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Liste des abréviations et des sigles

*La description du nom des gènes ou protéines est en anglais afin de rester fidèle à leur abréviation.

%	Pourcent
°C	Degré Celius
AC	Adénylate Cyclase
ACTB	B-Actin
ADAMTS1	Adisintegrin And Metallopreteinase With Thrombospondin-Like Motif Type 1
AKT	Protein Kinase B
ANKRD1	Ankyrin Repeat Domain 1
ANXA1	Annexin A1
AR	Récepteur Aux Androgènes
AREG	Amphiregulin
aRNA	ARN amplifié
ARNm	Acide Ribonucléique Messager
ART	Technique De Reproduction Assistée
ATP	Adénosine Triphosphate
BAX	Bcl2 Associated X
BCL2	B-Cell Lymphoma 2
BCS	Indice de la condition de chair
BMKs	Biomarqueurs
BMP	Bone Morphogenetic Protein
BMP15	Bone Morphogenetic Protein 15
BMP6	Bone Morphogenetic Protein 6
BTC	Betacellulin
cAMP	Adénosine Monophosphate Cyclique
CCAC	Canadian Council On Animal Care
CCNA	Cyclin A
CCNB	Cyclin B
CCNB1	Cyclin B1
CDK1	Cyclin Dependent Kinase 1
CDKN1A	Cyclin Dependent Kinase Inhibitor 1a
cDNA	AND complémentaire
CEBPB	CCAAT/Enhancer Binding Protein Beta
CETA	Canadian Embryo Transfer Association
CKB	Creatine Kinase Brain Isoform
CO2	Dioxide de carbone
COC	Complexe Ovocyte-Cumulus
COX-1	Cyclooxygenase 1

COX-2	Cyclooxygénase-2
CREB1	Camp Responsive Element Binding Protein 1
CRISPLD2	Cystein-Rich Secretory Protein LCCL Domain Containing 2
CTSL1	Cathepsin L1
Cx	Connexine
Cyp11a1	Cytochrome P450 Family 11 Subfamily A
Cyp17a1	Cytochrome P450 17A1
Cyp19	Aromatase
DEGs	Gènes différentiellement exprimés
DMV	Médecin Vétérinaire
DNA	Acide Désoxyribonucléique
E2	Estradiol
eGC	Gonadotrophine Chorionique Équine
EGF	Epidermal Growth Factor
EIF2B2	Eukaryotic Translation Initiation Factor 2b Subunit Beta
ELAP	Emerging Leader In The Americas Program
ELMA	Embryogene LIMS And Microarray Analysis
ERBB2	Erb-B2 Recptor Tyrosine Kinase 2
EREG	Epiregulin
ERK	Extracellular Signal-Regulated Kinase
Erα	Récepteur Aux Estrogènes A
Erβ	Récepteur Aux Estrogènes B
ET	Transfère d'embryon
Fe2+	Ferreux
FOXO	Forkhead Box O
FSH	Hormone Follicu-Stimulante
FSHR	Récepteur À La FSH
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA-4	Gata Binding Protein 4
GDF9	Growth Differentiation Factor 9
GEO	Gene Expression Omnibus
GFPT2	Glutamine-Fructose-6- Phosphate Transaminase 2
GH	Growth Hormone
GMPc	Guanosine Monophosphate Cyclique
GnRH	Gonadotropin-Releasing Hormone
GO	Gene Ontology
GSDMB	Gasdermin B
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
GWAS	Genome-Wide Association Studies

H_2O_2	Peroxidase
HA	Acide Hyaluronique
HAS2	Hyaluronan Synthase Type 2
hGC	Gonadotrophine Chorionique Humaine
HMG-CoA	3-Hydroxy-3-Methyglutaryl Coenzyme A Reductase
HPG	Hypothalamus Pituitaire Gonade
Hsd17b1	Hydroxysteroid 17-Beta Dehydrogenase 1
Hsd3b1	3beta-Hydroxysteroid Dehydrogenase
IETS	International Embryo Transfer Society
IGF	Insulin Growth Factor
IGF2	Insulin-Like Growth Factor 2
IGFBP	Insulin Growth Factor Binding Protein
IGFR1	Insulin-Like Growth Factor 1 Receptor
IL-1A	Interleukin 1A
IL1B	Interleukine 1 Bêta
INFG	Interferon Gamma
INHA	Inhibin A
INHB	Inhibin B
INSIG1	Insulin-Induced Genes 1
INSIG2	Insulin-Induced Genes 2
INSR	Insulin Receptor
IPA	Ingenuity Pathway Analysis
IVC	Culture *In Vitro*
IVF	Fécondation *In Vitro*
IVM	Maturation *In Vitro*
IVP	Production *In Vitro*
IαI	Inter-A Trypsine Inhibitrice
JNK	C-Jun N-Terminal Kinase
K+	Potassium
KCNJ8	Potassium Voltage- Gated Channel Subfamily J Member 8
KCTD8	Potassium Channel Tetramerization Domain Containing 8
KDR	Kinase Insert Domain Receptor
KL	Facteur Ligand De C-Kit
KO-	Knock-Out
KRAS	Kristen Ras
KRT4	Keratin 4
LH	Hormone Lutéinisante
LHR	Récepteur à la LH
LIF	Leukemia Inhibiting Factor
LUM	Lumican

MAPK	Mitogen-Activated Protein Kinase
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-Activated Protein Kinase Kinase
MET	Maternal To Embryonic Transition
mg	Milligrame
MGARP	Mitochondria-Localized Glutamic Acid-Rich Protein
MII	Métaphase II
ml	Mililitre
mm	Milimètre
mM	Milimolaire
MMP	Métalloprotéases
MPF	Facteur De Promotion De La Maturation
mSOF	Modified Synthetic Oviduct Fluid
mTOR	Mammalian Target Of Rapamycin
MYC	Myelocytomatosis Proto-Oncogene, BHLH Transcription Factor
NF-kB	Kappa-Light-Chain-Enhancer Of Activated B Cells
NOTCH1	Notch Homolog 1
NR3C3	Nuclear Receptor Subfamily 3, Group C, Member 3
NRP1	Neuropilin 1
NSERC	Natural Sciences And Engineering Research Council Of Canada -
OMVQ	Collège des médecins vétérinaire du Québec
OPU	Récolte d'ovocyte «Ovum Pick-Up»
OSAP	Ovary Specific Acidic Protein
P2Y	Purinergic Receptor
P4	Progestérone
PANTHER	Protein Analysis Through Evolutionary Relationships
PAPP-A	Pregnancy-Associated Plasma Protein A
PCK2	Phosphoenolpyruvate Carboxykinase 2
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDE3A	Phosphodiestérase 3A
PEA15	Phosphoprotein Enriched In Astrocytes 15
PGC	Cellule germinale embryonnaires
PGE2	Prostaglandine E2
PGF_{2a}	Prostaglandine F2a
PGR	Récepteur à la progestérone
PI3K	Phosphoinositide 3-Kinase
PKA	Protéine Kinase A
PKC	Protein Kinase C
PMSG	Pregnant Mare Serum Gonadotropin

PPI	Protein–Protein Interactions
PRL	Prolactine
PTEN	Phosphatase And Tensin Homolog
PTX3	Pentraxine 3
RBP	Retinol-Binding Protein
REDIH	Reproduction, Early Development, And Impact On Health
RELN	Reelin
ROS	Reactive Oxygen Species
RPM	Rotation Per Minute
RT-qPCR	Reverse Transciptase Real Time PCR
SCAP	SREBF Cleavage-Activating Protein
SD	Standard Deviation
SF-1	Steroidogenic Factor 1
SF3A1	Splicing Factor 3 Subunit 1
SO	Stimulation Ovarienne
SREBF1	Sterol Regulatory Element Binding Transcription Factor 1
SREBF2	Sterol Regulatory Element Binding Transcription Factor 2
StAR	Steroidogenic Acute Regulatory Protein
STAT3	Signal Transducer And Activator Of Transcription 3
TBP	TATA-Box Binding Protein
TF	Transferrin
TFPI2	Tissue Factor Pathway Inhibitor 2
TFR2	Transferrin Receptor 2
TGF	Transforming Growth Factor
TGFb	Transforming Growth Factor B
TLH	HEPES-Buffered Tyrode’s Medium
TNF	Tumour Necrosis Factor
TNFIP-6	Tumor Necrosis Factor-Induced Protein-6
TP53	Tumor Protein 53
TSC	Tuberous Sclerosis Complex
TTR	Transthyretin
TZP	Projection Transzonale
uL	Microlitre
UTR	Région non transcrite de l'ARNm
V-Akt	Murine Thymoma Viral
VEGF	Facteur de croissance de la vascularisation endothéliale
VLDLR	Very-Low-Density-Lipoprotein Receptor
VNN1	Vanin 1
ZP	Zona pellucide

Remerciements

Le travail qui entoure la réalisation d'une thèse de doctorat représente de nombreuses heures de travail qui semble parfois impossible. Sans aucun doute l'achèvement de ma thèse fut loin d'être un travail solitaire, en effet il n'aurait pas été possible d'accomplir une fraction de mes projets de recherche sans l'aide et le soutien de nombreuses personnes dont la contribution ne sera pas oubliée.

J'ai beaucoup de reconnaissance et d'admiration envers Dr Marc André Sirard, mon directeur de thèse. Je vous remercie de m'avoir fait confiance malgré mes connaissances plutôt légères en productions animales, spécialement chez la vache laitière. Vous avez su être disponible pour me guider, m'encourager et me conseiller tout au long de mon projet de recherche et de mes études afin de m'aider à faire les bons choix et lancer ma carrière en recherche. De plus, je vous suis reconnaissant de m'avoir offert la flexibilité de travailler sur la ferme familiale durant le temps des récoltes. J'aimerais aussi remercier mon codirecteur, Dr Patrick Blondin pour vos conseils et votre perspective de l'industrie au cours de mon projet de recherche, ainsi que les membres de mon comité de thèse, de pré doc et du jury; Dr François Castonguay, Dr François Richard, Dr Claude Robert, Dr Jacques J. Tremblay et Dr Christopher Price pour vos contributions à mon parcours académique et scientifique.

Ce travail n'aurait pas été possible sans le soutien de l'Université Laval et des organismes subventionnaires. Je tiens spécialement à remercier tous les contribuables canadiens (plus précisément la classe moyenne) d'avoir participé au fond du CRSNG et du REDIH, qui m'ont offert des bourses d'études pour mon doctorat. Je veux aussi remercier le CRDSI de m'avoir accordé une bourse d'excellence et le RQR pour les bourses de voyages à des conférences scientifiques mémorables. Finalement, je remercie sincèrement la bande des Montagnais du Lac-Saint-Jean, les Mashteuiatsh d'avoir soutenu mes études grâce au programme Nishkatsh upahuatsh, merci; Tshinishkumitin.

J'aimerais aussi remercier Dr Claude Robert et Dr François Richard pour votre soutien et pour nos nombreuses conversations de science, de sport et de génétique. D'aucune façon il m'est impossible d'oublier de remercier les Isabelles[2]; Isabelle Dufort et Isabelle Gilbert, sans

qui le labo tomberait dans le néant total, vos conseils et vos habiletés en laboratoire (au pluriel avec plusieurs s) m'ont permis de passer à travers les nombreux défis et « troubleshootings » associés aux manipulations qui ne fonctionnent pas toujours comme l'on désire, spécialement dans le cas des dernières lames de micropuce… Je remercie aussi l'équipe de chez Boviteq, Anne-Marie, Rémi, Christian, FX, ainsi que les techniciens et techniciennes qui m'ont toujours accueilli chaleureusement lors de mes nombreuses visites. Je tiens aussi à remercier Yanick et Manon de chez EmrbyoTech, qui ont participé à mon projet de recherche via la récolte d'échantillons.

Évidemment, mon doctorat n'aurait pas été aussi « le fun » sans l'ensemble des étudiants présents et passés de notre laboratoire et du laboratoire de Dr Robert qui ont su rendre mes études aussi divertissantes et amicales. Merci de m'avoir intégré même si vous ne comprenez pas toujours mon franglais et merci pour le bel esprit d'équipe et de solidarité dans le bureau, toutes nos discussions et bien sûr les soirées cocktails du CRDSI, les « partys » de Noël de science animale, sans oublier le fameux méchoui. J'aimerais aussi apporter une attention spéciale à deux étudiants que j'ai eu la chance de superviser, Simon Lafontaine et Lia Rossi-Perazza pour leurs dévouements et leurs contributions à nos projets de recherche Genesis et Osiris.

Je remercie enfin celles et ceux qui me sont chers et que j'ai quelque peu délaissés ces derniers mois pour achever cette thèse. Mes bons « friends », Alexis et Méo, ainsi que mon frère Francis pour toutes nos soirées à faire n'importe quoi sauf de la science, ce qui m'a permis de bien me changer les idées. Je suis aussi reconnaissant à mes parents Philippe Landry et Chantal Gagnon Landry, qui ont tout fait pour m'aider, m'encourager et me soutenir tout au long de mes années d'études. Finalement, je veux sincèrement remercier ma conjointe, Lindsay Cormier, qui a quitté sa ville natale, son emploi, ses amis et sa famille à Moncton pour venir vivre « an adventure of a lifetime » à Québec avec moi. Sans aucun doute ta présence, ton soutien et notre amour sont les raisons qui m'ont motivé à persévérer dans mes études, à me surpasser et probablement à rester sain d'esprit de vivre dans une ville…

Merci à vous tous et tous ceux que j'ai assurément oublié de remercier !

Avant-propos

Chapitre 2:

Landry D.A., Bellefleur A.M., Labrecque R., Grand F.X., Vigneault C., Blondin P. and Sirard M.A. (2016). Effect of cow age on the in vitro developmental competence of oocytes obtained after FSH stimulation and coasting treatments. Theriogenology, 86(5), 1240-1246.

Cet article a été publié dans la revue Theriogenology. Date de soumission : 18 février 2016; Date de publication : 15 avril 2016. La version dans cette thèse est la même que celle publiée.

Dans ce chapitre, David A. Landry a contribué à l'élaboration du projet et est responsable de la réalisation de toutes les analyses, de l'interprétation des données et de l'écriture du manuscrit. Anne-Marie Bellefleur a contribué à la récupération des données rétrospectives. Rémi Labrecque, Christian Vigneault, François-Xavier Grand et Patrick Blondin ont participé à l'élaboration du projet. Marc-André Sirard a contribué à l'élaboration du projet, l'analyse et l'interprétation des données et à la révision finale du manuscrit. L'ensemble des coauteurs ont lu, participé à l'amélioration du manuscrit et approuvé la version finale du manuscrit.

Chapitre 3:

Landry D.A., Fortin C., Bellefleur A.M., Labrecque R., Grand F.X., Vigneault C., Blondin P. and Sirard, M. A. (2017). Comparative analysis of granulosa cell gene expression in association with oocyte competence in FSH-stimulated Holstein cows. Reproduction, Fertility and Development, 29(12), 2324-2335.

Cet article est publié dans la revue Reproduction, Fertility and Development. Date de soumission : 16 novembre 2016; Date de publication : 9 mars 2017. La version dans cette thèse est la même que celle publiée.

Dans ce chapitre, David A. Landry a effectué la réalisation de toutes les expériences, de l'analyse et de l'interprétation des données et de l'écriture du manuscrit. Cholé Fortin a participé à l'écriture d'une section du manuscrit et à la révision de tout le manuscrit. Anne-Marie Bellefleur a contribué à la récupération des données rétrospectives et des échantillons biologiques chez Boviteq. Rémi Labrecque, Christian Vigneault, François-Xavier Grand et Patrick Blondin ont participé à l'élaboration du projet. Marc-André Sirard a contribué à l'élaboration du projet, l'analyse, l'interprétation des données et à la révision finale du manuscrit. L'ensemble des coauteurs ont lu, participé à l'amélioration du manuscrit et approuvé la version finale du manuscrit.

Chapitre 4:

Landry D.A., Labrecque R., Grand F.X., Vigneault C., Blondin P. and Sirard M.A. (2017) Effect of heifer age on the granulosa cell transcriptome after ovarian stimulation. Reproduction, Fertility and Development, 30 (7), 980-990.

Cet article est publié dans la revue Reproduction, Fertility and Development. Date de soumission : 29 mars 2017; Date de publication : 17 novembre 2017. La version dans cette thèse est la même que celle publiée.

Dans ce chapitre, David A. Landry a effectué la réalisation de toutes les expériences, de l'analyse et de l'interprétation des données et de l'écriture du manuscrit. Rémi Labrecque, Christian Vigneault, François-Xavier Grand et Patrick Blondin ont participé à l'élaboration du projet. Marc-André Sirard a contribué à l'élaboration du projet, l'analyse, l'interprétation des données et à la révision finale du manuscrit. L'ensemble des coauteurs ont lu, participé à l'amélioration du manuscrit et approuvé la version finale du manuscrit.

Chapitre 5:

Landry D.A., Rossi-Perazza L., Lafontaine S., and Sirard M.A., (2018) Expression of atresia biomarkers in granulosa cells after ovarian stimulation in heifers. Reproduction, 156 (3), 239-248.

Cet article est publié dans la revue Reproduction. Date de soumission : 10 avril 2018. La version dans cette thèse est la même que celle publiée.

Dans ce chapitre, David A. Landry a contribué à l'élaboration du projet et a effectué l'analyse et de l'interprétation des données et de l'écriture du manuscrit. Lia Rossi-Perazza et Simon Lafontaine sont responsables de la réalisation des expériences, de l'analyse et de l'interprétation et ont contribués à l'écriture du manuscrit. Marc-André Sirard a contribué à l'élaboration du projet, l'analyse et l'interprétation des données et à la révision du manuscrit. L'ensemble des coauteurs ont lu, participé à l'amélioration du manuscrit et approuvé la version finale du manuscrit.

Chapitre 6:

Landry D. A. and Sirard M. A. (2018) Follicle capacitation: A meta-analysis to investigate the transcriptome dynamics following FSH decline in bovine granulosa cells., Biology of Reproduction.

Cet article est publié dans la revue Biology of Reproduction. Date de soumission : 31 janvier 2018; Date de publication : 14 avril 2018. La version dans cette thèse est la même que celle publiée, sauf pour les tables supplémentaires 6-2 et 6-3 qui ont été réduites de 2250 et 1550 DEGs, respectivement à 200 DEGs chacun pour une meilleure visualisation dans la thèse.

Dans ce chapitre, David A. Landry a contribué à l'élaboration du projet et est responsable de la réalisation de toutes les expériences, des analyses et de l'interprétation des données, de l'écriture du manuscrit et a participé à l'élaboration du projet. Marc-André Sirard a contribué à l'élaboration du projet, l'analyse et l'interprétation des données ainsi qu'à la révision finale du manuscrit.

1. Introduction

1.1.La biologie de la reproduction

La reproduction est au centre de la science de la vie; elle permet la survie des espèces, la diversité génétique et l'évolution dans les différents environnements. La biologie de la reproduction est l'étude de l'ensemble des processus fondamentaux qui sont orchestrés par les systèmes reproducteurs dans le but de préserver et de perpétuer les gènes chez toutes les espèces. Elle étudie les fonctions et le rôle des différents organes sexuels confectionnés par des milliers d'années d'évolution. De tous les types de reproduction possibles, les mammifères ont une reproduction sexuée, ce qui fait référence à deux systèmes reproducteurs distincts associés au sexe de l'individu, mâle et femelle et ayant des fonctions complètement différentes, mais un but unique de produire des cellules sexuelles appeler des gamètes. Les gamètes ne contiennent que la moitié des chromosomes d'une cellule normale diploïde et sont issus d'une division cellulaire réductionnelle, la méiose. Cette division permet d'augmenter la diversité génétique des gamètes grâce à l'enjambement (''crossing-over'') des chromosomes parents et de réduire le nombre de chromosomes à une seule copie, dite haploïde. Ainsi la méiose permet de former quatre gamètes haploïdes chez le mâle et un seul chez la femelle. Les gamètes produits par méiose seront complètement différents de la cellule mère, ce qui permet en autre la diversité génétique entre les générations et offre l'opportunité de créer de nouvelles combinaisons via des mutations pour s'adapter à l'environnement qui, lui-même, change d'autant, ce qu'on appelle en autre, l'évolution. Finalement, les gamètes mâles et femelles doivent se fusionner pour former un zygote diploïde ayant un nouveau génome dérivé des parents qui se développera en un nouvel individu.

En plus de produire des gamètes sexuels, les systèmes reproducteurs doivent être capables de permettre la rencontre des gamètes entre les individus grâce à un système parfois complexe d'entreposage, de libération et de livraison des gamètes via des organes génitaux externes et internes. Chez les mammifères, le système reproducteur femelle doit prendre en charge le développement du ou des embryons produits à la suite de la fusion des gamètes mâle et femelle, grâce à l'utérus. Évidemment, les systèmes reproducteurs sont finement régulés grâce aux différentes hormones sexuelles qui jouent un rôle essentiel au niveau de la

fonction sexuelle des différentes espèces.

1.2.Système reproducteur femelle

Chez les mammifères, la femelle joue un rôle beaucoup plus complexe que le mâle en termes de reproduction. Son système doit en autre synchroniser la production de gamètes avec la réceptivité de son utérus (Forde and Lonergan, 2012; Lonergan *et al.*, 2016) et, dans certains cas, en fonction des saisons (Dardente *et al.*, 2016). Son système doit aussi soutenir le développement d'un embryon s'il y a fécondation. Les ovaires et les voies génitales femelles constituent les organes génitaux internes, qui sont situés à l'intérieur de la cavité pelvienne. On retrouve les ovaires et les oviductes, en paires, l'utérus et le vagin. L'ovaire est constitué d'un cortex, qui renferme les gamètes et permet leur maturation, et d'une région médullaire plus profonde, qui contient les nerfs et les vaisseaux sanguins. Dans la région du cortex, on retrouve les follicules ovariens, qui sont des petites structures formées d'un œuf immature, l'ovocyte qui est enveloppé dans une couche de cellule somatique non -sexuelle aussi nommée cellule de la prégranulosa. La croissance d'un follicule défini par le terme folliculogenèse permettra en parallèle la croissance et la maturation d'un gamète femelle qui deviendra un ovule mature, ce processus de développement d'un gamète femelle est l'ovogenèse (Adams *et al.*, 2008). Les différentes étapes de la croissance du follicule et de l'ovule sont aussi dépendantes des hormones sexuelles qui agissent avant même la naissance chez les grands mammifères. Cependant, la production d'ovules matures ne se produit qu'à partir de la puberté.

1.3.Puberté

La puberté est une étape importante du développement sexuel chez les mammifères; elle est définie dans le temps par une suite d'évènements biologiques qui permettent de rendre fonctionnel le système de reproduction. La puberté amène aussi une multitude de modifications corporelles et comportementales due à la production accrue d'hormones sexuelles par les ovaires sous l'effet des gonadotrophines qui, avant la puberté, sont sécrétées en faible quantité. Chez la femelle, la puberté est caractérisée par une réponse spécifique de l'hypothalamus et de la glande pituitaire aux hormones gonadiques. Elle est marquée par l'augmentation de la sécrétion et de la pulsatilité des hormones gonadotrophines,

particulièrement l'hormone lutéinisante (LH), qui est responsable de la fin de la croissance folliculaire et du relâchement du gamète femelle (Rodriguez and Wise, 1989; Day and Nogueira, 2013) qui sera expliquée un peu plus en détail à la section 1.8 traitant du développement des follicules dépendants aux gonadotrophines. L'âge d'atteinte de la puberté chez les femelles est très variable selon les espèces, ainsi que la durée du cycle ovarien. Chez la vache laitière, *Bos Taurus*, le début de la puberté est très influencé par les facteurs génétiques et environnementaux comme la qualité et la quantité de nourriture disponible. Généralement, elle survient entre 9 et 12 mois d'âge dans les régions tempérées avec un cycle ovarien de 20 à 24 jours (Rodriguez and Wise, 1989; Fajersson *et al.*, 1991; Rodrigues *et al.*, 2002).

1.3.1. Hormones sexuelles

Pour tous les mammifères, le cycle ovarien est finement régulé par des hormones de nature protéique ou lipidique synthétisées par le système hypothalamo-hypophysaire, qui intègre les facteurs endogènes et exogènes et les traduit par des modifications de sécrétion des gonadotrophines. Ces mécanismes complexes faisant intervenir des facteurs hypothalamiques, hypophysaires et gonadiques sont ce qu'on appelle le système de régulation de l'axe HPG (Bentley *et al.*, 2010). Une caractéristique importante de la libération des gonadotrophines est le phénomène de sécrétion par changement rapide des concentrations plasmatiques à intervalles plus ou moins réguliers. Ce mode de sécrétion est appelé *sécrétion pulsatile*. Ainsi, l'hormone folliculo-stimulante (FSH) et l'hormone lutéinisante (LH) présentent un caractère pulsatile qui varie en fonction du cycle ovarien et selon l'espèce (Hillier, 2001). La sécrétion des gonadotrophines, FSH et LH sont sous la dépendance d'un facteur hypothalamique, l'hormone de libération des gonadotrophines hypophysaires (GnRH) est elle-même sous l'action des rétrocontrôles positifs et/ou négatifs des différentes hormones stéroïdiennes telles que l'œstrogène et la progestérone.

L'œstrogène et la progestérone sont des hormones de nature lipidique synthétisées à partir du cholestérol. L'œstrogène signifie « qui engendre œstrus » et la progestérone signifie « qui permet la gestation ». L'œstrogène a pour rôle majeur de provoquer l'œstrus qui est la période de chaleur, propice pour la fécondation chez les animaux (revue dans deCatanzaro, 2015), et

la progestérone est l'hormone du maintien de la gestation (revue dans Lonergan and Forde, 2015). Ces hormones jouent un rôle critique dans le follicule ovarien en assurant un rétrocontrôle du développemental folliculaire en synergie avec les gonadotrophines (LH et FSH). Leurs rôles au sein de la folliculogenèse seront expliqués en détail dans la section 1.8.1.

1.4.Le cycle ovarien

Au niveau de l'ovaire, le cycle ovarien ou cycle œstral est une série d'évènements périodiques qui mène à la croissance et à la libération d'un ou plusieurs œufs matures. Le cycle ovarien typique est divisé en différentes phases relative au stade de croissance des follicules; la phase folliculaire et la phase lutéale qui sont séparées par l'ovulation (Forde *et al.*, 2011). Tout d'abord, la phase folliculaire correspond à l'intervalle entre la régression du corps jaune et l'ovulation. Ensuite la phase lutéale qui correspond à la période entre l'ovulation et la régression du corps jaune, où le corps jaune est responsable de la production de progestérone et qui empêche le signal d'ovulation des cycles suivants. La durée du cycle ovarien est une caractéristique de l'espèce et montre une variance individuelle de 18 à 25 jours chez la vache (Saint-Dizier and Chastant-Maillard, 2014).

1.4.1. Phase folliculaire

La phase folliculaire du cycle ovarien est la période où le follicule croit en absence d'un corps jaune et sera capable d'ovulé. Cette phase peut être divisée en trois périodes successives : l'intervalle entre la régression du corps jaune et le début de l'œstrus (période de chaleur), l'intervalle entre le début de l'œstrus et le signal d'ovulation et l'intervalle entre le signal d'ovulation et l'ovulation elle-même. La phase folliculaire est généralement courte chez la vache, de 2 à 5 jours (Saint-Dizier and Chastant-Maillard, 2014), elle est caractérisée par le déclin de progestérone due à la régression du corps jaune du cycle précédent. Ainsi, le follicule dominant de la phase folliculaire sera ovulé grâce à l'absence de progestérone, ce qui permet la sécrétion de l'hormone LH. À l'ovulation, l'ovocyte s'échappera dans l'oviducte et sera prêt à la fécondation.

1.4.2. Ovulation

L'ovulation est la libération d'un ou plusieurs gamète(s) femelle(s) lors de la rupture d'un ou plusieurs follicule(s) préovulatoire(s), et se retrouve après la phase folliculaire et démarque le début de la phase lutéale. Le processus d'ovulation est contrôlé par les hormones de l'axe HPG et de la pulsatilité de la LH afin de provoquer des changements dans le follicule qui le prépare pour libérer l'ovocyte dans les trompes de Fallope. De façon générale, la rupture du follicule est dû à l'augmentation de la pression interne et de la fragilisation de la paroi du follicule.

1.4.3. Phase lutéale

Après l'ovulation et la perte du fluide folliculaire, le follicule collapse, les cellules restantes du follicule s'hypertrophient et deviennent une structure glandulaire qu'on appellera désormais le corps jaune (à cause de sa pigmentation jaune) qui finira par dégénérer s'il y a absence du signal de gestation, voir section 1.10.2. La fonction des cellules change subitement et ces dernières sécrètent parfois de l'œstrogène et toujours de la progestérone. Le sort du corps jaune dépend de la fécondation de l'ovule, s'il n'est pas fécondé, après 14 jours (chez la femme) et 17 jours (chez la vache) le corps jaune régressera, ce qui mènera à la fin de la phase lutéale (Saint-Dizier and Chastant-Maillard, 2014). Si l'ovule est fécondé, le corps jaune continuera à se développer et, selon l'espèce, il restera en place ou non jusqu'à la fin de la gestation afin de fournir les hormones nécessaires pour le soutien de la gestation.

1.5. Folliculogenèse

La phase folliculaire ne représente qu'une petite partie de la folliculogenèse et une fois entamée est irréversible. La succession des nombreuses étapes de la formation et l'activation des follicules primordiaux jusqu'à la formation d'un follicule préovulatoire capable d'ovulation durant la phase folliculaire est désignée par le terme folliculogenèse (figure 1-1 (Edson *et al.*, 2009). Il est intéressant de noter que la durée totale du développement folliculaire, de la sortie de la réserve folliculaire jusqu'au stade préovulatoire varie d'une espèce à l'autre et peut prendre jusqu'à plus de 200 jours chez la vache (Saint-Dizier and

Chastant-Maillard, 2014). Ce processus est en continu et chaque jour une portion de follicules primordiaux sont activés et débutent la folliculogenèse.

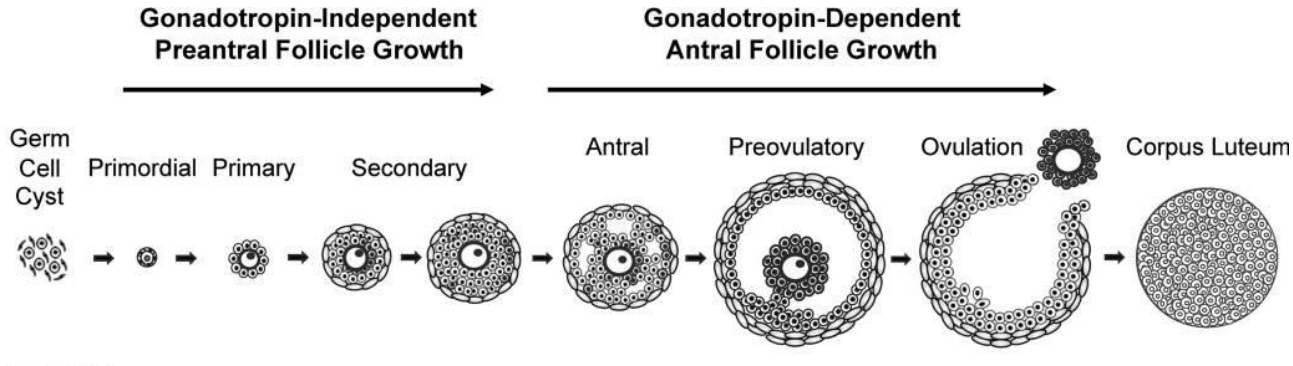


Figure 1-1: Le développement du follicule ovarien (Edson et al., 2009)

Classification des stades majeurs de la folliculogenèse indépendante et dépendante des gonadotrophines. Figure reproduite avec la permission de Elsevier © 2009.

Bien avant la phase folliculaire, le follicule primordial est activé à la suite d'un signal spécifique encore méconnue (voir section 1.7.1) et se transforme en un follicule primaire, ce qu'on appelle l'activation des follicules primordiaux. Ensuite, le follicule primaire se transforme en un follicule secondaire ou préantral, dans lequel les cellules somatiques forment plusieurs couches distinctes autour du gamète, qui sont les cellules de la granulosa et de la thèque. À partir de cette étape, la croissance des follicules deviendra strictement dépendante des hormones hypophysaires et durant chaque cycle ovarien un ou plusieurs follicules préantraux se développera en un follicule dominant capable d'ovulation s'il se retrouve dans la phase folliculaire (Hillier, 2001). Toutefois, seulement une faible proportion de follicules se rendra dans le stade préovulatoire,; plus de 99 % des follicules qui entreront dans la folliculogenèse seront éliminés et régresseront par le processus de l'atrésie folliculaire qui peut intervenir à tous les stades de la folliculogenèse (revue dans Matsuda *et al.*, 2012). Une telle sélection est importante afin de permettre l'ovulation des meilleurs ovocytes possible.

1.6.Formation des gamètes femelles : Stade embryonnaire

1.6.1. Cellules germinales embryonnaires (PGCs)

Chez les mammifères, la production de gamètes femelles débute lors du développement embryonnaire. La formation des ovocytes primaires à partir des PGCs peut être divisée en sept étapes : (1) la génération de cellules germinales primordiales (PGCs); (2) la migration des PGCs vers les futures gonades; (iii) la colonisation des PGCs dans les gonades par mitose; (iv) la différentiation des PGCs en ovogonies; (v) la prolifération des ovogonies; (vi) l'initiation de la méiose et (vii) l'arrêt de la méiose au stade diplotène de la prophase I (Saint-Dizier and Chastant-Maillard, 2014).

Tout d'abord, les cellules précurseurs des ovocytes primaires sont les cellules germinales primordiales qui se différencient lors de la période de gastrulation (Yeom *et al.*, 1996; Pesce *et al.*, 1998). Les cellules germinales sont capables de migrer vers les crêtes génitales par mouvements amiboïdes et chez les gros mammifères, par transport intravasculaire afin d'atteindre une plus grande distance (Wartenberg, 1983). Toutefois, le processus qui permet aux cellules de migrer à certains endroits spécifiques est encore incertain. Tout au long et même après leur migration, les PGCs poursuivent leur prolifération par mitose, formant ainsi des groupes d'ovogonies qui coloniseront les gonades du fœtus (Pepling, 2006). Certains facteurs sont reconnus pour réguler la différenciation des PGCs en ovogonies. Entre autres, les membres de la famille des ''transforming growth factor B'' (TGFβ), comme les ''Bone morphogenetic protein'' (BMP) sont essentiels et augmenteraient le nombre de PGCs chez la souris (Ying *et al.*, 2000; Ying and Zhao, 2001; Pesce *et al.*, 2002; Puglisi *et al.*, 2004). Il a aussi été démontré que la concentration de ces facteurs détermine le nombre de PGCs formés. De plus, la migration et la survie des PGCs est soutenue par la présence des récepteurs c-kit et son ligand Kit (facteur KL) (Matsui *et al.*, 1990) qui assurent aussi la prolifération des PGCs en combinaison avec le «leukemia inhibiting factor» (LIF) et LIF-like factor (Dolci *et al.*, 1993; Fleischman, 1993). Le profil d'expression du système KIT (récepteur et son ligand) correspond avec la prolifération et la migration des cellules germinales primordiales. Les souris mutantes de ce système ne démontrent aucune prolifération des PGCs et aucun signe de migration/colonisation des futures gonades (Buehr *et al.*, 1993).

Par la suite, les ovogonies se sépareront du nid et seront entourées par une couche de cellules somatiques, les cellules prégranulosa. Les ovogonies qui ne réussiront pas à s'entourer de cellules somatiques seront éliminées (Pepling and Spradling, 2001). Des facteurs stimulateurs de la méiose provoquent l'entrée des ovogonies en méiose où elles sont arrêtées lors de l'étape de la première prophase au stade diplotène afin de devenir des ovocytes primaires (Ginsburg *et al.*, 1990; Hunt and Hassold, 2008). À cette étape, l'ovocyte primaire est entouré d'une simple couche de cellule somatique, le tout enrobé dans une membrane appelée la membrane propria et le tout s'appelle le follicule primordial (Picton *et al.*, 1998).

1.6.2. Follicule primordial

À la naissance, l'ovaire va contenir son nombre maximal de follicules primordiaux qui peuvent rester en arrêt de la méiose et en dormance pour plus de 50 ans en attendant le signal pour reprendre le développement de l'ovocyte. On dénombre la quantité d'ovocytes non folliculaires à environ un maximum de 2 700 000 ovogonies chez la vache (Saint-Dizier and Chastant-Maillard, 2014). Après une forte dégénération de la plupart d'entre eux lors de leurs premières activités méiotiques, le nombre d'ovogonies qui sont devenues des ovocytes primaires après la méiose va diminuer de plus de 95 %. À la naissance on dénombre un compte maximum d'ovocytes folliculaires de 135 000 chez la vache (Erickson, 1966).

1.7. Développement des follicules : Phase indépendante des gonadotrophines

1.7.1. Transition des follicules primordiaux aux follicules primaires

Le recrutement/activation des follicules primordiaux dans le vaste groupe de la réserve ovarienne commence dès qu'ils sont formés et d'autres vont attendre des mois et des années avant leur démarrage. À la suite de la puberté, certains follicules qui commencent leurs développements pourront se rendre jusqu'à l'ovulation. L'activation du follicule primordial est un processus plus ou moins bien connu qui fait appel à un déséquilibre entre l'inhibition et l'activation du follicule primordial. Il est mal compris pourquoi chaque jour, une petite portion de follicules primordiaux commence leur développement pour devenir des follicules

primaires et de quelle façon ils sont sélectionnés. Toutefois, leur sélection semble faire intervenir les voies de signalisation PTEN (phosphatase and TENsin homolog), PI3K (phosphoinositide 3-kinase) et TSC/mTOR (tuberous sclerosis complex-mammalian target of rapamycin), qui sont connues pour être impliquées dans la régulation de l'activation et l'inhibition du démarrage de la croissance des follicules primordiaux (Reddy *et al.*, 2005). Suite à son activation, un follicule primordial devient un follicule primaire lorsque la simple couche de cellules de granulosa entourant l'ovocyte prolifère et devient cubique (Lintern-Moore and Moore, 1979). La transition du follicule primordial en follicule primaire est aussi accompagnée de l'augmentation du diamètre de l'ovocyte et du recrutement des cellules génitrices de la thèque à partir des cellules du stroma. Certains facteurs produits par les cellules de granulosa sont impliqués dans le recrutement des cellules du stroma (Hirshfield, 1991).

1.7.2. Transition des follicules primaires en follicules secondaires

Lorsque le follicule contient plus que deux couches de cellules de granulosa, il devient un follicule secondaire ou préantral. À cette étape, il y a une augmentation accrue du diamètre folliculaire qui passe de 20 µm à entre 150 et 400 µm, selon l'espèce (150 µm chez la vache et 200 µm chez la femme). Cette augmentation du diamètre folliculaire est principalement due à l'augmentation du diamètre de l'ovocyte jusqu'à son diamètre final de 120 µm, chez la vache et chez la femme)(van den Hurk *et al.*, 1997). L'augmentation du diamètre ovocytaire est due en partie à l'accumulation des réserves de protéines essentielles pour les futurs stades de maturation ainsi que les premiers jours de développement suite à la fécondation; cette étape représente une phase de cytodifférenciation (Picton *et al.*, 1998). En plus de l'augmentation du diamètre de l'ovocyte, les cellules de la granulosa prolifèrent ce qui augmente davantage la dimension du follicule et sa complexité. Les cellules de la stroma s'organisent sur la région externe de la membrane basale du follicule et deviendront les cellules de la thèque du follicule. La prolifération des cellules de la thèque mènera à la formation de deux couches distinctes, une couche interne, hautement vascularisée et une couche externe, appelée la capsule fibreuse. Les cellules de la thèque ont plusieurs rôle durant la folliculogenèse, elles sont responsable de la production d'androgène (voir section sur la

stéroïdogénèse) et supporte la croissance du follicule via la production d'une multitude de facteurs essentiels à la vascularisation (revue dans Young and McNeilly, 2010).

La transition du follicule primaire en follicule secondaire est totalement indépendante des gonadotrophines, même s'il est possible de détecter des récepteurs aux gonadotrophines à ce stade (Fortune and Eppig, 1979). La transition de primaire à secondaire est plutôt due aux facteurs intraovariens produits par le follicule (Kol and Adashi, 1995), ce qui inclut deux facteurs de la famille des facteurs de transformation et croissance bêta (TGFB, transforming growth factor beta), GDF9 (Elvin *et al.*, 1999; Nilsson and Skinner, 2002) et BMP15 (Yan *et al.*, 2001).

1.7.2.1. Zona Pellucida

Lors de la phase préantrale, l'ovocyte sécrète des glycoprotéines qui forment une couche condensée transparente autour de lui-même, qu'on appelle la zone pellucide. Cette couche peut devenir une matrice constituée entre autres, de trois types de glycoprotéines (ZP1, ZP2 et ZP3) et permet de séparer l'ovocyte des cellules de la granulosa qui l'entourent (Takagi *et al.*, 1989) sans altérer le réseau de communication.

1.7.2.2. Jonctions communicantes

Des contacts avec la granulosa et l'ovocyte restent maintenus à l'aide de ponts cytoplasmiques qui pénètrent la zona, connus sous le nom de projection transzonale (TZP) et forment un nombre incroyable de jonctions GAP créant ainsi un réseau d'échange et de communication fonctionnel essentiel au développement de l'ovocyte (Hussein *et al.*, 2006; Gilchrist *et al.*, 2008). Les jonctions intercellulaires sont composées de protéines de type connexine (Cx). Les jonctions communicantes GAP facilitent la communication bidirectionnelle entre l'ovocyte et les cellules de granulosa; elles permettent le transport de nutriments, de précurseurs métaboliques, de molécules d'informations (ARNm, hormones, neurotrophines et facteurs de croissance) et des signaux méiotiques (Buccione *et al.*, 1990).

On retrouve deux types de connexine soit la Cx37 et la Cx43 (Valdimarsson *et al.*, 1993). La Cx37 est majoritairement présente entre les cellules de granulosa et l'ovocyte tandis que la

Cx43 assure la communication entre les cellules du cumulus et de la granulosa et n'est pas présente entre l'ovocyte et les cellules du cumulus (Simon *et al.*, 1997). Ces deux connexines sont essentielles pour le bon développement du follicule; les souris mutantes nulles de la connexine 37 sont stérile et démontrent un arrêt du développement ovocytaire avant la phase de méiose, une perte de maturation folliculaire (arrêt à la phase préantrale), un développement inapproprié du corps jaune et un échec de l'ovulation (Simon *et al.*, 1997). Les souris mutantes de la connexine 43 meurt rapidement après la naissance et démontrent une déficience dans leurs cellules germinales et un arrêt de la croissance du follicule primaire, démontrant ainsi le rôle de la connexine 43 et des cellules de granulosa au début de la folliculogenèse (Juneja *et al.*, 1999).

1.8. Développement des follicules : Phase dépendante des gonadotrophines

D'un point de vue fonctionnel, la folliculogenèse peut se subdiviser en deux phases successives, la phase indépendante des gonadotrophines, qui représente plus de 75 % de la durée totale du développement folliculaire et la phase dépendante des gonadotrophines qui est néanmoins plus rapide. C'est au cours de la deuxième phase que l'ovocyte acquiert sa compétence méiotique et développementale.

1.8.1. Gonadotrophines et leurs récepteurs

Les gonadotrophines sont des hormones formées de deux sous-unités glycoprotéiques alpha et bêta qui agissent sur les fonctions des gonades. La sous-unité alpha est spécifique à l'espèce. De plus, elle est commune au sein des gonadotrophines de la même espèce. À l'opposé, la sous-unité bêta est spécifique à chaque gonadotrophine. Deux des hormones sont sécrétées par l'hypophyse antérieure et jouent un rôle essentiel et distinctif dans le système reproducteur : l'hormone folliculo-stimulante (FSH) et l'hormone lutéinisante (LH). Leurs actions dépendent de leurs liaisons et de l'activation de leurs récepteurs, le récepteur à la FSH (FSHR) et le récepteur à la LH (LHR). Il existe aussi une gonadotrophine chorionique semblable à la LH, sécrétée par le placenta. La chorionique humaine (hCG), sécrétée chez

les femmes enceintes et la chorionique équine (eCG), anciennement appelée PMSG (« Pregnant mare serum gonadotropin ») sécrétée chez la jument gestante.

À partir du follicule secondaire ou préantral, le follicule devient strictement dépendant de l'action des deux gonadotrophines FSH et LH d'une façon temporelle. En dessous d'un certain niveau de FSH ou de LH, les follicules ne peuvent plus se développer et entrent en atrésie, ainsi l'exposition des follicules préantraux à la FSH est favorable pour leurs survie (Cortvrindt *et al.*, 1997). Contrairement au stade primaire, le follicule exprime des récepteurs fonctionnels FSHR et LHR et est désormais strictement dépendant des gonadotrophines. L'analyse des récepteurs membranaires à la fin de la phase préantrale et au début de la phase antrale du follicule révèle que seulement les cellules de la thèque interne peuvent lier la LH tandis que seulement les cellules de la granulosa peuvent lier la FSH. Les souris mutantes nulles pour FSH et son récepteur (FSHR) sont toutes deux infertiles et démontrent un arrêt folliculaire au stade préantral (Burns *et al.*, 2001) et les ovaire sont significativement plus petit en plus de démontré des anormalie vaginale et utérin (Abel et al., 2000). Les souris mutantes du récepteur à la LH sont infertiles ayant aussi des follicules arrêtés au stade préantral (Zhang *et al.*, 2001).

1.8.2. Transition du follicule préantral au follicule antral

Chez les primates et le bovin, le follicule passe de la phase préantrale au début de la phase antrale lorsque son diamètre atteint 1 mm (Koering, 1983; Monniaux *et al.*, 1984). La formation d'un follicule antral capable d'ovuler est un processus qui est déterminé par sa prolifération cellulaire et sa capacité à répondre aux gonadotrophines. Ainsi, les cellules de la granulosa continuent de proliférer et atteignent plus de 5 à 6 couches, ce qui augmente davantage la taille du follicule. Un liquide visqueux (fluide folliculaire), produit par les cellules de la granulosa, commence à faire son apparition entre elles, dans ce qu'on appelle l'antrum folliculaire (une simple cavité) ce qui marque le début de la phase folliculaire antrale. Le fluide folliculaire contient une multitude d'éléments de régulation dérivés de la circulation sanguine et de la sécrétion des cellules folliculaires, c'est à dire, des gonadotrophines, des stéroïdes, des facteurs de croissance, des enzymes et plus. La production du fluide folliculaire est accentuée par la vascularisation folliculaire et la

perméabilité des vaisseaux sanguins. La formation de l'antrum n'est pas totalement comprise, mais il semble que la FSH (Mao *et al.*, 2002), la LH (Cortvrindt *et al.*, 1998), l' EGF (Gutierrez *et al.*, 2000), l'activine (Zhao *et al.*, 2001) et le système KL (Driancourt *et al.*, 2000) en sont responsables.

L'augmentation de l'antrum folliculaire sépare les cellules de granulosa et mène à la ségrégation de l'ovocyte en périphérie. Toutefois, l'ovocyte reste toujours entouré par une couche dense de cellules de la granulosa qui sera désormais appelée le cumulus oophorus. Les cellules du cumulus resteront toujours connectées aux cellules périphériques de granulosa (aussi nommé les granulosa murales) par une mince couche de cellules. Quant à lui, l'ovocyte synthétise de l'ARN et des protéines afin de se préparer à entrer dans la phase préovulatoire.

Lors de la phase préantrale, selon l'espèce, un seul follicule (mono-ovulatoire) ou quelques follicules (pluriovulatoire) seront sélectionné(s) pour devenir le ou les follicules dominants capable d'ovulation. Cette sélection se fait à partir du groupe de follicules recruté du groupe de follicules préantraux, connu comme étant le recrutement folliculaire. Chez certaines espèces, comme la vache laitière, le croissance des follicules préantraux se produit par vague folliculaire (Adams, 1999). Chez la vache, deux à trois vagues folliculaires sont observées lors du cycle œstral. La première vague commence au jour de l'ovulation, dans un cycle de deux vagues on observe le deuxième recrutement au jour 10, ayant un cycle œstral de 21 jours, tandis que dans un cycle de trois vagues on observe le deuxième recrutement le jour 8 et le troisième recrutement le jour 16, ayant un cycle œstral de 23 jours. Chez le bovin, les vagues folliculaires se produisent en continu durant la phase folliculaire et durant la phase lutéale. Toutefois, seulement le follicule présent durant la phase folliculaire sera ovulé due à la diminution de progestérone de la phase lutéale du cycle précédent (Fortune *et al.*, 2004).

1.8.3. Recrutement, sélection et dominance du follicule

Plusieurs follicules sont recrutés conjointement dans une vague en début de la phase folliculaire, mais un seul va échapper à l'atrésie pour devenir le follicule dominant (voir figure 1-2 (Forde *et al.*, 2011)). Seulement les follicules ayant un diamètre supérieur à 3-5

mm et exprimant les récepteurs à la FSH chez les cellules de la granulosa pourront être recrutés.

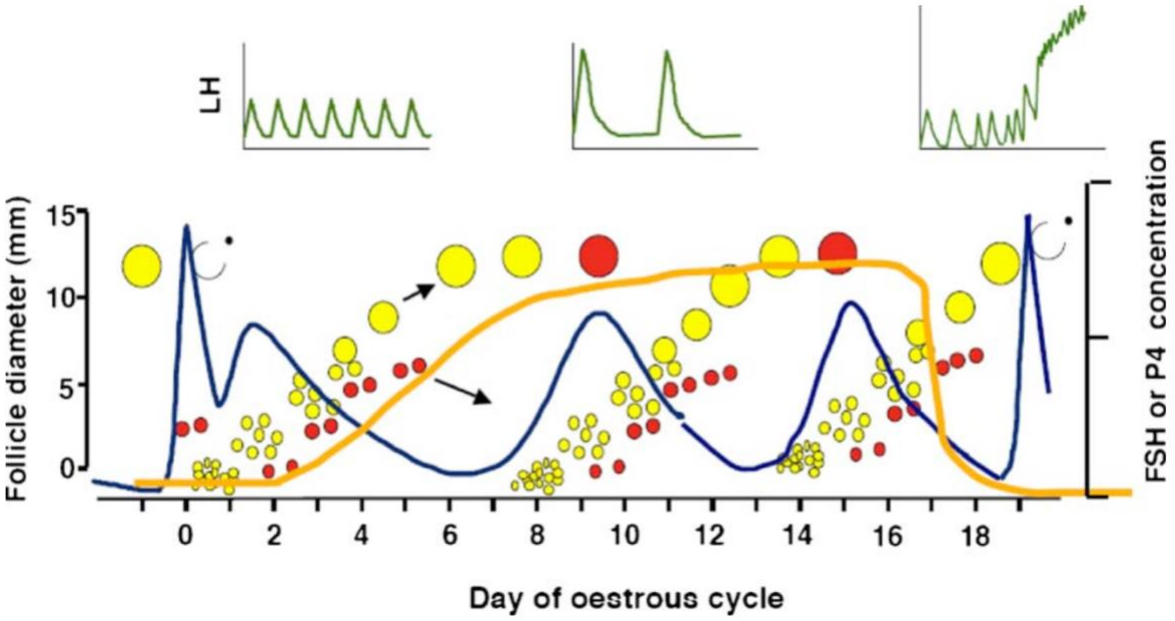


Figure 1-2: Dynamique folliculaire à trois vagues (Forde et al., 2011)

Représentation schématique d'un cycle ovarien à trois vagues folliculaires. Les cercles jaunes représentent les follicules en santé et les cercles rouges sont les follicules en atrésie. Chaque vague folliculaire débute à la suite d'une hausse de FSH (ligne bleue) et seulement durant la phase folliculaire, sans progestérone (P4, ligne orange) qui permettra la pulsatilité et le pic de LH (ligne verte). Figure reproduite avec la permission de Elsevier © 2011.

Le recrutement est initié par une légère augmentation de FSH plasmatique, ce qui active augmenter la croissance et la prolifération des cellules de la granulosa et la sécrétion d'œstradiol à partir des androgènes sécrétés par les cellules de la thèque, selon le concept de deux cellules et deux gonadotrophines (figure 1-3, voir section sur la stéroïdogénèse). L'augmentation de l'œstradiol et de l'inhibine jusqu'à un certain niveau dans la circulation sanguine mèneront à la réduction de la production de FSH par rétroaction négative, mettant fin au recrutement de cette vague. (Adams *et al.*, 1992). Tous les follicules ayant une dimension supérieure à 5 mm contribuent significativement à la régulation négative de la concentration de FSH (Gibbons *et al.*, 1997). La FSH, les androgènes et l'œstradiol stimulent la croissance des cellules de granulosa, la croissance du follicule antral et l'apparition de récepteurs à la LH à la surface des cellules de la granulosa. Une étude démontre que

l'augmentation de la durée et de la concentration de FSH augmentent le nombre de follicules recrutés (Gibbons *et al.*, 1997).

Un processus de sélection permet de déterminer le follicule de la cohorte qui sera destiné à devenir le follicule dominant et le nombre de follicules sélectionnés est dépendant du nombre d'ovulations de l'espèce. Le processus de sélection est passif et le premier follicule qui acquiert les récepteurs à la LH dans les cellules de granulosa deviendra le follicule dominant à un diamètre folliculaire d'environ 8.5 mm. (Ireland and Roche, 1987; Xu *et al.*, 1995). Cette sélection se fait par l'indépendance graduelle du follicule dominant à la FSH et l'acquisition nouvelle des récepteurs à la LH et sa dépendance par les cellules de granulosa.

Les follicules subordonnés seront éliminés par atrésie. Le follicule dominant ainsi que le plus gros follicule subordonné sont presque de la même dimension lors de leur recrutement, mais ils divergent graduellement. Cette étape est appelée la déviation (Ginther *et al.*, 1997). Il est pertinent de noter que même avant ce processus de sélection du dominant et de déviation, tous les follicules ayant un diamètre supérieur à 5 mm sont capables de devenir un follicule dominant chez le bovin (Gibbons *et al.*, 1997).

L'effet des gonadotrophines sur la déviation du follicule est influencé par l'action autocrine et paracrine des substances produites localement dans l'ovaire, en autre le système d'inhibine et d'activine, les IGFs et leurs protéines de liaisons IGFBPs, les androgènes et l'œstrogène. Brièvement, l'inhibine et l'activine produites par les cellules de granulosa ont une action modulatoire de la production d'androgènes des cellules de la thèque. L'expression de l'inhibine augmente progressivement relativement à l'activine durant la déviation du follicule, ce qui encourage l'action de la LH à produire des androgènes précurseurs de la synthèse d'œstradiol chez les cellules de la thèque (Hillier, 2001). De plus l'inhibine accentue l'effet des facteurs de croissance de l'IGF (IGF, Insulin-like growth factor). Les IGFs sont habituellement liés au IGFBPs, neutralisant ainsi leurs actions. L'action de la FSH sur les cellules de granulosa relâche une protéase PAPP-A (Pregnancy-associated plasma protein A) qui dégrade les IGFBPs et libère les IGFs (Spicer, 2004). IGF-1 et IGF-2 sont exprimés dans les cellules de granulosa et de la thèque respectivement (Khamsi and Roberge, 2001), et sont

détectables librement dans le fluide folliculaire suite à l'action de PAPP-A. Ces facteurs sont en synergie avec la FSH et la LH et stimulent la prolifération des cellules de la granulosa et la production d'œstrogène. En d'autres mots, les IGFs amplifient le signal de la FSH et LH et permettent au follicule dominant de rapidement synthétiser de l'œstradiol et ainsi de supprimer la FSH de la circulation par rétrocontrôle négatif et ainsi empêcher les follicules subordonnés de poursuivre leur maturation (Fortune *et al.*, 2004).

D'autres facteurs tels que les facteurs GDF9 et BMP15 sécrétés par l'ovocyte jouent aussi des rôles cruciaux au cours des différentes étapes de la folliculogenèse. Entre autres, GDF9 et BMP15 régulent la formation et assurent la fonction des cellules du cumulus. Seuls ou en combinaison avec la FSH stimulent la prolifération des cellules de granulosa lors des étapes de la dominance du follicule. GDF9 et BMP15, en combinaison avec BMP6, assurent l'inhibition de la production de progestérone par la FSH et ainsi prévient la lutéinisation prématurée du follicule (Vitt *et al.*, 2000; Otsuka *et al.*, 2001).

1.8.3.1. Stéroïdogenèse

Sous l'influence des gonadotrophines (FSH et LH), le follicule antral produit plus de 30 à 70 % des androgènes retrouvés chez la femelle. Les androgènes sont responsables de la continuité du développement folliculaire en induisant la prolifération et la différenciation des cellules de granulosa par les récepteurs aux androgènes (AR) et aux œstrogènes (Er α et β) (Findlay and Drummond, 1999). Les cellules de la thèque sont responsables de la synthèse d'androgènes à partir de l'acétate et/ou du cholestérol via leur activation par la LH. Les cellules de la granulosa sont incapables de former des androgènes; elles sont plutôt fournies en androgènes exogènes qui seront rapidement convertis en œstrogènes par l'action d'une aromatase Cyp19 (Hillier *et al.*, 1994). Cette aromatisation d'androgènes en œstrogènes est sous le contrôle de la stimulation par la FSH et par la LH lorsque les follicules acquièrent les LHR. Dans le follicule en développement, les androgènes proviennent seulement des cellules de la thèque, tandis que l'œstrogène provient d'une coopération dans laquelle les androgènes des cellules de la thèque sont aromatisés par les cellules de granulosa, ce qu'on appelle le concept de deux cellules et des deux gonadotrophines (figure 1-3) (Hillier *et al.*, 1994).

Les androgènes produits par le follicule en croissance sont fondamentaux pour son propre développement. Premièrement, avec la FSH, les androgènes stimulent la croissance des cellules de granulosa, la croissance du follicule antral et l'apparition des récepteurs à la LH à la surface des cellules de granulosa. L'œstrogène crée un rétrocontrôle positif sur la prolifération des cellules de la granulosa, ce qui résulte en une augmentation de la concentration d'œstrogène dans la circulation. Les souris mutantes du récepteur aux androgènes AR sont sous-fertiles et démontrent un nombre réduit de follicules antraux, une diminution du nombre d'ovulations et une forte mort cellulaire des cellules de granulosa (Hu *et al.*, 2004). Les souris mutantes conditionnelles du récepteur à l'œstrogène α (Erα) dans l'ovaire, démontrent un arrêt de la folliculogenèse au début du stade antral et sont infertiles tandis que les souris mutantes dans l'ovaire du récepteur Erβ sont fertiles (Emmen and Korach, 2003).

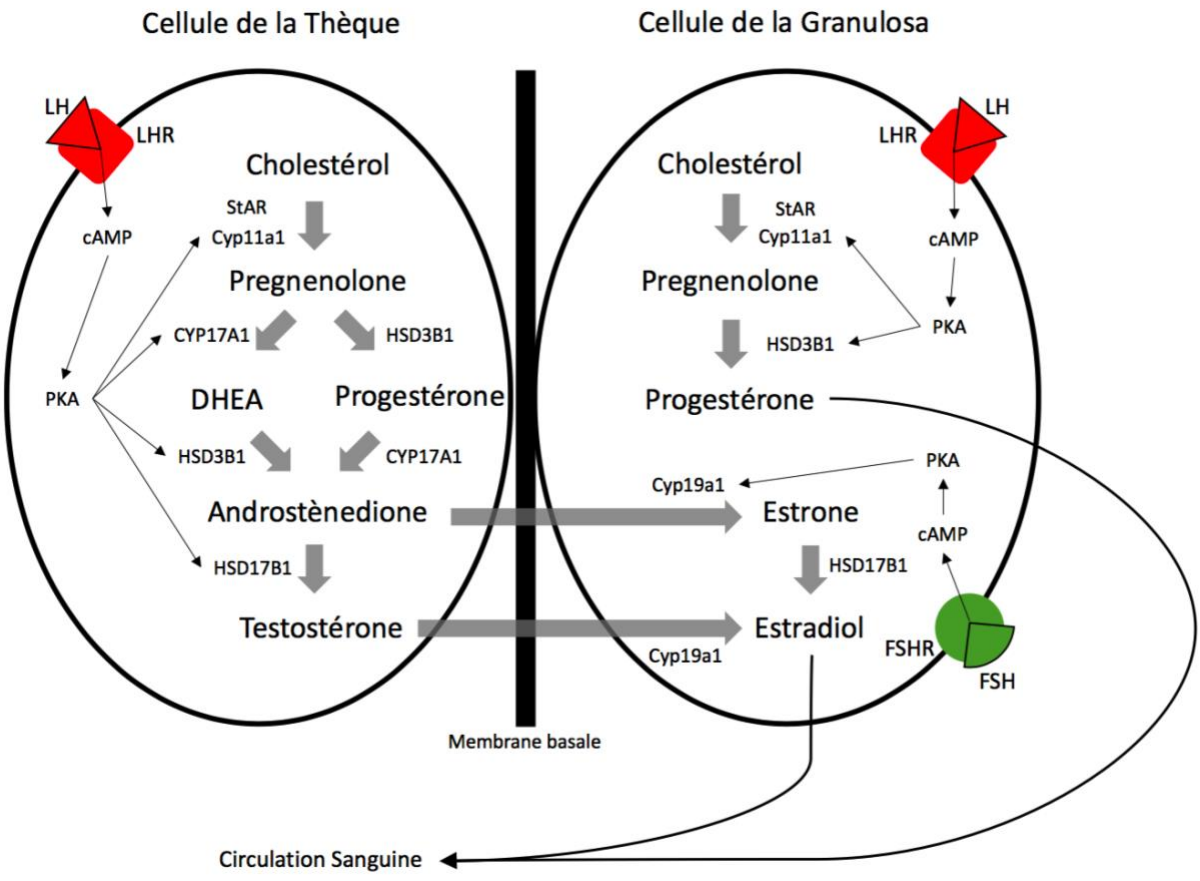


Figure 1-3: Concept des deux cellules et deux gonadotrophines

Avant l'acquisition des récepteurs à la LH chez les cellules de la granulosa, la FSH stimule la production de l'enzyme Cyp19a1, l'aromatase qui converti l'androstènedione ou la testostérone en oestrone et oestradiol, respectivement. Après l'acquisition des récepteurs à la LH, la cellule de granulosa acquiert la capacité de convertir le cholestérol en progestérone (P4) (Hillier *et al.*, 1994).

Les stéroïdes ne sont pas les seuls facteurs paracrines qui agissent sur la maturation du follicule antral, on retrouve aussi des cytokines, produites via l'activation de récepteurs aux gonadotrophines lors de la phase antrale. Les connaissances au niveau des cytokines impliquées dans la maturation du follicule sont limitées. Toutefois, on retrouve une balance entre certaines cytokines comme IL-1B et IL-6 ayant un rôle majeur dans la survie et la mort cellulaire des cellules de la granulosa (Matsuda *et al.*, 2012).

1.9. Phase préovulatoire : Pic préovulatoire et ovulation

L'ovulation est un processus complexe induit par le pic de LH au cours duquel il y a la reprise de la méiose chez l'ovocyte, la production de progestérone, l'expansion du cumulus, la rupture folliculaire et la libération de l'ovocyte.

1.9.1. Pic de LH

Le passage de la phase antrale dominant vers la phase préovulatoire du follicule dépend strictement de la pulsatilité et de l'augmentation brusque de l'hormone LH (pic de LH) (Hillier, 2001; Adams *et al.*, 2008). L'augmentation d'œstradiol produit sous l'influence de la LH par le follicule dominant permet d'initier l'augmentation de la LH pulsative et ainsi le pic de LH. Les pics préovulatoires de la FSH et de la LH sont la gâchette physiologique qui stimule les changements préovulatoires dans le follicule, l'ovulation et la lutéinisation des cellules folliculaires, ce qui mène à des changements drastiques de l'endocrinologie globale du follicule. Dans les 3 à 12 heures qui suivent l'exposition du follicule à une dose élevée de LH, des changements se produisent dans l'ovocyte. L'activation de la protéine G couplée au récepteur de la LH (LH-R) chez les cellules de la granulosa murale stimule la voie PKA/cAMP, ce qui induit le récepteur à la progestérone (PGR) et initie la lutéinisation du follicule et enclenche la production de progestérone (Robker *et al.*, 2009). Cette capacité

récemment acquise par les cellules de granulosa résulte en une augmentation des gènes associés à la production de progestérone, en autre : la protéine de régulation (StAR), P450scc, GATA-4, SF-1 et COX-1 (Devoto *et al.*, 2009) et ainsi une augmentation exponentielle de la progestérone et de la 17α-hydroxyprogestérone. Toutefois, cette augmentation rapide des progestines suppose que la machinerie cellulaire pour sa synthèse soit préalablement produite dans les cellules de granulosa. Suite au pic de LH, on observe aussi une rupture des liaisons entre l'ovocyte et les cellules du cumulus, processus appelé l'expansion du cumulus (voir section 1.9.3). On observe aussi une augmentation du fluide folliculaire ainsi que de la pression intrafolliculaire suite à l'augmentation de la perméabilité des vaisseaux sanguins (Salha *et al.*, 1998).

La reprise de la méiose chez l'ovocyte est enclenchée à la suite du pic de LH. Brièvement, suite au pic de LH, la voie de signalisation AMPc/PKA phosphoryle les jonctions Cx43 ce qui réduit les échanges entre les cellules de la granulosa. La fermeture des canaux intercellulaires mène directement à la baisse de GMPc dans l'ovocyte, ce qui permet à la phosphodiestérase 3A (PDE3A) d'hydrolyser l'AMPc dans l'ovocyte et d'activer le facteur de division (MPF) qui est responsable de la reprise de la méiose (Conti *et al.*, 2012). Lors de la division méiotique, la moitié des chromosomes sont séparés entre les cellules filles, mais la plupart du cytoplasme se dirige vers une seule cellule fille, qui est l'ovocyte secondaire. L'autre cellule qui contient la moitié des chromosomes et très peu de cytoplasme est appelée le corps polaire et sera expulsée. Cette division inégale permet de donner à l'ovocyte secondaire toute la machinerie cellulaire, l'ARN maternel et les protéines. Toutefois, l'ovocyte secondaire s'arrêtera en métaphase 2 lors de sa deuxième division méiotique. Chez la plupart des espèces, l'ovocyte sera expulsé du follicule lors de l'ovulation en arrêt méiotique.

1.9.2. Production de progestérone

Le pic de LH est aussi caractérisé par une augmentation de la sécrétion folliculaire d'androgène, plus spécifiquement la progestérone. L'augmentation d'androgène marque le changement des cellules de la thèque et des propriétés des cellules de granulosa qui ne convertira plus les androgènes en œstrogènes, mais plutôt synthétisera la progestérone. Suite

à la stimulation à la LH, les cellules de granulosa synthétisent directement de la progestérone. De plus, ces cellules perdent leurs récepteurs aux œstrogènes et à la FSH et acquièrent la capacité de lier la progestérone, ce qui active un rétrocontrôle positif sur la production de progestérone.

La progestérone est associée au maintien de la gestation, mais elle détient un rôle essentiel dans la physiologie du follicule. Son effet est reflété par sa liaison avec son récepteur à la progestérone (PGR), entre autres PGR-A et PGR-B qui sont membres de la grande famille des récepteurs nucléaires NR3C3 (Iwai *et al.*, 1990). Les PGRs sont des régulateurs importants de la période préovulatoire et ils sont essentiels pour la rupture folliculaire et la relâche de l'ovocyte (revue dans (Robker *et al.*, 2009) (voir section sur la rupture folliculaire). Les récepteurs PGRs sont induits par l'activation du récepteur à la LH couplé à la protéine G qui stimule l'adénylate cyclase (AC) et ainsi la voie de signalisation cAMP/PKA et MAPK (Salvador *et al.*, 2002). Le plus haut niveau d'expression des récepteurs PGRs est dans les cellules de la granulosa en lutéinisation, dans les cellules de la thèque et au début du corps jaune (Hild-Petito *et al.*, 1988).

Chez l'ovaire des souris KO-PGR, le follicule se développe normalement jusqu'à la période de l'ovulation où la souris démontre aucune ovulation (anovulation) et aucun follicule en rupture (Robker *et al.*, 2000a). Toutefois, le reste du follicule se développe de façon normale, les cellules du follicule entrent en lutéinisation et le follicule devient un corps jaune. De plus, les ovocytes récoltés des souris mutantes sont capables d'être fécondés et sont complètement compétents. Cette étude démontre que le récepteur PGR n'est pas essentiel pour la croissance et la lutéinisation et que le récepteur à la progestérone peut avoir un effet sans son ligand, la progestérone. Néanmoins, la liaison du récepteur PGR à la progestérone semble rester essentielle pour l'ovulation et la rupture folliculaire.

1.9.3. Expansion du cumulus

L'ovocyte dans le follicule antral est entouré d'une population de cellules somatiques, les cellules du cumulus. Cette population est issue des cellules de granulosa et sont liées les unes aux autres et avec l'ovocyte par des liaisons intercellulaires et des jonctions Gap assurant une

communication bidirectionnelle. Les cellules du cumulus répondent indirectement et différemment des cellules murales de la granulosa au pic de LH. Le pic de LH induit chez les cellules de la granulosa murales l'expression de trois gènes, l'amphiréguline (*Areg*), l'épiréguline (*Ereg*) et la betacelluline (*Btc*) qui induisent l'expression de gènes tels que l'enzyme de synthèse de l'hyaluronane de type 2 (*Has2*), la cyclooxygénase-2 (*Cox-2*), la prostaglandine-syhtétase 2 (*Ptgs2*) et la protéine 6 induite par le facteur de nécrose des tumeurs (*Tnfaip6,* «tumor necrosis factor alpha induced protein-6») essentiels pour la synthèse et la stabilisation d'une matrice unique qui enveloppe l'ovocyte et les cellules du cumulus produite par les cellules du cumulus (Hsieh et al., 2007). Ce processus est connu sous le nom de l'expansion du cumulus ou mucification, l'unité de base de cette matrice est l'acide hyaluronique (HA), un glycosaminoglycan composé d'une série de disaccarides d'acide glucuronique et de N-acétyle-glucosamine (Salustri *et al.*, 1992). L'HA est sécrété par les cellules du cumulus par l'enzyme HAS2. Le principal rôle de l'HA est de remplir l'espace intercellulaire afin d'assembler une matrice extracellulaire utile à l'ovulation et la fécondation subséquente (Russell and Salustri, 2006).

Chez le follicule préovulatoire, certaines protéines issues de la circulation sanguine ou localement synthétisées sont nécessaires pour les fonctions du cumulus, ce qui inclut la famille des inter-α trypsine inhibitrice (IαI), la protéine 6 induite par le facteur de nécrose des tumeurs (TNFAIP-6), la pentraxine 3 (PTX3) et le versican. Les facteurs IαI sont produits par les cellules hépatiques et sont présents dans la circulation sanguine et on les retrouve dans le fluide folliculaire lors de la phase préovulatoire, les IαI sont reconnus pour stabiliser la matrice du cumulus (Chen *et al.*, 1994). Tnfaip-6 et PTX3 sont des protéines produites par les cellules du cumulus lors de l'expansion du cumulus et ils sont aussi des stabilisateurs de la matrice (Salustri *et al.*, 2004; Rugg *et al.*, 2005). Tandis que le versican est majoritairement produit par les cellules de granulosa murale et est rapidement incorporé dans la matrice après le pic de LH (Russell *et al.*, 2003). Toutefois, le clivage du versican est requis par une protéase, ADAMTS1 (adisintegrin and metallopreteinase with thrombospondin-like motif type 1) pour la fonction normale du cumulus et son ovulation.

L'expression d'une enzyme limitante de la production de prostaglandine (PG), la COX-2 induit la production de prostaglandine E2 (PGE2) qui stimule la synthèse de l'AMPc dans

les cellules du cumulus et de granulosa afin d'induire l'expansion du cumulus *in vitro* (Eppig, 1981). La production de PG par l'expression de COX-2 est cruciale pour l'expansion du cumulus. D'autres facteurs tels que le facteur de croissance épidermique (EGF, epidermal growth factor) et le facteur de croissance transformateur (TGF, transforming growth factor) contribuent à la production de la matrice du cumulus (Tirone *et al.*, 1997)

En plus d'être important pour l'ovulation, la matrice extracellulaire du complexe ovocyte cumulus (COC) est essentielle à la fécondation. La matrice contient des molécules potentiellement chimiotactiques pour les spermatozoïdes (Eisenbach and Tur-Kaspa, 1999), la matrice joue aussi un rôle dans la capacitation du sperme, il agirait comme une barrière sélective contre les spermatozoïdes (Van Soom *et al.*, 2002). La matrice du COC facilite aussi le transport de l'ovocyte dans l'oviducte et permet l'adhésion entre le COC et épithélium tubaire (Lam *et al.*, 2000).

1.9.4. Rupture de la paroi folliculaire et ovulation

Lors de la fin de la phase préovulatoire, l'expansion rapide de l'antrum folliculaire par l'accumulation du fluide folliculaire entraine la formation d'une mince couche des cellules murales de granulosa, de la membrane basale et des cellules de la thèque; on appelle cette région le stigma. La position du follicule dans le cortex ovarien forme un renflement dans l'ovaire et à ce moment, le stigma devient de plus en plus mince et avascularisé due à la dégénération du tissu et au remodelage de la matrice extracellulaire au niveau de l'apex du follicule. Le remodelage de la matrice extracellulaire et la dégradation du stigma nécessitent l'implication contrôlée de certaines protéases telles que les métalloprotéases de la matrice (MMPs) responsables du contrôle de l'ovulation (Richards *et al.*, 1998). De plus, l'augmentation de progestérone intrafolliculaire stimule l'expression d'une autre métalloprotéase, ADAMTS1 et d'une cystéine protéase CTSL1 aussi cruciale pour dégrader le mur folliculaire du stigma (Robker *et al.*, 2000b).

La rupture du follicule devient inévitable due à l'augmentation de la pression intrafolliculaire et de l'amincissement de la paroi folliculaire au niveau de l'apex du follicule. Ainsi, le fluide

folliculaire s'échappe du follicule entrainant avec lui le complexe COC (l'ovocyte et sa masse de cellules du cumulus en expansion).

1.9.5. Réaction inflammatoire

L'ovulation chez les mammifères est un phénomène unique qui requiert la rupture d'un tissu sain à la surface de l'ovaire afin de libérer l'ovocyte. L'hypothèse que l'ovulation est comparable à une réaction inflammatoire fut initialement proposée par Espey en 1980. Le processus de l'ovulation est souvent comparé à une réaction inflammatoire, car elle implique l'action de protéases dans le remodelage du tissu, la présence des cellules immunitaires, de prostaglandines et d'agent du stress oxydatif (ROS). Chez le rat, on observe une augmentation du volume sanguin et un œdème du follicule dû à l'augmentation de la perméabilité et de la vascularisation du follicule qui commence quelques heures après le début de la phase préovulatoire (Tanaka *et al.*, 1989).

L'ovulation est rendue possible grâce à la sécrétion des protéases suite au pic préovulatoire et un système de contrôle d'antiprotéase (Robker *et al.*, 2000b; Bédard *et al.*, 2003). Les inhibiteurs de protéase sont essentiels afin de réduire les dommages aux tissus environnants du follicule lors de la dégradation de l'apex du follicule. L'ovulation est soutenue par la présence de cellules leucocytaires telles que macrophages, neutrophiles, lymphocytes, éosinophiles et des mastocytes (Bukulmez and Arici, 2000), qui agissent positivement sur l'ovulation en relâchant des enzymes qui digèrent la matrice extracellulaire et engendre le remodelage du follicule. Les interleukines (en autre l'interleukine 1 bêta, IL1B), qui sont des cytokines présentes lors de l'ovulation, régulent l'activité des cellules du follicule par la synthèse de protéases et de prostaglandines et sont impliquées dans la formation du corps jaune (Hurwitz *et al.*, 1993). De plus, l'HA péricellulaire et le versican sont connus pour être des inhibiteurs du dommage oxydatif chez les cellules du follicule lors de l'ovulation (Russell and Salustri, 2006)

1.10. Phase lutéale

Chez la plupart des mammifères, le corps jaune qui se forme lors de la phase lutéale a une durée de vie assez limitée, de 4 à 21 jours si la femelle n'a pas été fécondée. Sa régression (lutéolyse) permet la reprise de la phase folliculaire du cycle ovarien via la diminution de sa production de progestérone. Toutefois, s'il y a gestation, l'endomètre induira un blocage de la lutéolyse et la persistance du corps jaune cyclique en un corps jaune gestatif indispensable à l'établissement de la gestation (revue dans McCracken *et al.*, 1999).

1.10.1. Formation du corps jaune

Les parties résiduelles du follicule (granulosa et la thèque interne) se replient dans l'espace central qui fut libéré par l'ovocyte et se transforme morphologiquement et fonctionnellement en un tissu mésenchyme hautement stéroïdogénique, le corps jaune. Les cellules de la thèque forment des petites cellules lutéales tandis que les cellules de la granulosa s'hypertrophient pour former des grosses cellules lutéales, riches en mitochondries, réticulums endoplasmiques lisses, gouttelettes de lipides, appareils de Golgi, et selon l'espèce, riche en pigments (lutéine) donnant ainsi sa coloration jaunâtre. Cette transformation s'appelle la lutéinisation et elle est associée avec une augmentation de la production de progestatifs (Auletta and Flint, 1988). Le corps jaune est composé de cellules stéroïdogéniques (environ 50%) et de cellules non stéroïdogéniques, qui sont toutes les deux essentielles pour la synthèse et la sécrétion de stéroïdes sous l'influence de facteurs lutéotropes tels que l'action de la LH via son second messager l'AMPc et de la prolactine qui induit et maintient la présence des récepteurs de LH sur les cellules lutéales. La production basale de progestérone P est généralement plus grande dans les grosses cellules lutéales issues des cellules de granulosa (Carrasco *et al.*, 1996). Environ 30-40% des cellules du corps jaune sont des cellules endothéliales non stéroïdogéniques qui permettent l'élaboration d'un réseau vasculaire essentiel pour le transport des gonadotrophines et des substrats de la stéroïdogenèse (cholestérol). Le facteur de croissance de la vascularisation endothéliale (VEGF) est produit par les cellules lutéales et son inhibition prévient la production de progestérone et d'androgène, ce qui démontre son importance de sein de la formation du corps jaune (Fraser *et al.*, 2000).

1.10.2. Lutéolyse

Chez certaines espèces, la régression lutéale ou lutéolyse est causée par le retrait du complexe lutéotrophique, qui assure la survie du corps jaune. Chez d'autres espèces, c'est plutôt la production d'un facteur lutéolytique qui mène à l'élimination normale du corps jaune. Sauf chez les primates, la prostaglandine provenant de l'utérus (PGF_2-α) serait le facteur menant la régression du corps jaune.

La lutéolyse est associée avec une diminution marquée de la production de progestérone ainsi qu'une diminution marquée de la synthèse de la protéine StAR chez les cellules lutéales (Devoto *et al.*, 2009). Chez la femme la production de hCG du trophoblaste prévient la régression du corps jaune cyclique et ainsi permet la production de progestérone afin de supporter les premières semaines de grossesse en transformant le corps cyclique en corps jaune gestatif. Chez les ruminants, la transformation du corps jaune cyclique en corps jaune gestatif est assurée par l'intervention de l'embryon qui bloque l'action lutéolytique de l'utérus en inhibant la sécrétion de PGF2a et par le maintien de l'action lutéotrope des hormones hypophysaires (LH et PRL)

1.11. Concept de qualité des ovocytes

Chez les mammifères, il est bien connu que l'ovocyte dépend strictement du follicule pour sa croissance grâce à la communication bidirectionnelle tout au long de la folliculogenèse. Toutefois, l'atteinte de la taille finale de l'ovocyte ne reflète pas nécessairement sa capacité à former un embryon chez toutes les espèces. Ainsi il est important pour l'ovocyte d'acquérir une certaine maturation et sa compétence développementale lors des dernières étapes de la folliculogenèse, d'où vient le concept de la qualité de l'ovocyte. Selon la revue de littérature sur le concept de la qualité des ovocytes de Sirard et al., publiée en 2006, la compétence au développement de l'ovule se distingue par certaines caractéristiques développementales :

(1) L'habilité de l'ovocyte à reprendre sa méiose : Chez l'humain et le bovin, l'ovocyte acquiert la capacité de reprendre la méiose lorsqu'il atteint sa dimension finale, juste avant la formation de l'antrum (Fair *et al.*, 1995). Chez les mammifères, lorsque

l'ovocyte est enlevé du follicule à partir de la phase antrale, la méiose reprend de façon spontanée, car le processus d'arrêt méiotique est maintenu par le follicule grâce à la présence des jonctions communicantes (Zhang *et al.*, 2010). La reprise de la méiose suite à l'extraction de l'ovocyte du follicule est appelée la maturation spontanée (Edwards, 1965).

(2) L'habilité de l'ovocyte à se diviser suite à la fécondation : La capacité de clivage est pratiquement automatique chez l'ovocyte, ce programme est intrinsèque à l'ovocyte mature et peut même avoir lieu en absence de fécondation par un stimulus externe (Ware *et al.*, 1989). L'échec du clivage par l'ovocyte fécondé peut découler d'un spermatozoïde dysfonctionnel qui échoue l'activation de l'ovocyte ou à l'ovocyte lui-même qui n'a pas acquis la compétence de clivage.

(3) L'habilité de l'ovocyte à se développer au stade de blastocyste : La formation du blastocyste est un facteur important de la détermination de la qualité de l'ovule. À la suite de la fécondation, l'ovocyte doit former un blastocyste en 6 à 9 jours afin d'avoir une chance significative de s'implanter dans l'utérus, ce qui est fortement influencé par le statut du follicule d'où il provient. Une caractéristique importante de l'ovocyte est sa capacité à se diviser sans aucune activité transcriptionnelle jusqu'à la mise en place de son propre génome. Cette capacité unique repose sur l'accumulation et l'entreposage d'ARN essentiel pour la synthèse des différentes protéines essentielles aux premières étapes de développement de l'embryon. L'accumulation d'ARN est spécifique à l'ovocyte à partir du stade folliculaire antral. Il est intéressant de noter que la plupart des ovocytes qui ne se rendent pas au stade de blastocyste sont bloqués dans le stade ou tout près du stade de transition entre l'utilisation de l'ARN maternel à la production de l'ARN embryonnaire, au stade de transition entre le génome maternel au génome embryonnaire (MET; maternal to embryonic transition), ce qui arrive au stade de 8 à 16 cellules chez le bovin (Barnes and First, 1991) et au stade de 4 à 8 cellules chez l'humain. L'activité transcriptionnelle du nouveau génome de l'embryon est essentielle pour son développement après l'étape du MET. L'incompétence de l'embryon à transcrire son propre ARN à l'atteinte du MET serait

en partie due au manque de matériel d'activation maternelle du génome embryonnaire entreposé dans l'ovocyte (Vigneault *et al.*, 2004).

(4) L'habilité à induire une gestation et de la mettre à terme : les blastocystes ne sont pas tous capables d'induire une grossesse cependant, cet échec est parfois associé à la receveuse et non à la qualité de l'ovocyte. Néanmoins, il est connu que les blastocystes issus des ovocytes muris *in vitro* ont des taux plus faibles d'induire une grossesse que les blastocystes issus des ovocytes muris *in vivo* (Peterson and Lee, 2003), ce qui démontre l'importance de la maturation finale du follicule pour avoir un bon ovule.

1.12. Maturation de l'ovocyte

Les génomes de l'ovocyte et du spermatozoïde participent de façon égale à la création du nouveau génome embryonnaire. À la suite de la fécondation, le nouveau génome embryonnaire n'est pas tout à fait prêt pour la transcription et peut prendre plusieurs divisions avant d'être activé. Ainsi, il n'y a aucune transcription présente au début du développement du zygote et les premières divisions sont contrôlées par l'information maternelle présente dans l'ovocyte. Subséquemment, le génome embryonnaire n'a quasi aucun impact sur les premières étapes de son développement, tandis que la qualité de l'information maternelle joue un rôle majeur lors de cette période. Une autre caractéristique importante du zygote est l'origine de son cytoplasme qui est entièrement maternel, ainsi la maturation de l'ovocyte lors de la folliculogenèse est cruciale pour le bon développement de l'embryon et sa qualité. On retrouve différents niveaux de maturation de l'ovocyte; la maturation méiotique, la maturation cytoplasmique et la maturation moléculaire suite au pic des gonadotrophines préovulatoire (Sirard *et al.*, 2006).

1.12.1. Maturation nucléaire

Les ovocytes qui sont formés lors de la phase fœtale sont bloqués au stade de la vésicule germinale, en prophase I de la première division méiotique. Tout au long du développement folliculaire, les ovocytes sont en attente d'un signal qui leur permettra de reprendre leur première division méiotique. Ainsi, la maturation méiotique ou nucléaire est la cascade

d'évènements induits par le pic de LH, qui est le signal menant à la reprise de la méiose I et à l'arrêt de la seconde division méiotique au stade de métaphase II (MII) qui sera ovulé dans cette condition et sera capable d'être fécondé. Comme mentionné plus haut, le retrait de l'ovocyte de son follicule est aussi un signal qui active une pseudo-maturation nucléaire qui permet la reprise de la méiose I et son arrêt au stade de métaphase II, processus appeler la maturation spontanée (Edwards, 1965). Ce processus de pseudo-maturation suggère que la mécanique qui permet le maintien de l'arrêt méiotique est possible grâce aux communications avec les cellules du follicule (revue dans Conti *et al.*, 2012; Gilchrist 2011).

La maturation nucléaire est caractérisée par la rupture de la vésicule germinale (GVBD, germinal vesicle breakdown) qui se produit entre 4 et 8 heures post-LH (Kruip *et al.*, 1983) et qui est associée avec une diminution des activités de transcription de l'ovocyte (Fair *et al.*, 1995). Le niveau de condensation de la chromatine dans l'ovocyte est le facteur principal qui influence l'activité transcriptionnelle, qui passe d'une configuration de la chromatine diffuse à une configuration de la chromatine plus condensée et d'un nucléus défini (Tan *et al.*, 2009). La condensation de la chromatine peut être classifiée en différents stades; GV0, GV1, GV2 et GV3 (Lodde *et al.*, 2007).

Avant même que le programme de maturation nucléaire soit enclenché, la transcription est arrêtée pour plusieurs cycles cellulaires. Cette transformation démontre que la maturation moléculaire et cytoplasmique est terminée, et que l'ovocyte détient toute l'information nécessaire pour sa survie, sa fécondation et les premières étapes de son développement.

1.12.2. Maturation cytoplasmique

La maturation cytoplasmique et moléculaire doit être terminée avant la fin de la maturation méiotique et/ou du retrait de l'ovocyte du follicule. Les premiers signes de la maturation cytoplasmique sont observables quelques jours avant le pic de LH. On note la redistribution des organelles (mitochondries et granules corticaux) et la réorganisation des éléments du cytosquelette (Fair *et al.*, 1995). Au début de la maturation de l'ovocyte, les mitochondries sont retrouvées de façon homogène à travers du cytoplasme de l'ovocyte. Néanmoins lors de l'avancement de la maturation de l'ovocyte, les mitochondries se déplacent vers la région

périnucléaire, ce qui suggère que le processus de maturation nécessite plus d'énergie au niveau nucléaire (phosphorylation de protéines et synthèse de protéines) (Stojkovic *et al.*, 2001). De plus, la migration des granules corticaux vers le cortex est aussi un phénomène observé lors de la maturation du cytoplasme. Les granules corticaux sont essentiels afin de bloquer la polyspermie (Ducibella *et al.*, 1988). Finalement, l'apparition de gouttelettes lipidiques est une autre caractéristique observable lors de la maturation du cytoplasme afin d'emmagasiner des réserves d'énergie et de membranes pour les divisions embryonnaires.

1.12.3. Maturation moléculaire

Aucune transcription ne se produit entre le début de la maturation nucléaire et la transition du génome maternel au génome embryonnaire (MET) débute et que l'ARNm maternel est remplacé par l'ARNm embryonnaire (Barnes and First, 1991). Ainsi, l'ARNm et les protéines essentielles pour la maturation nucléaire, la fécondation et les premiers stades de développement de l'embryon doivent être emmagasinés dans l'ovocyte. Il n'est pas surprenant que la qualité de l'ARN maternel reflète directement la capacité développementale de l'embryon afin de réussir les premières étapes de son développement et d'activation son génome nouvellement créé.

Chez le bovin, la synthèse d'ARN dans l'ovocyte se produit durant l'ovogenèse, plus précisément au stade folliculaire préantral. Toutefois, une diminution graduelle et importante de l'activité transcriptionnelle sera observée chez les ovocytes au stade folliculaire antral lorsque l'ovocyte atteint sa taille maximale (Fair *et al.*, 1995, 1996). Malgré la diminution de la transcription, il est quand même possible de détecter la synthèse de novo entre la fin de sa croissance et l'arrêt complet de la transcription au stade GVBD (Hunter and Moor, 1987).

L'ovocyte doit entreposer des ARNm maternels tout au long de l'ovogenèse qui devra rester stable pour une période de plusieurs jours, jusqu'à l'activation du génome embryonnaire. Les ARNm accumulés ont une demi-vie de 12 jours chez la souris (Bachvarova and De Leon, 1980) et seront utilisés graduellement jusqu'au stade du MET. Dans le but de rester stable, les ARNm maternels vont subir une étape de déadénylation, qui raccourcie la queue poly-A à l'extrémité 3' de l'ARN pour permettre leur stabilisation et leur activation, ce qui sera

accompagné d'un rallongement de la queue poly-A. Ce processus de déadénylation et d'entreposage de l'ARNm dans l'ovocyte implique l'action de plusieurs éléments de régulation présents dans la région 3' non-transcrite (3'-UTR) de l'ARNm (Radford *et al.*, 2008; Chen *et al.*, 2011).

L'ovocyte commence à utiliser l'ARNm emmagasiné lors des phases précédentes dès son entrée en GVBD, ce qui enclenche son horloge biologique. Dû à sa quantité limitée d'ARNm de la réserve maternel, l'ovocyte a une durée de vie limitée puisque ses ARNm essentiels seront épuisés ou dégradés et insuffisant pour soutenir la fécondation et les premières divisions embryonnaires. Selon l'auteur qui propose le concept de la date d'expiration (Sirard, 2011). L'ovocyte est dépendent de ses ARNm pour le renouvèlement de ses protéines essentielles, et selon Sirard 2011 trois hypothèses sont proposées expliquant le principe d'expiration de l'ovocyte : (1) l'ARN requis pour la maintenance de l'ovocyte diminue et donc la survie de l'ovocyte (2) l'ARN en réserve pour les besoins de l'ovocyte ne sont pas maintenus, ainsi l'ovocyte peut entamé les premières étapes de la fécondation et du clivage, mais pas plus et (3) l'ARN spécifique de compétence est dégradé au fil du temps ce qui empêche les étapes embryonnaires, par exemple : l'activation du génome embryonnaire.

1.13. Techniques de reproduction assistée

1.13.1. Production animale et élevage sélectif

Par définition, la production animale est l'ensemble des techniques qui sont relatives à l'élevage des animaux et dont sont issus des produits propres à la consommation. L'élevage sélectif est au cœur de la production animale et elle permet aux éleveurs de sélectionner les meilleurs traits génétiques qui permettent d'augmenter leurs capacités de production de génération en génération. L'arrivée des techniques d'analyse génétique a ouvert une nouvelle ère à la production animale, l'agriculture moderne. Grâce au progrès génétique, il est possible d'augmenter la valeur génétique des animaux d'un troupeau afin d'être plus performant, plus efficace, plus rentable, et tout ça plus rapidement.

Avant l'arrivée des techniques de reproduction assistée, l'obtention de la descendance d'une vache laitière pouvait prendre jusqu'à 65 mois. Ce long intervalle est principalement dû au long cycle reproducteur femelle; pour mesurer le potentiel de production d'une vache laitière, celle-ci doit d'abord atteindre sa puberté (12 mois en moyenne), être inséminée et gestante (9 mois) et compléter un cycle complet de lactation (10 mois). De plus, la génisse à laquelle elle aura donné naissance devra être testée afin que les données des descendants puissent être attribuées aux géniteurs (Schaeffer, 2006; Jonas and de Koning, 2015). Avec l'arrivée de la génomique, les critères de production et de reproduction ont été associés avec des marqueurs génétiques grâce à une série d'études pangénomique (GWAS, genome-wide association studies). Ainsi, il est possible d'obtenir une prédiction génomique sur les performances et le potentiel reproducteur de l'animal avant même sa naissance.

Même avec la sélection génomique, l'industrie a développé de nouvelles stratégies afin d'augmenter le gain génétique et la production d'animaux de haute valeur génétique : (1) La manipulation hormonale utilisée en concert avec le transfert d'embryons permet à une seule vache d'augmenter la quantité de descendances et leurs génétiques en modulant l'origine de la semence. Ensuite, les embryons ayant les marqueurs génomiques favorables seront transférés dans des vaches porteuses de moindre valeur génétique, ce qui permet l'augmentation de la production d'une descendance de haut niveau. (2) L'utilisation de jeunes donneuses d'ovocytes dans un protocole de transfert d'embryons est aussi une technique qui permet de réduire davantage le temps entre les générations. Cette technique d'utilisation de génisse permet l'utilisation de gamètes et le début d'une gestation chez une vache porteuse avant même que la génisse elle-même ait atteint sa puberté.

1.13.2. Manipulation hormonale et transfert d'embryons

Comme il sera présenté dans les prochaines sections, la qualité de l'ovocyte est associée au degré de différenciation du follicule duquel il provient, ce qui est un facteur clé du succès en reproduction assistée. De plus, la manipulation de la reproduction femelle est une question de synchronisation, les ovaires doivent être stimulés dans au bon moment, juste après le début d'une nouvelle vague folliculaire et la récolte des ovocytes doit se faire dans un follicule muri pour une période de temps spécifique, ce qu'on appelle le coasting. Ainsi une panoplie de

facteurs intervient dans le contrôle, la croissance et la maturation des gamètes femelles afin de produire un ovocyte de qualité, synonyme de succès en reproduction assistée.

1.13.3. Stimulation ovarienne chez la vache

Les techniques de reproduction assistées se sont aussi développées afin de traiter l'infertilité chez les animaux d'élevages de haute valeur génétique, et dans les programmes de gain génétique. Dans l'industrie laitière canadienne, les problèmes de reproduction sont la cause majeure et représentent environ 15% des vaches réformées (*Gouvernement Canadien, 2012, Industrie laitière canadienne en chiffre)*. De plus, les vaches ayant une haute valeur génétique sont en forte demande et représentent un marché lucratif pour la reproduction assistée bovine.

Les vaches subissent des protocoles d'hyperstimulation ovarienne pour ensuite être inséminées ou encore les ovocytes sont récoltés par des techniques telles la ponction folliculaire transvaginale par ultrasonographie (Bousquet *et al.*, 1999) qui a remplacé la laparoscopie (Sirard and Lambert, 1986) et l'aspiration chirurgicale (Brackett *et al.*, 1982). Les ovocytes récoltés par ponction folliculaire (OPU, Ovum pick-up) sont maturés, fécondés et développés in vitro jusqu'aux stades de blastocyste pour être implantés dans des femelles porteuses de moindre valeur génétique, ce qu'on appelle le transfert d'embryons (Phillips and Jahnke, 2016) ou simplement congelés pour être transférés ultérieurement. Ainsi, la même vache peut donner un nombre remarquablement élevé d'embryons dans une même année.

Le protocole général de stimulation hormonale chez les vaches utilise deux injections de FSH exogène par jour pour une durée de trois jours, étant donnée la demi-vie relativement courte de la FSH exogène (Ben-Menahem, 2018). Le synchronisation de l'initiation de la stimulation ovarienne (SO) est primordial dans le cycle ovarien de la vache, le traitement doit commencer juste après l'émergence de la vague folliculaire; simplement une journée avant ou après diminue significativement la réponse de l'ovaire (Nasser *et al.*, 1993). Étant donné que certaines vaches ont deux ou trois vagues de croissance folliculaire dans un cycle ovarien, il est très difficile de contrôler le temps optimal de l'initiation de la SO chez un groupe de vaches. Il est possible de contrôler le cycle ovarien et d'induire une vague de

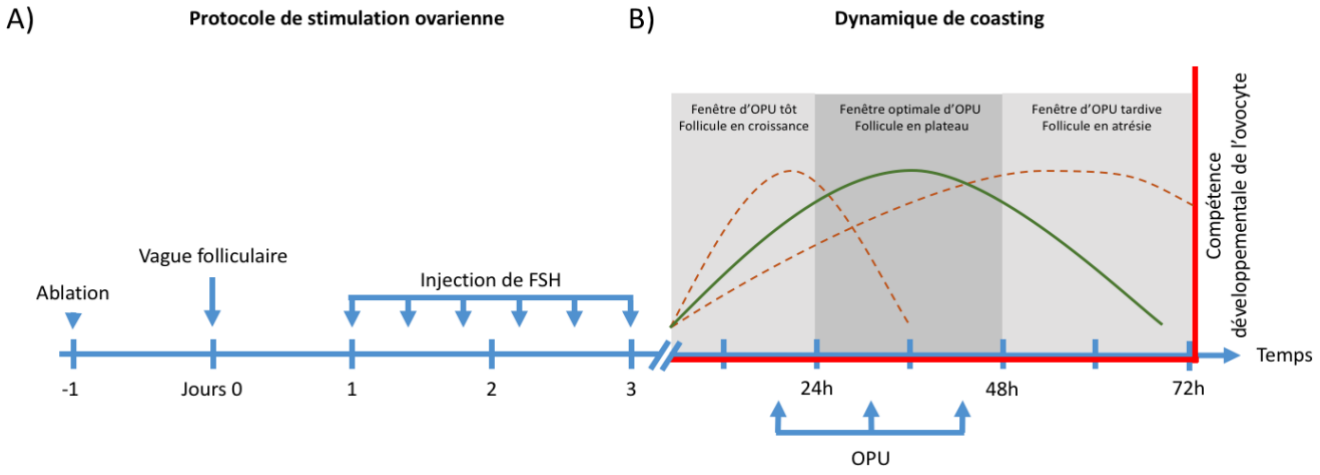
croissance folliculaire chez la vache, ce qui simplifie le processus. L'habilité d'induire le début d'une vague folliculaire permet d'initier des traitements de SO à un troupeau sans prendre note de leurs cycles œstraux (Mapletoft *et al.*, 2002). Le protocole le plus utilisé en Amérique du Sud pour synchroniser l'émergence des vagues folliculaires chez les vaches est l'utilisation d'œstradiol et de progestérone 4 jours avant le traitement aux gonadotrophines (Bó *et al.*, 1995). Le traitement à l'œstradiol supprime le relâche de FSH et induit l'atrésie folliculaire, lorsque l'œstradiol est métabolisé, la FSH augmente et une nouvelle vague folliculaire émerge avec une nouvelle cohorte uniforme de follicules entre 3 et 5mm. En Amérique du Nord et en Europe, l'utilisation d'hormones est interdite pour la synchronisation des vagues folliculaires. Au Canada, l'utilisation de la ponction transvaginale par ultrason des follicules dominants est utilisée pour synchroniser les animaux. En fait, l'ablation des follicules dominants permettra de retirer leurs effets négatifs sur les autres follicules en croissance et de permettre une nouvelle montée de FSH et ainsi une nouvelle vague folliculaire (Berfelt *et al.*, 1994). De façon générale, le protocole de SO peut débuter 4 jours après l'initiation de la synchronisation des vagues folliculaires par l'œstradiol et la progestérone et 2 jours après l'ablation des follicules dominants (Bó and Mapletoft, 2014).

Tout au long du protocole de synchronisation et de stimulation ovarienne, la vache restera sous l'effet d'un implant de progestérone qui permet la libération de progestérone empêchant ainsi l'ovulation des follicules. De ce fait, les follicules seront récoltés directement dans l'ovaire après une période de temps sans aucune stimulation (appelé le coasting) et les ovocytes seront transférés au laboratoire pour la maturation *in vitro*, la fécondation *in vitro* et seront mis en culture *in vitro* pour 6 à 7 jours jusqu'à ce que l'embryon soit au stade de blastocyste.

1.13.4. Coasting

En 1996, Blondin et al., ont découvert qu'une période sans stimulation aux gonadotrophines entre la stimulation ovarienne et la ponction des ovaires était favorable pour l'augmentation de la compétence de l'ovocyte comparativement aux ovocytes toujours en croissance avec 11 % vs 32 % de taux de blastocystes. Ainsi le contexte folliculaire est crucial pour la maturation de l'ovocyte et que la croissance rapide des follicules sous l'influence de la FSH

induite par la SO a d'abord des effets négatifs qui retarde l'acquisition de la compétence au développement de l'ovocyte (Blondin *et al.*, 1996). Il fut proposé que la stimulation ovarienne force le follicule à se développer rapidement et ne laisse aucun temps à l'ovocyte d'acquérir sa compétence. Ainsi, le follicule nécessite une phase sans stimulation à la FSH avant la ponction des ovocytes et cette période de temps est appelée le « coasting » (figure 1-4). Plusieurs études ont par la suite amélioré le protocole (Blondin *et al.*, 1997a, b; Sirard *et al.*, 1999) tandis que Blondin et al. en 2002 ont déterminé que le temps optimal de coasting était de 48h comparativement à 33 h après la dernière injection de FSH avant la ponction des ovocytes, ce qui a donné un taux 80 % de blastocystes (Blondin *et al.*, 2002). Une autre étude



plus récente de la même équipe de recherche a démontré que le temps optimal de coasting de l'ovocyte est entre 44 h et 68 h avec le meilleur résultat de blastocyste à un temps de 54 +/- 7 h (Nivet *et al.*, 2012).

Figure 1-4: Protocole de stimulation et dynamique du coasting chez la vache

A) Profils du protocole de stimulation ovarienne générale chez les vaches laitières, ablation (retrait des follicules supérieurs à 5 mm) et six injections de FSH exogène. B) Compétence développementale générale de l'ovocyte (ligne verte) en fonction du temps de la récolte des ovocytes après la dernière injection de FSH. Les lignes pointillées sont les variances possibles des différents animaux. OPU signifie les différentes périodes de récoltes des ovocytes (Landry et al., 2018).

La période de coasting entraine la différentiation d'un plus grand nombre de follicules qui détiennent des signes similaires à un follicule en début d'atrésie. Ces conditions folliculaires

similaires à un cycle naturel miment très bien les mêmes conditions de LH basal retrouvées lors de la période avant l'ovulation. Les études supportent fortement que l'ovocyte acquière sa compétence lors de cette période sans gonadotrophine après le pic de FSH et avant celui de LH. Il est important de mentionner que la réduction de FSH, 4 à 5 jours avant l'ovulation est physiologique chez les vaches en cycle normal (Cooke *et al.*, 1997; Stricker *et al.*, 2006).

1.13.5. Stimulation ovarienne chez la génisse

L'autre stratégie utilisée de concert avec la sélection génomique consiste à faire entrer des génisses prépubères dans un protocole de SO suivie par un prélèvement d'ovocytes (OPU) par aspiration transvaginale. Cette technique permet en autre de diminuer le temps entre les générations et de produire une descendance de haute valeur génétique plus rapidement. La stimulation ovarienne, le coasting et le transfert d'embryon peuvent être accomplis sans aucun effet néfaste chez les donneuses prépubères (Evans *et al.*, 1994; Presicce *et al.*, 1997; Khatir *et al.*, 1998). En fait, présentement, les mêmes techniques de stimulation ovarienne, de coasting ainsi que de culture des ovocytes et des embryons utilisées chez les vaches adultes sont aussi utilisées chez les jeunes vaches (Boviteq). Toutefois, le pourcentage d'embryons qui se développe à partir d'ovocytes de génisses est significativement plus faible que les ovocytes qui proviennent des vaches adultes (Looney *et al.*, 1995; Revel *et al.*, 1995; Kauffold *et al.*, 2005). En 2005, une étude a démontré que les génisses de plus de 10 mois d'âge avaient un taux de blastocystes comparable aux vaches adultes (14 à 18 mois d'âge) tandis que les donneuses de moins de 10 mois (6 à 9 mois) produisent moins d'embryons transférables et plus d'ovocytes dégénérés. Toutefois, l'étude n'a pas utilisé la technique de coasting, mais simplement une stimulation ovarienne suivie d'une insémination artificielle et d'un lavage de l'utérus (Ax *et al.*, 2005). Cette diminution d'embryons viables est néfaste tant en termes de perte de progrès génétiques potentiels pour la race, mais aussi en termes de pertes de profit pour les compagnies de génétiques bovines.

1.14. Évaluation de la compétence des ovocytes

Les cliniques de fertilité ainsi que l'industrie utilisent des méthodes d'évaluation de la compétence de l'ovocyte non invasive n'affectant pas l'ovocyte. Les observations

développementales vues dans la section du concept de qualité de l'ovocyte sont entre autres des techniques d'évaluation de la compétence, ce qui comprend le clivage des cellules, le développement jusqu'au stade de blastocyste en 5-9 jours et le développement à terme de l'embryon ainsi que sa santé. Toutefois, les embryologistes doivent choisir l'embryon qui semble avoir la meilleure compétence en fonction des critères développementaux pour le transfert et l'implantation.

1.14.1. Méthodes morphologiques

Les critères de sélection d'ovocytes compétents les plus utilisés dans l'industrie sont basés sur les différents aspects morphologiques du complexe ovocyte-cumulus une fois retiré du follicule. L'évaluation de la structure du cumulus, du nombre de couches et de son organisation et l'évaluation du cytoplasme de l'ovocyte furent proposée comme un système de classification efficace (Blondin and Sirard, 1995). Les COCs sont classifiés selon un système à six différentes classes en continu. Les classes 1 et 2 représentent les COCs ayant un cytoplasme homogène entouré de plusieurs couches de cellules du cumulus compactes. Les classes 3 à 6 sont la portion du spectre où les COCs ont un cytoplasme de plus en plus granuleux avec un cumulus incomplet ou en expansion progressive, la classe 6 étant une expansion de 100%. Les ovocytes de la classe 3, c'est-à-dire ceux qui présente un début d'expansion dans les cellules du cumulus et du début de granulation dans le cytoplasme de l'ovocyte sont ceux qui réussissent le mieux à se développer jusqu'au stade 16 cellules par rapport aux autres classes (Blondin and Sirard, 1995). Ces travaux ont permis de démontrer que le début de l'atrésie, observé dans la classe 3, a un effet bénéfique sur la compétence de l'ovocyte.

1.14.2. Méthodes moléculaires

Au cours des dernières années, les scientifiques ont accordé beaucoup d'importance à la recherche de critères moléculaires fiables pour définir le niveau de qualité de l'ovocyte et permettre la sélection du ou des meilleurs ovocytes afin d'augmenter le taux de succès en reproduction assistée.

L'acquisition de la compétence au développement de l'ovocyte est associée avec le développement du follicule. Il est connu que l'ovocyte communique avec les cellules

environnantes, en autre les cellules du cumulus et les cellules de granulosa et que la destinée de l'ovocyte repose sur l'intégrité des signaux du follicule et de sa dynamique de développement. Il fut démontré que les ovocytes récupérés cinq jours après l'émergence d'une vague folliculaire, durant la phase de plateau (phase entre le pic de FSH et de LH) se développent significativement plus jusqu'au stade de blastocyste comparativement aux ovocytes récupérés durant la phase de croissance (phase sous l'effet de la FSH, deux jours après l'émergence d'une vague folliculaire) et aux ovocytes récupérés en phase d'atrésie avancée (phase où les follicules régressent dû à leurs persistances dans l'ovaire, sept jours après l'émergence d'une vague folliculaire (Vassena *et al.*, 2003). De plus, la phase de plateau correspond au moment où les follicules sont soumis aux premiers signes d'atrésie.

Grâce aux techniques moléculaires, il devient possible d'utiliser les cellules folliculaires telles que les cellules de la granulosa afin de caractériser le statut du follicule d'origine des ovocytes et de prédire la compétence des ovocytes. Plusieurs études récentes suggèrent que le niveau de transcrit de certains gènes candidats (biomarqueurs) chez les cellules de la granulosa sont associés à la compétence développementale et le taux de succès en ART (Hamel *et al.*, 2010; Jiang *et al.*, 2010; Nivet *et al.*, 2013, 2016; Uyar *et al.*, 2013; Kordus and LaVoie, 2017).

1.14.2.1. Analyse des cellules de la granulosa

Il est bien connu que les cellules de granulosa communiquent de façon bidirectionnelle avec l'ovocyte lors de la folliculogenèse. Les cellules de granulosa jouent un rôle essentiel dans la différenciation du follicule menant aux conditions optimales pour la croissance de l'ovocyte, la maturation de l'ovocyte et l'ovulation (D'Aurora *et al.*, 2016). La qualité des ovocytes peut être reliée à l'étape finale de maturation du follicule menant ainsi à un ovocyte de première qualité permettant une gestation saine. Dès le début des années 2000, une étude de Robert *et al.*, 2001 a démontré la présence de gènes qui seraient associés avec la compétence au développement de l'ovocyte, ce qui supporte l'idée que le transcriptome des cellules de granulosa pourrait prédire la qualité de l'ovocyte et du succès en ART. Les cellules de granulosa sont habituellement jetées lors de la récolte des ovocytes, ainsi l'étude de l'expression des gènes associés à la compétence au développement de l'ovocyte dans les

cellules de granulosa pourrait potentiellement devenir une méthode non invasive de routine pour déterminer la qualité folliculaire.

Une étude préliminaire de Nivet *et al.*, 2013 a démontré le rôle de la différenciation folliculaire en association avec la compétence des ovocytes dans un contexte de coasting. Tout d'abord, il fut établi que le meilleur temps de coasting chez la vache sexuellement mature se retrouve entre 44 h et 68 h qui est la période de coasting optimale. Le temps de coasting de 20 h est associé avec une différentiation folliculaire tôt (Early differentiation) et le temps de coasting de 92 h est associé avec une différentiation folliculaire tardive (Late differentiation), ce qui représente les fenêtres maximales de récolte des ovocytes (voir figure 1-5). L'expérience de type micropuce de Nivet et al (2012) a permis l'analyse du transcriptome des cellules de granulosa des follicules des différents stades de compétence associés au coasting ; tôt, optimal et tard. Pour ce faire, la technologie des micropuces fut utilisée afin de comparer l'expression des gènes entre différents contrastes pour les temps de coasting, entre 20, 44, 68 et 92 h. Cette analyse a permis de découvrir les différentes fonctions cellulaires et les voies de signalisation associées avec le statut des follicules d'origine des ovocytes compétents et moins compétents, figure 1-5.

Deux autres études similaires ont aussi permis d'identifier les fonctions biologiques et les voies de signalisation associer avec le statut des follicules de tailles différentes dans les stades de croissance, de plateau et d'atrésie (Douville and Sirard, 2014; Girard *et al.*, 2015b). Dans les deux cas, les études ont découvert plusieurs gènes candidats spécifiques au différents stades du follicule afin de distinguer la dynamique des follicules d'origines.

Les différentes études qui portent sur les biomarqueurs des cellules de granulosa démontrent que les cellules de granulosa obtenues lors de l'aspiration transvaginale des ovocytes ont le potentiel de donner l'information sur la qualité du follicule et de l'ovocyte. La récolte des cellules de granulosa à la suite de l'aspiration, au lieu de les jeter, permettrait de diagnostiquer la qualité de la stimulation ovarienne et le statut du follicule aspiré en lien avec la compétence au développement de l'ovocyte.

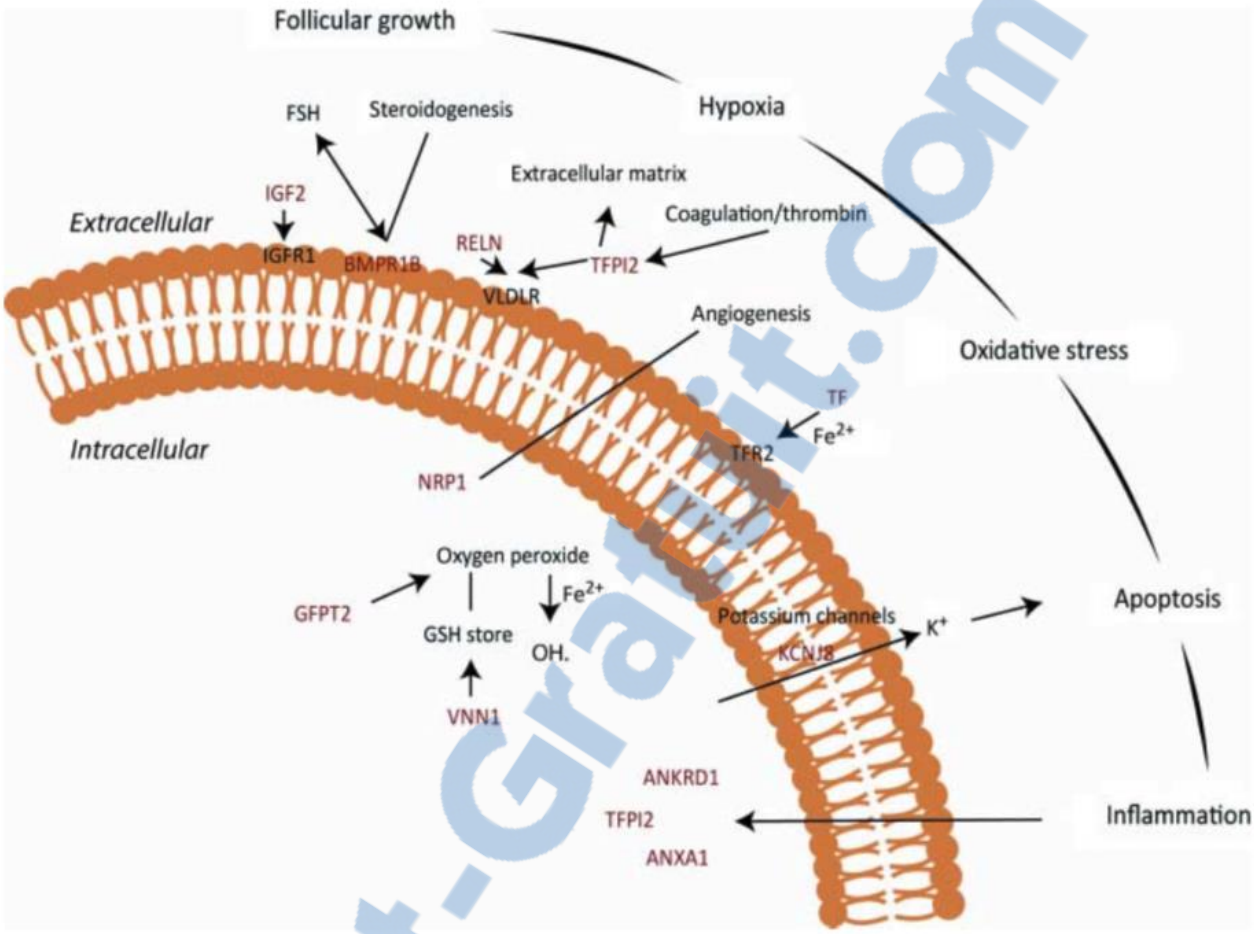


Figure 1-5: Expression des gènes dans la cellule de granulosa lors du coasting

Rôle fonctionnel des marqueurs de coasting entre 20, 44, 68 et 92 h. En rouge sont les marqueurs découverts dans l'étude Nivet et al., 2013, en noir les molécules et les fonctions biologiques. Figure reproduite avec permission © 2013, Bioscientifica.

1.15. Hypothèse et objectifs

Au cours des dernières années, le développement des techniques de reproduction assistée a eu un impact significatif sur nos pratiques et nos stratégies en reproduction. L'industrie a bénéficié d'une multitude d'améliorations des techniques par une meilleure compréhension de la physiologie reproductive des animaux. Nos habilitées à s'interroger sur l'expression des gènes exprimés grâce à la transcriptomique, nous mènent vers de nouvelles perspectives dans le développement et l'amélioration des techniques de la reproduction animale. Cette nouvelle information peut maintenant servir à mieux diagnostiquer les causes des mauvaises réponses ovariennes chez les différentes donneuses. Dans l'industrie bovine, près d'un animal sur trois présente une réponse sous optimale en termes de qualités ou de quantités d'ovules et il est important de comprendre la cause afin de mieux adapter la stimulation ovarienne suivante (Hall *et al.*, 2013). Notre *hypothèse de recherche* stipule qu'une signature moléculaire des cellules de la granulosa est associée avec la compétence au développement optimal de l'ovocyte. De plus, l'analyse de l'expression des gènes chez les cellules de la granulosa permettrait d'identifier le statut de différentiation des follicules de mauvaises donneuses dans le but d'optimiser le prochain protocole ovarien.

L'objectif général de cette thèse était de créer un profil du parfait follicule et les outils nécessaires afin de générer un plan de stimulation ovarienne pour chaque animal. Pour ce faire, nous avons intégré l'information du transcriptome des cellules de granulosa issue de stimulation ovarienne suivie du coasting chez des vaches et génisses commerciales dans le but d'associer la signature transcriptomique des cellules du follicule avec la compétence au développement de l'ovocyte. De plus, nous avons utilisé la bio-informatique afin de faire des méta-analyses dans le but de caractériser l'acquisition de la compétence au développement des ovocytes au niveau des cellules de granulosa chez la vache laitière.

Le premier objectif du projet était de déterminer si l'âge des donneuses d'ovocytes avait un effet sur le potentiel de développement des ovocytes à la suite d'une stimulation ovarienne et du coasting. Après chaque stimulation ovarienne, l'information sur chaque animal est enregistrée, on retrouve le nombre de follicules récoltés, leurs tailles, le nombre d'ovocytes mis en maturation, le nombre d'embryons clivé, le nombre de morulas et finalement le

nombre d'ovocytes en maturation qui se sont développés en blastocyste (le nombre d'embryons viable). Ainsi, nous avons comparé toute l'information disponible pour chaque groupe d'âge afin d'identifier à partir de quel âge les animaux répondre moins bien au traitement ovarien.

Le second objectif qui était de comparer l'expression des gènes chez les mauvaises et les bonnes donneuses d'ovocyte, en deux modèles de comparaison : (1) Les mauvaises donneuses d'ovocytes sexuellement mature en contraste avec les bonnes donneuses sexuellement mature, et (2) les mauvaises jeunes donneuses d'ovocyte (moins de 11 mois d'âge) en contraste avec les bonnes donneuses sexuellement mature. Pour ce faire, nous avons récolté des cellules de la granulosa de plus de 500 animaux ayant reçu une SO suivie de coasting entre les âges de 5 mois jusqu'à 9 ans. Le transcriptome des cellules de granulosa fut comparé à l'aide des micropuces dans le but d'identifier des marqueurs d'incompétence et de compétence associés avec les différents statuts de différentiation du follicule. De plus, des méta-analyses à partir des micropuces de cette thèse et de plusieurs autres études ont été réalisées afin d'identifier des biomarqueurs ensuite validés en RT-qPCR. Plusieurs analyses statistiques ont permis d'identifier les groupes de follicules d'origine des ovocytes incompétents en différents groupes.

Le troisième objectif était de caractériser l'expression des gènes dans les cellules de la granulosa des génisses pré-, péri- et postpubères dans le but d'identifier les différences physiologiques associées à la compétence développementale des ovocytes en fonction de l'âge de la donneuse d'ovocytes. Pour ce faire, dix génisses prépubères furent utilisées selon trois différentes stimulations ovariennes à trois différents âges de 8, 11 et 14 mois. Ainsi, chaque génisse fut le contrôle d'elle-même étant donné que chaque ovocyte a été fécondé avec la semence du même taureau. Le transcriptome de chaque groupe d'âge a ensuite été comparé suivant la même approche méthodologique.

2. Effect of cow age on the in vitro developmental competence of oocytes obtained following FSH stimulation/coasting treatments.

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Cet article a été publié dans la revue « Theriogenology » sous la référence suivante :
Landry D.A., Bellefleur A.M., Labrecque R., Grand F.X., Vigneault C., Blondin P. and Sirard M.A. (2016). Effect of cow age on the in vitro developmental competence of oocytes obtained after FSH stimulation and coasting treatments. Theriogenology, 86(5), 1240-1246.

2.1.Résumé

L'utilisation d'ovocytes de jeunes donneuses dans un programme de fécondation *in vitro* et de transfert d'embryons représente une opportunité unique d'accélérer le gain génétique des vaches laitières en réduisant le temps entre les générations. Dans cette étude, nous avons enquêté sur la relation entre l'âge de la donneuse et la compétence au développement des ovocytes chez la femelle Holstein âgée de 5 à 18 mois suite à une stimulation ovarienne suivi du coasting. Les résultats démontrent un nombre significativement plus élevé de petits follicules (5-6 mm) chez les donneuses de 5 à 10 mois et un plus grand nombre de follicules moyen (7-10 mm) chez les donneuses âgées de 6 à 7 mois. Nos analyses statistiques démontrent aussi que le nombre total de follicules est significativement ($p < 0.05$) plus élevé chez les donneuses âgées de 5 à 8 mois et tend à l'être ($p=0.053$) chez les donneuses de 9 mois d'âge comparativement aux vaches sexuellement matures. Néanmoins, les ovocytes qui sont récoltés chez les donneuses âgées de 5 à 10 mois produisent significativement moins d'embryons qui se rendent au stage de morulas et de blastocyste comparativement aux vaches sexuellement matures. En résumé, nos résultats démontrent qu'un plus grand nombre d'ovocytes peut être obtenu à partir de jeunes donneuses, mais leur faible compétence développementale réduit ce gain.

2.2. Abstract

The use of oocytes obtained from younger donors for *in vitro* fertilisation (IVF) followed by embryo transfer (ET) represents an opportunity to accelerate genetic gain by reducing generation time. In this study, we investigated the relationship between donor age and the in vitro developmental competence of oocytes obtained from Holstein females (aged 5–18 months) following FSH stimulation and coasting. The follicle size patterns showed a significantly higher total number of small follicles (5–6 mm) from donors aged 5–10 months and a higher total number of medium-sized follicles (7–10 mm) in donors aged 6–7 months compared to sexually mature cows. Our analysis also revealed that the total number of follicles was significantly higher ($p < 0.05$) in donors aged 5–8 months and tended to be higher ($p = 0.053$) in 9-month-old donors. However, oocytes obtained from donors aged 5–10 months yielded fewer embryos reaching the morula and blastocyst stages. In summary, our results demonstrate that a higher number of oocytes can be obtained from younger animals, but lower developmental competence negates this gain.

2.3. Introduction

Over the past decade, the development of *in vitro* production of embryos from young donors has become a valuable tool for enhancing genetic gain in dairy cattle. Combined with genomic improvement of bulls, the progressive use of oocyte donor genomic analysis at an early age has changed the approach to improving the next generation of dairy cows. The use of peripubertal donors for in vitro fertilization and embryo transfer (IVF-ET) represents a significant opportunity to accelerate gains in genetic merit and to compress the time interval between generations. It is well established that peripubertal cattle can provide oocytes that yield healthy embryos *in vitro* and *in vivo* (Armstrong *et al.*, 1992; Majerus *et al.*, 1999; Palma *et al.*, 2001).

Ovarian stimulation, embryo transfer and pregnancy can be achieved without detrimental physiological effects on the prepubertal donor (Evans *et al.*, 1994; Presicce *et al.*, 1997; Khatir *et al.*, 1998). Oocytes can be collected by repeated ovum pick-up (OPU) on calves without affecting subsequent reproductive or lactation performances (Majerus *et al.*, 1999; Ax *et al.*, 2005). Normal oocyte development up to the transferable blastocyst stage and beyond can be achieved in peripubertal animals using protocols already established for adult oocytes by combining ovarian stimulation (OS), OPU, *in vitro* maturation (IVM), fertilization or culture (IVC) and embryo transfer (Evans *et al.*, 1994; Presicce *et al.*, 1997; Khatir *et al.*, 1998) .

Many studies show that bovine embryos can be produced successfully *in vitro* using oocytes from sexually immature calves as young as 2–4 months old (Majerus *et al.*, 1999; Palma *et al.*, 2001; Kauffold *et al.*, 2005). A number of studies have investigated developmental differences between embryos obtained from calf and adult oocytes with or without ovarian stimulation. No significant differences in percent maturation, fertilization and early cleavage after IVF have been shown, but reduced blastocyst yield and significantly greater embryo loss after ET have been associated with younger donors (Looney *et al.*, 1981; Revel *et al.*, 1995; Presicce *et al.*, 1997; Khatir *et al.*, 1998; Kauffold *et al.*, 2005). Ax et al showed that 10-month-old animals produced oocytes with similar developmental competence and provided transferable embryos in yields comparable to post-pubertal cyclic animals (16–18

months old) while younger animals (6-9 months old) produced fewer transferable and total embryos (Ax *et al.*, 2005). However, embryos originating from younger donors and developing to day 7 were of similar quality grade and had gene transcription levels similar to those of sexually mature donors (Torres *et al.*, 2015). Studies of *Bos indicus* have also shown a higher number of embryos from 10-month-old animals in comparison with younger donors with or without ovarian stimulation (Camargo *et al.*, 2005; Bernal U. *et al.*, 2011).

Although oocytes from young donors have lower ability to produce good quality blastocysts, superovulation in calves has been shown to increase oocyte developmental competence almost to the same level as in mature animals (Majerus *et al.*, 1999). Different studies comparing oocyte competence in non-stimulated calves versus hormonally stimulated calves suggest that stimulation increases the percentage of usable oocytes (cumulus oocyte complex or COC), the cleavage rate, and the percentage of embryos that reach the morula and blastocyst stages (Armstrong *et al.*, 1994; Revel *et al.*, 1995; Presicce *et al.*, 1997).

In cattle, ovarian stimulation followed by a gonadotropin-free resting period called "coasting" has been shown to constitute an efficient regimen for generating oocytes of higher quality and for increasing the yield of blastocysts. The use of coasting in ovarian stimulation protocols has produced blastocyst yields as high as 80% in mature cows (Blondin *et al.*, 2002; Nivet *et al.*, 2012). The coasting period creates a progressive follicular hypoxia associated with an increase in follicular apoptosis and inflammation (Nivet *et al.*, 2013). This early follicular atresia mimics several pre-ovulatory changes in the dominant follicle and is associated with improved oocyte competence (Sirard, 2011).

The objective of this present study is to determine whether or not donor age affects the developmental potential of oocytes obtained from calves and heifers following a commercial protocol of ovarian stimulation and coasting.

2.4.Material and methods

2.4.1. Experimental design

This retrospective study was performed on data gathered for 1031 *Bos taurus* calves or heifers (Holstein breed) aged 5–18 months. All ovarian stimulations, *in vitro* fertilizations, and *in vitro* culture were performed from January 2012 to July 2015 in a controlled commercial environment. The number of follicles, the follicle size and oocyte developmental competence after IVF were investigated in order to identify any link between donor age and oocyte quality. The animals were grouped according to nominal age: 5 months (n = =22); 6 months (n = 57); 7 months (n = 102); 8 months (n = 131); 9 months (n = 189); 10 months (n = 190); 11 months (n = 131); 12 months (n = 103). Each age group contained animals aged up to one day less than the next age group (1 month = 30 days). The control (sexually mature) group contained animals aged 16–18 months (n = 106).

2.4.2. Chemicals

All reagents and media supplements used in these experiments were obtained from Sigma-Aldrich Co. unless otherwise indicated.

2.4.3. Ovarian stimulation treatment and oocyte recovery

The dominant follicle was aspirated 36–48 h before hormone administration. Animals were stimulated for 3 days with FSH. According to the body condition score (BCS), FSH was administered in 5 x 30 mg, 6 x 30 mg or 6 x 40 mg doses of NIH Folltropin-V (Bioniche Animal Health, Belleville, ON, Canada), followed by a coasting (no FSH) period of 19, 30 or 43 h. Using trans-vaginal ultrasonography, follicle diameters were measured and COCs were collected by trans-vaginal puncture under epidural anesthesia, using an 18G needle and COOK aspiration unit (COOK Medical, Bloomington, IN, USA). COCs and granulosa cells were collected in warm HEPES-buffered Tyrode's medium (TLH) containing heparin (10 IU/ml) and transferred to the laboratory for IVM.

2.4.4. In vitro maturation

The COCs were placed in HEPES-buffered TLH solution (supplemented with 10% bovine serum, 0.2 mM pyruvate, and 50 mg/ml gentamicin) and washed three times to remove follicular fluid. Healthy COCs were placed in droplets (50 µl) of maturation medium under mineral oil. Maturation medium was composed of TCM199 (Gibco 11150059, Invitrogen Life Technologies), 10% fetal bovine serum (FBS, Wisent Bioproducts), 0.2 mM pyruvate, 50 mg/ml gentamycin, 0.5 mg/ml FSH (Folltropin-V, Bioniche Animal Health, Belleville, ON, Canada), 5 mg/ml luteinizing hormone (Lutropin, Bioniche) and 1 mg/ml prostaglandin E_2. Maturation droplets were incubated for 24 h at 38.5C with 5% CO_2 in maximal humidity.

2.4.5. IVF

After 24 h of IVM, COCs were collected and washed twice in TLH medium before being transferred in groups of 5 to droplets (48 µl) under mineral oil. The droplets consisted of modified Tyrode's lactate (TL) medium supplemented with fatty-acid-free BSA (0.6% w/v), pyruvic acid (0.2 mM), heparin (2 µg/ml) and gentamycin (50 mg/ml). Oocytes were transferred under mineral oil 15 min prior to adding semen. To stimulate sperm motility, penicillamine, hypotaurine and epinephrine (2 µl, 1 mM and 250 mM respectively) were added to each droplet. Selected spermatozoa (Semex, Canada) stored in liquid nitrogen were thawed for 1 min in water at 35.8 C, laid on a discontinuous gradient (45 % over 90 %) of BoviPure (Nidacon Laboratories AB, Göthenborg, Sweden) and centrifuged at 600 xg for 5 min. The supernatant containing the cryoprotectant and dead spermatozoa was discarded, and the pellet was then re-suspended in 1 ml of modified TL and centrifuged at 300 xg for 2 min. The re-suspended spermatozoa were counted on a hemocytometer and diluted with IVF medium to obtain a final concentration of $1x10^6$ cells/ml. Finally, 2 µl of sperm suspension were added to the droplets containing the matured COCs. The fertilization medium was incubated at 38.5 °C for 18–22 h in a humidified atmosphere of 94.5 % air and 5.5 % CO_2.

2.4.6. In vitro culture

For culture, groups of 10 embryos were placed in droplets (10 µl) of modified synthetic oviduct fluid (mSOF) with non-essential amino acids, 3 mM EDTA, and 0.4 % fatty-acid-

free BSA (ICP bio, Auckland, New Zealand) under embryo-tested mineral oil. The embryo culture dishes were incubated at 38.5 C with 6.5 % CO_2, 5 % O_2, and 88.5 % N_2 in 100 % humidity. Embryos were transferred to new droplets (10 µl) of mSOF containing non-essential and essential amino acids 72 h post-fertilization. At 120 h post-fertilization, embryos were transferred to droplets (20 µl) of mSOF containing non-essential and essential amino acids in order to prevent toxicity due to ammonium concentration and nutrient depletion caused, respectively, by amino acid degradation and embryo metabolism. Blastocyst development was examined on days 7 and 8 post-fertilization.

2.4.7. Statistical analysis

One-way ANOVA with Dunnett post-test analyses were performed on follicle population, oocyte developmental competence and embryo development using Graph Pad Prism 6 (GraphPad Software, San Diego California USA). Normal curves were obtained using the normal distribution formula in Microsoft Excel.

2.5. Results

2.5.1. Effect of donor age on the number and size of follicles recovered

Figure 2-1 shows the numbers of follicles in diameter ranges from 5–6 mm to > 15 mm, aspirated from calves or heifers at different ages, after ovarian stimulation with FSH according to a commercial protocol. One-way ANOVA shows a significantly higher number of 5–6 mm follicles ($p < 0.01$ or lower according to the number of asterisks) at all ages except 11 and 12 months old in comparison to the control age (sexually mature animals). The number of 7–10 mm follicles was also significantly higher ($p < 0.05$) in animals 6 and 7 months old compared to the control group, but did not differ significantly from that observed at 5, 8, 9, 10, 11 and 12 months (among which the lowest p value was 0.118). Follicles in the 11–15 mm diameter range were significantly more numerous only in 5-month-old animals ($p = 0.02$).

2.5.2. Effect of donor age on oocyte developmental competence

Figure 2-2 shows the number of follicles aspirated per cycle and oocyte progression up to blastocyst formation at day 7. The oocytes undergoing maturation were selected on the basis of morphological criteria from the total number of follicles aspirated, which was significantly higher ($p < 0.01$) in donors aged 5 to 8 months compared to the control group and tended to be higher ($p = 0.053$) in 9-month-old animals. The significance was greater ($p < 0.0001$) when the comparison was limited to donors aged 5 to 7 months. However, this number only tended to be different in animals aged 8 and 9 months ($p = 0.094$ and 0.051 respectively). The number of oocytes that cleaved after IVF was also higher in donors aged 5 to 7 months ($p < 0.001$) but was not significantly different from the rest ($p = 0.09$). The number of oocytes that reached the morula and blastocyst stages nevertheless did not differ significantly for any of the age groups (lowest p values being 0.164 and 0.124 respectively).

2.5.3. Effect of donor age on morula and blastocyst formation

The percentages of embryos that reached the cleavage, morula and blastocyst stages after IVF (Figure 2-3) are expressed relative to the number of oocytes in maturation and not to the total number of follicles aspirated. Cleavage did not differ significantly from the control group for any age range (lowest p value = 0.135). In contrast, morula and blastocyst formation was significantly lower among embryos obtained from oocyte donors 5–10 months old ($p \leq 0.05$). However, different periods of coasting after ovarian stimulation did not affect the rate of blastocysts in our donors (supplemental figure 2-1).

2.5.4. Variability of individual responses to ovarian stimulation in terms of total number of aspirated follicles and blastocyst yield

Figure 2-4 shows the mean, standard deviation and coefficient of variation for each oocyte donor age group, and compares the groups in terms of the distribution of total numbers of follicles aspirated and blastocyst yields. Variability between individuals was great in all groups. The standard deviation (SD) decreased as donor age increased for the total number of aspirated follicles, and increased with donor age for blastocyst yield. In the case of the number of aspirated follicles, 2 distinct groupings were distinguishable. The distribution was

notably broader among heifers aged 5–7 months, hence greater variability (SD) compared to those aged 8 months or older, which constituted a grouping with a smaller population distribution (Figure 2-4 B). However, these distinctions were not apparent in the case of blastocyst yield (Figure 2-4 C).

2.6. Discussion

This is the first study in which oocyte donor age, follicle population and embryo outcome were measured for *Bos taurus* using a large dataset compiled in a commercial setting. The first significant observation is that the number of small-to-medium-sized follicles was greater in younger donors, resulting in a greater number of total follicles aspirated after ovarian stimulation in comparison with sexually mature donors. Younger donors therefore provided a greater number of oocytes that were still undergoing maturation during IVM. These oocytes did not yield greater numbers of morulae or blastocysts in comparison with those obtained from adult cows. Based on the initial number of oocytes in maturation and the final number that reached stages beyond cleavage, the yields of morulae and blastocysts were significantly lower in the case of younger cows.

Greater numbers of smaller follicles in younger donors have been observed in previous studies (Brogliatti *et al.*, 1997; Gandolfi *et al.*, 1998; Bols *et al.*, 1999; Palma *et al.*, 2001; Kauffold *et al.*, 2005; D'Occhio *et al.*, 2013). Early studies suggested that the initial potential for oocyte developmental competence is acquired at a follicle size of approximately 3 mm (Blondin and Sirard, 1995) and that the ability to develop into a blastocyst is positively correlated with follicle diameter (Blondin and Sirard, 1995; Hagemann *et al.*, 1999). However, it has been shown in a more recent and properly controlled study of post-stimulation coasting that the correlation with competence is not linear, that oocytes from medium-sized follicles (7–10 mm) give better average results compared to those from larger or smaller follicles (Nivet *et al.*, 2012). In view of this, oocytes from medium-sized follicles should be better suited to developing beyond the cleavage stage than smaller (5–6 mm) or larger (> 11 mm) follicles. The greater number of smaller follicles in younger donors could explain the lower frequency with which the resulting embryos developed beyond the cleavage stage (i.e. to the morula and blastocyst stages, Figure 2-3).

Kauffold et al (2005) showed that developmental competence of oocytes obtained from calves 2–4 months old is related to the diameter of the follicle of origin. Oocytes from follicles greater than 8 mm in diameter have a greater developmental potential than those from follicles smaller than 8 mm (Kauffold *et al.*, 2005). However, only 2 follicle size groups

were examined; no distinction was made between large (> 11 mm) and medium-sized (8–10 mm) follicles.It would be interesting to determine whether or not large follicles contain oocytes of higher quality than medium-sized follicles. Several other studies suggest lower developmental competence of oocytes from younger animals, most showing that calves and cows provide similar numbers reaching cleavage while calves or heifers provide fewer reaching the morula and blastocyst stages (Looney *et al.*, 1995; Revel *et al.*, 1995; Bols *et al.*, 1999; Oropeza *et al.*, 2004; Camargo *et al.*, 2005; Torres *et al.*, 2015), albeit without specifying follicle diameters.

However, based on our results, some younger donors (6 and 7 months old) have a greater number of follicles in the optimal range for quality (7–10 mm) but do not yield blastocysts accordingly, in fact yield them in significantly lower numbers in comparison to control animals. Previous studies have shown that relative protein expression is lower in oocytes from calves compared to those from cycling cows. Lévesque and Sirard (1994) demonstrated that the protein expression pattern in oocytes obtained from calves (1.5 months old) was generally similar to that in defective oocytes obtained from cycling cows, and that the expression patterns in the associated cumulus cells differed. In a similar study (Gandolfi *et al.*, 1998), a significant decrease in protein synthesis and different protein expression patterns were noted in IVM oocytes from calves aged 2–4 months in comparison with those from adult cows. In addition, embryos constructed from calf M-II chromosomes by fusion into adult ooplasts were more likely to cleave and slightly more likely to become blastocysts compared to embryos made from adult M-II chromosomes fused into calf oocytes (Salamone *et al.*, 2001). Furthermore, embryos constructed from calf ooplasm and adult cumulus cells (as nuclear donor) were less likely to cleave or reach the morula and blastocyst stages than embryos generated using adult ooplasts (Salamone *et al.*, 2001). These findings further suggest that ooplasmic and molecular maturation are key components of acquisition of oocyte developmental competence, and that maturation could be compromised in younger donors, which would explain why we observe in some younger donors a greater number of follicles in the size range associated with good oocyte quality, but less developmental competence.

Oocytes originating from younger donors are known to be less competent, some authors reporting a relationship between follicle size and competence in calves while others note a direct effect of lower-quality ooplasm and altered patterns of protein expression in heifers (Lévesque and Sirard, 1994; Gandolfi *et al.*, 1998; Salamone *et al.*, 2001; Kauffold *et al.*, 2005). We also observed high variability between individuals in terms of the number of oocytes selected for maturation and total transferable embryos. Revel et al (Revel *et al.*, 1995) reported that the number of blastocysts developed from individual treated calves was highly variable, 15% of calves producing no embryos, 30% producing four, and 20% producing more than six. This is also a result of the highly variable number of oocytes recovered from individual animals, which ranged from 10 to 78 oocytes. Taneja et al., 2000, reported similar high variability for the number of follicles aspirated (ranging from 5 to 164) and the number of total and usable oocytes recovered (ranging from 4 to 152) among younger donors (2 and 4 months old). Majerus et al., 1999 reported that oocytes can be recovered repeatedly in calves without affecting their growth or the onset of puberty, although large variation between donors is a major concern. A similar observation was made by Armstrong et al., 1994, who observed highly variable responses to ovarian stimulation treatments in very young calves.

In the present study, we showed two different groups of donors in terms of variability: animals aged from 5 to 7 months and animals over 8 months old (Figure 2-4B). Variability was greater in the former group, with the number of follicles/oocytes ranging from 0 to 67 oocytes per OPU. It is interesting this variability was not reflected in blastocyst yield, suggesting that oocytes from animals younger than 8 months can yield transferable embryos in greater numbers. We believe that these animals have the potential to produce more oocytes per cycle and may provide a greater number of transferable embryos. We propose that the main factor decreasing oocyte developmental competence in younger animals is not strictly age-related but rather individual ovarian dynamics. We therefore believe that studying the components of variation on an individual basis could allow correction of the ovarian stimulation (OS) as well as optimisation of the coasting period in order to obtain a significant improvement in embryo outcome.

In conclusion, our results suggest that super-ovulation and oocyte recovery for embryo transfer can reach a very high success rate in all peripubertal calves, but the optimal response is observed at 11–12 months, at which age there is no significant difference in terms of morulas and embryos rate from the results obtained using sexually mature animals. We also show that animals younger than 9 months old provide a larger number of oocytes per cycle. Although the overall efficiency of IVF procedures (embryos percentage) is lower when using younger donors, the greater numbers of oocytes collected counter-balance the lower ratio of embryo formation and results in the same number of transferable embryos as obtained from sexually mature cows. However, the main concern with super-ovulation in peripubertal calves and adult animals remains individual variability, which should be addressed using individual stimulation regimens or OPU timing.

2.7. Acknowledgements

This work was funded by the Natural Sciences and Engineering Research Council of Canada - Collaborative Research and Development (NSERC-CRD grant no. RDCPJ461697-13). D.A. Landry received studentship support from NSERC and REDIH (CIHR Training program in Reproduction, Early Development, and Impact on Health).

2.8. Disclosure

Authors declare no potential conflict of interests.

2.9.References

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2.10. Figures

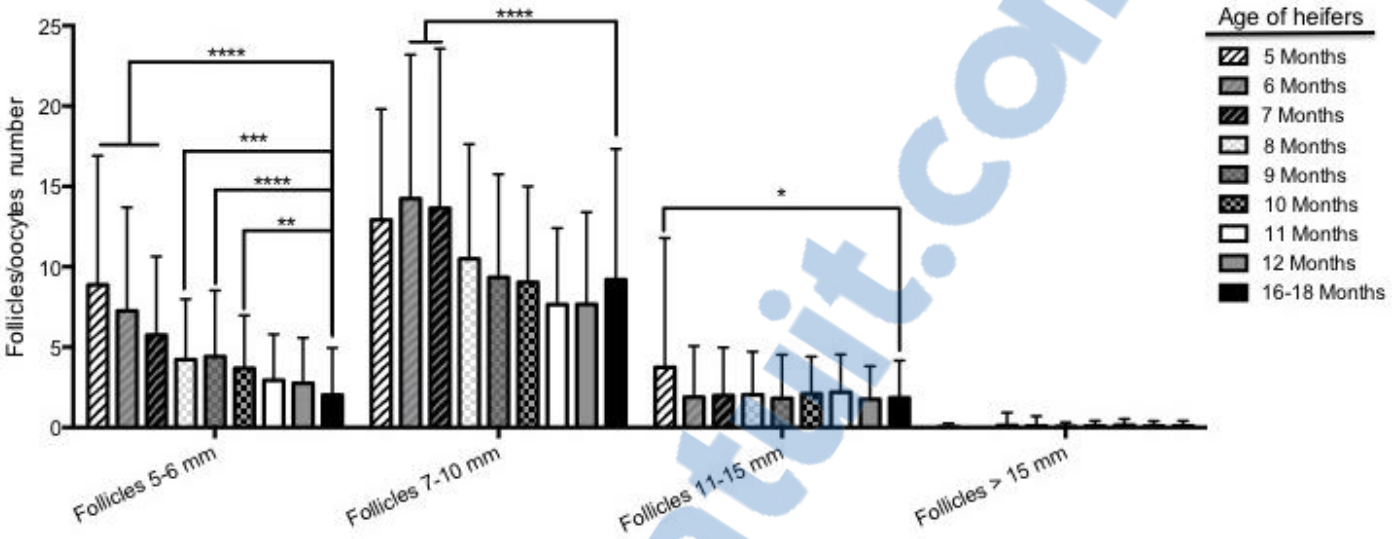


Figure 2-1: Effect of oocyte donor age on the size category distribution of follicles counted after FSH-induced superovulation as practiced in commercial dairy cattle (Holstein) production

''Follicle number'' refers to the average total oocytes recovered by OPU. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

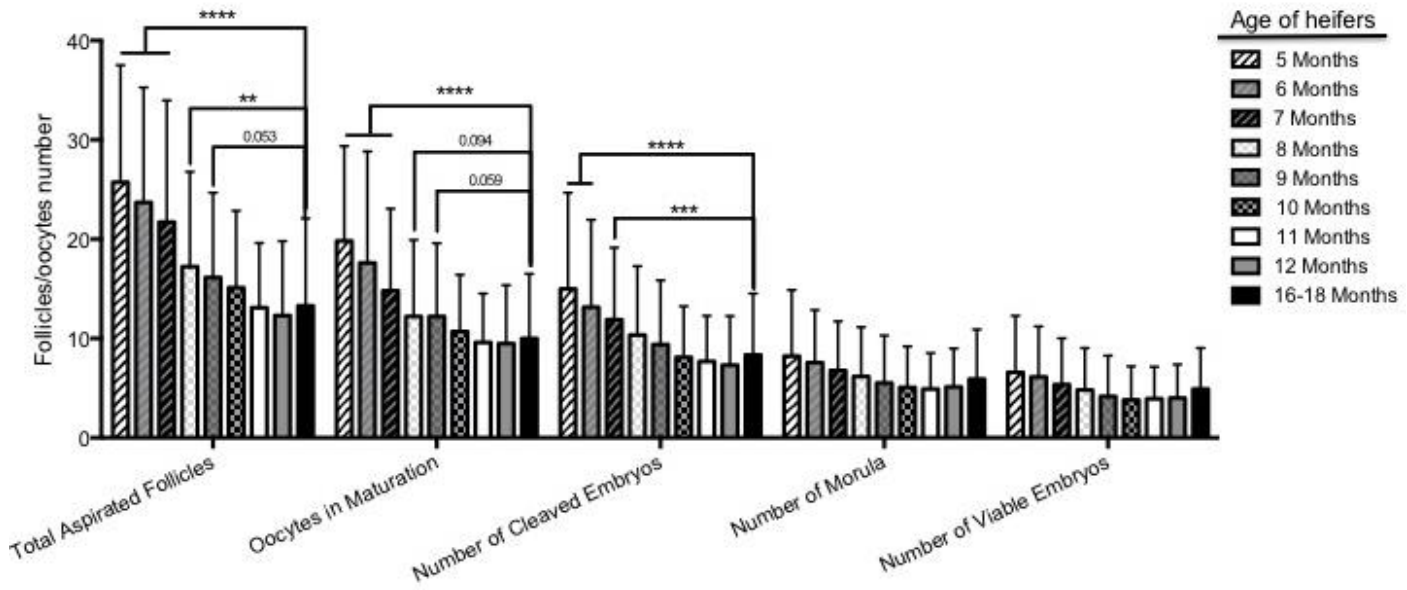


Figure 2-2: Effect of donor age on the developmental competence of IVM/IVF oocytes collected from FSH-stimulated cycling Holstein calves or heifers

‘‘Follicle/oocyte’’ refers to average total numbers aspirated, usable for IVM, cleaving after IVF or reaching the morula/blastocyst stages. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

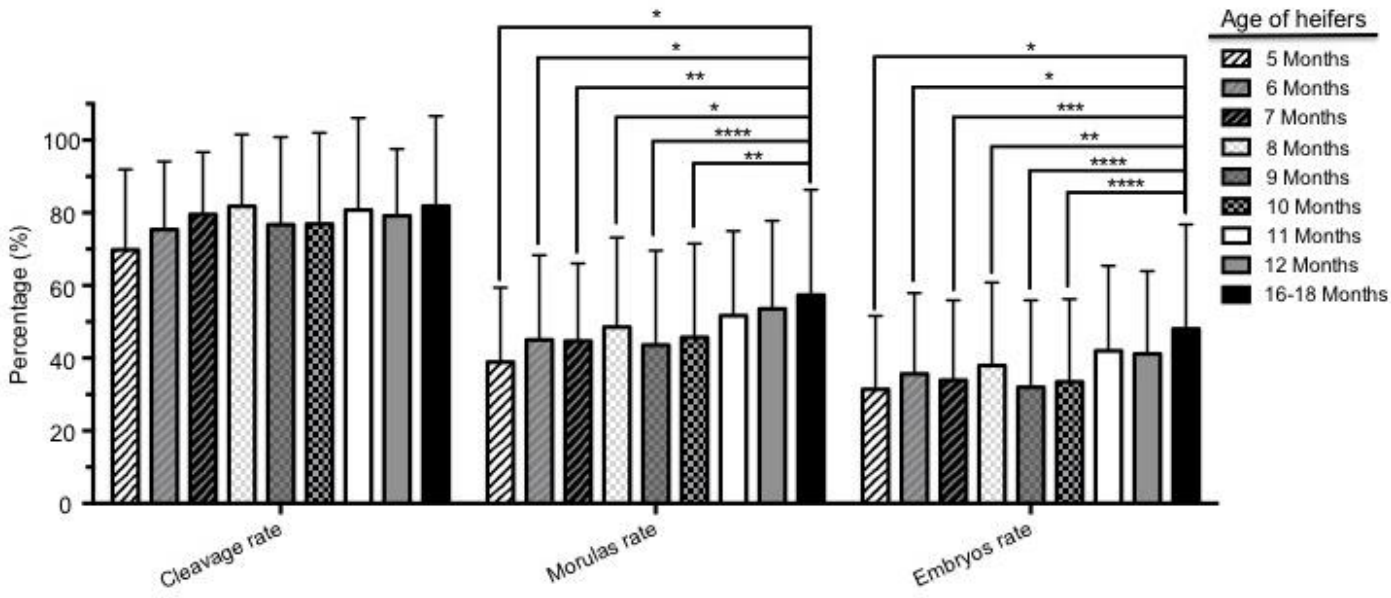


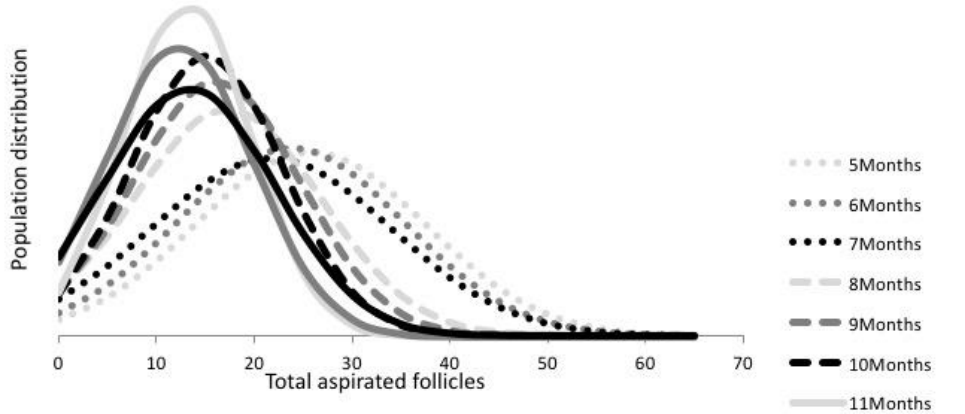
Figure 2-3: Development of Holstein cattle embryos following IVF of oocytes recovered from donors aged 5 to 18 months after FSH-induced superovulation as practised in commercial production

‘‘Percentage’’ refers to mature fertilized oocytes reaching the cleavage, morula or embryo stages, expressed as an average ratio. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

A)

		Variability comparisons between ages					
		Total aspirated follicles			Blastocysts rate		
Oocyte donors age	n	Mean	SD	CV (%)	Mean (%)	SD (%)	CV(%)
5 Months	22	25.74	11.77	45.73%	31.44	20.22	64.30%
6 Months	57	23.70	11.58	48.86%	35.64	22.30	62.56%
7 Months	102	21.71	12.25	56.43%	33.93	22.01	64.87%
8 Months	131	17.23	9.56	55.45%	37.93	22.90	60.38%
9 Months	189	16.15	8.54	52.88%	31.99	23.92	74.75%
10 Months	190	15.11	7.74	51.24%	33.47	22.80	68.13%
11 Months	131	13.11	6.50	49.60%	42.03	23.37	55.60%
12 Months	103	12.32	7.47	60.66%	41.18	22.79	55.33%
16 to 18 Months	106	13.30	8.78	66.03%	48.03	28.73	59.82%

B)



C)

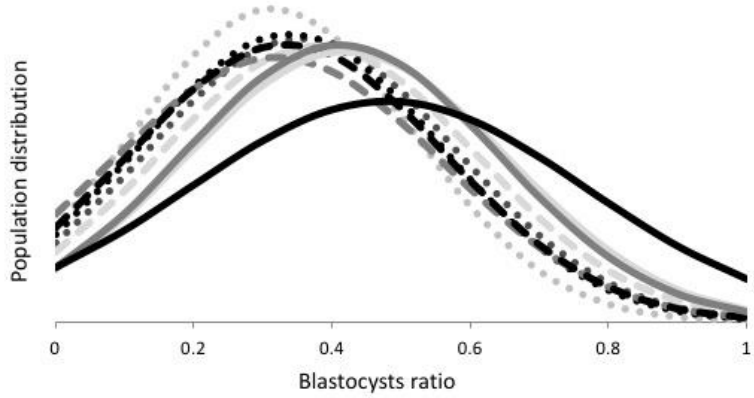
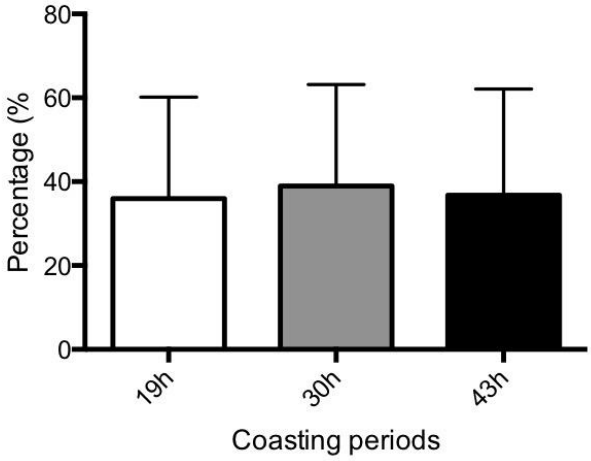


Figure 2-4: Relationship between oocyte donor age and the variance of the total aspirated follicles and blastocyst yield in commercial production of cattle embryos (Holstein)

(A) Mean, standard deviation and coefficient of variation; (B) Normal curves (relative frequencies of the measured averages).



Supplemental figure 2-1: Relationship between coasting periods and the percentage of embryos

‘‘Percentage’’ refers to mature fertilized oocytes reaching the embryo stages, expressed as an average ratio. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

3. Granulosa cell gene expression, oocyte competence and embryo production in FSH-stimulated Holstein cows

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

Landry D.A., Fortin C., Bellefleur A.M., Labrecque R., Grand F.X., Vigneault C., Blondin P. and Sirard, M. A. (2017). Comparative analysis of granulosa cell gene expression in association with oocyte competence in FSH-stimulated Holstein cows. Reproduction, Fertility and Development, 29(12), 2324-2335.

3.1.Résumé

La stimulation ovarienne à l'aide de FSH exogène suivie par une période de coasting est une technique efficace afin d'augmenter le nombre d'ovocytes compétents pour la production d'embryons *in vitro* chez le bovin. Néanmoins, la qualité des ovocytes obtenus à la suite de la stimulation ovarienne et du coasting varie considérablement d'un animal à l'autre. L'objectif de cette étude est de mieux comprendre les conditions folliculaires qui sont associées avec une mauvaise compétence développementale des ovocytes. Les cellules de la granulosa de 94 vaches Holstein dans un environnement commercial de production d'embryons furent récoltées à la suite d'une stimulation ovarienne et du coasting. Les analyses en micropuce démontrent 120 gènes qui sont différentiellement exprimés d'au moins 1.5 fois dans la comparaison des donneuses d'ovocytes compétents (> 75 % devenant des blastocystes) contre les donneuses d'ovocytes incompétents (< 35 %). Grâce à ''Ingenuity Pathway Analysis'' (IPA), nous avons démontré les principales fonctions biologiques et les régulateurs en amont potentiellement régulés chez les donneuses d'ovocytes incompétents. La majorité d'eux sont impliqués dans la prolifération cellulaire, l'apoptose, le métabolisme des lipides, la disponibilité de rétinol et la signalisation de l'insuline. En résumé, nous avons démontré que la différence dans la maturité du follicule lors de la récolte des ovocytes peut expliquer les différences dans la compétence finale de l'ovocyte en fonction de chaque animal. Nous avons aussi démontré une déficience dans le métabolisme des lipides et dans la signalisation du rétinol chez les cellules de la granulosa des donneuses d'ovocytes incompétents.

3.2. Abstract

Ovarian stimulation with exogenous FSH followed by FSH withdrawal or 'coasting' is an effective means of increasing the number of oocytes obtainable for the in vitro production of cattle embryos. However, the quality of the oocytes thus obtained varies considerably from one cow to the next. The aim of the present study was to gain a better understanding of the follicular conditions associated with low oocyte developmental competence. Granulosa cells from 94 Holstein cows in a commercial embryo production facility were collected following ovarian stimulation and coasting. Microarray analysis showed 120 genes expressed with a differential of at least 1.5 when comparing donors of mostly competent (> 75 % becoming blastocysts) with donors of mostly incompetent oocytes (< 35 %). Using ingenuity pathway analysis, we revealed the main biological functions and potential upstream regulators that distinguish donors of mostly incompetent oocytes. These are involved in cell proliferation, apoptosis, lipid metabolism, retinol availability and insulin signalling. In summary, we demonstrated that differences in follicle maturity at collection could explain differences in oocyte competence associated with individual animals. We also revealed deficiencies in lipid metabolism and retinol signalling in granulosa cells from donors of mostly incompetent oocytes.

3.3. Introduction

Assisted reproductive technologies (ART) have been developed as a solution to infertility in humans and domestic animals, including dairy cows. An offshoot of ART is the *in vitro* production (IVP) of embryos, which has become a valuable tool for reproducing high-performance animals in large numbers and enhancing genetic gain in the herd. In large mammals, a major limitation of the success of IVF is the developmental competence that oocytes acquire during the period leading up to their collection from follicles (Sirard *et al.*, 2006). Therefore, follicular conditions and molecular markers associated with competence have been the focus of most research on oocyte quality (Nivet *et al.*, 2013, 2016). Studies such as these have demonstrated the importance of controlling levels of FSH and particularly the timing of FSH withdrawal in order to maximise the yield of competent oocytes. In mammals, a follicular wave occurs following an increase in endogenous FSH (Adams *et al.*, 1992) and culminates in deviation towards a single dominant follicle (Ginther *et al.*, 1989, 1997) that suppresses FSH secretion (Ireland and Roche, 1987). The declining FSH concentration causes atresia in subordinate follicles while the dominant follicle expresses LH receptor and thereby becomes no longer dependent on FSH, but on LH (Ali *et al.*, 2001). The FSH concentration can be maintained by providing the hormone exogenously, bringing about a condition known as ovarian superstimulation (OS), which rescues most of the subordinate follicles in the growing cohort, resulting in multiple dominant follicles, each capable of ovulation. However, many oocytes thus obtained are of poor quality (for a review, see Lonergan *et al.*, 2016). Nevertheless, there has been one major improvement to the stimulation regimen, namely FSH withdrawal (coasting), which has increased the average developmental competence significantly (Sirard *et al.*, 1999; Blondin *et al.*, 2002). FSH coasting is described as the period of time between the final injection of exogenous FSH and the ovum pick-up (OPU) procedure, during which a temporary general improvement in oocyte developmental competence occurs (Nivet *et al.*, 2012). Follicle dynamics during coasting are now better understood, and optimising the duration of this period is key to improving the success of ART. Oocyte quality increases abruptly at the beginning of coasting, reaches a maximum or plateau and then decreases as the follicles begin to undergo atresia if progesterone is maintained to inhibit the LH surge (Nivet *et al.*, 2012). These studies highlight the importance of proper differentiation of follicle status during coasting if we are

to provide an optimal environment for the acquisition of maximal oocyte developmental competence.

Ovarian follicle maturation is a complex process involving bidirectional communication between somatic cells and germ cells. Granulosa cells and other somatic cells making up the follicle play a role in oocyte maturation while the oocyte, in turn, promotes proliferation and differentiation of follicular cells (El-Hayek and Clarke, 2016). Developmental competence is known to vary throughout the process, and the quality of the oocyte is thus linked inextricably to the physiological status of the follicle at the time of collection (Sirard *et al.*, 2006). Because granulosa cells are in constant communication with the oocyte, they should be a source of reliable indicators of follicle status, and specific markers of oocyte quality have been found in granulosa and cumulus cells of cattle (Nivet *et al.*, 2013; Bunel *et al.*, 2014), humans, rats, mice and pigs (for a review, see Chronowska, 2014).

Although some cows respond to the OS–coasting treatment by yielding oocytes that reach the blastocyst stage *in vitro* 100% of the time (Blondin *et al.*, 2002; Nivet *et al.*, 2012), the response of individual cows is variable, which is a major concern for businesses that provide cattle embryos (Landry *et al.*, 2016). The goal of the present study was to determine the follicle gene expression associated with poor oocyte developmental competence and to identify specific markers of follicle status. Based on analysis of the granulosa cell transcriptome, we reveal conditions that compromise oocyte quality in association with early and late stages of coasted follicle differentiation. In addition, we found a deficiency in lipid metabolism in some animals that yield oocytes with lower developmental competence.

3.4.Materials and methods

3.4.1. Ethics statement

The clinical procedures and industrial practices used in Boviteq follow the established cattle reproduction management practices, which have been approved by the College of Veterinary Surgeons of Quebec (OMVQ), the Canadian Embryo Transfer Association (CETA) and the International Embryo Transfer Society (IETS). This company follows the Canadian Council

on Animal Care (CCAC) guidelines for farm animals and the research projects do not involve the use of exclusive animals for research purposes, and neither does it involve the implementation of new animal procedures to obtain additional biological samples other than the ones used in their routine commercial activities. The study did not require handling animals on university premises.

3.4.2. Experimental design

The present study was performed on granulosa cells gathered from 94 elite Holstein cycling cows subjected to ovarian stimulation treatment as described below and selected randomly in a controlled commercial environment for embryo production (mean (± s.d.) age of cows 34 ± 29 months; range 12–108 months). Of the 94 animals used in the present study, only eight were kept in remote farms; those animals were not used in the microarray analysis and none was lactating during the embryo transfer (ET) program. The gamete and granulosa collection procedures took place between December 2013 and December 2014. Animals were divided into three groups according to their percentage of oocytes that reached the blastocyst stage after IVF, namely good, middle and poor developmental competence, with a ratio of blastocysts >0.75, between 0.74 and 0.36 and <0.35 respectively. Sixteen animals from the good and the poor developmental groups were selected for microarray analysis and all 94 animals were used in real-time quantitative PCR (qPCR) validation according to their oocyte developmental group.

3.4.3. Chemicals

Unless stated otherwise, all reagents and media supplements used in these experiments were obtained from Sigma-Aldrich.

3.4.4. Ovarian stimulation treatment and granulosa cell collection

The protocol for ovarian stimulation and OPU was essentially the same as described previously (Landry *et al.*, 2016). Briefly, the dominant follicle was aspirated 36–48 h before hormone administration. Animals were stimulated for 3 days with FSH. According to animal age, body condition score (BCS) and/or based on previous stimulation, FSH was

administered in five 30-mg, six 30-mg or six 40-mg doses of NIH Folltropin-V (Bioniche Animal Health), followed by a coasting (no FSH) period of 19, 30 or 43 h, accordingly, to animal age, body condition score and/or based on previous stimulation (Figure 3-1). Using transvaginal ultrasonography, follicle diameters were measured and cumulus–oocyte complexes (COCs) were collected by transvaginal puncture under epidural anaesthesia, using an 18-G needle and COOK aspiration unit (COOK Medical). COCs and granulosa cells were collected in warm HEPES-buffered Tyrode's medium (TLH) containing heparin (10 IU mL^{-1}) and transferred to the laboratory for IVM.

3.4.5. In vitro maturation

COCs were placed in TLH (supplemented with 10% bovine serum, 0.2 mM pyruvate and 50 mg mL^{-1} gentamycin) and washed three times with TLH to remove follicular fluid. Healthy COCs were placed in 50-µL droplets of maturation medium under mineral oil. The composition of the maturation medium was as follows: TCM-199 (Gibco 11150059; Invitrogen Life Technologies), 10% fetal bovine serum (FBS; Wisent Bioproducts), 0.2 mM pyruvate, 50 mg mL^{-1} gentamycin, 0.5 mg mL^{-1} FSH (Folltropin-V; Bioniche Animal Health), 5 mg mL^{-1} LH (Lutropin; Bioniche Animal Health) and 1 mg mL^{-1} prostaglandin E_2. Maturation droplets were incubated for 24 h at 38.5 °C under 5 % CO_2 in maximal humidity.

3.4.6. *In vitro* fertilisation

After 24 h IVM, COCs were collected and washed twice in TLH before being transferred in groups of five to 48-µL droplets under mineral oil. The droplets consisted of modified Tyrode's lactate (TL) medium supplemented with fatty acid-free BSA (0.6 % w/v), pyruvic acid (0.2 mM), heparin (2 µg mL^{-1}) and gentamycin (50 mg mL^{-1}). Oocytes were transferred under mineral oil 15 min before the addition of semen. To stimulate sperm motility, 2 µL penicillamine, 1 mM hypotaurine and 250 mM adrenaline were added to each droplet. Selected spermatozoa (Semex) stored in liquid nitrogen were thawed for 1 min in water at 35.8 °C, laid on a discontinuous gradient (45 % over 90 %) of BoviPure (Nidacon Laboratories) and centrifuged at 600 *g* for 5 min at 26 °C. The supernatant containing the

cryoprotectant and dead spermatozoa was discarded, and the pellet was then resuspended in 1 mL modified TL and centrifuged at 300 *g* for 2 min at 26 °C. The suspended spermatozoa were counted on a haemocytometer and diluted with IVF medium to obtain a final concentration of 1×10^6 cells mL^{-1}. Finally, 2 µL sperm suspension was added to the droplets containing the matured COCs. Samples were incubated in the fertilisation medium at 38.5 °C for 18–22 h in a humidified atmosphere of 94.5 % air and 5.5 % CO_2.

3.4.7. *In vitro* culture

For culture, groups of 10 embryos were placed in 10-µL droplets of modified synthetic oviducal fluid (mSOF) with non-essential amino acids, 3 mM EDTA and 0.4 % fatty acid-free BSA (ICPbio) under embryo-tested mineral oil. The embryo culture dishes were incubated at 38.5 °C with 6.5 % CO_2, 5 % O_2 and 88.5 % N_2 at 100 % humidity. Embryos were transferred to new 10-µL droplets of mSOF containing non-essential and essential amino acids 72 h after fertilisation. At 120 h after fertilisation, embryos were transferred to 20 µL droplets of mSOF containing non-essential and essential amino acids in order to prevent toxicity due to ammonium concentration and nutrient depletion caused by amino acid degradation and embryo metabolism respectively. Blastocyst development was examined on Days 7 and 8 after fertilisation. Granulosa cells were collected and pooled for each animal within 5 min after OPU and oocyte retrieval, then washed twice with phosphate-buffered saline (PBS) at 4 °C and snap frozen for RNA extraction.

3.4.8. RNA extraction and amplification

Total RNA was extracted from each sample and purified using an AllPrep DNA/RNA/miRNA Universal kit (Qiagen). The purity and concentration of the extracted RNA were analysed using a Bioanalyzer (Agilent Technologies). All extracts were of good quality with an RNA integrity number > 8.3. For the microarray, 5 ng purified RNA from each individual sample was amplified using T7 RNA polymerase with the RiboAmp HS Plus RNA Amplification Kit (Thermo Fisher). After two amplification rounds of 6 h each, the amplified RNA (aRNA) output was quantified using a NanoDrop ND-1000 device (NanoDrop Technologies).

3.4.9. Sample labelling and microarray hybridisation

Cows were divided into two groups based on oocyte developmental competence: (1) poor, meaning blastocysts were obtained less than 35 % of the time; and (2) good, meaning blastocysts were obtained more than 75 % of the time. Sixteen animals were selected randomly from each group and four were pooled into four replicates for each group to reduce the effects of individual variation and to create a contrast based on oocyte developmental competence (Pool1 vs Pool5; Pool2 vs Pool6; Pool3 vs Pool7 and Pool4 vs Pool8 see Table S1, available as Supplemental Material to this paper). For each pool, 4 µg aRNA was labelled with Cy3 and Cy5 using the ULS Fluorescent Labelling Kit for Agilent arrays (Kreatech Diagnostics). The labelled product was then purified with the Pico-Pure RNA Isolation Kit (Thermo Fisher) but without DNase treatment. Labelling efficiency was measured using the NanoDrop ND-1000. Samples were hybridised on an EmbryoGENE bovine 44K microarray (Robert *et al.*, 2011). Overall, four hybridisations in dye swap, corresponding to a total of 32 cows, were performed. A total of 825 ng of each sample labelled with Cy3 and Cy5 was incubated in a solution containing 10× blocking agent and 25× fragmentation buffer in a volume of 55 µL at 60 °C for 15 min, with samples put on ice immediately after. An equal volume of 2× GEx Hybridisation Buffer HI-RPM (Agilent) was added to obtain a total volume of 110 µL. The hybridisation mix (100 µL) was added onto the array and hybridisation was performed at 65°C for 17 h using an Agilent hybridisation chamber in a rotary oven. Slides were washed with Gene Expression Wash Buffer 1 containing 0.005% Triton X-102 for 3 min at room temperature and then transferred to Gene Expression Wash Buffer 2 containing 0.005 % Triton X-102 for 3 min at 42 °C. Final washes with acetonitrile for 10 s at room temperature (19–21 °C), followed by Stabilisation and Drying Solution (Agilent) for 30 s at room temperature (19–21 °C), were performed before air drying of the slides.

3.4.10. Microarray data analysis

Slides were scanned using the Tecan PowerScanner microarray scanner and features extracted using ArrayPro 6.4 (Media Cybernetics). Microarray data were subjected to a

simple background subtraction, normalised within array (Loess) and between array (Quantile) and analysed statistically with the Limma package using FlexArray version 1.6.1(Genome Quebec, Montreal, Canada). Differences were considered statistically significant at two-sided $P < 0.05$. Microarray data have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE88893 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE88893, accessed Oct 19, 2016).

3.4.11. Complementary DNA preparation and real-time PCR

Real-time qPCR was performed to validate the microarray results. All 94 animals were included in the acquisition and the stability of the reference genes used to establish gene expression and microarray validation. However, only 13 cows from the good donors' group and 27 cows from the poor donors' group were compared in the analysis of gene differential expression. Most of the genes of interest were selected on the basis of the symmetrical raw change in expression level and $P < 0.05$, a positive change indicating upregulation in the poor donor group. Other genes identified in previous studies that were associated with negative energy balance and coasting periods (Nivet *et al.*, 2013; Girard *et al.*, 2015a) were also examined in the present analysis. RNA (385 ng) extracted from granulosa cells was reverse transcribed using a q-Script Flex cDNA synthesis kit (Quanta Biosciences) with oligo dT (20) primers according to the manufacturer's recommendations. The primers were designed using the IDT PrimerQuest tool (available at the Integrated DNA Technologies, URL: https://www.idtdna.com/Primerquest) based on sequences obtained using the UMD3.1/bosTau5 assemble version of the bovine genome (Zimin AV et al., 2009) and on microarray results. The specificity of each primer pair was confirmed by measuring the mobility of the amplified fragment in a 1.2% agarose standard electrophoresis gel and sequencing. Primer sequences and accession numbers are provided in Table 1. LightCycler 480 SYBR Green I Master and the LightCycler 480 (Roche Diagnostics) were used. The PCR conditions for all genes were as follows: denaturing cycle for 10 min at 95 °C, followed by 50 cycles of denaturing at 95 °C for 10s, annealing for 10 s and extension at 72 °C for 20 s, then melting curve analysis (95 °C for 1 s, 65 °C for 1 s and a step cycle from 72 to 97 °C at 0.11 °C s^{-1}) and a final cooling step at 4 °C.

3.4.12. Statistical analysis of real-time qPCR results

Data analysis was performed using the LightCycler 480 Software 1.5.0 SP4 (version 1.5.0.39; Roche Diagnostics). Analysis of the stability of housekeeping genes in granulosa cells was performed using GeNorm VBA applet software (Biogazelle, Belgium), as described previously (Vandesompele *et al.*, 2002). The most stable reference genes were identified by stepwise exclusion of the least stable gene and recalculating the M values. Two housekeeping genes, namely β-actin (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were found to be the most stable to normalise our data ($M < 1.5$, the threshold suggested in the software). Normalised data cleaned using the Rout method of outlier removal were analysed using the Mann–Whitney non-parametric test. Follicular population and blastocyst outcome were analysed using unpaired multiple *t*-tests and unpaired *t*-tests respectively. All statistical analyses were performed using GraphPad Prism version 6.00 (GraphPad Software).

3.4.13. Ingenuity pathway analysis

The limma data for all 27 633 probes on the EmbryoGENE bovine array targeting a transcript in the contrast were uploaded into the ingenuity pathway analysis (IPA) software (Ingenuity Systems; www.ingenuity.com, accessed Oct 19, 2016) for analysis of the functional implications of the patterns of differential expression in terms of gene–molecule interactions. A 'core analysis' was performed using default parameters with a threshold of 1.5 for the change in expression (symmetrical fold change) and 0.05 for *P*. For each functional category, IPA calculates a *Z*-score that is positive when predicting that a physiological activity increases and negative when predicting that it decreases. *Z*-scores ≥ 2 or ≤ -2 indicate that the trend is statistically significant. The *P*-value, calculated by Fisher's exact test, measures the likelihood that the association between genes and a given function is random (due to chance). Finally, the upstream section of the core analysis was used to determine which upstream regulators in our contrast were likely activated or inhibited.

3.5. Results

3.5.1. *In vitro* embryo production

Based on blastocyst yield following IVF of oocytes, cows subjected to the OS–coasting procedure were divided into two groups: 13 good donors (oocyte developmental competence considered high; i.e. blastocyst stage reached at least 75% of the time) and 27 poor donors (oocyte developmental competence considered low; i.e. blastocyst stage reached less than 35% of the time). Fifty-four cows fell in the middle ground and were therefore not used in the analysis. There were no significant differences in follicle number or size between the good donor and poor donor groups (Figure 3-2). There was also no significant effect of the length of the coasting period on blastocyst development rate ($P = 0.32$; data not shown). Animals selected for microarray are listed in Table S1; there were no significant differences in the size and number of follicles aspirated or in the number of oocytes maturing. As expected, the number of viable embryos and the percentage of embryos differed ($P < 0.05$) between the pools of good donors and the pools of poor donors.

3.5.2. Microarray analysis

To investigate differences in the transcriptomes of granulosa cells taken from poor and good donors, a custom-made EmbryoGENE microarray slide was used. Of the 21 139 reference genes, 15 024 were considered expressed in poor donors and 15 608 expressed in good donors, based on the signal exceeding a threshold defined as the mean negative control plus twice the standard deviation. Based on the criteria for significant change (fold change ≥ 1.5; $P < 0.05$), 120 probes showed differential expression in the comparison of poor and good donors (Figure 3-3), with 72 of those probes corresponding to an official gene symbol (Table S2).

3.5.3. Real-time qPCR

Confirmation of the microarray results was obtained in the form of real-time qPCR data for transcripts selected on the basis of differential expression in poor donors, profile during the coasting period and known function in or outside the ovary. Six genes were thus quantified: keratin 4 (*KRT4*), lumican (*LUM*), phosphoprotein enriched in astrocytes 15 (*PEA15*),

phosphoenolpyruvate carboxykinase 2 (*PCK2*), sterol regulatory element binding transcription factor 2 (*SREBF2*) and transthyretin (*TTR*). *LUM* was significantly overexpressed and *KRT4*, *PCK2*, *SREBF2* and *TTR* were significantly underexpressed in poor relative to good donors (Figure 3-4). Although *PEA15* was underexpressed in poor donors, the effect was not significant ($P = 0.20$). *PCK2* showed reverse regulation in the real-time qPCR compared with the microarray analysis, whereas the results for the other genes were consistent with the microarray analysis. The opposite regulation of *PCK2* can be explained by the higher number of animals used in the PCR analysis.

3.5.4. Ingenuity pathway analysis

Comparison of poor with good donors using IPA revealed a significant association between a certain group of genes expressed differentially in granulosa cells and certain biological functions. The latter include cell death and survival, gene expression, organismal survival, cellular assembly and organisation and lipid metabolism (Figure 3-5). The top 50 most significant canonical pathways are presented in Table 3-2. IPA also revealed a cascade of upstream transcriptional regulators that may explain the observed changes in gene expression. Setting the activation *Z*-score at 2 or –2 and P at < 0.05, 22 potential upstream regulators are predicted to be activated or inhibited in granulosa cells of poor donors, Table 3-3

3.6. Discussion

The goal of the present study was to investigate the effects of a fertility stimulation procedure on the physiological status of ovarian follicles in donors of oocytes that fail to yield blastocysts following IVF. In the case of dairy cattle, such failure could be due to any of several factors, including suboptimal health, nutritional status, post-initial aspiration follicle population, collecting oocytes before or after the optimal FSH coasting period and genetic changes affecting gonadotropin receptors. In order to decrease the effect of individual bias on the genomic response to the OS–coasting procedure, donors of incompetent oocytes were pooled, as were donors of competent oocytes. The background noise due to this grouping makes the observed differential expression all the more noteworthy, increases the likelihood that the genes thus identified are associated with a specific cause of incompetence and makes the association of the differential expression of genes, canonical pathways and biological functions with early, late or timely differentiation of follicle cells more compelling.

IPA pointed to essential biological functions that are affected primarily in poor donors. Cell death and survival, organismal survival, gene expression and cell growth and proliferation are the main biological functions affected in granulosa cells from poor donors. These functions are well known aspects of granulosa cell dynamics in cows undergoing OS followed by FSH withdrawal. The effect of coasting after OS plays a critical role in determining the physiological status of the follicle and thus the acquisition of oocyte developmental competence (Blondin *et al.*, 2002; Nivet *et al.*, 2012). A previous study showed that 44–68 h of FSH withdrawal is optimal for the differentiation of follicles containing maximally competent oocytes and that oocytes collected from under- or over-differentiated follicles (i.e. too early or too late) are less competent (Nivet *et al.*, 2012). Follicle cell growth and proliferation signals have been associated with early follicle differentiation. The period of maximal oocyte competence is associated with finely tuned angiogenesis, inflammation and early atresia signals, whereas the subsequent period of declining competence is associated with apoptotic signals and decreased cell viability (Nivet *et al.*, 2013; Bunel *et al.*, 2014; Douville and Sirard, 2014; Girard *et al.*, 2015b). Our data revealed that signalling from both early and late differentiated follicles are found in the poor

donor group and show specific well known genes and networks from previous studies, as described below.

Functional analysis revealed that canonical pathways such mammalian target of rapamycin (mTOR) and insulin-like growth factor 1 (IGF1) signalling responded significantly with a *Z*-score that tended to be negative in poor donors. Based on the *P*-value in IPA, a significant value suggests an association between the genes of interest and a given process or pathway that is not likely due to random chance (Krämer *et al.*, 2013). Pathways involving mTOR integrate both intracellular and extracellular signals to function as regulators of cell growth, proliferation and survival (Laplante and Sabatini, 2009). It is well known that mTOR signalling is an important pathway in the promotion of ovarian follicle development (Cheng *et al.*, 2015). Growth factors are known to stimulate mTOR signalling through increases in the phosphorylation activity of protein kinase B, also known as AKT (Inoki *et al.*, 2002; Potter *et al.*, 2002). IGF1 regulates multiple ovarian functions, including granulosa cell growth and proliferation, steroidogenesis and the suppression of apoptosis (Balasubramanian *et al.*, 1997; Devoto *et al.*, 1999; Hu *et al.*, 2004; Kwintkiewicz and Giudice, 2009; Mani *et al.*, 2010). These actions of IGF1 are mediated by the IGF1 receptor, which stimulates phosphorylation of several downstream signalling factors including phosphatidylinositol 3-kinase (PI3K)/AKT (Hu *et al.*, 2004; Mani *et al.*, 2010; Mack *et al.*, 2012). In contrast, phosphatase and tensin homologue (PTEN) inactivates AKT and attenuates IGF1-induced cell proliferation in human granulosa cells, suggesting that it may be a trigger for differentiation transition (Goto *et al.*, 2009; Makker *et al.*, 2014). In addition, IPA upstream analysis, a powerful tool for characterising gene upregulation or downregulation with a specific trigger, such as a gene or compound, suggested that PTEN is a significant upstream regulator activated in poor donors. Together, the decreased mTOR and IGF1 signalling and increased PTEN signalling in poor donors may indicate that follicles were becoming over-differentiated.

Conversely, IPA also revealed that apoptosis, cell death and autophagy were somewhat depressed in the poor donor group, suggesting that oocytes were collected too early and that the follicles were under-differentiated and still in the growth phase with less apoptotic

signalling compared with good donors. A previous study demonstrated that under-differentiated follicles expressed signalling pathways that are associated with cell proliferation, angiogenesis and extracellular molecules (Nivet *et al.*, 2013). A major upstream regulator identified by the present analysis is the oncosuppressor tumor protein 53 (TP53), although several downstream genes consistent with its activation or its inhibition were also identified, reducing its *Z*-score to zero. TP53 plays a major role in the regulation of cell cycle progression, such as cell cycle arrest and apoptotic response to cellular stress and damage (Liwak *et al.*, 2012), as well as atresia in bovine granulosa cells (Hatzirodos *et al.*, 2014). Because both early and late differentiation are contributing to poor donor status, the upstream regulator may seem to be neutralised but, in fact, reveals downstream target genes, such as cell progression genes, that can be used to analyse the follicular status of individual cows.

As potential markers of apoptotic follicles, *LUM* and *PEA15* are up- and downregulated respectively, but only *LUM* significantly so. LUM is an extracellular matrix proteoglycan involved in invasiveness, proliferation, migration, apoptosis and angiogenesis in various cell types (Nikitovic *et al.*, 2008; Williams *et al.*, 2010). Overexpression of LUM has been associated recently with cumulus cells from bovine embryos with limited developmental potential (Bunel *et al.*, 2015) and LUM reportedly inhibits melanoma cell migration and cell growth by inhibiting matrix metalloproteinase (MMP) 14 and subsequently inducing apoptosis. However, LUM function varies, depending on cell type (Niewiarowska *et al.*, 2011; Stasiak *et al.*, 2016). In granulosa cells, MMPs contribute to the degradation of extracellular matrix components, and increased expression of the active form of MMP14 coincides with ovulation in both primates and rodents (Puttabyatappa *et al.*, 2014). Heightened expression of *LUM*, and hence inhibition of MMP14, suggests a follicle that is still in the early stages of differentiation (under-differentiated) and not yet ready for ovulation. PEA15 is a death effector domain (DED)-containing protein that functions as a negative regulator of apoptosis (Ramos *et al.*, 2000)r. Some have reported that PEA15 is an AKT substrate and that its phosphorylation and stabilisation by AKT contributes to AKT-mediated survival signalling. In addition, PEA15 binds extracellular signal-regulated kinase (ERK) 1/2 and thereby abolishes the nuclear translocation of these kinases (Trencia *et al.*,

2003; Gervais *et al.*, 2006). Inactivation of ERK1/2 by the inhibitor UO126 (mitogen-activated protein kinase kinase (MEK) 1/2 inhibitor) appears to inhibit FSH stimulation of steroidogenic acute regulatory protein (StAR) expression and subsequent progesterone synthesis and to simultaneously increase FSH-induced P450 aromatase and subsequent oestradiol production in rat granulosa cells (Moore *et al.*, 2001; Yu *et al.*, 2005). Reduced *PEA15* expression suggests a follicle undergoing atresia or precocious differentiation (over-differentiation).

Lipid metabolism is another major biological function that was found to be affected in donors of poor-quality oocytes. Steroidogenic cells such as granulosa cells do not store significant quantities of steroidogenic hormones and rely on stimulation to initiate *de novo* synthesis of steroids, a process that requires a constant supply of cholesterol as a precursor. Cholesterol in granulosa cells can be derived from two sources: (1) *de novo* synthesis in the endoplasmic reticulum; and (2) mobilisation of lipid droplets, the latter being preferable (for a review, see Shen *et al.*, 2016). Thus, lipid metabolism is a critical and essential function of granulosa cells. IPA revealed P2Y receptor signalling to have a high *P*-value in poor donors. P2Y signalling is known to be involved in lipid metabolism to induce metabolic or vascular events (Martinez *et al.*, 2015). IPA also revealed four major players in lipid and cholesterol homoeostasis that are significantly affected in poor donors. Upstream activation of insulin-induced genes 1 and 2 (*INSIG1* and *INSIG2* respectively) is predicted in poor donors, as is upstream inhibition of sterol regulatory element-binding transcription factor 1 (SREBF1 or SREBP1) and SREBF cleavage-activating protein (SCAP). The INSIG proteins regulate the activity of SREBPs, SCAP and 3-hydroxy-3-methyglutaryl coenzyme A reductase (HMG-CoA reductase) and play an important role in cholesterol homoeostasis. Both INSIG1 and INSIG2 bind SREBPs and SCAP in the endoplasmic reticulum, thereby inhibiting the release of the SREBP–SCAP complex and preventing the transcription of SREBP target genes whose products are needed for the uptake and synthesis of cholesterol, fatty acid, phospholipids and triglycerides (Krapivner *et al.*, 2008; for a review, see Dong and Tang, 2010). In addition, SREBP1 and SREBP2 are known to increase in response to FSH and to regulate cholesterol and lipid biosynthesis (Liu *et al.*, 2009), whereas FSH, IGF1 and lipoproteins synergistically enhance progesterone biosynthesis (Veldhuis *et al.*, 1988; LaVoie *et al.*, 1999).

The *PCK2* gene encodes a rate-limiting mitochondrial enzyme known as phosphoenolpyruvate carboxykinase 2, which catalyses the GTP-dependent conversion of oxaloacetate to phosphoenolpyruvate required for both gluconeogenesis and glyceroneogenesis (for a review, see Beale *et al.*, 2007). Gluconeogenesis is regulated by glucagon (released when blood glucose is low) and by insulin through the effects of protein kinase A (PKA) and cAMP in response to fluctuations in food availability. The function of glucagon is to generate glucose, whereas glyceroneogenesis generates precursors for triglyceride synthesis from pyruvate and carbon dioxide, a process that is essential for proper lipid metabolism. Pyruvate is a major intermediate in glycolysis and the most ubiquitous energy source derived from monosaccharide metabolism. It is the main energy source for the oocyte, and the granulosa cells could support oocyte quality by providing this intermediate via direct cumulus transfer. The fact that expression of *PCK2* is generally lower in follicles associated with lower embryo quality may be an indication of higher blood glucose. In addition, high doses of pyruvate decrease oestradiol production, and deficient gluconeogenesis and glyceroneogenesis due to weak expression of PCK2 could lead to accumulation of pyruvate (Campbell *et al.*, 2010).

Tretinoin is a pharmaceutical form of all-*trans* retinoic acid, an upstream regulator of functions related to cell proliferation, which IPA predicts will be inhibited. Retinol (vitamin A) and its physiological metabolite retinoic acid are also important regulators of embryonic development (Ross *et al.*, 2000) and modulators of follicle development, oocyte development and ovarian steroidogenesis (Brown *et al.*, 2003; Girard *et al.*, 2015b). Transthyretin (TTR) belongs to a group of proteins that bind and transport thyroid hormones and is involved indirectly in carrying retinol. TTR forms a complex with retinol and retinol-binding protein (RBP) to transport the hormones from the serum into the follicle and oocyte (Vieira and Schneider, 1993), and these proteins are found in follicular fluid (Schweigert and Zucker, 1988; Anahory *et al.*, 2002; Schweigert *et al.*, 2006). In cows, increased blood concentrations of β-hydroxybutyrate 60 days post partum are associated with negative energy balance, and the dominant follicles of these animals also show insufficient retinoic acid stimulation and low oocyte quality (Girard *et al.*, 2015a).

KRT4 is an intermediate filament cytoskeletal protein that has an essential function in maintaining the structural integrity of the epidermis (Bragulla and Homberger, 2009; Sakamoto *et al.*, 2011) and is regulated by retinoids in human keratinocytes (Törmä, 2011). Downregulation of *KRT4* is consistent with its cellular assembly and organisational functions, which are affected in poor donors, suggesting impaired follicular development due to a lack of retinoid signalling. In dairy cows, lower *KRT4* gene expression and a decrease in retinoids in granulosa cells have also been associated with a strongly negative energy balance (Girard *et al.*, 2015a).

In summary, we observed two distinct physiological states in follicles in association with early and late collection of oocytes, both compromising oocyte developmental competence. Another cause of poor oocyte quality observed in some cows is a major deficiency in lipid metabolism. By identifying suitable markers of follicle status, and hence oocyte developmental competence, animal reproductive health status could be monitored and suitable adjustments could be made to the next ovarian stimulation or FSH coasting period in order to increase the yield of healthy blastocyst embryos.

3.7. Declaration of interest

Authors declare no potential conflict of interests.

3.8. Funding

This work was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) – Collaborative Research and Development (grant no. RDCPJ461697-13). D. A. Landry received studentship support from NSERC and REDIH (CIHR (Canadian Institutes of Health Research) Training Program in Reproduction, Early Development, and Impact on Health).

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3.10. Figures

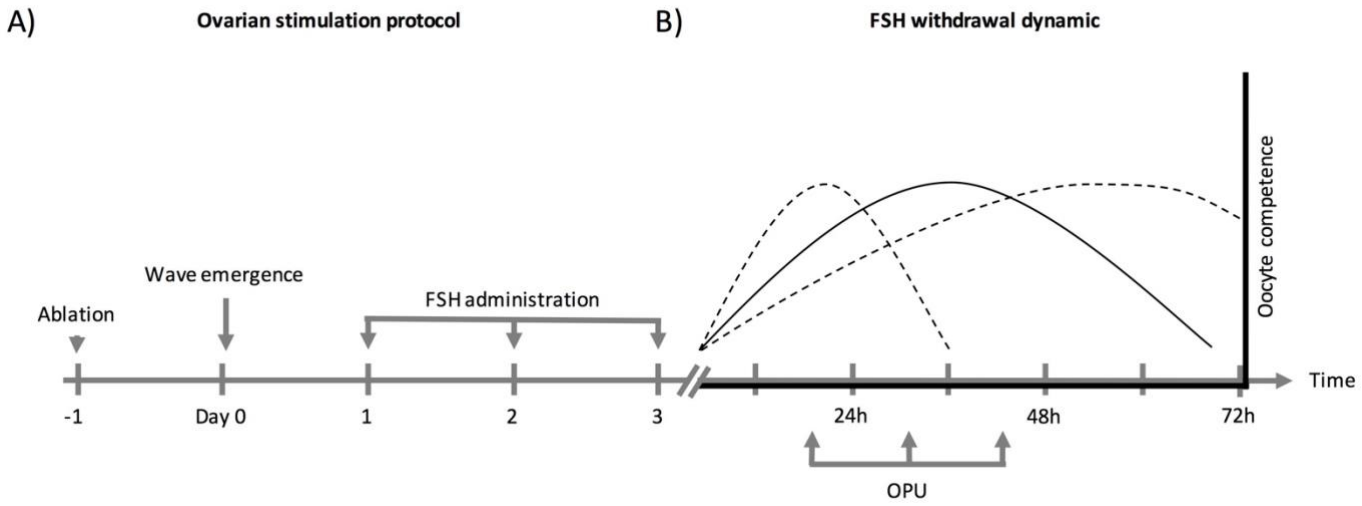

Figure 3-1: Ovarian stimulation regimen and coasting dynamic in dairy cattle

(*a*) Timing of FSH injections for ovarian stimulation in Holstein cows (*Bos taurus*) following follicle ablation by ultrasound. (*b*) Oocyte developmental competence observed previously as a function of time elapsed following the final FSH injection (coasting; Nivet *et al.*, 2012). The solid line represents the average and dashed lines represent individual variations. OPU, ovum pick-up.

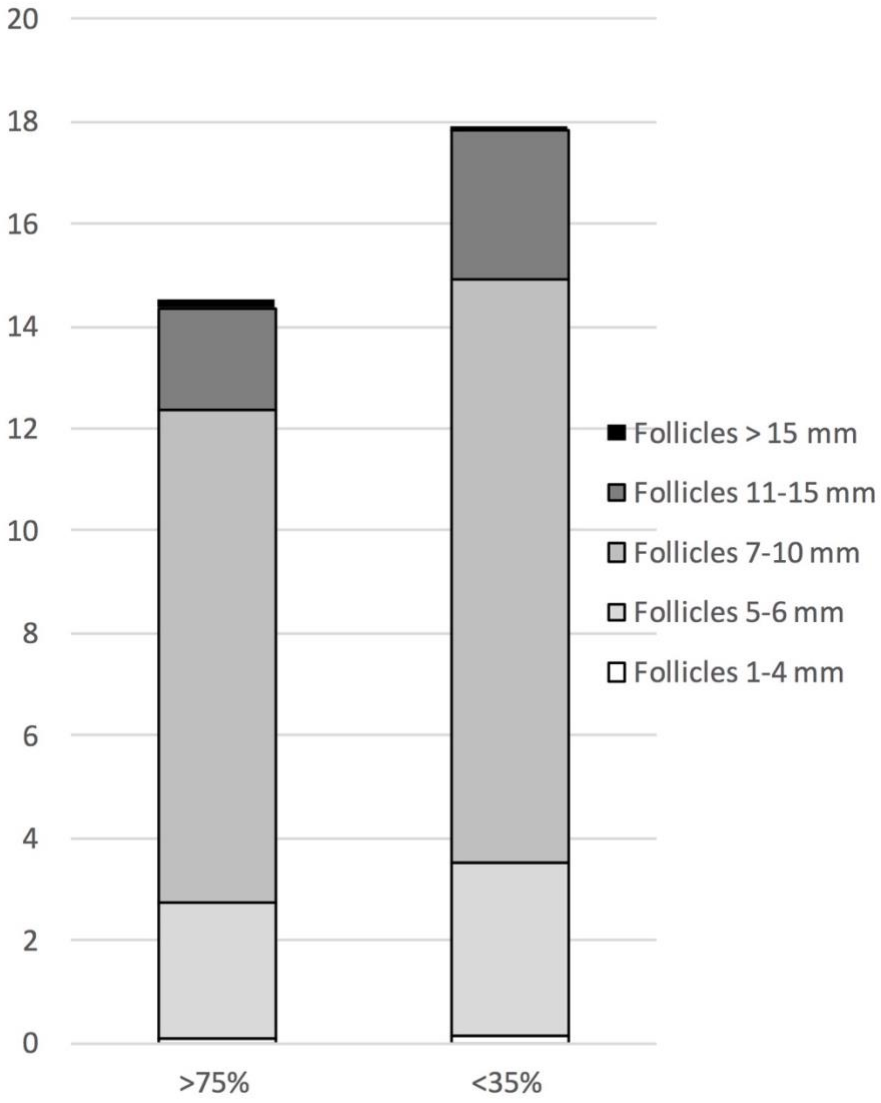

Figure 3-2: Follicle distribution in FSH-stimulated Holstein cattle

Number and size distribution of follicles in ovaries of cows rated as donors of competent or incompetent oocytes (>75% or <35% forming blastocysts respectively) after ovarian stimulation followed by a coasting period. There was no significant difference in the total number or the number in each size category between the two groups.

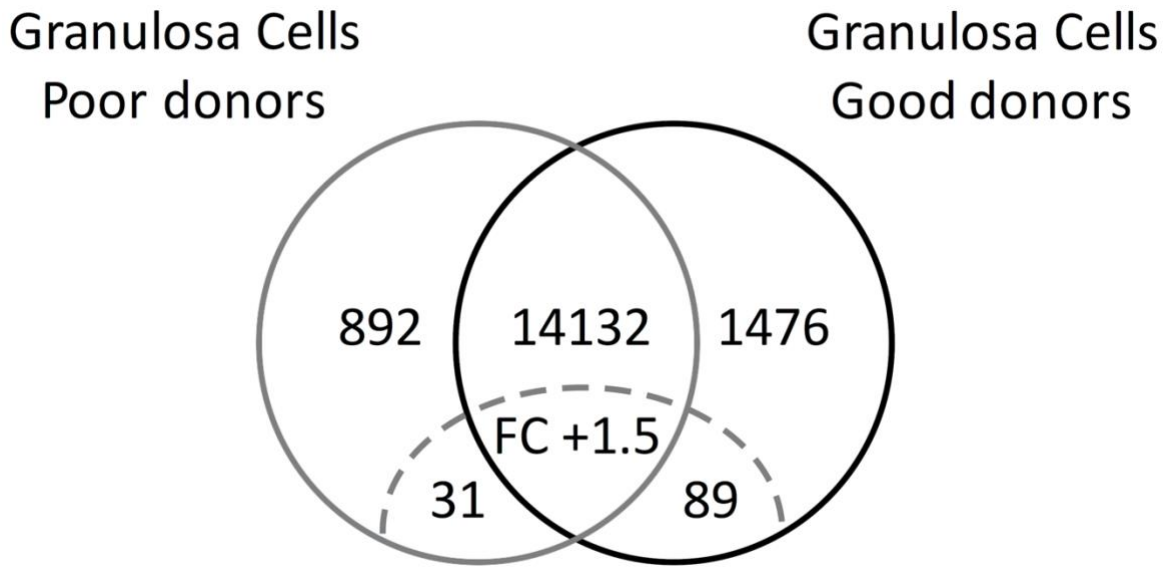

Figure 3-3: Venn diagram showing the number of genes detected in microarray

The number of genes expressed (microarray probe signal intensity above the defined threshold) in granulosa cells of ovarian follicles in FSH-stimulated Holstein cows rated as donors of competent or incompetent oocytes (good and poor donors respectively) are compared in a Venn diagram. The number of probes expressing at least a 1.5-fold change (FC +1.5 with $P < 0.05$) of the level noted in the other group appear at the bottom.

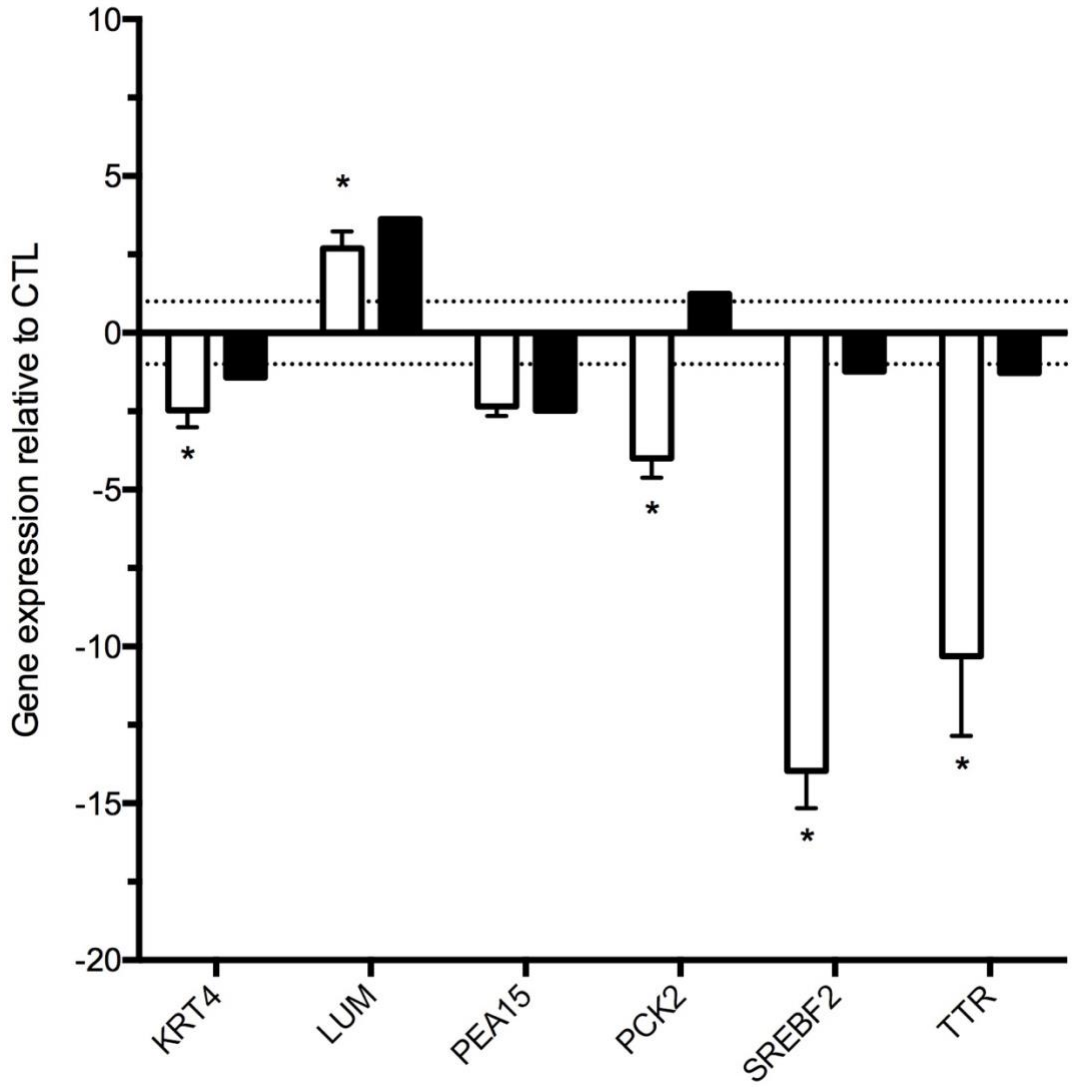

Figure 3-4: Expression of selected genes by FSH-stimulated Holstein cows rated as poor donors of competent oocytes relative to expression in cows rated as good donors

Genes were quantified by real-time quantitative polymerase chain reaction (qPCR; open columns) and microarray probes (filled columns). Real-time qPCR values are given as the mean ± s.e.m. Asterisks indicate significant differences in real-time qPCR values between poor and good donors ($P < 0.05$). The dashed lines represent a fold change of 1.5. *KRT4*, keratin 4; *LUM*, lumican; *PCK2*, phosphoenolpyruvate carboxykinase 2; *PEA15*, phosphoprotein enriched in astrocytes 15; *SREBF2*, sterol regulatory element binding transcription factor 2; *TTR*, transthyretin.

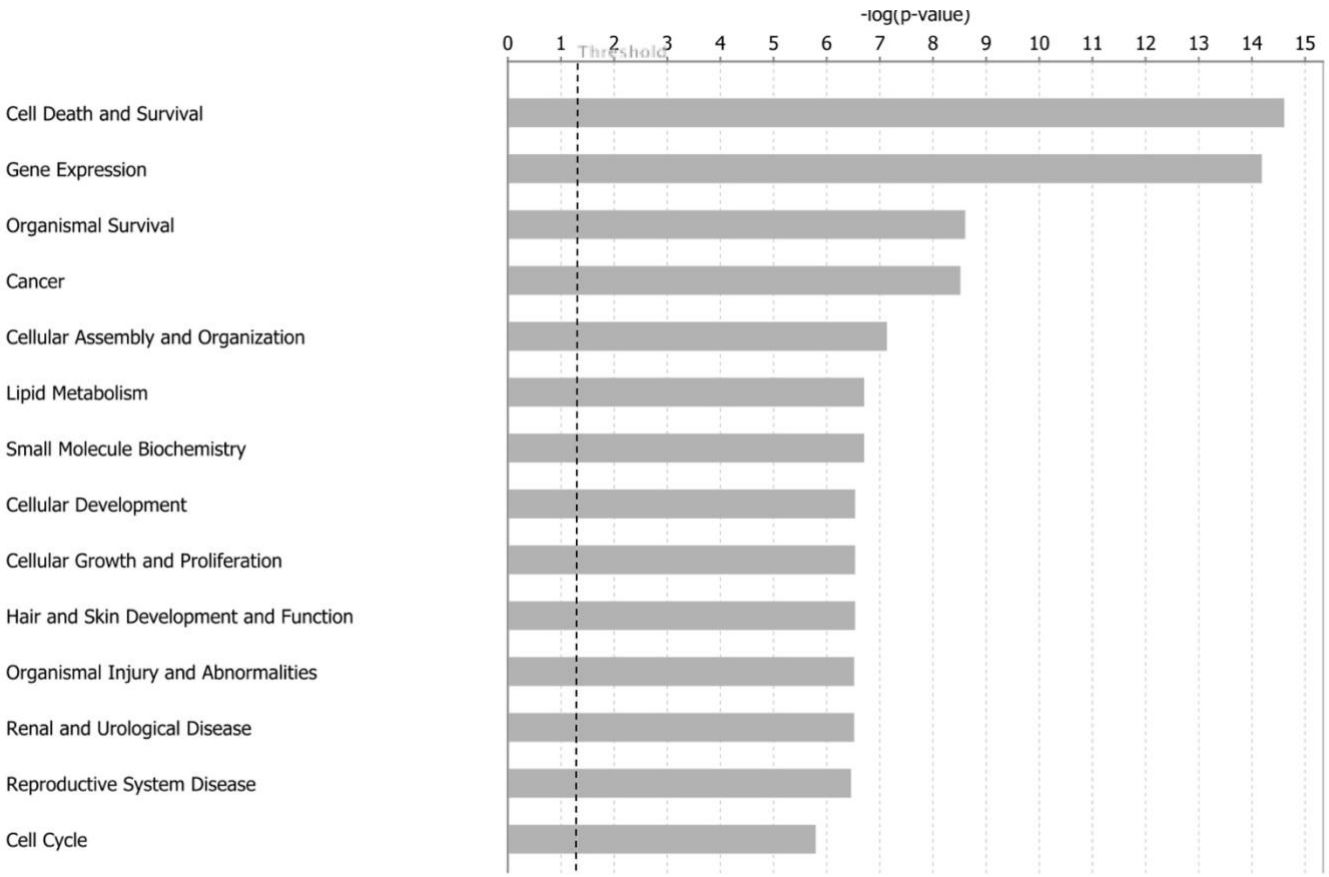

Figure 3-5: Biological functions in granulosa cells associated with incompetent oocyte

The 14 most significant biological functions in granulosa cells of FSH-stimulated Holstein cows rated as poor donors of competent oocytes, as determined by ingenuity pathway analysis and ranked by statistical significance (*P*-value). The dashed line represents the threshold for a significant *P*-value.

3.11. Tables

Table 3-1: Primers used in real-time polymerase chain reaction experiments

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *ACTB*, β-actin; *KRT4*, keratin 4; *LUM*, lumican; *PEA15*, phosphoprotein enriched in astrocytes 15; *PCK2*, phosphoenolpyruvate carboxykinase 2; *SREBF2*, sterol regulatory element binding transcription factor 2; *TTR*, transthyretin

Gene Symbol	Primer sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	GenBank Accession no.
GAPDH	Forward: CCAACGTGTCTGTTGTGGATCTGA	218	57	NM_001034034.2
	Reverse: GAGCTTGACAAAGTGGTCGTTGAG			
ACTB	Forward: ATCGTCCACCGCAAATGCTTCT	102	57	NM_173979.3
	Reverse: GCCATGCCAATCTCATCTCGTT			
KRT4	Forward: TCTGCTTCCTTCACCTCTAC	230	57	NM_001098385.1
	Reverse: GGGACACATGACCCTGATAA			
LUM	Forward: TAGTCTGCCACCTGATATGT	250	57	XM_005206042.2
	Reverse: GCCTGCCTTTCATCTTCTC			
PEA15	Forward: AGCCACAACAAGCTGGACAA	174	57	NM_001075456.1
	Reverse: GTACTTCTTGGCACTGGGGA			
PCK2	Forward: CCATTGACGCCATCATCTTTG	272	57	NM_001205594.1
	Reverse: GAACCAGTTGACGTGGAAGA			
SREBF2	Forward: TAATTGTCCTGTTCCTTCCC	306	57	NM_001205600.1
	Reverse: CTTCGATACTAGTGCCCATC			
TTR	Forward: CTCCTTTGTCTCGCTGGACT	229	57	NM_173967.3
	Reverse: ATTTGTCCTCTGTGGTGAGCC			

Table 3-2: Fifty most differentially expressed canonical pathways in granulosa cells from FSH-stimulated Holstein cows rated as poor donors of competent oocytes

The statistical significance of the pathways, their ratio (i.e. the number of differentially expressed genes that map to the pathway divided by the total number of genes that map to the canonical pathway) and *Z*-score, as identified by ingenuity pathway analysis, are also shown. CCR3, C-C motif chemokine receptor 3; CXCR4, C-X-C chemokine receptor type 4; ERK, extracellular signal-regulated kinase; FLT3, Fms related tyrosine kinase 3; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GnRH, gonadotrophin-releasing hormone; HGF, hepatocyte growth factor; HIF1α, hypoxia-inducible factor-1α; IGF1, insulin-like growth factor 1; IL, interleukin; JAK, Janus tyrosine kinases; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB; NRF2, nuclear factor E2 related factor 2; PAK, p-21 activated kinase; PI3K, phosphatidylinositol 3-kinase; PPARα, peroxisome proliferator-activated receptor α; RAR, retinoic acid receptor; RXR, retinoid X receptor; STAT, signal transducers and activators of transcription; TR, T-cell receptor

Ingenuity canonical pathways	–log(*P*-value)	Ratio	*Z*-score
Protein ubiquitination pathway	3.8700	0.1350	
mTOR signalling	3.6900	0.1440	–0.626
Wnt/β-catenin signalling	3.4900	0.1470	–0.775
Pancreatic adenocarcinoma signalling	3.3200	0.1670	–1.291
Aryl hydrocarbon receptor signalling	3.2900	0.1510	–1.500
14-3-3-mediated signalling	3.2300	0.1600	
P2Y purinergic receptor signalling pathway	3.2100	0.1550	
CCR3 signalling in eosinophils	3.1000	0.1560	
ERK/MAPK signalling	3.0800	0.1360	
Protein kinase A signalling	3.0100	0.1130	
Relaxin signalling	2.9400	0.1450	
Breast cancer regulation by stathmin1	2.8800	0.1320	
Renal cell carcinoma signalling	2.8200	0.1780	–1.414
Oestrogen-dependent breast cancer signalling	2.7300	0.1820	
Androgen signalling	2.6300	0.1490	–0.577
p70S6K signalling	2.5800	0.1440	–1.000
Erythropoietin signalling	2.5100	0.1710	
Gap junction signalling	2.4900	0.1310	
PI3K/AKT signalling	2.4600	0.1410	0.943
CXCR4 signalling	2.4100	0.1310	–1.091
JAK/STAT signalling	2.4000	0.1670	–1.732
PAK signalling	2.3800	0.1540	–0.535
Phospholipase C signalling	2.3700	0.1170	–0.816
LPS-stimulated MAPK signalling	2.3500	0.1640	–0.577
RAR activation	2.3500	0.1260	
TR/RXR activation	2.3400	0.1520	
PPARα/RXRα activation	2.3200	0.1250	0.471

NF-κB activation by viruses	2.3000	0.1620	–1.155
Hypoxia signalling in the cardiovascular system	2.3000	0.1690	
Ceramide signalling	2.2700	0.1550	–1.387
Endothelin-1 signalling	2.2600	0.1240	–1.279
Thrombin signalling	2.2600	0.1220	–1.279
Prostate cancer signalling	2.1800	0.1510	
NRF2-mediated oxidative stress response	2.1300	0.1220	–0.302
Renin–angiotensin signalling	2.1000	0.1360	–0.500
Signalling by Rho family GTPases	2.1000	0.1140	–1.043
HGF signalling	2.0900	0.1390	–0.535
p53 signalling	2.0900	0.1430	
IGF1 signalling	2.0600	0.1410	–1.667
IL-15 production	2.0200	0.2220	
IL-3 signalling	2.0100	0.1550	
UVB-induced MAPK signalling	1.9800	0.1700	–0.707
IL-8 signalling	1.9400	0.1180	–1.279
Growth hormone signalling	1.9200	0.1510	0.000
Neuregulin signalling	1.9100	0.1400	–1.265
GnRH signalling	1.8900	0.1260	–0.243
FLT3 signalling in haematopoietic progenitor cells	1.8700	0.1490	
HIF1α signalling	1.8700	0.1350	
fMLP signalling in neutrophils	1.8500	0.1300	–0.258

Table 3-3: Predicted upstream regulators in granulosa cells from FSH-stimulated Holstein cows rated as poor donors of competent oocytes

The expression differential (based on microarray probe), molecule type, predicted activation state, *Z*-score and statistical significance (*P*-value of overlap between the dataset and the genes that are regulated by the upstream), as identified by ingenuity pathway analysis, are also shown. CYP7A1, cytochrome P450 family 7 subfamily A member 1; EGF, epidermal growth factor; INSIG1, insulin-induced gene 1; INSIG2, insulin-induced gene 2; INSR, insulin receptor; JAK1, janus kinase 1; MAP4K4, mitogen-activated protein kinase kinase kinase kinase 4; MKL2, myocardin like 2; MMP14, matrix metallopeptidase 14; NPC1, intracellular cholesterol transporter 1; NRG1, neuregulin 1; POR, cytochrome P450 oxidoreductase; PPARGC1B,peroxisome proliferator-activated receptor gamma, coactivator 1 beta; PTEN, phosphatase and tensin homologue; SCAP, SREBF cleavage-activating protein; SREBF1, sterol regulatory element binding transcription factor 1.

Upstream regulator	Fold change	Molecule type	Predicted activation state	Activation *Z*-score	$P_{overlap}$
INSIG1	–1.165	Other	Activated	3.069	0.000 802 000
MAP4K4		Kinase	Activated	2.706	0.000 038 100
NRG1	1.095	Growth factor	Activated	2.447	0.000 519 000
INSIG2	1.063	Other	Activated	2.384	0.000 214 000
NPC1	1.239	Transporter	Activated	2.144	0.001 440 000
POR	–1.241	Enzyme	Activated	2.060	0.000 000 558
PTEN	1.208	Phosphatase	Activated	2.046	0.005 870 000
MKL2	–1.155	Transcription regulator	Activated	2.000	0.017 300 000
Lipid		Chemical: endogenous mammalian	Activated	2.000	0.031 400 000
Hydrogen peroxide		Chemical: endogenous mammalian	Inhibited	–2.029	0.000 037 300
JAK1	–1.052	Kinase	Inhibited	–2.200	0.041 600 000
MMP14	–1.287	Peptidase	Inhibited	–2.213	0.031 400 000
Adenosine triphosphate7B	–1.018	Transporter	Inhibited	–2.333	0.000 136 000
EGF	–1.076	Growth factor	Inhibited	–2.432	0.000 337 000
CYP7A1		Enzyme	Inhibited	–2.433	0.000 214 000
INSR	–1.134	Kinase	Inhibited	–2.454	0.000 000 006
Testosterone propionate		Chemical drug	Inhibited	–2.616	0.026 800 000
Tretinoin		Chemical: endogenous mammalian	Inhibited	–2.637	0.000 817 000
PPARGC1B		Transcription regulator	Inhibited	–2.646	0.009 680 000
SREBF1	–1.482	Transcription regulator	Inhibited	–2.652	0.011 600 000
SCAP	–1.003	Other	Inhibited	–3.262	0.000 475 000

Supplemental data

Supplemental Table 3-1: Animal selection for the microarray analysis

Follicle sizes, total number of aspirated follicles, number of oocytes in maturation and number of viable embryos (blastocyst) are reported. The percentage of embryos refers to the ratio between the number of oocytes in maturation and the number of viable embryos.

OPU Date	Cow I.D	Age at OPU	SPO Program	Coasting Duration	Follicles 1-4 mm	Follicles 5-6 mm	Follicles 7-10 mm	Follicles 11-15 mm	Follicles > 15 mm	Total Aspirated Follicles	Oocytes in Maturation	Number of Viable Embryos	Embryos (0%)
	Pool #1												
2014-02-18	Good1	14 months	P150F	30	0	1	6	3	0	10	5	4	80
2014-02-18	Good2	14 months	P150F	19	0	1	13	2	0	16	11	8	73
2014-02-04	Good3	14 months	P150F	30	0	1	2	6	0	9	9	7	78
2013-12-03	Good4	3 years	P240F	43	0	0	5	5	2	12	5	4	80
Average		**19.5**	-	-	**0**	**0.75**	**6.5**	**4**	**0.5**	**11.75**	**7.5**	**5.75**	**78**
	Pool #2												
2014-10-27	Good1	21 months	P180F	43	1	3	8	5	0	17	12	9	75
2014-10-27	Good2	3 years	P240F	43	0	5	15	0	0	20	11	9	82
2014-11-10	Good3	21 months	P180F	43	0	2	18	2	0	22	23	20	87
2014-12-01	Good4	23 months	P180F	43	0	7	12	1	0	20	17	13	76
Average		**25.25**	-	-	**0.25**	**4.25**	**13.25**	**2**	**0**	**19.75**	**15.75**	**12.75**	**80**
	Pool #3												
2014-02-11	Good1	16 months	P150F	30	0	6	14	0	0	20	12	10	83
2014-12-08	Good2	15 months	P180F	30	0	6	8	2	0	16	11	8	73
2014-12-15	Good3	8 years	P240F	30	0	4	10	0	0	14	7	6	86
2014-12-15	Good4	18 months	P180F	43	0	1	3	2	0	6	4	3	75
Average		**36.25**	-	-	**0**	**4.25**	**8.75**	**1**	**0**	**14**	**8.5**	**6.75**	**79**
	Pool #4												
2014-10-20	Good1	8 years	P240F	30	0	0	8	2	0	10	5	5	100
2014-11-24	Good2	16 months	P180F	43	0	5	11	0	0	16	10	7	70
2014-11-24	Good3	8 years	P240F	30	0	4	9	1	0	14	9	7	78
2014-02-04	Good4	13 months	P150F	30	0	3	19	0	0	22	18	13	72
Average		**55.25**	-	-	**0**	**3**	**11.75**	**0.75**	**0**	**15.5**	**10.5**	**8**	**80**
	Pool #5												
2013-12-10	Poor1	12 months	P150F	30	0	0	9	2	0	11	11	3	27
2014-12-08	Poor2	22 months	P180F	43	0	1	8	1	0	10	7	2	29
2014-10-20	Poor3	13 months	P180F	30	0	1	9	2	0	12	10	1	10
2014-10-27	Poor4	6 years	P240F	43	0	13	39	0	0	52	41	9	22
Average		**29.75**	-	-	**0**	**3.75**	**16.25**	**1.25**	**0**	**21.25**	**17.25**	**3.75**	**22**
	Pool #6												
2014-11-24	Poor1	22 months	P180F	43	0	5	15	5	0	25	21	6	29
2014-12-01	Poor2	5 years	P240F	43	1	1	5	1	0	8	6	0	0
2013-12-10	Poor3	13 months	P150F	30	0	4	19	8	0	31	23	7	30
2014-12-15	Poor4	18 months	P180F	43	0	2	7	4	0	13	12	3	25
Average		**28.25**	-	-	**0.25**	**3**	**11.5**	**4.5**	**0**	**19.25**	**15.5**	**4**	**21**

	Pool #7												
2014-11-10	Poor1	21 months	P180F	43	2	2	1	0	0	5	7	2	29
2014-12-08	Poor2	3 years	P240F	43	0	6	12	4	0	22	14	3	21
2014-11-10	Poor3	6 years	P240F	43	0	15	38	8	0	61	43	15	35
2014-11-10	Poor4	15 months	P180F	30	0	1	4	3	0	8	5	0	0
Average		**36**	-	-	**0.5**	**6**	**13.75**	**3.75**	**0**	**24**	**17.25**	**5**	**21**
	Pool #8												
2014-10-20	Poor1	3 years	P240F	43	0	0	12	6	0	18	12	3	25
2013-12-10	Poor2	13 months	P150F	30	0	3	11	3	0	17	16	5	31
2013-12-10	Poor3	16 months	P150F	30	0	0	6	3	0	9	5	0	0
2014-11-24	Poor4	3 years	P240F	43	0	7	11	1	0	19	15	4	27
Average		**25.25**	-	-	**0**	**2.5**	**10**	**3.25**	**0**	**15.75**	**12**	**3**	**21**
ANOVA		-	-	-	N.S	N.S	N.S	N.S	N.S	N.S	N.S	(P<0.05)	(P<0.05)

Supplemental Table 3-2: Differentially expressed genes in the comparison of poor versus good donors

List of the 72 genes differentially expressed (fc ≥ 1.5, p < 0.05) with the raw symmetrical fold-change and statistical significance (p-value).

Gene Symbol	Fold change	P-value	Gene Symbol	Fold change	P-value
LUM	3.627317	0.002474616	JUN	-1.567378	0.01911778
GPX6	2.556556	0.02261067	TRPM6	-1.593232	0.0300385
RAB38	1.931243	0.000230169	TMEM79	-1.593997	0.03413238
CARTPT	1.925002	0.01329396	ABCA3	-1.604337	0.000738508
IGSF11	1.900717	0.000136452	MMP7	-1.608459	0.000641934
SLC38A2	1.83982	0.000765694	SETX	-1.630486	0.005143528
USP50	1.833847	0.01008011	CHST4	-1.646124	0.02726936
CLU	1.710803	0.03357212	KIAA1737	-1.650971	0.003634006
MAL2	1.660993	0.000405846	WWC1	-1.659587	0.003127952
HMCN1	1.648876	0.007514724	SERPINB4	-1.660379	0.01873367
CDH2	1.641244	0.02838836	RUNX1T1	-1.676873	0.02851859
CCNB1IP1	1.620082	0.000244699	GIMAP6	-1.67727	0.03265761
TMEFF2	1.575506	0.0164174	PLA2G6	-1.688972	0.000105436
ZNF391	1.557867	0.01001603	INHA	-1.689603	0.01086921
FEZ1	1.531702	0.004950878	CLEC2L	-1.707389	0.002858025
RGS7	1.510848	0.001700443	PIGR	-1.712788	0.00338853
FSD1	1.505096	0.03899792	NUDT4	-1.752517	0.009658898
SLC37A1	-1.503263	0.004494458	C28H10orf57	-1.75681	0.001520848
TMUB1	-1.50387	0.004922629	ZNF295	-1.794232	0.007276165
UPLP	-1.507879	0.01948026	FGFR2	-1.798736	0.01002178
RARA	-1.508098	0.01500485	PLAC8	-1.815297	0.008775145
IFI27L2	-1.509744	0.001927701	KRAS	-1.82904	4.08E-05
SLC30A7	-1.519336	0.005060526	VNN2	-1.867502	0.02137052
CHADL	-1.519963	0.000379435	ABHD8	-1.873638	0.001373457
MECP2	-1.525504	0.000928258	MGST2	-1.887881	0.01275033
IMPACT	-1.526818	0.01920168	PACSIN1	-1.928247	9.60E-05
NT5M	-1.529137	0.000434609	IGLL1	-1.969435	0.01641942
PDE6G	-1.540464	0.009066276	SERPINA1	-2.100363	0.03108778
EML2	-1.540464	0.04889933	BOLA	-2.104153	0.008118577
TNRC6A	-1.54114	0.01155914	BoLA	-2.244453	0.01292849
PLAC9	-1.542396	0.002436764	DMBT1	-2.344823	0.01072773
OGDH	-1.543249	0.00050238	PEA15	-2.48329	0.007978792
POFUT2	-1.545419	0.0284294	JSP.1	-2.575469	0.006995262

TRAPPC9	-1.54944	0.005462395	BOLA-N	-2.732387	0.006076131
CEACAM19	-1.553534	0.01446992	LY6G6C	-2.815814	0.001143032
TGFB3	-1.553553	0.03002349	PTP4A3	-2.850533	0.002682053

4. Effect of heifer age on the granulosa cell transcriptome after ovarian stimulation

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Cet article a été publié dans la revue « Reproduction, Fertility and Development» sous la référence suivante :
Landry D.A., Labrecque R., Grand F.X., Vigneault C., Blondin P. and Sirard M.A. (2017) Effect of heifer age on the granulosa cell transcriptome after ovarian stimulation. Reproduction, Fertility and Development, 30 (7), 980-990.

4.1.Résumé

La sélection génomique accélère considérablement le gain génomique chez la vache laitière. La diminution du temps entre les générations en utilisant des gamètes de jeunes donneuses accélère davantage les programmes d'amélioration génétique. Même si la stimulation ovarienne est possible sans aucun inconvénient chez les donneuses pré-pubère ayant des embryons in vitro viables, la compétence au développement des ovocytes est plus faible que chez les vaches sexuellement matures. Le but de cette étude est de démontrer à quel moment la compétence au développement des ovocytes est acquise en fonction de l'âge de la génisse. Dix génisses Bos Taurus Holstein péri-pubères ont subi une stimulation ovarienne avec coasting à l'âge de 8, 11 (moyenne de 10.8) et 14 (moyenne de 13.7) mois. Les ovocytes récoltés furent fécondés avec la semence d'un seul taureau, ainsi chaque génisse fut le contrôle d'elle-même. L'analyse du transcriptome des cellules de la granulosa récoltées lors de la récolte des ovocytes fut analysée en micropuce et l'expression différentielle des gènes fut mesurée en PCR en temps réel. L'analyse des principales composantes des résultats de micropuce ont démontré que l'expression des gènes est différente chez les jeunes donneuses. De plus, en utilisant ''Ingenuity Pathway Analysis'' et ''NetworkAnalyst'' il a été possible d'identifier les principales fonctions biologiques qui sont affectées chez les jeunes donneuses. Nos résultats suggèrent que la différenciation cellulaire, la signalisation de l'inflammation et de l'apoptose sont moins apparentes chez les donneuses péri-pubères. Ces traits physiologiques ont été associés à une faible concentration basale d'hormone lutéinisante.

4.2. Abstract

Genomic selection is accelerating genetic gain in dairy cattle. Decreasing generation time by using younger gamete donors would further accelerate breed improvement programs. Although ovarian stimulation of peripubertal animals is possible and embryos produced *in vitro* from the resulting oocytes are viable, developmental competence is lower than when sexually mature cows are used. The aim of the present study was to shed light on how oocyte developmental competence is acquired as a heifer age. Ten peripubertal *Bos taurus* Holstein heifers underwent ovarian stimulation cycles at the ages of 8, 11 (mean 10.8) and 14 (mean 13.7) months. Collected oocytes were fertilised *in vitro* with spermatozoa from the same adult male. Each heifer served as its own control. The transcriptomes of granulosa cells recovered with the oocytes were analysed using microarrays. Differential expression of certain genes was measured using polymerase chain reaction. Principal component analysis of microarray data revealed that the younger the animal, the more distinctive the gene expression pattern. Using ingenuity pathway analysis (IPA) and NetworkAnalyst (www.networkanalyst.ca), the main biological functions affected in younger donors were identified. The results suggest that cell differentiation, inflammation and apoptosis signalling are less apparent in peripubertal donors. Such physiological traits have been associated with a lower basal concentration of LH.

4.3. Introduction

Ovarian stimulation (OS), *in vitro* embryo production and embryo transfer (ET) are among the assisted reproduction technologies (ARTs) that have been improving genetic gain in dairy cattle. Over the past decade, the development of juvenile ET programs has significantly accelerated genetic enhancement by compressing the time interval between generations (García-Ruiz *et al.*, 2016). It is well established that ovarian stimulation, ET and pregnancy can be achieved in peripubertal cattle without detrimental effects on physiology, on the onset of puberty or on subsequent reproductive and lactation performance (Armstrong *et al.*, 1992; Khatir *et al.*, 1998; Majerus *et al.*, 1999; Ax *et al.*, 2005).

In *Bos taurus*, puberty is characterised by specific responses of the hypothalamus and pituitary gland to gonadal steroids and by a subsequent increase in pulsatile secretion of LH leading to the first ovulation (Rodriguez and Wise, 1989; Day and Nogueira, 2013). The onset of puberty in dairy heifers is strongly affected by genetic and environmental factors, as well as by food quality and availability, and occurs generally around 9–12 months of age in temperate climates (Rodriguez and Wise, 1989; Fajersson *et al.*, 1991).

Although ovarian stimulation is possible in peripubertal animals, it is well documented that calf oocytes are less competent than oocytes from sexually mature cows (Gandolfi *et al.*, 1998; Salamone *et al.*, 2001; Kauffold *et al.*, 2005; Landry *et al.*, 2016). It has been demonstrated that oocytes from heifers older than 10 months have similar developmental competence and yield transferable embryos in numbers comparable to those from postpubertal cyclic animals (16–18 months old), whereas those from younger animals (6–9 months old) yield fewer embryos (Ax *et al.*, 2005). In a retrospective comparison with sexually mature cows, we observed only a slight difference: similar developmental competence of oocytes from heifers beginning at 11 months of age. We also demonstrated that younger donors (5–9 months old) had greater numbers of total follicles but did not provide greater numbers of blastocysts, resulting in significantly lower embryo yields compared with sexually mature cows (Landry *et al.*, 2016).

Several research groups have suggested that oocytes obtained from younger donors have altered patterns of protein expression and poor ooplasm quality. In general, calf oocytes present a protein pattern similar to that of defective cow oocytes and cumulus cells (Lévesque and Sirard, 1994), and a significant decrease in protein synthesis and incoherent protein expression patterns compared with adult oocytes (Gandolfi *et al.*, 1998). Transferring calf MII chromosomes into adult ooplasm results in more competent embryos than does transferring adult MII chromosomes into calf oocytes (Salamone *et al.*, 2001). These findings suggest compromised cytoplasmic and molecular maturation in oocytes from younger donors and that follicular cells appear to play a central role in oocyte maturation (Sirard *et al.*, 2006).

The aim of the present study was to characterise the changes that occur in the granulosa cell transcriptome as a heifer grows and to relate these to physiological changes that underlie the success of assisted reproduction in bovine. To determine whether differences observable in follicles are associated with age, we compared the effects of ovarian stimulation on granulosa cells recovered from the same individual animals aged 8, 11 (peripubertal stage) and 14 months (after puberty). Based on our analysis, we suggest that biological functions associated primarily with cell differentiation, cell survival and death, inflammation and apoptosis signalling are disrupted in younger heifers and that this is possibly a consequence of lower basal LH.

4.4.Materials and methods

4.4.1. Ethics statement

The clinical procedures and industrial practices used in Boviteq follow the established cattle reproduction management practices, which have been approved by the College of Veterinary Surgeons of Quebec (OMVQ), the Canadian Embryo Transfer Association (CETA) and the International Embryo Transfer Society (IETS). This company follows the Canadian Council on Animal Care (CCAC) guidelines for farm animals and the research projects do not involve the use of exclusive animals for research purposes, and nor do they involve the implementation of new animal procedures to obtain additional biological samples other than

the ones used in their routine commercial activities. This study did not require handling animals on university premises.

4.4.2. Chemicals

All reagents and media supplements used in the present study were of tissue culture grade and were obtained from Sigma-Aldrich, unless specified otherwise.

4.4.3. Animals

Prepubertal *B. taurus* Holstein heifers (n = 10) were used for this study. Each heifer underwent three ovarian stimulation cycles, allowing three gamete collections, at the ages of 8, 11 (mean 10.8) and 14 (mean 13.7) months. The gametes were fertilised *in vitro* with spermatozoa from a single collection and freezing batch of one adult bull. Each heifer was its own control for the measurement of age effects. The steps involving live animals were performed in a commercial IVF setting, namely at Boviteq, a centre specialised in bovine embryo transfer and other ARTs involved in research and development.

4.4.4. Ovarian stimulation and gamete collection

The protocol for ovarian stimulation and ovum pick-up was essentially as described previously (Nivet *et al.*, 2013). Briefly, each heifer was first treated with progesterone (Zoetis) in order to repress dominant follicles and thereby reduce the risk of spontaneous ovulation. The dominant follicle (diameter ≥5 mm) was aspirated 36 h before administration of hormones. The ovarian stimulation program consisted of six injections of 30 mg FSH (Folltropin-V; Vetoquinol N.-A) administered at 12-h intervals. Ovum pick-up was performed 43 h after the final FSH injection, this being the optimal length of the FSH withdrawal or coasting period in mature animals (Nivet *et al.*, 2012). Using transvaginal ultrasonography, follicular diameters were measured and cumulus–oocyte complexes (COCs) were collected by ovum pick-up under epidural anaesthesia (COOK Medical). Granulosa cells and COCs were collected in warm HEPES-buffered Tyrode's medium (TLH) containing Hepalean (10 IU mL^{-1}) and transferred to the laboratory for IVM. These procedures were performed from October 2014 through May 2015.

4.4.5. IVM

COCs were placed in TLH supplemented with 10% bovine serum, 0.2 mM pyruvate and 50 mg mL^{-1} gentamicin and washed three times with TLH to remove follicular fluid. Healthy COCs were placed in droplets (50 µL) of maturation medium under mineral oil. Maturation medium was composed of TCM199 (Invitrogen Life Technologies), 10% fetal bovine serum (FBS, Wisent Bioproducts), 0.2 mM pyruvate, 50 mg mL^{-1} gentamicin, 5 mg mL^{-1} FSH (Folltropin-V, Bioniche Animal Health, Belleville, Ontario, Canada), 0.5 mg/mL LH (Lutropin; Bioniche) and 1 mg mL^{-1} oestradiol. Maturation droplets were incubated for 24 h at 38.5 °C with 5 % CO_2 in maximal humidity.

4.4.6. IVF

COCs incubated for 24 h were washed twice in TLH before being transferred in groups of five to droplets (48 µL) of medium under mineral oil. This medium consisted of modified Tyrode's lactate (TL) medium supplemented with 0.6% (w/v) fatty acid-free BSA, 0.2 mM pyruvic acid, 2 µg mL^{-1} heparin and 50 mg mL^{-1} gentamicin. Oocytes were transferred 15 min before semen addition, and 2 µL of a solution containing penicillamine (2 mM), hypotaurine (1 mM) and adrenaline (250 mM) was added to each droplet to stimulate sperm motility. Semen stored in liquid nitrogen (Centre d'Insémination Artificielle du Québec) was thawed for 1 min in water at 35.8°C, added to a discontinuous gradient (45–90%) of BoviPure (Nidacon Laboratories) and centrifuged at 600 *g* for 5 min at room temperature. The supernatant containing the cryoprotectant and dead spermatozoa was discarded and the pellet was resuspended in 1 mL modified TL, centrifuged at 300 *g* for 2 min at room temperature and resuspended for counting on a haemocytometer and then diluted with IVF medium to a count of 2.5×10^7 cells mL^{-1}. Finally, 2 µL of this suspension was added to each droplet containing matured COCs to a final concentration of 1×10^6 cells mL^{-1}. The fertilisation medium was incubated at 38.5 °C for 15–18 h in a humidified atmosphere of 95 % air and 5 % CO_2. Semen from a single bull and from a single ejaculate was used for all heifers in order to avoid male-associated bias.

4.4.7. In vitro culture

For *in vitro* culture (IVC), embryos were placed in groups of 10 in droplets (10 µL) of modified synthetic oviductal fluid (mSOF) with non-essential amino acids, 3 mM EDTA and 0.4 % fatty acid-free BSA (ICP Bio) under embryo-tested mineral oil (#8410; Sigma-Aldrich). The embryo culture dishes were incubated at 38.5 °C with 6.5 % CO_2, 5 % O_2, and 88.5 % N_2 in 100 % humidity. Embryos were transferred to fresh mSOF droplets containing non-essential and essential amino acids 72 h after fertilisation and to larger droplets (20 µL) 120 h after fertilisation in order to avoid effects due to nutrient depletion and accumulation of toxic levels of ammonium.

Follicle size, total number of aspirated follicles, number of oocytes placed in maturation, number of viable embryos and cleavage percentage were recorded for assessment of animal reproductive performance. These data were analysed using repeated-measures one-way analysis of variance (ANOVA) with Dunnett's post test in order to establish significance. Differences were considered to be statistically significant at the 95 % confidence level ($P < 0.05$, two-tailed)

4.4.8. RNA extraction and amplification

Total RNA was extracted from granulosa cells using the AllPrep DNA/RNA/miRNA Universal kit (Quiagen, Tonronto, ON, Canada). Total RNA integrity and concentration were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies) with the RNA PicoLab Chip (Agilent Technologies). Samples were amplified linearly to generate enough material for hybridisation. Antisense (a) RNA was produced using the RiboAmp®HS^{Plus} RNA Amplification kit (Thermo Fisher Scientific). After two amplification rounds of 6 h each, the aRNA output was quantified using the NanoDrop ND-1000 (Nano-Drop Technologies).

4.4.9. Sample labelling and microarray hybridisation

Transcriptome analysis was performed on granulosa cells from four heifers (Boviteq IDs 10989, 10991, 10994 and 10995; see Table S1, available as Supplemental Material to this

paper) using microarray hybridisation. Heifers were selected on the basis of the number of embryos after ovarian stimulation. For each sample, 2 µg aRNA was labelled using the ULS Fluorescent Labelling Kit for Agilent arrays (Cy3/Cy5; Kreatech Diagnostics). The labelled product was then purified with the Arcturus Pico-Pure RNA Isolation Kit (Applied Biosystems, ThermoFisher). Labelling efficiency was measured using the Nano-Drop ND-1000. Samples from the four biological replicates (heifers) were hybridised on the EmbryoGENE bovine 44 K microarray slide (Agilent) (Robert *et al.*, 2011). The hybridisations were performed according to the following design: the two peripubertal age periods were compared individually with the control age (i.e. 8 vs 14 months; and 11 vs 14 months) for a total of two comparisons per animal. Overall, eight hybridisations were performed, corresponding to the four biological replicates and the two comparisons. A total of 825 ng of each labelled aRNA sample (Cy3 and Cy5) was incubated in a solution containing 2× Blocking Agent and 1× Fragmentation Buffer (Agilent) in a volume of 55 µL at 60°C for 15 min and then immediately put on ice. Then, 55 µL of 2× GEx Hybridisation Buffer HI-RPM (Agilent) was added to bring the total volume to 110 µL. The hybridisation mix (100 µL) was added to the array and hybridisation was performed at 65°C for 17 h using an Agilent hybridisation chamber in a rotating oven. Slides were washed for 3 min at room temperature with Gene Expression Wash Buffer 1 containing 0.005% Triton X-102 and then for 3 min at 42°C with Gene Expression Wash Buffer 2 containing 0.005% Triton X-102. Final washes were performed with acetonitrile for 10 s at room temperature and then with Stabilisation and Drying Solution (Agilent) for 30 s at room temperature before slides were air dried. Slides were scanned using the Tecan PowerScanner microarray scanner (Tecan Group) and features were extracted using ArrayPro 6.4 (Media Cybernetics). Another study was performed in parallel with this one, with the same settings and animals, to compare the blastocyst transcriptome (Morin-Doré *et al.*, 2017).

4.4.10. Microarray data analysis

Microarray data were subjected to a simple background subtraction, normalised within array (Loess) and between arrays (quantile), and analysed statistically with the Limma package (BioC, R) using FlexArray version 1.6.1 (McGill University and Génome Québec). Differences were considered statistically significant for two-sided $P < 0.05$. Microarray data

were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE93703 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE93703, public on Jan 18, 2017).

A between-group analysis (BGA; Culhane *et al.*, 2002) using R software (R Foundation for Statistical Computing) was performed in order to separate the granulosa cell samples in relation to their groups, whereas the heat map was created using NetworkAnalyst (McGill) on normalized data (Xia *et al.*, 2015).

4.4.11. Functional analysis

FlexArray version 1.6.1 was used to perform functional enrichment analysis of specific gene lists based on a cut-off relative change in expression level. The gene lists were then analysed using ingenuity pathway analysis (IPA; Ingenuity Systems; http://www.ingenuity.com, accessed Oct 26, 2016) and NetworkAnalyst (Xia *et al.*, 2015). All statistically significant genes ($P < 0.05$; change greater than ±1.5-fold) were uploaded to the application and each identifier was mapped to its corresponding object in the respective database.

4.4.12. Preparation of cDNA and quantitative real-time polymerase chain reaction

To confirm microarray analysis results, real-time quantitative polymerase chain reaction (qPCR) was performed on cDNA. Briefly, RNA (507 ng) from granulosa cells (all samples) was reverse transcribed using a qScript Flex cDNA Synthesis Kit (Quanta Biosciences) with a mixture of oligo(dT) and random primers according to the manufacturer's recommendations. Five candidate genes were selected according to the differences in their expression levels compared with the reference condition and their relevance to embryo development processes: BCL2-like1 (*BCL2L1*), creatine kinase B (*CKB*), forkhead box A2 (*FOXA2*), tumour necrosis factor-α-induced protein 6 (*TNFAIP6*) and insulin-like growth factor 2 (*IGF2*). The primers used for real-time qPCR are listed in Table S2 and were designed using the IDT PrimerQuest tool (http://www.idtdna.com/primerquest/home/index,

accessed Mar 26, 2016) from sequences obtained using *B. taurus* (taxid: 9913) reference RNA sequences (refseq_rna) and results from our microarray analysis. To confirm the specificity of each pair of primers, electrophoresis on a standard 1.2% agarose gel was performed for each amplified fragment. The PCR products were then purified with the QIAquick PCR purification kit (Qiagen), quantified using the NanoDrop ND-1000 and sequenced. The products were then used to create standard curves for quantification experiments, with dilutions ranging from 2×10^{-4} to 2×10^{-8} ng nL^{-1}. Real-time qPCR was performed on a LightCycler 480 (Roche Diagnostics) using SYBR incorporation. Each real-time qPCR, in a final volume of 20 µL, contained the cDNA, 0.25 mM each primer and 1× SYBR mixture (Light-Cycler 480 SYBR Green I Master; Roche Diagnostics). The PCR conditions used for all genes were as follows: denaturing for 10 min at 95°C, followed by 50 PCR cycles of denaturing at 95°C for 10 s, annealing for 10 s (Table S2) and extension at 72°C for 20 s, with melting curve analysis at 95°C and a final cooling step at 40°C. cDNA was quantified using LightCycler 480 software version 1.5 (Roche Diagnostics) by comparison against the standard curves. Specificity of the PCR was confirmed by melting curve analysis provided by the LightCycler software.

4.4.13. Statistical analysis of real-time qPCR results

Analysis of gene expression stability in the two groups of granulosa cells (8 vs 14 months; 11 vs 14 months) from the different heifers was performed using GeNorm VBA applet software (BioGazelle, Belgium), as described previously (Vandesompele *et al.*, 2002). The most stable reference genes were identified by stepwise exclusion of the least stable gene and recalculating the M values. After GeNorm analysis, β-actin (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were designated as the most stable genes, with M values <1.5, as recommended in the software. Data normalisation was performed based on GeNorm normalisation using *ACTB* and *GAPDH* as reference genes (Vandesompele *et al.*, 2002). GraphPad Prism 6 (GraphPad Software) was used to evaluate differences in mRNA expression. Normalised data cleaned using the Rout method of outlier removal were analysed using paired-measures one-way ANOVA with Tukey's post test in order to establish significance. Differences were considered to be statistically significant at the 95% confidence level ($P < 0.05$, two-tailed). Values are presented as the mean ± s.e.m.

4.5. Results

4.5.1. Reproductive performance of animals

Follicle size, the numbers of follicles, number of oocytes placed in maturation, viable embryos (blastocysts) and embryo yield were noted for each animal that underwent ovarian stimulation followed by coasting. These data are presented for the four animals used in the microarray analysis in each age group in Table S1. Animal age had no significant effect on these values. All information related to reproductive performance from all 10 heifers at the three ages studied is shown in Figure 4-1 and 4-2. A repeated-measure one-way ANOVA between 8, 11 and 14 months of age showed no significant differences. Dunnett's multiple comparisons test revealed a tendency ($P = 0.079$) for lower embryo yield in younger animals (8 months) compared with the control (Figure 4-2). These results differ slightly from previous findings that embryo yield was significantly lower when comparing 8- but not 11-month-old animals with sexually mature animals (Landry *et al.*, 2016). However, the relatively small number of animals used in the present study may have attenuated the statistical significance of the effect of age. A power analysis suggests that the sample size should be at least $n = 53$ (power 0.8, $P = 0.05$, effect size 0.25) to detect a moderate significant change (QFAB Bioinformatics 2015; http://www.anzmtg.org/stats/PowerCalculator, accessed 24 Mar 2017).

4.5.2. Microarray analysis

BGA was performed based on the intensity data normalised within (Loess) and between (quantile) arrays of the contrasts of 8 versus 14 months and 11 versus 14 months (Figure 4-3a). Based on the signal intensity corresponding to each gene symbol, BGA analysis was used to isolate each animal in three groups accordingly to their age. One clear observation is that all animals in the 8-month-old group are further from the 14-month-old group compared with the 11-month-old group, which was closer to the control. The heat maps show more differences in terms of differentially expressed genes (DEG) in the 8 versus 14 month groups (Figure 4-3b) compared with the 11 versus 14 month groups (Figure 4-3c).

Microarray analysis of DEGs confirmed this observation. Based on the $P < 0.05$ and 1.5-fold criteria, there were 1421 and 1307 up- and downregulated genes in the comparison of 8 versus 14 months, compared with 435 up- and 342 downregulated genes in the comparison of 11 versus 14 months (Figure 4-4). Thus, the larger age difference increased the number of DEGs considerably.

4.5.3. Real-time qPCR validation

Microarray data were validated using real-time qPCR. Figure 4-5 shows the real-time qPCR validation values as mean relative expression compared with the reference group (14 months) and their statistical significance in the transcriptomic analysis and the real-time qPCR analysis. Five candidate genes were selected on the basis of differential expression relative to the control condition and relevance to granulosa cell function.

4.5.4. Functional analysis (IPA) and NetworkAnalyst

Uploading data into the IPA program allowed identification of upstream regulators, canonical pathways and biological functions (Figure 4-6). IPA upstream analysis is a powerful tool for characterising gene up- or downregulation with a specific trigger, such as a gene or compound, and calculates a *z*-score. The activation *z*-score is a statistical approach that determines whether an upstream regulator is determined by the regulation direction associated with the relationship from the regulator to the gene in the dataset. A positive *z*-score predicts that a regulator or a physiological activity is increased and a negative *z*-score predicts that it is decreased; *z*-scores ≥ 2 or ≤ -2 indicate that the trend is statistically significant. The *P*-value, calculated by Fisher's exact test, measures the likelihood that the apparent association between genes and a given function is random (due to chance). Finally, the upstream section of the core analysis was used to determine which upstream regulators in our contrasts were most likely to be stimulated or inhibited. These are shown in Table S3 for 8 versus 14 months and 11 versus 14 months.

NetworkAnalyst is a visual and network-based analysis of gene expression data (e.g. microarray) that allows investigation of the mechanism underlying or driving differences in

a complex dataset. Analysis of protein–protein interactions (PPI) by NetworkAnalyst was used to complement the IPA findings and to determine the biological processes associated with the major nodes (protein) and their interactions within the dataset.

Among the DEGs from both comparisons, a comparative analysis was performed to identify those that appear specifically in one comparison. Thus, we were able to identify specific DEGs associated with important pathways and upstream regulators that are peculiar to 8- and 11-month-old heifers compared with their references. Results of upstream regulator analysis for the comparison of 8 versus 14 months and 11 versus 14 months, as well as pathway analysis for the comparison of 8 versus 14 months are given in Table S4. Pathway analysis of DEGs in 11- compared with 14-month-old heifers showed no significant result.

4.6. Discussion

To obtain high-quality oocytes from young donors, it is important to understand the process that occurs in follicular cells during the acquisition of oocyte competence before and after puberty. In order to elucidate the interplay between the follicular environment before puberty and the lower oocyte competence observed in younger donors, we used genomics to investigate associations among the gene expression profiles of follicular cells aspirated from peripubertal heifers before and after puberty. This original approach allows observation of individual variations against a background of reduced genetic noise. The results thus obtained demonstrate that the gene expression profile of peripubertal donors differs from that observed in more mature donors.

The most obvious finding of the present study is the greater number of differentially expressed genes in the 8- versus 14-month-old comparison than in the 11- versus 14-month-old comparison. The distinction is also shown by BGA analysis, in which gene expression at 8 months of age appears much further from that of the same animal at 14 months of age (Figure 4-2). The apparent non-linearity in the disposition of the groups between the ages of 8, 11 and 14 months suggests there is no linear change in gene expression in granulosa cells before and after puberty. The smaller differences between 11 and 14 months shown in BGA and heat map analysis are in agreement with previous studies in which it was demonstrated

that animals 10–11 months old produced oocytes of quality similar to those obtained from sexually mature animals, unlike heifers <10 months old (Ax *et al.*, 2005; Landry *et al.*, 2016). Several researchers have also demonstrated a difference in oocyte developmental capability between calves and adult cows with or without ovarian stimulation (Looney *et al.*, 1995; Revel *et al.*, 1995; Presicce *et al.*, 1997; Khatir *et al.*, 1998; Kauffold *et al.*, 2005).

The use of bioinformatics software such as IPA and NetworkAnalyst to interpret the DEG profiles pointed to essential biological functions and subnetworks that are active mainly in younger donors. Most of these biological functions are well known characteristics of follicular dynamics under ovarian stimulation and coasting. Further analysis of the genes and upstream regulators revealed central nodes, signalling pathways and essential biological processes and subnetworks in granulosa cell function. The functional analysis will have more power by using a fold change of 1.5 to increase the number of DEGs even if the change at each gene is lower, the addition of 10–100 variable genes in a given direction will indicate a clear shift in a specific pathway and strengthen the significance of the functional analysis (Dalman *et al.*, 2012).

At first glance, the biological function analysis seems to suggest that follicles in 8-month-old coasting heifers are growing more actively than at the reference age in terms of the decrease in cell death and the increase in cell viability. However, upstream regulator analysis showed specific biomarkers of the plateau phase, such as the growth hormone (GH), prolactin (PRL) and tumor suppressor 53 (TP53). GH and PRL have been associated previously with maximal oocyte competence (Nivet *et al.*, 2013) and TP53 has been associated with the plateau stage (Douville and Sirard, 2014). The phosphatidylinositol 3-kinase (PI3K) complex, which is associated with cellular proliferation and growth (Chalhoub and Baker, 2009), is predicted to be inhibited in 8-month-old heifers, suggesting that these follicles are not really in a phase of more active growth compared with the reference. Notch homolog 1 (NOTCH1) is also predicted to be inhibited and, in granulosa cells, inhibition of NOTCH signalling suppressed AKT phosphorylation and thus cell proliferation (Wang *et al.*, 2016). The AKT group is also an upstream regulator and was downregulated in the comparison of 8 versus 14 month olds (*z*-score = 1.8). Another gene predicted to be inhibited in younger heifers is GATA4, a

member of the GATA family of transcriptional regulatory proteins involved positively in expression of steroidogenesis genes and a key regulator of granulosa cell differentiation and proliferation (Bennett *et al.*, 2013; Padua *et al.*, 2014). The absence of NOTCH1 and GATA4 was observed only in 8-month-old heifers. The aromatase gene (*CYP19A1*) is downregulated in both arrays (8 vs 14 months and 11 vs 14 months). The aromatase (*CYP19A1*) gene expression is known to be significantly less active during the plateau versus growing stage and during the atretic versus plateau stage in granulosa cells from bovine follicles between 5 and 9 mm in diameter (Douville and Sirard, 2014), further suggesting that the follicles in these heifers (8 and 11 months old) are not in the growing phase compared with their reference, but most likely still in the plateau phase.

A major characteristic of the plateau phase is the beginning of follicle differentiation, a critical step in the preparation of the dominant follicle for ovulation and for acquisition of oocyte competence. During FSH coasting, the optimal phase is characterised by increased hypoxia, inflammation and apoptosis signalling, thus mimicking the LH surge in the preovulatory follicle (Sirard *et al.*, 2006). NetworkAnalyst suggested that the biological process of responding to follicular hypoxia at 8 months of age involves differential expression at central nodes such as kinase insert domain receptor (KDR), tumour necrosis factor (TNF), murine thymoma viral (V-Akt) homolog-2 (AKT2) and Kristen ras (KRAS). Granulosa cells sustain follicle growth by releasing proangiogenic factors such as vascular endothelial growth factor (VEGF) in order to promote angiogenesis (Siemeister *et al.*, 1998; Greenaway *et al.*, 2004). VEGF exerts its cellular effects through its tyrosine kinase receptors Flt-1 (VEGFR1) and KDR (VEGFR2), whereas KDR is the principal mediator of angiogenic effects. VEGF interacts with KDR and exerts cytoprotective effects against apoptotic cell death and follicular atresia in bovine granulosa cells (Greenaway *et al.*, 2004) and human granulosa cell tumours (Färkkilä *et al.*, 2011). It has been shown that blocking KDR results in an antiangiogenic effect and effectively blocks tumour growth in human umbilical vein endothelial cells (Rastelli *et al.*, 2011). TNF is a critical modulator of granulosa cell differentiation, steroidogenesis, inflammation and apoptosis signalling (Spicer, 1998; Sakumoto *et al.*, 2003; Glister *et al.*, 2014). It has been shown that TNF reduces VEGF expression via protein kinase C in human granulosa cells, thus balancing angiogenesis and

vascular permeability in preovulatory follicles (Machelon and Nome, 1999). *AKT2* is an oncogene highly expressed in ovarian cancer and associated with angiogenesis and tumour growth (Jiang *et al.*, 2016). *KRAS* is another oncogene that alters granulosa cell fate by preventing differentiation, proliferation, ovulation and apoptosis. However, the effect of KRAS is dependent on the stage of granulosa cell differentiation (Fan *et al.*, 2008) and is likely involved in plateau equilibrium.

The ovulation process has often been compared to an inflammatory reaction involving many cytokines, both pro- and anti-inflammatory. In both the present comparisons, regulation of cytokine biosynthesis was one of the most significant biological processes identified by NetworkAnalyst. In preovulatory follicles, the LH surge induces numerous genes related to inflammation (Richards *et al.*, 2002). In fact, even without the LH surge, such as in the coasting procedure used on cows, FSH removal creates a follicular environment that involves oxidative stress and the expression of inflammation genes (Nivet *et al.*, 2013). Ovarian stimulation for 4 days has been shown to alter the expression of genes related to angiogenesis and to activate oxidative stress response genes in bovine granulosa cells just before ovulation (Dias *et al.*, 2014). Ovarian stimulation is clearly a process that generates reactive oxygen species (ROS) as a result of both steroid production and cell growth and especially towards ovulation, which is likely explained by macrophage recruitment after the LH surge (Hanukoglu, 2006).

Although formation of ROS within mitochondria is a major internal trigger of apoptotic cell death resulting in the release of cytochrome *c* and activation of caspase-3, it is also essential for the ovulatory process (Bras *et al.*, 2005; Shkolnik *et al.*, 2011). Adding antioxidants to mouse and rat granulosa cells cultured in the presence of LH reduced the expression of genes essential for inflammation, thus preventing ovulation (Shkolnik *et al.*, 2011; Park *et al.*, 2012). It has been shown that hydrogen peroxide alone can upregulate the expression of inflammation-related and ovulatory genes, thus mimicking the effect of LH, specifically CCAAT/Enhancer binding protein beta (*CEBPB*) (also known as *TCT5)*, hyaluronan synthase 2 (*HAS2*) and *TNFAIP6* (Shkolnik *et al.*, 2011), suggesting a critical role of ROS signalling in ovulation. Although ROS are generally considered cytotoxic, H_2O_2 also acts as

an essential intracellular messenger (Forman *et al.*, 2010). Cytokines such as interleukin (IL)-1 and TNF are known mediators of the preovulatory effects of ROS following the LH surge (Shkolnik *et al.*, 2011). During the ovarian inflammatory response, expression of the proinflammatory cytokine IL-1 is augmented in granulosa cells (Simón *et al.*, 1994; Son and Roby, 2006) and TNF has been shown to induce inflammatory responses and initiate apoptosis in bovine granulosa cells (Sakumoto *et al.*, 2003; Glister *et al.*, 2014). Expression of both these genes is associated with an increase in oocyte developmental competence in the coasting follicle (Nivet *et al.*, 2013). Expression of IL-1A, IL-1B, TNF and TNFAIP66 is significantly downregulated, and CREBPB ($z = -1.6$) and hyaluronic acid are predicted to be inhibited in the 8 versus 14 month array, but not in the 11 versus 14 month comparison, thus supporting the potential deficiency in ROS generation, H_2O_2 signalling and inflammatory response observed in younger peripubertal heifers.

Another modulator of cell fate that is inhibited in 8-month-old heifers is the mitogen-activated protein kinase (MAPK) group, which includes extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK; also known as MAPK8) and p38 (also known as MAPK14), well characterised as a modulator of proliferation, differentiation, steroidogenesis, stress response, inflammation and apoptosis signalling in granulosa cells (Pearson *et al.*, 2001; Peter and Dhanasekaran, 2003). MAPKs are involved in transducing a wide variety of extracellular stimuli, including growth factor, hormone and cytokine signals. In cow ovaries, increased levels of ERK promote the survival of dominant follicles during FSH decline (Ryan *et al.*, 2007). ERK also plays a crucial role in monkey granulosa cell proliferation (Uma *et al.*, 2003) and in steroidogenesis (for a review, see Manna and Stocco, 2011). In granulosa cells, p38 also has a critical role in steroidogenesis, and gonadotropins have been shown to promote steroidogenic acute regulatory protein (StAR) expression and steroidogenesis by activating both p38 and ERKs (for a review, see Manna and Stocco, 2011). However, p38 in monkeys is also activated during granulosa cell apoptosis (Uma *et al.*, 2003). In bovine granulosa cells, apoptosis is associated with prolonged phosphorylation of p38 and JNK (Guerrero-Netro *et al.*, 2015) and p38 is known to mediate the expression of IL-1 and TNF (IPA database, www.analysis.ingenuity.com accessed on Oct 26, 2016), both

involved in inflammation and apoptosis signalling. This is consistent with the lack of inflammation process observed in young animals.

Figure 4-7 summarises the biological process involved in age-specific biological process. Insulin and VEGF signalling are among the DEGs noted in 8-month-old heifers. Based on upstream analysis, FSH and LH are both predicted to be inhibited at this age. In addition, several LH signalling downstream actions appear to be inhibited significantly, such as response to ROS accumulation, proinflammatory cytokine and prostaglandin receptors, prosteroidogenic transcription factors and apoptotic signals (Table S4). Indeed, basal LH has a significant effect on the granulosa cell transcriptome during the dominant phase, as reviewed previously (Sirard, 2016). Conversely, upstream analysis of DEGs found specifically in 11-month-old heifers (Table S4) suggests more basal LH, resulting in fewer differences compared with their references (PRL, cyclic adenosine monophosphate (cAMP), signal transducer and activator of transcription 3 (STAT3) and cAMP responsive element binding protein 1 (CREB1)). Moreover, upstream regulators such as p38 (z = 1.7), prostaglandin E_2 (z = 1.6) and H_2O_2 (z = 1.25) have *P*-values that predict a tendency to be activated, which suggests an optimal environment for follicular differentiation as the animals reach puberty.

4.6.1. Conclusion and remarks

In conclusion, granulosa cells from 8-month-old heifers contain a set of compromised essential signalling molecules that may play pivotal roles in cell differentiation, inflammation and early apoptosis, which are all necessary for an optimal oocyte environment in coasting, as summarised in Figure 4-8. The downregulation of critical genes suggests that ovulation signalling is weaker at this age. It is possible that peripubertal animals have insufficient basal LH. It has been shown previously that inducing such a condition artificially in mature animals using a gonadotrophin-releasing hormone antagonist during the coasting period can significantly reduce oocyte quality (Labrecque and Sirard, 2014; Sirard, 2016). It is known that basal LH is lower before puberty but gradually increases as puberty approaches. Overall, peripubertal donors exhibit less cell differentiation, inflammation and apoptosis signalling, which could be associated with a lower basal concentration of LH.

4.7. Acknowledgements and funding

The Natural Sciences and Engineering Research Council of Canada – Collaborative Research and Development (NSERC-CRD grant no. RDCPJ461697–13) provided funding for this study. D. A. Landry received studentship support from NSERC and REDIH (Canadian Institutes of Health Research (CIHR) Training program in Reproduction, Early Development, and Impact on Health).

4.8. Declaration of interest

The authors declare no potential conflicts of interest.

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4.10. Figures

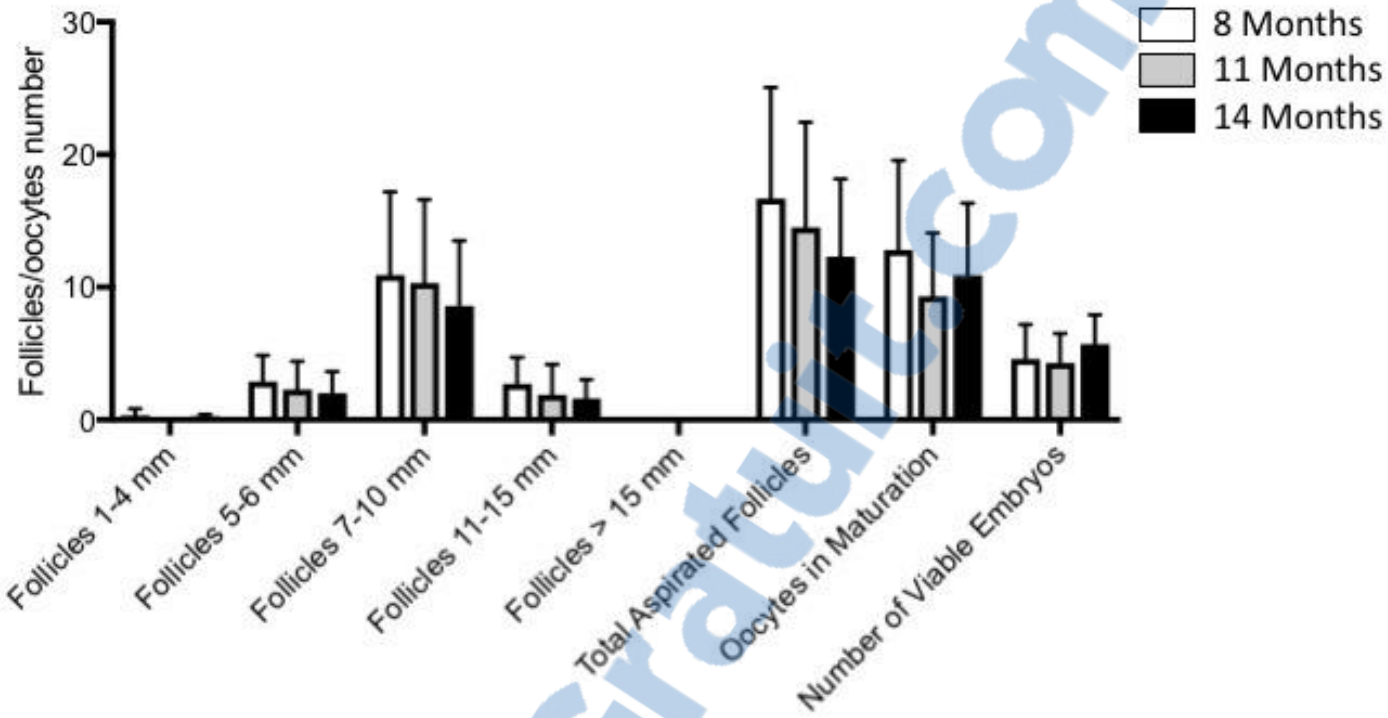

Figure 4-1: Effect of oocyte donor age on the size category distribution of follicles

Number of follicles was counted after FSH-induced superovulation and the developmental competence of IVM and IVF oocytes collected from the same 10 Holstein heifers at 8, 11 and 14 months of age. 'No. follicles or oocytes' refers to the average total numbers aspirated, usable for IVM or reaching blastocyst stages. Data are the mean ± s.d.

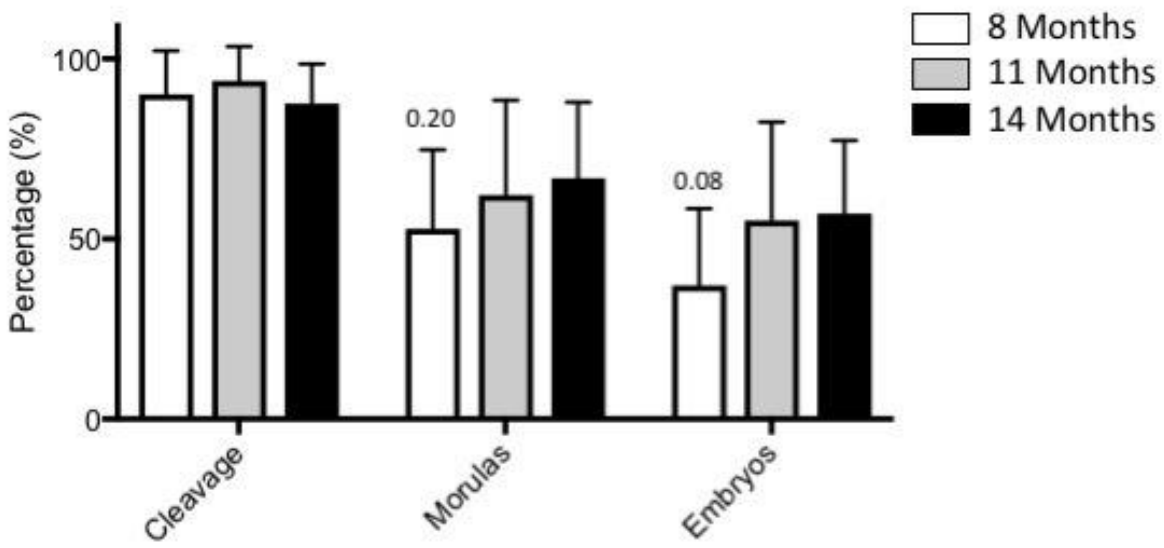

Figure 4-2: Development competence of heifer oocytes after conventional IVF

Oocytes were recovered from the 10 donors at 8, 11 and 14 months of age after FSH-induced superovulation. 'Percentage' refers to mature fertilised oocytes reaching the cleavage, morula or embryos stages, expressed as an average ratio of the number of cleaved, morula or blastocyst (embryos) to the number of oocyte placed in maturation. Data are the mean ± s.d. No significant change was found and p-values lower than 0.2 are identified on top of the column if applicable.

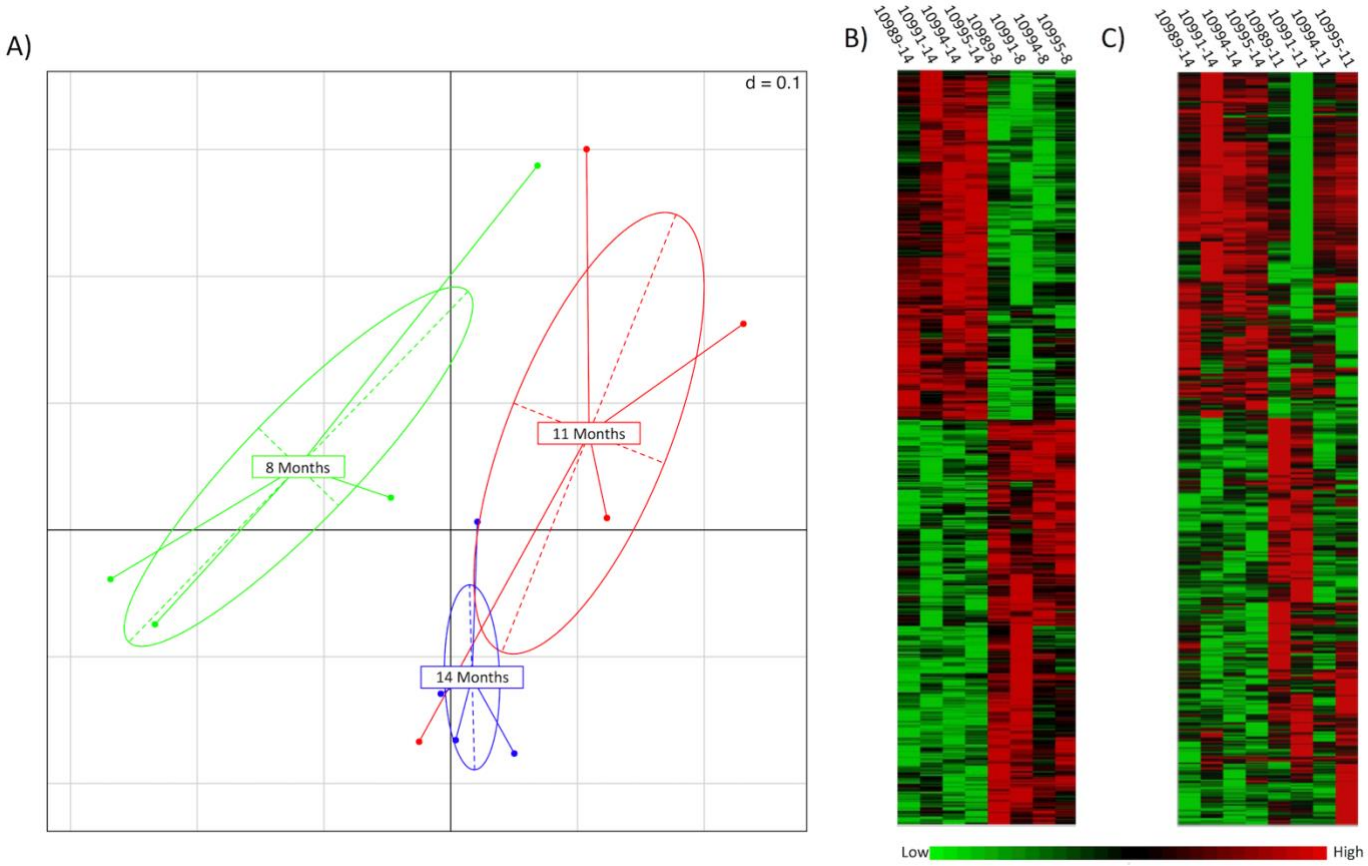

Figure 4-3: Between group analysis and heatmaps analysis from the microarrays

(*a*) Between group analysis (BGA) of data for all probes using R software (R Foundation for Statistical Computing) showing the three Holstein heifer age groups, namely 8, 11 and 14 months. Given the design of the experiment, two sets of data were available for the 14-month-old groups, which were pooled before the quantile normalisation using R software. (*b*, *c*) Two heat maps illustrating the spatial expression of differentially expressed genes from each animal (10989, 10991, 10994 and 10995 with their age after the dash) in comparison of 8 versus 14 months (*b*) and 11 versus 14 months (*c*) using NetworkAnalyst (McGill). The data were normalised within arrays (Loess) using Flex array and between arrays (quantile) using NetworkAnalyst.

Figure 4-4: Venn diagram showing the number of genes detected in the microarrays

The number of genes expressed differentially, defined as microarray probe signal intensity increasing (upregulated) or decreasing (downregulated) at least 1.5-fold ($P < 0.05$), in granulosa cells in ovarian follicles of FSH-stimulated Holstein were compared between heifers at 8 and 11 months of age. The reference expression level was measured in heifers at 14 months of age.

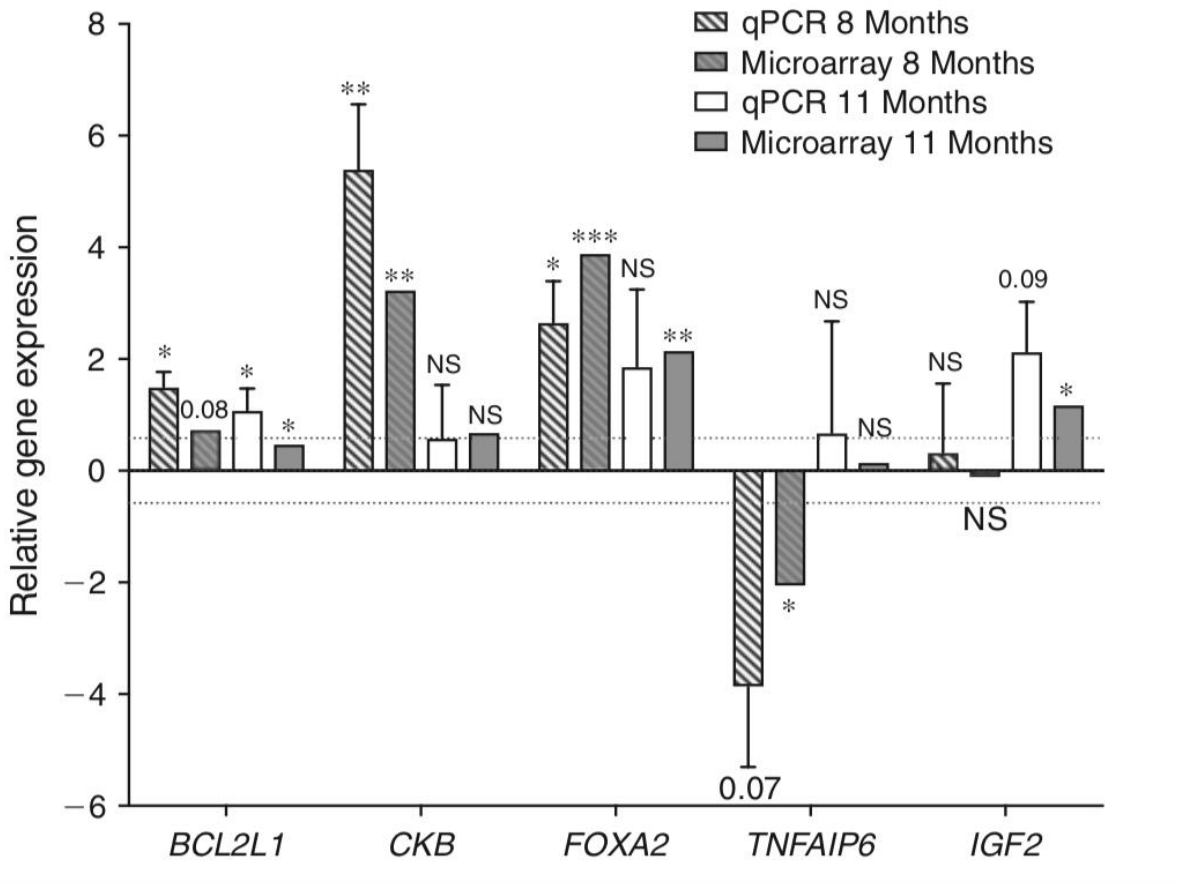

Figure 4-5: RT-qPCR validation for the microarrays analysis

Log2-transformed relative (to the reference age of 14 months) expression of five selected genes in FSH-stimulated Holstein heifers at 8 and 11 months of age, as quantified by real-time quantitative polymerase chain reaction (qPCR) and using microarray probes (Microarray). *BCL2L1*, BCL2-like 1; *CKB*, creatine kinase B; *FOXA2*, forkhead box A2; *TNFAIP6*, tumour necrosis factor α-induced protein 6; *IGF2*, insulin-like growth factor 2. The real-time qPCR values are the mean (± s.e.m.) relative expression compared with the reference groups. The dotted lines represent a change of 1.5-fold (log2 of 0.585). Data are the mean ± s.e.m. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with the reference age of 14 months.

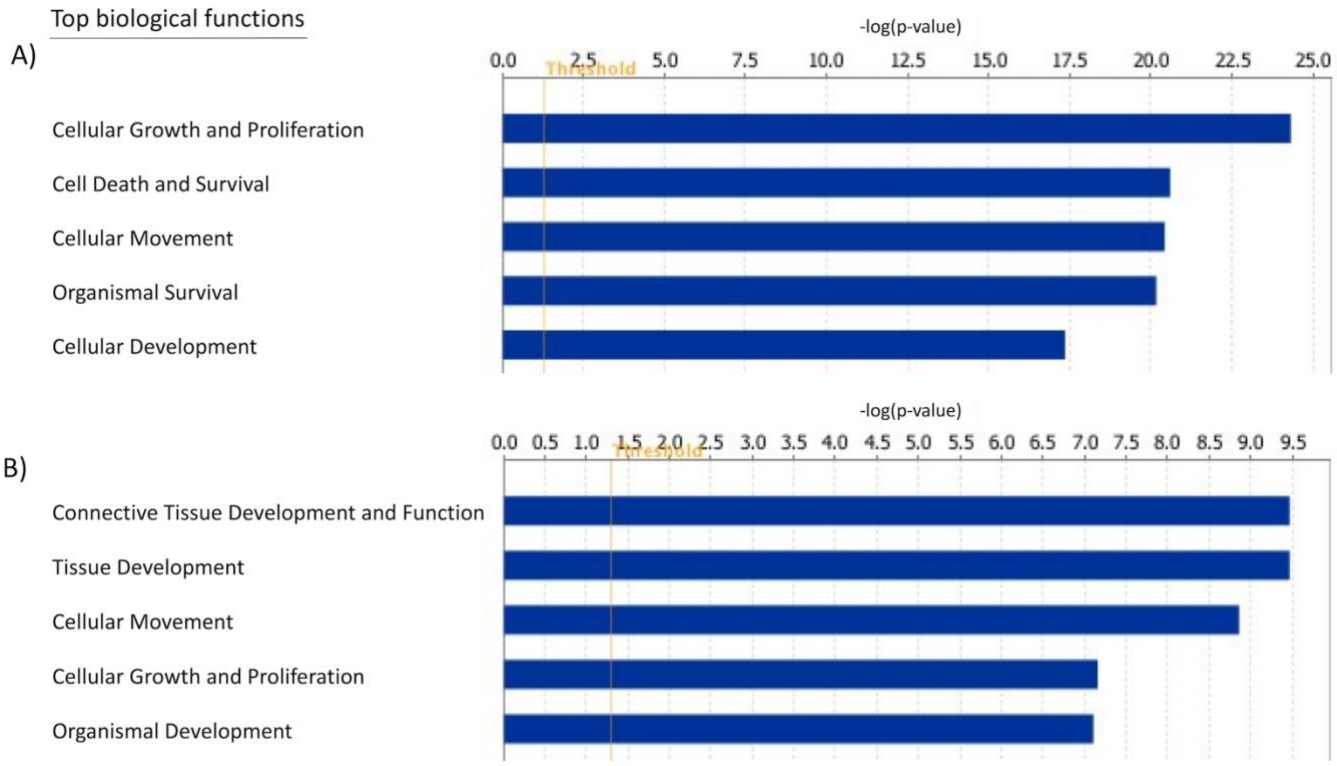

Figure 4-6: Biological functions in granulosa cells associated with heifers age

The five most significant biological functions in granulosa cells of FSH-stimulated Holstein heifers at (*a*) 8 and (*b*) 11 months of age, as determined by ingenuity pathway analysis and ranked by statistical significance (*P*-values). The threshold of significance ($P = 0.05$) is indicated (vertical lines).

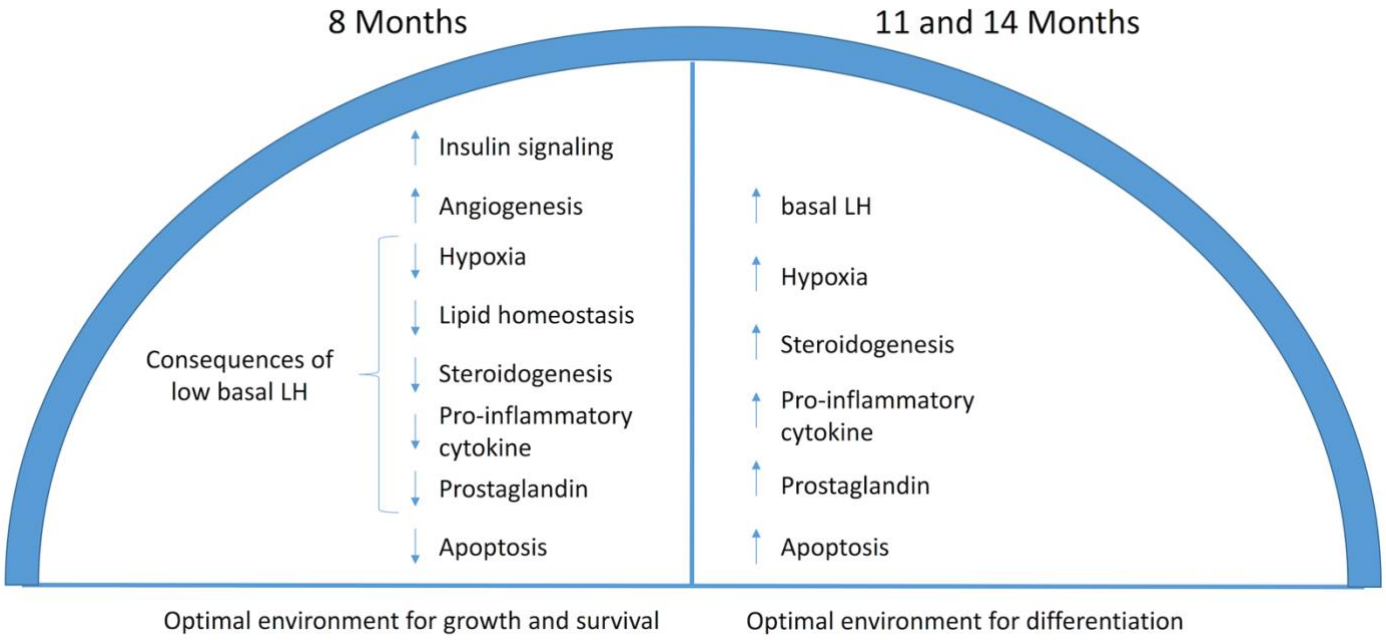

Figure 4-7: Functional roles of genes and upstream regulators involved in age-specific biological processes in FSH-stimulated Holstein heifers

Roles associated with heifers at 8 months of age are in the left side and at 11 months of age are in the right side. The arrows represent the up- or downregulation of the biological function.

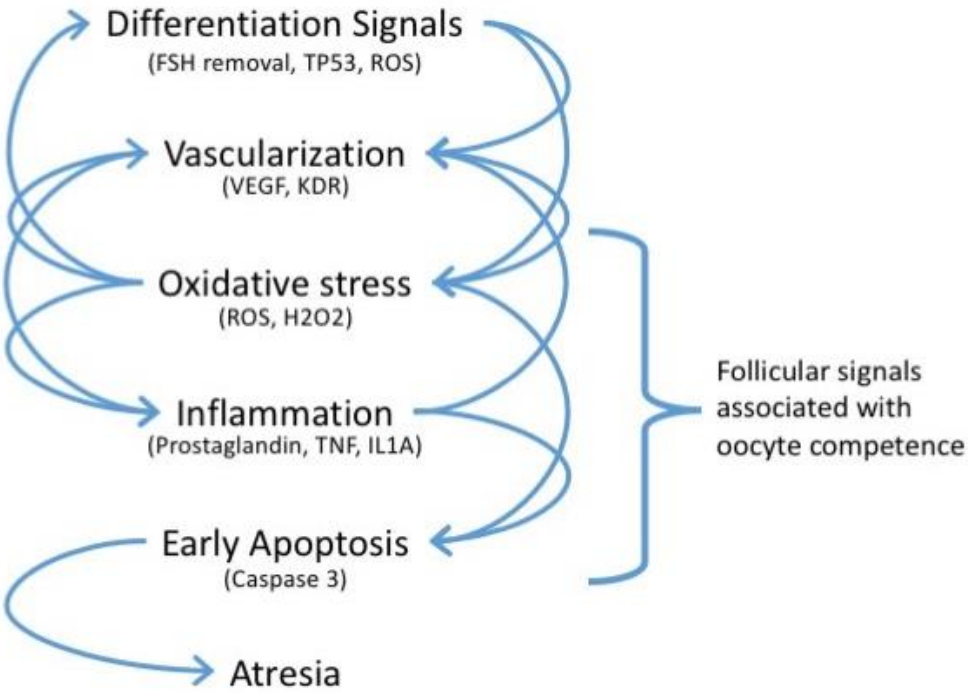

Figure 4-8: Mechanistic representation of signalling interactions following FSH withdrawal in FSH-stimulated Holstein heifers

As described previously (Nivet *et al.*, 2012), oxidative stress, inflammation and early apoptosis are associated with optimal oocyte competence. Proteins and molecules appearing in parentheses are key regulators of their respective biological process. TP53, tumor protein 53; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; KDR, kinase insert domain receptor; TNF, tumour necrosis factor; IL-1A, interleukin 1A.

Supplemental data

Supplemental Table 4-1: Selection of the ten animals for the microarray analysis

Animals selected for the present study with follicle size, total number of aspirated follicles, number of oocytes in maturation and number of viable embryos (blastocysts). The percentage of embryos refers to the ratio between the number of oocytes in maturation and the number of viable embryos.

OPU Date	Name	Age at OPU (months)	Total Aspirated Follicles	Follicles 1-4 mm	Follicles 5-6 mm	Follicles 7-10 mm	Follicles 11-15 mm	Follicles > 15 mm	Oocytes in Maturation	Number of Viable Embryos	Embryos %
	Group 8 months										
2014-10-13	10989	7.75	15	0	4	10	1	0	12	7	58 %
2014-10-13	10990	7.72	16	2	2	9	3	0	7	3	43 %
2014-10-13	10991	7.98	10	0	2	6	2	0	9	6	67 %
2014-10-13	10992	7.98	9	0	0	9	0	0	9	4	44 %
2014-10-13	10993	8.71	15	0	2	11	2	0	11	6	55 %
2014-10-13	10994	8.02	27	0	2	20	5	0	20	9	45 %
2014-10-13	10995	7.65	23	0	2	16	5	0	23	5	22 %
2014-10-13	10996	7.79	32	0	7	21	4	0	23	4	17 %
2014-10-13	10997	8.28	15	0	5	5	5	0	10	2	20 %
2014-10-13	10998	8.54	5	0	3	2	0	0	4	0	0 %
	Average	**8.04**	**16.7**	**0.2**	**2.9**	**10.9**	**2.7**	**0**	**12.8**	**4.6**	**37.09%**
	Group 11 months										
2015-01-07	10989	10.58	13	0	4	9	0	0	10	7	70 %
2015-01-07	10990	10.55	17	0	0	11	6	0	11	4	36 %
2015-01-07	10991	10.81	7	0	3	4	0	0	8	7	88 %

2015-01-07	10992	10.81	5	0	1	4	0	0	2	2	100 %
2015-01-07	10993	11.53	6	0	1	5	0	0	2	1	50 %
2015-01-07	10994	10.84	16	0	0	11	5	0	10	5	50 %
2015-01-07	10995	10.48	31	0	3	25	3	0	10	7	70 %
2015-01-07	10996	10.61	18	0	2	13	3	0	15	2	13 %
2015-01-07	10997	11.10	11	0	2	7	2	0	8	4	50 %
2015-01-07	10998	11.37	21	0	7	14	0	0	17	4	24 %
	Average	**10.87**	**14.5**	**0**	**2.3**	**10.3**	**1.9**	**0**	**9.3**	**4.3**	**50.07%**
	Group 14 months										
2015-04-01	10989	13.34	12	0	4	6	2	0	11	5	45 %
2015-05-11	10990	13.31	7	0	0	7	0	0	7	4	57 %
2015-04-01	10991	13.57	3	0	2	0	1	0	2	1	50 %
2015-04-01	10991	14.88	10	0	1	7	2	0	10	4	40 %
2015-04-01	10992	13.57	5	1	1	2	1	0	3	2	67 %
2015-04-01	10993	14.29	9	0	2	5	2	0	8	8	100 %
2015-04-01	10994	13.60	23	0	0	18	5	0	19	8	42 %
2015-04-01	10995	13.24	13	0	2	10	1	0	12	8	67 %
2015-04-01	10996	13.37	11	0	3	8	0	0	10	4	40 %
2015-04-01	10997	13.86	11	0	2	7	2	0	8	6	75%
2015-04-01	10998	14.13	22	0	5	16	1	0	21	8	38 %
	Average	**13.74**	**11.5**	**0.1**	**2**	**7.8**	**1.5**	**0**	**10.1**	**5.6**	**57.11%**

Supplemental Table 4-2: Primers used in real-time PCR experiments

Gene Symbol	Primer sequence		Product size (pb)	Annealing temperature	GenBank Accession Number
GAPDH	Fwd:	CCAACGTGTCTGTTGTGGATCTGA	218	57	NM_001034034.2
	Rev:	GAGCTTGACAAAGTGGTCGTTGAG			
ACTB	Fwd:	ATCGTCCACCGCAAATGCTTCT	102	57	NM_173979.3
	Rev:	GCCATGCCAATCTCATCTCGTT			
BCL2L1	Fwd:	GCA GGT ATT GGT GAG TCG GAT	116	57	NM_001077486.2
	Rev:	TGT TCC CGT AGA GTT CCA CAA			
CKB	Fwd:	CCA ATA GCC ACA ACA CGC TG	322	57	NM_001015613.1
	Rev:	TTG AGG TCG GTC TTG TGC TC			
FOXA2	Fwd:	GAACAAAGCGGGCCTGGATACC	447	57	XM_002692160.4
	Rev:	GACTTCCCTGCAACGACAGCAA			
TNFAIP6	Fwd:	CAAGGGCAGAGTTGGATACC	230	57	NM_001007813.2
	Rev:	TGTGCCAGTAGCAGATTTGG			
IGF2	Fwd:	CACGCGCAGAACACCAAGTCAT	140	57	NM_174087.3
	Rev:	TGGGATTGCGAGCGATAAAGGT			

Supplemental Table 4-3: Upstream Analysis identified by Ingenuity Pathway Analysis

A) In granulosa cells from 8-month-old heifers after FSH stimulation and coasting, compared to themselves at 14 months of age. B) In granulosa cells from 11-month-old heifers after FSH stimulation and coasting, compared to themselves at 14 months of age. The predicted upstream regulator, the expression differential (based on microarray probe), molecule type, predicted activation state, z score and statistical significance of the overlap (p value) are shown.

A)

Upstream Regulator	Exp Fold Change	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
beta-estradiol		chemical - endogenous mammalian	Activated	3.067	9.13E-23
tretinoin		chemical - endogenous mammalian	Activated	2.889	2.30E-10
TCF7L2	1.549	transcription regulator	Activated	2.446	2.38E-05
Growth hormone		group	Activated	2.439	1.02E-04
SP1	-1.804	transcription regulator	Activated	2.438	1.32E-08
KLF4		transcription regulator	Activated	2.438	6.79E-08
Ins1		other	Activated	2.274	6.37E-06
TP53		transcription regulator	Activated	2.256	4.26E-22
LDLR	-1.768	transporter	Activated	2.21	3.04E-04
PRDM1		transcription regulator	Activated	2.19	1.71E-03
SOD1	-2.013	enzyme	Activated	2.132	2.68E-06
CD3E	-2.118	transmembrane receptor	Activated	2.116	1.01E-03
hexachlorobenzene		chemical toxicant	Activated	2.111	6.22E-05
IFNA2		cytokine	Activated	2.039	4.55E-06
F2	5.346	peptidase	Activated	2.037	1.36E-06
PRL		cytokine	Activated	2.029	2.01E-10
NR5A2	-2.697	ligand-dependent nuclear receptor	Activated	2.01	2.16E-03
PDGF BB		complex	Activated	2.005	1.22E-06
IL19		cytokine	Inhibited	-2	7.20E-03
BTRC		enzyme	Inhibited	-2	1.56E-02
LRP5		transmembrane receptor	Inhibited	-2	1.56E-02
IL12A		cytokine	Inhibited	-2.022	1.62E-04
SPI1		transcription regulator	Inhibited	-2.023	4.39E-05
hyaluronic acid		chemical - endogenous mammalian	Inhibited	-2.06	1.59E-02
CLEC7A		transmembrane receptor	Inhibited	-2.161	4.68E-03
PI3K (complex)		complex	Inhibited	-2.169	2.86E-05

SENP1		peptidase	Inhibited	-2.2	2.00E-03
PRKCQ	-2.025	kinase	Inhibited	-2.236	1.24E-02
CD2		transmembrane receptor	Inhibited	-2.284	1.34E-02
P38 MAPK		group	Inhibited	-2.363	3.47E-04
NR0B2		ligand-dependent nuclear receptor	Inhibited	-2.387	2.25E-02
SOX7		transcription regulator	Inhibited	-2.449	1.20E-02
GRB2		kinase	Inhibited	-2.449	2.25E-02
NOTCH1	-2.431	transcription regulator	Inhibited	-2.517	5.29E-04
KRAS	4.066	enzyme	Inhibited	-2.545	5.47E-13
PAX1		transcription regulator	Inhibited	-2.646	5.50E-03
STAT5B		transcription regulator	Inhibited	-2.75	4.95E-02
STAT5A		transcription regulator	Inhibited	-2.795	6.28E-05
GATA4		transcription regulator	Inhibited	-3.553	1.75E-03

B)

Upstream Regulator	Exp Fold Change	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
EGF		growth factor	Activated	2.728	1.52E-06
PRL		cytokine	Activated	2.705	8.20E-05
PTH		other	Activated	2.608	1.16E-06
TP53		transcription regulator	Activated	2.459	8.35E-05
IL10RA		transmembrane receptor	Activated	2.449	5.07E-03
TGFB1		growth factor	Activated	2.4	2.20E-06
TO-901317		chemical reagent	Activated	2.365	1.65E-04
EGR2		transcription regulator	Activated	2.353	1.23E-04
tretinoin		chemical - endogenous mammalian	Activated	2.229	2.95E-06
Ins1		other	Activated	2.207	5.56E-03
NCOA2		transcription regulator	Activated	2.186	2.07E-05
CTGF		growth factor	Activated	2.18	1.79E-04
NR5A2		ligand-dependent nuclear receptor	Activated	2.178	7.57E-05
PGR		ligand-dependent nuclear receptor	Activated	2.174	1.34E-02
Vegf		group	Activated	2.138	9.94E-04
hydrogen peroxide		chemical - endogenous mammalian	Activated	2.077	4.74E-04
forskolin		chemical toxicant	Activated	2.026	2.43E-07
8-bromo-cAMP		chemical reagent	Activated	2.008	3.31E-05

Growth hormone		group	Activated	2	4.13E-03
miR-16-5p		mature microrna	Inhibited	-2.621	7.70E-05

Supplemental Table 4-4: Upstream and pathway analysis identified by Ingenuity Pathway Analysis

A) Upstream regulators identified by IPA from the differential gene expression peculiar to 8-month-old heifers after FSH stimulation and coasting, relative to 14-month-olds. B) Upstream regulators identified by IPA from differential gene expression peculiar to 11-month-old heifers after FSH stimulation and coasting, relative to 14-month-olds. Predicted upstream regulator, expression differential (based on microarray probe), molecule type, predicted activation state, z score and statistical significance of the overlap (p value) are shown. C) Pathway analysis (IPA) of differential gene expression peculiar to 8-month-old heifers after FSH stimulation and coasting, relative to 14-month-olds. The affected canonical pathway, p value, ratio (based on DEGs) and the z score are shown.

A)

Upstream Regulator	Exp Fold Change	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
beta-estradiol		chemical - endogenous mammalian	Activated	3.184	8.80E-18
beta-carotene		chemical - endogenous mammalian	Activated	2.515	3.07E-06
CREM		transcription regulator	Activated	2.45	1.36E-02
ADRB		group	Activated	2.429	1.15E-02
NPPB		other	Activated	2.425	4.45E-02
IL10RA		transmembrane receptor	Activated	2.401	1.05E-01
ABCA1		transporter	Activated	2.383	3.18E-04
LIF		cytokine	Activated	2.259	1.51E-01
INSIG2		other	Activated	2.236	7.58E-03
SYVN1		transporter	Activated	2.232	3.02E-04
IRF7		transcription regulator	Activated	2.225	4.75E-01
PCGEM1		other	Activated	2.207	2.77E-01
INSIG1	-1.575	other	Activated	2.203	6.97E-06
ADRB2		g-protein coupled receptor	Activated	2.19	1.14E-01
SKIL		transcription regulator	Activated	2.155	1.34E-02
WNT1		cytokine	Activated	2.136	1.51E-03
POR	1.827	enzyme	Activated	2.132	1.05E-04
GFI1		transcription regulator	Activated	2.073	6.85E-05
CD38	1.547	enzyme	Activated	2.02	6.79E-08
Igm		complex	Activated	2.005	8.54E-04
ACTL6A		other	Inhibited	-2	2.12E-01
MBTD1	1.718	other	Inhibited	-2	4.36E-01
FSH		complex	Inhibited	-2.032	9.11E-07
KMT2D		transcription regulator	Inhibited	-2.047	6.55E-04

P38 MAPK		group	Inhibited	-2.05	2.06E-03
KAT5		transcription regulator	Inhibited	-2.121	3.23E-01
caspase		group	Inhibited	-2.138	2.91E-03
Ige		complex	Inhibited	-2.143	4.56E-01
GSK3B		kinase	Inhibited	-2.144	4.58E-02
SREBF1		transcription regulator	Inhibited	-2.149	4.04E-06
ICOS		transmembrane receptor	Inhibited	-2.158	2.18E-01
MSTN		growth factor	Inhibited	-2.167	4.74E-01
SLC16A3		transporter	Inhibited	-2.173	4.99E-04
TLR5		transmembrane receptor	Inhibited	-2.176	2.25E-01
ATF2		transcription regulator	Inhibited	-2.186	2.38E-01
RICTOR		other	Inhibited	-2.208	1.07E-06
Lh		complex	Inhibited	-2.213	2.75E-04
CSF1R		kinase	Inhibited	-2.213	7.62E-03
USP7	1.675	peptidase	Inhibited	-2.216	2.78E-02
hyaluronic acid		chemical - endogenous mammalian	Inhibited	-2.217	1.11E-01
BMP7		growth factor	Inhibited	-2.224	4.29E-02
RELA		transcription regulator	Inhibited	-2.231	6.13E-06
T3-TR-RXR		complex	Inhibited	-2.236	2.94E-01
ARHGAP21		other	Inhibited	-2.236	3.30E-01
IL12A		cytokine	Inhibited	-2.257	1.91E-03
uric acid		chemical - endogenous mammalian	Inhibited	-2.297	1.79E-01
KRAS	2.039	enzyme	Inhibited	-2.318	3.44E-09
ADAM17	-1.562	peptidase	Inhibited	-2.338	7.33E-02
PRKAA2		kinase	Inhibited	-2.359	3.12E-03
AXIN1		other	Inhibited	-2.377	6.19E-03
ZEB1		transcription regulator	Inhibited	-2.383	7.33E-02
CEBPD	2.08	transcription regulator	Inhibited	-2.414	1.08E-02
PLAGL1		transcription regulator	Inhibited	-2.423	1.17E-03
MTORC1		complex	Inhibited	-2.433	3.27E-02
RASSF5		other	Inhibited	-2.556	8.43E-05
PTGER2		g-protein coupled receptor	Inhibited	-2.577	2.01E-03
Gsk3		group	Inhibited	-2.578	3.21E-02
NR5A1		ligand-dependent nuclear receptor	Inhibited	-2.664	6.35E-02
D-fructose		chemical - endogenous mammalian	Inhibited	-2.815	2.87E-02
palmitic acid		chemical - endogenous mammalian	Inhibited	-2.92	8.49E-06
MTOR		kinase	Inhibited	-3.003	2.92E-08

B)

Upstream Regulator	Exp Fold Change	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
PRL		cytokine	Activated	2.752	4.35E-05
cyclic AMP		chemical - endogenous mammalian	Activated	2.589	2.18E-04
STAT3		transcription regulator	Activated	2.571	7.33E-04
MKNK1		kinase	Activated	2.236	1.12E-04
CREB1		transcription regulator	Activated	2.232	4.03E-03
butyric acid		chemical - endogenous mammalian	Activated	2.183	1.71E-02
ESR1		ligand-dependent nuclear receptor	Activated	2.144	3.14E-04
KLF3		transcription regulator	Inhibited	-2	7.21E-02
IRF3		transcription regulator	Inhibited	-2	8.18E-03
IRF7		transcription regulator	Inhibited	-2.219	1.02E-03

C)

Ingenuity Canonical Pathways	p-value	Ratio	z-score
MIF-mediated Glucocorticoid Regulation	4.37E-02	2.00E-01	2.646
Fc Epsilon RI Signaling	2.75E-02	1.52E-01	2.357
VEGF Signaling	1.45E-02	1.65E-01	2.324
EIF2 Signaling	3.47E-02	1.37E-01	2.324
FcÎ³ Receptor-mediated Phagocytosis in Macrophages and Monocytes	4.79E-02	1.52E-01	2.324
Phospholipase C Signaling	5.89E-03	1.47E-01	2.309
Signaling by Rho Family GTPases	2.04E-02	1.37E-01	2.268
Rac Signaling	8.71E-04	1.92E-01	2.132
Thrombin Signaling	7.08E-04	1.67E-01	2.121
Insulin Receptor Signaling	2.24E-02	1.50E-01	2.065
Cyclins and Cell Cycle Regulation	3.24E-02	1.67E-01	-2.111
RhoGDI Signaling	1.95E-02	1.45E-01	-2.132
PTEN Signaling	1.58E-04	2.07E-01	-3

5. Expression of atresia biomarkers in granulosa cells after ovarian stimulation in heifers

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Cet article est publié dans la revue « Reproduction » sous la référence suivante :
Landry D.A., Rossi-Perazza L., Lafontaine S., and Sirard M.A., (2018) Expression of atresia biomarkers in granulosa cells after ovarian stimulation in heifers. Reproduction, 156 (3), 239-248.

5.1.Résumé

L'utilisation de jeunes donneuses de gamètes dans un programme de sélection génétique chez la vache laitière avance significativement les gains génétiques en diminuant l'intervalle entre les générations. La stimulation ovarienne et la pratique de retrait de l'hormone folliculo-stimulante (FSH), aussi appelé coasting, sont des protocoles intensivement utilisés chez les génisses pré-pubères sans aucun effet néfaste sur les performances subséquente de reproduction, mais produisent généralement moins d'embryons que les vaches sexuellement matures. Toutefois, les résultats de transfert d'embryons récents ne montrent aucune différence entre le taux de blastocyste chez les donneuses pré-pubères et les donneuses sexuellement matures, mais démontrent une différence significative dans leurs périodes de coasting. Le but de cette étude était d'identifier de bons biomarqueurs dans les cellules de la granulosa capable de distinguer les follicules ayant une différenciation optimale des follicules trop différentiées et en atrésie afin d'obtenir l'information sur la dynamique du coasting chez les donneuses pré et post-pubères. Nous avons intégré des données transcriptomiques issues des bases de données publiques dans une méta-analyse de type ''vote-counting'' afin d'élucider les changements moléculaires dans les cellules de la granulosa lors d'une différentiation poussée du follicule et lors de l'atrésie. Grâce à la méta-analyse, nous avons découvert l'expression des gènes associée avec la mort folliculaire et nous avons identifié des biomarqueurs potentiels sur le statut des cellules de la granulosa. L'analyse des six biomarqueurs sélectionnés entre les donneuses pré- et post-pubères a démontré que les jeunes donneuses expriment plus les marqueurs d'atrésie après la même période de coasting que les donneuses sexuellement matures. Ainsi, nous avons découvert que les jeunes donneuses ont une dynamique différente de coasting possiblement due à une insuffisance dans la signalisation de l'hormone lutéinisante (LH).

5.2. Abstract

The use of younger gamete donors in dairy cattle genetic selection programs significantly accelerates genetic gains by decreasing the interval between generations. Ovarian stimulation and the practice of follicle-stimulating hormone (FSH) withdrawal, also known as coasting, are intensively used in pre-pubertal heifers without detrimental effects on subsequent reproductive performance but generally with lower embryo yields. However, recent data from embryo transfer programs showed similar embryo yields in younger and sexually mature animals but with a significant difference in the coasting period. The aim of the present study was to identify a set of granulosa cell biomarkers capable of distinguishing optimal follicle differentiation from late differentiation and atresia in order to assess the differences in coasting dynamics between pre- and post-pubertal donors. We integrated transcriptomic data sets from a public depository and used vote counting meta-analysis in order to elucidate the molecular changes occurring in granulosa cells during late follicle differentiation and atresia. The meta-analysis revealed the gene expression associated with follicle demise, and most-importantly, identified potential biomarkers of that status in bovine granulosa cells. The comparison of the expression of six biomarkers between pre- and post-pubertal donors revealed that younger donors had more signs of atresia after the same period of coasting. We found different follicular dynamics following coasting in younger donors. It is possible that younger donors are less capable to sustain follicular survival most likely due to insufficient LH signaling.

5.3. Introduction

The manipulation of reproductive hormones requires knowledge of ovarian physiology, which is mainly based on a network of extracellular and intracellular molecular interactions controlled by follicle-stimulating hormone (FSH) and luteinizing hormone (LH). One of the functions of FSH is the tonic stimulation of granulosa cells via its receptor FSHR, which increases intracellular cAMP formation and activation of genes required for proliferation and differentiation (Hillier, 2001). Then, tonic stimulation of granulosa cells from dominant follicles by LH via its receptor LHR prior to its surge mimics some of the FSH actions while its surge triggers ovulation (Hillier, 2001; Nivet et al., 2017). Therefore, the fate of follicles is based on the granulosa cell responses to circulating hormones (Matsuda et al., 2012). Understanding these interactions is crucial to define new approaches for pharmaceutical manipulation of ovarian function and to improve fertility treatments.

In cattle, assisted reproductive technologies (ART) such as in vitro fertilization and embryo transfer were created and have been used for animals of high commercial value. One important discovery that has significantly increased embryo yields in dairy cows is ovarian stimulation (OS) followed by FSH withdrawal, also known as coasting (Sirard et al., 1999; Blondin et al., 2002). This technique is based on the maintenance of a high concentration of gonadotropin by injection of exogenous FSH for three days followed by a period of time with no exogenous FSH before the ovum pick-up (OPU) procedure. During this time, a temporary improvement in oocyte developmental competence generally occurs (Nivet et al., 2012). Indeed, the final oocyte competence acquisition occurs between the FSH decline and the pre-ovulation LH surge artificially induced by coasting (Sirard et al., 2006). Interestingly, oocyte quality increases at the beginning of coasting, then reaches a maximum after 2 days and plateaus, and then decreases as the follicle begins to undergo atresia and late differentiation (Nivet et al., 2012). However, coasting dynamics and the quality of oocytes following coasting vary significantly between animals and according to their sexual maturity (Landry et al., 2016, 2017).

Although ovarian stimulation is possible in peri-pubertal or even pre-pubertal heifers, it is well documented that embryo yields are significantly lower compared to sexually mature

cows (Gandolfi et al., 1998; Salamone et al., 2001; Kauffold et al., 2005; Landry et al., 2016). Multiple essential biological functions such as cell differentiation, cell survival and death, inflammation, and apoptosis signalling are affected in peri-pubertal heifers (Landry et al., 2017). Knowing that basal LH is lower before puberty and gradually increases as heifers reach puberty (Rodriguez and Wise, 1989; Day and Nogueira, 2013), it was proposed that the unsatisfactory reproduction rates in peri-pubertal heifers is possibly related to the insufficiency in LH signalling (Landry et al., 2017).To support this concept it is possible to identify a number of genes that were associated with atresia and late differentiation in previous studies (Nivet et al., 2013; Douville and Sirard, 2014; Girard et al., 2015). By performing a meta-analysis of transcriptomic data from optimal and late differentiated and atretic follicles, we discovered a number of potential biomarkers to assess the follicular status during coasting. These targets were validated on granulosa cell samples from animals of different ages and different coasting times to test the hypothesis. We found that, indeed, insufficient LH signaling in younger donors during coasting forces follicles to undergo atresia much sooner compared to follicles from sexually mature cows during the same period of coasting. Thus, the optimal window of oocyte competence following coasting is reduced and moved earlier in time.

5.4.Materials and methods

5.4.1. Ethics statement

The clinical procedures and industrial practices used at Boviteq follow the established cattle reproduction management practices, which have been approved by the College of Veterinary Surgeons of Quebec (OMVQ), the Canadian Embryo Technology Association (CETA), and the International Embryo Transfer Society (IETS). This company follows the Canadian Council on Animal Care (CCAC) guidelines for farm animals and the research projects do not involve the use of exclusive animals for research purposes or the implementation of new animal procedures, other than the ones used in their routine commercial activities, to obtain additional biological samples. This study did not require handling animals on university premises.

5.4.2. Data retrieval

Microarray gene expression data from earlier studies of granulosa cells (Gene Expression Omnibus number: GSE110578, GSE40916, GSE63904, GSE63918, GSE63919, and GSE56145) were retrieved from the ELMA database and pooled together for meta-analysis. All datasets were generated using the EmbryoGENE bovine microarray and were analyzed using the following contrasts: heifers with low blastocyst yields vs cows with optimal blastocyst yields, 44 vs. 92 h, and 68 vs. 92 h of FSH withdrawal, plateau vs. atretic follicles of 6-9mm and >9mm in diameter. All microarray experiments were validated in the original studies (Nivet *et al.*, 2013; Douville and Sirard, 2014; Girard *et al.*, 2015b). The data from each microarray were subjected to a simple background subtraction, normalized within array (Loess) and between array (Quantile), and analyzed statistically with the Limma package using FlexArray 1.6.1 (http://genomequebec.mcgill.ca/FlexArray)

5.4.3. Meta-analysis of biomarkers of atresia

Microarray meta-analysis was performed using the vote counting method (Tseng *et al.*, 2012; Rikke *et al.*, 2015). Briefly, differentially expressed (DE) genes are first selected based on a threshold to obtain a list of DE genes from each study. The vote for each gene is then calculated as the total number of times it occurs in all DE lists, and the final DE genes are

selected based on the minimal number of votes set by the user. In this study, DE genes with a threshold p-value lower than 0.1 from five different arrays were used in the vote counting meta-analysis. The vote counting strategy used in this study ranked biomarkers on the basis of one principal and two secondary criteria. The principal criterion was the number of supporting studies in which each microarray showed significant differential expression in the same direction for a given biomarker, and this counted as a vote in favor of that biomarker being real. Because vote counting frequently leads to ties, we also used two secondary criteria. One being that the first vote should be from the study contrasting good (over 70% embryo yield) and poor donors (lower than 35% embryo yield), in order to find biomarkers that are expressed in poor donors and in association with different stages of atresia. The second was the computation of a simple fold change average between all counts for a given biomarker to differentiate the most promising ones in the full set. The vote counting method was previously tested and approved as a strategy to rank biomarker candidates and is indeed an effective method for selecting biomarkers (Rikke *et al.*, 2015).

5.4.4. Analysis of biological functions and upstream regulators

In order to determine which biological functions were affected by the candidate biomarkers, the meta-analysis datasets were subjected to functional analysis using Ingenuity ® Pathway Analysis (IPA). Briefly, the lists of differentially expressed genes from the meta-analysis were uploaded into IPA ® and analyzed for major biological functions and potential upstream regulators. This attributed the probability of association between DE genes in the datasets and major biological functions affected. Also, IPA ® determines the upstream regulators of DE genes by referring to its database of previously known effects of different molecules (endogenous or exogenous) on target genes. Each upstream regulator has an overlap p value and an activation Z score. The activation z score is an overall score based on the known effects (up regulation or down regulation) of a molecule on each of its target genes. An upstream regulator is thus attributed an activated (Z-score > 2), inhibited (Z-score < -2), or uncertain state based on the observed changes in gene expression of known downstream targets. This analysis enabled us to identify major upstream regulators as a consequence of follicular atresia.

5.4.5. Ovarian stimulation treatment and granulosa cell collection

Samples were obtained according to a previously published study protocol of embryo production from Holstein Bos Taurus (Landry *et al.*, 2016). Briefly, each animal was first treated with progesterone (Zoetis) in order to reduce the risk of spontaneous ovulation. All large follicles (superior or equal to 5 mm) were aspirated 36-48 h prior to administration of hormones. The ovarian stimulation program consisted of six injections of NIH Folltropin-V (Bioniche Animal Health) administered at 12 h intervals. According to animal age, body weight and/or based on previous stimulations, FSH was administered in five 30-mg, six 30-mg, or six 40-mg doses of NIH Folltropin-V, followed by a coasting (no FSH) period of 30 or 43 h. Using transvaginal ultrasonography, follicular diameters were measured and cumulus-oocyte complexes (COCs) were collected by transvaginal puncture under epidural anesthesia (COOK Medical, Bloomington, IN, USA) using an 18-G needle and COOK aspiration unit (COOK Medical). Granulosa cells and COCs were collected in warm HEPES-buffered Tyrode's medium (TLH) containing Hepalean (10 Ul/ml). After removing the oocytes and cumulus, granulosa cells were purified by centrifugation (4500 RPM for 1 minute) and the cell pellets were snap frozen on dry ice before RNA extraction. The OPU procedure was performed in a commercial IVF setting, namely at Boviteq (Saint-Hyacinthe, QC, Canada), a center specialized in bovine embryo transfer and other assisted reproduction technologies involved in research and development.

5.4.6. RNA extraction

Total RNA was extracted from granulosa cells from 32 heifers and 28 cows using the *RNeasy mini kit* (Qiagen), following the protocol recommended by the manufacturer. Total RNA integrity and concentration were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) with the RNA NanoLab Chip (Agilent Technologies). All extracts were of good quality with RNA integrity numbers higher than 8.9.

5.4.7. Complementary DNA preparation and quantitative real-time polymerase chain reaction

To confirm the microarray analysis results, quantitative real-time polymerase chain reaction (qRT-PCR) was performed on cDNA. Briefly, RNA (325 ng) from granulosa cells (all samples) was reverse-transcribed using a qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) with a mixture of oligo dT and random primers according to the manufacturer's recommendations. The primers used for qRT-PCR are listed in (supplemental table 1) and were designed using the IDT PrimerQuest tool (http://www.idtdna.com/primerquest/home/index) from sequences obtained using *Bos taurus* (taxid: 9913) reference RNA sequences (refseq_rna) and results from our meta-analysis. To confirm the specificity of each pair of primers, electrophoresis on a standard 1.2 % agarose gel was performed for each amplified fragment. The PCR products were then purified with the QIAquick PCR purification kit (Qiagen), quantified using the NanoDrop ND-1000, and sequenced. The products were then used to create standard curves for quantification experiments, with dilutions ranging from 2 x 10^{-4} to 2 x 10^{-8} ng/nL. Real-time PCR was performed on a LightCycler 480 (Roche Diagnostics, Laval, QC, Canada) using SYBR incorporation in order to analyze gene expression stability in the two groups of granulosa cells (heifers with 30 h of coasting vs cows with 30 h of coasting; heifers with 43 h of coasting vs cows with 43 h of coasting). Each qRT-PCR reaction, in a final volume of 10 µL, contained the complementary DNA, 0.25 mM of each primer, and 1X SYBR mixture (Light-Cycler 480 SYBR Green I Master, Roche Diagnostics). The PCR conditions used for all genes were as follows: denaturing for 10 min at 95 °C; 50 PCR cycles (denaturing, 95 °C for 10 s; annealing (Supplemental Table 2) for 10 s; extension, 72 °C for 20 s), a melting curve (95 °C), and a final cooling step at 40 °C. Complementary DNA quantifications were performed using LightCycler 480 software version 1.5 (Roche Diagnostics) by comparison with the standard curves. Polymerase chain reaction specificity was confirmed by melting-curve analysis provided by the LightCycler software. The data were normalized through geNORM, and the most stable reference genes were identified by the stepwise exclusion of the least stable gene and recalculating the M values. Four housekeeping genes, namely B-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), splicing factor 3 subunit 1 (*SF3A1*), and TATA-Box binding protein (*TBP*) were found to be the most stable

genes with M values ≤ 1.5 as recommended by the software to normalize our data. Normalized data were cleaned using the Rout method of outlier removal and were analyzed using unpaired T test in order to establish significance. Differences were considered to be statistically significant at the 95 % confidence level ($p < 0.05$). Values are presented as mean ± standard error of the mean. All statistical analyses were performed using Graphpad Prism version 7.00 (Graphpad software).

5.5. Results

Using the vote counting method, we performed transcriptome meta-analyses of four independent microarray studies (five microarray datasets in total). Each individual array analyzed a different follicular status from the optimal period toward follicular atresia. The DE genes were proposed as potential biomarker candidates and were submitted to functional analysis within IPA and validation by real time PCR.

In order to explore the effects of coasting time in young and older donors, data from 331 dairy cattle subjected to OS and coasting were randomly selected and divided into two groups based on age: 170 pre-pubertal heifers (ages were less than 10 months) and 161 post-pubertal heifers (ages between 12 and 18 months). There was no significant difference between the blastocyst rates of pre- and post-pubertal animals. However, the duration of coasting period required to achieve the same blastocyst yield in the two groups was significantly different (chi-square < 0.0001) (figure 5-1). A summary of the 30 most differentially expressed genes are shown in table 5-1.

5.5.1. Meta-Analysis

Our hypothesis suggests that younger donors have a lower capability to sustain follicle growth during FSH withdrawal and this leads to follicular atresia. Thus, we compared the DE genes of a total of four microarrays that specifically compared granulosa cells from the optimal coasting period to granulosa cells from late-differentiation, and granulosa cells from plateau follicles to granulosa cells from atretic follicle of 6-9 mm and >9 mm diameter. Using a vote counting meta-analysis in order to identify potential biomarkers, a total of 193 DEGs

were significantly associated with late follicle differentiation and/or atresia in young donors (supplemental table 2).

5.5.2. Functional Analysis

Using the datasets of modulated genes from the meta-analysis above, Ingenuity Pathway Analysis revealed a significant association between a group of genes expressed differentially in granulosa cells and certain biological functions. The latter include cell death and survival, cellular development, connective tissues development, and cellular growth and proliferation (figure 5-2). In addition, Ingenuity Pathway Analysis generated statistically significant predictions for increased (z-score > 2) or decreased (z-score < −2) activity of upstream transcriptional regulators that are the most activated or inhibited (Table 1A) and the most significant (Table 1B) in association with the average fold change of the DE genes from the meta-analysis.

5.5.3. Validation of Meta-analysis Gene Expression

Confirmation of the microarray and meta-analysis results was obtained in the form of real-time qPCR data for transcripts selected on the basis of differential expression in the poor pre-pubertal donors and on the basis of the meta-analysis. Six candidate biomarkers were selected according to the difference in their expression levels compared to the optimal condition and their relevance in follicular atresia: Mitochondria-localized glutamic acid-rich protein (*MGARP*) also known as the ovary specific acidic protein (*OSAP*), Gasdermin B (*GSDMB*), Creatine kinase brain isoform (*CKB*), Potassium channel tetramerization domain containing 8 (*KCTD8*), Cystein-rich secretory protein LCCL domain containing 2 (*CRISPLD2*), and Inhibin A (*INHA*) (figure 5-3). The expression patterns of all candidate genes were consistent with the meta-analysis results.

5.6. Discussion

The results of this study showed that pre-pubertal donors have different follicular dynamics following FSH withdrawal and reach follicular atresia sooner than post-pubertal donors. With the accumulation of transcriptomic data from microarray experiments, it was possible to use meta-analysis to further investigate the transcriptional regulation of genes across multiple studies and obtain biologically significant information (Tseng *et al.*, 2012). Using the vote counting method for meta-analysis of differentially expressed genes (DEGs) (Rikke *et al.*, 2015), we compared granulosa cells from optimal follicles to granulosa cells from late differentiated and atretic follicles in superstimulated cows.

Interestingly, embryo rates from pre-pubertal animals, younger than 8 months of age, increased significantly (from 30% to 40% blastocysts) over the last few years (Landry *et al.*, 2016, 2017 and this paper), and this is a reflection of the empirical adaptation of coasting time. We previously demonstrated that oocytes originating from heifers younger than 10 months of age are less competent compared to oocytes from sexually mature cows (Landry *et al.*, 2016). Before the use of coasting, many studies demonstrated that bovine embryos could be produced successfully *in vitro* using oocytes from sexually immature animals (Majerus *et al.*, 1999; Palma *et al.*, 2001) but with a significant reduction in embryos yields (Revel *et al.*, 1995; Presicce *et al.*, 1997; Khatir *et al.*, 1998). In the present study, the embryo yield was similar between pre- and post-pubertal cows. However, the coasting period was significantly different between the two groups, suggesting that a shorter period of FSH withdrawal in pre-pubertal animal results in different follicle dynamics and a better embryo outcome. Our meta-analysis may explain the differences as we found differentially expressed genes between optimal and late differentiation and atretic follicles that characterize the follicle status in pre- and post-pubertal animals.

5.6.1. Gene analysis

In this study, the genes were analyzed in a standard contrast between pre- and post-pubertal oocyte donors. In this context, depending on the selected biomarkers, they can be associated with optimal timing or late differentiation and atresia. The mitochondria-localized glutamic acid-rich protein (MGARP), also known as the ovary specific acidic protein (OSAP), is up-

regulated in dominant follicles (Liu *et al.*, 2009). Although the detailed regulatory mechanisms are not yet fully understood, it is known that MGARP is a mitochondrial transmembrane protein that operates during steroidogenesis (Matsumoto *et al.*, 2009). It was proposed that MGARP functions as a transporter for the precursors of steroidogenesis and/or helps the newly synthesized steroids exit mitochondria (Zhou *et al.*, 2011). Moreover, its expression increased in the same proportion as sex hormone levels (Zhou *et al.*, 2011). This association of *MGARP* with steroidogenesis is consistent with our data demonstrating its down regulation in atretic follicles and its association with lower progesterone production as predicted by IPA.

The gene encoding for Cystein-rich secretory protein LCCL domain containing 2 (CRISPLD2) is also regulated by progesterone as its expression was significantly increased following progesterone injection in mice (Sriraman *et al.*, 2010; Yoo *et al.*, 2014). Moreover, the suppression of CRISPLD2 was associated with the down regulation of some important extracellular matrix genes (Zhang *et al.*, 2016) which suggests a potential role in the regulation of the ECM enzymes and proteins following the increase in progesterone. Interestingly, CRISPLD2 also inhibited inflammation by the modulation of interleukin IL1-B and IL6 (Zhang *et al.*, 2016). The lack of *CRISPLD2* expression contributes to the increase in inflammation and potentially the increase in ROS since atresia is an inflammatory process of cellular death. This suggests that after the same time of coasting, younger donors are more advanced in atresia signaling compared to mature cows.

The inhibins (Inhibin A and Inhibin B) are glycoprotein hormones from the transforming growth factor B (TGF-B) family. Inhibin A (INHA) is mainly secreted by differentiated granulosa cells under LH during the pre-ovulatory phase (Welt *et al.*, 1999; Welt and Schneyer, 2001) while Inhibin B (INHB) is secreted by developing granulosa cells (Broekmans *et al.*, 2009). The expression of *INHA* was detected in granulosa cells from dominant follicles and in the corpus luteum, but not in small antral follicles (Schwall *et al.*, 1990). Thus, the level of INHA depends on gonadotropin stimulation and on the stage of follicle development (Welt and Schneyer, 2001). The expression of *INHA* decreased faster (at 43 h of coasting) in young animals, supporting the hypothesis of a potential lack of LH

action. Taken together, these results suggest that in younger donors, the absence of progesterone, potentially due to the lack of LH signaling, down regulate essential genes that control steroidogenesis and inflammation, leading to the promotion of cellular death and atresia.

Younger donors also expressed multiple atresia biomarkers. The creatine kinase (CK), brain isoform (CKB) is a fundamental enzyme for cellular energy due to its mechanism of storing, buffering, and transporting high-energy phosphates from the mitochondria to places that require energy (Zervou et al., 2017). The apoptosis process can be ATP-dependent or not, but most commonly, follicular atresia occurs by caspase activation, an ATP-dependent process (Tsujimoto, 1997). Based on the high demand of ATP for atresia and the fundamental role of CKB in the maintenance of cellular energy, we can suggest that CKB levels are expected to be increased in atretic follicles. Moreover, a recent study demonstrated higher expression of CKB in pre-pubertal donors compared to post-pubertal cows (Landry et al., 2017). Similarly, the potassium channel tetramerization domain 8 (KCTD8) gene is part of a voltage-gated potassium channel that affects the permeation of potassium (K+) through the channel pore (Paus et al., 2012). A reduction of intracellular levels of K+ may be one of the permissive signals for apoptosis (Yu, 2003). Low intracellular K+ decreased the DNA-binding activity of anti-apoptotic transcription factors and increased the binding activity of the pro-apoptotic transcription factors (p53 and forkhead) (Yang et al., 2006). Several classes of potassium channels are expressed in granulosa cells (Mason et al., 2002; Traut et al., 2009) and it was previously suggested that the loss of potassium in oocytes and granulosa cells may be involved in initiating the cascade of events leading to their demise (Perez et al., 2000). Suppressing the potassium efflux in whole cells prevents the activation of the nuclease and caspase activity whereas enhancing the efflux of K+ activate those apoptotic enzymes (Hughes and Cidlowski, 1999). This suggests that a lower level of KCTD8 would reduce the intracellular level of K+ by modulating its permeability in granulosa cells and may lead the follicle to atresia in pre-pubertal donors. On the opposite, it is possible that a high concentration of KCTD8 prevent atresia in older animals by conserving normal level of K+ in granulosa cells.

Gasdermin B (GSDMB) is one of the four members of the Gasdermin family and plays a role in the regulation of epithelial proliferation by acting as a tumor suppressor (Hergueta-Redondo *et al.*, 2014). Interestingly, GSDMB is a regulator of TGF-B1 expression which has dual functions with a role in granulosa cell proliferation, differentiation, and apoptosis (Kale *et al.*, 2013). We suggest that GSDMB is an important mediator of cellular death in granulosa cells and its expression increases apoptotic signals and TGFB to move the follicle toward atresia.

5.6.2. Functional Analysis

We also performed functional analysis of the DE gene dataset to describe the biological functions and the upstream regulators associated with the follicle demise. One of the most important factors in folliculogenesis is the balance between cell survival and cell death. It comes to no surprise that biological functions such as cell death and survival, cellular development, growth and proliferation were modulated during granulosa cell late differentiation and atresia. Indeed, apoptosis signaling plays a critical role in the maintenance of the ovarian function in order to remove most follicles that are not suited for ovulation. The first sign of atresia is the initiation of apoptosis in mural granulosa cells which involves both intrinsic and extrinsic signaling (Carou *et al.*, 2017). A complete description of apoptotic pathways in granulosa cells is not available yet, but a spectrum of pro- and anti-apoptosis molecules are finely regulated by hormones such as gonadotropins, growth factors, cytokines, and estrogens (Matsuda *et al.*, 2012). Interestingly, the upstream regulator analysis revealed some potential modulators of atresia.

The initiation of follicular atresia is orchestrated by the depletion of key survival-promoting factors and/or by specific death ligand molecules (Matsuda-Minehata *et al.*, 2006). In granulosa cells, estradiol is produced by the aromatase under FSH and has various actions such as promoting folliculogenesis and inhibiting cell apoptosis (see review Rosenfeld *et al.*, 2001). We believe that the decline in FSH and estradiol before the LH surge is a trigger for the initiation of follicle differentiation (Sirard *et al.*, 2006). Thus, the follicle produces cytokines in order to increase inflammation and vascularization which are activated similarly in early apoptosis (Hatzirodos *et al.*, 2014). If the LH receptor is expressed in the dominant follicle, the LH surge will trigger differentiation and ovulation. On the other hand, the

absence of LHR and/or LH signaling will push the follicle toward atresia following a progressive depletion of survival factors initiated by FSH, estradiol and/or basal LH. Follicle atresia was increased in cows treated with an antagonist to GnRH (Cetrotide) that removes basal LH during FSH withdrawal (Nivet *et al.*, 2017), suggesting a crucial role of basal LH in follicle survival. It is interesting to notice that early signs of atresia has a positive effect on oocyte developmental competence (Sirard *et al.*, 1999; Vassena *et al.*, 2003) which is characteristic of the first step in the initiation of follicle differentiation following FSH decline or withdrawal (Sirard *et al.*, 2006).

Follicular atresia is also induced by death ligand-receptors such as the tumor necrosis factor (TNF) signaling (Matsuda *et al.*, 2012; Yamamoto *et al.*, 2015). In a recent meta-analysis, we determined that TNF is an important regulator of cell differentiation associated with optimal oocyte developmental competence but also associated with atresia in follicle persistence (Landry et al., 2018). Indeed, TNF is a multifunctional pro-inflammatory cytokine that mediates a wide range of biological functions by binding to two specific receptors (type I and II). Type I receptor contains an intracellular death domain and type II induces gene expression, cell survival, growth and differentiation (Sakumoto et al., 2003). Tumor necrosis factor plays a broad role during the preovulatory period as it induced ovulation in perfused rat ovaries and the effect was further increased by the addition of LH (Brännström et al., 1995). On the other hand, TNF expression was increased to promote the demise of unruptured follicles (Yamamoto *et al.*, 2015), necrosis being the result of an inflammatory reaction (Yang *et al.*, 2015). However, inflammation also plays a role during folliculogenesis as it is well accepted that mammalian ovulation is comparable to an inflammatory reaction (Jabbour *et al.*, 2009). In this study, we showed that atresia involves multiple cytokines inducing inflammation. The follicle persistence without LH surge may cause an inflammatory cascade through the non-canonical pathway of NF-kB which stimulates the production of pro-inflammatory cytokines such as IL-1B, IL6, INFG, and angiogenesis factors (Machelon and Nome, 1999; Bhattacharyya *et al.*, 2010; Santulli *et al.*, 2015; Yang *et al.*, 2015) that modulate follicular regression.

5.6.3. Conclusion

The FSH decline before the LH surge plays a crucial role during oocyte developmental competence acquisition which can be reproduced artificially by FSH withdrawal. However, we showed that pre- and post-pubertal cows have different dynamics following coasting (figure 5-4) and younger donors are less capable of sustaining follicular survival, most likely due to their lack in LH signaling. Thus, it is recommended that FSH withdrawal be shorter in younger donors in order to achieve higher blastocyst rates. Moreover, we provided a set of new biomarkers that will help to characterize the follicular status of atresia in bovine granulosa cells. Those biomarkers will provide new insight in the evaluation of ovarian stimulation regimens.

5.7. Acknowledgement

We would like to thank Boviteq for allowing the use of granulosa cells and data from their heifers and cows.

5.8. Funding

The Natural Sciences and Engineering Research Council of Canada - Collaborative Research and Development (NSERC-CRD grant no. RDCPJ461697-13) provided funding for this study. D.A. Landry received studentship support from NSERC and REDIH (CIHR Training program in Reproduction, Early Development, and Impact on Health). L.R-P. received a studentship support from ELAP (Emerging Leader in the Americas Program).

5.9. Declaration of interest

The authors declare no potential conflicts of interest.

5.10. References

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5.11. Figures

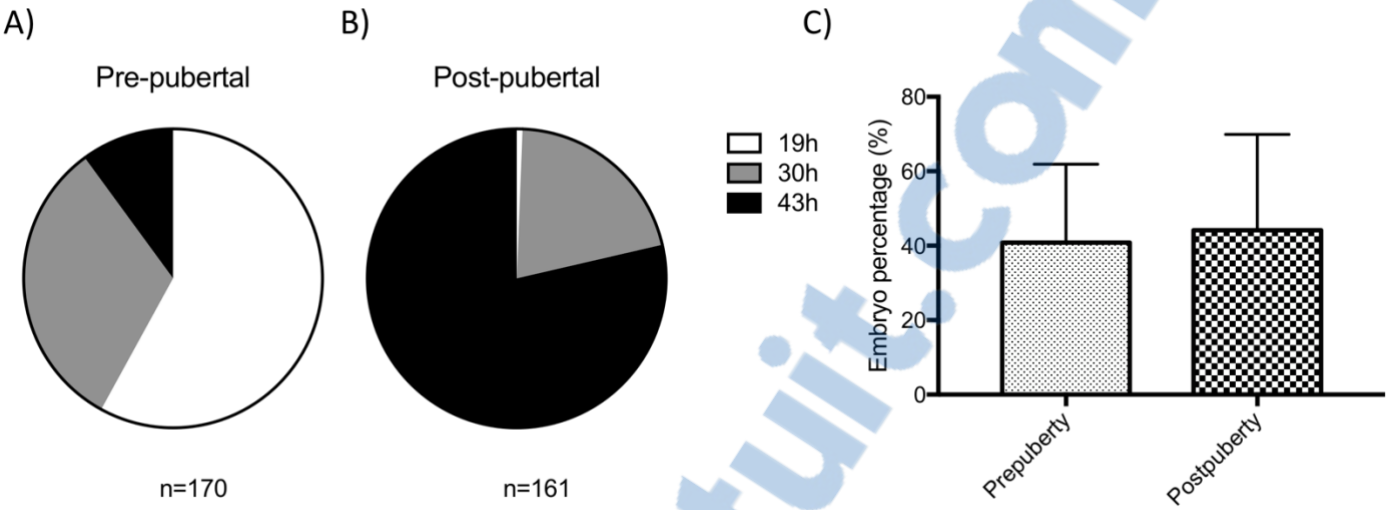

Figure 5-1: Relationship between oocyte donor age and coasting period length during ovarian stimulation regimens

Coasting period were reported from A) 170 pre-pubertal heifers and B) 161 post-pubertal cows. For the same animals, C) the mean embryo percentages 7 days following in vitro fertilization of all oocytes matured were also reported.

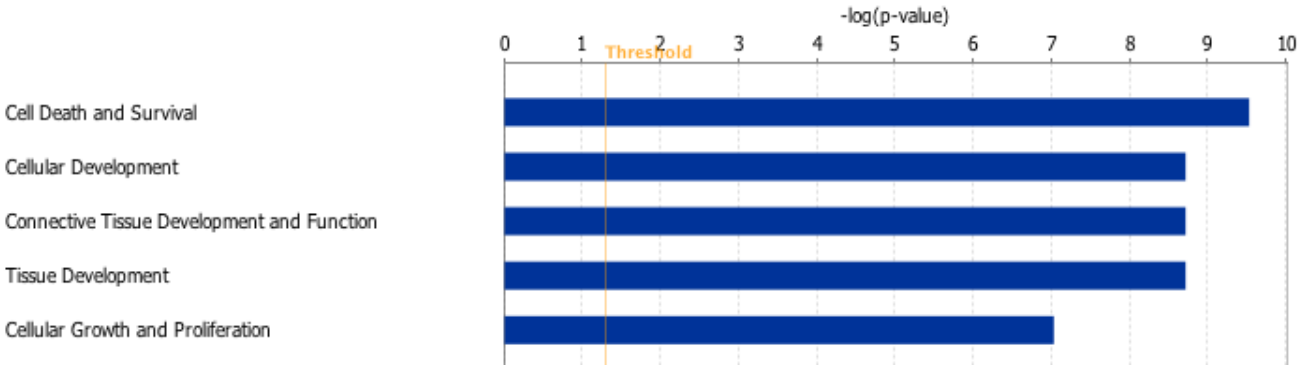

Figure 5-2: Biological functions analysis from Ingenuity Pathway Analysis

The five most significant biological functions from the meta-analysis datasets of the differentially expressed genes (DEGs) in granulosa cells associated with follicle late differentiation and atresia. The threshold line represents the significant P-value.

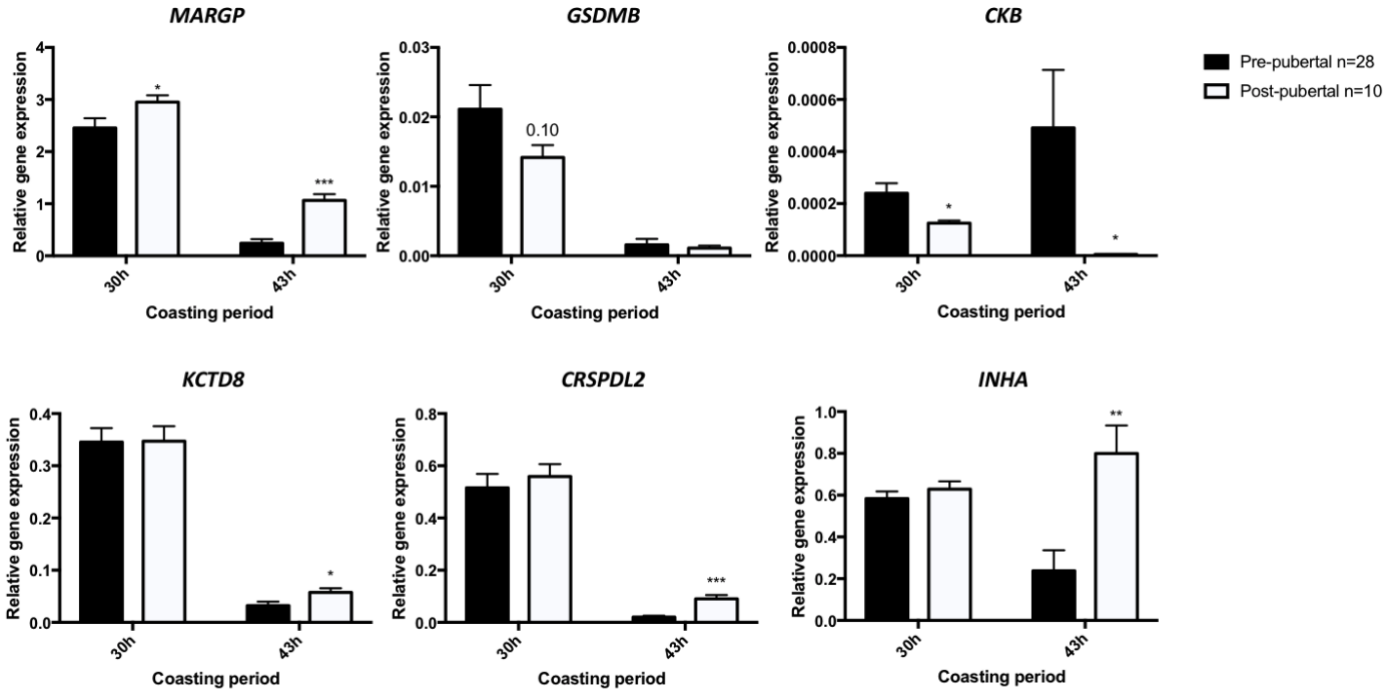

Figure 5-3: Gene expression levels of six selected biomarkers

Gene expression levels of six selected biomarkers were used to assess the atresia level at A) 30 h and B) 43 h of coasting in pre- and post-pubertal bovine granulosa cells. RT-qPCR values are mean relative expression with standard error of the mean compared to the reference groups (black bar). Significant differences were calculated using unpaired T test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

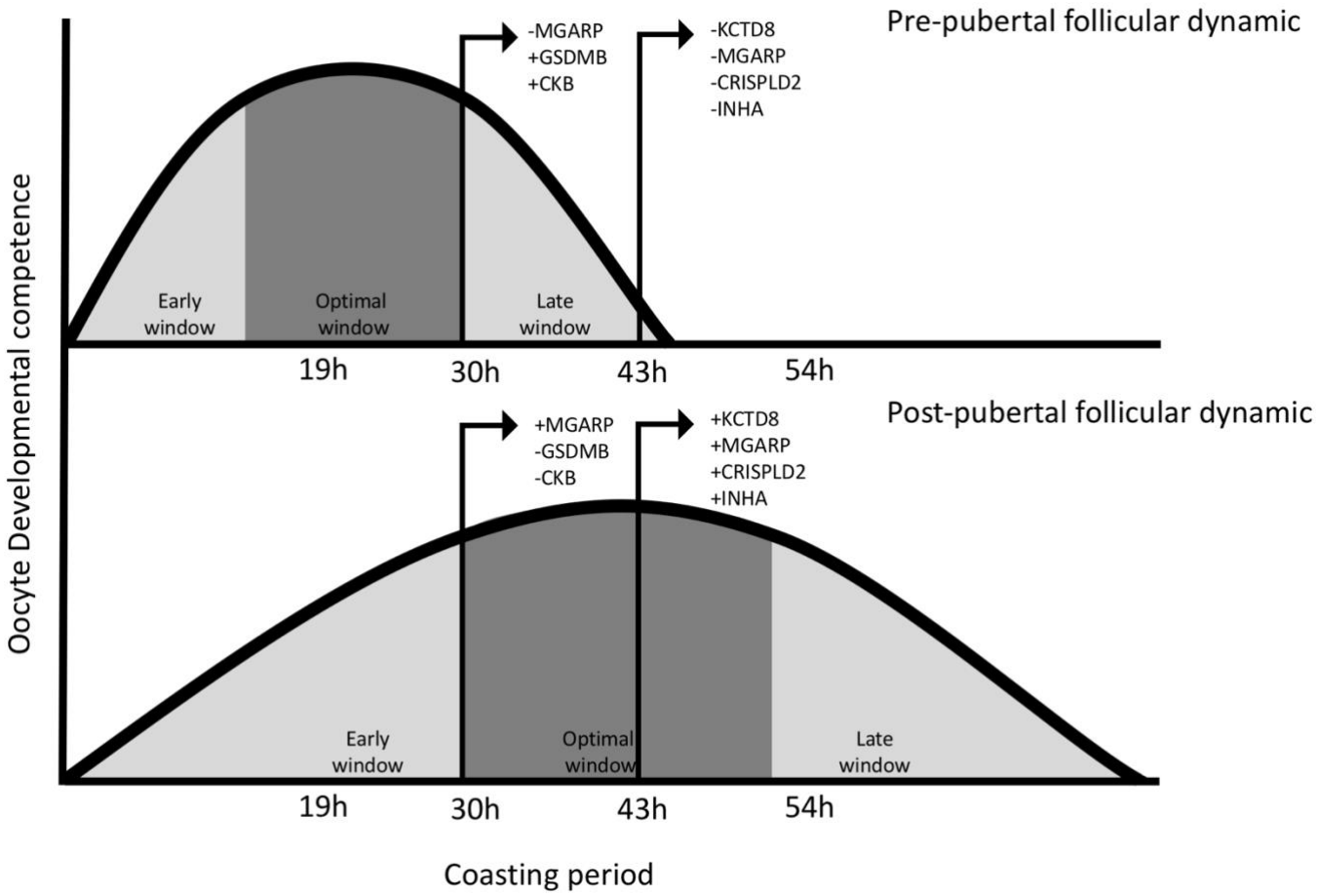

Figure 5-4: Schematization of follicle dynamics following coasting in pre- and post-pubertal donors

The oocyte developmental competence is represented by the black curve across the coasting period. Each specific phase of follicle dynamics is described inside the curve as follows: early, optimal, and late windows of oocyte developmental competence. The arrows represent the expression of the selected biomarkers, a plus or negative (+/-) sign represents an increase or decrease in gene expression compared to the other group for the same period of coasting.

5.12. Tables

Table 5-1: Top 30 most differentially expressed genes from the granulosa cell's meta-analysis.

The top 15 most down regulated genes and the top 15 most up-regulated genes in the meta-analysis of granulosa cells are shown with their average fold change and p-value. A complete list of DEGs is available in supplementary table 2.

Gene Symbol	Average FC	Average P-Value	Gene Symbol	Average FC	Average P-Value
CALB2	-3.80	0.0287743	SH3RF1	2.37	0.0212858
NMB	-3.17	0.0083525	FOLR2	2.38	0.0395062
INHA	-2.90	0.0223478	CLCA2	2.40	0.0393079
SUSD4	-2.62	0.0214671	CKB	2.41	0.0235166
GSTA5	-2.51	0.0298568	DEFB4	2.41	0.0096596
TBC1D8	-2.39	0.0154918	GSDMB	2.46	0.0261716
GRB14	-2.36	0.0277227	ETS2	2.52	0.0166111
OSAP	-2.34	0.0205689	CD5L	2.74	0.0355574
ECRG4	-2.27	0.0171964	SWAP70	2.77	0.0571574
NAP1L5	-2.22	0.0092056	IL1A	2.96	0.0040150
CITED1	-2.21	0.0052008	CTSS	3.26	0.0236380
TOX2	-2.20	0.0268395	PLAT	3.60	0.0300998
LRRC17	-2.19	0.0158701	TRIB1	3.70	0.0242082
CRISPLD2	-2.12	0.0547113	DEFB5	3.76	0.0253889
FDFT1	-2.06	0.0070817	FABP4	4.02	0.0304273

Table 5-2: Predicted upstream regulators in granulosa cells from the meta-analysis datasets of the differentially expressed genes (DEGs) in granulosa cells associated with follicle late differentiation and atresia

A) Most activated and B) most significant upstream regulators. For each upstream regulator, molecule type, predicted activation state, Z-score, and statistical significance (P-value of overlap between the dataset and the genes that are regulated by the upstream regulator) are shown in the table. Red font color is not significant.

A)

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
NFkB	complex	Activated	2.183	1.45E-01
CXCL12	cytokine	Activated	2	2.59E-02
STAT1	transcription regulator		1.982	1.33E-01
IL1A	cytokine		1.965	4.73E-02
IL6	cytokine		1.942	6.06E-02
IL1B	cytokine		1.913	7.56E-04
CSF2	cytokine		1.912	5.46E-03
CREB1	transcription regulator		1.89	1.41E-02
APP	other		1.769	2.24E-04
TLR4	transmembrane receptor		1.756	5.49E-03
TNF	cytokine		1.629	6.61E-05
IFNG	cytokine		1.616	1.78E-03
AHR	ligand-dependent NR		-1.741	1.40E-02
progesterone	chemical - endogenous		-1.757	2.78E-03
PSEN1	peptidase		-1.969	7.53E-03

B)

Upstream Regulator	Molecule Type	Activation z-score	p-value of overlap
beta-estradiol	chemical - endogenous mammalian	0.993	9.24E-07
Vegf	group	-0.786	3.05E-05
TNF	cytokine	1.629	6.61E-05
TP53	transcription regulator	0.623	8.87E-05
HRAS	enzyme		1.25E-04
APP	other	1.769	2.24E-04
ACKR1	g-protein coupled receptor		2.56E-04
VEGFA	growth factor	0.818	3.13E-04
HIC1	transcription regulator	0	4.07E-04
IL10RA	transmembrane receptor	-0.788	4.07E-04
ESR1	ligand-dependent nuclear receptor	0.612	4.34E-04
FSH	complex		5.27E-04

HNF1B	transcription regulator	-0.152	6.01E-04
Lh	complex		7.51E-04
IL1B	cytokine	1.913	7.56E-04

5.13. Supplemental data

Supplemental Table 5-1: List of the primer sequence used in this study

Primers used in real-time PCR experiments

Gene Symbol		Primer sequence	Product size (pb)	Annealing temperature	GenBank Accession Number
GAPDH	Fwd:	CCAACGTGTCTGTTGTGGATCTGA	218	57	NM_001034034.2
	Rev:	GAGCTTGACAAAGTGGTCGTTGAG			
ACTB	Fwd:	ATCGTCCACCGCAAATGCTTCT	102	57	NM_173979.3
	Rev:	GCCATGCCAATCTCATCTCGTT			
EIF2B2	Fwd:	GAACTCATACTCTAGCACTGG	263	57	NM_001015593.1
	Rev:	GTAGATGTAGGAAGGTGCATT			
SF3A1	Fwd:	TGTGTCCCTCTTGCTGAGTT	211	57	NM_001081510.1
	Rev:	ATCGCATCCTACAGGGCATT			
TBP	Fwd:	GCCTTGTGCTTACCCACCAACAGTTC	200	57	NM_001075742.1
	Rev:	TGTCTTCCTGAAACCCTTCAGAATAGGG			
MGARP	Fwd:	GAGAACGAAAGCCCTGGTGA	182	57	NM_001166611.1
	Rev:	ACCTATCAGCCCCTCTACGG			
GSDMB	Fwd:	CCTAAGCATTCTCCCCACCAG	372	57	NM_001244217.1
	Rev:	TACTCCTCCACACTCCTCAGT			
CKB	Fwd:	CCA ATA GCC ACA ACA CGC TG	322	57	NM_001015613.1
	Rev:	TTG AGG TCG GTC TTG TGC TC			
KCTD8	Fwd:	CCCCCACCAGACAAACGTAG	343	57	NM_001205725.1
	Rev:	TCCCACATCCACTGTTCACG			
CRISPLD2	Fwd:	CTCCCGTGTTTGGAAGCAAC	158	57	NM_001100299.1
	Rev:	CTGGACTCCATTCCTGAGCG			
INHA	Fwd:	CCTGCTGGTACTGACATCCG	154	57	NM_174094.4
	Rev:	GGAACAGAGAGGACAACGCA			

Supplemental Table 5-2: Meta-analysis dataset of gene expression in granulosa cells

Dataset of the 193 differentially expressed genes (DEGs) (Gene symbol, average P-value, and average fold change) from the vote counting meta-analysis in granulosa cells associated with late differentiated and atretic follicles.

Gene Symbol	Average FC	Average P-Value	Gene Symbol	Average FC	Average P-Value
CALB2	-3.80	0.0287743	KRTCAP3	-0.66	0.0367490
NMB	-3.17	0.0083525	BZW2	-0.66	0.0489644
INHA	-2.90	0.0223478	EZR	-0.55	0.0220740
SUSD4	-2.62	0.0214671	MAN2A1	-0.55	0.0309362
GSTA5	-2.51	0.0298568	NR3C1	-0.54	0.0040755
TBC1D8	-2.39	0.0154918	SLC9A9	-0.54	0.0246601
GRB14	-2.36	0.0277227	TMEM20	-0.54	0.0225278
OSAP	-2.34	0.0205689	RPESP	-0.52	0.0322770
ECRG4	-2.27	0.0171964	FIS1	-0.51	0.0193048
NAP1L5	-2.22	0.0092056	RAD51L1	-0.50	0.0044160
CITED1	-2.21	0.0052008	GALNTL4	-0.49	0.0377356
TOX2	-2.20	0.0268395	DDX58	-0.49	0.0036787
LRRC17	-2.19	0.0158701	AKT2	-0.49	0.0179924
CRISPLD2	-2.12	0.0547113	ZCCHC6	-0.48	0.0570225
FDFT1	-2.06	0.0070817	ZNRD1	-0.48	0.0500529
HCN1	-2.04	0.0478313	RYBP	-0.47	0.0012541
ASPN	-2.03	0.0167076	GTF2H1	-0.45	0.0158073
ANKRD43	-1.96	0.0226655	MGRN1	-0.45	0.0110483
RUNX1T1	-1.91	0.0174847	C10H15orf44	-0.45	0.0052258
PRSS7	-1.88	0.0141327	PPARD	-0.44	0.0145882
ARSK	-1.88	0.0141056	WWC2	-0.43	0.0258851
PNMAL1	-1.80	0.0279399	ENPP5	-0.40	0.0317226
MRPS6	-1.80	0.0161873	MYL9	-0.39	0.0359502
LINGO2	-1.79	0.0113555	FAM20B	-0.34	0.0249846
TSPAN5	-1.79	0.0273578	IGSF11	-0.20	0.0281256
PTP4A3	-1.75	0.0209941	TSPAN13	0.02	0.0095529
TMEM170A	-1.73	0.0283870	LEPREL1	0.05	0.0430965
EPDR1	-1.72	0.0154840	PTPRN2	0.09	0.0367277
ARL6IP1	-1.72	0.0111317	C6H4ORF31	0.13	0.0584451
NRP1	-1.71	0.0395603	ALDOA	0.22	0.0145777
TWSG1	-1.71	0.0081287	SMS	0.33	0.0322363
NFE2L1	-1.69	0.0235676	MGC139448	0.35	0.0199929
PABPC4	-1.67	0.0214266	PTP4A1	0.38	0.0054035
SIL1	-1.65	0.0094717	KIAA1737	0.39	0.0221162
KCTD8	-1.64	0.0419704	OSBP	0.42	0.0586494

ATG13	-1.63	0.0145833	CALM	0.43	0.0043048
HYDIN	-1.63	0.0394835	LYZ2	0.46	0.0563957
KCTD8	-1.62	0.0427328	C19H17orf61	0.46	0.0396184
ABCA3	-1.61	0.0221019	TSPAN13	0.52	0.0074315
POLR1E	-1.60	0.0026519	PGLYRP2	0.54	0.0364691
CITED1	-1.59	0.0107580	MTDH	0.54	0.0259399
PRKD1	-1.58	0.0401817	SLC1A3	0.57	0.0264575
WHSC2	-1.57	0.0275636	ALDH1A1	0.58	0.0074987
C9ORF59	-1.57	0.0036411	TMEM176B	0.62	0.0397135
PNPLA2	-1.56	0.0321817	UTP15	0.62	0.0231346
TCFL5	-1.55	0.0047045	TTR	0.63	0.0470544
CBR4	-1.55	0.0061798	NUDT4	0.73	0.0050981
RAB13	-1.53	0.0091895	TPI1	0.80	0.0141795
MED21	-1.53	0.0457721	TIMD4	0.81	0.0220824
TMEM47	-1.52	0.0044314	ORMDL3	0.86	0.0245242
MRP63	-1.52	0.0242387	UBD	0.89	0.0497601
CEP110	-1.52	0.0186988	EFEMP2	0.93	0.0332493
PLA2G6	-1.51	0.0153260	SLC16A1	1.01	0.0326137
HSD17B1	-1.49	0.0340757	WARS	1.31	0.0252784
MTHFD1L	-1.49	0.0389205	RGS7	1.38	0.0297991
PACSIN1	-1.49	0.0114976	IFI16	1.39	0.0406436
PCDHA13	-1.49	0.0316034	MDK	1.39	0.0525996
PDCL	-1.47	0.0448324	SFRP1	1.40	0.0149335
ZNF618	-1.46	0.0145791	EHD4	1.40	0.0625118
LNP1	-1.46	0.0273032	PRDX6	1.45	0.0347095
TRIB2	-1.46	0.0385150	OPA1	1.46	0.0365148
PGCP	-1.45	0.0312064	EFNA1	1.46	0.0339898
ECE1	-1.45	0.0374273	SMPDL3A	1.47	0.0167775
KCTD8	-1.44	0.0504443	ISG12(B)	1.48	0.0480523
SAMM50	-1.44	0.0230862	LGR4	1.49	0.0273115
ZNF672	-1.42	0.0190836	S100A4	1.49	0.0417019
PREPL	-1.41	0.0070495	SCARF1	1.49	0.0196837
SPA17	-1.40	0.0220769	HMGB1	1.50	0.0100221
SSR2	-1.38	0.0274604	GDPD3	1.53	0.0418635
MECP2	-1.38	0.0317037	PRKCD	1.55	0.0318717
NKIRAS1	-1.37	0.0248427	ADORA2B	1.55	0.0847319
NR5A2	-1.35	0.0529538	PPA1	1.58	0.0700169
LDOC1	-1.35	0.0098359	SH3PXD2B	1.61	0.0130034
PSMB4	-1.35	0.0482079	FOXA3	1.64	0.0306299
COPG2	-1.35	0.0413241	ARRB1	1.67	0.0261544
C10H14ORF1	-1.34	0.0227871	HSPH1	1.68	0.0266232
NCOR1	-1.33	0.0292689	HCLS1	1.71	0.0233302
FLJ10803	-1.32	0.0276457	METRNL	1.76	0.0389838
NDUFAB1	-1.32	0.0408252	TMEM2	1.84	0.0400451

CHRD	-1.32	0.0322711	SNCA	1.91	0.0378071
FHIT	-1.31	0.0414949	PMP22	1.97	0.0366259
SCCPDH	-1.31	0.0226703	CFP	1.99	0.0248797
BRI3	-1.31	0.0145388	STK17A	2.15	0.0149472
MOSC2	-1.31	0.0410792	LTF	2.19	0.0688645
MSMB	-1.30	0.0296006	CA2	2.28	0.0694440
TRIP4	-1.30	0.0306016	TNFRSF21	2.32	0.0149943
HS3ST5	-1.29	0.0349051	SH3RF1	2.37	0.0212858
TNPO1	-1.17	0.0096326	FOLR2	2.38	0.0395062
TRIM68	-1.05	0.0283483	CLCA2	2.40	0.0393079
IL33	-1.05	0.0176295	CKB	2.41	0.0235166
SEMA6D	-0.99	0.0445520	DEFB4	2.41	0.0096596
JUN	-0.98	0.0213550	GSDMB	2.46	0.0261716
SIRT2	-0.87	0.0205483	ETS2	2.52	0.0166111
UNC13D	-0.87	0.0480907	CD5L	2.74	0.0355574
MTPN	-0.86	0.0050705	SWAP70	2.77	0.0571574
MTPN	-0.85	0.0034926	IL1A	2.96	0.0040150
ALDH9A1	-0.82	0.0298293	CTSS	3.26	0.0236380
RPL39	-0.77	0.0122146	PLAT	3.60	0.0300998
PON3	-0.70	0.0326124	TRIB1	3.70	0.0242082
LRRC49	-0.70	0.0210543	DEFB5	3.76	0.0253889
AMACR	-0.69	0.0243830	FABP4	4.02	0.0304273

6. Follicle capacitation: A meta-analysis to investigate the transcriptome dynamics following FSH decline in bovine granulosa cells.

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Cet article a été publié dans la revue « Biology of Reproduction » sous la référence suivante : Landry D. A. and Sirard M. A. (2018) Follicle capacitation: A meta-analysis to investigate the transcriptome dynamics following FSH decline in bovine granulosa cells., Biology of Reproduction.

6.1.Résumé

Au cours des dernières années, beaucoup de progrès ont été faits afin d'améliorer la production d'embryons après une stimulation ovarienne chez les animaux domestiques. La pratique du retrait de l'hormone folliculo-stimulante (FSH), défini par la période de temps entre la dernière injection de FSH et la récolte des ovocytes, a augmenté considérablement les taux d'embryons chez la vache laitière. Depuis, les changements spécifiques à l'expression des gènes chez les cellules de la granulosa furent associés avec l'augmentation et de la diminution de la compétence développementale des ovocytes à la suite du retrait de la FSH. Dans cette étude, nous avons intégré des grands ensembles de données obtenus à partir des bases de données publiques en utilisant une technique de méta-analyse en vue d'élucider les changements moléculaires dans les cellules de granulosa à la suite de la diminution de FSH en association avec la compétence développementale des ovocytes. La méta-analyse a démontré l'expression de gènes spécifique qui est issue de la diminution du signal de prolifération et de l'augmentation du signal de différentiation et du début de l'atrésie folliculaire. De plus, la diminution de FSH induit un état d'hypoxie cellulaire et déclenche l'expression des molécules pro-inflammatoire, ce qui résulte en un début d'atrésie et mime l'effet du pic de l'hormone lutéinisante (LH) lors de l'ovulation. Afin de bien identifier et caractériser cette période, nous proposons d'utiliser le terme de ''capacitation folliculaire'' dans le but de se référer aux changements fonctionnels qui préparent la machinerie moléculaire du follicule au pic de LH et à l'ovulation à la suite du déclin de FSH. Durant cette période, le follicule transmet à l'ovocyte la compétence au développement afin de produire un embryon viable. Toutefois, si cette période n'est pas rapidement suivie par un pic de LH, le signal de l'apoptose fera augmenter l'atrésie folliculaire, la dégénération du follicule et la diminution de la qualité de l'ovocyte.

6.2. Abstract

In recent years, exciting progress was made to improve the embryo outcome after ovarian stimulation in domestic animals. The practice of follicle-stimulating hormone (FSH) withdrawal, which is defined as the period of time between the last injection of FSH and oocyte retrieval, resulted in embryo yields significantly superior. Since then, specific changes in the transcriptome of granulosa cells were associated with the increase and also the decline in oocyte developmental competence following the FSH decline. In this study, we integrated large data sets from a public depository using a meta-analysis in order to elucidate the molecular changes occurring in granulosa cells following FSH decline in association with oocyte developmental competence. The meta-analysis revealed that the gene expression patterns observed during this period resulted from the downregulation of proliferative signals, and the upregulation of differentiation signals and early apoptotic signals. Additionally, FSH decline induced cellular hypoxia and triggered the expression of pro-inflammatory molecules which resulted in early atresia and mimicked the luteinizing hormone (LH) surge signaling to ovulation. To characterize this unique differentiation period, we suggest using the term "follicle capacitation" to refer to the functional changes occurring within the follicle in order to prepare the molecular machinery for the LH surge and ovulation following FSH decline. During this period, the follicle confers the oocyte with developmental competence to become a viable embryo. However, if this period is not rapidly followed by a LH surge, apoptosis signals are increased to generate follicular atresia and decrease oocyte quality.

6.3. Introduction

Over the last two decades, research on follicle development and oocyte physiology resulted in major improvements in assisted reproduction technologies (ART) such as ovarian stimulation, in vitro maturation, and in vitro fertilization followed by embryonic development. The success of ART is primarily based on the ability to generate one or many good gametes that will successively develop into healthy offspring. It was clearly demonstrated that the origin of the oocyte influences the developmental potential of the embryo in bovine (Sirard *et al.*, 2006; Biase, 2017), humans (Jones *et al.*, 2008; Virant-Klun *et al.*, 2013)s and rodents (Xue *et al.*, 2013). Most importantly, the oocyte developmental competence is acquired during late folliculogenesis and is strongly associated with the follicle status (Krisher, 2004; Sirard *et al.*, 2006; Adams *et al.*, 2008).

In mammals, antral follicles are recruited in a wave-like manner in response to an increase in follicle stimulating hormone (FSH) following the previous ovulation or regression of a dominant follicle. The growth of the recruited follicles is FSH-dependent until the selection of the dominant follicle (Adams *et al.*, 1992, 2008; Fortune *et al.*, 2004), which occur when the follicles reach a size of approximately 8.5 mm. The selection or deviation of the dominant follicle is a passive event that begins for the first follicle to express the luteinizing hormone (LH) receptor (LHR) (Ginther *et al.*, 1989). As FSH declines naturally, the dominant follicle switches its dependency from FSH to LH resulting in a LH-dependent plateau phase of non-exponential growth and follicular survival support until the LH surge (Ali *et al.*, 2001). The other follicles that did not acquire the LHRs are designated as subordinate as the FSH level decreases and the LH level increases. The absence of LHR and the decline in circulating FSH causes the subordinate follicles to slow their growth and will eventually result in atresia, unless the dominant follicle is regress or is physically removed, then the second larger follicle will replace the dominant (Ginther *et al.*, 1997, 2003). In the absence of pregnancy or progesterone, the LH surge will occur and will trigger the process of ovulation and follicular differentiation (luteinization) into a highly vascular structure: the corpus luteum. In cattle, if progesterone levels remain high, the LH pulses will not increase and the dominant follicle will not ovulate and undergo atresia leading to a new wave of antral follicles as FSH increases (Mihm and Evans, 2008; Robker *et al.*, 2009; Forde *et al.*, 2011).

Ovarian stimulation takes advantage of the capability of all subordinate follicles to become dominant. By injecting exogenous FSH at the right moment after recruitment and for a few days, the subordinate follicles can be rescued from atresia and can become sub-dominant follicles capable of ovulation. Moreover, several studies demonstrated that bovine oocytes originating from the plateau phase just before the LH surge are associated with higher blastocyst rates (Douville and Sirard, 2014; Girard *et al.*, 2015b). This phase can be artificially induced in cattle by withdrawing FSH for a few days (coasting) after ovarian stimulation (Blondin *et al.*, 2002) resulting in almost 100% embryo yields in bovine (Nivet *et al.*, 2012). The coasting period creates a progressive granulosa cells gene expression that mimics several pre-ovulatory changes in the dominant follicle and is associated with improved oocyte competence (Sirard, 2011)

In this study, we hypothesized that the FSH decline after deviation (or FSH withdrawal after ovarian stimulation) is part of the molecular changes occurring in granulosa cells in preparation for ovulation and is essential to increase oocyte developmental competence. The aim of this study was to better understand the granulosa cell transcriptome and its regulation after FSH decline or withdrawal and to provide new biomarkers of follicular status.

6.4.Materials and Methods

6.4.1. Data retrieval

Microarray gene expression data from earlier studies of granulosa cells (Gene Expression Omnibus numbers: GSE40916, GSE63904, GSE63918, GSE63919, and GSE56145) were retrieved from the EmbryoGENE LIMS and Microarray Analysis (ELMA) platform for storage and analysis of microarray data and pooled together for meta-analysis. All datasets were generated using the EmbryoGENE bovine microarray and were analyzed after 20, 44, 68, and 92 h of FSH coasting and from growing, plateau and, atretic follicles 6-9 mm and >9 mm of diameter from slaughterhouse ovaries. All microarray experiments were confirmed in their respective publication (Nivet *et al.*, 2013; Douville and Sirard, 2014; Girard *et al.*, 2015b). The data from each microarray were exported after background correction and normalization within each array (loess) using FlexArray 1.6.1 (http://genomequebec.mcgill.ca/FlexArray).

6.4.2. Microarray meta-analysis association with FSH decline

Microarray meta-analysis was performed using the network-based visual analytics for gene expression profiling, meta-analysis, and interpretation (NetworkAnalyst). Data processing involved a log2 transformation, the conversion of all gene probes to "Entrez ID" and a quantile normalization as recommended by the program workflow in order to perform statistical analysis (Xia *et al.*, 2015). NetworkAnalyst analyzes individual microarray datasets separately and subsequently performs meta-analysis. Differential expression analysis of each dataset was performed individually within Flexarray using a moderated *t* test based on the Limma algorithm for reference purposes only. Meta-analysis was performed in NetworkAnalyst using Fisher's method ($p < 0.05$) to identify genes expressed differentially (DEGs) between follicles associated with natural or artificial FSH decline. Fisher's method of meta-analysis combines p values from the multiple datasets independently of the sample size within each study.

6.4.3. Network-based meta-analysis

Briefly, separate lists of meta-analysis DEGs during the acquisition of competence and the competence decrease were submitted to the network analysis in which a search algorithm was performed to identify the protein that directly interacts with the given list. The resulting nodes and their interaction partners were returned to build the network. The network construction was restricted to contain original seed proteins only, using zero-order analysis in NetworkAnalyst. Important nodes can be identified based on their positions within the network with two established node centrality measurements: degree centrality and betweenness centrality. The degree of a node is the number of connections it has to other nodes and the betweenness measures the number of shortest paths going through the node from the global network structure.

6.4.4. Analysis of biological functions and upstream regulators

The PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system web-based software was used to group overrepresented functions of differentially expressed genes into functional classifications (Mi *et al.*, 2017). Complementary to PANTHER, and in order to establish which biological functions were affected by FSH decline, the meta-analysis datasets were subjected to functional analysis using Ingenuity® Pathway Analysis (IPA). Briefly, the list of differentially expressed genes from the meta-analysis between the periods associated with high competence (plateau and optimal coasting period) and those associated with lower competence (Growing and Atretic, and early and late coasting) were uploaded into IPA® and analyzed for major biological functions and potential upstream regulators. This attributed the probability of association between genes in the dataset and major biological functions affected. Also, IPA® determined the upstream regulators of DEGs by referring to its database of previously known effects of different molecules (endogenous or exogenous) on target genes. Each upstream regulator had an overlap p value and an activation Z score. The activation score is an overall score based on the known effects (up regulation or down regulation) of a molecule on each of its target genes. An upstream regulator is thus attributed an activated (Z-score > 2), inhibited (Z-score < 2), or uncertain state based on the observed changes in gene expression of known

downstream targets. This analysis enabled us to identify major upstream regulators as a consequence of FSH decline and the absence of a LH surge.

6.4.5. Animals, ovarian stimulation and granulosa cells collection

Granulosa cells were obtained from 186 randomly selected heifers and cows (Holstein breed) aged from 6 to 130 months other than those included in the meta-analysis. All ovarian stimulation, in vitro fertilizations, and in vitro culture were performed from March 2015 to April 2017 in a controlled commercial environment. The protocol for ovarian stimulation and OPU was essentially the same as described previously (Landry *et al.*, 2016). Briefly, the dominant follicle was aspirated 36–48h before hormone administration. Animals were stimulated for 3 days with FSH. According to animal age, body condition score (BCS) and/or based on previous stimulation, FSH was administered in five 30-mg, six 30-mg or six 40-mg doses of NIH Folltropin-V (Bioniche Animal Health), followed by a coasting (no FSH) period of 19, 30 or 43 h (Figure 6-1). Using transvaginal ultrasonography, cumulus–oocyte complexes (COCs) were collected by transvaginal puncture under epidural anesthesia, using an 18-G needle and COOK aspiration unit (COOK Medical). COCs and granulosa cells were collected in warm HEPES-buffered Tyrode's medium (TLH) containing heparin (10 IU mL 1) and transferred to the laboratory for in vitro production. Oocyte developmental competence was measured in each animal by the number of oocytes placed in maturation that reaches the blastocyst stage following fertilization. Granulosa cells were collected within 10-15 minutes after the ovum pick-up (OPU), centrifuge at 4500 RPM for 1 minute at room temperature, snap freeze and conserved at -80 degrees until RNA extraction.

6.4.6. Reverse transcriptase quantitative PCR

Total RNA was extracted from each sample and purified using the RNeasy mini kit (Qiagen). The purity and concentration of the extracted RNA were analyzed using a Bioanalyzer (Agilent Technologies). All extracts were of good quality with an RNA integrity number > 8. A total of 325 ng of RNA was reverse-transcribed using a q-Script FlexTM cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) with oligo dT(20) and random primers following the manufacturer's recommendations. The primers used for real-time RT-PCR

were designed using the IDT PrimerQuestTM tool (available on the Integrated DNA technologies website) from sequences obtained using the UMD3.1/bosTau5 assembled version of the bovine genome. To confirm the specificity of each pair of primers, electrophoresis on a standard 1.2% agarose gel was performed for each amplified fragment, and the sequenced PCR template was then used to prepare a standard curve, which was included in each reaction. Primer sequences and annealing temperatures are shown in Supplemental Table S1. Real-time PCR was performed using LightCycler 480 SYBR Green I Master and the LightCycler 480TM (Roche Diagnostics, Laval, QC, Canada). Based on the fact that each single microarray has been already confirmed and publish, the samples were not run in technical replicate thus, each gene of interest for each animal was run in a single well. The PCR conditions used for all genes were as follows: denaturing cycle for 10 min at 95 °C; 50 PCR cycles (denaturing, 95 °C for 10s; annealing for 10 sec; extension, 72 °C for 20 sec), a melting curve (95 °C for 1 sec, 65 °C for 1 sec and a step cycle starting at 72 °C up to 97 °C at 0.11 °C /sec) and a final cooling step at 4 °C. Data were normalized through geNORM, and the most stable reference genes were identified by the stepwise exclusion of the least stable gene and recalculating the M values. *ACTB*, *GAPDH*, *EIF2B2*, *SF3A1* and *TBP* were the most stable genes with M values < 1.5 as recommended by the software. One-way ANOVA with the Tukey's post-test was performed on normalized data using GraphPad Prism version 7.00 (GraphPad Software, San Diego, CA).

6.5.Results

Using NetworkAnalyst, we performed transcriptome meta-analyses of three independent microarray studies (eight microarray datasets in total). Each individual array analysed granulosa cells from a different follicular status during natural FSH decline or FSH withdrawal. The genes in granulosa cells that were differentially expressed in association with the follicular status and/or the differentiation degree were submitted to network and bio-functional analyses within NetworkAnalyst, PANTHER and Ingenuity Pathway Analysis, respectively.

6.5.1. Network-based meta-analysis of genes associated with oocyte competence

Oocyte competence is associated with the plateau phase in slaughterhouse follicles and with the period from 44 to 68 h of FSH withdrawal (coasting) after ovarian stimulation. Thus, we compared the gene expression in granulosa cells from the period of higher oocyte competence, the plateau phase and the optimal differentiation, to the gene expression in granulosa cells associated with the periods of lower oocyte competence which are the follicular growing and atretic status for medium (6-9 mm) and large (>9 mm) follicles, and the early and late differentiation after coasting. Using NetworkAnalyst, the meta-analysis Fisher's test revealed a total of 2,248 differentially expressed (DE) genes in the granulosa cells from the period of acquisition of competence leading to the plateau and the optimal coasting periods (supplemental table 2); and a total of 1,589 DE genes associated with the oocyte competence decrease period leading to atresia and late differentiation (supplemental table 3) that are common to the three groups (coasted, 6-9mm plateau, and >9 mm plateau follicles) ($p<0.05$).

Network analysis of highly connected hub proteins was conducted in the same web-based NetworkAnalyst software. In order to simplify the network of protein nodes, the major nodes in granulosa cells were extracted and displayed separately with their neighbour nodes for the period of increased and decreased competence (Figure 6-2 and Figure 6-3). In terms of network topology measures, PCNA (Proliferating Cell Nuclear Antigen) and TP53 (Tumor protein 53) were identified as the most significantly down- and up-regulated hub genes, respectively (betweenness of 62365 and 870010, respectively; degree centrality of 42 and 40, respectively) in granulosa cells of follicle associated with the acquisition of oocyte competence (Figure 6-2). Whereas, CDK1 (Cyclin Dependent Kinase 1) and CDKN1A (Cyclin Dependent Kinase Inhibitor 1A) were identified as the most down- and up-regulated hub genes, respectively (betweenness of 52211and 4907, respectively; degree centrality of 69 and 24, respectively) in granulosa cells of follicle associated with oocyte competence decreases (Figure 6-3).

The biological processes of DE genes were also analyzed in NetworkAnalyst with the gene ontology (GO) enrichment analysis database ($p<0.05$). The biological processes included phagocytosis, transferase activity, cell adhesion, regulation of transcription in the top list of the most significant processes, as well as the apoptotic process, JAK/STAT cascade, and angiogenesis within the list of significant processes (FDR <0.05).

6.5.2. Functional Analysis

Using the datasets of modulated genes from both meta-analyses, IPA Function analysis generated statistically significant predictions for increased (z-score > 2) or decreased (z-score < −2) activity in several cellular functions. The IPA allowed the identification of predicted regulators that were most likely activated or inhibited in granulosa cells from follicle associated with the acquisition and decrease of oocyte developmental competence after FSH decline (summarized in figure 6-4, full list in supplemental table 4). For each meta-analysis (competence acquisition and competence decrease), gene symbols associated with the meta-analysis combined t-statistic ($P<0.05$) and their expression level were uploaded separately to the PANTHER functional annotation and enrichment analysis (Mi *et al.*, 2017), and the top twenty most significant biological process enrichment scores are summarized in table 1.

6.5.3. Validation of Meta-analysis Gene Expression

The genes included in the RT-qPCR analysis were *PCNA* (Proliferating Cell Nuclear Antigen), *CDK1* (Cyclin Dependent Kinase 1), *CCNB1* (Cyclin B1), *CDKN1A* (Cyclin Dependent Kinase Inhibitor 1A), and *VNN1* (Vanin 1). The expression patterns of all candidate genes were consistent with the meta-analysis results (figure 6-5). Embryo yields were also compared accordingly to the coasting period with no significant changes.

6.6. Discussion

With the accumulation of transcriptomic data from microarray experiments and advances in statistical methods, it is now possible to use meta-analysis to further investigate the transcriptional regulation of genes across multiple conditions and obtain biologically

significant information (Tseng *et al.*, 2012). Using the Fisher's combined P-values method for meta-analysis of differentially expressed genes (DEGs) (Xia *et al.*, 2015), we studied granulosa cell gene expression following the natural decline in FSH after deviation and after FSH withdrawal in superstimulated cows which are both associated with a higher oocyte developmental competence (Blondin *et al.*, 2002). Based on microarray data from three different studies (Nivet *et al.*, 2013; Douville and Sirard, 2014; Girard *et al.*, 2015b), this meta-analysis described the gene regulation and the biological functions involved in the acquisition of oocyte competence after FSH decline and FSH withdrawal. This study provided new insights to better understand oocyte competence acquisition after FSH decline, and provided new biomarkers for the assessment of follicular status. We also present the new concept of follicle capacitation, in which the follicle prepares its molecular machinery for the LH surge following FSH decline and provides the oocyte with a signal to acquire developmental competence.

6.6.1. Effect of FSH decline on oocyte developmental competence

The period between the exponential follicular growth under FSH and the trigger for ovulation (LH surge) is known as the plateau phase where follicular growth is more the result of the accumulation of follicular fluid than of cellular division. It is mainly during this phase that the process of cell differentiation begins. Interestingly, oocytes enclosed in follicles in the plateau phase have significantly higher developmental competence (Sirard *et al.*, 2006; Sirard, 2016). Based on the PANTHER enrichment analysis, FSH decline and withdrawal resulted in an over representation of multiple biological processes related to cell cycle, cell division, cellular respiration, mitochondrial functions, and apoptosis.

NetworkAnalyst meta-analysis identified PCNA, CDK1, TP53, and CDKN1A as the most central nodes in granulosa cells during oocyte competence acquisition following FSH decline and withdrawal. Commonly used as marker of cell proliferation, PCNA is a DNA polymerase accessory protein which is involved in Okazaki fragment joining, DNA repair, and chromatin assembly, and forms complexes with other proteins involved in cell cycle regulation such as the cyclin-dependant-kinase (CDK) (Tsurimoto, 1999; Mailand *et al.*, 2013). Many studies showed that FSH increases PCNA gene expression in rat, chicken, cow, and human granulosa

cells (Fricke *et al.*, 1997; El-Hefnawy and Zeleznik, 2001; Lin *et al.*, 2011; Liu *et al.*, 2014); and interestingly, PCNA interacts with cyclin-dependant-kinase 1 (CDK1) in the control of the cell cycle (Maga and Hübscher, 2003). Also known as a cell division control protein, CDK1 is partnered with cyclin A (CCNA) and cyclin B (CCNB) during the late S/G2 phase and these complexes are required to ensure the successful completion of mitosis (Brown *et al.*, 2015). The addition of FSH to cultured granulosa cells resulted in the accumulation of CDK1 (also known as CDC2) (Pavlová *et al.*, 2013) which is a marker of proliferation. Interestingly, CDK1 expression was associated with lower oocyte quality in ewes compared to sexually mature lamb (Lin *et al.*, 2017). We demonstrated that the expression of PCNA, CCNB1, and CDK1 was higher early in coasting and progressively decreased during FSH withdrawal as oocyte developmental competence increased. The proteins ERBB2 (Erb-B2 Recptor Tyrosine Kinase 2) and MYC (Myelocytomatosis Proto-Oncogene, BHLH Transcription factor) were the two top inhibited upstream regulators and are both involved in the proliferation of granulosa cells under FSH and after the LH surge to promote the formation of the corpus luteum (Piontkewitz *et al.*, 1997; Noma *et al.*, 2011). On the other hand, the tumor suppressor TP53 encodes a transcription factor that is activated in response to several forms of cellular stress, acts as a negative regulator of cellular growth, induces cell cycle arrest by affecting MYC and CDK (Brown *et al.*, 2007), and most importantly is an important inducer of apoptosis (Soussi and Wiman, 2015). As the most upregulated hub gene in the acquisition of competence and the top upstream regulator, TP53 is a central regulator of this period. Interestingly, TP53 is regulated by various protein kinases including Phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (AKT), ERBB2, CDKN1A, and CDK that also regulates PCNA (Sirotkin *et al.*, 2009); thus, it is possible that some of these protein kinases might have some interrelationships with PCNA and TP53, as the downregulation of one can result in the accumulation of the other as previously demonstrated (Livneh, 2006).

The transcription factor TP53 was associated with follicular atresia as well as the apoptosis of granulosa cells by depletion of cell survival factors such as FSH, Insulin-like growth factor (IGF), Insulin receptor (INSR), and estradiol (E2) (Matsuda *et al.*, 2012; Hatzirodos *et al.*, 2014). In granulosa cells, estradiol has various functions such as promoting folliculogenesis and inhibiting cell apoptosis (see review (Rosenfeld *et al.*, 2001)). The actions of FSH, IGF,

and INSR synergistically enhance follicular development, proliferation, cell survival, and promote estradiol production in the growing follicle (Armstrong and Webb, 1997; Richards *et al.*, 2002; Mani *et al.*, 2010). PI3K/AKT signaling is the central anti-apoptotic intracellular signal transduction pathway that is initiated by these factors (Sun *et al.*, 2003; Hu *et al.*, 2004; Mani *et al.*, 2010; Matsuda *et al.*, 2012). Its anti-apoptotic effect is mediated in part by phosphorylating the forkhead box O (FOXO) subfamily of transcription factors. When survival-promoting factors are depleted, forkhead box O1 (FOXO1) and O3 (FOXO3) are dephosphorylated and translocated to the nucleus where they inhibit cell proliferation and estrogen production, and enhance the pro-apoptotic factors TP53 and BCL2 Associated X (BAX) (Park *et al.*, 2005; Matsuda *et al.*, 2011, 2012; Wang *et al.*, 2012). Two members of the FOXO family including FOXO3 were predicted to be activated in the dataset associated with optimal oocyte competence following FSH decline or withdrawal, further supporting the central role of TP53 and apoptotic signalling.

Acting as a central mediator of TP53, cyclin-dependent kinase inhibitor 1A (CDKN1A) functions as a cyclin-dependent kinase inhibitor in response to DNA damage or other cellular stresses (Warfel and El-Deiry, 2013). However, its function in granulosa cells remains unclear and its expression was upregulated after the LH surge to decrease proliferation and promote final differentiation (Wissing *et al.*, 2014). Moreover, the inhibition of CDKN1A by the microRNA miR-93 promoted granulosa cell proliferation in human granulosa cells line KGN (Jiang *et al.*, 2015) suggesting a role in the regulation of granulosa cells growth and differentiation which is supported by its differential expression throughout coasting. Taken together, the upstream analysis clarified a signaling pathway that includes TP53, FOXO members, and CDKN1A following FSH decline in the early follicular atresia induction. Furthermore, by arresting cell division, TP53 promotes the differentiation and the reduction of cell proliferation. If at the same time LH is maintained, it provides an anti-apoptotic activity enabling the follicle to continue its growth and produce estradiol, but if LH is removed atresia occurs rapidly (Nivet *et al.*, 2017).

Indeed, LH support during the FSH decline period is crucial for the oocyte developmental competence (reviewed in Sirard, 2016). The suppression of LH expression using a

gonadotropins-releasing hormone (GnRH) antagonist during FSH withdrawal resulted in less competent oocytes after IVF (Labrecque *et al.*, 2014). A recent study demonstrated that the removal of LH support during FSH withdrawal (with a GnRH antagonist) immediately stops follicular growth, creates a precocious plateau (no growth), and leads to rapid granulosa cell atresia (Nivet *et al.*, 2017). Interestingly, the pattern of expression of multiple genes and upstream regulators that are associated with the decline in oocyte competence resembles the pattern of expression when the atresia is induced by the absence of LH signalling due to GnRH antagonist treatment. Among the genes involved, tumor necrosis factor alpha (TNF-a), an activator of the extrinsic caspase pathway signalling, is activated after the optimal/plateau phase to enter follicular atresia. It is possible that LH acts as a stimulator of inflammation to prepare the follicle for ovulation, but it is believed that LH supports follicular growth and promotes follicular differentiation during FSH withdrawal by a possible PKA/PKC (protein kinase) signaling (Flynn *et al.*, 2008; Karlsson *et al.*, 2010; Sirard, 2016; Tremblay and Sirard, 2017). It is also possible that FSH decline or withdrawal induces oxidative stress within the follicle (Tilly and Tilly, 1995; Tsai-Turton and Luderer, 2006), which promotes inflammation and cell differentiation in preparation for the LH surge.

The enrichment analysis revealed that the decline in FSH seems to alter the mitochondrial oxidoreduction function and to decrease the anti-oxidant capability which creates a cellular oxidative stress environment in granulosa cells. Angiogenesis is essential during folliculogenesis to better oxygenate the multiple layers of the follicle. Granulosa cells regulate angiogenesis by releasing pro-angiogenesis molecules and complexes such as the vascular endothelial growth factor (VEGF) in the surrounding stroma under the influence of FSH and LH to induce growth of pre-existing vessels. Tsai-Turton and Luderer (Tsai-Turton and Luderer, 2006) demonstrated that FSH reduced the accumulation of reactive oxygen species (ROS) in cultured granulosa cells and also increased the production of the antioxidant glutathione. They also observed a rise in ROS after the removal of FSH that initiated the apoptotic cascade. A similar rise in ROS was observed after the LH surge which was involved in the increase of the pro-inflammatory cytokines TNF-a, Interleukin 1 (IL1) (Kodaman and Behrman, 2001), and CDKN1A (Wissing *et al.*, 2014), further suggesting that FSH decline or withdrawal increases hypoxia within granulosa cells and triggers pro-inflammatory and

pro-apoptotic molecules resulting in an early state of atresia, and to some extent, this mimics ovulation signaling (Shkolnik *et al.*, 2011).

The production of cytokines in the ovary and their effects on some ovarian processes strongly suggest that cytokines are important mediators of ovarian functions. The expression of TNF-a was repetitively shown in granulosa cells at different stages of follicular growth including follicle persistence (Stassi *et al.*, 2017). This is consistent with our data that showed an increase in the activation of TNF-a after FSH decline/removal and a further increase in TNF-a activity during follicle persistence in the absence of gonadotropins. Previous studies demonstrated that, depending on the stage of development, TNF-a can regulate granulosa cell differentiation, ovulation, or apoptosis (Glister *et al.*, 2014; Silva *et al.*, 2017). Interestingly, the perfusion of TNF-a in rat ovaries induced ovulation and the effect was further increased by the addition of LH (Brännström *et al.*, 1995), further suggesting that TNF-a has a role to promote differentiation in absence of FSH but only to a certain extent, whereas TNF-a activation increases in un-ruptured follicle to induce follicular senescence (Yamamoto *et al.*, 2015). Based on this study, we suggest that TNF-a is increased following FSH decline in order to prepare the follicle for differentiation (follicle capacitation) and the LH surge. However, the persistence of the follicle without LH surge is interpreted as an un-ruptured follicle and further increases apoptosis to signal follicular atresia.

As many others demonstrated, early signs of atresia have a positive effect on oocyte developmental competence (Sirard *et al.*, 1999; Vassena *et al.*, 2003). This may be partly explained by the similarity in ultra-structure observed between oocytes undergoing pre-maturation and oocytes undergoing early atresia (Assey *et al.*, 1994). Embryo production was doubled when post-mortem ovaries were kept at 30 °C for 4 h instead of 2 h due to the production of ROS and the initiation of atresia which positively affected oocyte developmental competence (Blondin *et al.*, 1997a). However, if oocytes are kept in compromised follicles in vivo for too long without the LH surge to trigger ovulation, the expression of pro-inflammation and pro-apoptosis molecules increases further. As previously demonstrated, the expression of VNN1, a marker of lower developmental competence which is linked to apoptosis and/or oxidative stress (Nivet *et al.*, 2013), was increased late in

coasting and was also associated with the decrease in developmental competence in this study. Among the most significant activated upstream molecules, TGFB1 is known to limit follicle development in the presence of FSH (Rosairo *et al.*, 2008), to reduce steroidogenesis in human and rat granulosa cells (Fang *et al.*, 2014; Bai *et al.*, 2017), repress luteinisation, and promote apoptosis in bovine granulosa cells (Zheng *et al.*, 2009). Indeed, the TGFB superfamily was implicated at multiple levels in the regulation of ovarian function depending on the species studied and the stage of follicular development (reviewed in (Knight and Glister, 2006)). In the present study, our data suggest that TGFB1 is associated with the follicular differentiation leading to optimal oocyte competence, and the persistence of the follicle without an LH surge further activates TGFB1 to promote follicular atresia.

6.6.2. Conclusion

We demonstrated the gene expression in granulosa cells associated with the acquisition of oocyte competence following the FSH decline that occurs naturally or artificially (FSH withdrawal). In both cases, the granulosa cells have a dynamic gene expression pattern that prepares the follicle machinery for the LH surge and ovulation. The term follicle capacitation refers to the functional changes occurring within the follicle to prepare for the LH surge and ovulation; it involves a decrease in cell proliferation and an increase in differentiation signaling triggered by the FSH decline. Thus, inducing FSH withdrawal (coasting) after ovarian stimulation is beneficial for the oocyte as it allows time for follicle capacitation, resulting in higher embryo yields. The lack of FSH support downregulated proliferative signals such as PCNA, CDK1, and CCNB1, and increased the expression of CDKN1A and VNN1 which could become good indicators of the follicular status following FSH decline using granulosa cells. Finally, we suggest that the decline in FSH induces cellular hypoxia resulting in the increase of pro-inflammation cytokines and early apoptotic signals, and that the period of follicle capacitation is crucial for the acquisition of oocyte developmental competence. This study provides a better knowledge of ovarian physiology, which is key to improve the assisted reproductive technologies such as the coasting regimen. We also discovered new biomarkers to assess if the follicular status is early, optimal or late following coasting.

6.7. Acknowledgement

We would like to thank Lia Rossi-Perazza, DMV for the compilation of all RT-qPCR analysis and Boviteq for allowing the use of granulosa cell data from their heifers and cows.

6.8. Grant support

The Natural Sciences and Engineering Research Council of Canada - Collaborative Research and Development (NSERC-CRD grant no. RDCPJ461697-13) provided funding for this study. D.A. Landry received studentship support from NSERC and REDIH (CIHR Training program in Reproduction, Early Development, and Impact on Health).

6.9. Declaration of interest

The authors declare no potential conflicts of interest.

6.10. References

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6.11. Figures

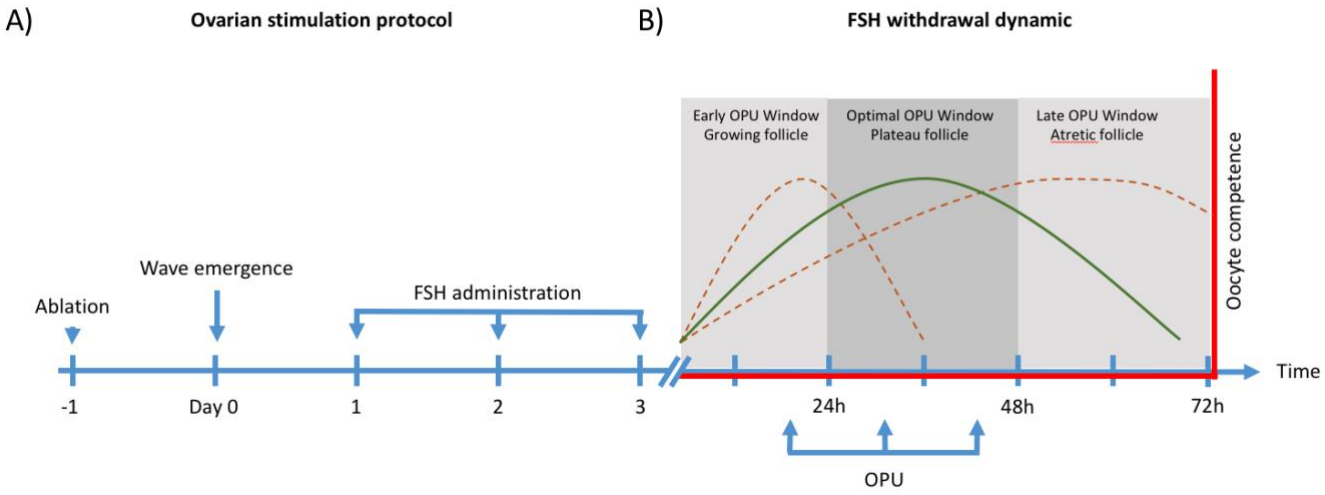


Figure 6-1: Ovarian stimulation regimen and coasting dynamic associated with follicle differentiation in dairy cattle

A) Timing of FSH injections for ovarian stimulation in Holstein cows (*Bos taurus*) following follicle ablation by transvaginal ultrasound. B) Oocyte developmental competence observed as a function of time elapsed following the final FSH injection (coasting). The solid line represents the average and dash lines represent individual variations. Figure was taken from Landry et al., (Landry *et al.*, 2017) and adapted for better visualization of the early, optimal and late ovum pick up (OPU) windows following coasting.

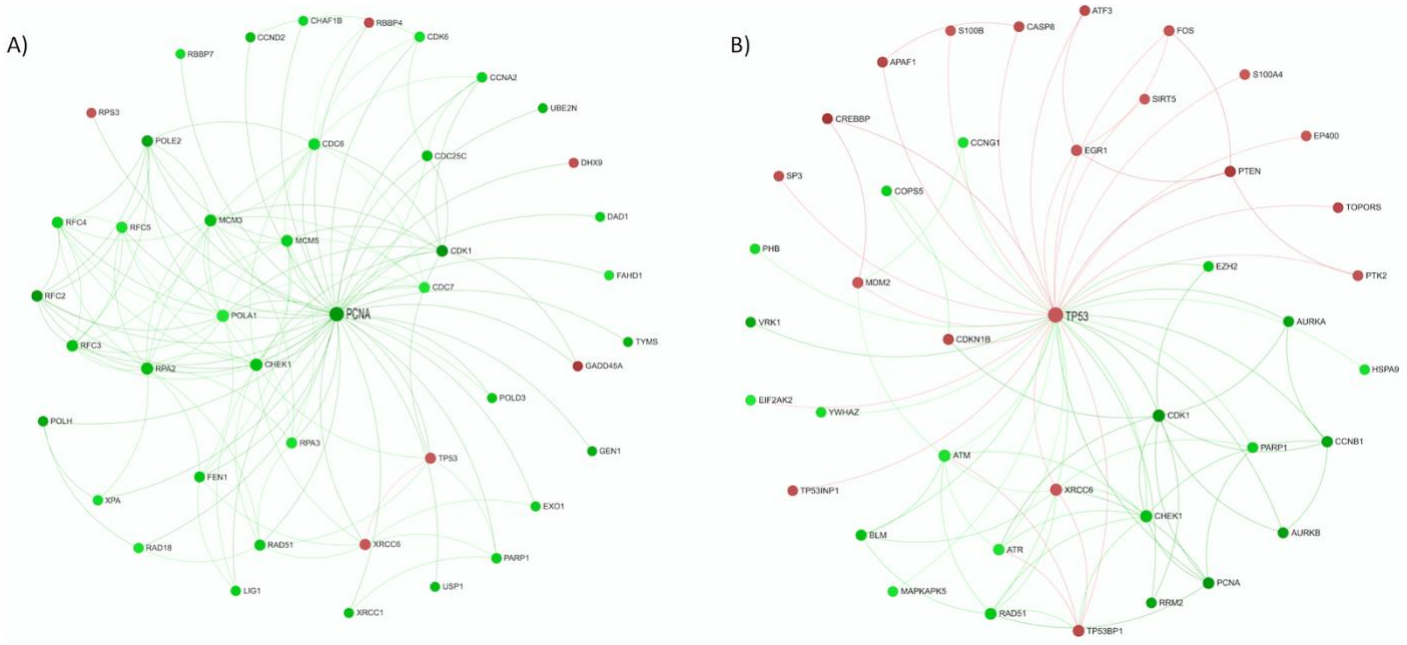


Figure 6-2: Network analysis of differentially expressed genes (DEGs) in bovine granulosa cells derived from follicles associated with developmental competence acquisition after FSH decline

The differentially regulated genes were analyzed separately in NetworkAnalyst and zero-order protein-protein interaction networks were constructed. Interaction networks of highly connected hub genes were extracted from the original network to highlight protein nodes connecting significant hub genes such as the *PCNA* network (A), and the *TP53* network (B).

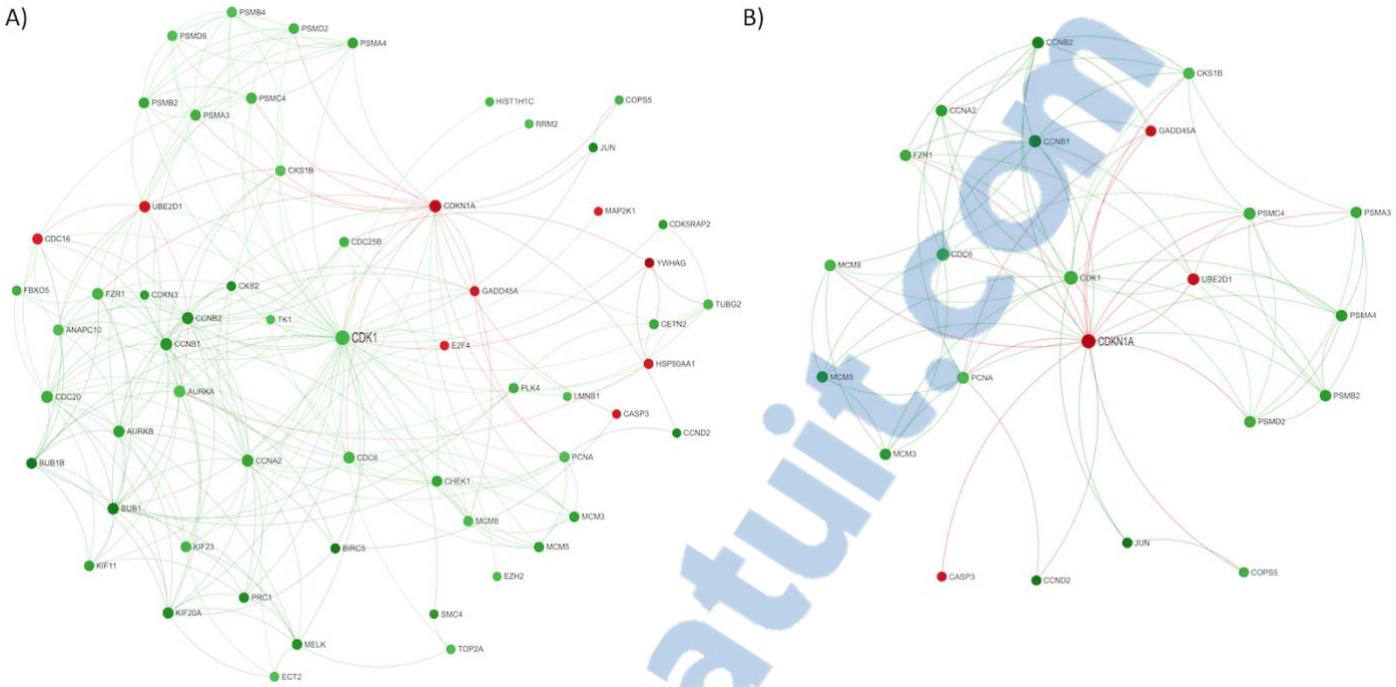
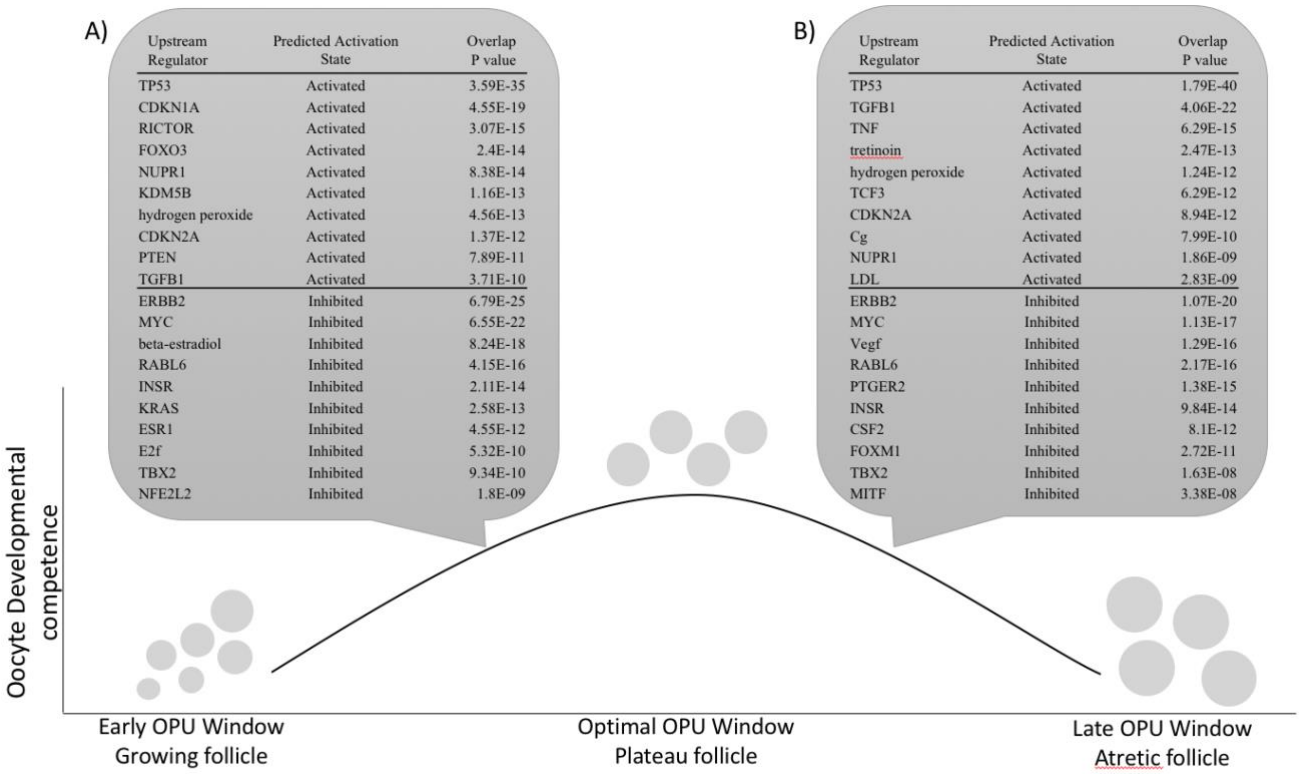


Figure 6-3: Network analysis of differentially expressed genes (DEGs) in bovine granulosa cells derived from follicles associated with developmental competence decrease after FSH decline

The differentially regulated genes were analyzed separately in NetworkAnalyst and zero-order protein-protein interaction networks were constructed. Interaction networks of highly connected hub genes were extracted from the original network to highlight protein nodes connecting significant hub genes such as the *CDK1* network (A), and the *CDKN1A* network (B).



A)

Upstream Regulator	Predicted Activation State	Overlap P value
TP53	Activated	3.59E-35
CDKN1A	Activated	4.55E-19
RICTOR	Activated	3.07E-15
FOXO3	Activated	2.4E-14
NUPR1	Activated	8.38E-14
KDM5B	Activated	1.16E-13
hydrogen peroxide	Activated	4.56E-13
CDKN2A	Activated	1.37E-12
PTEN	Activated	7.89E-11
TGFB1	Activated	3.71E-10
ERBB2	Inhibited	6.79E-25
MYC	Inhibited	6.55E-22
beta-estradiol	Inhibited	8.24E-18
RABL6	Inhibited	4.15E-16
INSR	Inhibited	2.11E-14
KRAS	Inhibited	2.58E-13
ESR1	Inhibited	4.55E-12
E2f	Inhibited	5.32E-10
TBX2	Inhibited	9.34E-10
NFE2L2	Inhibited	1.8E-09

B)

Upstream Regulator	Predicted Activation State	Overlap P value
TP53	Activated	1.79E-40
TGFB1	Activated	4.06E-22
TNF	Activated	6.29E-15
tretinoin	Activated	2.47E-13
hydrogen peroxide	Activated	1.24E-12
TCF3	Activated	6.29E-12
CDKN2A	Activated	8.94E-12
Cg	Activated	7.99E-10
NUPR1	Activated	1.86E-09
LDL	Activated	2.83E-09
ERBB2	Inhibited	1.07E-20
MYC	Inhibited	1.13E-17
Vegf	Inhibited	1.29E-16
RABL6	Inhibited	2.17E-16
PTGER2	Inhibited	1.38E-15
INSR	Inhibited	9.84E-14
CSF2	Inhibited	8.1E-12
FOXM1	Inhibited	2.72E-11
TBX2	Inhibited	1.63E-08
MITF	Inhibited	3.38E-08

Figure 6-4: Upstream regulators identified by Ingenuity Pathway Analysis (IPA) from the meta-analysis datasets of the differentially expressed genes (DEGs) in granulosa cells

Meta-analysis was made in associated with the follicles from the periods of oocyte competence acquisition (A) and decrease (B). For a better visualization, upstream regulator data are presented in the form of a table associated with the transition between the oocyte developmental competence windows after FSH decline. The solid line represents the global developmental competence of the oocyte associated with the follicles status (growing, plateau, and atretic follicle; or early, optimal, and late differentiation).

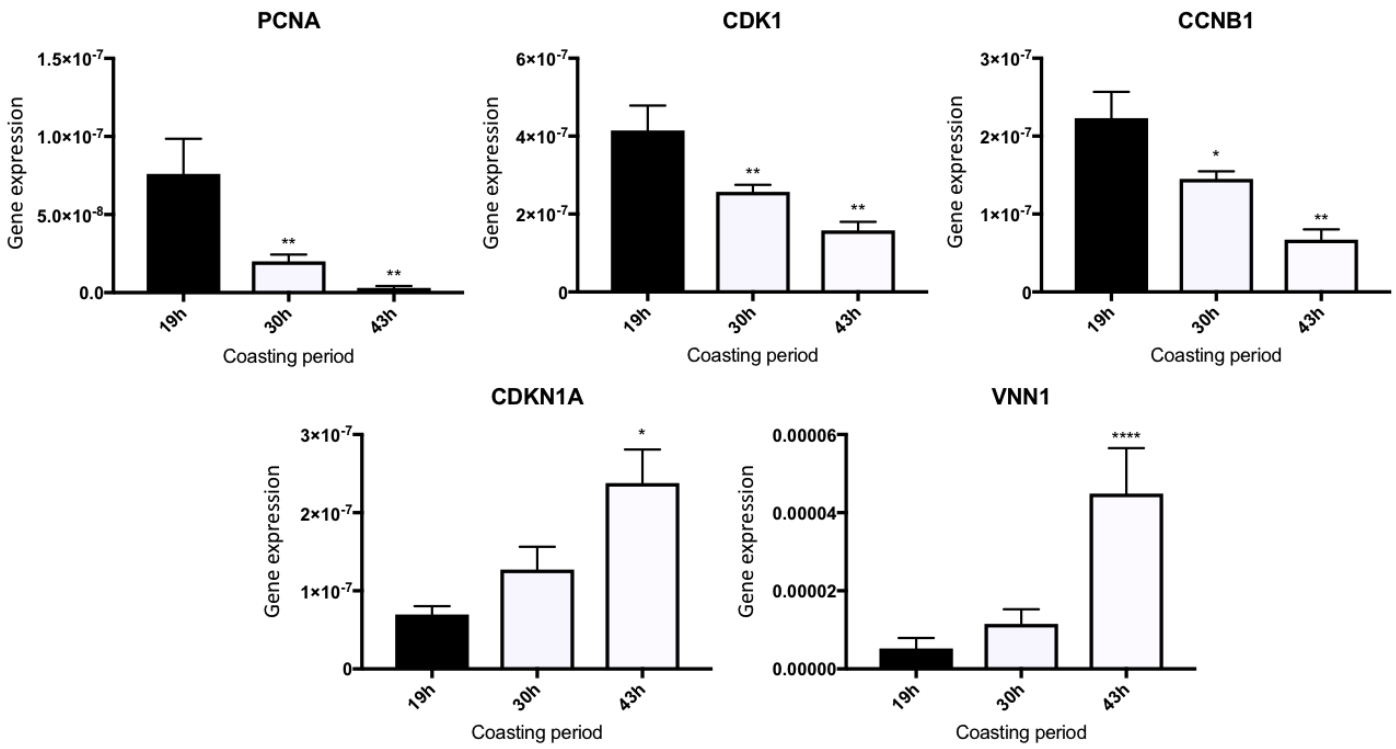


Figure 6-5: Gene expression levels of five selected biomarkers were used to validate the meta-analysis results in bovine granulosa cells following coasting for 19, 30, and 43 hours

RT-qPCR values are mean relative expression with standard error of the mean compared to the reference groups (black bar). The results were normalized using geNorm values calculated using endogenous *ACTB*, *GAPDH*, *EIF2B2*, *SF3A1*, and *TBP* transcript levels. Significant differences were calculated using the one-way ANOVA with Tukey's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

6.12. Table

Table 6-1: Biological processes in granulosa cells from the meta-analysis datasets

PANTHER results for biological processes in granulosa cells from the meta-analysis datasets associated with the acquisition of oocyte developmental competence (A) and its decreased (B). For each meta-analysis dataset, the top twenty enriched biological processes GO terms and its number in parentheses are presented. The reference list consists of the mapped IDs by PANTHER that are particular to the GO term (Cho and Campbell, 2000), and the number of hits is the number of genes in the meta-analysis dataset that map to this annotation data category. Fold enrichment gives the overrepresentation. The p-values were calculated by PANTHER software based on the binomial distribution; they were adjusted based on Bonferroni correction for multiple testing.

A) GO biological process complete	**Ref. list**	**# hits**	**Fold Enrich.**	**Adjust. P-value**
protein K11-linked ubiquitination (GO:0070979)	20	11	6.19	1.96E-02
mitochondrial respiratory chain complex I assembly (GO:0032981)	41	16	4.39	1.06E-02
mitochondrial translational elongation (GO:0070125)	79	29	4.13	2.94E-06
mitochondrial translational initiation (GO:0070124)	79	29	4.13	2.94E-06
mitotic sister chromatid segregation (GO:0000070)	88	27	3.45	4.39E-04
DNA-dependent DNA replication (GO:0006261)	90	24	3	2.51E-02
cell division (GO:0051301)	263	67	2.87	6.39E-10
protein folding (GO:0006457)	151	37	2.76	5.51E-04
cellular respiration (GO:0045333)	126	30	2.68	1.57E-02
cofactor biosynthetic process (GO:0051188)	123	29	2.65	2.77E-02
response to oxidative stress (GO:0006979)	227	49	2.43	2.35E-04
purine ribonucleoside triphosphate metabolic process (GO:0009205)	176	38	2.43	7.77E-03
rRNA processing (GO:0006364)	162	35	2.43	2.00E-02
response to endoplasmic reticulum stress (GO:0034976)	180	38	2.37	1.30E-02
regulation of mitotic cell cycle phase transition (GO:1901990)	183	38	2.34	1.90E-02
small molecule biosynthetic process (GO:0044283)	299	62	2.33	1.67E-05
coenzyme metabolic process (GO:0006732)	192	39	2.28	2.35E-02
ribonucleoside monophosphate metabolic process (GO:0009161)	198	40	2.27	2.01E-02
DNA repair (GO:0006281)	340	68	2.25	1.19E-05
regulation of apoptotic signaling pathway (GO:2001233)	305	59	2.18	4.29E-04

B) GO biological process complete	**Ref. list**	**# hits**	**Fold Enrich.**	**Adjust. P-value**
mitotic sister chromatid segregation (GO:0000070)	88	19	3.45	3.63E-02
cell division (GO:0051301)	263	50	3.04	1.01E-07
response to oxidative stress (GO:0006979)	227	34	2.4	3.53E-02
actin cytoskeleton organization (GO:0030036)	370	52	2.25	8.79E-04

regulation of protein catabolic process (GO:0042176)	282	39	2.21	4.61E-02
purine-containing compound metabolic process (GO:0072521)	317	43	2.17	2.54E-02
vasculature development (GO:0001944)	373	48	2.06	2.85E-02
apoptotic process (GO:0006915)	549	68	1.98	1.17E-03
protein localization to organelle (GO:0033365)	435	53	1.95	4.21E-02
negative regulation of protein modification process (GO:0031400)	452	55	1.95	2.93E-02
regulation of cell migration (GO:0030334)	559	66	1.89	8.96E-03
regulation of cell cycle (GO:0051726)	779	86	1.77	3.54E-03
carbohydrate derivative metabolic process (GO:1901135)	781	84	1.72	1.31E-02
cellular response to stress (GO:0033554)	1117	120	1.72	7.82E-05
regulation of transferase activity (GO:0051338)	720	77	1.71	4.31E-02
epithelium development (GO:0060429)	752	80	1.7	3.43E-02
protein transport (GO:0015031)	865	91	1.68	1.19E-02
small molecule metabolic process (GO:0044281)	1286	134	1.67	6.21E-05
negative regulation of signal transduction (GO:0009968)	874	91	1.67	1.79E-02
organonitrogen compound biosynthetic process (GO:1901566)	1128	114	1.62	3.86E-03

6.13. Supplemental data

Supplemental Table 6-1: Primers used in real-time PCR experiments

Gene Symbol		Primer sequence	Product size (pb)	Annealing temperature	GenBank Accession Number	Primer Efficiency
GAPDH	Fwd:	CCAACGTGTCTGTTGTGGATCTGA	218	57	NM_001034034.2	1.856
	Rev:	GAGCTTGACAAAGTGGTCGTTGAG				
ACTB	Fwd:	ATCGTCCACCGCAAATGCTTCT	102	57	NM_173979.3	1.921
	Rev:	GCCATGCCAATCTCATCTCGTT				
EIF2B2	Fwd:	GAACTCATACTCTAGCACTGG	263	57	NM_001015593.1	1.914
	Rev:	GTAGATGTAGGAAGGTGCATT				
SF3A1	Fwd:	TGTGTCCCTCTTGCTGAGTT	211	57	NM_001081510.1	1.876
	Rev:	ATCGCATCCTACAGGGCATT				
TBP	Fwd:	GCCTTGTGCTTACCCACCAACAGTTC	200	57	NM_001075742.1	1.937
	Rev:	TGTCTTCCTGAAACCCTTCAGAATAGGG				
PCNA	Fwd:	CTAGCCGTGTCATTGCGACT	176	57	NM_001034494.1	1.951
	Rev:	GCTGCACCAAGGAGACATGA				
CDK1	Fwd:	GGCACTCCCAATAATGAAGTGTGG	135	57	NM_174016.2	2.026
	Rev:	CGAGAGCAGATCCAAGCCATTT				
CCNB1	Fwd:	TTTTAGTCTGGGTCGCCCTC	166	57	NM_001045872.1	1.995
	Rev:	AAAAGCTCCCGCTGCAATCT				
CDKN1A	Fwd:	GAGGGACAGTGTGACACCAG	245	57	NM_001098958.2	2.005
	Rev:	AGGATGCTACAGGAGCTGGA				
VNN1	Fwd:	TGGCACGTTTGGAACCCAGTAT	223	57	NM_001024556.2	2.064
	Rev:	CTTTGGATTGAGCCTTAGCGCTTG				

Supplemental Table 6-2: Meta-analysis dataset of gene expression in granulosa cells from the oocyte competence acquisition period.

Dataset of the top 200 differentially expressed genes (DEGs) (Combined T-value ≥ 20, Combined p-value < 0.05) from the meta-analysis using NetworkAnalyst with the Fisher's method in granulosa cells associated with the follicle from the transition of growing to plateau, and early to optimal differentiation, both associated with the acquisition of oocyte developmental competence.

Official Gene symbol	Combined T-stat	Combined P-value	Official Gene symbol	Combined T-stat	Combined P-value
JMJD1C	77.7	1.59E-09	NT5DC1	-44.5	2.72E-05
NRP1	72.9	4.95E-09	POLR3H	-44.6	2.65E-05
MYOF	69.3	1.53E-08	RAP1GDS1	-44.7	2.58E-05
CCNI	63.1	9.41E-08	CENPX	-44.8	2.52E-05
HBP1	61.8	1.27E-07	DNAJC30	-44.9	2.46E-05
PAM	61.4	1.39E-07	BID	-45.0	0.000023
RSAD2	60.9	1.57E-07	LSM3	-45.1	2.25E-05
PARP9	60.7	1.61E-07	SELENOW	-45.2	2.21E-05
SRP72	58.2	3.92E-07	ACSL3	-45.2	2.18E-05
TARBP1	57.5	4.73E-07	NSL1	-45.3	2.17E-05
ATP1A1	57.1	5.32E-07	MRPL34	-45.3	2.12E-05
BMPR1B	56.4	6.78E-07	RBM18	-45.4	2.08E-05
PTPRM	56.0	7.64E-07	ATP2B1	-45.5	2.03E-05
YPEL5	55.5	8.68E-07	IDE	-45.7	1.89E-05
CITED2	54.5	1.22E-06	CENPK	-45.7	1.89E-05
PLEKHA5	54.3	1.26E-06	CYC1	-45.9	1.75E-05
TPBG	54.2	1.3E-06	LBR	-45.9	1.74E-05
SLC38A2	54.0	1.36E-06	DBF4	-46.2	1.61E-05
FYN	53.9	1.42E-06	UBE2N	-46.5	1.44E-05
LEPROT	53.0	1.83E-06	TOPBP1	-46.5	1.44E-05
GRASP	52.6	2.13E-06	PSMA5	-46.9	1.24E-05
TAF7	52.5	2.2E-06	COQ3	-47.0	1.22E-05
UBE2E3	52.3	2.32E-06	TACC3	-47.1	1.16E-05
PNRC1	51.9	2.6E-06	GSG2	-47.2	1.14E-05
ITM2B	51.4	3.07E-06	TBC1D10B	-47.2	1.12E-05
GPR137B	51.4	3.07E-06	NXT2	-47.4	1.05E-05
FBXO32	51.4	3.07E-06	ZMAT2	-47.5	1.03E-05
CAPRIN1	51.2	3.32E-06	PSMA6	-47.8	9.4E-06
IER5	51.1	3.32E-06	UCHL3	-48.0	8.84E-06
SDC2	51.0	3.32E-06	TCF19	-48.1	8.59E-06
GPC3	51.0	3.32E-06	ECT2	-48.1	8.59E-06
STX12	50.7	3.75E-06	NCAPG2	-48.1	8.59E-06
BEND6	50.6	3.82E-06	PSMA4	-48.2	8.25E-06
LAMB1	50.5	4.02E-06	DEPDC1	-48.5	7.57E-06
PDZD8	49.6	5.41E-06	CDC25B	-48.9	6.66E-06
ROBO1	49.6	5.41E-06	LSM4	-49.0	6.38E-06
CCNK	49.5	5.41E-06	TYMS	-49.2	0.000006
CIRBP	49.4	5.48E-06	NUF2	-49.4	5.48E-06

RPUSD4	49.2	0.000006	USP5	-49.4	5.48E-06
PNRC2	49.1	6.04E-06	NUSAP1	-49.5	5.41E-06
ITM2C	48.8	6.93E-06	TUBG2	-49.6	5.41E-06
VRK2	48.6	7.51E-06	RRM1	-49.7	5.25E-06
ZFAND5	48.5	7.57E-06	TUBA8	-49.8	5.07E-06
TARS	48.4	7.87E-06	MELK	-50.0	4.76E-06
MYH11	48.4	7.87E-06	TIMM8B	-50.0	4.74E-06
DBT	48.3	8.2E-06	TBCA	-50.1	4.51E-06
DTX3	48.2	8.25E-06	PCYT2	-50.3	4.23E-06
ANGPT2	47.9	9.14E-06	NCAPG	-50.8	3.52E-06
SCPEP1	47.8	9.3E-06	LDHA	-51.0	3.32E-06
MYLIP	47.6	9.81E-06	RNASEH2C	-51.0	3.32E-06
ARMCX2	47.6	9.87E-06	GEN1	-51.1	3.32E-06
PGRMC1	47.5	1.03E-05	PGP	-51.1	3.32E-06
IER3	47.2	1.12E-05	ASF1B	-51.5	3.04E-06
PDCD6IP	47.2	1.14E-05	ITGA2	-51.8	2.8E-06
TRIM24	47.0	1.21E-05	XRCC2	-52.0	2.6E-06
TBC1D4	46.8	1.26E-05	KIF15	-52.0	2.58E-06
FLCN	46.7	1.35E-05	MANF	-52.2	2.47E-06
PRKAG3	46.4	1.49E-05	UBE2L3	-52.5	2.2E-06
ERAP1	46.4	1.51E-05	VRK1	-52.8	1.96E-06
SLC37A1	46.2	1.62E-05	KIF20A	-53.2	1.75E-06
CSNK2A2	46.1	1.64E-05	POLH	-53.2	1.75E-06
ZRANB1	46.1	1.67E-05	HADHB	-53.3	1.74E-06
VNN1	46.1	1.67E-05	CCNB2	-53.3	1.72E-06
UBE2D1	46.1	1.67E-05	BCS1L	-53.5	1.6E-06
RLF	46.0	1.71E-05	POLE2	-53.8	1.42E-06
TRIP12	45.7	1.88E-05	ELOC	-54.3	1.26E-06
SNTB2	45.6	1.91E-05	AURKA	-54.3	1.26E-06
ANTXR2	45.5	2.01E-05	CKS2	-54.4	1.26E-06
TTC3	45.4	2.08E-05	CKAP2	-54.6	1.22E-06
NAGA	45.2	2.18E-05	FANCI	-55.1	9.76E-07
PDK4	45.1	2.25E-05	RRM2	-55.2	9.6E-07
LAP3	44.8	2.47E-05	CCNB1	-55.3	9.42E-07
AKAP1	44.8	0.000025	BOLA3	-55.5	8.68E-07
TOP2B	44.8	0.000025	NCAPH	-55.7	8.16E-07
RCN2	44.2	3.02E-05	KIF11	-55.9	7.64E-07
STXBP3	44.2	3.09E-05	PRC1	-56.3	6.87E-07
IFIH1	44.1	3.13E-05	SKA3	-56.4	6.78E-07
IFIT5	44.0	3.24E-05	TUBA1A	-57.4	4.73E-07
GOLPH3	44.0	3.27E-05	IFT20	-57.4	4.73E-07
RIN2	43.7	3.67E-05	DSN1	-57.7	4.51E-07
BEND5	43.5	3.85E-05	PIF1	-57.8	4.5E-07
MAN1A1	43.4	3.93E-05	BIRC5	-58.7	3.18E-07
CROT	43.4	3.93E-05	UQCRQ	-58.9	2.98E-07
SF3B1	43.1	4.49E-05	BUB1B	-58.9	2.98E-07
EIF2A	43.0	4.58E-05	BUB1	-59.1	2.98E-07
METAP1	42.8	4.81E-05	AURKB	-59.3	2.83E-07
TMEM59	42.8	4.99E-05	CKS1B	-60.8	1.57E-07

SEC24D	42.7	4.99E-05	ASPM	-61.3	1.39E-07
PDCD4	42.6	0.000053	GMNN	-61.4	1.39E-07
Foxp1	42.5	5.39E-05	ATP5J	-61.9	1.27E-07
RPSA	42.4	5.59E-05	RFC2	-62.3	1.18E-07
SERINC3	42.4	5.64E-05	KIF2C	-62.7	1.02E-07
GAB1	42.3	5.68E-05	PCNA	-63.1	9.41E-08
CASP6	42.3	5.75E-05	CDK1	-63.2	9.41E-08
UCK1	42.1	6.02E-05	FIGNL1	-63.7	8.87E-08
HERPUD1	42.1	6.05E-05	KPNA2	-64.0	8.53E-08
APPL2	42.1	6.11E-05	DLGAP5	-65.3	5.27E-08
GEM	42.0	6.41E-05	RMI2	-66.5	3.51E-08
AIMP1	41.9	6.54E-05	TUBA3E	-66.5	3.51E-08

Supplemental Table 6-3: Meta-analysis dataset of gene expression in granulosa cells from the oocyte competence decrease period.

Dataset of the top 200 differentially expressed genes (DEGs) (Combined T-value ≥ 20, Combined p-value < 0.05) from the meta-analysis using NetworkAnalyst with the Fisher's method in granulosa cells associated with the follicle from the transition of plateau to atretic, and optimal to late differentiation, both associated with the decrease of oocyte developmental competence.

Official Gene symbol	Combined T-stat	Combined P-value	Official Gene symbol	Combined T-stat	Combined P-value
COL12A1	59.5	2.01E-06	INHBB	-39.1	3.08E-04
MAP3K8	58.2	2.01E-06	NAP1L5	-39.1	3.03E-04
STC1	56.4	2.91E-06	MMAB	-39.2	3.03E-04
RND3	55.4	3.48E-06	PIGS	-39.2	3.03E-04
GPSM1	55.1	3.48E-06	ARHGEF9	-39.2	3.02E-04
RIN2	52.3	7.22E-06	NUF2	-39.2	3.02E-04
APOD	51.7	8.64E-06	IHH	-39.3	2.95E-04
GFPT2	51.5	9.22E-06	SEC61B	-39.4	2.89E-04
EPHA2	51.4	9.22E-06	COLQ	-39.4	2.86E-04
LYSMD2	51.1	9.68E-06	EBP	-39.6	2.67E-04
TP53BP2	50.5	1.13E-05	GCLC	-39.6	2.66E-04
PHC2	50.0	1.35E-05	OXSM	-39.6	2.66E-04
ISG20L2	49.6	1.55E-05	TMEM42	-39.7	2.59E-04
ANKRD1	49.4	1.58E-05	RAB26	-39.9	2.46E-04
CDH2	48.8	1.95E-05	PRR14	-40.1	2.24E-04
SRP14	48.4	2.05E-05	LAMC2	-40.2	2.22E-04
FOSL1	47.4	2.91E-05	CALB2	-40.2	2.22E-04
CORO1C	47.2	3.09E-05	CCNB1	-40.2	2.22E-04
IGF2R	46.8	3.50E-05	STIL	-40.2	2.22E-04
BAMBI	46.5	3.81E-05	SMYD3	-40.2	2.22E-04
PCOLCE2	46.4	3.86E-05	STYXL1	-40.2	2.22E-04
SDC4	45.8	4.65E-05	CKAP2	-40.3	2.22E-04
TMOD1	45.8	4.65E-05	LRRC2	-40.4	2.19E-04
SPOCK2	45.7	4.77E-05	UQCC2	-40.6	2.03E-04
PTX3	45.6	4.96E-05	CENPF	-40.8	1.87E-04
TIMP1	45.1	5.89E-05	TMEM55A	-41.0	1.76E-04
KRT18	44.9	6.04E-05	UPK1B	-41.1	1.72E-04
CTSS	44.5	7.08E-05	SEL1L3	-41.1	1.68E-04
XIRP1	44.4	7.10E-05	SMC4	-41.3	1.57E-04
NFKBIA	44.3	7.26E-05	MELK	-41.4	1.53E-04
CASP8	43.9	8.08E-05	GMNN	-41.7	1.43E-04
DKK3	43.7	8.60E-05	CRISPLD2	-42.0	1.31E-04
ID2	43.6	8.82E-05	PRR15	-42.2	1.21E-04
CFL1	43.4	9.16E-05	TMEM229B	-42.2	1.21E-04
SERPINB8	43.1	9.90E-05	KIF15	-42.3	1.18E-04
TUBB6	43.1	9.91E-05	HCN1	-42.4	1.17E-04
FSCN1	43.0	1.00E-04	RAP2A	-42.5	1.11E-04
MYB	43.0	1.01E-04	ECHS1	-42.6	1.08E-04

JAG1	42.9	1.01E-04	RPS8	-42.6	1.08E-04
DCAF6	42.8	1.04E-04	PDIA3	-42.7	1.06E-04
LGMN	42.8	1.05E-04	PFKP	-42.7	1.06E-04
GPNMB	42.3	1.21E-04	RPS19	-42.8	1.04E-04
NMT2	42.0	1.29E-04	VRK1	-42.9	1.01E-04
DDAH1	42.0	1.30E-04	GSTM3	-43.1	9.90E-05
MAOB	41.7	1.41E-04	VWA1	-43.2	9.58E-05
ELP3	41.7	1.44E-04	CCNB2	-43.2	9.58E-05
UAP1	41.5	1.50E-04	TCF19	-43.2	9.58E-05
SPP1	41.5	1.53E-04	MTCP1	-43.3	9.46E-05
GATM	41.4	1.57E-04	NDUFS4	-43.3	9.46E-05
RAP1B	41.3	1.60E-04	CKS2	-43.4	9.16E-05
NUFIP1	41.1	1.68E-04	NUSAP1	-43.6	8.65E-05
DDX5	41.0	1.76E-04	TNPO1	-43.7	8.56E-05
AMD1	40.9	1.76E-04	PRC1	-43.8	8.45E-05
COQ10B	40.4	2.19E-04	BLVRB	-43.9	8.11E-05
YWHAG	40.3	2.22E-04	CNRIP1	-44.3	7.26E-05
CCDC82	40.2	2.22E-04	KIF20A	-44.3	7.24E-05
SWAP70	40.2	2.22E-04	STBD1	-44.4	7.13E-05
DOCK7	40.2	2.22E-04	NOSTRIN	-44.6	6.92E-05
GPC1	40.0	2.31E-04	JUN	-44.9	6.04E-05
TNFRSF21	39.6	2.66E-04	PPARG	-44.9	6.04E-05
SERPINE1	39.4	2.82E-04	INHBA	-45.0	6.03E-05
REXO2	39.2	3.03E-04	NDUFAF3	-45.1	5.89E-05
HNRNPAB	39.1	3.03E-04	GUCA1A	-45.7	4.77E-05
CHMP4B	39.1	3.08E-04	ASPM	-45.9	4.64E-05
CAMK2N2	38.9	3.24E-04	ATXN7L3	-46.1	4.35E-05
MEF2A	38.9	3.25E-04	CKAP2L	-46.2	4.22E-05
FRMD8	38.5	3.78E-04	CCND2	-46.4	3.86E-05
MARCKSL1	38.5	3.81E-04	ETNK2	-46.6	3.68E-05
DBNDD2	38.4	3.85E-04	SQLE	-46.8	3.50E-05
TP53INP1	38.4	3.85E-04	SYNGR2	-47.7	2.56E-05
PLXDC2	38.4	3.93E-04	PGF	-47.8	2.50E-05
CNN1	38.3	3.93E-04	DYSF	-47.8	2.50E-05
CRABP2	38.2	4.12E-04	STAMBPL1	-48.1	2.30E-05
LUM	38.2	4.16E-04	GFRA1	-48.4	2.05E-05
IL22RA1	38.1	4.20E-04	NDC80	-48.4	2.05E-05
NKAIN1	37.9	4.54E-04	STRA6	-48.4	2.05E-05
DHX9	37.9	4.54E-04	GRB14	-48.5	2.05E-05
CLDND1	37.9	4.59E-04	CITED1	-48.8	1.95E-05
TNFRSF12A	37.8	4.70E-04	CCNF	-49.4	1.58E-05
VAT1	37.6	5.06E-04	KRT2	-49.9	1.35E-05
ACTL6A	37.4	5.50E-04	BUB1	-50.5	1.13E-05
PLAT	37.2	5.74E-04	FST	-50.6	1.13E-05
CHD4	37.1	5.98E-04	MRPS6	-51.0	9.97E-06
EPB41L3	37.1	5.98E-04	BUB1B	-51.1	9.68E-06
APP	37.1	6.01E-04	AMH	-52.3	7.22E-06
GNS	37.1	6.03E-04	ST3GAL4	-52.4	7.22E-06
CSNK1G2	37.0	6.06E-04	CA8	-52.4	7.22E-06

OLR1	37.0	6.06E-04	BIRC5	-53.0	6.31E-06
FARP1	37.0	6.06E-04	NDUFV2	-53.6	5.28E-06
STAR	36.9	6.23E-04	TMEM106C	-54.0	4.67E-06
SLITRK2	36.8	6.37E-04	DYNLT1	-54.1	4.57E-06
MUSK	36.8	6.55E-04	H2AFZ	-54.8	3.61E-06
AOX1	36.7	6.66E-04	RPA3	-55.2	3.48E-06
SPEN	36.6	6.95E-04	KCNJ8	-55.2	3.48E-06
LEPROT	36.4	7.26E-04	KIF18A	-55.3	3.48E-06
PIGW	36.2	7.92E-04	TRIB2	-56.6	2.91E-06
IGF2	36.1	8.09E-04	TNNI3	-57.5	2.35E-06
RELB	36.1	8.09E-04	INHA	-58.2	2.01E-06
NT5C2	36.1	8.09E-04	LDHA	-58.4	2.01E-06

Supplemental Table 6-4: Upstream regulator analysis on the datasets from the granulosa cell's meta-analysis.

Full list of upstream regulators identified by IPA from the differentially expressed genes (DEGs) associated with the period of oocyte competence acquisition (A) and decrease (B) in bovine granulosa cells. Predicted upstream regulator, molecule type, predicted activation state, z score, and statistical significance of the overlap (p value) are shown.

A)

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
TP53	transcription regulator	Activated	6.512	3.59E-35
CDKN2A	transcription regulator	Activated	5.95	1.37E-12
RICTOR	other	Activated	5.744	3.07E-15
NUPR1	transcription regulator	Activated	5.42	8.38E-14
tretinoin	chemical - endogenous mammalian	Activated	5.152	7.27E-08
IFNG	cytokine	Activated	4.193	1.72E-03
MAP4K4	kinase	Activated	4.122	7.38E-10
SMARCB1	transcription regulator	Activated	4.046	9.81E-06
MKNK1	kinase	Activated	4.025	3.60E-03
TNF	cytokine	Activated	3.903	6.54E-05
FOXO3	transcription regulator	Activated	3.762	2.40E-14
INSIG1	other	Activated	3.605	4.04E-03
STAT4	transcription regulator	Activated	3.591	5.43E-03
uric acid	chemical - endogenous mammalian	Activated	3.575	1.12E-03
NLRP3	other	Activated	3.567	1.27E-05
INHA	growth factor	Activated	3.407	5.84E-06
cholesterol	chemical - endogenous mammalian	Activated	3.332	1.11E-02
GATA1	transcription regulator	Activated	3.276	1.69E-04
PAF1	other	Activated	3.162	2.79E-02
SPARC	other	Activated	3.13	8.65E-03
JUNB	transcription regulator	Activated	3.111	3.23E-02
F2R	g-protein coupled receptor	Activated	3.102	1.06E-02
KDM5A	transcription regulator	Activated	3.086	2.28E-05
EDN1	cytokine	Activated	3.058	3.63E-02
PRL	cytokine	Activated	3.053	2.47E-07
BNIP3L	other	Activated	2.998	2.96E-07
NGF	growth factor	Activated	2.997	2.86E-02
IFNA2	cytokine	Activated	2.987	3.95E-03

POR	enzyme	Activated	2.919	2.96E-06
RBL2	other	Activated	2.919	7.50E-05
NFKB1	transcription regulator	Activated	2.886	4.23E-02
Pkc(s)	group	Activated	2.88	4.22E-03
SIRT1	transcription regulator	Activated	2.867	4.51E-02
IFNB1	cytokine	Activated	2.865	2.56E-02
DYRK1A	kinase	Activated	2.818	1.16E-03
FOXO4	transcription regulator	Activated	2.815	2.48E-06
INSIG2	other	Activated	2.768	4.56E-05
F7	peptidase	Activated	2.759	1.48E-02
ABCA1	transporter	Activated	2.711	1.58E-02
F2	peptidase	Activated	2.639	1.31E-02
IL1B	cytokine	Activated	2.563	1.50E-02
TCF7L2	transcription regulator	Activated	2.551	1.11E-07
KDM5B	transcription regulator	Activated	2.524	1.16E-13
Irgm1	other	Activated	2.523	3.18E-06
Rb	group	Activated	2.475	5.80E-07
CYP51A1	enzyme	Activated	2.449	1.15E-03
PTEN	phosphatase	Activated	2.443	7.89E-11
LDL	complex	Activated	2.434	9.88E-05
BMP	group	Activated	2.433	1.18E-02
HDAC2	transcription regulator	Activated	2.383	3.54E-02
Creb	group	Activated	2.377	3.01E-02
ID3	transcription regulator	Activated	2.369	3.23E-02
HOXA9	transcription regulator	Activated	2.345	4.09E-05
ELK1	transcription regulator	Activated	2.339	1.46E-02
P38 MAPK	group	Activated	2.33	8.96E-03
DOCK8	other	Activated	2.324	4.44E-03
PRKCA	kinase	Activated	2.311	2.91E-02
TGFB1	growth factor	Activated	2.281	3.71E-10
hydrogen peroxide	chemical - endogenous mammalian	Activated	2.28	4.56E-13
OSM	cytokine	Activated	2.259	3.25E-09
CDKN1A	kinase	Activated	2.245	4.55E-19
SRF	transcription regulator	Activated	2.242	3.94E-02
Ck2	complex	Activated	2.219	1.08E-03
PARP9	enzyme	Activated	2.2	4.13E-02
TGFBR2	kinase	Activated	2.19	8.38E-04
SAMSN1	other	Activated	2.183	1.24E-02

PRKCD	kinase	Activated	2.156	4.38E-02
MAPK7	kinase	Activated	2.145	1.08E-02
LONP1	peptidase	Activated	2.138	1.01E-03
SASH1	other	Activated	2.138	1.40E-02
FGF2	growth factor	Activated	2.135	3.87E-03
EIF3E	other	Activated	2.111	2.06E-03
PGR	ligand-dependent nuclear receptor	Activated	2.107	7.50E-05
GNA12	enzyme	Activated	2.107	4.09E-03
NPPB	other	Activated	2.105	1.30E-04
IL6	cytokine	Activated	2.103	1.46E-02
GNRH	group	Activated	2.081	9.35E-03
TCF4	transcription regulator	Activated	2.079	9.87E-05
corticosterone	chemical - endogenous mammalian	Activated	2.074	2.68E-02
MEF2D	transcription regulator	Activated	2.058	4.10E-03
USF1	transcription regulator	Activated	2.038	2.72E-02
SCARB1	transporter	Activated	2.013	5.61E-03
NPSR1	g-protein coupled receptor	Activated	2	1.18E-04
DDX17	enzyme	Activated	2	2.14E-02
CLCA2	ion channel	Activated	2	2.95E-02
CLCN5	ion channel		1.982	1.51E-02
CHRM1	g-protein coupled receptor		1.981	3.92E-02
GSK3B	kinase		1.956	7.81E-03
MB21D1	enzyme		1.951	2.95E-02
AMH	growth factor		1.946	3.88E-03
PEX5L	ion channel		1.937	1.30E-04
TOP1	enzyme		1.932	3.32E-03
AVP	other		1.919	7.85E-03
mannose	chemical - endogenous mammalian		1.89	6.59E-05
IKBKB	kinase		1.89	7.82E-04
VHL	transcription regulator		1.869	2.03E-06
Lh	complex		1.846	4.16E-10
F3	transmembrane receptor		1.833	1.99E-02
ADCYAP1	other		1.795	4.32E-05
EP300	transcription regulator		1.792	2.32E-02
PTGS2	enzyme		1.788	9.71E-03
IFNAR1	transmembrane receptor		1.772	1.20E-02
GDF2	growth factor		1.771	1.60E-03
IPMK	kinase		1.715	2.21E-02
NGFR	transmembrane receptor		1.714	6.34E-04

FZD8	g-protein coupled receptor		1.71	2.80E-03
LMNB1	other		1.709	5.43E-04
MAP3K1	kinase		1.698	3.10E-03
ELF4	transcription regulator		1.697	1.12E-03
CD28	transmembrane receptor		1.639	2.11E-05
SLC29A1	transporter		1.633	1.05E-02
CPE	peptidase		1.625	3.88E-03
EIF2AK3	kinase		1.614	1.71E-07
MAPK14	kinase		1.608	2.68E-03
Cg	complex		1.601	2.84E-04
DDX58	enzyme		1.598	1.53E-02
GnRH analog	biologic drug		1.596	3.01E-09
EIF2AK2	kinase		1.574	1.39E-03
E2F6	transcription regulator		1.539	8.29E-06
UXT	transcription regulator		1.538	2.51E-04
CSF2RB	transmembrane receptor		1.536	1.51E-02
WT1	transcription regulator		1.524	1.39E-07
CSF1R	kinase		1.51	1.18E-03
SLC9A3R1	other		1.504	2.62E-02
ATF6	transcription regulator		-1.516	8.45E-04
NRF1	transcription regulator		-1.53	3.16E-03
ANXA2	other		-1.542	1.79E-02
MTDH	transcription regulator		-1.546	2.73E-02
CRTC2	other		-1.546	3.32E-02
Gsk3	group		-1.557	4.09E-03
MDM2	transcription regulator		-1.581	9.62E-03
TSC2	other		-1.623	2.84E-06
STAT5B	transcription regulator		-1.632	3.65E-02
SAFB2	other		-1.633	3.32E-02
COL18A1	other		-1.699	1.84E-03
L-histidine	chemical - endogenous mammalian		-1.724	6.80E-04
EIF4E	translation regulator		-1.729	2.95E-05
APOE	transporter		-1.744	2.97E-02
VEGFB	growth factor		-1.755	3.17E-02
PPARGC1B	transcription regulator		-1.789	4.34E-03
MED1	transcription regulator		-1.797	3.98E-05
ELAVL1	other		-1.825	1.37E-06
HGF	growth factor		-1.827	9.70E-14
PPARGC1A	transcription regulator		-1.863	1.30E-02

PPARG	ligand-dependent nuclear receptor		-1.871	2.04E-03
CCNE1	transcription regulator		-1.912	7.27E-03
phosphate	chemical - endogenous mammalian		-1.956	1.24E-02
HSF2	transcription regulator		-1.976	1.49E-05
KMT2E	enzyme		-1.982	1.49E-03
TOPBP1	other		-1.988	3.92E-02
ATG16L1	enzyme	Inhibited	-2	9.69E-03
SMOC2	other	Inhibited	-2	1.48E-02
COPS8	other	Inhibited	-2	1.48E-02
DBI	other	Inhibited	-2	2.95E-02
Esrra	transcription regulator	Inhibited	-2.017	9.88E-06
ESR1	ligand-dependent nuclear receptor	Inhibited	-2.039	4.55E-12
AHR	ligand-dependent nuclear receptor	Inhibited	-2.091	6.06E-06
SIRT2	transcription regulator	Inhibited	-2.121	2.32E-04
TASP1	peptidase	Inhibited	-2.169	3.32E-03
EGFR	kinase	Inhibited	-2.174	2.96E-08
MITF	transcription regulator	Inhibited	-2.174	5.16E-06
TFAP2C	transcription regulator	Inhibited	-2.178	1.97E-02
SERCA	group	Inhibited	-2.191	6.76E-05
NCL	other	Inhibited	-2.2	1.76E-02
SOX2	transcription regulator	Inhibited	-2.202	1.68E-02
CDK1	kinase	Inhibited	-2.213	1.42E-04
CKS1B	kinase	Inhibited	-2.219	2.16E-04
SMURF2	enzyme	Inhibited	-2.219	2.02E-02
CYP7A1	enzyme	Inhibited	-2.236	1.51E-02
MYCBP2	enzyme	Inhibited	-2.236	2.02E-02
beta-estradiol	chemical - endogenous mammalian	Inhibited	-2.238	8.24E-18
PCGEM1	other	Inhibited	-2.279	2.06E-03
KRAS	enzyme	Inhibited	-2.308	2.58E-13
TFRC	transporter	Inhibited	-2.4	9.04E-04
CCND1	transcription regulator	Inhibited	-2.408	5.02E-08
ERBB4	kinase	Inhibited	-2.433	7.05E-03
AURKB	kinase	Inhibited	-2.449	1.37E-02
CDK2	kinase	Inhibited	-2.449	2.13E-02
S100A6	transporter	Inhibited	-2.53	4.66E-04
RASSF5	other	Inhibited	-2.538	3.36E-04
testosterone	chemical - endogenous mammalian	Inhibited	-2.559	1.07E-03
RARA	ligand-dependent nuclear receptor	Inhibited	-2.592	4.95E-05
NFE2L2	transcription regulator	Inhibited	-2.619	1.80E-09

SMAD7	transcription regulator	Inhibited	-2.631	1.68E-03
ESRRA	ligand-dependent nuclear receptor	Inhibited	-2.644	4.43E-03
EP400	other	Inhibited	-2.725	1.02E-06
IL15	cytokine	Inhibited	-2.726	1.21E-06
NFE2L1	transcription regulator	Inhibited	-2.828	4.37E-03
MMP3	peptidase	Inhibited	-2.837	2.55E-03
E2F2	transcription regulator	Inhibited	-2.887	3.28E-07
SREBF2	transcription regulator	Inhibited	-2.962	4.03E-08
TRIM24	transcription regulator	Inhibited	-3	1.10E-02
AREG	growth factor	Inhibited	-3.027	2.49E-05
CSF2	cytokine	Inhibited	-3.062	2.03E-08
CCNK	kinase	Inhibited	-3.065	6.34E-04
MAX	transcription regulator	Inhibited	-3.133	1.58E-07
SREBF1	transcription regulator	Inhibited	-3.137	1.98E-02
PTGER2	g-protein coupled receptor	Inhibited	-3.173	3.51E-09
E2F3	transcription regulator	Inhibited	-3.357	9.61E-06
SCAP	other	Inhibited	-3.456	7.54E-07
TAL1	transcription regulator	Inhibited	-3.541	2.68E-02
E2f	group	Inhibited	-3.575	5.32E-10
FOXM1	transcription regulator	Inhibited	-4.041	3.67E-09
MYC	transcription regulator	Inhibited	-4.109	6.55E-22
ANGPT2	growth factor	Inhibited	-4.415	4.82E-03
INSR	kinase	Inhibited	-4.433	2.11E-14
TBX2	transcription regulator	Inhibited	-4.869	9.34E-10
RABL6	other	Inhibited	-5.031	4.15E-16
ERBB2	kinase	Inhibited	-5.397	6.79E-25

B)

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
RICTOR	other	Activated	5.031	2.95E-07
TP53	transcription regulator	Activated	4.774	1.79E-40
NUPR1	transcription regulator	Activated	4.754	1.86E-09
tretinoin	chemical - endogenous mammalian	Activated	3.732	2.47E-13
Tgf beta	group	Activated	3.584	3.40E-05
PRL	cytokine	Activated	3.377	1.55E-08
CDKN2A	transcription regulator	Activated	3.285	8.94E-12
cholesterol	chemical - endogenous mammalian	Activated	3.101	3.73E-02

GnRH analog	biologic drug	Activated	2.994	1.26E-08
TNF	cytokine	Activated	2.964	6.29E-15
BMP6	growth factor	Activated	2.843	4.19E-04
ETS1	transcription regulator	Activated	2.746	4.28E-02
FOXO4	transcription regulator	Activated	2.745	1.20E-07
BNIP3L	other	Activated	2.688	3.97E-08
Irgm1	other	Activated	2.688	1.37E-06
TNFSF11	cytokine	Activated	2.685	1.92E-06
Nfat (family)	group	Activated	2.673	2.34E-03
SMARCB1	transcription regulator	Activated	2.658	1.68E-05
hydrogen peroxide	chemical - endogenous mammalian	Activated	2.65	1.24E-12
CREM	transcription regulator	Activated	2.65	3.22E-04
COMMD1	transporter	Activated	2.646	1.33E-03
BMP4	growth factor	Activated	2.622	9.94E-04
AMH	growth factor	Activated	2.611	2.22E-03
PRKCD	kinase	Activated	2.552	5.37E-04
aldosterone	chemical - endogenous mammalian	Activated	2.547	1.36E-03
TCF3	transcription regulator	Activated	2.492	6.29E-12
POR	enzyme	Activated	2.467	1.39E-04
SELPLG	other	Activated	2.449	3.17E-02
ABCA1	transporter	Activated	2.42	3.34E-04
MYD88	other	Activated	2.407	7.75E-05
BMPR1A	kinase	Activated	2.385	1.97E-02
STK11	kinase	Activated	2.366	2.65E-05
LDL	complex	Activated	2.359	2.83E-09
oblimersen	biologic drug	Activated	2.345	1.85E-02
ERK	group	Activated	2.341	5.38E-04
MAPK3	kinase	Activated	2.333	2.95E-02
TGFB1	growth factor	Activated	2.29	4.06E-22
KDM5B	transcription regulator	Activated	2.278	1.44E-07
MAPK14	kinase	Activated	2.273	4.72E-06
Cg	complex	Activated	2.268	7.99E-10
VGLL3	other	Activated	2.236	1.11E-03
CYP51A1	enzyme	Activated	2.236	1.70E-03
HRG	other	Activated	2.236	1.33E-02
PAF	chemical - endogenous mammalian	Activated	2.233	1.46E-03
CXCL12	cytokine	Activated	2.229	4.56E-02
NPPB	other	Activated	2.228	2.19E-04

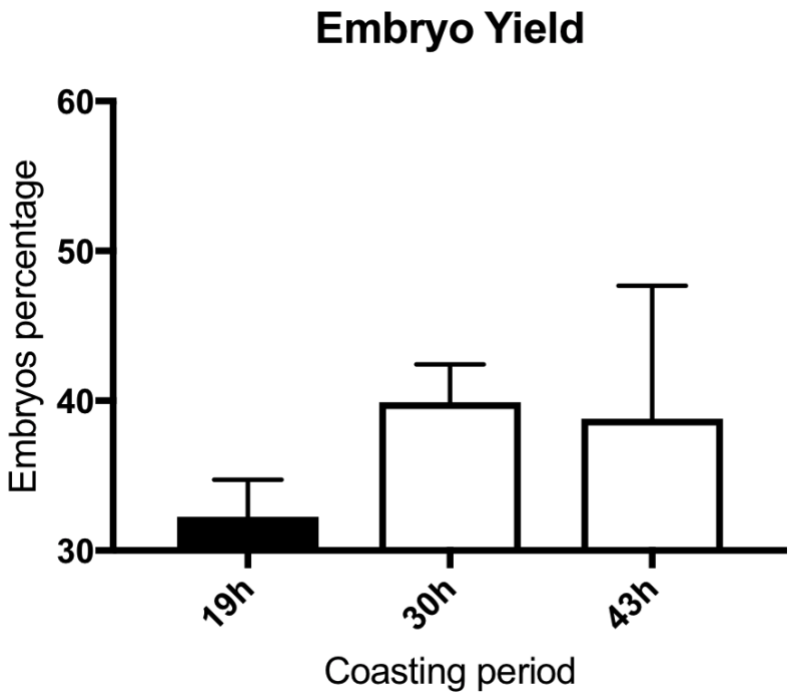
TGFB3	growth factor	Activated	2.225	4.32E-03
LMNB1	other	Activated	2.219	3.66E-03
Smad2/3-Smad4	complex	Activated	2.208	6.36E-03
INSIG2	other	Activated	2.2	3.49E-03
INHA	growth factor	Activated	2.192	4.21E-07
NOTCH1	transcription regulator	Activated	2.173	5.43E-04
IFNB1	cytokine	Activated	2.169	1.80E-03
SP1	transcription regulator	Activated	2.149	8.36E-08
MFSD2A	transporter	Activated	2.138	1.08E-04
IFNG	cytokine	Activated	2.134	9.80E-07
GDF2	growth factor	Activated	2.118	2.11E-04
EGLN	group	Activated	2.101	3.57E-07
POMC	other	Activated	2.086	4.36E-02
NLRP3	other	Activated	2.061	2.68E-05
cholic acid	chemical - endogenous mammalian	Activated	2.056	4.84E-02
XDH	enzyme	Activated	2.053	2.00E-04
TNFSF13B	cytokine	Activated	2.04	4.26E-04
OSM	cytokine	Activated	2.035	3.81E-09
SIRT1	transcription regulator	Activated	2.028	1.91E-03
RELB	transcription regulator	Activated	2.012	1.09E-03
WISP2	growth factor	Activated	2.008	7.30E-04
TGM2	enzyme	Activated	2.001	4.61E-02
RLIM	enzyme	Activated	2	4.33E-03
IRAK3	kinase	Activated	2	3.24E-02
CTSS	peptidase	Activated	2	4.51E-02
SMAD4	transcription regulator		1.995	9.21E-05
DYRK1A	kinase		1.992	7.48E-04
D-glucose	chemical - endogenous mammalian		1.987	1.10E-12
PML	transcription regulator		1.986	1.30E-04
PLC	group		1.982	3.24E-02
PEX5L	ion channel		1.98	1.70E-03
PTTG1	transcription regulator		1.98	9.18E-03
PGR	ligand-dependent nuclear receptor		1.977	9.54E-10
F7	peptidase		1.976	7.72E-03
TNFSF12	cytokine		1.972	4.07E-02
Jnk	group		1.965	2.94E-04
TCF12	transcription regulator		1.964	1.85E-02
NFATC2	transcription regulator		1.958	2.90E-03

MAP4K4	kinase		1.957	3.05E-03
ECSIT	transcription regulator		1.941	2.81E-03
PIN1	enzyme		1.941	3.56E-03
KITLG	growth factor		1.939	7.96E-08
JAK1	kinase		1.936	9.37E-03
Collagen Alpha1	group		1.934	6.41E-03
F2	peptidase		1.931	4.98E-07
IL31	other		1.929	4.70E-02
SELP	transmembrane receptor		1.89	1.08E-02
CDKN1A	kinase		1.889	3.02E-16
NGF	growth factor		1.879	5.15E-04
TCF7L2	transcription regulator		1.876	6.14E-04
Smad	complex		1.859	1.73E-03
AGT	growth factor		1.839	1.78E-14
NFKB1	transcription regulator		1.819	1.55E-05
MKNK1	kinase		1.807	7.13E-03
PAX7	transcription regulator		1.805	6.95E-04
CTNNB1	transcription regulator		1.801	1.28E-06
ADRB	group		1.794	3.51E-06
TP63	transcription regulator		1.793	3.48E-05
SOX4	transcription regulator		1.785	9.73E-04
JUNB	transcription regulator		1.763	3.98E-02
PDGF BB	complex		1.762	4.89E-12
IL6	cytokine		1.754	7.46E-11
STAT5a/b	group		1.744	1.60E-02
Rb	group		1.731	3.41E-06
GDF9	growth factor		1.727	4.87E-05
ZAP70	kinase		1.718	2.07E-02
KEAP1	transcription regulator		1.709	6.43E-03
SMARCA4	transcription regulator		1.702	2.60E-06
IL3	cytokine		1.688	3.66E-05
norepinephrine	chemical - endogenous mammalian		1.67	9.49E-03
corticosterone	chemical - endogenous mammalian		1.662	4.12E-02
thyroid hormone	chemical - endogenous mammalian		1.66	9.73E-04
E2F6	transcription regulator		1.633	4.64E-03
RARB	ligand-dependent nuclear receptor		1.633	4.97E-03
CHADL	other		1.633	1.75E-02

UTP	chemical - endogenous mammalian		1.617	6.27E-05
NFkB (complex)	complex		1.606	1.40E-04
SOX11	transcription regulator		1.603	4.45E-03
TSH	complex		1.596	3.59E-04
IL1A	cytokine		1.596	4.55E-04
CEBPA	transcription regulator		1.577	5.05E-05
SPP1	cytokine		1.563	5.69E-06
CSF1	cytokine		1.56	7.28E-04
RELA	transcription regulator		1.548	4.82E-04
TLR4	transmembrane receptor		1.54	1.30E-07
IFNAR1	transmembrane receptor		1.537	3.28E-02
PDGFRA	kinase		1.52	6.73E-04
HBEGF	growth factor		1.516	3.62E-02
SPI1	transcription regulator		1.507	2.07E-02
RETNLB	other		1.5	1.32E-04
AR	ligand-dependent nuclear receptor		-1.512	2.15E-10
CBX5	transcription regulator		-1.604	1.21E-02
NOG	growth factor		-1.628	3.66E-03
PRNP	other		-1.632	3.93E-05
MBTPS1	peptidase		-1.633	1.75E-04
EIF4G1	translation regulator		-1.633	4.27E-03
NFE2L2	transcription regulator		-1.636	2.87E-04
ELAVL1	other		-1.637	2.27E-05
APOE	transporter		-1.645	4.24E-03
MDM2	transcription regulator		-1.661	1.06E-02
UCP1	transporter		-1.691	5.74E-03
NUMB	other		-1.715	3.93E-02
INHBA	growth factor		-1.72	2.70E-05
CCNK	kinase		-1.725	2.05E-02
DBI	other		-1.732	9.05E-03
CDK2	kinase		-1.732	3.62E-02
MMP14	peptidase		-1.78	7.72E-03
TRAF2	enzyme		-1.796	1.69E-02
CAT	enzyme		-1.8	3.66E-03
DICER1	enzyme		-1.901	1.53E-02
MYCN	transcription regulator		-1.903	4.13E-07
ZNF217	transcription regulator		-1.912	6.15E-03
MAPT	other		-1.94	2.05E-09

SATB1	transcription regulator		-1.944	2.20E-03
ITGA6	transmembrane receptor		-1.951	3.93E-02
FOXG1	transcription regulator		-1.954	1.70E-03
MTOR	kinase		-1.978	9.56E-06
LIN9	other		-1.98	1.63E-03
BLOC1S5	other		-1.98	3.24E-02
CKS1B	kinase		-1.981	8.64E-04
TASP1	peptidase		-1.982	6.41E-03
SMOC2	other	Inhibited	-2	4.33E-03
ACTB	other	Inhibited	-2	6.36E-03
CYP7A1	enzyme	Inhibited	-2	2.09E-02
SIRT2	transcription regulator	Inhibited	-2	3.93E-02
CBX7	other	Inhibited	-2	4.70E-02
TAL1	transcription regulator	Inhibited	-2.034	1.70E-02
SREBF1	transcription regulator	Inhibited	-2.055	2.11E-03
Vegf	group	Inhibited	-2.107	1.29E-16
S100A6	transporter	Inhibited	-2.121	9.14E-04
PPARGC1A	transcription regulator	Inhibited	-2.149	7.19E-04
FBXO32	enzyme	Inhibited	-2.191	4.44E-02
ASAH1	enzyme	Inhibited	-2.216	4.51E-02
NELFB	other	Inhibited	-2.236	1.11E-03
MED1	transcription regulator	Inhibited	-2.325	2.24E-06
PIK3R1	kinase	Inhibited	-2.39	4.45E-03
ZEB2	transcription regulator	Inhibited	-2.395	1.75E-02
HOXC8	transcription regulator	Inhibited	-2.433	2.39E-02
BMI1	transcription regulator	Inhibited	-2.587	3.34E-04
CSF2	cytokine	Inhibited	-2.635	8.10E-12
ESRRA	ligand-dependent nuclear receptor	Inhibited	-2.699	4.46E-06
CNR1	g-protein coupled receptor	Inhibited	-2.714	7.80E-03
HLX	transcription regulator	Inhibited	-2.784	2.27E-03
E2F2	transcription regulator	Inhibited	-2.79	3.25E-06
MYC	transcription regulator	Inhibited	-2.795	1.13E-17
Alpha catenin	group	Inhibited	-2.89	2.78E-03
SREBF2	transcription regulator	Inhibited	-2.95	4.22E-06
INSR	kinase	Inhibited	-2.967	9.84E-14
AREG	growth factor	Inhibited	-3.144	4.87E-06
SCAP	other	Inhibited	-3.216	2.09E-05
EP400	other	Inhibited	-3.268	5.02E-07
E2f	group	Inhibited	-3.348	1.13E-05

ERBB2	kinase	Inhibited	-3.421	1.07E-20
TBX2	transcription regulator	Inhibited	-3.508	1.63E-08
PTGER2	g-protein coupled receptor	Inhibited	-3.576	1.38E-15
FOXM1	transcription regulator	Inhibited	-4.267	2.72E-11
RABL6	other	Inhibited	-4.271	2.17E-16
MITF	transcription regulator	Inhibited	-4.453	3.38E-08



Supplemental Figure 6-1: Embryo percentage in association with the coasting period

Embryo yields were calculated as the mean percentage with standard error of the mean for each coasting time compared to the reference group (less coasting).

7. Conclusion générale

Le développement des nouvelles techniques de reproduction assistée a considérablement amélioré le nombre d'embryons viables produits chez le bovin laitier. L'industrie canadienne de production d'embryons bovins a profité des nombreuses percées dans le milieu de la reproduction assistée, ce qui lui a permis à Boviteq de devenir un leader mondial dans le domaine. Depuis les dernières années, les techniques de production d'embryons bovins font face à plusieurs défis, ce qui inclut la grande variabilité individuelle aux traitements ovariens et l'efficacité de ces traitements ovariens chez les jeunes donneuses, qui ont une compétence développementale de l'ovocyte plus faible que chez les vaches adultes. Il est bien connu que la qualité de l'ovocyte est directement liée à la santé du follicule et à son degré de différenciation. Au cours de cette thèse, nous avons étudié l'expression des gènes dans les cellules de la granulosa pour comprendre le phénomène de différentiation dynamique du follicule précédant la récupération des ovocytes. Au cours de l'aspiration des follicules, les cellules de la granulosa murales se détachent de la paroi folliculaire et sont aspirées avec les complexes ovocyte-cumulus. En temps normal, les cellules de la granulosa qui se retrouve dans le milieu d'aspiration sont jetées après la sélection et le lavage des ovocytes. Mais dans le cadre de nos études, elles furent ramassées dans le but d'identifier la santé et le statut folliculaire au moment de la récolte des ovocytes, et ce, sans compromettre l'intégrité des complexes ovocyte-cumulus et la production d'embryons.

L'un des aspects majeurs abordés au cours de cette thèse est la variabilité individuelle chez les donneuses, ce qui réduit le potentiel de production d'embryons de certaines vaches donneuses élites. Mes travaux de recherche ont démontré une grande variabilité individuelle sur la compétence au développement des ovocytes chez la vache laitière et qu'il est possible d'améliorer le système de production d'embryons en comparant directement les bonnes et les mauvaises donneuses afin d'identifier les sources de variation. Cette partie du projet de recherche a permis de découvrir la signature moléculaire des cellules de granulosa associée avec la compétence au développement de l'ovocyte, ce qui a rendu possible de produire un plan de la chronologie de l'expression des gènes dans le but de caractériser le follicule. Nous avons aussi démontré que dans la plupart des cas, la faible compétence au développement des ovocytes est attribuée à une mauvaise différentiation du follicule, ce qui explique

davantage la variabilité interindividuelle. Ainsi, nous avons proposé que le synchronisme de la période de coasting a un impact significatif sur la qualité des ovocytes et qu'elle constitue la cause majeure du mauvais taux d'embryons chez les vaches sexuellement matures. Par la suite, nous avons découvert dix nouveaux biomarqueurs, ayant la capacité de classifier les vaches qui échouent à produire un nombre satisfaisant d'embryons après la stimulation ovarienne dans deux groupes possibles; les follicules insuffisamment différenciés et les follicules trop avancés. Ce diagnostic simple va permettre à l'industrie de modifier le traitement suivant, soit le temps de coasting des donneuses afin de synchroniser la récolte de l'ovocyte avec la différenciation optimale des follicules (Figure supplémentaire 7-1). Cette nouvelle technologie ouvre la possibilité à l'industrie de se tourner vers les stimulations ovariennes personnalisées, ce qui va augmenter la capacité à générer des embryons de qualité supérieure.

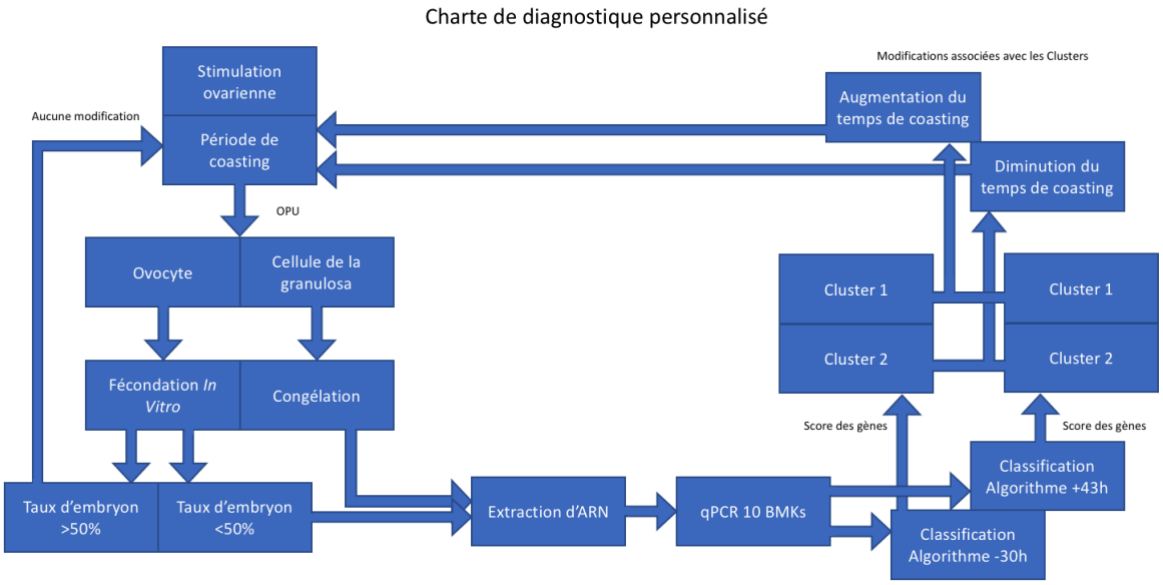


Figure 7-1 : Schéma de la charte de procédures à suivre pour la stimulation ovarienne personnalisée avec l'utilisation des algorithmes de coasting

Tout d'abords, la donneuse suit un protocole de stimulation ovarienne avec un temps de coasting général. À la suite de l'aspiration des ovocytes (OPU) les cellules de la granulosa seront conservées à -80 Celsius tandis que l'ovocyte sera fécondé in vitro. Si le taux d'embryon est supérieur à 50%, le protocole général est adéquat pour l'individu. Au contraire, si le taux d'embryon est inférieur à 50%, l'ARN sera extrait des cellules de la granulosa pour en faire le test des biomarqueurs (BMKs) en PCR en temps réel. Selon le temps de coasting que l'individu a reçu, l'algorithme de moins de 30h ou de plus de 43h de coasting sera

utilisé afin de classifier les animaux dans des clusters. Chaque cluster est associé avec une modification au temps de coasting et selon le score de l'algorithme, calculer sur l'expression des biomarqueurs le temps de coasting sera augmenter ou diminuer pour le prochain cycle de reproduction assistée.

L'autre aspect majeur abordé au cours de cette thèse, est la compréhension de la dynamique folliculaire des jeunes donneuses d'ovocytes ce qui devient de plus en plus important dans les programmes de gain génétique. Tout d'abord, nos travaux ont permis de définir précisément l'âge auquel le taux d'embryons est significativement différent chez les donneuses péri-pubères. En ayant cette information, il fut possible de se concentrer sur des groupes d'âge spécifiques et de mieux comprendre la dynamique de leurs follicules, ce qui nous a permis de découvrir un nouvel aspect physiologique de la dynamique de coasting qui est plus courte chez les génisses pré-pubères. Cette différence de dynamique chez les jeunes donneuses est le résultat d'une insuffisance dans la signalisation de l'hormone lutéinisante (LH), qui est essentielle lors du coasting pour permettre une différenciation adéquate du follicule. Ainsi, la meilleure compréhension de la croissance folliculaire et de la dynamique de coasting chez la génisse pré-pubère a permis à l'industrie d'adapter le protocole de stimulation par la réduction du temps de coasting des jeunes donneuses. De plus, dans un contexte de nutrition, nos travaux de recherche ont aussi démontré une déficience métabolique particulière chez les vaches et les jeunes donneuses, ce qui a poussé l'industrie à revoir le régime d'alimentaire des génisses avec succès.

Grâce aux travaux réalisés au cours de cette thèse, nous avons offert à Boviteq de l'information cruciale sur le statut des follicules de certaines donneuses d'ovocytes, ce qui a permis à l'industrie d'apporter une multitude de changements aux programmes de stimulation ovarienne, de coasting et même d'un point de vue de la nutrition des animaux pré-pubères et sexuellement matures. De ce fait, l'industrie a réussi à augmenter la qualité des ovocytes et leurs taux d'embryons chez les donneuses prépubères et est maintenant en mesure de personnaliser les traitements ovariens chez les donneuses sexuellement matures et ce, au travers des nombreuses installations et cliniques satellites de Boviteq. Les technologies que Boviteq a développées d'eux-mêmes ou en collaboration avec l'Université Laval ont permis à la compagnie de prendre la tête dans l'innovation de la reproduction bovine. Ainsi, la capacité de produire des gamètes de qualité chez les génisses pré-pubères va permettre à

Boviteq d'accélérer davantage le gain génétique des vaches laitières et de maintenir leur position stratégique dans l'innovation mondiale de la reproduction bovine, ce qui représente des milliers de dollars pour l'industrie canadienne.

D'un point de vue fondamental, nous avons établi le modèle physiologique d'un parfait follicule en associant l'expression des gènes de la cellule de granulosa en fonction de la compétence développementale de l'ovocyte. Nous avons aussi proposé un nouveau terme physiologique au développement du follicule entre le déclin de FSH et le pic de LH, ce qui est la période de capacitation du follicule. Cette période fait référence au changement moléculaire et fonctionnel qui se produit dans les cellules de la granulosa dans le but de préparer le follicule pour le pic de LH et l'ovulation. Avec l'identification des biomarqueurs de cette période, il sera possible de développer d'autre test diagnostique capable d'identifier la qualité de maturation du follicule et ainsi d'augmenter notre capacité à produire des ovocytes de meilleure qualité chez le bovin et potentiellement chez l'humain. Finalement, les informations fondamentales sur la signalisation de la croissance et de l'atrésie du follicule, spécialement lors de l'acquisition de la compétence au développement de l'ovocytes va permettre aux chercheurs de mieux comprendre cette période cruciale et d'améliorer les techniques de reproduction assistées.

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