the processes needed for protein cycle: gene expression, posttranslational modification, protein folding, amino acid metabolism, and protein degradation. These results are similar to those previously described by Alvarez *et al.* (2012) who reported that morphologically good quality porcine COCs had a higher protein content compared with those of a lower quality. This greater protein content would require an optimal protein synthesis machinery and could explain why the processes involved in the protein cycle were upregulated in the CC-Blast. Moreover, in the present study, the amino acid metabolism and protein degradation IPA biofunctions were not associated with the 2- to 8-cell fate. An incorrect regulation of the protein synthesis machinery or amino acid metabolism could be detrimental to the COC. For example, a change in the availability of specific amino acids like glutamine may result in a huge impact on energy distribution between cumulus expansion and oocyte feeding with required pyruvate (Sutton-McDowall *et al.* 2004).

Another biofunction that is highlighted in the CC-Blast but not in CC-2-8-cells is the “free radical scavenging”. This function is important to protect the COC from the detrimental free radical production, which results from follicle metabolism. In fact, previous research has shown that fewer oocytes could be retrieved from follicles with granulosa cells producing a higher level of reactive oxygen species (ROS) and the embryos resulting from those follicles implanted at a decreased rate in human (Jancar *et al.* 2007). Therefore, the use of antioxidant agents, such as sodium selenite, during *in vitro* follicle culture has improved follicle, oocyte, and embryo development in mice (Abedelahi *et al.* 2010). Furthermore, in the context of *in vitro* embryo production, several culture conditions induced increased ROS production (Martin-Romero *et al.* 2008). This implies that COCs need the appropriate molecules to confront this hostile *in vitro* oxidative environment to be able to further develop into an embryo. In addition, the presence of higher superoxide dismutase activity was associated with successful pregnancies in human (Matos *et al.* 2009). Therefore, it seems consistent to find the free radical scavenging biofunction to be upregulated in the COCs with the highest potential. The fact that the COCs with the lowest potential were missing this biofunction could in part explain their destiny by a lack of free radicals regulation.

Among the top six upregulated biofunctions in the CCs of the 2- to 8-cell fate-associated COCs, the biofunctions cell death and survival can be linked to the absence of the free radical scavenging biofunction and to a certain extent contribute to the low potential of these COCs. In a

recent review (Devine *et al.* 2012), the authors concluded that antral follicles are highly sensitive to apoptosis induced by oxidative stress.

Subsequent to microarray analysis, 12 genes were selected to undergo qRT-PCR validation: six genes for each COC fate. Among these genes, four had a significantly higher expression in the CCs associated with the 2- to 8-cell fate, and although microarray analysis trends were confirmed with a significant correlation value, only one gene exhibited a significantly higher expression associated with the blastocyst fate. The high correlation ( $r^2 = 0.5955$ ) between the arrays and PCR experiments is shown in [Figure 5-5](#). Cumulus cells associated with the 2- to 8-cell fate displayed a higher expression of AGPAT9, CLIC3, KRT8, and LUM, whereas CCs associated with the blastocyst fate had a higher expression of GATM. Cumulus-oocyte complexes were recovered from 2 to 6mm follicles. At this size, follicles are still growing or are already in the atresia process (Blondin *et al.* 1995). The presence of those two statuses could explain a part of the gene expression variability between the different pools of the same fate.

The enzyme AGPAT9 is a 1-aclyglycerol-3-phosphate-O-acyltransferase that catalyzes the conversion of lysophosphatic acid to phosphatidic acid, which is involved in lipid metabolism (Kent *et al.* 1995). The isoform AGPAT9 has been identified as a key regulator of lipid accumulation in adipocytes (Kent *et al.* 1995). The overexpression of the AGPAT9 gene, which is involved in cell growth and proliferation *via* the mammalian target of rapamycin pathway activation (Tang *et al.* 2006), is consistent with the upregulation of the cellular growth and proliferation biofunction associated with the 2- to 8-cell fate. In our context, it could indicate that the cumulus biopsies associated with developmental failure originated from growing follicles containing oocytes with reduced developmental competence (Blondin *et al.* 1996).

The chloride intracellular channel protein (CLIC3) is involved in cellular processes such as chloride ion concentration control, cell volume regulation, and apoptosis stimulation in response to cellular stress (Murthi *et al.* 2012). The involvement of CLIC3 in apoptosis is consistent with the upregulation of the cell death and survival IPA biofunction found in CC associated with the 2- to 8-cell fate.

The gene KRT8 codes for keratin 8 protein, an intermediate filament protein. The knockdown of KRT8 in mice resulted in a reduced litter size and in the death of the pups at birth (Baribault *et al.* 1993, Jaquemar *et al.* 2003). Although KRT8 can be linked to the cellular assembly and organization,

which are upregulated in CC with 2- to 8-cell fate; KRT8, in association with KRT18, can exert protection against Fas-induced apoptosis in a simple epithelium (Gilbert *et al.* 2001) and has also been associated with apoptosis in the liver (Nakamichi *et al.* 2005). The CC-2-8-cells may have to face a higher apoptosis rate than the CC-Blast and respond by increasing KRT8 expression.

The gene LUM encodes for the extracellular matrix–secreted protein lumican, which is a small leucine-rich proteoglycan. In bovine oocyte, LUM was overexpressed after *in vitro* maturation (Mamo *et al.* 2011). Lumican roles seem to vary between cell types (Williams *et al.* 2010, Nikitovic *et al.* 2008, Zeltz *et al.* 2010). However, the authors propose lumican to be involved in invasiveness, proliferation, migration, adhesion, apoptosis, and angiogenesis. Given the variability of tissue roles proposed for lumican and the lack of information on its role in the COC, we can only note the overexpression of lumican in CC associated with 2- to 8-cell fate.

The GATM gene (or AGAT) encodes an L-arginine–glycine amidinotransferase, a mitochondrial enzyme that produces guanidinoacetate, the first step of creatine synthesis. Creatine synthesis can be triggered when tissues are deprived of oxygen such as during follicular growth (Fischer *et al.* 1992). Higher expression of creatine kinase B (CKB) in human CCs has been associated with embryos of good quality (Lee *et al.* 2010). Furthermore, CKB knockdown in an ovarian cancerous cell line resulted in inhibition of proliferation and induction of apoptosis under hypoglycemia or hypoxia conditions (Li *et al.* 2013), hypoxia-like in follicular growth phase. A reduction in glucose consumption and lactate production and an increase in ROS production (detrimental to developmental potential as mentioned previously) were also observed in the CKB knockdown. The GATM enzyme is also involved in the synthesis of ornithine, which participates in the regulation of nitrogen levels in cells (Wu *et al.* 1998). Several studies revealed the impact of nitrogen species on oocyte developmental competence. For example, the Day 11 hatched blastocyst rate of oocytes obtained by ovum pick up from cows receiving urea supplementation in their diet was lower than that for the control group (64.3% vs. 83.5%) (Ferreira *et al.* 2011). In another study comparing a low-NH<sub>3</sub>- and a high-NH<sub>3</sub>-generating diet, *in vitro* culture of oocytes from the high-NH<sub>3</sub>-generating diet resulted in a reduced blastocyst production compared with oocytes from the low-NH<sub>3</sub>-generating diet (10.9% vs. 20.6%) (Sinclair *et al.* 2000). The GATM gene expression also increased with FSH withdrawal progression during hormonal treatment (Bunel *et al.* 2013). An adapted regulation of nitrogen and oxidative species seems to have a role in oocyte developmental competence, suggesting a key role for GATM.

## **5.6. Conclusion**

By using a fully individual embryo production system, we were able to identify a new set of genes expressed by CCs, which could be indicative of what makes the difference between a high-potential COC that is able to reach the blastocyst stage and a COC that will fail to develop. Such information may then be used to modify the culture system to improve the quality of oocytes at the time of fertilization. These new insights on CC gene expression also provide the opportunity to understand what makes a high-potential COC but also it could be used to improve *in vitro* the embryo yield as a result of selecting only the good quality COC.

## 5.7. Materials and methods

All reagents and media supplements used in these experiments were of tissue-culture grade and were obtained from Sigma Chemicals Co. (St. Louis, MO, USA or Diegem, Belgium) unless otherwise indicated.

### 5.7.1. *In vitro* culture and CC recovery

#### 5.7.1.1. Collection of COCs

Cow ovaries of mixed breeds from a nearby slaughterhouse (Hoogstraten, Belgium) were collected and transported to the laboratory as soon as possible after slaughter. After three washes in warm saline solution supplemented with 5% kanamycin, COCs from follicles 2 to 6mm in diameter were collected and washed in a HEPES-Tyrode's Albumin Lactate Pyruvate solution containing 114mM of NaCl, 3.1mM of KCl, 0.3mM of Na<sub>2</sub>HPO<sub>4</sub>, 2.1mM of CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.4mM of MgCl<sub>2</sub>·6H<sub>2</sub>O, 2mM of sodium bicarbonate, 1mM of pyruvate, 36mM of lactate, 1mM of HEPES, 0.0001% phenol red, 0.4mg/mL of BSA, and 50mg/mL of gentamicin. Only COCs with homogeneous cytoplasm surrounded by five or more unexpanded CC layers (quality grade I) were selected for *in vitro* maturation.

#### 5.7.1.2. *In vitro* embryo production: single or group IVP system control

Before maturation, a small biopsy of the compact CCs (comprising CCs from the center to the periphery) of the selected grade I COCs destined for individual IVP culture was removed with a scalpel and a needle and individually snap-frozen for RNA analysis (eight replicates: eight pools of five COCs, four pools for embryo at blastocyst stage [CC-Blast] and four pools for embryo arrested at 2- to 8-cell stage [CC-2-8-cells]). Some COCs were matured in groups of 50 (as an *in vitro* embryo production control) in 500mL in 4-well plates (Nunc) or singly in 24-well plates (Nunc) in 20mL tissue culture medium 199 supplemented with 0.4mM of glutamine, 0.2mM of sodium pyruvate, 0.1mM of cysteamine, 50mg/mL of gentamicin, 20ng/mL of EGF for 24h, and subsequently fertilized in groups of 100 in 500mL or individually in 20mL fertilization medium with 106 spermatozoa/mL sperm for 18 to 22h in humidified air with 5% CO<sub>2</sub> at 38.5°C. The frozen sperm, originating from the same ejaculate for all replicates, from a bull with proven *in vitro* fertility was thawed in a warm water bath for 1min and separated on a discontinuous Percoll gradient (90%–45%). The fertilization medium consisted of 114mM of NaCl, 3.1mM of KCl, 0.3mM of Na<sub>2</sub>HPO<sub>4</sub>, 2.1mM of CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.4mM of MgCl<sub>2</sub>·6H<sub>2</sub>O, 25mM of sodium bicarbonate, 1mM of pyruvate, 36mM of lactate, 0.0001% phenol red,

6mg/mL of BSA, 50mg/mL of gentamicin, and 0.72U/mL of heparin. After fertilization, presumptive zygotes were denuded by pipetting the zygotes up and down with a glass pipette in 2mL of HEPES-Tyrode's Albumin Lactate Pyruvate. They were then cultured in groups of approximately 25 in 50mL or individually with a small lump of homologous CCs (undefined number of cells) in 20mL of synthetic oviductal fluid (SOF) supplemented with ITS (10mg/mL of insulin, 5.5mg/mL of transferrin, and 6.7ng/mL of selenium) and 2% BSA under mineral oil in a modular incubator with an atmosphere of 90% N<sub>2</sub>, 5% CO<sub>2</sub>, and 5% O<sub>2</sub> at 38.5°C. The SOF medium consisted of 108mM of NaCl, 7.2mM of KCl, 1.2mM of KH<sub>2</sub>PO<sub>4</sub>, 0.8mM of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.6% v:v sodium lactate, 25mM of NaHCO<sub>3</sub>, 0.0266mM of phenol red, 0.73mM of sodium pyruvate, 1.78mM of CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.34mM of trisodium citrate dihydrate, 2.755mM of myoinositol, 3% v:v MEM 50× (minimal essential medium) (Life Technologies, Merelbeke, Belgium), 1% v:v MEM 100× (nonessential amino acid solution) (Life Technologies), 0.4mM of glutamine and 50mg/mL of gentamicin. The SOF medium was not refreshed during the whole culture period. All culture plates with media were equilibrated in a CO<sub>2</sub> incubator for at least 2h before use. Cleavage and blastocyst development were determined on an individual basis at 48h and 8 days after fertilization, respectively. Cleavage rates were calculated based on the initial total number of oocytes, and blastocyst rates were calculated based on both the initial total number of oocytes and the total number of cleaved zygotes. Subsequently, the CCs, which were individually snap-frozen before maturation, were thawed and pooled per five at the RNA extraction time according to the developmental competence of the COCs: CC from oocytes that developed to the blastocyst stage and CC from oocytes that stopped developing at the 2- to 8-cell stage after 7 days of culture.

#### ***5.7.1.3. Embryo quality assessment: total cell number (4',6'-diamidino-2-phenylindole [DAPI]) and apoptotic cell ratio (Terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL])***

To determine the total cell number and apoptotic cell ratio, individually produced (n = 24) and control group (n = 24) expanded embryos were stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and 40,60-diamidino-2-phénylindole (DAPI). Blastocysts were washed in warm PBS (Life Technologies) supplemented with poly vinyl pyrrolidone (PVP) (1mg/mL) before fixation in 4% paraformaldehyde overnight. All incubation steps occurred at room temperature in the dark unless otherwise noted and embryos were washed in 1mg/mL of PVP between each incubation step. After washing in 1mg/mL of PVP, the blastocysts were incubated in 0.5% Triton-X for 80 minutes. Subsequently, positive (artificial DNA denaturation, TUNEL and DAPI staining) and negative (artificial DNA denaturation, DAPI staining) controls were incubated with 50U/mL DNase (Roche, Vilvoorde, Belgium) for 1h. Blastocysts (except negative controls) were then stained with a TUNEL enzyme–labeling mix (Roche) for 75min at 37°C and DAPI staining for 10min. Finally, the

blastocysts were washed in PVP, mounted on glass slides and observed under a fluorescent microscope. For each blastocyst, the individual total cell number (blue nuclei, DAPI) and the total number of apoptotic cells (green nuclei, TUNEL) were determined. The apoptotic cell ratio was defined as the percentage of apoptotic cells of the total cell number.

#### **5.7.1.4. Statistical analysis**

Cleavage and blastocyst rates were analyzed using a binary logistic regression model. If the interaction term was not significant, it was excluded from the final model. Blastocyst total cell numbers and apoptotic rates were analyzed using a mixed model ANOVA and presented as means  $\pm$  standard error of the mean. The effects of treatment, replicate, and the interaction between these two factors were assessed. Values of  $P < 0.05$  were considered statistically significant.

### **5.7.2. Analysis of RNA**

#### **5.7.2.1.1. Extraction of RNA**

Cumulus cell total RNA was extracted with the PicoPure RNA Isolation kit (Life Technologies, Foster City, CA, USA) following the manufacturer's protocol. Genomic DNA contamination was removed using DNase treatment directly on the columns (Qiagen, Mississauga, ON, Canada), whereas nucleic acids were bound to the column. Total RNA was eluted with 15mL of elution buffer.

A 2100 Bioanalyzer apparatus was used to assess RNA concentration and quality (Agilent Technologies, Santa Clara, CA, USA) with RNA Pico Chips (Agilent Technologies). All analyzed samples had an RNA integrity number 7 or greater.

#### **5.7.2.2. Processing of RNA for microarray analysis**

Three of the eight biological replicates underwent microarray analysis and 3ng of their total RNA were amplified using T7 RNA polymerase (RiboAmp HS RNA Amplification kit; Life Science, Foster City, CA, USA) according to the manufacturer's instructions. Indirect labeling using Universal linker system anti-RNA (ULS aRNA) Fluorescent Labeling was used with a fixed amount of 3mg aRNA per array (ULS Fluorescent Labeling Kit for Agilent arrays; Kreatech Biotechnology, Amsterdam, the Netherlands) according to the manufacturer's protocol. The PicoPure RNA Isolation kit was used to purify aRNA samples (Life Science). Amplification and labeling efficiency were assessed using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Each comparison was made using a two-color dye swap on Agilent-manufactured EmbryoGENE slides (Robert *et al.* 2011). Hybridizations were performed according to the manufacturer's protocol (Agilent) using 825ng

of labeled aRNA per replicate. After the washing steps, slides were scanned with a PowerScanner (Tecan, Männedorf, Switzerland) and the ArrayPro 6.3 software was used to analyze images (MediaCybernetics, Bethesda, MD, USA).

#### ***5.7.2.3. Microarray data normalization and statistical analysis***

Signal intensity data files were normalized and analyzed using the FlexArray 1.6.1 software (Genome Quebec, URL: <http://genomequebec.mcgill.ca/FlexArray>). The first step in the data processing was to perform a simple background subtraction. Data were then normalized for dye bias using a within-array Loess, and a between-array « quantile » normalization was then performed to minimize array effects. Normalized data were assessed using e-Bayes moderated t test (Linear Models for Microarray) included in the FlexArray software to calculate fold changes of probe intensity. A False Discovery Rate algorithm (Benjamini-Hochberg) was finally applied.

Positive probe signals were determined on normalized data by establishing a significant cutoff threshold based on a degree of confidence associated with the variability of the negative controls. This cutoff threshold was calculated as the mean background plus two times the standard deviation. All the data equal to or lower than the cutoff threshold determined previously were not considered in the inventory analysis.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and are accessible through Gene Expression Omnibus Series accession number GSE55368 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55368>).

#### ***5.7.2.4. Processing of RNA for quantitative reverse transcription-polymerase chain reaction and statistical analysis***

The eight biological replicates underwent quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis. Using the qScript Flex complementary DNA Synthesis Kit with a mix of oligo-dT (Quanta Biosciences, Gaithersburg, MD, USA), 1ng of extracted total RNA was reverse transcribed according to the manufacturer's instructions. At the end of the RT, 20mL of nuclease-free water was added to the final 20mL RT reaction. Primers used for qRT-PCR were designed from sequences based on the UMD3.1/bosTau5 assembled version of the bovine genome using the integrated DNA technology PrimerQuest web interface (<http://www.idtdna.com>).

For each sample and each primer pair, 2mL of complementary DNA was used to perform quantitative PCR on a LightCycler 480 apparatus with LightCycler 480 SYBR Green I Master (Roche Applied Science, Laval, QC, Canada). Reactions were performed in 96-well plastic plates in a final volume of 20mL (Roche Applied Science). The reaction mix contained 7mL of PCR grade water, 10mL

of SYBR Green I Master, and 0.25mM of each primer. For each candidate gene tested, the product was purified on a 2% agarose gel, extracted with the QIAquick Gel Extraction kit (Qiagen), quantified with a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies), and sequenced to confirm the specificity of each primer pair. The standard curves for quantification experiments consisted of five standards of the purified and confirmed PCR products diluted from  $2 \times 10^{-4}$  to  $2 \times 10^{-8}$ ng/mL. The PCR conditions used for all genes were as follows: an initial denaturing step of 10min at 95°C followed by 50 PCR cycles (denaturing, 95°C for 10s; annealing at the adapted temperature for 10s; elongation, 72°C for 20s; acquisition at the adapted temperature for 1s), a melting curve at 95°C for 1s, 65°C for 1s, and a step cycle starting at 65 up to 97°C at 0.11°C/s and a final cooling step at 40°C for 10s. Data normalization was performed with GeNORM normalization factor (Vandesompele *et al.* 2002) from expression values of three housekeeping genes (Actin, beta, Glyceraldehyde-3-phosphate dehydrogenase, Periphilin 1) and after GeNORM processing only Actin, beta and Periphilin 1 were kept as reference genes. To determine the statistically significant differences in mRNA contents between the two COC fates, an unpaired *t* test with a P < 0.05 was performed using Prism 5.02 (GraphPad software, La Jolla, CA, USA).

#### **5.7.2.5. Biofunction analysis**

Microarray data were analyzed with the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems; <http://www.ingenuity.com>). Lists of differentially expressed genes were generated from the microarray experiments using a 1.5-fold change cutoff and a value of P < 0.05. Ingenuity Pathway Analysis identified the biofunctions that were the most significant to the data set. The significance of the association between the data set and the biofunctions was assessed in two steps: (1) a ratio of the number of molecules from the data set that fit to the biofunction divided by the total number of molecules that are involved in the biofunctions was established; (2) Fisher's exact test was performed to calculate a P-value determining the probability that the association between the genes in the data set and the biofunction would occur only by chance.

## **5.8. Acknowledgments**

This work was funded by the Natural Sciences and Engineering Research Council (NSERC) (NETGP340825-06) of Canada. A. Bunel was supported by TGF-96122-CIHR Training Program in Reproduction, Early Development, and the Impact on Health (REDIH). E.P.A. Jorssen was supported by a Research grant from the Belgian Government (Federale Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu, Cel Contractueel Onderzoek) "Embryoscreen RF6222."

Rapport Gratuit

## 5.9. References

- Abedelahi A, Salehnia M, Allameh AA, Davoodi D. 2010. Sodium selenite improves the *in vitro* follicular development by reducing the reactive oxygen species level and increasing the total antioxidant capacity and glutathione peroxide activity. *Hum Reprod* 25(4):977-985.
- Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ. 2001. Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction* 121(5):647-653.
- Alvarez GM, Dalvit GC, Cetica PD. 2012. Influence of the cumulus and gonadotropins on the metabolic profile of porcine cumulus-oocyte complexes during *in vitro* maturation. *Reproduction in domestic animals* 47(5):856-864.
- Assidi M, Dieleman SJ, Sirard MA. 2010. Cumulus cell gene expression following the LH surge in bovine preovulatory follicles: potential early markers of oocyte competence. *Reproduction* 140(6):835-852.
- Assou S, Haouzi D, De Vos J, Hamamah S. 2010. Human cumulus cells as biomarkers for embryo and pregnancy outcomes. *Molecular human reproduction* 16(8):531-538.
- Assou S, Haouzi D, Mahmoud K, Aouacheria A, Guillemin Y, Pantesco V, Reme T, Dechaud H, De Vos J, Hamamah S. 2008. A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. *Molecular human reproduction* 14(12):711-719.
- Auclair S, Uzbekov R, Elis S, Sanchez L, Kireev I, Lardic L, Dalbies-Tran R, Uzbekova S. 2013. Absence of cumulus cells during *in vitro* maturation affects lipid metabolism in bovine oocytes. *American journal of physiology Endocrinology and metabolism* 304(6):E599-613.
- Baribault H, Price J, Miyai K, Oshima RG. 1993. Mid-gestational lethality in mice lacking keratin 8. *Genes & development* 7(7A):1191-1202.
- Bayasula, Iwase A, Kobayashi H, Goto M, Nakahara T, Nakamura T, Kondo M, Nagatomo Y, Kotani T, Kikkawa F. 2013. A proteomic analysis of human follicular fluid: comparison between fertilized oocytes and non-fertilized oocytes in the same patient. *Journal of assisted reproduction and genetics* 30(9):1231-1238.
- Bertoldo MJ, Nadal-Desbarats L, Gerard N, Dubois A, Holyoake PK, Grupen CG. 2013. Differences in the metabolomic signatures of porcine follicular fluid collected from environments associated with good and poor oocyte quality. *Reproduction* 146(3):221-231.
- Bettegowda A, Patel OV, Lee KB, Park KE, Salem M, Yao J, Ireland JJ, Smith GW. 2008. Identification of novel bovine cumulus cell molecular markers predictive of oocyte competence: functional and diagnostic implications. *Biology of reproduction* 79(2):301-309.
- Blondin P, Coenen K, Guilbault LA, Sirard MA. 1996. Superovulation can reduce the developmental competence of bovine embryos. *Theriogenology* 46(7):1191-1203.
- Blondin P, Sirard MA. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Molecular reproduction and development* 41(1):54-62.
- Bunel A, Nivet AL, Blondin P, Vigneault C, Richard FJ, Sirard MA. 2013. Cumulus cell gene expression associated with pre-ovulatory acquisition of developmental competence in bovine oocytes. *Reproduction, fertility, and development*.
- Devine PJ, Perreault SD, Luderer U. 2012. Roles of reactive oxygen species and antioxidants in ovarian toxicity. *Biology of reproduction* 86(2):27.
- Edgar R, Domrachev M, Lash AE. 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic acids research* 30(1):207-210.
- Eppig JJ. 2001. Oocyte control of ovarian follicular development and function in mammals. *Reproduction* 122(6):829-838.
- Ferreira FA, Gomez RG, Joaquim DC, Watanabe YF, de Castro e Paula LA, Binelli M, Rodrigues PH. 2011. Short-term urea feeding decreases *in vitro* hatching of bovine blastocysts. *Theriogenology* 76(2):312-319 e311.
- Feuerstein P, Cadoret V, Dalbies-Tran R, Guerif F, Bidault R, Royere D. 2007. Gene expression in human cumulus cells: one approach to oocyte competence. *Hum Reprod* 22(12):3069-3077.
- Feuerstein P, Puard V, Chevalier C, Teusan R, Cadoret V, Guerif F, Houlgate R, Royere D. 2012. Genomic assessment of human cumulus cell marker genes as predictors of oocyte developmental competence: impact of various experimental factors. *PloS one* 7(7):e40449.
- First NL, Leibfried-Rutledge ML, Sirard MA. 1988. Cytoplasmic control of oocyte maturation and species differences in the development of maturational competence. *Progress in clinical and biological research* 267:1-46.
- Fischer B, Kunzel W, Kleinstein J, Gips H. 1992. Oxygen tension in follicular fluid falls with follicle maturation. *European journal of obstetrics, gynecology, and reproductive biology* 43(1):39-43.

- Gebhardt KM, Feil DK, Dunning KR, Lane M, Russell DL. 2011. Human cumulus cell gene expression as a biomarker of pregnancy outcome after single embryo transfer. *Fertility and sterility* 96(1):47-52 e42.
- Gilbert S, Loranger A, Daigle N, Marceau N. 2001. Simple epithelium keratins 8 and 18 provide resistance to Fas-mediated apoptosis. The protection occurs through a receptor-targeting modulation. *The Journal of cell biology* 154(4):763-773.
- Gilchrist RB, Ritter LJ, Armstrong DT. 2004. Oocyte-somatic cell interactions during follicle development in mammals. *Animal reproduction science* 82-83:431-446.
- Goovaerts IG, Leroy JL, Jorssen EP, Bols PE. 2010. Noninvasive bovine oocyte quality assessment: possibilities of a single oocyte culture. *Theriogenology* 74(9):1509-1520.
- Goovaerts IG, Leroy JL, Langbeen A, Jorssen EP, Bosmans E, Bols PE. 2012. Unravelling the needs of singly *in vitro*-produced bovine embryos: from cumulus cell co-culture to semi-defined, oil-free culture conditions. *Reproduction, fertility, and development* 24(8):1084-1092.
- Goovaerts IG, Leroy JL, Van Soom A, De Clercq JB, Andries S, Bols PE. 2009. Effect of cumulus cell coculture and oxygen tension on the *in vitro* developmental competence of bovine zygotes cultured singly. *Theriogenology* 71(5):729-738.
- Hamamah S. 2011. [Omics as a tool for ART]. *Gynecologie, obstetrique & fertilité* 39(1):1-2.
- Hamel M, Dufort I, Robert C, Gravel C, Leveille MC, Leader A, Sirard MA. 2008. Identification of differentially expressed markers in human follicular cells associated with competent oocytes. *Hum Reprod* 23(5):1118-1127.
- Hamel M, Dufort I, Robert C, Leveille MC, Leader A, Sirard MA. 2010. Genomic assessment of follicular marker genes as pregnancy predictors for human IVF. *Molecular human reproduction* 16(2):87-96.
- Hasegawa J, Yanaihara A, Iwasaki S, Mitsukawa K, Negishi M, Okai T. 2007. Reduction of connexin 43 in human cumulus cells yields good embryo competence during ICSI. *Journal of assisted reproduction and genetics* 24(10):463-466.
- Hasegawa J, Yanaihara A, Iwasaki S, Otsuka Y, Negishi M, Akahane T, Okai T. 2005. Reduction of progesterone receptor expression in human cumulus cells at the time of oocyte collection during IVF is associated with good embryo quality. *Hum Reprod* 20(8):2194-2200.
- Iager AE, Kocabas AM, Otu HH, Ruppel P, Langerveld A, Schnarr P, Suarez M, Jarrett JC, Conaghan J, Rosa GJ, Fernandez E, Rawlins RG, Cibelli JB, Crosby JA. 2012. Identification of a novel gene set in human cumulus cells predictive of an oocyte's pregnancy potential. *Fertility and sterility*.
- Jancar N, Kopitar AN, Ihann A, Virant Klun I, Bokal EV. 2007. Effect of apoptosis and reactive oxygen species production in human granulosa cells on oocyte fertilization and blastocyst development. *Journal of assisted reproduction and genetics* 24(2-3):91-97.
- Jaqueamar D, Kupriyanov S, Wankell M, Avis J, Benirschke K, Baribault H, Oshima RG. 2003. Keratin 8 protection of placental barrier function. *The Journal of cell biology* 161(4):749-756.
- Kent C. 1995. Eukaryotic phospholipid biosynthesis. *Annual review of biochemistry* 64:315-343.
- Lee MS, Liu CH, Lee TH, Wu HM, Huang CC, Huang LS, Chen CM, Cheng EH. 2010. Association of creatin kinase B and peroxiredoxin 2 expression with age and embryo quality in cumulus cells. *Journal of assisted reproduction and genetics* 27(11):629-639.
- Li L, Ferin M, Sauer MV, Lobo RA. 2011. Serum and follicular fluid ghrelin levels negatively reflect human oocyte quality and *in vitro* embryo development. *Fertility and sterility* 96(5):1116-1120.
- Li XH, Chen XJ, Ou WB, Zhang Q, Lv ZR, Zhan Y, Ma L, Huang T, Yan YB, Zhou HM. 2013. Knockdown of creatine kinase B inhibits ovarian cancer progression by decreasing glycolysis. *The international journal of biochemistry & cell biology* 45(5):979-986.
- Mamo S, Carter F, Lonergan P, Leal CL, Al Naib A, McGettigan P, Mehta JP, Evans AC, Fair T. 2011. Sequential analysis of global gene expression profiles in immature and *in vitro* matured bovine oocytes: potential molecular markers of oocyte maturation. *BMC genomics* 12:151.
- Martin-Romero FJ, Miguel-Lasobras EM, Dominguez-Arroyo JA, Gonzalez-Carrera E, Alvarez IS. 2008. Contribution of culture media to oxidative stress and its effect on human oocytes. *Reproductive biomedicine online* 17(5):652-661.
- Matoba S, Fair T, Lonergan P. 2010. Maturation, fertilisation and culture of bovine oocytes and embryos in an individually identifiable manner: a tool for studying oocyte developmental competence. *Reproduction, fertility, and development* 22(5):839-851.
- Matos L, Stevenson D, Gomes F, Silva-Carvalho JL, Almeida H. 2009. Superoxide dismutase expression in human *cumulus oophorus* cells. *Molecular human reproduction* 15(7):411-419.
- Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. 2002. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* 296(5576):2178-2180.

- McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P, Matzuk MM. 2004. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum Reprod* 19(12):2869-2874.
- Murthi P, Stevenson JL, Money TT, Borg AJ, Brennecke SP, Gude NM. 2012. Placental CLIC3 is increased in fetal growth restriction and pre-eclampsia affected human pregnancies. *Placenta* 33(9):741-744.
- Nakamichi I, Toivola DM, Strnad P, Michie SA, Oshima RG, Baribault H, Omary MB. 2005. Keratin 8 overexpression promotes mouse Mallory body formation. *The Journal of cell biology* 171(6):931-937.
- Nikitovic D, Katonis P, Tsatsakis A, Karamanos NK, Tzanakakis GN. 2008. Lumican, a small leucine-rich proteoglycan. *IUBMB life* 60(12):818-823.
- Nivet AL, Bunel A, Labrecque R, Belanger J, Vigneault C, Blondin P, Sirard MA. 2012. FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction* 143(2):165-171.
- Nivet AL, Vigneault C, Blondin P, Sirard MA. 2013. Changes in granulosa cells' gene expression associated with increased oocyte competence in bovine. *Reproduction* 145(6):555-565.
- Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M. 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* 303(5658):682-684.
- Robert C, Nieminen J, Dufort I, Gagne D, Grant JR, Cagnone G, Plourde D, Nivet AL, Fournier E, Paquet E, Blazejczyk M, Rigault P, Juge N, Sirard MA. 2011. Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays. *Molecular reproduction and development* 78(9):651-664.
- Seli E, Robert C, Sirard MA. 2010. OMICS in assisted reproduction: possibilities and pitfalls. *Molecular human reproduction* 16(8):513-530.
- Sinclair KD, Kuran M, Gebbie FE, Webb R, McEvoy TG. 2000. Nitrogen metabolism and fertility in cattle: II. Development of oocytes recovered from heifers offered diets differing in their rate of nitrogen release in the rumen. *Journal of animal science* 78(10):2670-2680.
- Sirard MA, Coenen K. 2006. In vitro maturation and embryo production in cattle. *Methods Mol Biol* 348:35-42.
- Su YQ, Sugiura K, Eppig JJ. 2009. Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism. *Seminars in reproductive medicine* 27(1):32-42.
- Sutton-McDowall ML, Gilchrist RB, Thompson JG. 2004. Cumulus expansion and glucose utilisation by bovine cumulus-oocyte complexes during *in vitro* maturation: the influence of glucosamine and follicle-stimulating hormone. *Reproduction* 128(3):313-319.
- Sutton-McDowall ML, Gilchrist RB, Thompson JG. 2010. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* 139(4):685-695.
- Tang W, Yuan J, Chen X, Gu X, Luo K, Li J, Wan B, Wang Y, Yu L. 2006. Identification of a novel human lysophosphatidic acid acyltransferase, LPAAT-theta, which activates mTOR pathway. *Journal of biochemistry and molecular biology* 39(5):626-635.
- Vajta G, Peura TT, Holm P, Paldi A, Greve T, Trounson AO, Callesen H. 2000. New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. *Molecular reproduction and development* 55(3):256-264.
- van Montfoort AP, Geraedts JP, Dumoulin JC, Stassen AP, Evers JL, Ayoubi TA. 2008. Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. *Molecular human reproduction* 14(3):157-168.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* 3(7):RESEARCH0034.
- Williams KE, Fulford LA, Albig AR. 2010. Lumican reduces tumor growth via induction of fas-mediated endothelial cell apoptosis. *Cancer microenvironment : official journal of the International Cancer Microenvironment Society* 4(1):115-126.
- Wu G, Morris SM, Jr. 1998. Arginine metabolism: nitric oxide and beyond. *The Biochemical journal* 336 (Pt 1):1-17.
- Zeltz C, Brezillon S, Kapyla J, Eble JA, Bobichon H, Terryn C, Perreau C, Franz CM, Heino J, Maquart FX, Wegrowski Y. 2010. Lumican inhibits cell migration through alpha2beta1 integrin. *Experimental cell research* 316(17):2922-2931.
- Zhang X, Jafari N, Barnes RB, Confino E, Milad M, Kazer RR. 2005. Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. *Fertility and sterility* 83 Suppl 1:1169-1179.

## 5.10. Tables

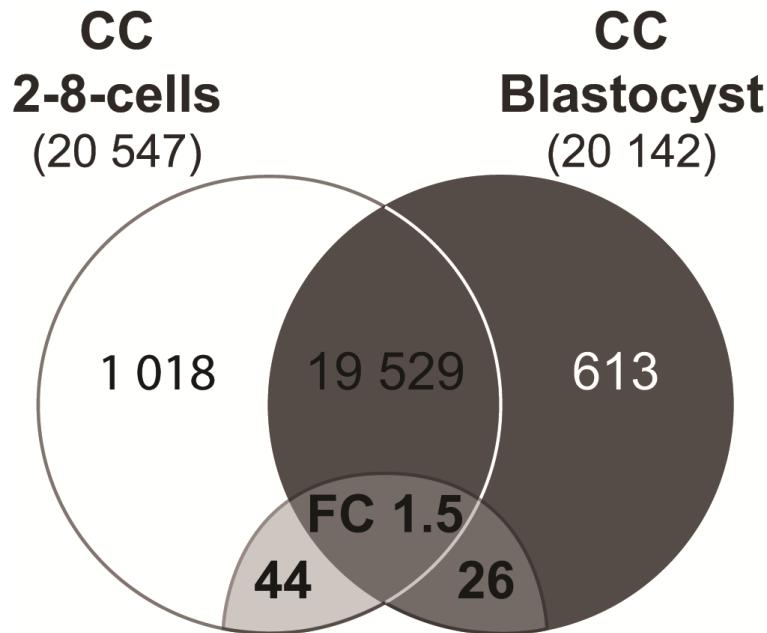
**Table 5-1. Cleavage and Blastocyst rates.**

Cleavage (48 hours p.i.) and blastocyst (8 days p.i.) rates, mean cell number and mean apoptotic cell ratio of control in group matured, fertilized, and cultured COCs (GGG) and of fully individually produced embryos (SSS).

Treatment	N	N cleavage (%)	N total blastocyst (%)	Mean cell number ± SD	Mean apoptotic cell ratio ± SD
<b>GGG</b>	631	461 (73.1)	197 (31.2)	141.7 ± 15.0	3.2 ± 2.2
<b>SSS</b>	249	187 (75.1)	69 (27.7)	150.5 ± 10.4	2.8 ± 1.4

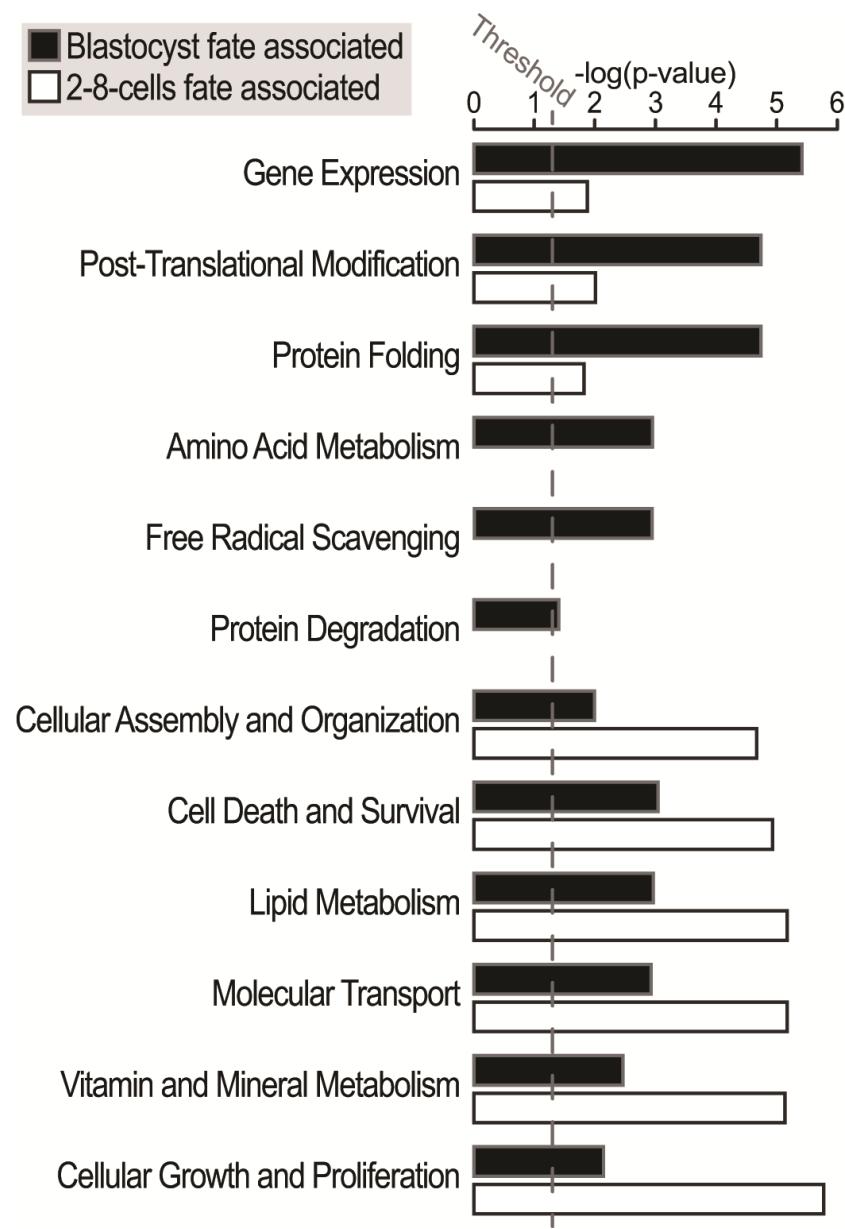
Abbreviation: p.i., post insemination.

## 5.11. Figures



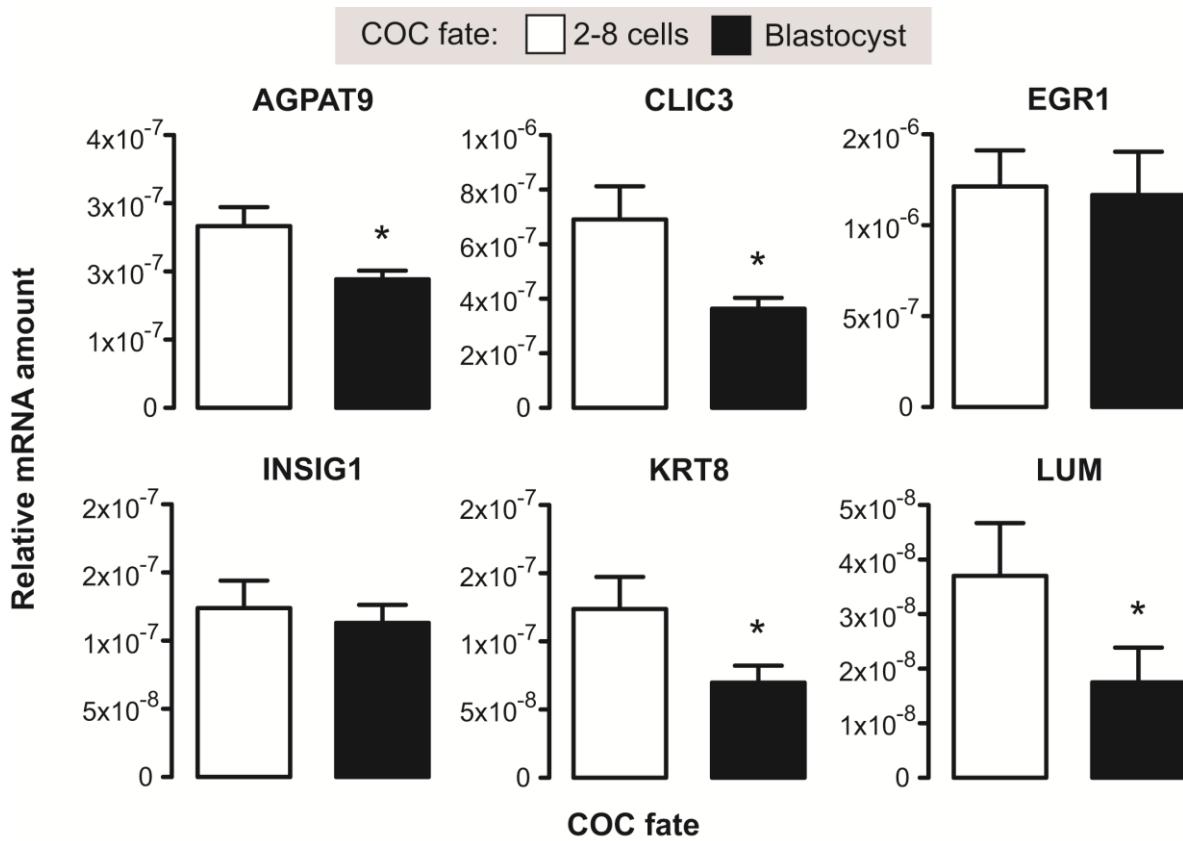
*Figure 5-1. Venn diagram representing the number of expressed genes in bovine CCs depending on the fate of their oocyte, i.e. 2- to 8-cells arrested embryos or Day 8 blastocysts.*

The diagram indicates the number of probes corresponding to unique gene symbols with at least one probe having an intensity signal higher than the defined threshold (background intensity plus two times its standard deviation for each condition). Numbers between brackets indicate the total gene number for each corresponding condition. Numbers on the bottom represent the number of probes differentially expressed with a 1.5-FC cutoff and  $P < 0.05$ .  $n = 3$ . CC, cumulus cell; FC, fold change.



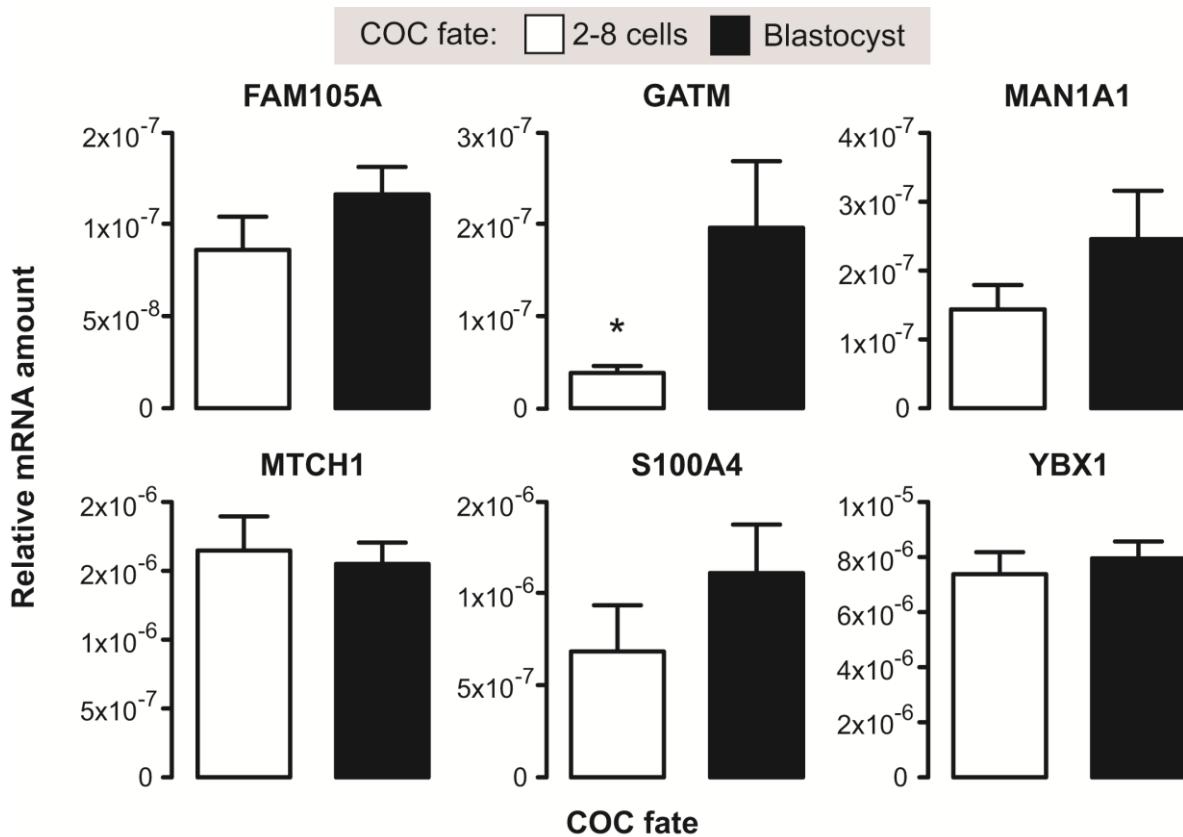
**Figure 5-2. Top 12 Ingenuity Pathway Analysis biofunctions in bovine cumulus cells**

(top six of each cumulus-oocyte complex fate category) and their significance. n = 3.



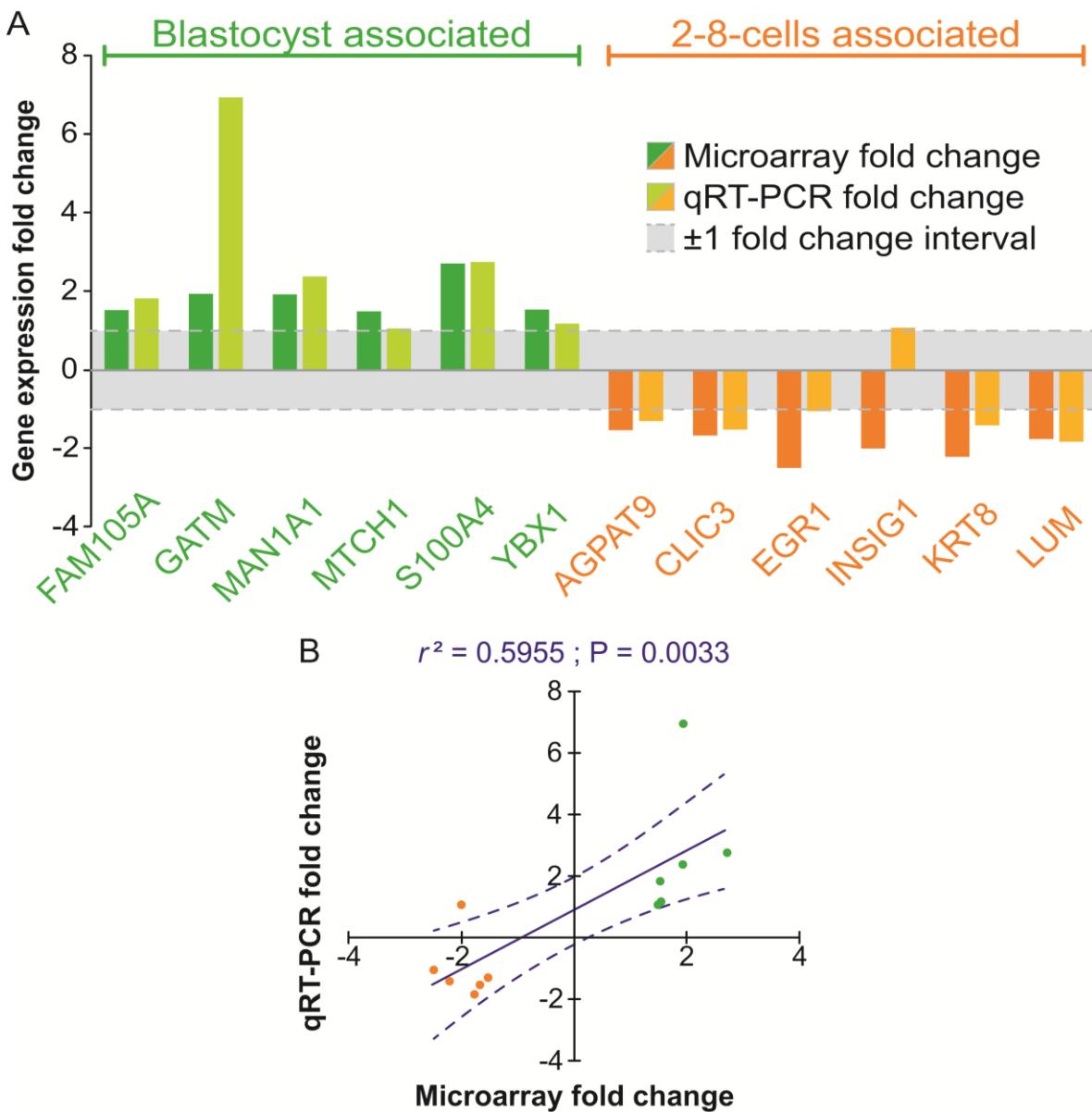
**Figure 5-3. Quantification of selected genes by qRT-PCR in CCs from each COC fate category.**

These six genes were upregulated in the embryo arrested at 2- to 8-cell stage (CC-2-8-cells) according to the microarray data.  $n = 8$  replicates and data are the mean  $\pm$  standard error of the mean, \* $P < 0.05$ . AGPAT9, 1-acylglycerol-3-phosphate O-acyltransferase 9; CC, cumulus cell; CLIC3, Chloride intracellular channel 3; COC, cumulus-oocyte complex; EGR1, Early growth response 1; INSIG1, Insulin induced gene 1; KRT8, Keratin 8; LUM, Lumican; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.



**Figure 5-4. Quantification of selected genes by qRT-PCR in CCs for each COC fate category.**

These six genes were upregulated in the embryo at blastocyst stage (CC-Blast) according to the microarray data.  $n = 8$  and data are the mean  $\pm$  standard error of the mean, \* $P < 0.05$ . CC, cumulus cell; COC, cumulus-oocyte complex; FAM105A, Family with sequence similarity 105, member A; GATM, Glycine amidinotransferase; MAN1A1, Mannosidase alpha, class 1A, member 1; MTCH1, Mitochondrial carrier 1; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; YBX1, Y box binding protein 1.



**Figure 5-5. Bovine cumulus cell gene expression fold change comparison**

(A) and correlation (B) between microarray and qRT-PCR for 12 selected genes associated with the blastocyst or the 2- to 8-cell fate.

$n = 8$ . qRT-PCR, quantitative reverse transcription–polymerase chain reaction. (For interpretation of the references to color in this Figure, the reader is referred to the web version of this article.)

## 6. CONCLUSION ET DISCUSSION GÉNÉRALES

Ces dernières décennies, l'amélioration des techniques de reproduction assistée a suscité un grand intérêt pour le recours à ces techniques tant chez l'humain que chez le bovin. En retour, l'intensification de la pratique de la procréation assistée incite à davantage de recherche afin d'optimiser les protocoles et ainsi obtenir de meilleurs résultats que ce soit en termes de quantité, mais surtout en termes de qualité des embryons produits et des naissances consécutives aux cycles de stimulation/fécondation. La qualité d'un embryon dépendant de la qualité de son ovocyte originel, sans pour autant négliger le rôle du spermatozoïde et la composition des milieux de culture, la compréhension la plus approfondie et juste possible des mécanismes régissant la qualité ovocytaire est fondamentale. Pour ce faire, nous nous sommes engagés dans l'étude des cellules adjointes de l'ovocyte : les cellules du cumulus, plus particulièrement dans l'exploration et l'analyse de leur transcriptome.

Dans un premier temps, nous avons obtenu un portrait de l'évolution de l'expression génique du cumulus au cours de la phase finale d'acquisition de la compétence ovocytaire au développement. Ainsi, les gènes dont l'expression varie progressivement et parallèlement à la qualité ovocytaire, évaluée *via* les taux de blastocystes, seraient vraisemblablement des acteurs dans l'acquisition des compétences de l'ovocyte. En outre en étirant la période de différenciation folliculaire par l'allongement du sevrage en FSH avant la ponction ovocytaire, nous avons constaté que la compétence ovocytaire diminue ([Nivet et al. 2012](#)). L'expression génique du cumulus reflète cet amoindrissement de compétence. Les gènes impliqués tant dans l'acquisition que dans la perte de compétence de l'ovocyte pourraient alors servir à orienter l'optimisation des conditions de procréation. Cette optimisation pourrait passer par exemple par : l'ajustement des durées de stimulation ovarienne afin d'obtenir un profil d'expression génique reconnu comme étant idéal vis-à-vis de la compétence et de la qualité ovocytaire ; l'ajout de molécules régulant adéquatement l'expression des gènes identifiés comme devant être soit exprimés soit réprimés, afin de mimer au mieux les conditions physiologiques, particulièrement dans le cas de la maturation *in vitro*.

Dans un deuxième temps, en utilisant un inhibiteur de la production de LH, nous avons montré l'influence de cette LH sur le transcriptome du cumulus. Cette méthode fréquemment utilisée en stimulation ovarienne, avec notamment du Cétrorelix, un antagoniste de la GnRH inhibant la production de LH, permet de prévenir une ovulation prématurée des follicules. Bien que les taux de blastocystes associés à ce protocole ne soient pas significativement différents en présence ou en

absence de Cetrorelix ([Labrecque et al. 2014](#)), le transcriptome du cumulus, lui, est significativement remanié. D'ailleurs d'autres études indiquent que l'absence de LH en fin de maturation folliculaire pourrait avoir un effet à long terme. En effet, de faibles taux de LH ont été reliés à une hausse des taux d'échec précoce de la gestation ([Chen et al. 2016](#)), et l'utilisation de Cétrorelix à plus de résorption embryonnaire ([Bittner et al. 2011](#)). Comme le montre la possible inhibition, en absence de LH, de fonctions en lien avec la transcription, la production d'énergie, la synthèse protéique ou encore le métabolisme des acides aminés dans les cellules du cumulus, le rôle de la LH, même à des concentrations dites basales, au moment de la maturation finale du follicule, et donc de l'ovocyte, ne doit pas être sous-estimé.

La troisième étape a été de vérifier si l'influence de la LH, constatée précédemment, passait par l'expression du récepteur à la LH dans les cellules du cumulus bovin. Pourtant, l'absence de ce récepteur pendant la maturation *in vitro* met en doute une possible action directe de la LH sur le cumulus. Ceci suggère plutôt un rôle d'intermédiaire pour la granulosa qui elle exprime le récepteur à la LH ([Eppig et al. 1997](#)) et transmettrait ainsi le signal LH au cumulus par des mécanismes encore à éclaircir. Ainsi, l'intérêt de l'ajout de LH dans les milieux de maturation encore pratiqué par certains groupes chez le bovin paraît inutile. Ces observations illustrent un système gonadotropique en deux parties au sein du follicule avec le granulosa qui est capable de répondre à la LH et le cumulus qui lui répondrait davantage à la FSH. En parallèle, nous avons également montré l'expression d'enzymes stéroïdogènes, sans influence du récepteur à la LH, par les cellules du cumulus. Et bien que la production de progestérone n'ait pas été vérifiée ici, les cellules du cumulus bovin semblent avoir la capacité de répondre à une stimulation par la P4 puisqu'elles expriment son récepteur. Enfin, la variation de l'expression du régulateur de l'expression du récepteur à la LH, MVK, au sein du cumulus au cours de la maturation incite à plus de recherche quant à son rôle dans ces cellules.

Finalement, nous avons associé l'expression génique du cumulus au destin embryonnaire de son complexe-ovocyte cumulus d'origine. De cette manière nous avons obtenu le profil transcriptomique de cellules du cumulus associées à un ovocyte compétent et celui de cellules du cumulus associées à un ovocyte incompétent. Suite à l'analyse de ces profils, plusieurs processus importants dans le soutien à l'ovocyte se sont démarqués. Par exemple, l'expression génique des cumuli associés à la formation d'un blastocyste est affiliée à une modification post-traductionnelle, un replis des protéines, un métabolisme des acides aminés, une dégradation des protéines ou encore une épuration des radicaux libres plus importants que pour les cumuli associés à un embryon ayant échoué. Les

résultats de cette étude éclaircissent sur ce qui fait d'un cumulus un « bon cumulus » pour son ovocyte, et a permis d'obtenir des gènes pouvant servir à la prédition du devenir d'un COC.

Ce travail de doctorat apporte ainsi de nouvelles connaissances fondamentales concernant l'expression génique des cellules du cumulus bovin, notamment au regard de la compétence ovocytaire au développement. Plusieurs portraits transcriptomiques complets du cumulus ont ainsi été dressés et des gènes marqueurs de compétence/incompétence en sont ressortis. Ces bio-marqueurs pourraient éventuellement être utilisés à des fins de prédition du destin d'un COC dès le début de la maturation *in vitro*, ou bien comme indicateurs de l'(in)adéquation des milieux de culture ou de la stimulation ovarienne.

Le premier portrait transcriptomique du cumulus obtenu lors de ce travail, est un portrait que l'on pourrait qualifier de « tri-dimensionnel », en ceci qu'il a été établi pour trois périodes différentes : l'*acquisition*, le *maintien* et la *perte* de la compétence au développement. Il est alors possible d'explorer les gènes ou groupes de gènes du cumulus impliqués dans chacun de ces trois processus. Par ailleurs, le cumulus se trouvant à l'interface entre l'ovocyte et le reste du follicule (liquide folliculaire, granulosa et thèque), son transcriptome reflète à la fois l'état de compétence de l'ovocyte et l'état de différenciation dans lequel le follicule est engagé (en cours de maturation, mature, ou sur le déclin). Toutefois, de par son organisation anatomique, il est possible qu'un léger décalage temporel de l'expression génique en réponse à un stimulus ait lieu au sein du follicule. La granulosa étant en première ligne pour recevoir les signaux extérieurs au follicule (hormones, température, oxygénation...), elle y répond avant le cumulus, qui lui-même y répondra avant l'ovocyte et *vice versa* concernant les signaux en provenance dudit ovocyte. De la même manière qu'on observe l'atrésie progresser de la granulosa vers l'ovocyte, ou l'inhibition de l'expression du récepteur à la LH s'étioler de l'ovocyte vers la granulosa, la réponse à la stimulation ovarienne, par exemple, pourrait être observée à un temps *t* dans le transcriptome de la granulosa, mais pas encore dans celui du cumulus, ni dans celui de l'ovocyte. En cumulant les informations obtenues sur l'*acquisition*, le *maintien* et la *perte* de compétence ovocytaire au travers du cumulus, aux informations en lien avec le *destin* du COC, il est possible d'affiner encore d'avantage la description du bagage transcriptomique que doit porter un cumulus pour être capable de jouer correctement son/ses rôles auprès de l'ovocyte. Finalement, le transcriptome ne donne malheureusement qu'une partie de l'information concernant une cellule *fonctionnelle*. Pour avoir le schéma de fonctionnement dans son ensemble, il faut notamment adjoindre à la transcriptomique ses co-équipières que sont la génomique, l'épi-génomique, la protéomique, la métabolomique et autres « omics ».

L'accroissement continu des connaissances sur la mystérieuse organisation du COC, et même du follicule dans son ensemble, permet d'optimiser, ou de perfectionner, les protocoles de stimulation ovarienne, ainsi que les milieux de culture *in vitro* utilisés au cours de la procréation assistée ; et donc, *in fine*, la qualité des ovocytes et embryons cultivés en vue d'obtenir une progéniture saine et vigoureuse.

Enfin, au-delà de la production *in vitro* d'embryons, plus nous comprendrons et combinerons les différentes disciplines en lien avec l'étude des mécanismes reproductifs (femelle et mâle), plus nous serons en mesure d'adapter l'environnement et le mode de vie des individus pour favoriser leur reproduction. On sait déjà par exemple, que pour certains animaux comme les ovins, la durée du cycle jour/nuit influence les cycles de reproduction ([Malpaux et al. 1997](#)), ou encore que la chaleur diminue la fertilité des vaches ([De Rensis et al. 2017](#)). De même, les effets de la nutrition sur la reproduction animale et humaine, tant femelle que mâle d'ailleurs, sont de plus en plus reconnus et étudiés ([Leroy et al. 2015; Zebeli et al. 2015; Adewoyin et al. 2017; Gaskins and Chavarro 2017; Guan and Martin 2017](#)). D'ailleurs, les connexions entre le métabolisme et l'axe hypothalamo-hypophysio-gonadique dans son entiereté sont également explorées ([Dupont et al. 2010; Evans and Anderson 2017; Manfredi-Lozano et al. 2017](#)). Les effets de l'activité physique font aussi l'objet de recherche ([Harrison et al. 2016](#)). Finalement, les effets de l'exposition à certains composants chimiques de notre environnement – appelés perturbateurs endocriniens – sur la reproduction sont passés au crible ([Minguez-Alarcon et al. 2016; Ziv-Gal and Flaws 2016](#)).

Les années à venir devraient ainsi voir des changements importants se mettre en place dans la gestion de la reproduction, et ce tant pour les élevages que pour les couples désirant un enfant.

## BIBLIOGRAPHIE

- Abd El Naby WS, Hagos TH, Hossain MM, Salilew-Wondim D, Gad AY, Rings F, Cinar MU, Tholen E, Looft C, Schellander K, Hoelker M, Tesfaye D. 2013. Expression analysis of regulatory microRNAs in bovine cumulus oocyte complex and preimplantation embryos. *Zygote* 21(1):31-51.
- Adamiak SJ, Powell K, Rooke JA, Webb R, Sinclair KD. 2006. Body composition, dietary carbohydrates and fatty acids determine post-fertilisation development of bovine oocytes in vitro. *Reproduction* 131(2):247-258.
- Adewoyin M, Ibrahim M, Roszaman R, Isa MLM, Alewi NAM, Rafa AAA, Anuar MNN. 2017. Male Infertility: The Effect of Natural Antioxidants and Phytochemicals on Seminal Oxidative Stress. *Diseases* 5(1).
- Aerts JM, Bols PE. 2010b. Ovarian follicular dynamics. A review with emphasis on the bovine species. Part II: Antral development, exogenous influence and future prospects. *Reproduction in domestic animals = Zuchthygiene* 45(1):180-187.
- Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. 2004. Cytoskeleton. In: Molecular biology of the cell, 4th ed. Artmed.
- Alvarez GM, Casiro S, Gutnisky C, Dalvit GC, Sutton-McDowall ML, Thompson JG, Cetica PD. 2016. Implications of glycolytic and pentose phosphate pathways on the oxidative status and active mitochondria of the porcine oocyte during IVM. *Theriogenology* 86(9):2096-2106.
- Anderson E, Albertini DF. 1976. Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. *The Journal of cell biology* 71(2):680-686.
- Anguita B, Jimenez-Macedo AR, Izquierdo D, Mogas T, Paramio MT. 2007. Effect of oocyte diameter on meiotic competence, embryo development, p34 (cdc2) expression and MPF activity in prepubertal goat oocytes. *Theriogenology* 67(3):526-536.
- Auclair S, Uzbekov R, Elis S, Sanchez L, Kireev I, Lardic L, Dalbies-Tran R, Uzbekova S. 2013. Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. *American journal of physiology Endocrinology and metabolism* 304(6):E599-613.
- Augustin R, Pocar P, Navarrete-Santos A, Wrenzycki C, Gandolfi F, Niemann H, Fischer B. 2001. Glucose transporter expression is developmentally regulated in in vitro derived bovine preimplantation embryos. *Molecular reproduction and development* 60(3):370-376.
- Baker TG. 1963. A Quantitative and Cytological Study of Germ Cells in Human Ovaries. *Proceedings of the Royal Society of London Series B, Biological sciences* 158:417-433.
- Baruselli PS, Ferreira RM, Sales JN, Gimenes LU, Sa Filho MF, Martins CM, Rodrigues CA, Bo GA. 2011. Timed embryo transfer programs for management of donor and recipient cattle. *Theriogenology* 76(9):1583-1593.
- Beg MA, Ginther OJ. 2006. Follicle selection in cattle and horses: role of intrafollicular factors. *Reproduction* 132(3):365-377.
- Bergh C, Bryman I, Nilsson L, Janson PO. 1998. Results of gonadotrophin stimulation with the option to convert cycles to in vitro fertilization in cases of multifollicular development. *Acta obstetricia et gynecologica Scandinavica* 77(1):68-73.
- Berry DP, Friggins NC, Lucy M, Roche JR. 2016. Milk Production and Fertility in Cattle. *Annual review of animal biosciences* 4:269-290.
- Biason-Lauber A. 2010. Control of sex development. *Best practice & research Clinical endocrinology & metabolism* 24(2):163-186.
- Bilodeau S, Fortier MA, Sirard MA. 1993. Effect of adenylate cyclase stimulation on meiotic resumption and cyclic AMP content of zona-free and cumulus-enclosed bovine oocytes in vitro. *Journal of reproduction and fertility* 97(1):5-11.
- Bittner AK, Horsthemke B, Winterhager E, Grummer R. 2011. Hormone-induced delayed ovulation affects early embryonic development. *Fertility and sterility* 95(7):2390-2394.
- Blondin P, Bousquet D, Twagiramungu H, Barnes F, Sirard MA. 2002. Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biology of reproduction* 66(1):38-43.
- Blondin P, Coenen K, Guilbault LA, Sirard MA. 1996a. Superovulation can reduce the developmental competence of bovine embryos. *Theriogenology* 46(7):1191-1203.
- Blondin P, Dufour M, Sirard MA. 1996b. Analysis of atresia in bovine follicles using different methods: flow cytometry, enzyme-linked immunosorbent assay, and classic histology. *Biology of reproduction* 54(3):631-637.
- Blondin P, Sirard MA. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Molecular reproduction and development* 41(1):54-62.

- Bornslaeger EA, Schultz RM. 1985. Regulation of mouse oocyte maturation: effect of elevating cumulus cell cAMP on oocyte cAMP levels. *Biology of reproduction* 33(3):698-704.
- Braw-Tal R, Yossefi S. 1997. Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. *Journal of reproduction and fertility* 109(1):165-171.
- Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ. 1990. FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Developmental biology* 138(1):16-25.
- Burnik Papler T, Vrtacnik Bokal E, Maver A, Kopitar AN, Lovrecic L. 2015. Transcriptomic Analysis and Meta-Analysis of Human Granulosa and Cumulus Cells. *PloS one* 10(8):e0136473.
- Caballero J, Gilbert I, Fournier E, Gagne D, Scantland S, Macaulay A, Robert C. 2014. Exploring the function of long non-coding RNA in the development of bovine early embryos. *Reproduction, fertility, and development* 27(1):40-52.
- Campbell JM, Lane M, Vassiliev I, Nottle MB. 2013. Epiblast cell number and primary embryonic stem cell colony generation are increased by culture of cleavage stage embryos in insulin. *The Journal of reproduction and development* 59(2):131-138.
- Cetica P, Pintos L, Dalvit G, Beconi M. 2002. Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation in vitro. *Reproduction* 124(5):675-681.
- Cetica P, Pintos L, Dalvit G, Beconi M. 2003. Involvement of enzymes of amino acid metabolism and tricarboxylic acid cycle in bovine oocyte maturation in vitro. *Reproduction* 126(6):753-763.
- Chen CD, Chiang YT, Yang PK, Chen MJ, Chang CH, Yang YS, Chen SU. 2016. Frequency of low serum LH is associated with increased early pregnancy loss in IVF/ICSI cycles. *Reproductive biomedicine online* 33(4):449-457.
- Colonna R, Mangia F. 1983. Mechanisms of amino acid uptake in cumulus-enclosed mouse oocytes. *Biology of reproduction* 28(4):797-803.
- Colton SA, Downs SM. 2004. Potential role for the sorbitol pathway in the meiotic dysfunction exhibited by oocytes from diabetic mice. *Journal of experimental zoology Part A, Comparative experimental biology* 301(5):439-448.
- Curry TE, Jr., Mann JS, Huang MH, Keeble SC. 1992. Gelatinase and proteoglycanase activity during the periovulatory period in the rat. *Biology of reproduction* 46(2):256-264.
- Dan-Goor M, Sasson S, Davarashvili A, Almagor M. 1997. Expression of glucose transporter and glucose uptake in human oocytes and preimplantation embryos. *Human reproduction* 12(11):2508-2510.
- Davis BJ, Lennard DE, Lee CA, Tiano HF, Morham SG, Wetsel WC, Langenbach R. 1999. Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1beta. *Endocrinology* 140(6):2685-2695.
- De Felici M, Klinger FG, Farini D, Scaldaferri ML, Iona S, Lobascio M. 2005. Establishment of oocyte population in the fetal ovary: primordial germ cell proliferation and oocyte programmed cell death. *Reproductive biomedicine online* 10(2):182-191.
- de Loos F, Kastrop P, Van Maurik P, Van Beneden TH, Kruip TA. 1991. Heterologous cell contacts and metabolic coupling in bovine cumulus oocyte complexes. *Molecular reproduction and development* 28(3):255-259.
- De Rensis F, Lopez-Gatius F, Garcia-Isprierto I, Morini G, Scaramuzzi RJ. 2017. Causes of declining fertility in dairy cows during the warm season. *Theriogenology* 91:145-153.
- Dehennaut V, Lefebvre T, Leroy Y, Vilain JP, Michalski JC, Bodart JE. 2009. Survey of O-GlcNAc level variations in *Xenopus laevis* from oogenesis to early development. *Glycoconjugate journal* 26(3):301-311.
- Diaz FJ, Wigglesworth K, Eppig JJ. 2007. Oocytes determine cumulus cell lineage in mouse ovarian follicles. *Journal of cell science* 120(Pt 8):1330-1340.
- Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P. 1998. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proceedings of the National Academy of Sciences of the United States of America* 95(23):13612-13617.
- Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* 383(6600):531-535.
- Downs SM, Humpherson PG, Leese HJ. 1998. Meiotic induction in cumulus cell-enclosed mouse oocytes: involvement of the pentose phosphate pathway. *Biology of reproduction* 58(4):1084-1094.

- Drahorad J, Tesarik J, Cechova D, Vilim V. 1991. Proteins and glycosaminoglycans in the intercellular matrix of the human cumulus-oophorus and their effect on conversion of proacrosin to acrosin. *Journal of reproduction and fertility* 93(2):253-262.
- Driancourt MA. 2001. Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. *Theriogenology* 55(6):1211-1239.
- Dunning KR, Akison LK, Russell DL, Norman RJ, Robker RL. 2011. Increased beta-oxidation and improved oocyte developmental competence in response to l-carnitine during ovarian in vitro follicle development in mice. *Biology of reproduction* 85(3):548-555.
- Dunning KR, Anastasi MR, Zhang VJ, Russell DL, Robker RL. 2014a. Regulation of fatty acid oxidation in mouse cumulus-oocyte complexes during maturation and modulation by PPAR agonists. *PloS one* 9(2):e87327.
- Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL. 2010. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biology of reproduction* 83(6):909-918.
- Dunning KR, Russell DL, Robker RL. 2014b. Lipids and oocyte developmental competence: the role of fatty acids and beta-oxidation. *Reproduction* 148(1):R15-27.
- Dunning KR, Watson LN, Zhang VJ, Brown HM, Kaczmarek AK, Robker RL, Russell DL. 2015. Activation of Mouse Cumulus-Oocyte Complex Maturation In Vitro Through EGF-Like Activity of Versican. *Biology of reproduction* 92(5):116.
- Dupont J, Maillard V, Coyral-Castel S, Rame C, Froment P. 2010. Ghrelin in female and male reproduction. *International journal of peptides* 2010.
- Edwards RG. 1965. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature* 208(5008):349-351.
- Emori C, Sugiura K. 2014. Role of oocyte-derived paracrine factors in follicular development. *Animal science journal = Nihon chikusan Gakkaiho* 85(6):627-633.
- Eppig JJ, Pendola FL, Wigglesworth K, Pendola JK. 2005. Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: amino acid transport. *Biology of reproduction* 73(2):351-357.
- Eppig JJ, Wigglesworth K, Pendola F, Hirao Y. 1997. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biology of reproduction* 56(4):976-984.
- Eppig JJ, Wigglesworth K, Pendola FL. 2002. The mammalian oocyte orchestrates the rate of ovarian follicular development. *Proceedings of the National Academy of Sciences of the United States of America* 99(5):2890-2894.
- Erickson BH. 1966. Development and senescence of the postnatal bovine ovary. *Journal of animal science* 25(3):800-805.
- Erickson GF, Shimasaki S. 2000. The role of the oocyte in folliculogenesis. *Trends in endocrinology and metabolism: TEM* 11(5):193-198.
- Espey LL. 1980. Ovulation as an inflammatory reaction--a hypothesis. *Biology of reproduction* 22(1):73-106.
- Espey LL, Richards JS. 2002. Temporal and spatial patterns of ovarian gene transcription following an ovulatory dose of gonadotropin in the rat. *Biology of reproduction* 67(6):1662-1670.
- Evans AC, Adams GP, Rawlings NC. 1994. Endocrine and ovarian follicular changes leading up to the first ovulation in prepubertal heifers. *Journal of reproduction and fertility* 100(1):187-194.
- Evans MC, Anderson GM. 2017. Neuroendocrine integration of nutritional signals on reproduction. *Journal of molecular endocrinology* 58(2):R107-R128.
- Fair T. 2003. Follicular oocyte growth and acquisition of developmental competence. *Animal reproduction science* 78(3-4):203-216.
- Fair T, Hyttel P, Greve T. 1995. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Molecular reproduction and development* 42(4):437-442.
- Fenwick J, Platteau P, Murdoch AP, Herbert M. 2002. Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos in vitro. *Human reproduction* 17(2):407-412.
- Ferguson EM, Leese HJ. 2006. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. *Molecular reproduction and development* 73(9):1195-1201.
- Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA, Navarro PA. 2009. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology* 71(5):836-848.

- Filion F, Bouchard N, Goff AK, Lussier JG, Sirois J. 2001. Molecular cloning and induction of bovine prostaglandin E synthase by gonadotropins in ovarian follicles prior to ovulation in vivo. *The Journal of biological chemistry* 276(36):34323-34330.
- Findlay JK, Drummond AE. 1999. Regulation of the FSH Receptor in the Ovary. *Trends in endocrinology and metabolism*: TEM 10(5):183-188.
- Frank LA, Sutton-McDowall ML, Brown HM, Russell DL, Gilchrist RB, Thompson JG. 2014. Hyperglycaemic conditions perturb mouse oocyte in vitro developmental competence via beta-O-linked glycosylation of heat shock protein 90. *Human reproduction* 29(6):1292-1303.
- Fulka J, Jr., First NL, Moor RM. 1998. Nuclear and cytoplasmic determinants involved in the regulation of mammalian oocyte maturation. *Molecular human reproduction* 4(1):41-49.
- Galli C, Lazzari G. 2008. The manipulation of gametes and embryos in farm animals. *Reproduction in domestic animals = Zuchthygiene* 43 Suppl 2:1-7.
- Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luoro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH, Ritvos O. 2000. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nature genetics* 25(3):279-283.
- Gaskins AJ, Chavarro JE. 2017. Diet and fertility: a review. *American journal of obstetrics and gynecology*.
- Gerris JM. 2005. Single embryo transfer and IVF/ICSI outcome: a balanced appraisal. *Human reproduction update* 11(2):105-121.
- Gilchrist RB, Lane M, Thompson JG. 2008. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Human reproduction update* 14(2):159-177.
- Ginther OJ, Beg MA, Bergfelt DR, Donadeu FX, Kot K. 2001. Follicle selection in monovular species. *Biology of reproduction* 65(3):638-647.
- Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. 1998. Pulsatility of systemic FSH and LH concentrations during follicular-wave development in cattle. *Theriogenology* 50(4):507-519.
- Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. 1999. Selection of the dominant follicle in cattle: establishment of follicle deviation in less than 8 hours through depression of FSH concentrations. *Theriogenology* 52(6):1079-1093.
- Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. 2000. Selection of the dominant follicle in cattle: role of estradiol. *Biology of reproduction* 63(2):383-389.
- Ginther OJ, Kot K, Kulick LJ, Wiltbank MC. 1997. Emergence and deviation of follicles during the development of follicular waves in cattle. *Theriogenology* 48(1):75-87.
- Grisart B, Massip A, Dassy F. 1994. Cinematographic analysis of bovine embryo development in serum-free oviduct-conditioned medium. *Journal of reproduction and fertility* 101(2):257-264.
- Guan Y, Martin GB. 2017. Cellular and molecular responses of adult testis to changes in nutrition: novel insights from the sheep model. *Reproduction* 154(5):R133-R141.
- Guidobaldi HA, Teves ME, Unates DR, Anastasia A, Giojalas LC. 2008. Progesterone from the cumulus cells is the sperm chemoattractant secreted by the rabbit oocyte cumulus complex. *PloS one* 3(8):e3040.
- Guo XY, Liu XM, Jin L, Wang TT, Ullah K, Sheng JZ, Huang HF. 2017. Cardiovascular and metabolic profiles of offspring conceived by assisted reproductive technologies: a systematic review and meta-analysis. *Fertility and sterility* 107(3):622-631 e625.
- Gutnisky C, Dalvit GC, Pintos LN, Thompson JG, Beconi MT, Cetica PD. 2007. Influence of hyaluronic acid synthesis and cumulus mucification on bovine oocyte in vitro maturation, fertilisation and embryo development. *Reproduction, fertility, and development* 19(3):488-497.
- Hagemann LJ. 1999. Influence of the dominant follicle on oocytes from subordinate follicles. *Theriogenology* 51(2):449-459.
- Han SJ, Conti M. 2006. New pathways from PKA to the Cdc2/cyclin B complex in oocytes: Wee1B as a potential PKA substrate. *Cell cycle* 5(3):227-231.
- Harris SE, Leese HJ, Gosden RG, Picton HM. 2009. Pyruvate and oxygen consumption throughout the growth and development of murine oocytes. *Molecular reproduction and development* 76(3):231-238.
- Harrison CL, Brown WJ, Hayman M, Moran LJ, Redman LM. 2016. The Role of Physical Activity in Preconception, Pregnancy and Postpartum Health. *Seminars in reproductive medicine* 34(2):e28-37.
- Harvey EB. 1935. Cleavage without Nuclei. *Science* 82(2125):277.
- Hashimoto N, Kishimoto T. 1988. Regulation of meiotic metaphase by a cytoplasmic maturation-promoting factor during mouse oocyte maturation. *Developmental biology* 126(2):242-252.
- Hashimoto S, Minami N, Yamada M, Imai H. 2000. Excessive concentration of glucose during in vitro maturation impairs the developmental competence of bovine oocytes after in vitro fertilization:

- relevance to intracellular reactive oxygen species and glutathione contents. Molecular reproduction and development 56(4):520-526.
- Hennet ML, Combelles CM. 2012. The antral follicle: a microenvironment for oocyte differentiation. Int J Dev Biol 56(10-12):819-831.
- Hirshfield AN. 1991. Theca cells may be present at the outset of follicular growth. Biology of reproduction 44(6):1157-1162.
- Hlinka D, Kalatova B, Uhrinova I, Dolinska S, Rutarova J, Rezacova J, Lazarovska S, Dudas M. 2012. Time-lapse cleavage rating predicts human embryo viability. Physiological research 61(5):513-525.
- Holm P, Shukri NN, Vajta G, Booth P, Bendixen C, Callesen H. 1998. Developmental kinetics of the first cell cycles of bovine in vitro produced embryos in relation to their in vitro viability and sex. Theriogenology 50(8):1285-1299.
- Hosoe M, Shioya Y. 1997. Distribution of cortical granules in bovine oocytes classified by cumulus complex. Zygote 5(4):371-376.
- Hung WT, Hong X, Christenson LK, McGinnis LK. 2015. Extracellular Vesicles from Bovine Follicular Fluid Support Cumulus Expansion. Biology of reproduction 93(5):117.
- Hussein TS, Froiland DA, Amato F, Thompson JG, Gilchrist RB. 2005. Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. Journal of cell science 118(Pt 22):5257-5268.
- Ireland JJ. 1987. Control of follicular growth and development. Journal of reproduction and fertility Supplement 34:39-54.
- Ireland JJ, Roche JF. 1982. Effect of progesterone on basal LH and episodic LH and FSH secretion in heifers. Journal of reproduction and fertility 64(2):295-302.
- Ireland JJ, Roche JF. 1983. Growth and differentiation of large antral follicles after spontaneous luteolysis in heifers: changes in concentration of hormones in follicular fluid and specific binding of gonadotropins to follicles. Journal of animal science 57(1):157-167.
- Irving-Rodgers HF, van Wezel IL, Mussard ML, Kinder JE, Rodgers RJ. 2001. Atresia revisited: two basic patterns of atresia of bovine antral follicles. Reproduction 122(5):761-775.
- Isobe N, Terada T. 2001. Effect of the factor inhibiting germinal vesicle breakdown on the disruption of gap junctions and cumulus expansion of pig cumulus-oocyte complexes cultured in vitro. Reproduction 121(2):249-257.
- Jewgenow K, Braun BC, Dehnhard M, Zahmel J, Goeritz F. 2017. Research on reproduction is essential for captive breeding of endangered carnivore species. Reproduction in domestic animals = Zuchthygiene 52 Suppl 2:18-23.
- Jiang Z, Wang Y, Lin J, Xu J, Ding G, Huang H. 2017. Genetic and epigenetic risks of assisted reproduction. Best practice & research Clinical obstetrics & gynaecology 44:90-104.
- Juengel JL, Hudson NL, Berg M, Hamel K, Smith P, Lawrence SB, Whiting L, McNatty KP. 2009. Effects of active immunization against growth differentiation factor 9 and/or bone morphogenetic protein 15 on ovarian function in cattle. Reproduction 138(1):107-114.
- Kaivo-Oja N, Mottershead DG, Mazerbourg S, Myllymaa S, Duprat S, Gilchrist RB, Groome NP, Hsueh AJ, Ritvos O. 2005. Adenoviral gene transfer allows Smad-responsive gene promoter analyses and delineation of type I receptor usage of transforming growth factor-beta family ligands in cultured human granulosa luteal cells. The Journal of clinical endocrinology and metabolism 90(1):271-278.
- Kaneko T, Saito H, Toya M, Satio T, Nakahara K, Hiroi M. 2000. Hyaluronic acid inhibits apoptosis in granulosa cells via CD44. Journal of assisted reproduction and genetics 17(3):162-167.
- Kawashima I, Liu Z, Mullany LK, Mihara T, Richards JS, Shimada M. 2012. EGF-like factors induce expansion of the cumulus cell-oocyte complexes by activating calpain-mediated cell movement. Endocrinology 153(8):3949-3959.
- Kruip TAM, Cran DG, van Beneden TH, Dieleman SJ. 1983. Structural changes in bovine oocytes during final maturation in vivo. Gamete Research 8(1):29-47.
- Kumar TR, Wang Y, Lu N, Matzuk MM. 1997. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nature genetics 15(2):201-204.
- Labrecque R, Vigneault C, Blondin P, Sirard MA. 2014. Gene expression analysis of bovine oocytes at optimal coothing time combined with GnRH antagonist during the no-FSH period. Theriogenology 81(8):1092-1100.
- Lam X, Gieseke C, Knoll M, Talbot P. 2000. Assay and importance of adhesive interaction between hamster (*Mesocricetus auratus*) oocyte-cumulus complexes and the oviductal epithelium. Biology of reproduction 62(3):579-588.

- Lawrence Y, Whitaker M, Swann K. 1997. Sperm-egg fusion is the prelude to the initial Ca<sup>2+</sup> increase at fertilization in the mouse. *Development* 124(1):233-241.
- Lee TF, Lee RK, Hwu YM, Chih YF, Tsai YC, Su JT. 2010. Relationship of follicular size to the development of intracytoplasmic sperm injection-derived human embryos. *Taiwanese journal of obstetrics & gynecology* 49(3):302-305.
- Lee YS, VandeVoort CA, Gaughan JP, Midic U, Obradovic Z, Latham KE. 2011. Extensive effects of in vitro oocyte maturation on rhesus monkey cumulus cell transcriptome. *American journal of physiology Endocrinology and metabolism* 301(1):E196-209.
- Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, Rao CV. 2001. Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. *Mol Endocrinol* 15(1):184-200.
- Leroy JL, Valckx SD, Jordaeens L, De Bie J, Desmet KL, Van Hoeck V, Britt JH, Marei WF, Bols PE. 2015. Nutrition and maternal metabolic health in relation to oocyte and embryo quality: critical views on what we learned from the dairy cow model. *Reproduction, fertility, and development* 27(4):693-703.
- Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G, Van Soom A. 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction* 130(4):485-495.
- Li J, Cao Y, Xu X, Xiang H, Zhang Z, Chen B, Hao Y, Wei Z, Zhou P, Chen D. 2015. Increased New lncRNA-mRNA Gene Pair Levels in Human Cumulus Cells Correlate With Oocyte Maturation and Embryo Development. *Reproductive sciences* 22(8):1008-1014.
- Li J, Mao G, Xia G. 2012. FSH modulates PKAI and GPR3 activities in mouse oocyte of COC in a gap junctional communication (GJC)-dependent manner to initiate meiotic resumption. *PloS one* 7(9):e37835.
- Li R, Norman RJ, Armstrong DT, Gilchrist RB. 2000. Oocyte-secreted factor(s) determine functional differences between bovine mural granulosa cells and cumulus cells. *Biology of reproduction* 63(3):839-845.
- Lippincott-Schwartz J, Roberts TH, Hirschberg K. 2000. Secretory protein trafficking and organelle dynamics in living cells. *Annual review of cell and developmental biology* 16:557-589.
- Little CM. 2010. One consequence of infertility treatment: multifetal pregnancy. *MCN Am J Matern Child Nurs* 35(3):150-155.
- Lonergan P, Fair T, Forde N, Rizos D. 2016. Embryo development in dairy cattle. *Theriogenology* 86(1):270-277.
- Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P, Boland MP. 1999. Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *Journal of reproduction and fertility* 117(1):159-167.
- Lonergan P, Monaghan P, Rizos D, Boland MP, Gordon I. 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. *Molecular reproduction and development* 37(1):48-53.
- Luberda Z. 2005. The role of glutathione in mammalian gametes. *Reprod Biol* 5(1):5-17.
- Luciano AM, Lodde V, Beretta MS, Colleoni S, Lauria A, Modina S. 2005. Developmental capability of denuded bovine oocyte in a co-culture system with intact cumulus-oocyte complexes: role of cumulus cells, cyclic adenosine 3',5'-monophosphate, and glutathione. *Molecular reproduction and development* 71(3):389-397.
- Luciano AM, Lodde V, Franciosi F, Tessaro I, Corbani D, Modina S. 2012. Large-scale chromatin morpho-functional changes during mammalian oocyte growth and differentiation. *European journal of histochemistry : EJH* 56(3):e37.
- Lundin K, Bergh C, Hardarson T. 2001. Early embryo cleavage is a strong indicator of embryo quality in human IVF. *Human reproduction* 16(12):2652-2657.
- Lussier JG, Matton P, Dufour JJ. 1987. Growth rates of follicles in the ovary of the cow. *Journal of reproduction and fertility* 81(2):301-307.
- Maalouf SW, Liu WS, Pate JL. 2016. MicroRNA in ovarian function. *Cell and tissue research* 363(1):7-18.
- Macaulay AD, Gilbert I, Caballero J, Barreto R, Fournier E, Tossou P, Sirard MA, Clarke HJ, Khandjian EW, Richard FJ, Hyttel P, Robert C. 2014. The gametic synapse: RNA transfer to the bovine oocyte. *Biology of reproduction* 91(4):90.
- Macaulay AD, Gilbert I, Scantland S, Fournier E, Ashkar F, Bastien A, Saadi HA, Gagne D, Sirard MA, Khandjian EW, Richard FJ, Hyttel P, Robert C. 2016. Cumulus Cell Transcripts Transit to the Bovine Oocyte in Preparation for Maturation. *Biology of reproduction* 94(1):16.
- Machaca K. 2007. Ca<sup>2+</sup> signaling differentiation during oocyte maturation. *Journal of cellular physiology* 213(2):331-340.
- Mahi-Brown CA, Yanagimachi R. 1983. Parameters influencing ovum pickup by oviductal fimbria in the golden hamster. *Gamete Research* 8(1):1-10.

- Malpaux B, Viguie C, Skinner DC, Thiery JC, Chemineau P. 1997. Control of the circannual rhythm of reproduction by melatonin in the ewe. *Brain research bulletin* 44(4):431-438.
- Manfredi-Lozano M, Roa J, Tena-Sempere M. 2017. Connecting metabolism and gonadal function: Novel central neuropeptide pathways involved in the metabolic control of puberty and fertility. *Frontiers in neuroendocrinology*.
- Martin O, Friggens NC, Dupont J, Salvetti P, Freret S, Rame C, Elis S, Gatien J, Disenhaus C, Blanc F. 2013. Data-derived reference profiles with corepresentation of progesterone, estradiol, LH, and FSH dynamics during the bovine estrous cycle. *Theriogenology* 79(2):331-343 e331-334.
- Masui Y, Markert CL. 1971. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *The Journal of experimental zoology* 177(2):129-145.
- Matousek M, Carati C, Gannon B, Mitsube K, Brannstrom M. 2001. Changes in intrafollicular pressure in the rat ovary by nitric oxide and by alteration of systemic blood pressure. *European journal of obstetrics, gynecology, and reproductive biology* 98(1):46-52.
- Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. 2002. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* 296(5576):2178-2180.
- McEvoy TG, Sinclair KD, Young LE, Wilmut I, Robinson JJ. 2000. Large offspring syndrome and other consequences of ruminant embryo culture in vitro: relevance to blastocyst culture in human ART. *Human fertility* 3(4):238-246.
- Merchant-Larios H, Chimal-Monroy J. 1989. The ontogeny of primordial follicles in the mouse ovary. *Progress in clinical and biological research* 296:55-63.
- Mihm M, Austin EJ. 2002. The final stages of dominant follicle selection in cattle. *Domestic animal endocrinology* 23(1-2):155-166.
- Mihm M, Baker PJ, Ireland JL, Smith GW, Coussens PM, Evans AC, Ireland JJ. 2006. Molecular evidence that growth of dominant follicles involves a reduction in follicle-stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle. *Biology of reproduction* 74(6):1051-1059.
- Minguez-Alarcon L, Hauser R, Gaskins AJ. 2016. Effects of bisphenol A on male and couple reproductive health: a review. *Fertility and sterility* 106(4):864-870.
- Modina S, Luciano AM, Vassena R, Baraldi-Scesi I, Lauria A, Gandolfi F. 2001. Oocyte developmental competence after in vitro maturation depends on the persistence of cumulus-oocyte communications which are linked to the intracellular concentration of cAMP. *Italian journal of anatomy and embryology = Archivio italiano di anatomia ed embriologia* 106(2 Suppl 2):241-248.
- Moor RM, Smith MW, Dawson RM. 1980. Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. *Experimental cell research* 126(1):15-29.
- Moreno RD, Schatten G, Ramalho-Santos J. 2002. Golgi apparatus dynamics during mouse oocyte in vitro maturation: effect of the membrane trafficking inhibitor brefeldin A. *Biology of reproduction* 66(5):1259-1266.
- Moussaddykine S, Assou S, Dechaud H, Hamamah S. 2012. [Other actors in the oocyte and follicular growth: the role of microRNAs in the cumulus-oocyte dialog]. *Gynecologie, obstetrique & fertilité* 40(3):170-173.
- Nelson SM. 2017. Prevention and management of ovarian hyperstimulation syndrome. *Thrombosis research* 151 Suppl 1:S61-S64.
- Nishimoto H, Matsutani R, Yamamoto S, Takahashi T, Hayashi KG, Miyamoto A, Hamano S, Tetsuka M. 2006. Gene expression of glucose transporter (GLUT) 1, 3 and 4 in bovine follicle and corpus luteum. *The Journal of endocrinology* 188(1):111-119.
- Nivet AL, Bunel A, Labrecque R, Belanger J, Vigneault C, Blondin P, Sirard MA. 2012. FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction* 143(2):165-171.
- Norris RP, Ratzan WJ, Freudzon M, Mehlmann LM, Krall J, Movsesian MA, Wang H, Ke H, Nikolaev VO, Jaffe LA. 2009. Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development* 136(11):1869-1878.
- Nuttinck F, Peynot N, Humblot P, Massip A, Dessy F, Flechon JE. 2000. Comparative immunohistochemical distribution of connexin 37 and connexin 43 throughout folliculogenesis in the bovine ovary. *Molecular reproduction and development* 57(1):60-66.
- Ogawa H, Wu Q, Komiyama J, Obata Y, Kono T. 2006. Disruption of parental-specific expression of imprinted genes in uniparental fetuses. *FEBS letters* 580(22):5377-5384.
- Ohta N, Saito H, Kuzumaki T, Takahashi T, Ito MM, Saito T, Nakahara K, Hiroi M. 1999. Expression of CD44 in human cumulus and mural granulosa cells of individual patients in in-vitro fertilization programmes. *Molecular human reproduction* 5(1):22-28.

- Oktem O, Oktay K. 2008. Stem cells: a perspective on oocytes. *Annals of the New York Academy of Sciences* 1127:20-26.
- Ozil JP. 1998. Role of calcium oscillations in mammalian egg activation: experimental approach. *Biophysical chemistry* 72(1-2):141-152.
- Paczkowski M, Silva E, Schoolcraft WB, Krisher RL. 2013. Comparative importance of fatty acid beta-oxidation to nuclear maturation, gene expression, and glucose metabolism in mouse, bovine, and porcine cumulus oocyte complexes. *Biology of reproduction* 88(5):111.
- Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M. 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* 303(5658):682-684.
- Pavlok A, Lucas-Hahn A, Niemann H. 1992. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. *Molecular reproduction and development* 31(1):63-67.
- Pelland AM, Corbett HE, Baltz JM. 2009. Amino Acid transport mechanisms in mouse oocytes during growth and meiotic maturation. *Biology of reproduction* 81(6):1041-1054.
- Perkel KJ, Tscherner A, Merrill C, Lamarre J, Madan P. 2015. The ART of selecting the best embryo: A review of early embryonic mortality and bovine embryo viability assessment methods. *Molecular reproduction and development* 82(11):822-838.
- Perret BP, Parinaud J, Ribbes H, Moatti JP, Pontonnier G, Chap H, Douste-Blazy L. 1985. Lipoprotein and phospholipid distribution in human follicular fluids. *Fertility and sterility* 43(3):405-409.
- Pincus G, Enzmann EV. 1935. THE COMPARATIVE BEHAVIOR OF MAMMALIAN EGGS IN VIVO AND IN VITRO : I. THE ACTIVATION OF OVARIAN EGGS. *The Journal of experimental medicine* 62(5):665-675.
- Pontes JH, Silva KC, Basso AC, Rigo AG, Ferreira CR, Santos GM, Sanches BV, Porcionato JP, Vieira PH, Faifer FS, Sterza FA, Schenk JL, Seneda MM. 2010. Large-scale in vitro embryo production and pregnancy rates from Bos taurus, Bos indicus, and indicus-taurus dairy cows using sexed sperm. *Theriogenology* 74(8):1349-1355.
- Prochazka R, Nagyova E, Brem G, Schellander K, Motlik J. 1998. Secretion of cumulus expansion-enabling factor (CEEF) in porcine follicles. *Molecular reproduction and development* 49(2):141-149.
- Racowsky C, Satterlie RA. 1985. Metabolic, fluorescent dye and electrical coupling between hamster oocytes and cumulus cells during meiotic maturation in vivo and in vitro. *Developmental biology* 108(1):191-202.
- Rahe CH, Owens RE, Fleeger JL, Newton HJ, Harms PG. 1980. Pattern of plasma luteinizing hormone in the cyclic cow: dependence upon the period of the cycle. *Endocrinology* 107(2):498-503.
- Richards JS, Midgley AR, Jr. 1976. Protein hormone action: a key to understanding ovarian follicular and luteal cell development. *Biology of reproduction* 14(1):82-94.
- Rieger D, Loskutoff NM. 1994. Changes in the metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes in vitro. *Journal of reproduction and fertility* 100(1):257-262.
- Rienzi L, Vajta G, Ubaldi F. 2011. Predictive value of oocyte morphology in human IVF: a systematic review of the literature. *Human reproduction update* 17(1):34-45.
- Robert C, Nieminen J, Dufort I, Gagne D, Grant JR, Cagnone G, Plourde D, Nivet AL, Fournier E, Paquet E, Blazejczyk M, Rigault P, Juge N, Sirard MA. 2011. Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays. *Molecular reproduction and development* 78(9):651-664.
- Rüsse I. 1983. Oogenesis in cattle and sheep. *Bibliotheca anatomica* 24:77-92.
- Rüsse I, Sinowitz F. 1991. Gametogenese & Harn- und Geschlechtsorgane. In: I. R, F. S, editors. *Lehrbuch der Embryologie der Haustiere*: Verlag Paul Parey, Berlin, Hamburg. p 51, 70 & 314.
- Russell DL, Ochsner SA, Hsieh M, Mulders S, Richards JS. 2003. Hormone-regulated expression and localization of versican in the rodent ovary. *Endocrinology* 144(3):1020-1031.
- Russell DL, Salustri A. 2006. Extracellular matrix of the cumulus-oocyte complex. *Seminars in reproductive medicine* 24(4):217-227.
- Saito H, Kaneko T, Takahashi T, Kawachiya S, Saito T, Hiroi M. 2000. Hyaluronan in follicular fluids and fertilization of oocytes. *Fertility and sterility* 74(6):1148-1152.
- Salha O, Nugent D, Dada T, Kaufmann S, Levett S, Jenner L, Lui S, Sharma V. 1998. The relationship between follicular fluid aspirate volume and oocyte maturity in in-vitro fertilization cycles. *Human reproduction* 13(7):1901-1906.
- Salustri A, Garlanda C, Hirsch E, De Acetis M, Maccagno A, Bottazzi B, Doni A, Bastone A, Mantovani G, Beck Peccoz P, Salvatori G, Mahoney DJ, Day AJ, Siracusa G, Romani L, Mantovani A. 2004. PTX3 plays a

- key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization. *Development* 131(7):1577-1586.
- Salustri A, Yanagishita M, Hascall VC. 1989. Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification. *The Journal of biological chemistry* 264(23):13840-13847.
- Salustri A, Yanagishita M, Underhill CB, Laurent TC, Hascall VC. 1992. Localization and synthesis of hyaluronic acid in the cumulus cells and mural granulosa cells of the preovulatory follicle. *Developmental biology* 151(2):541-551.
- Sanderson MP, Keller S, Alonso A, Riedle S, Dempsey PJ, Altevogt P. 2008. Generation of novel, secreted epidermal growth factor receptor (EGFR/ErbB1) isoforms via metalloprotease-dependent ectodomain shedding and exosome secretion. *Journal of cellular biochemistry* 103(6):1783-1797.
- Sato N, Kawamura K, Fukuda J, Honda Y, Sato T, Tanikawa H, Kodama H, Tanaka T. 2003. Expression of LDL receptor and uptake of LDL in mouse preimplantation embryos. *Molecular and cellular endocrinology* 202(1-2):191-194.
- Schatten G, Simerly C, Schatten H. 1986. Microtubules in mouse oocytes, zygotes, and embryos during fertilization and early development: unusual configurations and arrest of mammalian fertilization with microtubule inhibitors. *Annals of the New York Academy of Sciences* 466:945-948.
- Schoenfelder M, Einspanier R. 2003. Expression of hyaluronan synthases and corresponding hyaluronan receptors is differentially regulated during oocyte maturation in cattle. *Biology of reproduction* 69(1):269-277.
- Schultz RM. 1985. Roles of cell-to-cell communication in development. *Biology of reproduction* 32(1):27-42.
- Senbon S, Hirao Y, Miyano T. 2003. Interactions between the oocyte and surrounding somatic cells in follicular development: lessons from in vitro culture. *The Journal of reproduction and development* 49(4):259-269.
- Shitsukawa K, Andersen CB, Richard FJ, Horner AK, Wiersma A, van Duin M, Conti M. 2001. Cloning and characterization of the cyclic guanosine monophosphate-inhibited phosphodiesterase PDE3A expressed in mouse oocyte. *Biology of reproduction* 65(1):188-196.
- Sirard MA. 2001. Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology* 55(6):1241-1254.
- Sirard MA, First NL. 1988. In vitro inhibition of oocyte nuclear maturation in the bovine. *Biology of reproduction* 39(2):229-234.
- Sirard MA, Richard F, Blondin P, Robert C. 2006. Contribution of the oocyte to embryo quality. *Theriogenology* 65(1):126-136.
- Sirois J, Dore M. 1997. The late induction of prostaglandin G/H synthase-2 in equine preovulatory follicles supports its role as a determinant of the ovulatory process. *Endocrinology* 138(10):4427-4434.
- Sirois J, Fortune JE. 1988. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. *Biology of reproduction* 39(2):308-317.
- Smith MF, McIntush EW, Smith GW. 1994. Mechanisms associated with corpus luteum development. *Journal of animal science* 72(7):1857-1872.
- Smitz JE, Cortvriendt RG. 2002. The earliest stages of folliculogenesis in vitro. *Reproduction* 123(2):185-202.
- Sohel MM, Hoelker M, Noferesti SS, Salilew-Wondim D, Tholen E, Looft C, Rings F, Uddin MJ, Spencer TE, Schellander K, Tesfaye D. 2013. Exosomal and Non-Exosomal Transport of Extra-Cellular microRNAs in Follicular Fluid: Implications for Bovine Oocyte Developmental Competence. *PloS one* 8(11):e78505.
- Spindler RE, Pukazhenthil BS, Wildt DE. 2000. Oocyte metabolism predicts the development of cat embryos to blastocyst in vitro. *Molecular reproduction and development* 56(2):163-171.
- Steeves TE, Gardner DK. 1999. Metabolism of glucose, pyruvate, and glutamine during the maturation of oocytes derived from pre-pubertal and adult cows. *Molecular reproduction and development* 54(1):92-101.
- Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Goncalves PB, Wolf E. 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biology of reproduction* 64(3):904-909.
- Sturmey RG, O'Toole PJ, Leese HJ. 2006. Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. *Reproduction* 132(6):829-837.
- Su YQ, Sugiura K, Li Q, Wigglesworth K, Matzuk MM, Eppig JJ. 2010. Mouse oocytes enable LH-induced maturation of the cumulus-oocyte complex via promoting EGF receptor-dependent signaling. *Mol Endocrinol* 24(6):1230-1239.

- Su YQ, Sugiura K, Wigglesworth K, O'Brien MJ, Affourtit JP, Pangas SA, Matzuk MM, Eppig JJ. 2008. Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. *Development* 135(1):111-121.
- Su YQ, Wu X, O'Brien MJ, Pendola FL, Denegre JN, Matzuk MM, Eppig JJ. 2004. Synergistic roles of BMP15 and GDF9 in the development and function of the oocyte-cumulus cell complex in mice: genetic evidence for an oocyte-granulosa cell regulatory loop. *Developmental biology* 276(1):64-73.
- Sugimura S, Akai T, Hashiyada Y, Somfai T, Inaba Y, Hirayama M, Yamanouchi T, Matsuda H, Kobayashi S, Aikawa Y, Otake M, Kobayashi E, Konishi K, Imai K. 2012. Promising system for selecting healthy in vitro-fertilized embryos in cattle. *PLoS one* 7(5):e36627.
- Sugimura S, Kobayashi N, Okae H, Yamanouchi T, Matsuda H, Kojima T, Yajima A, Hashiyada Y, Kaneda M, Sato K, Imai K, Tanemura K, Arima T, Gilchrist RB. 2017. Transcriptomic signature of the follicular somatic compartment surrounding an oocyte with high developmental competence. *Scientific reports* 7(1):6815.
- Sugiura K, Pendola FL, Eppig JJ. 2005. Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Developmental biology* 279(1):20-30.
- Sugiura K, Su YQ, Li Q, Wigglesworth K, Matzuk MM, Eppig JJ. 2010. Estrogen promotes the development of mouse cumulus cells in coordination with oocyte-derived GDF9 and BMP15. *Mol Endocrinol* 24(12):2303-2314.
- Sun GW, Kobayashi H, Suzuki M, Kanayama N, Terao T. 2003. Follicle-stimulating hormone and insulin-like growth factor I synergistically induce up-regulation of cartilage link protein (Crtl1) via activation of phosphatidylinositol-dependent kinase/Akt in rat granulosa cells. *Endocrinology* 144(3):793-801.
- Sunderland SJ, Crowe MA, Boland MP, Roche JF, Ireland JJ. 1994. Selection, dominance and atresia of follicles during the oestrous cycle of heifers. *Journal of reproduction and fertility* 101(3):547-555.
- Sutton-McDowall ML, Gilchrist RB, Thompson JG. 2004. Cumulus expansion and glucose utilisation by bovine cumulus-oocyte complexes during in vitro maturation: the influence of glucosamine and follicle-stimulating hormone. *Reproduction* 128(3):313-319.
- Sutton-McDowall ML, Gilchrist RB, Thompson JG. 2010. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* 139(4):685-695.
- Sutton ML, Cetica PD, Beconi MT, Kind KL, Gilchrist RB, Thompson JG. 2003a. Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes. *Reproduction* 126(1):27-34.
- Sutton ML, Gilchrist RB, Thompson JG. 2003b. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Human reproduction update* 9(1):35-48.
- Tarazona AM, Rodriguez JI, Restrepo LF, Olivera-Angel M. 2006. Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced in vitro. *Reproduction in domestic animals = Zuchthygiene* 41(1):5-11.
- Thibault C, Szollosi D, Gerard M. 1987. Mammalian oocyte maturation. *Reproduction, nutrition, developpement* 27(5):865-896.
- Thomas RE, Armstrong DT, Gilchrist RB. 2004. Bovine cumulus cell-oocyte gap junctional communication during in vitro maturation in response to manipulation of cell-specific cyclic adenosine 3',5'-monophosphate levels. *Biology of reproduction* 70(3):548-556.
- Thompson JG, Lane M, Gilchrist RB. 2007. Metabolism of the bovine cumulus-oocyte complex and influence on subsequent developmental competence. *Society of Reproduction and Fertility supplement* 64:179-190.
- Tomek W, Torner H, Kanitz W. 2002. Comparative analysis of protein synthesis, transcription and cytoplasmic polyadenylation of mRNA during maturation of bovine oocytes in vitro. *Reproduction in domestic animals = Zuchthygiene* 37(2):86-91.
- Tong XH, Xu B, Zhang YW, Liu YS, Ma CH. 2014. Research resources: comparative microRNA profiles in human corona radiata cells and cumulus oophorus cells detected by next-generation small RNA sequencing. *PLoS one* 9(9):e106706.
- Toole BP. 2004. Hyaluronan: from extracellular glue to pericellular cue. *Nature reviews Cancer* 4(7):528-539.
- Trigatti B, Rayburn H, Vinals M, Braun A, Miettinen H, Penman M, Hertz M, Schrenzel M, Amigo L, Rigotti A, Krieger M. 1999. Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology. *Proceedings of the National Academy of Sciences of the United States of America* 96(16):9322-9327.
- Trounson A, Anderiesz C, Jones G. 2001. Maturation of human oocytes in vitro and their developmental competence. *Reproduction* 121(1):51-75.

- Turley EA, Noble PW, Bourguignon LY. 2002. Signaling properties of hyaluronan receptors. *The Journal of biological chemistry* 277(7):4589-4592.
- Urrego R, Rodriguez-Osorio N, Niemann H. 2014. Epigenetic disorders and altered gene expression after use of Assisted Reproductive Technologies in domestic cattle. *Epigenetics* 9(6):803-815.
- Valiunas V, Polosina YY, Miller H, Potapova IA, Valiuniene L, Doronin S, Mathias RT, Robinson RB, Rosen MR, Cohen IS, Brink PR. 2005. Connexin-specific cell-to-cell transfer of short interfering RNA by gap junctions. *The Journal of physiology* 568(Pt 2):459-468.
- Van Blerkom J, Davis P, Alexander S. 2000. Differential mitochondrial distribution in human pronuclear embryos leads to disproportionate inheritance between blastomeres: relationship to microtubular organization, ATP content and competence. *Human reproduction* 15(12):2621-2633.
- van der Westerlaken LA, van der Schans A, Eyestone WH, de Boer HA. 1994. Kinetics of first polar body extrusion and the effect of time of stripping of the cumulus and time of insemination on developmental competence of bovine oocytes. *Theriogenology* 42(2):361-370.
- Van Hoeck V, Leroy JL, Arias Alvarez M, Rizos D, Gutierrez-Adan A, Schnorbusch K, Bols PE, Leese HJ, Sturmey RG. 2013. Oocyte developmental failure in response to elevated nonesterified fatty acid concentrations: mechanistic insights. *Reproduction* 145(1):33-44.
- Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PE, Leroy JL. 2011. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *PloS one* 6(8):e23183.
- Van Soom A, Van Vlaenderen I, Mahmoudzadeh AR, Deluyker H, de Kruif A. 1992. Compaction rate of in vitro fertilized bovine embryos related to the interval from insemination to first cleavage. *Theriogenology* 38(5):905-919.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* 3(7):RESEARCH0034.
- Vassena R, Mapletoft RJ, Allodi S, Singh J, Adams GP. 2003. Morphology and developmental competence of bovine oocytes relative to follicular status. *Theriogenology* 60(5):923-932.
- Watson AJ, De Sousa P, Caveney A, Barcroft LC, Natale D, Urquhart J, Westhusin ME. 2000. Impact of bovine oocyte maturation media on oocyte transcript levels, blastocyst development, cell number, and apoptosis. *Biology of reproduction* 62(2):355-364.
- Webb RJ, Bains H, Cruttwell C, Carroll J. 2002. Gap-junctional communication in mouse cumulus-oocyte complexes: implications for the mechanism of meiotic maturation. *Reproduction* 123(1):41-52.
- Wells L, Whelan SA, Hart GW. 2003. O-GlcNAc: a regulatory post-translational modification. *Biochemical and biophysical research communications* 302(3):435-441.
- Winterhager E, Kidder GM. 2015. Gap junction connexins in female reproductive organs: implications for women's reproductive health. *Human reproduction update* 21(3):340-352.
- Wonnacott KE, Kwong WY, Hughes J, Salter AM, Lea RG, Garnsworthy PC, Sinclair KD. 2010. Dietary omega-3 and -6 polyunsaturated fatty acids affect the composition and development of sheep granulosa cells, oocytes and embryos. *Reproduction* 139(1):57-69.
- Wu Y, Wu J, Lee DY, Yee A, Cao L, Zhang Y, Kiani C, Yang BB. 2005. Versican protects cells from oxidative stress-induced apoptosis. *Matrix biology : journal of the International Society for Matrix Biology* 24(1):3-13.
- Xu XF, Li J, Cao YX, Chen DW, Zhang ZG, He XJ, Ji DM, Chen BL. 2015. Differential Expression of Long Noncoding RNAs in Human Cumulus Cells Related to Embryo Developmental Potential: A Microarray Analysis. *Reproductive sciences* 22(6):672-678.
- Yang MY, Fortune JE. 2008. The capacity of primordial follicles in fetal bovine ovaries to initiate growth in vitro develops during mid-gestation and is associated with meiotic arrest of oocytes. *Biology of reproduction* 78(6):1153-1161.
- Yerushalmi GM, Salmon-Divon M, Yung Y, Maman E, Kedem A, Ophir L, Elemento O, Coticchio G, Dal Canto M, Mignini Renzinu M, Fadini R, Hourvitz A. 2014. Characterization of the human cumulus cell transcriptome during final follicular maturation and ovulation. *Molecular human reproduction* 20(8):719-735.
- Yokoo M, Miyahayashi Y, Naganuma T, Kimura N, Sasada H, Sato E. 2002. Identification of hyaluronic acid-binding proteins and their expressions in porcine cumulus-oocyte complexes during in vitro maturation. *Biology of reproduction* 67(4):1165-1171.

- Yoshino O, McMahon HE, Sharma S, Shimasaki S. 2006. A unique preovulatory expression pattern plays a key role in the physiological functions of BMP-15 in the mouse. *Proceedings of the National Academy of Sciences of the United States of America* 103(28):10678-10683.
- Young LE, Sinclair KD, Wilmut I. 1998. Large offspring syndrome in cattle and sheep. *Rev Reprod* 3(3):155-163.
- Yudin AI, Cherr GN, Katz DF. 1988. Structure of the cumulus matrix and zona pellucida in the golden hamster: a new view of sperm interaction with oocyte-associated extracellular matrices. *Cell and tissue research* 251(3):555-564.
- Zebeli Q, Ghareeb K, Humer E, Metzler-Zebeli BU, Besenfelder U. 2015. Nutrition, rumen health and inflammation in the transition period and their role on overall health and fertility in dairy cows. *Research in veterinary science* 103:126-136.
- Ziv-Gal A, Flaws JA. 2016. Evidence for bisphenol A-induced female infertility: a review (2007-2016). *Fertility and sterility* 106(4):827-856.
- Zuelke KA, Brackett BG. 1992. Effects of luteinizing hormone on glucose metabolism in cumulus-enclosed bovine oocytes matured in vitro. *Endocrinology* 131(6):2690-2696.
- Zuelke KA, Brackett BG. 1993. Increased glutamine metabolism in bovine cumulus cell-enclosed and denuded oocytes after in vitro maturation with luteinizing hormone. *Biology of reproduction* 48(4):815-820.