Analyses épigénétique et transcriptomique sur embryons bovins obtenus à partir d'ovocytes de donneuses péri-pubères

Mémoire

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Résumé

Avec l'arrivée des techniques de reproduction assistée et de la génomique, le progrès génétique chez les bovins laitiers est plus rapide que jamais, favorisant maintenant l'utilisation d'animaux de plus en plus jeunes pour la reproduction. Cette situation aurait possiblement un impact sur la qualité des embryons, affectant potentiellement la génisse de la génération suivante. Ce projet vise à documenter l'effet de l'âge sur la qualité de l'embryon et, en l'occurence, à identifier des pistes pour corriger la situation.

Dix jeunes femelles Holstein ont subi trois cycles de stimulation ovarienne (8, 11, 14 mois). Les ovules ont ensuite été fécondés *in vitro* (semence d'un même taureau adulte), générant trois lots d'embryons par animal. Grâce à la plateforme EmbryoGENE, il fut possible de mesurer l'expression génique ainsi que l'état de méthylation de l'ADN au stade blastocyste.

En premier lieu, l'analyse transcriptomique des contrastes selon l'âge (8 vs 14 mois et 11 vs 14 mois) a permis de dénombrer 242 gènes différentiellement exprimés pour le premier contraste et 296 pour le deuxième. Parmi les voies géniques affectées par l'âge, on retrouve notamment les voies mTOR et PPAR, ainsi que la voie de réponse au stress oxydatif médiée par NRF2. Pour sa part, l'analyse épigénétique a permis d'identifier 5787 régions différentiellement méthylées pour le premier contraste et 3658 pour le deuxième. Il est possible d'observer une tendance à l'hyperméthylation chez les embryons obtenus à partir de donneuses de 8 mois, alors qu'une hypométhylation du génome plus marquée est notée chez les embryons provenant des donneuses de 11 mois. Le premier constat est que les embryons sont marginalement affectés par l'âge de la donneuse et que la qualité s'avère très bonne dès 8 mois. Les résultats suggèrent une cause métabolique pour expliquer les différences observées, trahissant un impact plus grand des conditions *in vitro* sur les embryons produits par les plus jeunes donneuses.

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Liste des abréviations

5caC	5-carboxylcytosine
5fc	5-formylcytosin
5hmc	5-hydroxyméthylcytosine
5mC	5-methylcytosine
A	Adénine
ABCA1	ATP Binding Cassette Subfamily A Member 1
ABHD13	Abhydrolase domain containing 13
ACSL4	Acyl-CoA Synthetase Long Chain Family Member 4
ACSS1	Acyl-CoA synthetase short-chain family member 1
ACTE	Association canadienne de Transfert d'Embryons
ACTβ	Actine Beta
ACVR2A	Activin A Receptor Type 2A
ADAMTS2	ADAM Metallopeptidase with Thrombospondin Type 1 Motif 2
ADN	Acide désoxyribonucléique
ADNg	ADN génomique
ADNmt	Acide désoxyribonucléique mitochondriale
AFP	Alpha Fetoprotein
AHR	Aryl Hydrocarbon Receptor
Aicda	Activation-induced cytosine deaminase
AIRE	Autoimmune Regulator
AKT1	Serine-Threonine Protein Kinase
AMBP	Alpha-1-Microglobulin/Bikunin Precursor
AMH	Hormone antimüllérienne
ANK3	Ankyrin 3
ANOVA	Analyse de la variance
AREG	Amphiregulin
ARN	Acide ribonucléique
ARNa	Acide ribonucléique anti-sense
ARNm	Acide ribonucléique messager
ARNTL	Aryl Hydrocarbon Receptor Nuclear Translocator Like
ART	Techniques de reproduction assistée
ATM	ATM Serine/Threonine Kinase
ATP	Adenosine triphosphate
B2M	Beta-2-microglobulin
B4GALT1	Beta-1,4-Galactosyltransferase 1
BAD	BCL2 Associated Agonist Of Cell Death
BAX	BCL2 Associated X, Apoptosis Regulator
BCL2	BCL2, Apoptosis Regulator
BCL2L2	BCL2 Like 2
BCL-XL	BCL2 Like 1
BER	Base excision repair
βFGF	Fibroblast growth factor β
BMP	Bone morphogenetic protein
BMP15	Bone morphogenetic protein 15

BMP4	Bone Morphogenetic Protein 4
BMP8B	Bone Morphogenetic Protein 8b
BMPR1B	Bone Morphogenetic Protein Receptor Type 1B
BRPF1	Bromodomain and PHD finger-containing protein 1
BRWD1	Bromodomain and WD Repeat Domain Containing 1
BSG	Basigin
BSO	Buthionine sulfoximine
BSX	Brain Specific Homeobox
C	Cytosine
C5H12orf45	Chromosome 5 Open Reading Frame 45
CBS	Cystathionine-Beta-Synthase
CCAC	Canadian Council on Animal Care
CCDC60	Coiled-Coil Domain Containing 60
CCIL	Centre canadien d'information laitière
CCND2	Cyclin D2
CD81	CD81 Molecule
CD9	CD9 Molecule
CDC25B	Cell Division Cycle 25B
CDK2	Cyclin Dependent Kinase 2
CDK4	Cyclin Dependent Kinase 4
CDKN1B	Cyclin Dependent Kinase Inhibitor 1B
CDKN1C	Cyclin Dependent Kinase Inhibitor 1C
CEBPβ	CCAAT/Enhancer Binding Protein Beta
CENPB	Centromere Protein B
CETA	Canada Embryo Transfer Association
CGα	Glycoprotein Hormones, Alpha Polypeptide
CGP	Cellule germinale primordiale
CHUK	Conserved helix-loop-helix ubiquitous kinase
CIDR	Controlled Internal Drug Release
CKS2	CDC28 Protein Kinase Regulatory Subunit 2
CLEC16A	C-type lectin domain family 16 member A
CLYBL	Citrate lyase beta like
CO_2	Dioxyde de carbone
COC	Complexe ovocyte-cumulus
COX8B	Cytochrome c oxidase subunit 8B
CpA	Cytosine phosphorylée, suivie d'une adénine
CPE	Carboxypeptidase E
CPEB1	Cytoplasmic Polyadenylation Element Binding Protein 1
CpG	Cytosine phosphorylée, suivie d'une guanine
CpT	Cytosine phosphorylée, suivie d'une thymine
CREB1	Cyclic AMP-responsive element-binding protein 1
CRTC1	CREB Regulated Transcription Coactivator 1
CSF	Facteur cytostatique
CSF1	Colony Stimulating Factor 1
CSF2	Colony Stimulating Factor 2
CSK	C-src tyrosine kinase
CTNNB1	Catenin β1

CTSL2	Cathepsin L2
CXCL14	Chemokine (C-X-C motif) ligand 14
Cy3	Cyanine 3
Cy5	Cyanine 5
CYP11A1	Cytochrome P450 Family 11 Subfamily A Member 1
CYP19A1	Cytochrome P450 Family 19 Subfamily A Member 1
DAZAP1	DAZ Associated Protein 1
DAZL	Deleted in Azoospermia Like
DDR2	Discoidin Domain Receptor Tyrosine Kinase 2
DDX23	DEAD-Box Helicase 23
DEG	Gène différentiellement exprimé
DGAT1	Diacylglycerol O-acyltransferase 1
DHCR24	24-Dehydrocholesterol Reductase
DLGAP5	DLG Associated Protein 5
DMC1	DNA Meiotic Recombinase 1
DMR	Régions différentiellement méthylées
DNMT	DNA methyltransferase
DNMT1	DNA methyltransferase 1
DNMT1o	DNA methyltransferase 1, oocyte-specific
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
DNMT3L	DNA methyltransferase 3L
DOK5	Docking protein 5
DPPA3	Developmental Pluripotency Associated 3
DRAM1	DNA-damage regulated autophagy modulator 1
eCG	Gonadotrophine chorionique équine
EDMA	EmbryoGENE DNA methylation array
EGF	Epidermal growth factor
EGR1	Early Growth Response 1
EIF4E2	Eukaryotic translation initiation factor 4E family member 2
EMBV3	EmbryoGENE bovine microarray
ERBB2	Erb-b2 receptor tyrosine kinase 2
ERCC1	ERCC Excision Repair 1, Endonuclease Non-Catalytic Subunit
ERCC2	ERCC Excision Repair 2, TFIIH Core Complex Helicase Subunit
EREG	Epiregulin
ESR1	Estrogen receptor 1
ET	Transfert embryonnaire
F0	Génération parentale
F1	Première génération
F2	Deuxième génération
FANCA	Fanconi Anemia Complementation Group A
FANCC	Fanconi Anemia Complementation Group C
FANCG	Fanconi Anemia Complementation Group G
FANCL	Fanconi Anemia Complementation Group L
FARS2	Phenylalanyl-tRNA synthetase 2, mitochondrial
FC	Fold change
FDR	False discovery rate

FMN2	Formin 2
FOXB1	Forkhead Box B1
FOXO3A	Forkhead Box O3
FSH	Hormone folliculo-stimulante
FSHR	Follicle Stimulating Hormone Receptor
FST	Follistatin
FZD4	Frizzled Class Receptor 4
G	Guanine
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDF9	Growth differentiation factor 9
GDI1	GDP Dissociation Inhibitor 1
GGT1	Gamma-Glutamyltransferase 1
GHR	Growth Hormone Receptor
GJA1	Gap Junction Protein Alpha 1
GJA4	Gap Junction Protein Alpha 4
GLP1	Glucagon Like Peptide 1
Glut3	Glucose transporter 3
Glut8	Glucose transporter 8
GnRH	Gonadotropin-releasing hormone
GNRHR	Gonadotropin Releasing Hormone Receptor
GO	Gene Ontology
GPR3	G Protein-Coupled Receptor 3
GRK6	G protein-coupled receptor kinase 6
GTF3C1	General Transcription Factor IIIC Subunit 1
H1	Histone 1
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
H5	Histone 5
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDAC1	Histone deacetylase 1
HDAC2	Histone deacetylase 2
HDAC3	Histone deacetylase 3
HDAC8	Histone deacetylase 8
HIF1A	Hypoxia-inducible factor 1-alpha
HMSH4	MutS Homolog 4
HMX3	H6 Family Homeobox 3
HNF1A	HNF1 Homeobox A
HNF4α	Hepatocyte nuclear factor 4 alpha
HNRNPA1L2	Heterogeneous Nuclear Ribonucleoprotein A1-like 2
HOXA10	Homeobox A10
HOXA11	Homeobox A11
HP1	Heterochromatin protein
HSF1	Heat shock factor 1
HSF2	Heat Shock Transcription Factor 2

HTF	Human tubule fluid
ICM	Masse cellulaire interne
ICR	Imprinting control region
IETS	International Embryo Transfer Society
IGF1	Insulin-like growth factor 1
IGF2	Insulin-like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
IL11RA	Interleukin 11 Receptor Subunit Alpha
IL6ST	Interleukin 6 Signal Transducer
IMMP2L	Inner Mitochondrial Membrane Peptidase Subunit 2
INHα	Inhibin Alpha Subunit
INHBβ	Inhibin Beta B Subunit
INPPL1	Inositol Polyphosphate Phosphatase-Like 1
INSIG1	Insulin induced gene 1
INSL3	Insulin Like 3
IP_3	Inositol triphosphate
IP_3R	Inositol triphosphate (récepteur)
IPA	Ingenuity Pathway Analysis
IRS2	Insulin Receptor Substrate 2
IVC	Culture *in vitro*
IVF	Fécondation *in vitro*
IVM	Maturation *in vitro*
KISS1	KiSS-1 Metastasis Suppressor
KISS1R	KISS1 Receptor
KIT	Récepteur tyrosine kinase
KIT	KIT Proto-Oncogene Receptor Tyrosine Kinase
KITL	Ligand KIT
KLHL12	Kelch-like family member 12
LEP	Leptin
LEPR	Leptin Receptor
LFNG	LFNG O-Fucosylpeptide 3-Beta-N-Acetylglucosaminyltransferase
LH	Hormone luténéisante
LHX1	LIM Homeobox 1
LHX8	LIM Homeobox 8
LIF	LIF, Interleukin 6 Family Cytokine
LINE	Long-interspersed repetitive elements
LM-PCR	Ligation-mediated polymerase chain reaction
LPAR3	Lysophosphatidic Acid Receptor 3
LTR	Long terminal repeats
MAPK	Mitogen-activated protein kinase
MBD4	Methyl biding protein 4
MCI	Masse cellulaire interne
MEI1	Meiotic Double-Stranded Break Formation Protein 1
MGC134093	Hypothetical protein MGC134093
MLH1	MutL Homolog 1
MLH3	MutL Homolog 3
MOS	MOS Proto-Oncogene, Serine/Threonine Kinase

MPF	M-phase promoting factor
MSH5	MutS Homolog 5
Msxl	Msh homeobox 1
mTOR	Mammalian target of rapamycin
MYC	Myc proto-oncogene protein
MYST4 (MORF)	Histone (K-lysine) acetyltransferase
NANOG	Nanog Homeobox protein
NANOS3	Nanos C2HC-Type Zinc Finger 3
NBN	Nibrin
NCOA1	Nuclear Receptor Coactivator 1
NDF1P1	Nedd4 family interacting protein 1
NDP	NDP, Norrin Cystine Knot Growth Factor
NER	Nucleotide excision repair
NLRP5	NLR Family Pyrin Domain Containing 5
NLRP9	NLR Family Pyrin Domain Containing 9
NNAT	Neuronatin
NOBOX	NOBOX Oogenesis Homeobox
NOS1	Nitric Oxide Synthase 1
NOS3	Nitric Oxide Synthase 3
NPAS3	Neuronal PAS Domain Protein 3
NPEPPS	Aminopeptidase Puromycin Sensitive
NPM2	Nucleophosmin/Nucleoplasmin 2
NPR2	Natriuretic Peptide Receptor 2
NR2C2	Nuclear Receptor Subfamily 2 Group C Member 2
NR5A2	Nuclear Receptor Subfamily 5 Group A Member 2
NRF2	Nuclear factor (erythroid derived) like 2
NRIP1	Nuclear Receptor Interacting Protein 1
OAS1D	Oligoadenylate Synthetase 1
OCT4	Octamer-binding transcription factor 4
OMVQ	Ordre des Médecins Vétérinaires du Québec
OPU	Ovum pick-up
OTX1	Orthodenticle Homeobox 1
OVOL1	Ovo Like Transcriptional Repressor 1
OXT	Oxytocin/Neurophysin I Prepropeptide
OXTR	Oxytocin Receptor
PABPC1	Poly(A) binding protein, cytoplasmic 1
PAX8	Paired Box 8
PBS	Phosphate-buffered saline
PCR	Réaction en chaîne par polymérase
PCYT1B	Phosphate Cytidylyltransferase 1, Choline, Beta
PDE3A	Phosphodiesterase 3A
PDE4D	Phosphodiesterase 4D
PEG3	Paternally expressed gene 3
PGF2α	Prostaglandine F2α
PIGA	Phosphatidylinositol Glycan Anchor Biosynthesis Class A
PLAU	Plasminogen activator, urokinase
PLCB1	Phospholipase C Beta 1

PMS2	PMS1 Homolog 2, Mismatch Repair System Component
PMSG	Pregnant mare serum gonadotropin
POU1F1	POU Class 1 Homeobox 1
PPAR	Peroxysome proliferator-activated receptor
PRDM1	PR/SET Domain 1
PRDX1	Peroxiredoxin 1
PRDX2	Peroxiredoxin 2
PRL	Prolactin
PRLR	Prolactin Receptor
PRMT1	Protein arginine N-methyltransferase 1
PRMT4	Protein arginine N-methyltransferase 4
PRMT5	Protein arginine N-methyltransferase 5
PROP1	PROP Paired-Like Homeobox 1
PRPF6	Pre-mRNA-processing factor 6
PTGER2	Prostaglandin E Receptor 2
PTGFR	Prostaglandin F Receptor
PTGS2	Prostaglandin-Endoperoxide Synthase 2
PTX3	Pentraxin 3
RALY	RALY Heterogeneous Nuclear Ribonucleoprotein
RASL11B	RAS like family 11 member B
REC8	REC8 Meiotic Recombination Protein
ROS	Reactive oxygen species
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SAM	S-adenosylmethionine
SFRP1	Secreted frizzled related protein 1
SH2B1	SH2B Adaptor Protein 1
SHIP2	SH2-domain-containing inositol polyphosphate 5-phosphatase 2
SINE	Short-interspersed repetitive elements
SIRT1	Sirtuin 1
SIX3	SIX Homeobox 3
SLBP	Stem-loop binding protein
SLC6A4	Sodium-dependent serotonin transporter
SMAD1	SMAD Family Member 1
SMAD5	SMAD Family Member 5
SMAD6	SMAD family member 6
SMC1B	Structural Maintenance Of Chromosomes 1B
SOD1	Superoxide Dismutase 1
SOF	Synthetic oviduct fluid
SOHLH1	Spermatogenesis and Oogenesis Specific Basic Helix-Loop-Helix 1
SOHLH2	Spermatogenesis and Oogenesis Specific Basic Helix-Loop-Helix 2
SOX3	SRY-Box 3
SPO11	SPO11, Initiator of Meiotic Double Stranded Breaks
SRC	SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase
SRD5A1	Steroid 5 Alpha-Reductase 1
STAR	Steroidogenic Acute Regulatory Protein
STAT3	Signal Transducer And Activator Of Transcription 3
STRA8	Stimulated By Retinoic Acid 8

SULT1E1	Sulfotransferase Family 1E Member 1
Suv39H1	Histone-lysine N-methyltransferase SUV39H1
Suv39H2	Histone-lysine N-methyltransferase SUV39H2
Suv420H1	Histone methyltransferase H4-K20 specific
SYCP1	Synaptonemal Complex Protein 1
SYCP2	Synaptonemal Complex Protein 2
SYCP3	Synaptonemal Complex Protein 3
T	Thymine
TAF4B	TATA-Box Binding Protein Associated Factor 4b
TCF21	Transcription Factor 21
TDG	Thymine DNA glycosylase
TE	Trophectoderme
TERT	Telomerase Reverse Transcriptase
Tet	Ten-eleven translocation
Tet1	Ten-eleven translocation 1
Tet2	Ten-eleven translocation 2
Tet3	Ten-eleven translocation 3
TGFB1	Transforming growth factor β1
TGFBR2	Transforming growth factor beta receptor 2
THBS1	Thrombospondin 1
TIAL1	TIA1 Cytotoxic Granule Associated RNA Binding Protein Like 1
TKT	Transketolase
TNF	Tumor necrosis factor
TNFAIP6	TNF Alpha Induced Protein 6
TNFRSF1A	TNF Receptor Superfamily Member 1A
TOP3B	DNA Topoisomerase III Beta
TP53	Cellular tumor antigen p53
TRIP13	Thyroid Hormone Receptor Interactor 13
TRP73	Tumor Protein P73
TSHB	Thyroid Stimulating Hormone Beta
UBB	Ubiquitin B
UBE2A	Ubiquitin Conjugating Enzyme E2 A
UBE3A	Ubiquitin Conjugating Enzyme E3 A
UBR2	Ubiquitin Protein Ligase E3 Component N-Recognin 2
VDR	Vitamin D Receptor
WNT10A	Wnt family member 10
WNT4	Wnt Family Member 4
WNT7A	Wnt Family Member 7A
WT1	Wilms Tumor 1
YBX2	Y-Box Binding Protein 2
ZAR1	Zygote arrest 1
ZC3H7B	Zinc Finger CCCH-Type Containing 7B
ZFP36L2	ZFP36 Ring Finger Protein Like 2
ZFX	Zinc Finger Protein, X-Linked
ZP1	Zona Pellucida Glycoprotein 1
ZP2	Zona Pellucida Glycoprotein 2
ZP3	Zona Pellucida Glycoprotein 3

À mes parents, mes sœurs, mes grands-parents

qui veillent maintenant sur moi, mes amis et à tous ceux qui m'ont

encouragée et supportée dans cette aventure et à ce nouveau

chapitre de ma vie qui commence aujourd'hui.

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Avant-propos

Ce mémoire contient deux chapitres rédigés sous forme d'articles scientifiques, en anglais. Dans les deux cas, j'en suis l'auteure principale. Les co-auteurs sont les chercheurs Patrick Blondin, Christian Vigneault, François-Xavier Grand, Rémi Labrecque ainsi que Marc-André-Sirard, qui ont tous contribué de façon significative à ce projet de recherche.

Le premier article, portant le titre "Transcriptomic evaluation of bovine blastocysts obtained from peri-pubertal oocyte donors" a récemment été publié dans la revue scientifique *Theriogenology* (publié le 15 avril 2017; https://doi.org/10.1016/j.theriogenology.2017.01.005). Le deuxième article, soit "Epigenetic evaluation of bovine blastocysts obtained from peri-pubertal heifers at 8, 11 and 14 months using the same adult bull and *in vitro* fertilization" sera soumis pour publication.

Introduction

Avec près de 11 300 entreprises regroupant 1,4 millions d'animaux et produisant plus de 83 millions d'hectolitres de lait par année (CCIL, 2017), l'industrie laitière canadienne occupe actuellement le troisième rang du classement des productions agricoles basé sur l'importance des recettes économiques dégagées par les entreprises (CCIL, 2017). À lui seul, le Québec possède près de la moitié des fermes laitières canadiennes (5546, en 2016) et un cheptel de plus de 500 000 bêtes, pour une production d'environ 30 millions d'hectolitres de lait (CCIL, 2017). La production laitière par vache augmente (CCIL, 2017) et cette situation est favorisée par certains paramètres tels l'alimentation, la régie d'élevage, le confort, l'efficacité de la reproduction et l'amélioration génétique des animaux.

Avec l'arrivée des techniques de reproduction assistée et de la génomique, le progrès génétique chez les bovins laitiers est plus rapide que jamais. Les avancées technologiques importantes et la forte pression de sélection observée entraînent l'utilisation d'animaux de plus en plus jeunes à des fins reproductives, dans le but de maximiser le progrès génétique, en valorisant les meilleurs sujets et en réduisant l'intervalle entre les générations (Doormaal, 2012; Lohuis, 1995b). Entre autres, la stimulation hormonale de l'ovaire, le transfert embryonnaire et la fécondation *in vitro* permettent de devancer la mise en reproduction des jeunes vaches. Il est possible de constater que le marché de l'embryon gagne en importance depuis quelques années de sorte que le nombre total d'embryons bovins transférables produits s'élevait à près de 115 000 en 2015, par rapport à 83 550 en 2000 (ACTE, 2017). Près de 13 000 embryons bovins ont été exportés en 2015, générant un revenu pour l'industrie laitière de plus de 7.1 millions $, comparativement à 3,5 millions $ en 2002 (CCIL, 2017). Les centres de fécondation *in vitro*, pour leur part, ont permis la production de plus de 9 400 embryons viables en 2015 (ACTE, 2017). Ce contexte pose de nouvelles questions quant à la qualité des embryons obtenus avec des mères de plus en plus jeunes et le développement des outils d'analyse permettent aujourd'hui d'explorer le fonctionnement des gènes dès le stade embryonnaire de même que la programmation épigénétique associée à la méthylation de l'ADN.

Le présent mémoire sera divisé en trois sections; tout d'abord, le Chapitre 1 consistera en une revue de littérature qui présentera, sous la forme d'un portrait se voulant le plus complet possible, les connaissances actuelles en lien avec la reproduction bovine femelle et l'épigénétique. L'accent sera d'abord porté sur le développement des gamètes femelles ainsi que sur la structure et la fonction de l'ovaire. Par la suite, le concept d'épigénétique et les principes guidant la mise en place des modifications post-transcriptionnelles de l'ADN seront présentés. La programmation épigénétique de l'embryon sera ensuite abordée, après quoi l'importance de la modulation hormonale du cycle œstral chez le bovin et les diverses techniques de reproduction assistée seront discutées. Finalement, il apparaît pertinent de rassembler les connaissances actuelles sur la physiologie reproductive des animaux pré-pubères et péri-pubères ainsi que sur les caractéristiques que présentent leurs ovocytes et, éventuellement leurs embryons. Les Chapitres 2 et 3 présenteront, sous forme d'articles scientifiques, les expériences, les résultats et les conclusions tirées suite aux analyses transcriptomiques et épigénétiques, permettant de comparer la qualité des embryons provenant de donneuses péri-pubères et adultes.

CHAPITRE 1: Revue de littérature

Partie I

1.1 Structure et fonction ovarienne

Bien que l'intérêt pour l'étude de la fonction reproductrice femelle se soit manifesté tôt dans l'histoire (5^{e} siècle avant JC), ce n'est qu'au milieu des années 1600 que la fonction de l'ovaire est décrite pour la première fois. En 1827, l'ovule est enfin observé chez le chien; à ce moment, l'œuf de mammifère est défini (Cobb, 2006). L'ovaire est un organe particulier, qui a été étudié sous tous ses angles et qui fait toujours l'objet de recherches en reproduction. Il s'agit d'un organe ovale dont la taille varie entre 1,5 et 5 cm de longueur et dont le diamètre fait entre 1 et 3 cm chez le bovin (Ball and Peters, 2004). Sa structure est dynamique, s'adaptant au cours du cycle à la croissance folliculaire. Chez les mammifères, son anatomie complexe lui permet d'assurer ses fonctions reproductrices et endocriniennes. En effet, il est maintenant bien connu que les deux ovaires travaillent ensemble de façon à assurer la production des gamètes femelles (ovocytes), le développement folliculaire et le maintien d'un environnement favorable à une éventuelle gestation (Adams et al., 2008).

La zone médullaire forme le prolongement de l'hile et est composée de tissu conjonctif lâche (Gougeon, 2003). Alors que cette dernière assure l'innervation et la vascularisation sanguine et lymphatique de l'organe, le cortex ovarien, pour sa part, est formé de tissu de soutien fibreux et permet l'activité folliculaire (Zamboni, 1974). Il est ainsi possible d'observer trois types de structures au sein du cortex, trahissant l'implication de l'ovaire dans le déroulement de la formation et le relâchement des gamètes femelles, soient les follicules, les ovocytes et les corps jaunes. Finalement, l'ovaire est recouvert d'un épithélium cubique de surface, en dessous duquel se trouve un tissu conjonctif dense, la tunique albuginée (Ownby, 2002).

Les follicules sont des structures rondes remplies de fluide (liquide folliculaire) dont le diamètre peut atteindre entre 12 et 20 mm au moment de l'ovulation chez le bovin (Fortune et al., 1991; Ginther et al., 1996; Rodgers and Irving-Rodgers, 2010). Plusieurs follicules sont visibles sur chaque ovaire et chacun d'eux contient un

ovocyte, qui pourra potentiellement être ovulé, et est formé de trois types cellulaires entourant le gamète femelle, soient les cellules du cumulus, de granulosa et de la thèque (Rodgers and Irving-Rodgers, 2010). Le follicule procure à l'ovocyte un environnement propice à sa croissance et à son développement, en plus d'assurer la production d'hormones stéroïdiennes (Lunenfeld et al., 1975). Les follicules observés à la surface de l'ovaire ne sont pas tous au même stade de développement. En effet, il est possible de distinguer à la fois des follicules primordiaux, primaires, secondaires et tertiaires, ou pré-ovulatoires (Rodgers and Irving-Rodgers, 2010). Le follicule dit dominant est le plus gros follicule visible et c'est généralement celui qui sera sélectionné et qui assurera la prochaine ovulation. Chez le bovin, considérant que plusieurs follicules se développent à chaque vague folliculaire et qu'au terme de chaque cycle, un seul sera ovulé, il est important de noter que plus de 99% de ces follicules régresseront et se désintègreront (atrésie) sans être ovulés (Magre, 2011). Chez la femelle pré-pubère, on rapporte que le cortex ovarien présente des ovocytes primaires entourés d'une seule couche de cellules de support. La zone médullaire de l'ovaire immature, pour sa part, ne contient que certaines structures nerveuses et quelques vaisseaux sanguins (Ball and Peters, 2004).

Les corps jaunes, ou *corpus luteum*, sont également visibles à la surface de l'ovaire. En effet, un nouveau corps jaune est observé suite à chaque cycle œstral, à l'endroit même où a eu lieu la dernière ovulation, sur l'un ou l'autre des deux ovaires (Ownby, 2002). Le *corpus luteum* est une structure protubérante possédant une texture dense, celui-ci ayant comme fonction de produire la progestérone, qui permettra le soutien de la gestation (Ownby, 2002).

Finalement, l'ovaire assure également une fonction endocrinienne, qui implique le contrôle de la sécrétion d'œstrogène et de progestérone principalement; ces hormones stéroïdiennes produites par les follicules en réponse aux gonadotrophines (LH et FSH) sont synthétisées à partir du cholestérol, impliquant les cellules de la thèque interne et les cellules de granulosa (Gougeon, 2003; Ho et al., 2003; Roberts and Skinner, 1990). La gonade femelle agit sous le contrôle de la glande pituitaire, qui

elle-même est régulée par l'hypothalamus, le centre de contrôle des fonctions physiologiques (Ball and Peters, 2004).

1.2 Ovogenèse et folliculogenèse

Les processus de développement de l'ovaire, de l'ovule (ovogenèse) et du follicule (folliculogenèse) sont complémentaires et étroitement liés. Alors que l'ovogenèse est connue pour être initiée tôt dans le développement fœtal chez les mammifères, la folliculogenèse, quant à elle, débute peu avant la naissance chez le bovin (Peters and McNatty, 1980). Dès sa naissance, la jeune femelle possède déjà, dans sa réserve ovarienne, un nombre limité d'ovocytes (Bryskov AG, 1994). Cette réserve est considérée comme maximale dès l'arrêt des divisions mitotiques des futures gamètes. Les opinions demeurent partagées en ce qui concerne l'hypothèse que cette réserve puisse être renouvelable (Paulini et al., 2014) Bien que le nombre soit variable d'une étude à l'autre, Erickson (1966b), cité par Paulini et al. (2014), rapporte qu'il y aurait près de 2 700 000 cellules germinales dans l'ovaire de l'embryon bovin après 110 jours de gestation, alors que ce nombre diminuerait à 68 000 après la naissance, soit à 13 jours de vie. À ce moment, les cellules germinales ont atteint le stade d'ovocyte primaire, caractérisé par un arrêt de la première méiose des ovogonies au stade diplotène de la Prophase I. Cet arrêt méiotique sera maintenu jusqu'à la puberté (Erickson, 1966b; Monniaux, 2009; Picton, 2001).

Les premiers signes de formation de l'ovaire sont observés après environ 30 jours de gestation chez le bovin (Smith et al., 2014). L'ovogenèse est caractérisée par quatre étapes clés: la migration des cellules germinales primordiales (CGP) et la colonisation des crêtes génitales; la prolifération mitotique des ovogonies; la division méiotique des ovocytes et, finalement; l'ovulation et la fécondation des ovules (Gougeon, 2003; Russe, 1983; Smitz and Cortvrindt, 2002). Les cellules germinales primordiales sont totipotentes et cette condition semble être assurée, jusqu'à la première division méiotique de la future gonade, par l'expression de certains facteurs de transcription tels OCT4 (Pesce et al., 1998). Il importe de comprendre que l'ovogenèse est un processus très spécialisé; en effet, les cellules germinales primordiales, qui sont diploïdes, se différentient pour devenir des ovogonies qui, elles, se multiplient par

mitose. Celles-ci poursuivent leur maturation et deviennent éventuellement des ovocytes primaires qui entreront en méiose et, finalement, des ovules haploïdes aptes à être fécondés (van den Hurk and Zhao, 2005).

La folliculogenèse, pour sa part, réfère au processus par lequel les ovocytes s'entoureront de cellules somatiques et formeront des follicules qui se développeront pour atteindre, successivement les stades de follicules primordiaux, primaires, secondaires et, finalement, tertiaires ou pré-ovulatoires, sous l'influence des gonadotrophines FSH et LH (Paulini et al., 2014). Chez le bovin, les follicules subiront ensuite les processus de recrutement, de sélection et de dominance, pour, ultimement, ne mener qu'à une seule ovulation; en d'autres mots, il s'agit du processus de croissance et de maturation, qui sépare le moment où le follicule quitte la réserve ovarienne et l'ovulation (Paulini et al., 2014). Ainsi, comme le rapporte Monniaux et al. (2009), les gamètes haploïdes sont formés suite à un processus méiotique discontinu. Cette méiose est d'abord initiée au stade fœtal, puis est arrêtée au stade prophase de sa première division, alors que se développent les follicules ovariens. La méiose reprend ensuite peu avant l'ovulation, pour s'interrompre une deuxième fois au stade métaphase de la deuxième division. La méiose sera complétée que s'il y a fécondation (Monniaux et al., 2009).

Il est raisonnable de penser qu'une bonne compréhension au processus de différentiation des gamètes femelles chez le bovin ainsi que des changements structurels qui définissent l'ovocyte et qui le rendent compétent est essentielle pour le développement et l'amélioration des techniques de reproduction assistée (Paulini et al., 2014).

1.2.1 Chronologie de la formation de l'ovocyte et du follicule

À compter de la puberté, il est possible de constater que la différentiation des gamètes femelles se produit en étroite relation avec le processus de folliculogenèse (Magre, 2011). Tel que mentionné précédemment, ce processus assure la sélection et la croissance des follicules ovariens. Les follicules progressent ainsi du stade primordial au stade pré-ovulatoire. La folliculogenèse est alors divisée en deux étapes

importantes, soient la folliculogenèse basale et la folliculogenèse terminale, ces deux étapes étant dépendantes de la présence d'ovocytes (Magre, 2011; Monniaux et al., 2009).

La première étape de la folliculogenèse assure les premières phases de croissance des follicules et ce, jusqu'à l'atteinte du stade follicule secondaire (Monniaux, 2009). Dans la plupart des cas (Fair et al., 1995), elle confère à l'ovocyte sa compétence méiotique, le rendant apte à reprendre sa division méiotique, qui a été bloquée en prophase I (Monniaux et al., 2009). Cette première phase de croissance est supportée par l'action de différents facteurs considérés comme locaux, qui ont un mode d'action autocrine ou paracrine (Magre, 2011). En effet, l'hormone antimüllérienne (AMH), sécrétée par les follicules en croissance, certains facteurs de croissance tels GDF9 (*growth differentiation factor 9*) et BMP15, sécrétés par l'ovocyte et KITL agissent de façon à favoriser la croissance et le développement des follicules et des gamètes femelles (Magre, 2011). Plusieurs études ont démontré que cette première phase de la folliculogenèse est complètement indépendante de l'action de la FSH. En effet, différents protocoles impliquant l'hypophysectomie, la désensibilisation hypophysaire ou encore l'invalidation ou le *knock-out* du gène responsable de l'expression de la chaîne β de la FSH chez différentes espèces ont prouvé cette théorie (Monniaux et al., 2009; Kumar et al., 1997; Hillier, 1994).

La folliculogenèse dite terminale, pour sa part, permet au follicule de poursuivre son développement à partir du stade de follicule secondaire et ce, jusqu'à l'ovulation. La différenciation des follicules et des gamètes est alors orchestrée par les gonadotrophines FSH et LH (Armstrong, 1986; Hillier, 2004). Ces dernières sont sécrétées par l'hypophyse et permettent, successivement, les processus de recrutement, de sélection et de dominance folliculaire (Magre, 2011; Monniaux et al., 2009). Certains facteurs de croissance, protéases et stéroïdes sont également impliqués dans le bon déroulement de la folliculogenèse terminale (McGee and Hsueh, 2000). Cette deuxième phase de la folliculogenèse fera l'objet de la section 1.2.3.

La transition du follicule secondaire vers le follicule tertiaire (antral) est marquée par l'accumulation de liquide folliculaire entre les cellules de granulosa, ce qui conduira éventuellement à la formation de l'antre. Il va sans dire qu'à cette étape, l'interaction entre l'ovocyte et les cellules qui l'entourent est critique, alors que plusieurs facteurs de croissance sont impliqués. Cette transition du stade préantral au stade antral est critique pour le futur du follicule. En effet, cette capacité du follicule à compléter cette différentiation déterminera s'il poursuivra sa croissance ou non (atrésie) (Paulini et al., 2014).

Le follicule est officiellement nommé follicule antral lorsque l'ovocyte est entouré d'au moins 6 couches de cellules de granulosa (follicule de près de 0,2 mm chez le bovin - (Monniaux et al., 2009)) et qu'il possède une cavité antrale remplie de liquide folliculaire complètement différenciée (Adams et al., 2008). Les follicules antraux peuvent être observés chez le fœtus dès les jours 220 et 240 de gestation chez le bovin (Erickson, 1966b). À ce stade, l'antre sépare l'ovocyte, entouré du cumulus, des cellules de granulosa murales qui tapissent, pour leur part, la membrane du follicule (Armstrong, 1986; Motta et al., 2003). Les thèques externes et internes enveloppent le follicule et sont séparées des cellules de granulosa par la lame basale (Motta et al., 2003). Les différents types cellulaires composant le follicule possèdent des caractéristiques morphologiques et fonctionnelles qui leurs sont propres (Magre, 2011).

Pour sa part, la thèque externe est composée de tissu fibreux principalement alors que la thèque interne est constituée de cellules supportant un réseau de vaisseaux sanguins et sécrétant des androgènes (Armstrong 1986). Le follicule antral est généralement visible à la surface de l'ovaire (Ball and Peters, 2004). De plus, son développement jusqu'au stade pré-ovulatoire est caractérisé par l'accroissement du volume de l'antre, alors que l'ovocyte ne prend plus d'expansion (Monniaux et al., 2009). Le follicule développe également une sensibilité accrue et une dépendance à certaines hormones, ce qui favorisera le recrutement et, éventuellement, sa sélection puis, dans quelques cas, son ovulation. Ainsi, le follicule antral possède les récepteurs pour FSH, LH, EGF, bFGF et IGF1 (Beg and Ginther, 2006; Wandji et al., 1996).

À ce stade, les cellules de granulosa possèdent plus de corps lipidiques, de mitochondries, de ribosomes, d'appareils de Golgi, de vésicules et de réticulum endoplasmique lisse que ce qui a précédemment été observé chez les follicules secondaires (Sharma et Sawhney, 1999). Ceci permettraient de soutenir la production de plus en plus importante d'hormones stéroïdiennes en plus d'assurer le métabolisme des lipides et la signalisation calcique du récepteur œstrogène via les mitochondries (Sharma et Sawhney, 1999, tel que cité par (Paulini et al., 2014)). Les organites cellulaires sont alors disposés au centre du cytoplasme du follicule antral (Hyttel et al., 1997). Les mêmes observations sont rapportées chez les cellules de la thèque interne, qui présentent cependant un noyau allongé (O'Shea et al., 1978; Sharma et al., 1996). L'antre du follicule pré-ovulatoire sert à l'accumulation des produits de sécrétion des cellules de granulosa et de la thèque (Monniaux et al., 2009). Les granules corticaux, quant à eux, migrent progressivement vers la surface interne de la membrane de l'ovocyte, endroit où elles déverseront leur contenu enzymatique le moment venu (Gulyas, 1980). Il est important de noter que ces derniers seront produits jusqu'au moment de l'ovulation (Gulyas, 1980).

Tout au long de leur développement, les ovocytes et les follicules répondent à divers molécules et facteurs. Les principaux acteurs dans les processus d'ovogenèse et de folliculogenèse ont été présentés précédemment. À ceux-ci s'ajoutent plusieurs autres éléments, codés par des gènes précis. La Figure 1.1 présente ces principaux gènes impliqués dans la folliculogenèse, observés, pour la majorité chez la souris (Matzuk et Lamb, 2008). Ces gènes sont considérés comme essentiels, leur absence étant associée à un phénotype d'infertilité.

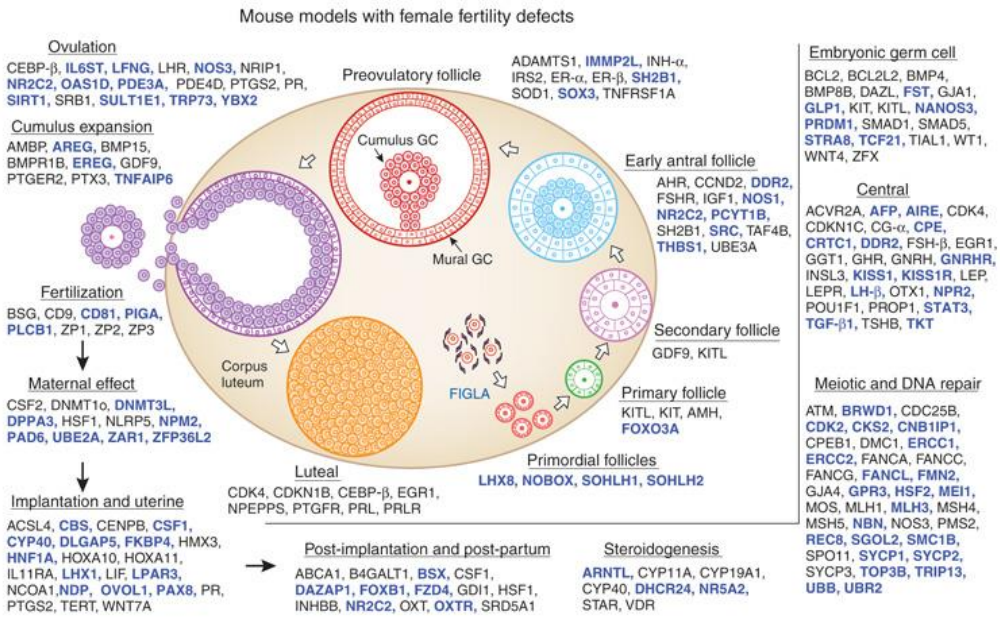

Figure 1.1. Gènes impliqués dans le processus de folliculogenèse chez la souris. Ces gènes sont considérés comme essentiels puisqu'ils génèrent un phénotype d'infertilité (*tiré, avec permission, de Macmillan Publishers Ltd: [Nature Medicine] (Matzuk and Lamb, 2008), copyright 2008*).

La Figure 1.2, quant à elle, présente l'histologie du follicule ovarien. Il est possible de discerner les follicules à différents stades de développement ainsi que les différents types cellulaires composant ces derniers et l'ovaire. Au stade pré-ovulatoire, le follicule a un diamètre de taille variant entre 15 et 20 mm chez le bovin (van den Hurk and Zhao, 2005) et contient un ovocyte bien visible entouré d'une zone pellucide bien différentiée et des cellules du cumulus avec lesquelles il entretient un lien étroit (Fair et al., 1997). Jusqu'au pic de LH, le noyau de l'ovocyte est maintenu en arrêt méiotique, au stade de vésicule germinale. Les cellules de granulosa sont adjacentes aux cellules du cumulus; celles-ci sont non-vascularisées et alimentées par diffusion passive. On distingue deux populations de cellules de granulosa, formant la granulosa antrale et la granulosa murale (Greenwald, 1988). Monniaux (2009) rapportent l'importance du follicule pour la croissance de l'ovocyte, mais aussi pour l'acquisition de sa compétence à la fécondation et au développement.

La lame basale isole les cellules de granulosa des cellules de la thèque en constituant une barrière à l'irrigation sanguine. Pour leur part, les cellules de la thèque sont caractérisées comme étant vascularisées et fibroélastiques et assureront un rôle crucial dans la production d'hormones stéroïdiennes, particulièrement de l'œstradiol. Elles sont divisées de façon à former deux tissus, soient la thèque interne et la thèque externe (Armstrong 1986).

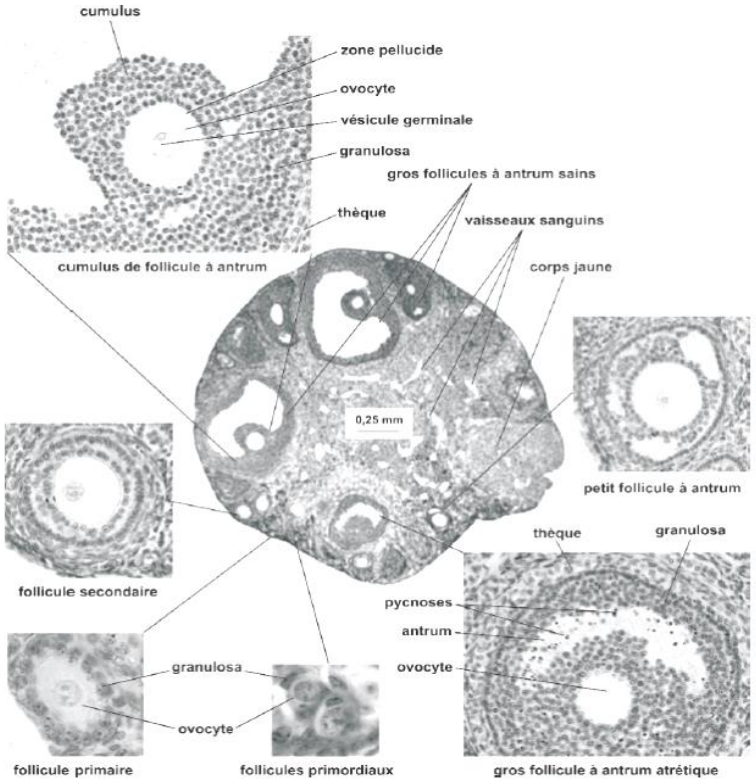

Figure 1.2. Histologie du follicule ovarien (tiré, avec permission, de Monniaux et al. 2009).

Pour sa part, l'ovocyte poursuit son développement pour finalement atteindre sa maturité au stade métaphase de la deuxième division méiotique. À ce moment, on observe un deuxième arrêt de la méiose, alors que ce dernier sera maintenu jusqu'au moment de la fécondation (Motlik and Kubelka, 1990; Pincus and Enzmann, 1935).

L'ovocyte forme, avec les cellules qui l'entourent, le complexe ovocyte-cumulus (COC). Avec les cellules de la granulosa, cette association forme un syncytium électro-physiologique qui possède plusieurs particularités. Il est d'abord possible de

noter la présence de jonctions ouvertes de type gap entre l'ovocyte et les cellules du cumulus, conférant une certaine perméabilité moléculaire. L'existence et l'efficacité de ces jonctions communicantes ont d'ailleurs été démontrées, entre autres, par Santiquet et al. (2012) chez le porc. Gershon et al. (2008) ont résumé les connaissances sur les jonctions de type gap dans l'ovaire.

Grâce à cette efficacité de communication et cette sensibilité de l'ovocyte à son environnement, il est possible pour le COC de coordonner plusieurs évènements. C'est le cas de l'ovogenèse, la folliculogenèse, la stéroïdogenèse, l'apoptose, l'arrêt puis la reprise de la méiose, la maturation de l'ovocyte (tant *in vitro* que *in vivo* - (Gershon et al., 2008) ainsi que la régulation du remodelage de la chromatine et de la transcription au cours des premières étapes de maturation de l'ovocyte bovin *in vitro* (Luciano et al., 2011).

1.2.2 Cycle œstral bovin

Le bovin est une espèce monotoque qui a un mode de reproduction poly-œstral non saisonnier et qui présente un cycle d'une durée moyenne de 21 jours (Ball and Peters, 2004). La puberté, définie par l'apparition du premier œstrus, généralement observée entre les âges de 12 et 14 mois chez les bovins, cet âge pouvant varier entre les individus (Ferrell, 1982; Ball and Peters, 2004). En effet, quelques facteurs peuvent influencer l'âge à la puberté, soient la race, l'alimentation, le poids et la saison. Le cycle œstral est divisé en deux périodes, folliculaire (3 à 4 jours) et lutéale (17 à 18 jours), et quatre phases distinctes, soient le pro-œstrus, l'œstrus, le métœstrus et le diœstrus (Ball and Peters, 2004). La phase folliculaire fait référence à l'intervalle entre la régression du corps jaune et l'ovulation, alors que la phase lutéale correspond à la période suivant l'ovulation, jusqu'à la lutéolyse. Par convention, l'ovulation a lieu au jour 0 du cycle œstral.

Le bon déroulement du cycle œstral bovin est assuré par un mécanisme hormonal finement orchestré. L'hypothalamus contrôle la reproduction chez le bovin en relâchant certains facteurs tels la GnRH, qui agit alors sur la glande pituitaire de

façon à stimuler le relâchement des gonadotrophines FSH et LH (Ball and Peters, 2004).

La phase folliculaire comprend le pro-œstrus (jours 19 à 21 du cycle œstral) et l'œstrus (jour 0) et est caractérisée par la croissance, le développement et la sélection des follicules (Ball and Peters, 2004). Le cycle œstral observé chez la vache est caractérisé par la présence de deux à trois vagues de développement folliculaire (plus de 95% des vaches), conduisant ultimement à une seule ovulation (Adams et al., 2008; Rajakoski, 1960; Savio et al., 1988; Sirois and Fortune, 1988). Le pro-œstrus a une durée qui varie entre 1 et 2 jours et c'est au cours de cette période que la femelle commence à adopter un comportement caractéristique, qui mènera ultimement à sa réceptivité sexuelle (Ball and Peters, 2004). Cette phase, au cours de laquelle se développent les follicules ovariens, se solde par une augmentation significative caractéristique de la concentration d'œstrogène, qui atteindra un pic quelques heures avant celui de la LH (Hillier, 1994). L'œstrogène, libéré par les cellules de la granulosa grâce à l'action de l'insuline, favorisera l'apparition du comportement sexuel en plus de préparer le système reproducteur à la fécondation et créer des conditions favorables à la libération de LH (Ball and Peters, 2004; Katz et al., 1980). La LH est d'abord libérée de façon pulsée et, dans les 24 à 32 heures précédant l'ovulation, sa concentration augmente considérablement (Ball and Peters, 2004). La sécrétion pulsée de LH mimerait et serait influencée par la sécrétion de GnRH, qui présente le même patron. Clarke and Cummins (1982) ont démontré cette relation chez la brebis.

La sécrétion de FSH suit sensiblement le même patron, mais son pic de concentration est moins important que celui de la LH (Hillier, 1994). La sécrétion de FSH s'étend sur une période plus longue, diminuant graduellement après l'ovulation. Pendant le pro-œstrus, la vulve de la vache gonfle légèrement, prend une coloration rosée et son épithélium épaissit (Gordon, 2011). La muqueuse et le tissu vaginal subissent également des modifications et permettent la sécrétion des phéromones propres aux chaleurs (Gordon, 2011). Du mucus s'échappe de la vulve. De plus, le comportement

de la vache change, de sorte qu'il est possible de la voir plus nerveuse et alerte. Dans plusieurs cas, elle tentera même de monter ses congénères (Gordon, 2011).

Entre 10 et 12 heures avant l'ovulation, la vache est réceptive sexuellement (Gordon, 2011) et acceptera la monte du mâle pour une période durant en moyenne 7 heures; c'est l'œstrus (Ball and Peters, 2004). La fin de l'œstrus est marquée par le pic de LH, qui permet la sélection finale du follicule qui sera ovulé (Armstrong, 1986; Hillier, 1994). En effet, l'œstradiol libéré par le follicule pré-ovulatoire agit positivement sur l'hypothalamus de façon à induire le pic de LH qui contribuera à déclencher l'ovulation (Armstrong, 1986). Le pic, puis la baisse de LH, synchronisés avec la hausse de progestérone créent les conditions favorables à l'ovulation (Armstrong, 1986).

L'ovulation consiste en l'expansion des cellules du cumulus, suivie de la rupture du follicule ovarien et en la libération de son contenu dans le tractus reproducteur femelle (Saint-Dizier, 2014). À ce moment, l'ovocyte demeure entouré des cellules du cumulus (Ball and Peters, 2004). Tel que mentionné précédemment, chez le bovin, l'ovocyte est libéré par l'ovaire alors qu'il est maintenu en arrêt méiotique; les gonadotrophines agissent alors de façon à stimuler la reprise de la méiose (la section 1.2.4 permettra une meilleure compréhension de ce phénomène). Cette reprise de la division méiotique de l'ovocyte est caractérisée par la rupture de la vésicule germinale, la condensation chromatique et l'expulsion du premier globule polaire (Crozet, 1991). Ces changements assurent la maturation nucléaire de l'ovocyte. On observera par la suite que la division de l'ovocyte s'arrête à nouveau, au stade métaphase II et cet arrêt sera maintenu jusqu'à la fécondation (Crozet, 1991).

Suite à l'ovulation, il y a formation du corps jaune, qui génère une cicatrice sur l'ovaire, là où l'ovulation a eu lieu (Magre, 2011). À ce moment, les cellules de granulosa évoluent, sous l'influence du pic de LH, pour devenir des cellules lutéales (Magre, 2011). C'est alors que débute la phase lutéale du cycle œstral bovin (Figure 1.3). Il est maintenant bien connu que la progestérone est alors libérée par le corps jaune et que celle-ci a comme rôle de préparer l'utérus à l'arrivée de l'embryon et ainsi à une éventuelle gestation (Saint-Dizier, 2014). Le corps jaune empêche également

toute autre ovulation, en exerçant un rétrocontrôle sur l'hypothalamus (Ball and Peters, 2004). La LH est également sécrétée pendant la phase lutéale du cycle œstral et ce, de façon pulsatile à fréquence peu élevée (Ball and Peters, 2004).

Le metœstrus suit l'œstrus. Celui-ci a une durée de 3 à 4 jours et représente la portion post-œstrus de la phase lutéale (Ball and Peters, 2004). Cette phase est initiée lorsque la vache exprime une baisse de réceptivité sexuelle (Gordon, 2011). Le diœstrus suit le metœstrus et réfère à la période pour laquelle un corps jaune est présent et actif, sécrétant la progestérone. Cette dernière période s'étend généralement du jour 5 (la progestérone commence à être relâchée) au jour 18 du cycle œstral bovin (Figure 1.3). La progestérone atteint un pic de concentration au jour 8 après quoi ce niveau élevé est maintenu jusqu'à la fin du dioestrus (Ball and Peters, 2004)

La fin du dioestrus marque la fin de la phase lutéale et, par le fait même le cycle œstral bovin. Cette dernière étape se solde par la régression du corps jaune (à compter du jour 18 du cycle) en l'absence de gestation (Ball and Peters, 2004). Il est alors primordial qu'il y ait communication entre l'utérus, l'ovaire et l'hypophyse de façon à reprendre le cycle. La lutéolyse du corps jaune est causée, entre autres, par la prostaglandine PGF2α, dont la libération est stimulée par l'ocytocine produite par le corps jaune lui-même (Wathes and Swann, 1982). L'ocytocine agit sur l'endomètre qui, lui, produit la PGF2α (Wathes and Swann, 1982). La progestérone exerce également un rétro-contrôle négatif sur l'expression de ses propres récepteurs, causant une diminution de la réponse à la progestérone (Saint-Dizier, 2014). Ces facteurs combinés causeront la régression du corps jaune. Cette étape est suivie par l'apoptose des cellules lutéales et, éventuellement, la formation d'un corps blanc sur l'ovaire, celui-ci n'étant pratiquement qu'un amas de tissu conjonctif (Saint-Dizier, 2014). Considérant qu'il y ait gestation, il n'y a pas de production de PGF2α par l'utérus et donc pas de baisse de progestérone.

La Figure 1.3 présente le cycle œstral bovin. Il est possible de discerner les courbes de concentrations des principales hormones impliquées dans le cycle de reproduction de la vache, soient la FSH, la LH, l'œstrogène et la progestérone.

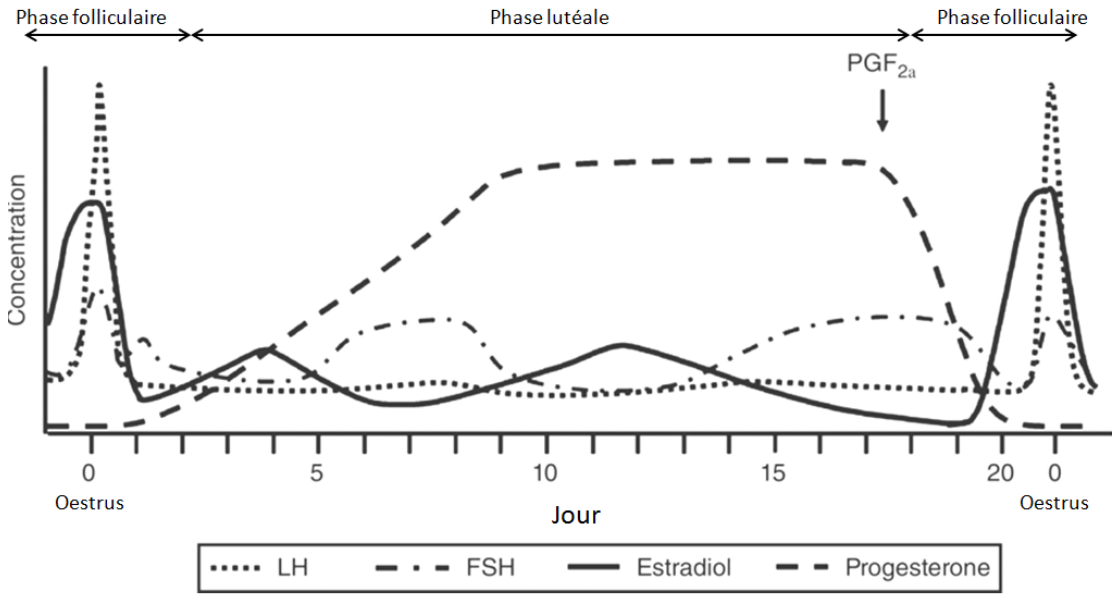

Figure 1.3. Cycle œstral bovin (*tiré, avec permission, de John Wiley and Sons: [Bovine reproduction] (Hopper, 2014), copyright 2014*).

1.2.3 Croissance folliculaire et mécanismes conduisant à l'ovulation

Tel que mentionné précédemment, la dynamique de la croissance folliculaire (folliculogenèse terminale) conduisant à l'ovulation chez les mammifères implique la présence préalable de plusieurs follicules dans l'ovaire et une synchronisation hormonale bien orchestrée. L'ovaire contient un pool de quelques milliers de follicules (Erickson, 1966). Entre 40 et 60 follicules sont donc disponibles par cycle, selon les espèces. Lorsque le follicule atteint le stade de développement caractérisé par le début de la formation de l'antre (follicule antral), soit à un diamètre de 2 à 3 mm, celui-ci devient dépendant de la FSH produite par le cerveau pour survivre et continuer de croître. La phase de recrutement est ainsi caractérisée par la présence d'un pic de FSH (Adams et al., 1992; van den Hurk and Zhao, 2005).

Parmi ceux qui répondent à la FSH, il sera possible de constater qu'un follicule se développera plus rapidement que les autres. C'est au cours de la phase de sélection que ce follicule est alors remarqué pour la première fois et défini comme étant le follicule dominant (Ginther et al., 1996; Ginther et al., 2003; Monniaux, 2009). Chez le bovin, le premier qui atteindra le seuil de 8,5 mm deviendra, dans la plupart des cas, le follicule dominant (Ginther et al., 2000). Ce follicule pré-ovulatoire est donc le premier à acquérir des récepteurs LH et devient avantagé par rapport aux autres (Gong et al., 1995; Monniaux, 2009). Dès qu'il devient exclusivement dépendant à la LH (début de la phase de différentiation), le follicule dominant relâche des quantités plus importantes d'inhibine et d'œstradiol (Monniaux, 2009). Les concentrations élevées d'inhibine exercent une rétroaction négative sur la glande pituitaire, ce qui provoque une baisse de FSH (Martin et al., 1991; Guilbault et al., 1993; Ireland et al., 1994). L'œstradiol, pour sa part, agit positivement sur l'hypothalamus de façon à induire le pic de LH qui contribuera à déclencher l'ovulation (Ginther et al., 1996; Webb et al., 1999). Les autres follicules ne présentent pas la machinerie nécessaire pour survivre en s'approvisionnant de LH ne pourront survivre sans FSH. Ainsi, la baisse de FSH causée par le rétrocontrôle exercé, entre autres, par le follicule dominant causera l'atrésie des autres follicules, soit près de 99% d'entre eux (Bergfelt et al., 2000; Ginther et al., 1996). Cette boucle de contrôle rétro-positif fait en sorte que le follicule qui domine se développera encore plus rapidement que les autres, en réprimant ces derniers, ce qui donne lieu à une course inégale vers la différentiation complète. Il s'agit du processus de dominance. De plus, le follicule subalterne produit des androgènes. Le follicule dominant en a ainsi moins à synthétiser par lui-même pour subvenir à ses besoins. Le follicule subalterne, pour sa part, ne sera pas détruit immédiatement. En effet, il survivra quelques heures supplémentaires et prendra la relève du follicule dominant, si nécessaire (Ko et al., 1991).

Les hauts niveaux de progestérone préviendront l'ovulation pour une durée définie. Lorsque la progestérone chutera, en fin de cycle, les conditions deviendront permissives, ce qui conduira à l'ovulation du follicule dominant. Bien que certaines différences soient observées d'une espèce à l'autre, la physiologie de la dominance folliculaire est sensiblement la même. En effet, l'évolution a permis une préservation

de cette caractéristique, tant pour les espèces mono-ovulantes que poly-ovulantes. Ginther (2016) et son équipe récemment publié une revue détaillée présentant la théorie de la sélection folliculaire chez le bovin.

La Figure 1.4 résume les différentes étapes par lesquelles les follicules transitent et ce, du début de leur croissance jusqu'à l'ovulation.

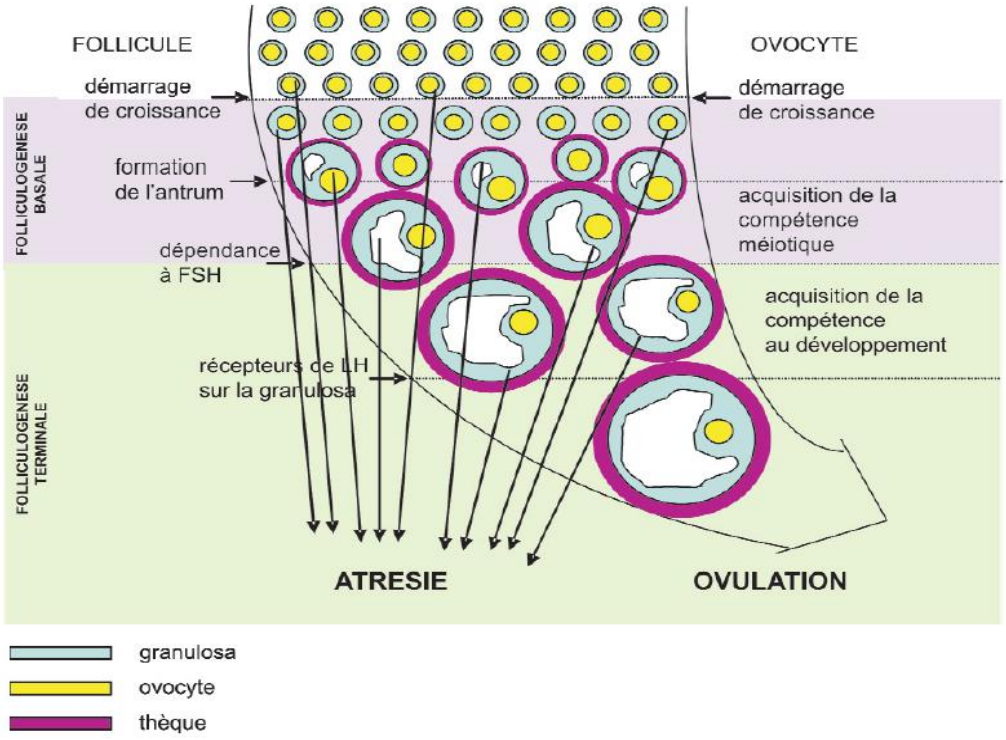

Figure 1.4. Dynamique de la croissance folliculaire (tiré, avec permission de Monniaux (2009)).

1.3 Fécondation

Suite au pic de LH, l'ovocyte subit les dernières étapes de maturation. Au terme des étapes de maturation nucléaire, cytoplasmique et moléculaire, l'ovocyte deviendra ultimement haploïde et sera, à ce stade, en mesure de supporter la fécondation puis les premières étapes du développement de l'embryon (Saint-Dizier, 2014).

À sa sortie du follicule, l'ovocyte est capté par l'oviducte et sera acheminé vers la jonction de l'isthme et l'ampoule, là où la fécondation aura lieu. La fécondation est

caractérisée comme étant la fusion des gamètes mâle et femelle, conduisant à la formation d'un embryon (Saint-Dizier, 2014).

Le processus de fécondation est initié lorsque les spermatozoïdes traversent le cumulus entourant l'ovocyte (Saint-Dizier, 2014). Ces derniers se lient ensuite à la zone pellucide de l'ovocyte; il y a alors réaction de l'acrosome. Le gamète mâle traverse par la suite la zone pellucide pour se fusionner directement à la membrane plasmique de l'ovocyte. Finalement, il y a internalisation du contenu spermatique dans le cytoplasme du gamète femelle. Cette étape est suivie de la décondensation du noyau; à ce moment, il est possible de constater la présence de deux pronoyaux, un mâle et un femelle (Saint-Dizier, 2014). Au moment de la fécondation, il y a reprise de la méiose, rendue possible grâce à l'inactivation du MPF (M-phase promoting factor) et du CSF (cytostatic factor) (Monniaux, 2009). L'entrée du spermatozoïde, coïncidant avec la fin de la deuxième division méiotique et l'expulsion du deuxième globule polaire, permet l'activation de l'ovocyte (Monniaux, 2009).

Parmi tous les ovocytes produits par l'ovaire au cours de son développement, certains seront considérés comme bons et seront fécondés alors que d'autres ne permettront pas de produire un embryon viable. Un ovocyte est considéré comme étant compétent au développement par sa capacité à reprendre la méiose, à se diviser suite à la fécondation, à se développer jusqu'au stade blastocyste, à induire une grossesse et à engendrer des descendants vivants et en santé (Sirard et al., 2006). Plusieurs éléments semblent influencer la qualité de l'ovocyte et sa compétence tels que, par exemple, la qualité et la taille du follicule (Blondin and Sirard, 1995), la dynamique de croissance folliculaire (Vassena et al., 2003), la stimulation hormonale (Blondin et al., 2002; Nivet et al., 2012) et, les conditions de culture *in vitro*.

Étant donné l'étroite relation entre l'ovocyte et son follicule, ce dernier influence directement la survie et la qualité du gamète de par l'importance et la qualité du stockage, la régulation post-transcriptionnelle d'ARNm ainsi que la synthèse de protéines (Memili et al., 1998). Ces éléments seront essentiels pour supporter les premières étapes du développement embryonnaire, avant l'activation du génome. Chez le bovin, la transcription du génome maternel a lieu jusqu'au moment où

l'ovocyte atteint une taille de 110 pm, permettant à ce dernier d'accumuler les ARNm nécessaires au développement embryonnaire jusqu'à 8 cellules (Fair et al., 1997; Renard, 1998).

Il a été démontré que les ovocytes obtenus à partir de follicules dominants (> 13 mm) se développent jusqu'au stade blastocyste et ce, à des taux significativement plus élevés que ceux provenant de plus petits follicules (entre 3 et 8 mm) (Hagemann et al., 1999). De plus, Donnison and Pfeffer (2004) ont rapporté que plusieurs gènes, incluant Oct4, Msxl, NDF1P1, Cyclin A2 et SLBP étaient quantitativement modulés chez des ovocytes provenant de follicules de plus petits que 2 mm comparativement à plus grands que 5mm étaient comparés.

Par ailleurs, alors que les follicules plus petits ne possèdent pas la machinerie moléculaire leur permettant de supporter la fécondation et les premières étapes du développement, il n'y aurait pas de différence observée quant au taux de développement embryonnaire entre les follicules de 3 mm de diamètre et ceux de plus de 5 mm en système *in vitro* (Blondin et al., 1996; Blondin and Sirard, 1995).

Considérant que la maturation de l'ovocyte est une étape essentielle au bon déroulement de sa fécondation, il importe que les milieux de culture *in vitro* présentent des caractéristiques s'apparentant à celles des milieux physiologiques, sans quoi l'efficacité de la technique pourrait être grandement diminuée, si celle-ci n'échoue pas. En contexte *in vivo*, la maturation nucléaire se produit en réponse au pic de LH alors que, selon les espèces, la reprise de la méiose *in vitro* peut résulter de la simple extraction de l'ovocyte de son environnement folliculaire. On noterait ainsi chez les ovocytes produits *in vitro* des différences à l'échelle ultra-structurale qui trahiraient une maturation incomplète (Sirard et al., 2006).

L'ovocyte obtient sa compétence progressivement depuis son stade début antral, en réponse à des signaux hormonaux consécutifs (FSH, LH) (Ginther et al., 1998). Contrairement à l'ovocyte lui-même, les cellules du cumulus possèdent des récepteurs pour la FSH et assurent ainsi la réponse à cette stimulation hormonale. Par la suite, la réponse du follicule à la LH pulsée signale l'acquisition de la compétence

finale (différentiation) (Ginther et al., 1998). En effet, une étude démontre que la présence de cetrotide (antagoniste de GnRH) inhibe l'action de la LH, causant ainsi la perte de compétence du follicule (Labrecque et al., 2014).

Partie II

La fécondation de l'ovocyte par le spermatozoïde donne lieu à la formation du zygote, puis de l'embryon. Cet embryon subit tout un processus de développement avant son implantation dans l'utérus de la femelle, qui a lieu au jour 20 de gestation chez le bovin (Saint-Dizier, 2014). Le passage d'une génération à la deuxième implique une reprogrammation du génome de l'embryon. Ce processus nécessite que les génomes des deux parents soient déméthylés dans les cellules germinales. Cette étape sera suivie d'une nouvelle vague de méthylation qui assurera la configuration du génome de l'embryon lui-même.

2.1 Développement embryonnaire

La formation du zygote, puis de l'embryon est amorcée à la suite de la fécondation. À ce moment, l'ovocyte est activé (Saint-Dizier, 2014). La première étape du développement embryonnaire consiste en la formation des pronoyaux, rendue possible à la suite de la complétion du premier cycle cellulaire, en passant par les phases S, G2 et M (Saint-Dizier, 2014). Le zygote est alors formé d'une seule cellule et les pronoyaux sont visibles dans le cytoplasme du futur embryon jusqu'au premier clivage. Une série de divisions cellulaires s'ensuit, en passant par les stades (i) 2 à 4 cellules, (ii) 4 à 8 cellules et finalement (iii) 8 à 16 cellules (Massicotte et al., 2006; Saint-Dizier, 2014). L'embryon bovin possédant 8 à 16 cellules devient alors morula précoce, puis morula compacte lorsqu'il possède entre 16 et 32 cellules (Massicotte et al., 2006). Jusqu'au stade morula, les cellules totipotentes vivent plusieurs cycles de division cellulaire avant d'amorcer leur première étape de différentiation (Massicotte et al., 2006). Finalement, l'embryon atteint le stade blastocyste, où il est alors nommé jeune blastocyste (ou blastocyste précoce), blastocyste en expansion puis blastocyste en éclosion (Massicotte et al., 2006). La Figure 1.5 illustre bien le processus de développement embryonnaire, présentant les étapes clés de sa différentiation vers le stade blastocyste.

Le blastocyste consiste en une structure composée d'un épithélium externe, nommé trophectoderme (Saint-Dizier, 2014). Cette couche cellulaire renferme une cavité

remplie de fluide (blastocœle) dans laquelle on retrouve un petit amas de cellules désigné sous l'appellation *masse cellulaire interne* (MCI). Alors que le trophectoderme constituera en partie le placenta, la masse cellulaire interne, pour sa part, donnera naissance à tous les tissus embryonnaires et au sac vitellin (Saint-Dizier, 2014).

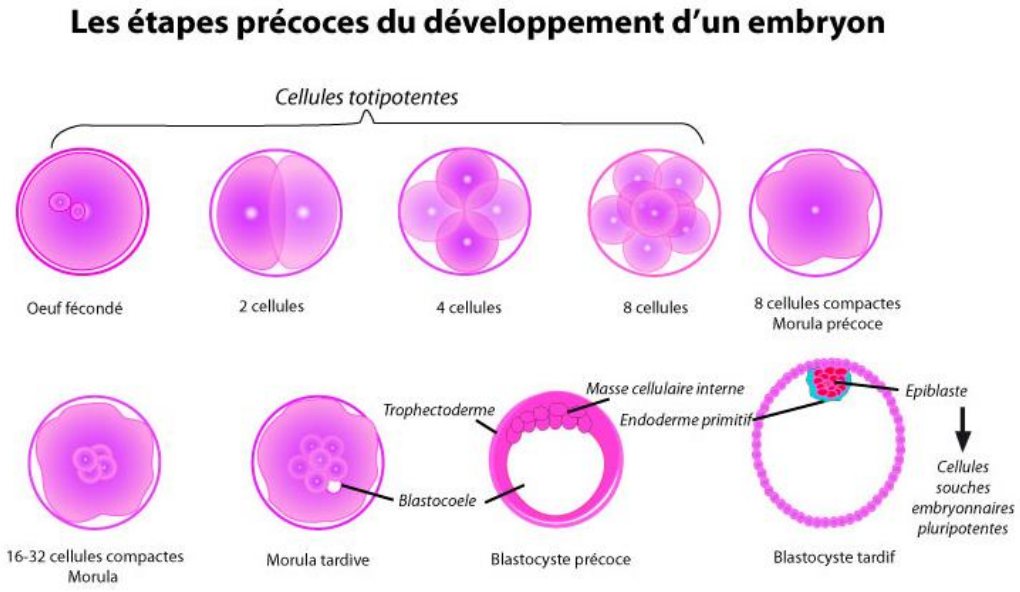

Figure 1.5. Développement embryonnaire (tiré, avec permission, de Démarchez (2011); https://biologiedelapeau.fr/spip.php?article60).

Les premières étapes du développement de l'embryon sont caractérisées par l'absence de transcription génique (Jeanblanc et al., 2008). Dans le but de subvenir aux besoins de l'embryon, le gamète femelle crée, pendant sa croissance, une réserve d'ARNm et de protéines dans laquelle l'embryon puise jusqu'à l'activation de son propre génome (Jeanblanc et al., 2008; Wassarman and Kinloch, 1992). L'embryon a alors besoin de ces transcrits puisque ceux-ci codent, entre autres, pour des facteurs impliqués dans la transcription, le remodelage de la chromatine, les modifications épigénétiques et dans les fonctions nécessaires au développement embryonnaire précoce (Li et al., 2010). Les ARN maternels seront également impliqués dans l'activation du génome embryonnaire (Wassarman and Kinloch, 1992). Étant donné le fait que l'embryon soit dépendant de l'ovocyte avant de pouvoir synthétiser par lui-même les éléments dont il

a besoin pour survivre et croître, il va de soi que tout mauvais fonctionnement ou dérangement dans la maturation ou le développement du gamète femelle pourra potentiellement affecter le développement de l'embryon.

2.2 Activation du génome embryonnaire

Les transcrits maternels qui supportent le développement embryonnaire précoce sont synthétisés et stockés dans l'ovocyte durant sa croissance (Wassarman and Kinloch, 1992). Le génome de l'embryon est d'abord mis en place grâce à la contribution de l'ovocyte et du spermatozoïde, puis activé au stade 8 à 16 cellules chez le bovin (Saint-Dizier, 2014). À ce moment, la synthèse protéique est assurée par l'expression de gènes dans les noyaux des cellules embryonnaires. Cette transcription débute suite à l'activation de l'ARN polymérase II, induite par une cascade de phosphorylation et de déphosphorylation, initiée par un programme qui débute à la fécondation (Memili and First, 1998).

Les changements épigénétiques tels la méthylation de l'ADN (Daxinger and Whitelaw, 2012), la modification des histones (méthylation, acétylation...) et le remodelage de la chromatine entrent alors en jeu de façon à forger l'épigénome de l'embryon. Ceux-ci seront présentés plus en détails dans la Partie III de cette présente revue. Suite à la fécondation, les chromosomes maternels et paternels subiront un remodelage important qui conduira à des différences marquées entre les pronoyaux (Wang et al., 2014). L'ovule a la capacité de reprogrammer le génome mâle et ce, en plus du sien. Ce dernier subira donc une déméthylation active alors que l'ADN femelle sera déméthylé plus progressivement (Wang et al., 2014). La re-méthylation du génome embryonnaire se fait de façon plus progressive et ce, au fil des divisions cellulaires, de sorte que l'épigénome soit progressivement établi (Jeanblanc et al., 2008). À ce moment, l'embryon est particulièrement sensible aux conditions physiologiques et environnementales (Cagnone and Sirard, 2013; Gad et al., 2012).

L'activation du génome embryonnaire s'établit de façon progressive et est caractérisée par deux phases distinctes: la phase mineure et la phase majeure (Memili et al., 1998; Memili and First, 1999; Jeanblanc et al., 2008). La phase mineure de l'activation du

génome embryonnaire débute à la phase S du premier cycle cellulaire et présente une activité réduite. Quant à elle, la deuxième phase de l'activation du génome de l'embryon, la phase majeure, est caractérisée par une augmentation rapide de la synthèse des transcrits (Nothias et al. 1995). À ce moment, l'ARNm et les protéines qui sont formées par l'embryon sont utilisées et indispensables à la croissance et au développement de l'embryon (Memili et al., 1998; Memili and First, 1999). Il est ainsi possible de constater une baisse de la disponibilité des transcrits maternels accompagnée d'une hausse de la synthèse d'ARNm par l'embryon, ce phénomène étant décrit comme la transition maternelle-embryonnaire (Barnes and First, 1991; Memili et al., 1998; Vigneault et al., 2004).

D'autres évènements critiques auront lieu entre les stades 4 et 8 cellules, entre le stade 16 cellules et le stade morula et, finalement, entre les stades morula et blastocyste - il sera finalement possible de noter une diminution graduelle de la disponibilité des transcrits maternels (qui sont présents jusqu'au stade blastocyste, mais en faible quantité à ce moment) et une augmentation de la synthèse de transcrits par l'embryon. La Figure 1.6 présente bien cette situation. De plus, au fur et à mesure de la progression vers le stade blastocyste, le nombre et la fonction des gènes transcrits favorisent davantage le développement de l'embryon, permettant successivement la transcription, la traduction et, éventuellement, la différentiation et la prolifération cellulaire (Saint-Dizier, 2014).

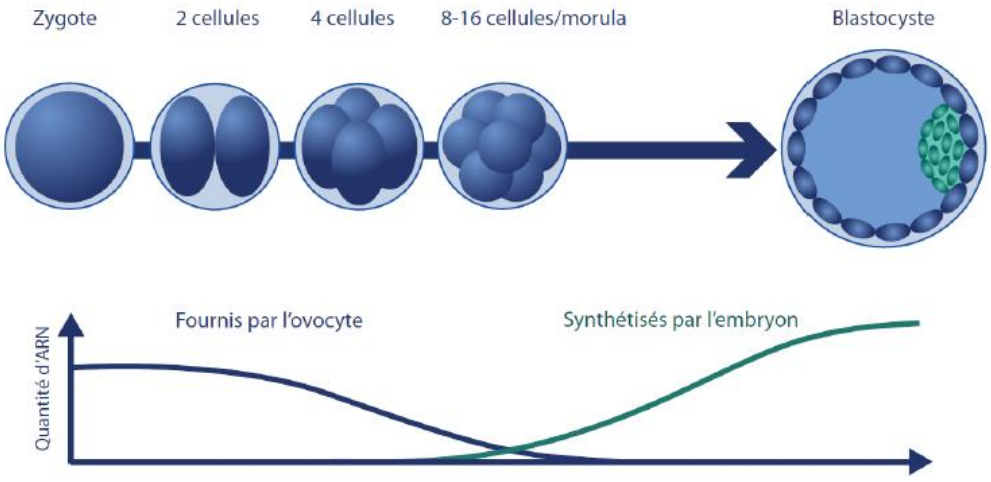

Figure 1.6. Transition materno-foetale (tiré, avec permission, de Cagnone (2013)).

L'activation du génome de l'embryon conduira à l'expression génique des cellules totipotentes. Ainsi, il est possible de constater qu'au stade blastocyste, il existe des différences de nature épigénétique entre les cellules de la masse cellulaire interne et celles du trophectoderme, alors que les premières présentent des marques plutôt répressives (Reik et al., 2003). Le développement d'un individu entier sera possible grâce à la mise en place de la formation des tissus embryonnaires et extra-embryonnaires. La pluripotence du bouton embryonnaire permet à l'embryon de se différentier et former les trois tissus embryonnaires, soient l'ectoderme, le mésoderme et l'endoderme (Reik et al., 2003).

Partie III

3.1 Principes génétiques

La séquence d'ADN est spécifique à chaque individu et code pour l'expression de milliers de gènes (près de 22 000 chez le bovin domestique, *Bos taurus* (Burt, 2009)), de façon à assurer le bon développement et toutes les fonctions physiologiques de l'organisme. La séquence d'ADN est la même pour toutes les cellules d'un même organisme. La régulation de l'expression génique est donc finement orchestrée et plusieurs mécanismes interviennent pour y jouer un rôle clé (Griffiths J.F., 2010).

3.1.1 La chromatine et l'expression génique

La chromatine désigne la structure complexe formée de l'ADN et de protéines, dans le noyau de chaque cellule d'un organisme eucaryote (Griffiths J.F., 2010) qui supporte l'information génétique (Huret, 2015). En effet, étant donné la longueur importante de la chaîne d'ADN, il est essentiel que celle-ci soit repliée et compactée, de façon à ce que toute l'information génétique puisse être contenue dans le noyau. La chromatine forme donc les chromosomes et est connue pour son implication dans l'expression des gènes. La Figure 1.7 illustre l'enroulement, puis la compaction de l'ADN pour former le chromosome. De façon à bien comprendre l'implication et l'efficacité de cette structure dans l'expression génique, il importe de connaître les principaux éléments qui la constituent.

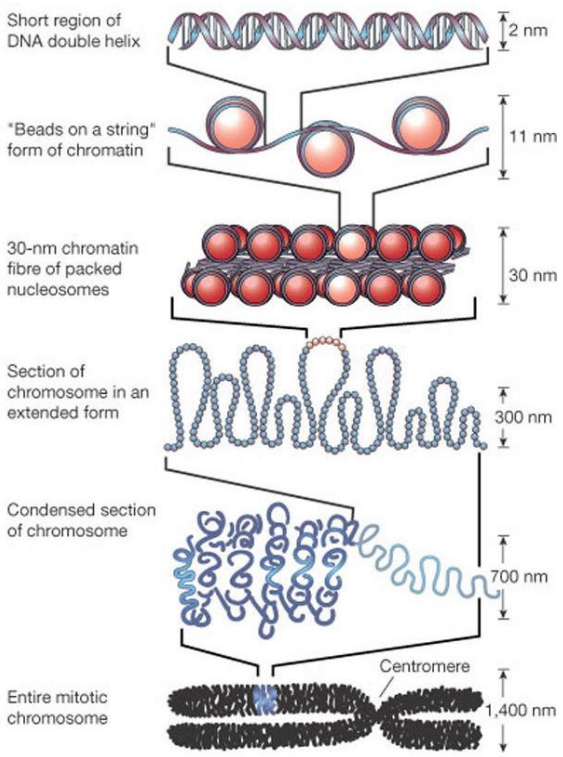

Figure 1.7. Compaction de l'ADN (*tiré, avec permission, de Macmillan Publishers Ltd: [Nature Publishing Group], (Felsenfeld and Groudine, 2003), copyright 2003*).

Tout d'abord, le nucléosome est considéré comme étant l'unité de base de la chromatine et est formé d'histones autour desquelles est enroulé l'ADN génomique (Huret, 2015). Il s'agit du premier niveau de compaction de l'ADN. Il existe quatre histones fondamentales, connues comme étant les histones H2A, H2B, H3 et H4, celles-ci étant considérées comme les principaux constituants protéiques de la chromatine (Cutter and Hayes, 2015). Au sein des nucléosomes, on retrouve deux copies de chaque histone fondamentale, formant un octamère autour duquel s'enroule une séquence de près de 147 paires de bases d'ADN (Cutter and Hayes, 2015). Cette configuration rend le nucléosome plus résistant à l'action de nucléases, entre autres, et sa structure dynamique permet l'interaction entre les histones et l'ADN et entre les histones adjacentes de façon à réguler l'accessibilité à l'ADN et ainsi, la transcription des gènes (Huret, 2015).

Les histones possèdent toutes une queue N-terminale qui est caractérisée par sa richesse en résidus lysine et arginine (Turner, 2001). Cette portion de l'histone est donc chargée positivement, ce qui favorise son contact étroit avec l'ADN qui, elle, porte une charge négative (groupements phosphate) ou encore avec le corps de l'histone voisine, qui est lui aussi chargé négativement (Turner, 2001). Bien qu'elles ne forment pas le cœur du nucléosome, les histones H1 et H5 sont également impliquées dans la formation de celui-ci. En effet, elles assurent la liaison des différents constituants, en plus de favoriser le rapprochement des nucléosomes (Huret, 2015; Robinson, 2006). Les nucléosomes sont disposés les uns à la suite des autres, donnant à l'ensemble histone/ADN l'apparence d'un collier de perles (Huret, 2015).

La chromatine peut être transcriptionnellement inactive ou active. Selon le cas, la conformation qu'elle adoptera sera différente. En effet, elle sera désignée sous l'appellation d'euchromatine si elle est sous forme ouverte, ou décondensée et ce, pendant l'interphase de la division cellulaire (Huret, 2015). L'euchromatine est retrouvée principalement dans le nucléoplasme de la cellule (Huret, 2015). Les fibres constituants l'euchromatine ont à ce moment une épaisseur de près de 11 nm (Zhang and Reinberg, 2001). Certaines marques dites épigénétiques sont alors présentes, touchant l'ADN et/ou les histones, et elles sont responsables de cet état de décompaction (Wu, 2010). Il sera ainsi possible pour la machinerie transcriptionnelle d'exercer ses fonctions et ainsi conduire à l'expression des gènes (Griffiths J.F., 2010). Le concept d'épigénétique sera présenté plus en détails dans la section 2.2.

On parle d'hétérochromatine lorsque la chromatine est condensée et compacte, rendant l'expression des gènes impossible, en raison d'un encombrement de macromolécules rendant les séquences codantes inaccessibles à la machinerie transcriptionnelle (Tartof, 1989). L'hétérochromatine est localisée autour du noyau et son état de compaction est maintenu tout au long du cycle cellulaire (Huret, 2015). Les fibres constituant l'hétérochromatine ont alors 30 nm d'épaisseur (Zhang and Reinberg, 2001). Cette forme de chromatine peut être constitutive ou encore facultative (Brown, 1966).

L'hétérochromatine constitutive présente une configuration très compactée et les séquences qu'elle contient ne codent que pour très peu de gènes (Huret, 2015). Les télomères et les centromères sont deux exemples de régions prises en charge par l'hétérochromatine (Huret, 2015; Yasmineh, 1970). Pour sa part, l'hétérochromatine facultative contient des régions codantes et, contrairement à l'hétérochromatine constitutive, elle est variante d'une cellule à l'autre tout en dépendant du développement embryonnaire (Brown, 1966). Un exemple bien documenté de ce mécanisme est celui du corpuscule de Barr, produit suite à l'inactivation d'un des deux chromosomes X chez la femelle, qui est maintenu dans un état hétérochromatique (Huret, 2015).

3.2 Épigénétique

Le concept d'épigénétique fait référence à l'ensemble des facteurs qui influencent l'expression des gènes d'un individu, sans modifier la séquence des nucléotides qui composent son code génétique. À ce jour, plusieurs éléments de la littérature soutiennent que les modifications épigénétiques sont héritables et surviennent dans la première semaine suivant l'union des gamètes, au cours de laquelle l'embryon en début de développement y est particulièrement sensible (Wang et al., 2014). À ce moment, les changements dans la méthylation de l'ADN (méthylation et déméthylation) sont connus pour influencer le développement et la santé du futur individu, en plus d'influencer le phénotype de ses descendants (Evertts et al., 2010; Turek-Plewa and Jagodzinski, 2005).

Comme la séquence d'ADN est identique pour toutes les cellules d'un même individu, les fonctions spécifiques à chaque cellule appartenant à un tissu sont dictées par l'activation de certains gènes et par l'inactivation ou la répression des autres (Turek-Plewa and Jagodzinski, 2005). Ainsi, le génome de toutes les cellules d'un même individu est le même; par contre, l'épigénome de chaque type cellulaire est différent, reflétant sa fonction au sein d'un tissu particulier (Turek-Plewa and Jagodzinski, 2005). Parmi les marques épigénétiques les plus connues et les plus documentées, on retrouve la méthylation de l'ADN et les modifications post-transcriptionnelles des histones. Ces marques n'affectent pas la séquence des

nucléotides qui composent l'ADN, mais peuvent influencer l'expression génique et le phénotype de l'individu. C'est donc l'établissement de ces marques épigénétiques qui permettent la différentiation cellulaire (Evertts et al., 2010; Turek-Plewa and Jagodzinski, 2005).

3.2.1 Méthylation de l'ADN

La méthylation de l'ADN consiste en un transfert d'un groupement méthyl à partir du composé S-adenosylmethionine (SAM) vers un nucléotide de l'ADN, la cytosine, tel que présenté à la Figure 1.8 (Yang et al., 2004). Cette modification affecte principalement les cytosines localisées dans les complexes CpG (dinucléotides); en effet, Ng et Bird (1999), tel que cité par Turek-Plewa and Jagodzinski (2005), rapportent que 60 à 90% des cytosines retrouvées dans les dinucléotides CpG sont méthylées chez les mammifères. Les ilots CpG sont connus comme étant des fragments d'ADN dans lesquels on retrouve une concentration importante (60 à 70%) de bases C et G (Turek-Plewa and Jagodzinski, 2005), et ceux-ci sont particulièrement abondants au sein des séquences promotrices (Evertts et al., 2010). Les endroits affectés par la méthylation sont alors nommés *régions différentiellement méthylées* (DMRs).

La méthylation de l'ADN est essentielle pour le bon déroulement du processus d'embryogenèse et est marquée par son action à la position 5 des cytosines, formant le composé 5-methylcytosine (5mC) (Denis et al., 2011; Wu and Zhang, 2012). Cette modification épigénétique est également essentielle pour l'établissement de l'empreinte génomique, le maintien de la pluripotence, l'inactivation du chromosome X chez la femelle et le maintien de l'intégrité du génome (Bird, 2002).

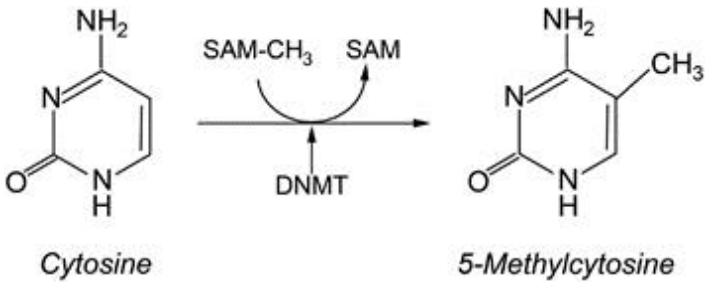

Figure 1.8. Transfert du groupement méthyl de SAM vers une cytosine, formant 5-methylcytosine (*tiré, avec permission, de Macmillan Publishers Ltd: [Heredity], (Gibney and Nolan, 2010), copyright 2010*).

Chez les mammifères, les patrons de méthylation sont établis au moment de la différenciation des cellules germinales et au cours de la période préimplantatoire des embryons (Denis et al., 2011; Wu and Zhang, 2012). Ces périodes sont caractérisées par d'importantes vagues de déméthylation et de re-méthylation jusqu'à l'établissement d'un patron stable (Denis et al., 2011; Wu and Zhang, 2012). Ce processus pousse la chromatine à adopter la forme d'euchromatine, la rendant transcriptionellement active (Turek-Plewa and Jagodzinski, 2005). Il est important de considérer qu'une faible méthylation correspond généralement à une forte expression du gène alors qu'une séquence hautement méthylée verra les gènes qu'elle code inactivés (Lev Maor et al., 2015).

En plus d'être essentielle à la régulation de la transcription des gènes et à la modification de la structure de la chromatine, la méthylation de l'ADN est également impliquée dans le développement embryonnaire et le marquage des gènes à empreinte. Elle est également importante pour le maintien des séquences codantes et prévient les dommages à l'ADN (Robertson, 2005). La méthylation anormale de l'ADN, résultant en une hyperméthylation ou hypométhylation de séquences clés peut causer l'apparition de maladies auto-immunes, de certains syndromes ou encore du cancer (Denis et al., 2011; Lucifero et al., 2007; Turek-Plewa and Jagodzinski, 2005).

3.2.2 Les DNMTs et leurs rôles

Les enzymes *DNA methyltransferases* (DNMTs) sont connues pour l'importance de leur rôle dans la programmation épigénétique et elles sont impliquées dans le bon déroulement du processus de méthylation de l'ADN (Denis et al., 2011). En établissant et en maintenant les patrons de méthylation aux endroits spécifiques dans le génome, elles contribuent ainsi au remodelage de la chromatine et à la régulation de l'expression génique au tout début du développement de l'embryon (Denis et al., 2011). Les DNMTs sont les seules enzymes connues actuellement permettant le transfert du groupement méthyl du SAM vers un nucléotide, soit la cytosine (Goll and Bestor, 2005). Chez les mammifères, trois méthyltransférases sont particulièrement importantes: DNMT1, DNMT3A et DNMT3B (Turek-Plewa and Jagodzinski, 2005). Celles-ci appartiennent à l'une ou l'autre de deux catégories, soient les DNMTs *de novo* (DNMT3A et DNMT3B) et les DNMTs de maintenance (DNMT1). Les DNMTs *de novo* permettent la méthylation des cytosines en 5mc après le processus de réplication et ce, à partir d'ADN non méthylé (Turek-Plewa and Jagodzinski, 2005). Pour leur part, les DNMTs de maintenance sont situées au niveau de la fourche de réplication et assurent la liaison du groupement méthyl à l'ADN hémiméthylé au moment de la réplication (Turek-Plewa and Jagodzinski, 2005). DNMT1 est responsable du maintien du patron de méthylation pour toute la durée de la vie cellulaire et de sa transmission d'une génération cellulaire à l'autre (Evertts et al., 2010; Turek-Plewa and Jagodzinski, 2005). DNMT3L est une protéine régulatrice qui agit de pair avec les autres DNMTs, mais qui ne possède, pour sa part, aucune activité enzymatique (Denis et al., 2011; Turek-Plewa and Jagodzinski, 2005).

La Figure 1.9 présente la structure des DNMTs chez les mammifères. Ceux-ci seront présentés plus en détails dans les prochaines lignes.

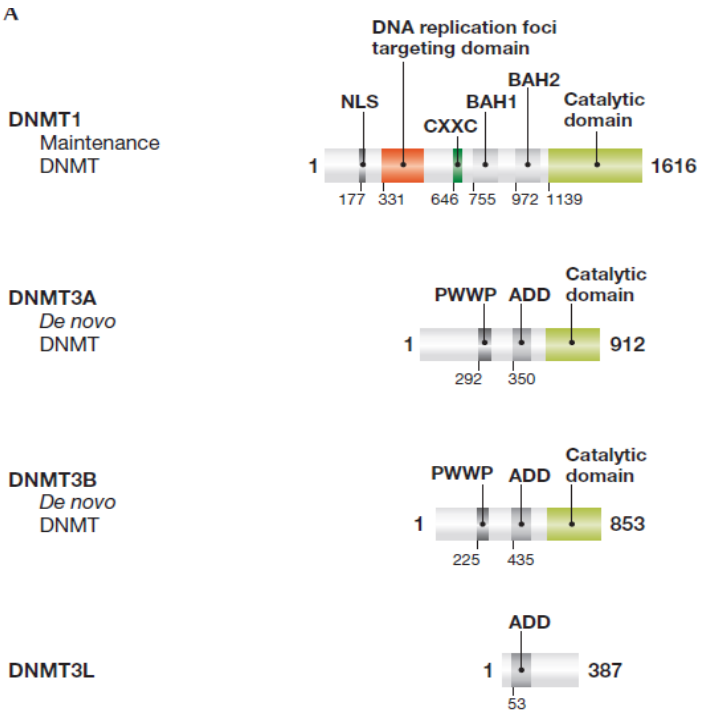

Figure 1.9: Structure des DNMTs chez les mammifères (*tiré, avec permission, de John Wiley and Sons: [EMBO Reports] (Denis et al. 2011), copyright 2011*).

Le domaine N de DNMT1 permet également la reconnaissance et la liaison de l'enzyme à d'autres protéines. L'expression de cette méthyltransférase est gérée par des promoteurs liés au sexe de sorte que, chez l'ovocyte, il est possible de retrouver une version tronquée de DNMT1, soit le transcrit DNMT1o (Kurihara et al., 2008). Cette dernière est observée dans le cytoplasme de l'ovocyte au cours de son développement et dans le cytoplasme des premières cellules l'embryon, avant l'implantation de ce dernier (Kurihara et al., 2008). DNMT1s assure, pour sa part, le maintien des patrons de méthylation chez l'embryon. Cette DNMT est d'abord associée au pronoyau maternel, puis éventuellement, au pronoyau paternel (Kurihara et al. 2008).

DNMT3 permet l'acquisition *de novo* des patrons de méthylation, pendant l'embryogenèse principalement. Cette méthyltransférase est également active au sein des cellules souches et des cellules germinales en développement, soit aux moments où les patrons de méthylation sont établis (Lucifero et al., 2007). Contrairement à

DNMT1, ces méthyltransférases DNMT3 ne démontrent aucune affinité particulière pour les substrats hémiméthylés (Lucifero et al., 2007). DNMT3A présente une préférence pour les sites de méthylation qui sont entourés de pyrimidines et peut aussi bien méthyler les régions riches en dinucléotides CpA et CpT, que les ilots CpG (Turek-Plewa and Jagodzinski, 2005). Pour sa part, DNMT3B joue un rôle dans la formation de tumeurs et est particulièrement impliqué dans la mise en place de méthylations des ilots CpG des séquences répétées au sein des régions péricentriques des chromosomes (Turek-Plewa and Jagodzinski, 2005).

3.2.3 Modifications post-traductionnelles des histones

Les queues N-terminales des histones sont la cible des modifications post-traductionnelles de la chromatine (Huret, 2015). Ces dernières peuvent ainsi affecter "leurs charges, mais aussi l'accessibilité à l'ADN et les interactions protéines/protéines avec le nucléosome" (Huret, 2015). Parmi les modifications post-traductionnelles les plus étudiées, il est possible de noter l'acétylation et la méthylation des histones. La phosphorylation, l'ubiquitination et la SUMOylation sont également observées (Denis et al., 2011), mais ne seront cependant pas détaillées dans la présente revue. Si plus d'une modification est apportée aux histones d'une même séquence, on parle alors de l'établissement d'un code d'histones (Figure 1.10), et il va sans dire que ces modifications auront ainsi un impact sur l'expression génique (Cheung et al., 2000).

Il est maintenant connu que les histones peuvent être méthylées. Cette méthylation permettrait d'ailleurs la formation de l'hétérochromatine via l'action de différentes méthyltransférases sur des histones spécifiques, favorisant l'enroulement de l'ADN autour des histones et influençant ainsi l'expression de certains gènes et les réponses biologiques qui en découlent (Zhang and Reinberg, 2001). La méthylation affecte les lysines et les arginines des histones et permet généralement la mise en place de marques très stables (Zhang and Reinberg, 2001). De plus, "étant donné la diversité des modifications possibles sur une seule histone, le nombre de combinaisons potentielles sur un nucléosome composé d'un octamère d'histones est extrêmement élevé" (Nicolas, 2001). Le processus de méthylation des histones s'apparente à celui

de l'ADN, impliquant le SAM comme donneur de groupement méthyle (Rivera and Ross, 2013). Jusqu'à 3 groupements méthyles peuvent être transférés sur chaque résidu lysine alors que seulement deux sont permis sur les résidus arginines (Rivera and Ross, 2013). Alors que la méthylation des histones est souvent associée à la mise en place de marques répressives, il arrive que ce ne soit pas toujours le cas (Rivera and Ross, 2013). Aussi, il est important de noter que les histones méthylées et non méthylées ne sont pas très différentes les unes des autres d'un point de vue biochimique puisque la méthylation n'affecte pas la charge globale de la protéine (Nicolas, 2001).

Tout comme c'était le cas pour la méthylation de l'ADN, certaines méthyltransférases catalysent le processus de méthylation chez histones. Parmi les méthyltransférases les plus connues, on retrouve PRMT4, PRMT1, PRMT5, Suv39H1 et Suv39H2. PRMT4 agit comme co-activateur de la transcription des récepteurs nucléaires et méthyle spécifiquement l'arginine 17 de l'histone H3 (van Holde, 1989). PRMT1, pour sa part, méthyle l'arginine des protéines qui ont comme fonction de lier l'ARN alors qu'il a été démontré, *in vitro*, que PRMT5 méthyle les histones H4 et H2A (Pahlich et al., 2006; Bedford et al., 2009; Burgos et al., 2015). Finalement, Suv39H1 et Suv39H2 (liées à Su(vas)3-9) interviennent dans le *silencing* transcriptionnel, processus associé à l'hétérochromatine (Garcia-Cao et al., 2004). De plus, Suv39H1 est connu pour méthyler spécifiquement la lysine 9 de l'histone H3, trahissant l'importance de son implication dans la formation de l'hétérochromatine (Lehnertz et al., 2003). Ces histones méthyltransférases comptent parmi les seules dont le rôle a été rapporté tant *in vitro* que *in vivo* (Nicolas, 2001).

Figure 1.10 Histones sujettes aux modifications post-traductionnelles chez les mammifères (tiré de Lacoste et Côté, 2003, permission obtenue via *Érudit - https://www.erudit.org/fr/*)

La méthylation de la lysine 9 de l'histone H3 a attiré l'attention de différents groupes de chercheurs puisqu'elle met en place une marque de répression génique. En effet, la protéine HP1, qui jour un rôle important dans la mise en place de la chromatine, présente une affinité particulière pour ce site de méthylation (Shimada and Murakami, 2010). Cette protéine est d'ailleurs recrutée sur le nucléosome suite à la méthylation de la lysine 9 de H3 par Suv39H1 chez l'humain (D'Alessio and Szyf 2006). En effet, HP1 est en mesure de reconnaître la forme méthylée de l'histone H3, celle-ci devenant vraisemblablement le site de liaison de HP1 sur l'hétérochromatine. Cette liaison favoriserait la mise en place du *silencing* de la transcription génique (Bannister et al., 2001; O'Carroll et al., 2000). Shimada and Murakami (2010) rapportent que la phosphorylation de HP1 cause un changement dans la conformation de la chromatine de façon à ce que celle-ci inhibe l'expression génique.

Une autre modification post-transcriptionnelle bien connue est l'acétylation des histones. Le mécanisme permettant la mise en place de cette modification implique les histone acétyltransférases (HAT) et les histone désacétylases (HDAC), celles-ci

étant des co-activateurs et des co-répresseurs de la transcription, respectivement (Nicolas, 2001).

L'acétylation de la queue N-terminale est possible grâce au transfert de groupements acétyles en provenance de l'acétyle coenzyme A vers le résidu lysine de l'histone cible (Cheung et al., 2000). Ceci a pour but de neutraliser la charge positive de l'histone auquel le groupement acétyle s'associe, forçant un changement de conformation de la chromatine. Celle-ci s'ouvre pour laisser place à la machinerie transcriptionnelle qui, elle, permettra l'expression génique (Cheung et al., 2000). Jusqu'à 4 groupements acétyles peuvent être transférés sur chaque résidu lysine des histones et chez les mammifères, il est possible d'observer qu'il y a acétylation des histones H2A (lysines 5 et 9), H2B (K5, K12, K15 et K20), H3(K9, K14, K18 et K23) et H4 (K5, K8, K12 et K16) (Turner, 2001).

La désacétylation, pour sa part, cause un repliement et une condensation de la chromatine, rendant impossible la transcription génique (Cheung et al., 2000). La charge positive de l'histone est ainsi rétablie. La désacétylation fait appel aux HDACs, ceux-ci étant en mesure de détacher le groupement acétyle des lysines de l'histone (Cheung et al., 2000). En plus d'être impliquées dans le contrôle de l'expression génique, les HDACs joueraient également un rôle dans divers processus biologiques incluant la prolifération cellulaire, l'apoptose, l'inflammation et la carcinogenèse (Chen et al., 2015). À ce jour, quatre catégories de désacétylases ont été identifiées; les 18 HDACs maintenant connues sont ainsi classées selon les caractéristiques que présentent leurs domaines catalytiques. HDAC1, HDAC2, HDAC3 et HDAC8, appartenant à la classe 1, agissent au sein de toutes les cellules de l'organisme alors que les autres HDACs sont retrouvées chez certains tissus, de manière plus spécifique (Chen et al., 2015).

3.2.4 Déméthylation

L'épigénome est modelable (Wu and Zhang, 2012). Ainsi, malgré le fait que les marques associées à la méthylation soient considérées comme particulièrement stables, la déméthylation de l'ADN parvient à retirer, voire effacer ces dernières (Wu

and Zhang, 2012). Les déméthylases agissent principalement au moment des deux principales vagues de déméthylation, qui surviennent suite à la migration des cellules germinales vers les crêtes germinales et suite à la fécondation, supprimant une partie de l'information épigénétique en agissant sur la chromatine (Hill et al., 2014). Les patrons de méthylation portés par les génomes parentaux sont effacés de façon à ce que les cellules de leur descendant retrouvent leurs caractéristiques totipotentes (Reik and Walter, 2001). Parmi les marques de méthylation qui seront conservées, une partie correspondent aux *imprinting control regions* (ICRs). Ces régions contrôlent l'expression de gènes et peuvent être associées aux cellules germinales ou somatiques (Reik and Walter, 2001). Il est également important de savoir que le génome maternel est protégé de l'oxydation des groupements méthyles par le facteur Stella (Nakamura et al., 2007). Suite à la fécondation, Stella protège donc certains sites spécifiques du génome maternel, dont les ICRs gamétiques, à la déméthylation (Nakamura et al., 2007).

La déméthylation peut être soit passive, soit active (Figure 1.11) (Seisenberger et al., 2013; Smith and Meissner, 2013). Dans le premier cas, les groupements méthyles de l'ADN sont retirés alors que l'expression de DNMT1 est inhibée (Meissner et Walter, 2015). L'enzyme n'est ainsi plus en mesure d'ajouter des groupements méthyles sur les brins d'ADN nouvellement formés; ce type de déméthylation nécessite ainsi que l'inhibition de la méthyltransférase soit maintenue sur plus d'un cycle cellulaire (Hill et al., 2014; Wu, 2010).

Pour sa part, la déméthylation active de l'ADN est plus rapide et implique plusieurs mécanismes cellulaires. À ce jour, il a été démontré que cette déméthylation active implique, entre autres, certaines déaminases, les enzymes déméthylases BER (*base excision repair*) et TET (ten-eleven translocation), les protéines de liaison MBD (*methyl-CpG binding domain*) et quelques molécules jouant le rôle d'intermédiaires (Bhattacharya et al., 1999; Zhu et al., 2000; Morgan et al., 2004; Tahiliani et al., 2009; Popp et al., 2010; He et al., 2011; Ito et al., 2011).

Les déaminases impliquées dans le processus de déméthylation de l'ADN ont comme rôle de modifier les cytosines qui ont été préalablement méthylées. Parmi les

déaminases les plus connues, on retrouve Aicda (*activation-induced cytidine deaminase*). Aicda a été identifiée dans les cellules germinales, l'ovocyte et les tissus embryonnaires murins (Morgan et al., 2005). La déaminase joue un rôle dans le processus de déméthylation de l'ADN en modifiant 5mC en thymine, via le remplacement du groupement NH_2 par un atome d'oxygène (Hill et al., 2014). La thymine est par la suite reconnue par la glycosylase murine TDG (*thymine DNA glycosylase*) qui, elle se chargera de l'enlever (Morgan et al., 2005). Le même scénario a été observé chez le poisson-zèbre, mais impliquant, dans ce contexte MBD4 et un des enzyme BER (*base excision repair*) et NER (*nucleotide excision repair*) qui, elles, se chargeront de retirer la thymine. Celle-ci sera remplacée par une cytosine non méthylée (Hill et al., 2014; Meissner et al.). Cette combinaison d'enzymes ne semble pas être aussi active chez les mammifères (Page-Lariviere and Sirard, 2014).

Au cours des dernières années, l'implication des enzymes *ten-eleven translocation* (Tet1, Tet2 et Tet3) dans le processus de déméthylation a été démontrée. En effet, ces enzymes (désoxygénases) permettraient l'hydroxylation rapide de 5mC en 5-hydrométhylcytosine (5hmC), en 5-formylcytosine (5fc) et 5-carboxylcytosine (5caC) (Ito et al., 2010) grâce à leur domaine de liaison en doigts de zinc CXXC spécifique, essentiel pour l'interaction entre les enzymes et l'ADN (Hashimoto et al., 2010; Hill et al., 2014; Tahiliani et al., 2009). Tet3 est connu pour être davantage exprimé dans l'ovocyte et l'embryon préimplantatoire, par rapport à Tet1 et Tet2 (Gu et al., 2011). Le rôle majeur de Tet dans le processus de déméthylation est mis de l'avant puisqu'il a été démontré que les glycosylates sont incapables de reconnaître le produit de l'hydroxylation (He et al., 2011). 5fc et 5caC sont, pour leur part, reconnues par la TGD, permettant la déméthylation active (He et al., 2011; Maiti and Drohat, 2011). L'hydroxylation empêche DNMT1 de se lier au site de méthylation, inhibant l'action de l'enzyme et pourrait ainsi davantage jouer un rôle dans la déméthylation passive (Tahiliani et al., 2009). De plus, Tet3 serait incapable de se lier aux régions protégées par Stella, laissant croire que cet enzyme ne serait pas en mesure d'oxyder les ICRs dans le génome maternel (Nakamura et al., 2007). Par ailleurs, Page-Lariviere and

Sirard (2014) ont récemment démontré qu'Aicda et Tet1 ne seraient pas impliqués dans le processus de déméthylation de l'ADN, suite à la fécondation.

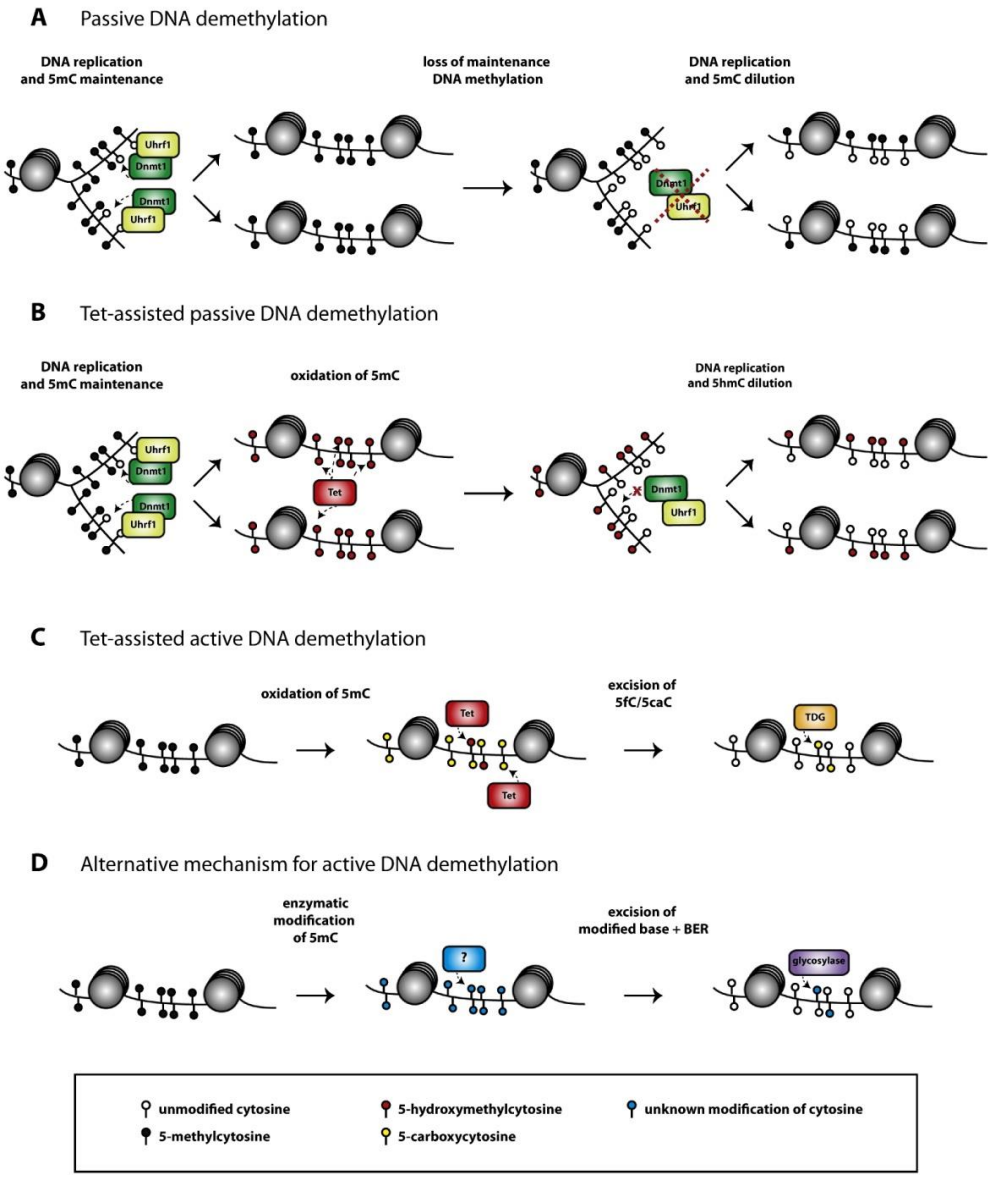

Figure 1.11. Mécanismes de déméthylation de l'ADN. (A) Déméthylation passive: L'inhibition de DNMT1 lors de la réplication de l'ADN provoque la déméthylation de l'ADN au bout de quelques cycles cellulaires; (B) Déméthylation passive impliquant Tet: la conversion de 5mC en 5hmC affecte l'activité de DNMT1; (C) Déméthylation active impliquant Tet: 5hmC peut être converti en 5fC et 5caC, composés qui sont par la suite hydroxylés; (D) mécanisme alternatif suggéré, impliquant les enzymes BER

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Le génome maternel est méthylé à 40% alors que le génome paternel l'est à 90% (Kobayashi et al., 2012). Ainsi, il a longtemps été soutenu que l'ADN maternel est déméthylé de façon passive alors que l'ADN paternel l'est activement, puisque les deux génomes doivent présenter des niveaux de méthylation équivalents au moment d'initier l'embryogenèse (Seisenberger et al., 2013). L'effacement est maintenu jusqu'à l'établissement, au cours de la gamétogénèse, des nouveaux patrons de méthylation liés au sexe (Fauque, 2013). Cependant, Wang et al. 2014 ont récemment démontré que le processus de déméthylation du génome maternel serait également actif. En effet, le groupe rapporte une disparition des 5-méthyl-cytosines, remplacées par des 5-hydrométhylcytosine, trahissant l'implication de TET (Wang et al., 2014).

3.2.5 Épigénétique trans-générationnelle

Les modifications épigénétiques peuvent être transmises d'une cellule mère aux cellules filles, influençant potentiellement le phénotype de ces dernières. Le mécanisme par lequel cette information est transférée d'une génération à l'autre n'est pas encore bien connu, mais impliquerait les gamètes et pourrait être affecté par l'environnement ou certaines conditions physiologiques (Daxinger and Whitelaw, 2012; Tarry-Adkins and Ozanne, 2011). Le principe repose sur le fait qu'une modification épigénétique causée par exposition environnementale à un paramètre d'influence, par exemple, subie par une femelle gestante (F0) affectera l'embryon qu'elle porte et qui se développe (F1) (Aiken and Ozanne, 2014; Jirtle and Skinner, 2007; Skinner, 2008). Étant donné que le développement des gamètes est initié tôt dans le processus de développement embryonnaire, leur différentiation et leur épigénome seront potentiellement affectés par cette même exposition environnementale. Dans ce cas, c'est l'individu de la génération suivante (F2) qui en subira les conséquences (Aiken and Ozanne, 2014; Jirtle and Skinner, 2007; Skinner, 2008). L'exemple le plus rapporté dans la littérature est celui du diabète de grossesse, où l'individu F1 ou F2 subit les conséquences d'une exposition maternelle à un

environnement métabolique défavorable (Gauguier et al., 1990). Une revue détaillée a récemment été publiée par Aiken and Ozanne (2014), faisant état des connaissances actuelles en lien avec la programmation développementale trans-générationnelle.

Quelques mécanismes selon lesquels la mère peut influencer le phénotype de ses descendants ont été proposés. Tout d'abord, la femelle transmet la moitié du bagage génétique ainsi que les marques épigénétiques y étant associées à sa descendance (Drake et al., 2005). L'altération de l'épigénome peut ainsi avoir des répercussions sur l'individu de la génération suivante (Drake et al., 2005). En plus des modifications apportées à l'épigénome via la méthylation de l'ADN et les modifications post-transcriptionnelles des histones, il a récemment été démontré que certains éléments répétés et transposables liés à l'ARN pourraient également être impliqués dans le processus de transmission épigénétique (Daxinger and Whitelaw, 2012).

Il a également été démontré que le comportement maternel post-natal pouvait influencer le patron épigénétique du nouveau-né. Le phénotype n'est ainsi pas directement transmis à la descendance, mais bien reprogrammé en réponse à un stimuli comportemental, par exemple, conservé d'une génération à l'autre (Champagne et al., 2006; Weaver et al., 2004). Il a également été démontré que le développement de la génération F2 peut-être influencé par un environnement utérin sous-optimal mis en place par l'individu F1 programmé (Aiken and Ozanne, 2014; Leese et al., 2008).

La transmission de l'information épigénétique d'une génération à l'autre peut également être assurée par l'intermédiaire des constituants du cytoplasme, plus particulièrement de la mitochondrie (Knudsen and Green, 2004). En effet, on sait que l'ADN mitochondrial (ADNmt) est transmis à la descendance via la lignée maternelle (Cummins, 2002). Or, il est maintenant aussi connu que la mitochondrie s'adapte en réponse à l'environnement métabolique, alors que le nombre de copies d'ADNmt varie selon l'importance de l'exposition à un stress oxydatif (Aiken et al., 2008). La reprogrammation précoce de la fonction mitochondriale pourrait ainsi avoir un impact sur le développement.

3.2.6 Empreinte parentale

Chez les mammifères, il est maintenant bien connu que l'union de deux génomes (maternel et paternel) permet le développement d'un embryon et éventuellement de l'individu. Ainsi, chacun des deux gamètes apporte son bagage génétique caractéristique et ceux-ci jouent un rôle complémentaire, de façon à assurer le futur du nouvel individu (Gabory and Dandolo, 2005). La marque que porte chaque gamète est connue comme étant *l'empreinte parentale*. Les gènes à empreinte sont donc essentiels pour le développement embryonnaire normal (Peters, 2014) et leur dysfonction est connue pour être associée à divers syndromes et types de cancers (Lim and Maher, 2010).

Ainsi, l'expression de certains gènes sera dépendante de son origine parentale, puisque les allèles parentaux d'un même gène n'ont pas nécessairement le même niveau d'expression (Daxinger and Whitelaw, 2012). Par exemple, les gènes à empreinte paternelle favoriseraient la croissance du fœtus alors que l'expression de gènes à empreinte maternelle la ralentirait, limitant les ressources à sa descendance afin de ne pas compromettre la survie de la mère (Charalambous et al., 2003; Moore and Haig, 1991). Chez les mammifères, étant donné que les deux gamètes ne portent pas les mêmes marques et caractéristiques, les deux sont nécessaires au développement de l'embryon. La parthénogenèse n'est donc pas possible dans de telles circonstances (Barlow and Bartolomei, 2014). Les gènes sujets à empreinte sont exprimés de façon mono-allélique et sont souvent situés les uns près des autres, sous forme de *clusters* dans les différentes régions chromosomiques, formant les *imprinting control regions* (ICRs) (Ferguson-Smith, 2011). Actuellement, près de 150 gènes à empreinte parentale ont été identifiés chez les mammifères (Bartolomei and Ferguson-Smith, 2011), alors qu'une trentaine seulement sont identifiés chez le bovin (Tian, 2014) .

La méthylation de l'ADN joue un rôle crucial dans l'établissement de l'empreinte parentale, en régulant l'expression ou encore la répression des allèles. Ainsi, si un gène porte l'empreinte paternelle, l'allèle paternelle sera exprimée alors que l'allèle

maternel subira une méthylation de sa chromatine et sera inactivé (Turek-Plewa and Jagodzinski, 2005). Parmi les différents gènes à empreinte, H19 sera exprimé grâce à l'activation de l'allèle maternelle alors que IGF2 sera activé si l'allèle paternel n'est pas réprimé. Contrairement à ce qu'on pourrait penser, l'empreinte parentale ne constitue pas une forme de transmission épigénétique trans-générationnelle. En effet, puisque les allèles doivent être reprogrammées à chaque génération, leur mémoire épigénétique est perdue après chaque génération (Daxinger and Whitelaw, 2012).

Partie IV

Le progrès génétique animal peut se faire rapidement ou encore s'échelonner sur plusieurs années. Chez les bovins laitiers, il est possible d'observer que ce progrès génétique se produit à grande vitesse, en raison de l'intensité croissante de la pression de sélection, de l'augmentation de la précision de sélection et de la diminution de l'intervalle entre les générations (Bouamra-Mechemache et al., 2008).

Depuis quelques années, la génomique prend de plus en plus d'importance pour des raisons économiques. Jusqu'à tout dernièrement, un simple test d'ADN à la naissance d'un veau permettait de prédire, avec une précision de 65-75%, les performances futures de cet animal (Murray, 2012). Bien que ce ne soit pas pratique courante, cette technique a été remplacée récemment par la pratique de biopsies embryonnaires, permettant l'identification des meilleurs sujets au tout début du développement de l'embryon (Swanson et al., 2007). Comme de gros investissements monétaires et près de 5 ans sont nécessaires pour obtenir la fiche complète et fiable d'un taureau lors d'un programme d'épreuve régulier (CIAQ, 1991), l'utilisation des tests génomiques gagne en popularité puisque ceux-ci permettent l'identification, en bas âge, des futurs taureaux élites (Swanson et al., 2007).

En plus du fait que les valeurs génétiques soient plus précises que les moyennes de parents, la génomique permet de cibler les meilleurs jeunes animaux parmi les milliers qui sont testés (Ponsart et al., 2013). Ainsi, l'utilisation de ces derniers dans les programmes de sélection permet une amélioration rapide du potentiel génétique du troupeau, en réduisant en plus l'intervalle de sélection entre les générations (Lohuis 1995). Pour l'industrie laitière, le développement des technologies de reproduction assistée telles que la stimulation ovarienne, la production d'embryons *in vitro* et le transfert embryonnaire, combiné à une meilleure efficacité des outils génomiques, facilite maintenant l'accès à la génétique supérieure.

4.1 Super-ovulation

Il est maintenant possible de favoriser la production de plus d'un follicule dominant lors d'un même cycle œstral chez le bovin. En effet, un traitement impliquant l'administration de FSH permet la croissance de plusieurs follicules à la fois, chacun d'eux ayant le potentiel de compléter sa maturation et d'être ovulé (Adams, 1994; Bo and Mapletoft, 2014). Il est possible d'induire une synchronisation des vagues folliculaires avant la stimulation ovarienne, en éliminant l'effet du follicule dominant (Jia et al., 2014). Un traitement de GnRH avant le début du protocole de stimulation provoque l'ovulation, puis l'émergence d'une nouvelle vague de développement folliculaire, qui pourra être synchronisée avec le traitement hormonal (Kohrarn et al., 1998a; Kohram et al., 1998b). Cette technique permet de connaitre le stade de développement des follicules au moment du début de la stimulation (Massicotte, 2000).

Il est intéressant de produire des follicules relativement gros, car ceci facilite leur récupération (Armstrong et al., 1997). L'utilisation de la FSH permet ainsi de synchroniser le développement des follicules, d'homogénéiser les follicules et d'obtenir plus d'ovocytes (Armstrong et al., 1997). Une période de 48h de *coasting* suivant 3 jours de stimulation FSH s'avère nécessaire pour favoriser le développement d'un ovule de qualité (Nivet et al., 2012). Le traitement implique l'apport quotidien en FSH pour une durée de quelques jours, la FSH ayant une durée de vie relativement courte, soit de près de 5h *in vivo* (Monniaux et al., 1983). Alors que la FSH est utilisé pour les adultes, l'eCG et la PMSG ont été utilisées dans les protocoles de super-ovulation chez les jeunes femelles pré-pubères; l'eCG, notamment, possède des propriétés lui conférant des effets s'apparentant à ceux de la FSH et de la LH simultanément (Murphy et Martinuk, 1991). Cette dernière a cependant une plus longue demi-vie dans l'organisme (40 h - Dieleman et al. 1993), de sorte que, souvent, seulement une ou deux injections sont nécessaires pour une même efficacité de la procédure (Armstrong et al., 1997). Armstrong et al. (1997) ont démontré que le traitement (7 jours), puis le retrait de progestagènes favoriseraient

une augmentation du nombre de follicules disponibles pour aspiration chez les animaux péri-pubères.

Il importe de noter que la stimulation ovarienne génère des réponses variables d'un individu à l'autre et le nombre de follicules à récupérer n'est pas garanti (Massicotte, 2000). De plus, quelques risques sont associés à la manipulation hormonale, qui pourrait, dans certaines situations causer le cancer, entre autres (van den Hurk and Zhao, 2005).

Les ovocytes produits suite à la stimulation hormonale sont fécondés *in vivo* par insémination artificielle (chez les adultes) ou encore récupérés pour fécondation *in vitro*, puis transférés chez des vaches receveuses. Ces deux techniques seront décrites aux sections 4.2.1 et 4.2.2. La collecte des embryons (dans le premier cas) ou des ovocytes (dans le deuxième) ne nécessite pas de manœuvres chirurgicales invasives.

4.2 Biotechnologies de la reproduction

4.2.1 Transfert embryonnaire

Le transfert embryonnaire et la fécondation *in vitro* gagnent également en popularité auprès des éleveurs de bovins laitiers (George 2013). Ces technologies sont basées sur le même concept de base. Celui-ci consiste à produire, chez une vache élite, un grand nombre d'ovules, suite à un programme de super-ovulation modulé par traitement hormonal. Ces ovules peuvent être récoltés après avoir été fécondés par insémination artificielle (transfert embryonnaire), ou encore fécondés à l'extérieur de l'organisme (ou *in vitro*). Dans les deux cas, les embryons sont ensuite transférés chez des vaches receveuses, qui assureront leur développement pendant près de 9 mois. Cette technologie permet de disperser rapidement la génétique des femelles élites puisque plusieurs embryons peuvent être produits par une même vache alors que le développement de ceux-ci est assuré par les mères-porteuses. Elle permet également de soutenir la production laitière des femelles de génétique moins impressionnante, en les utilisant comme receveuses, sans que leurs caractéristiques génétiques soient transmises à la génération suivante.

Grâce au transfert embryonnaire, il est maintenant possible de produire des jumeaux identiques en divisant l'embryon en tout début de développement. Ces derniers seront, par la suite, transférés chez deux femelles receveuses. Ainsi, ces embryons sont identiques sur le plan génétique, mais leurs épigénomes peuvent être différents en raison d'une exposition environnementale et métabolique différente (Shojaei Saadi et al., 2016).

4.2.2 Fécondation *in vitro*

Le processus de fécondation *in vitro* implique la récupération d'ovocytes par ponction ovarienne puis leur fécondation à l'extérieur de l'organisme, soit dans des milieux de culture adaptés à la maturation, puis la fécondation et enfin au développement des embryons. Ces protocoles ont été développés par l'utilisation de gamètes femelles dites "d'abattoir", obtenues suite à la mort des animaux. Des techniques simplifiées de collecte d'ovocytes ont été mises au point au cours des dernières décennies, de sorte que l'utilisation de donneuses vivantes est maintenant pratique courante. Tel que mentionné, la ponction trans-vaginale guidée par ultrasonographie est maintenant une technique populaire et utilisée couramment (Armstrong et al., 1997; Fry et al., 1998). Une à deux collectes par semaine sont possibles (Bage et al., 2003; Goodhand et al., 1999). La collecte des ovocytes suivant un protocole de stimulation ovarienne aux gonadotrophines augmente la qualité des ovocytes et donc l'efficacité du protocole de fécondation *in vitro*. Il est cependant nécessaire qu'ils subissent une étape de maturation *in vitro* (IVM) avant d'être fécondés (IVF) (Gordon, 1994).

Vingt-quatre heures sont allouées à la maturation *in vitro* des ovocytes bovins. L'aspiration et le lavage des ovocytes permettent le retrait du gamète du milieu inhibiteur, ce qui favorise la reprise automatique de la méiose. De plus, comme les ARN messagers et les longs ARN non codants peuvent être transférés des cellules du cumulus vers l'ovocyte (Macaulay et al., 2014), une maturation *in vitro* réalisée en l'absence des cellules du cumulus entourant l'ovocyte ne permet pas à ce dernier l'atteinte d'une maturité cytoplasmique optimale (Auclair et al., 2013; Sirard et al., 1988).

Par la suite, la fécondation *in vitro* elle-même est caractérisée par l'exposition de l'ovocyte à des spermatozoïdes capacités. Les milieux de culture contiennent de l'héparine, qui est responsable d'assurer cette capacitation des gamètes mâles chez le bovin (Ball and Peters, 2004). Les embryons sont ensuite transférés lorsqu'ils atteignent le stade blastocyste. Ainsi, 7 à 8 jours d'incubation dans le milieu de culture sont nécessaires avant que l'embryon puisse être implanté chez la femelle receveuse (Ball and Peters, 2004).

L'utilisation de la fécondation *in vitro* chez le bovin procure certains avantages intéressants. Tout d'abord, la réussite de cette technique ne dépend pas nécessairement de la mise en place d'un protocole de super-ovulation. En effet, la fécondation *in vitro* d'un seul ovule est envisageable. Au besoin, une stimulation modérée s'avère efficace (Nivet et al., 2012). Il est cependant recommandé d'avoir recours à la super-ovulation lorsque vient temps de produire un nombre important d'embryons. La fécondation *in vitro* offre également une alternative aux propriétaires de vaches qui ne répondent peu ou pas aux programmes de super-ovulation. Cependant, la susceptibilité à la polyspermie est accrue en contexte artificiel par rapport à la fécondation *in vivo* (Mizushima and Fukui, 2001). Les travaux de Blondin et al. (2002), puis de Nivet et al. (2012) ont permis d'améliorer la préparation de l'ovocyte, de façon à augmenter le taux de viabilité des embryons produits *in vitro*. En effet, 30 à 40% des ovocytes pouvaient atteindre le stade de blastocyste comparativement à plus de 65% maintenant avec le *coasting*.

Plusieurs équipes de recherche se sont penchées sur l'étude de la composition du milieu de culture des embryons depuis Whitten (1957), à la recherche des caractéristiques du milieu de culture idéal (Goto et al., 1988; Lu et al., 1988; Takada et al., 1990). Cependant, il va sans dire que, malgré tous les efforts et les améliorations proposées, le milieu de culture des embryons ne présente pas tout à fait les mêmes caractéristiques que l'environnement tubaire. À ce sujet, il importe de savoir qu'à ce moment, dans le contexte *in vitro*, l'embryon est particulièrement sensible et vulnérable aux modifications pouvant avoir lieu dans son environnement. Tel que mentionné précédemment, tout stress vécu par l'embryon peut se refléter dans

l'expression de ses gènes (Cagnone and Sirard, 2013; Gad et al., 2012). Un bon milieu de culture doit être optimisé pour bien répondre aux besoins métaboliques de l'embryon, en lui fournissant un approvisionnement approprié en eau, en oxygène (5%), en CO_2 (5%) et en bicarbonate, en plus de maintenir des niveaux optimaux de pH (7.4), de température (variable selon les espèces), de source d'énergie (pyruvate), de lipides et d'acides aminés essentiels. Certains milieux de culture, tels le SOF (Synthetic Oviduct Fluid) et le HTF (Human Tubule Fluid), ont été formulés de façon à ce que leur composition ressemble davantage à celle du fluide de l'oviducte (Quinn et al., 1985; Tervit et al., 1972).

Partie V

Étant donné la pression grandissante conduisant à l'utilisation de femelles de plus en plus jeunes à des fins reproductives, plusieurs équipes de recherche s'intéressent maintenant particulièrement à l'étude de la physiologie reproductive de ces jeunes animaux et aux caractéristiques que présentent leurs ovocytes et, éventuellement leurs embryons. Les jeunes vaches sont considérées pré-pubères lorsqu'elles sont âgées de moins de 8 mois, alors qu'elles entrent en période péri-pubertaire à l'âge de 8 mois et ce, jusqu'à l'âge de la puberté, soit 14 mois (Ferrell, 1982; Ball and Peters, 2004). Les prochaines lignes résument les principaux éléments propres aux jeunes animaux rapportés au cours des dernières années.

5.1 Caractéristiques de la reproduction des jeunes animaux

Tout d'abord, il est important de considérer que plusieurs éléments rendent la femelle immature inapte à se reproduire, à soutenir une gestation et à donner naissance à un veau vivant. Considérons tout d'abord le fait que la morphologie du système reproducteur du bovin immature présente des particularités qui le distinguent de celui de la vache adulte. En effet, chez l'animal pré-pubère, la majorité des structures reproductrices telles les oviductes, l'utérus, le cervix et le vagin sont sous-développées et peu, voire non fonctionnelles (Desjardins and Hafs, 1969). La plupart des organes reproducteurs se développent relativement lentement jusqu'à l'âge de 6 mois, pour ensuite prendre plus de volume et se différentier plus efficacement pendant les derniers mois séparant l'animal de la puberté. Desjardins and Hafs (1969) ont démontré que les structures reproductrices femelles sont en place et fonctionnelles au moment où la jeune femelle atteint l'âge de 10 mois. À ce stade, c'est le cerveau de la jeune femelle qui limite son activité sexuelle; ce n'est qu'au moment où la génisse atteint la puberté que celui-ci devient apte à prendre en charge le processus de maturation des gamètes. Il est important de noter que les ovaires et les ovocytes des animaux péri-pubères présentent des caractéristiques qui ressemblent davantage à celles observées chez les animaux adultes (Desjardins and Hafs, 1969). Selon les situations, la distinction phénotypique entre un animal péri-pubère et un animal pubère n'est pas toujours évidente.

L'axe hypothalamo-hypophysaire-ovarien ne devient fonctionnel qu'à la puberté. Avant ce moment, il existe une activité ovarienne presque normale, mais aucune ovulation (Duby, 1996; Massicotte, 2000). Certains groupes de recherche ont rapporté des patrons de vagues de croissance folliculaires et de dominance folliculaire similaires à ceux observés chez les vaches adultes (Duby, 1996; Evans, 1994). Duby et al. (1996) ont cependant démontré que les intervalles entre les vagues folliculaires sont plus courts chez les génisses pré-pubères. Pour leur part, les niveaux basaux de FSH et LH seraient similaires à ceux observés chez les animaux adultes, alors qu'il y aurait absence de pic de LH préovulatoire avant l'âge de 9 mois (Duby, 1996; Massicotte, 2000).

5.2 Qualité des ovocytes et compétence au développement

Bien qu'il soit possible d'obtenir un grand nombre d'ovocytes suite à une stimulation ovarienne (Erickson, 1966b) et de produire des embryons viables à partir d'ovocytes de femelles immatures (Armstrong et al., 1992; Majerus et al., 1999; Palma et al., 2001), les ovocytes de veaux sont considérés comme étant différents (de Paz et al., 2001), de moins bonne qualité et moins compétents que ceux des vaches adultes (Armstrong, 2001; Ax et al., 2005; Khatir et al., 1996; Khatir et al., 1998a; Majerus et al., 1999; Palma et al., 2001; Revel et al., 1995). La définition de la qualité de l'ovocyte dépend de la capacité du follicule à se développer et à se différentier. Rappelons que les cinq éléments clés, définissant la compétence ovocytaire sont la capacité 1) à reprendre la méiose; 2) à se diviser suite à la fécondation; 3) à se développer jusqu'au stade blastocyste; 4) à induire une gestation et à l'amener à terme et 5) à produire un veau vivant et en santé (Sirard et al., 2006).

La compétence moindre de l'ovocyte du veau par rapport à celui de la vache adulte a été observée, entre autres, par Gandolfi et al. (1998), qui ont évalué trois paramètres liés à la compétence *in vitro*, soient le diamètre ovocytaire, le métabolisme énergétique de l'ovocyte et la synthèse protéique de l'ovocyte et des cellules du cumulus et ce, chez des animaux âgés entre 10 et 14 semaines. Ils en sont venus à la

conclusion que l'ovocyte immature présente un diamètre plus petit que celui de la vache adulte. De plus, le gamète du veau métabolise la glutamine et le pyruvate moins rapidement que celui de la vache jusqu'à 24 heures de culture, alors que, par la suite, la vitesse de métabolisme est comparable pour les deux groupes d'animaux (Gandolfi et al., 1998). L'efficacité de synthèse protéique est également plus faible chez les ovocytes immatures (Gandolfi et al., 1998; Levesque and Sirard, 1994).

Kauffold et al. (2005) ont démontré, chez des veaux âgés entre 1 et 4 mois, que les ovocytes provenant des plus gros follicules, soient ceux dont le diamètre est supérieur à 8 mm), présentent une meilleure capacité à permettre à l'embryon de se développer jusqu'au stade blastocyste. Ceci suggère que les ovocytes de veaux acquièrent une meilleure compétence au développement grâce à un processus similaire à la pré-maturation chez la vache adulte; le *coasting* aurait ainsi un effet bénéfique sur la compétence ovocytaire des jeunes animaux (Landry et al., 2016). Afin de documenter la qualité des ovocytes sur le plan moléculaire, l'expression de certains gènes cibles a été mesurée par Romar et al. (2011). En effet, l'abondance des transcrits pour les gènes ZAR1, NLRP9, HSF1, PRDX1 et PRDX2 est rapportée comme étant significativement réduite chez les animaux immatures par rapport à ce qui est observé chez les vaches adultes. Les cellules du cumulus des jeunes femelles présentent également un profil différent de celui des vaches adultes et quelques groupes de recherche ont exploré leurs caractéristiques, à la recherche de marqueurs de qualité (Tatemoto et al., 1995; Kuwer et al., 1999; Bettegowda et al., 2008). Parmi ceux-ci, Bettegowda et al. (2008) ont rapporté un rôle fonctionnel pour les cathepsines, exprimées en plus grande proportion dans les cellules du cumulus de donneuses pré-pubères que chez les vaches adultes et suggèrent que l'abondance des ARNm associés permettrait de prédire la qualité de l'ovocyte.

Les ovocytes de jeunes donneuses présentent une compétence au développement réduite et une efficacité réduite à compléter leur maturation nucléaire et cytoplasmique, par rapport à ceux provenant de vaches adultes (Damiani et al., 1996; Gandolfi et al., 1998; Khatir et al., 1998a; Ptak et al., 2006; Salamone et al., 2001). Cette différentiation du follicule est complète lorsque l'animal atteint la puberté

(Khatir et al., 1996). Ainsi, il apparaît logique de penser qu'une différentiation incomplète pourrait être responsable de la qualité moindre des ovocytes observée chez les animaux pré-pubères et péri-pubères.

Les embryons provenant de femelles pré-pubères transférés chez des porteuses adultes produisent des veaux vivants et en santé, mais à des taux plus faibles que les embryons de vaches adultes. L'âge de la donneuse affecte ainsi la qualité de l'ovocyte et, éventuellement, de l'embryon (Khatir et al., 1998a; Majerus et al., 1999; Revel et al., 1995). De plus, lors d'une étude récente réalisée par notre équipe dans un contexte commercial, l'effet de l'âge a été observé autant sur le nombre de follicules que sur les taux de blastocystes (Landry et al., 2016). Aussi, l'utilisation d'outils génomiques a permis de confirmer la différence de qualité observée entre les embryons obtenus à différents stades de développement. Par exemple, Yoshida (2012) a démontré que l'expression génique présente des différences lorsque les embryons sont comparés aux stades 8-16 cellules et blastocystes. Cette observation suggère donc que l'embryon de l'animal immature dont le génome a été activé ressemble davantage à celui d'une vache adulte. Cette compétence amoindrie de l'embryon serait ainsi liée à sa dépendance à l'ovocyte et à l'abondance des transcrits qui lui sont fournis par celui-ci (Yoshida 2012).

Plusieurs études ont exploré différents moyens permettant d'améliorer la qualité des ovocytes des jeunes donneuses. Par exemple, alors que Cordova et al. (2011) ne rapportent aucun effet lié à l'ajout de leptine dans le milieu de maturation artificiel des ovocytes d'animaux prépubères, Jia et al. (2012), quant à eux, sont parvenus à démontrer que l'ajout de cette hormone favorise la maturation et le développement, tout en améliorant la qualité des embryons produits à partir des ovocytes maturés *in vitro* en présence de leptine. L'acquisition de la compétence au développement chez les ovocytes d'animaux péri-pubères est également affectée par l'âge des génisses et par le traitement hormonal (Armstrong et al., 1997; Ax et al., 2005; Palma et al., 2001; Presicce et al., 1997). Une étude a documenté l'effet d'une administration intra-ovarienne d'IGF1 sur la compétence au développement des ovocytes de génisses péri-pubertaires, l'expression des gènes (GLUT3, GLUT8, AKT1, BCL-XL, BAD et

BAX), chez des animaux âgés entre 7 et 10 mois *versus* 11 à 18 mois. L'abondance des transcrits des gènes a été notée comme étant affectée par l'âge et par le traitement à l'IGF1. De plus, l'apoptose est liée à l'âge de la donneuse et est notée plus particulièrement chez les embryons des animaux pré- et péri-pubères, contribuant à augmenter la mortalité embryonnaire après transfert (Zaraza et al., 2010). L'importance de l'apport en LH au cours des derniers jours précédant l'ovulation *in vivo* a également été démontrée par Labrecque et al. (2014).

5.3 Stimulation ovarienne

Il est possible d'observer la présence de vagues de développement folliculaires tôt dans le développement de la génisse. En effet, dès l'âge de deux semaines, une augmentation importante de la sécrétion de FSH est notée (Evans, 1994). Alors que les animaux atteignent l'âge de cinq mois, plusieurs groupes de recherche rapportent que l'augmentation du diamètre folliculaire et du nombre de follicules concorde avec une sécrétion élevée de FSH et LH (Duby, 1996; Evans, 1994; Massicotte, 2000).

Il est maintenant connu que les jeunes vaches répondent bien à la stimulation ovarienne et qu'il est aussi possible d'obtenir plus d'ovocytes suite à une stimulation ovarienne chez les jeunes donneuses, par rapport aux vaches adultes (Erickson, 1966b; Landry et al., 2016). Bien que Rick G (1996), Presicce et al. (1997) et Brogliatti GM (1999) aient obtenu des blastocystes à partir d'ovocytes de donneuses âgées entre 5 et 11 mois n'ayant pas subi de stimulation ovarienne, la plupart des études réalisées sur le sujet semblent unanimes: pour optimiser le succès de la fécondation *in vitro* des ovocytes de veaux, ceux-ci doivent être récupérés suite à une stimulation ovarienne aux gonadotrophines (Armstrong et al., 1994; Armstrong et al., 1997; Onuma and Foote, 1969; Revel et al., 1995). Considérant le fait que le cerveau ne prenne en charge la maturation des gamètes qu'au moment de la puberté (alors que les gamètes eux-mêmes ont développé la machinerie nécessaire pour répondre aux signaux hormonaux), plusieurs groupes de chercheurs se sont penchés sur l'impact de la stimulation ovarienne à l'aide d'hormones exogènes chez les animaux pré-pubères et péri-pubères. Les différentes équipes de recherche s'entendent pour dire qu'une

grande variabilité dans la réponse des individus au traitement hormonal est observée (Armstrong et al., 1992; Taneja et al., 2000; Jia et al., 2014).

La stimulation ovarienne des femelles prépubères fait appel à des protocoles de traitement à la FSH d'une durée variant entre 2 et 4,5 jours (Ax et al., 2005; Duby, 1996; Jia et al., 2014; Presicce et al., 1997; Salamone et al., 2001; Taneja et al., 2000) et nécessite des doses deux fois moins élevées de FSH que celles utilisées pour les vaches adultes (Massicotte, 2000).

Il a été rapporté que les ovaires de veaux âgés de 3 semaines peuvent répondre à une stimulation hormonale. Les ovocytes, récupérés par laparotomie, ont alors été considérés comme viables (Armstrong et al., 1992). Fry et al. (1998) ont vérifié l'effet d'un apport en GnRH suivant un programme de stimulation impliquant la FSH et la PMSG sur le développement folliculaire et la qualité de l'ovocyte en maturation *in vitro*. Leur étude a permis de démontrer un impact positif sur le développement folliculaire, alors que les animaux traités au GnRH possédaient un nombre plus élevé de follicules dont le diamètre est supérieur à 2 mm et de follicules récupérés par ponction trans-vaginale. Par contre, la qualité des ovocytes produits n'a pas été améliorée suite au traitement. Une grande variabilité entre les valeurs observées pour le groupe d'animaux est également rapportée (Fry et al., 1998). Massicotte (2000) a rapporté que 39% des follicules observés chez des femelles de 7 semaines sont viables; ces follicules ont la capacité de répondre à la stimulation ovarienne (Toner et al., 1992; Duby, 1996). Armstrong et al. (1994) ont démontré que la réponse des follicules provenant d'ovaires de veaux à la stimulation FSH augmente progressivement de 3 à 9 semaines d'âge et que les ovocytes récupérés à partir de ces follicules produisent des blastocystes, *in vitro*, à des taux similaires à ceux observés pour des femelles adultes.

5.4 Production d'embryons *in vivo* et *in vitro*

Tel que mentionné précédemment, plusieurs auteurs ont documenté la possibilité de produire des embryons viables à partir d'ovocytes de donneuses pré-pubères et péri-pubères (Armstrong et al., 1992; Majerus et al., 1999), que ce soit *in vivo* (Presicce et

al., 1997) ou encore *in vitro* (Armstrong, 2001; Palma et al., 2001). Les ovocytes des jeunes vaches peuvent maintenant être récupérés aisément grâce à la mise au point de diverses techniques telles l'aspiration trans-vaginale sous guidance ultrasonographique (Fry et al., 1998; Majerus et al., 1999; Presicce et al., 1997; Rick G, 1996) et la laparoscopie (Armstrong et al., 1992; Sirard and Lambert, 1985). Ceci facilite grandement leur utilisation dans les programmes de fécondation *in vitro*. Par contre, il est important de noter que l'aspiration trans-vaginale est moins efficace chez les animaux âgés de moins de 6 mois en raison de certaines contraintes physiologiques telle la taille du tractus reproducteur. Les sondes sont également moins bien adaptées pour une utilisation chez les veaux (Armstrong et al., 1997).

Palma et al. (2001) rapportent une faible quantité d'ovocytes récupérés suite à la stimulation ovarienne chez les très jeunes animaux et, ainsi, une efficacité à la fécondation *in vitro* plus faible que pour les vaches adultes. Il apparaît nécessaire d'utiliser d'importantes quantités de FSH dans les programmes de stimulation ovarienne, de façon à limiter l'impact de l'âge des animaux sur l'efficacité des programmes de fécondation *in vitro* (Palma et al., 2001). Alors qu'un apport en LH exogène s'avère efficace pour promouvoir le développement ovarien avant l'ovulation chez des animaux âgés entre 6 et 14 semaines, le traitement à la FSH permettrait d'améliorer l'efficacité de l'aspiration des follicules en favorisant le développement de plus gros follicules (Armstrong et al., 1997). En complément, Salamone et al. (2001), soutiennent que les ovocytes de génisses âgées de 6 mois ayant subi une stimulation ovarienne récupérés par laparoscopie ou aspiration folliculaire, puis maturés *in vitro* présentent une activité de MPF et MAPK ainsi qu'une quantité de récepteurs IP_3 (IP3R) plus faibles chez les ovocytes d'animaux immatures par rapport à ceux de femelles adultes. Cette situation traduit une inefficacité, voire une incapacité des ovocytes de veaux à compléter leur maturation cytoplasmique (Salamone et al., 2001).

Peu de groupes de chercheurs sont parvenus à transférer des embryons produits à partir d'ovocytes de veaux de moins de 5 mois. Parmi ceux-ci, Armstrong et al. (1997) et Taneja et al. (2000) sont parvenus à le faire, mais le nombre de gestations

venues à terme fut limité. En effet, les résultats obtenus démontrent un faible taux de mise bas, soit 7 sur les 84 embryons transférés. Pour sa part, le groupe de Majerus et al. (1999) a obtenu 3 veaux vivants sur les 20 transferts d'embryons réalisés. Chez les animaux utilisés (7 à 10 mois), les taux de blastocystes s'apparentaient davantage à ceux observés chez les femelles adultes, soient entre 5,1 et 11,1 blastocystes par donneuse chez les plus jeunes animaux (Majerus et al. 1999). L'effet de l'âge des génisses à leur première OPU a été documenté par Ax et al. (2005). Leur étude a démontré une augmentation de la qualité embryonnaire avec une augmentation de l'âge des animaux (8 à 10 mois).

Hypothèse et objectifs de recherche

Aujourd'hui, la stimulation hormonale de l'ovaire, le transfert embryonnaire et la fécondation *in vitro* permettent de devancer la mise en reproduction des jeunes vaches, accélérant le progrès génétique des troupeaux tout en valorisant les meilleurs sujets et en réduisant l'intervalle entre les générations. Il est ainsi primordial de prendre connaissance des particularités de la physiologie reproductive des animaux pré-pubères et péri-pubères ainsi que des caractéristiques que présentent leurs ovocytes et, éventuellement leurs embryons. On sait ainsi que les jeunes vaches répondent bien à la stimulation ovarienne et qu'il est possible d'obtenir plus d'ovocytes suite à la stimulation chez les jeunes donneuses, par rapport aux vaches adultes. Les ovocytes des jeunes donneuses sont cependant de moins bonne qualité et sont moins compétents que ceux des femelles pubères.

Le développement des outils d'analyse permet aujourd'hui d'explorer le fonctionnement des gènes dès le stade embryonnaire de même que la programmation épigénétique associée à la méthylation de l'ADN. Le concept d'épigénétique fait référence à l'ensemble des facteurs qui influencent l'expression des gènes d'un individu, sans modifier la séquence des nucléotides qui composent son code génétique. À ce jour, plusieurs éléments de la littérature soutiennent que les modifications épigénétiques sont héritables et surviennent dès la préparation des gamètes, pour se poursuivre durant la première semaine suivant l'union de ces derniers, au cours de laquelle l'embryon en début de développement y est particulièrement sensible. À ce moment, les changements dans la méthylation de l'ADN (méthylation et déméthylation) sont connus pour influencer le développement et la santé du futur individu, en plus d'influencer le phénotype de ses descendants. L'épigénome de l'embryon est modelable et évolue avec l'adaptation de l'individu en réponse à son environnement.

Les deuxième et troisième parties de ce mémoire ont comme but de valider ou de réfuter l'hypothèse suivante: les embryons obtenus à partir d'ovocytes de donneuses péri-pubères sont différents de ceux provenant de vaches adultes. L'objectif de ce projet consiste donc à documenter l'effet de l'âge sur la qualité de l'embryon sous

deux angles: l’analyse du transcriptome et de l'épigénome au stade blastocyste permettra ainsi d’identifier les éléments pouvant potentiellement affecter la compétence embryonnaire.

Cette étude est la première à analyser l'effet de l'âge sur la qualité de l'embryon bovin par analyse de l'expression des gènes et des perturbations épigénétiques. Dans un contexte d'élevage intensif, il importe particulièrement de bien comprendre les paramètres influençant la productivité, le potentiel génétique et les performances de reproduction des animaux. Ce projet de recherche a été réalisé en collaboration avec L'Alliance Boviteq Inc., un centre spécialisé en transfert embryonnaire et technologies de reproduction assistée chez les bovins.

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CHAPITRE 2: Transcriptomic evaluation of bovine blastocysts obtained from peri-pubertal oocyte donors

2.1 Résumé

Ce projet vise à documenter l'effet de l'âge sur la qualité de l'embryon bovin. Pour ce faire, dix jeunes femelles Holstein ont subi trois cycles de stimulation ovarienne, permettant trois collectes d'ovocytes (8, 11, 14 mois). Ces ovocytes ont ensuite été fécondés *in vitro*, générant trois lots d'embryons par animal. Grâce à la plateforme EmbryoGENE, il fut possible de mesurer l'expression génique au stade blastocyste. L'analyse de contraste selon l'âge a permis de dénombrer 242 gènes différentiellement exprimés pour le premier contraste (8 vs 14 mois) et 296 pour le deuxième (11 vs 14 mois). Le premier constat est que les embryons sont marginalement affectés par l'âge de la donneuse et que la qualité demeure très bonne dès 8 mois. Les résultats suggèrent une cause métabolique pour expliquer les différences observées, trahissant un impact plus grand des conditions *in vitro* sur les embryons produits par les plus jeunes donneuses.

2.2 Abstract

Assisted reproduction technologies (ART) and high selection pressure in the dairy industry are leading towards the use of younger females for reproduction, thereby reducing the interval between generations. This situation may have a negative impact on embryo quality, thus reducing the success rate of the procedures. This study aimed to document the effects of oocyte donor age on embryo quality, at the transcriptomic level, in order to characterize the effects of using young females for reproduction purpose.

Young Holstein heifers (n = 10) were used at three different ages for ovarian stimulation protocols and oocyte collections (at 8, 11 and 14 months). All of the oocytes were fertilized *in vitro* with the semen of one adult bull, generating three lots of embryos per animal. Each animal was its own control for the evaluation of the effects of age. The EmbryoGENE platform was used for the assessment of gene expression patterns at the blastocyst stage. Embryos from animals at 8 vs 14 months and at 11 vs 14 months were used for microarray hybridization. Validation was done by performing RT-qPCR on seven candidate genes.

Age-related contrast analysis (8 vs 14 mo and 11 vs 14 mo) identified 242 differentially expressed genes (DEGs) for the first contrast, and 296 for the second. The analysis of the molecular and biological functions of the DEGs suggests a metabolic cause to explain the differences that are observed between embryos from immature and adult subjects. The mTOR and PPAR signaling pathways, as well as the NRF2-mediated oxidative stress response pathways were among the gene expression pathways affected by donor age. In conclusion, the main differences between embryos produced at peri-pubertal ages are related to metabolic conditions resulting in a higher impact of *in vitro* conditions on blastocyts from younger heifers.

2.3 Introduction

In the dairy industry, the development of ovum pick up (OPU) methods, *in vitro* embryo production (IVM, IVF, IVC), and embryo transfer (ET), combined with the increased efficiency of genomic tools, made the use of young animals for reproduction purpose possible. The use of ultrasound to collect gametes from pre-pubertal and peri-pubertal hormonally stimulated animals is not recent (Brogliatti and Adams, 1996; Fry et al., 1998; Khatir et al., 1998b; Majerus et al., 1999; Presicce et al., 1997; Rick et al., 1996). While no deleterious effects are associated with ovarian stimulation or OPU on the health and production potential of young donors (Ax et al., 2005; Majerus et al., 1999; Presicce et al., 1997), genetic gain is certainly accelerated, as the best animals are identified early in life and the generation interval is considerably reduced (Doormaal, 2012; Lohuis, 1995a).

Although it is possible to recover more oocytes from the ovaries of young animals (Erickson, 1966a), it is well documented that calf oocytes are less competent compared to gametes from cows (> 14 months of age) (Armstrong, 2001; Damiani et al., 1996; Duby et al., 1996; Khatir et al., 1996; Presicce et al., 1997; Revel et al., 1995; Seidel et al., 1971). Despite the fact that healthy embryos can be obtained from pre-pubertal and peri-pubertal donors *in vivo* and *in vitro* (Armstrong et al., 1992; Majerus et al., 1999; Palma et al., 2001), several research groups explored the differences observed between calf and cow oocytes (and also embryos) such that some facts now support the following statements: (1) embryos from pre-pubertal calves transferred into adult recipients produce live and healthy offspring at a lower rate than embryos from adult donors, suggesting that donor age can affect oocyte and/or embryo quality (Ax et al., 2005; Khatir et al., 1998b; Majerus et al., 1999; Palma et al., 2001; Revel et al., 1995). In a recent study conducted by our team in a commercial setting, we have observed an age effect both on follicle number and on embryo rates (Landry et al., 2016); (2) oocytes from young donors have a reduced developmental competence, and a reduced efficiency to undergo nuclear and ooplasmic maturation (Gandolfi et al., 1998; Khatir et al., 1998a; Salamone et al., 2001), which affects embryo yield; (3) apoptosis is linked to oocyte donor age and is

increased in embryos from juvenile donors, contributing to higher rates of embryonic loss after embryo transfer (Zaraza et al., 2010); and (4) protein expression is reduced, even defective, in oocytes from young donors compared to oocytes from adult cows (Gandolfi et al., 1998; Levesque and Sirard, 1994).

Several studies explored methods to improve oocyte quality, but with variable success rates. In fact, hormonal stimulation of pre-pubertal and peri-pubertal calves may increase oocyte competence and embryo development potential (Armstrong et al., 1994; Majerus et al., 1999; Palma et al., 2001; Presicce et al., 1997; Revel et al., 1995). It was also reported, in pre-pubertal animals, that follicles > 8mm in diameter contained oocytes with a better ability to develop to the blastocyst stage *in vitro* (Kauffold et al., 2005). Oocyte developmental competence is therefore related to the age of the donor and can be improved by hormonal treatment (Duby et al., 1996; Looney et al., 1995).

The purpose of this study was to explore why embryos obtained from oocytes from young donors are less competent than those obtained from oocytes from adult cows. The gene expression pathways most significantly affected by age suggest that there is a metabolic cause responsible for the differences observed between embryos obtained from immature and adult subjects.

2.4 Material and methods

2.4.1 Chemicals

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

2.4.2 Animals

Prepubertal *Bos taurus* Holstein heifers (n=10) were used for this study. Each heifer underwent three ovarian stimulation cycles, resulting in three gamete collections, at the ages of 8, 11 (average 10.8) and 14 (average 13.7) months. The gametes were fertilized *in vitro* with spermatozoa from one adult male, producing three embryo lots per animal. Each heifer was its own control for the measurement of age effect. This

animal phase of the study was performed in an industrial IVF setting at Boviteq (Saint-Hyacinthe, QC, Canada), a specialized center for bovine embryo transfer and other assisted reproduction technologies involved in research and development.

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 our routine commercial activities. The study did not require handling animals on university premises.

2.4.3 Ovarian stimulation and gamete collection

The protocol for ovarian stimulation and ovum pick-up (OPU) was essentially the same as described by Nivet et al. (2012) and Labrecque et al. (2013). Briefly, each heifer was first treated with progesterone (CIDR) during the luteal phase in order to repress dominant follicles to reduce the risk of spontaneous ovulation. The dominant follicle was aspirated 36 h prior to administration of hormones. The ovarian stimulation program consisted of six injections of 30 mg FSH (Folltropin-V, Bioniche Animal Health, Belleville, ON, Canada) administered at 12 h intervals. Ovum pick up was performed 43 h after the last FSH injection, the optimal *coasting* (FSH starvation) period (Nivet et al., 2012). Using transvaginal ultrasonography, follicular diameters were measured and cumulus-oocyte complexes (COCs) were collected by OPU under epidural (COOK Medical, Bloomington, IN, USA). Granulosa cells and COCs were collected in warm HEPES-buffered Tyrode's medium (TLH) containing Hepalean (10 µL/mL) and transferred to the laboratory for IVM. The gamete collection procedures took place in the time period between October 2014 and May 2015.

2.4.3.1 *In vitro* maturation (IVM)

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 tubes of maturation medium composed of TCM199 (Invitrogen Life Technologies), 10% fetal bovine serum (FBS), 0.2 mM pyruvate, 50 mg/mL gentamicin, 5 mg/mL FSH (Bioniche Animal Health), 0.5 mg/mL LH (Lutropin, Bioniche Animal Health, Belleville, ON, Canada), and 1 mg/mL estradiol. Maturation droplets were incubated for 24 h at 38.5°C with 5% CO_2 in maximal humidity.

2.4.3.2 *In vitro* fertilization (IVF)

After 24 h of IVM, the COCs were collected and washed twice in TLH medium 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 0.6% (w/v) fatty acid-free BSA, 0.2 mM pyruvic acid, 2 µg/mL heparin, and 50 mg/mL gentamicin. Oocytes were transferred 15 min prior to semen addition and 2 µL of a solution containing penicillamine (2 mM), hypotaurine (1 mM), and epinephrine (250 mM) were added to each droplet to stimulate sperm motility. Semen from the same bull (Centre d'Insémination Artificielle du Québec, St-Hyacinthe, QC, Canada) was used for all of the IVF, for all of the heifers, in order to avoid any male-associated bias. The spermatozoa, previously stored in liquid nitrogen, were thawed for 1 min in 35.8°C water, added to a discontinuous gradient (45 over 90% BoviPure (Nidacon Laboratories AB, Göthenborg, Sweden)), and centrifuged at 600 X g for 5 min. The supernatant containing the cryoprotectant and the dead spermatozoa was discarded, and the pellet was then re-suspended in 1 mL of modified TL and centrifuged at 300 X g for 2 min. The re-suspended spermatozoa were counted on a hemocytometer and diluted with IVF medium to obtain a final concentration of 1 x 10^6 cells/mL. Finally, 2 µL of the sperm suspension were added to the droplets containing the matured COCs. The fertilization medium was incubated at 38.5°C for 15 to 18 h in a humidified atmosphere of 95% air and 5% CO_2.

2.4.3.3 *In vitro* culture (IVC)

After fertilization, presumptive zygotes were mechanically denuded, washed three times in TLH supplemented with fatty acid-free BSA and were then placed in groups of 10 in 10-µL droplets 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 (#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 new 10-µL droplets of mSOF containing non-essential and essential amino acids 72 h post-fertilization and to 20-µL droplets 120 h post-fertilization. This was to prevent toxicity due to increased ammonium concentration and to prevent nutrient depletion. Blastocyst development was assessed using industrial criteria for embryo transfer as recommended by the International Embryo Transfer Society at days 7 and 8 post-fertilization (Nivet et al., 2012). The embryos were finally snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Data such as follicle size, total number of aspirated follicles, number of oocytes in maturation, number of viable embryos, and cleavage percentage were recorded in order to assess the reproductive performance of the animals. Graph Pad Prism 6 (GraphPad Software, San Diego, California, USA) was used to perform paired-measures two-way ANOVA with Bonferroni post-test, or paired *t* tests in order to establish significance. Differences were considered to be statistically significant at the 95% confidence level ($p < 0.05$). Data are presented as mean ± standard error of the mean (SEM).

2.4.4 RNA extraction and amplification

Transcriptomic analysis was performed on blastocysts from four heifers (Boviteq IDs 10989, 10991, 10994 and 10995) using microarray hybridization. A total of five embryos were selected per heifer according to developmental stage and quality (visual appreciation), and pooled. Gene expression can be assessed by microarray analysis which measures the abundance of transcripts in embryos. Our group

contributed to the development of the EmbryoGENE transcriptomic platform, which has previously been used to assess the influence of the environment on embryo development (Gad et al., 2012; Robert et al., 2011).

Total RNA was extracted from the embryos using the *AllPrep DNA/RNA micro kit*, following the *Parallel gDNA and total RNA extraction* protocol. Total RNA integrity and concentration were evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) with the RNA PicoLab Chip (Agilent Technologies). The samples were linearly amplified to generate enough material for hybridization. Antisense RNA (aRNA) 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, Wilmington, DE).

2.4.5 Sample Labeling and Microarray Hybridization

For each sample, 2 µg of aRNA were labeled using the ULS Fluorescent Labeling Kit for Agilent arrays (Cy3/Cy5) (Kreatech Diagnostics, Amsterdam, Netherlands). The labeled product was then purified with the Pico-Pure RNA Isolation Kit. Labeling efficiency was measured using the Nano-Drop ND-1000. Samples from the four biological replicates (heifers) were hybridized on EmbryoGENE's bovine 44 K microarray (Robert et al., 2011). The hybridizations were performed according to the following design: for each heifer, the two peri-pubertal age periods were individually compared to the control age period (i.e., 8 months vs 14 months; 11 months vs 14 months) for a total of two comparisons per animal. Overall, eight hybridizations, corresponding to the four biological replicates and two comparisons, were performed. A total of 825 ng of each labeled sample (Cy3 and Cy5) were incubated in a solution containing 2X Blocking Agent and 1X Fragmentation Buffer in a volume of 55 µL at 60°C for 15 min and put on ice immediately after. Then, 55 µL of 2X GEx Hybridization Buffer HI-RPM were added for a total volume of 110 µL. The hybridization mix (100 µL) was added onto the array and hybridization was performed at 65°C for 17 h using an Agilent Hybridization chamber in a rotating 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 sec at room temperature, then with Stabilization and Drying Solution (Agilent) for 30 sec at room temperature, were performed before air drying of the slides. The slides were scanned using the Tecan PowerScanner microarray scanner (Tecan Group Ltd, Mǒnnerdorf, Switzerland) and features were extracted using ArrayPro 6.4 (Media Cybernetics, Bethesda, MD) (Labrecque et al., 2013).

2.4.6 Microarray data analysis

As described by Labrecque et al. (2014), microarray data were subjected to a simple background subtraction, normalized within array (Loess) and between array (Quantile), and statistically analyzed with the Limma package using FlexArray version 1.6.1 (Michal et al., 2007). Differences were considered statistically significant with a p-value less than 0.05. Microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE85438 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85438).

2.4.7 Functional analysis

FlexArray version 1.6.1 was used to perform functional enrichment analysis (Huang et al., 2009a, b) of specific gene lists based on fold-change cut-off. The gene lists were then analyzed with Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com). All statistically significant genes ($p < 0.05$; fold change > 1.5 or <-1.5) were uploaded to the application and each identifier was mapped to its corresponding object in the Ingenuity database. The functional analysis identified the biological functions that were the most significant to the molecules in the dataset (Labrecque et al., 2014).

2.4.8 Complementary DNA preparation and quantitative real-time polymerase chain reaction

To confirm the results from microarray analysis, quantitative real-time polymerase chain reaction (qRT-PCR) was performed on cDNA. Briefly, total RNA from three blastocysts was reverse transcribed using qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) with oligo dT following the manufacturer's recommendations. Seven candidate genes were selected according to the difference in their expression level compared to the reference condition and their relevance in embryo development processes: Acyl-CoA synthetase short-chain family member 1 (ACSS1), Poly(A) binding protein, cytoplasmic 1 (PABPC1), NANOG homeobox protein, Eukaryotic translation initiation factor 4E family member 2 (EIF4E2), histone methyltransferase H4-K20 specific (SUV420H1), RALY heterogeneous nuclear ribonucleoprotein, and Pre-mRNA-processing factor 6 (PRPF6). The primers used for qRT-PCR are listed in 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 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 absolute quantification experiments, with the following dilution sequence: 2×10^{-4}, 2×10^{-5}, 2×10^{-6}, 2×10^{-7} and 2×10^{-8} ng/mL. Real-time PCR was performed on a LightCycler 480 (Roche Diagnostics, Laval, QC, Canada) using SYBR incorporation. Each qRT-PCR reaction, in a final volume of 20 µL, contained the complementary DNA corresponding to 0.1 blastocyst, 0.25 mM of each primer, and 1X SYBR mix (Light-Cycler 480 SYBR Green I Master, Roche Diagnostics). The PCR conditions used for all genes were as follow: denaturing for 10 min at 95°C; 50 PCR cycles (denaturing, 95°C for 10 sec; annealing, (Table 2.1) for 10 sec; extension, 72°C for 20 sec), a melting-curve (95°C), and a final cooling step at 40°C.

Complementary DNA quantifications were performed with the 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 (Labrecque et al., 2014).

Gene	Forward primer (5'-3')	Reverse primer (3'-5')	Annealing temp. (°C)	Product size (bp)	Accession number
GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase)	CCAACGTGTCTGTTGTGGATCTGA	GAGCTTGACAAAGTGGTCGTTGAG	58	217	NM_001040552
CHUK (Conserved helix-loop-helix ubiquitous kinase)	TGATGGAATCTCTGGAACAGCG	TGCTTACAGCCCAACAACTTGC	57	180	NM_174021
ACSS1 (Acyl-CoA synthetase short-chain family member 1)	GTAGTGAAGCGTCTTCCAAA	GTCTAGGAACAAGCCGACTC	57	251	NM_174746
PABPC1 (Poly(A) binding protein, cytoplasmic 1)	GTCTCTCCGTTCTAAAGTTGATG	GGCATTTGCTCGGTACATAA	57	265	NM_174568
NANOG (Homeobox protein)	TTCAATTCCCAGCAGCAAATC	GACTCATTACCCTTCCCTCAAA	57	227	NM_001025344
EIF4E2 (Eukaryotic translation initiation factor 4E family member 2)	GATAATTCGACTGCGGAAGG	GGAAAGGATTATTGCCAGCTT	57	409	NM_001075795
SUV420H1 (Histone methyltransferase H4-K20 specific)	TAGACTCTCTTTCCTGTCTTC	CACTTTGGACGTTAAAGACT	57	214	NM_001076551
RALY (RALY Heterogeneous Nuclear Ribonucleoprotein)	CAGCAGGAGAAAGGCGTACA	CCAAGCCTGGGAATGTCCTG	57	259	NM_001014847
PRPF6 (Pre-mRNA-processing factor 6)	CTGAAGAACATCGCCAGCAC	ATCTTCACAGTGCGGTGGAAC	57	236	NM_001046375

Table 2.1 Information on primers used for RT-qPCR validation

2.4.9 Statistical analysis of quantitative RT-PCR results

For each gene tested, three biological replicates corresponding to embryo pools collected from eight cows (Boviteq IDs 10989, 10991, 10994, 10995 and 10990, 10992, 10993, 10996) were used. Analysis of gene expression stability in the two groups of embryos (8 vs 14 months; 11 vs 14 months) from the different heifers was

performed using the GeNorm VBA applet software, as described by Vandesompele et al. (2002). As reported by Goossens et al. (Goossens et al., 2005), the M value is an indicator of gene stability. The genes associated with low ***M*** values display more stable expression. On the contrary, genes with high M values show a greater variability in RNA expression (Goossens et al., 2005; Vandesompele et al., 2002). The most stable reference genes were then identified by the stepwise exclusion of the least stable gene and recalculating the M values (Labrecque et al., 2014). After GeNorm analysis, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Conserved helix-loop-helix ubiquitous kinase (CHUK) were the most stable genes with M-values less than 1.5 as recommended by the software. Beta-actin (ACTβ) was excluded from the analysis; its expression was variable in the datasets. Graph Pad Prism (version 5.0) was used to perform the evaluation of the messenger RNA (mRNA) differences by two-tailed paired non-parametric *t* test for the 8 vs 14 months and the 11 vs 14 months contrasts.

2.5 Results

2.5.1 Reproductive performance of the animals

First, the number and quality of blastocysts was assessed according to age. Each of the ten females was stimulated and underwent OPU procedure. The number of embryos generated for each animal is presented in Table 2.2.

Subject (Boviteq ID)	Embryos at 8 months	Embryos at 11 months	Embryos at 14 months
10989	7	7	(5 + 7)
10990	3	4	4
10991	6	7	(1 + 4)
10992	4	2	2
10993	6	1	8
10994	9	5	(8 + 14)
10995	5	7	(8 + 6)
10996	4	2	4
10997	2	4	6

10998	0	4	8
Average	4.6 ± 2.6	4.3 ± 2.2	8.5 ± 6.0

Table 2.2 Number of embryos produced *in vitro* per animal, per condition. The number of embryos obtained from the females at 8 months varied between 0 and 9, while 1 to 7 were produced at 11 months, and 2 to 22 at 14 months. Two OPU sessions were necessary to obtain enough embryos from some of the 14 month-old subjects (10989, 10991, 10994, and 10995).

Furthermore, data such as follicle size, total number of aspirated follicles, number of oocytes in maturation, number of viable embryos, and cleavage percentage were recorded. It was therefore possible to determine the impact of donor age on the size and number of aspirated follicles (Fig. 2.1), on oocyte developmental competence (Fig. 2.2), and on the ability to develop to the blastocyst stage (Fig. 2.3). A correlation analysis of the results demonstrated a direct negative relationship between age and the total number of aspirated follicles, with an r^2 value of -0.094 ($p > 0.05$).

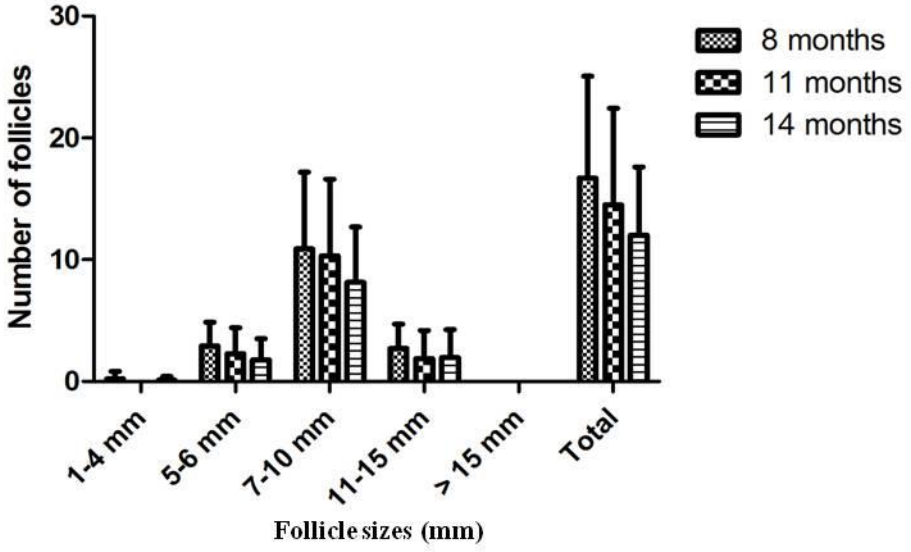

Figure 2.1 Effect of donor age on size and number of aspirated follicles. Follicles were classified according to size and grouped into five categories (1-4 mm, 5-6 mm, 7-10 mm, 11-15 mm and > 15 mm). An important proportion of the aspirated follicles had diameters varying between 7-10 mm

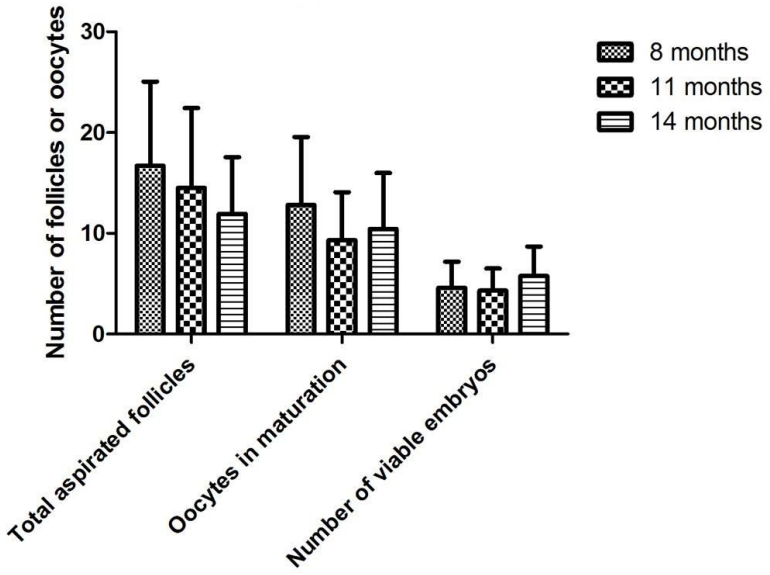

Figure 2.2 Effect of donor age on oocyte progression and developmental competence. The number of oocytes in maturation and the number of viable embryos produced in *vitro* were stable across time periods, although lower than the number of aspirated follicles. Hence, more follicles need to be collected from young animals in order to obtain the same number of oocytes in maturation, and eventually of embryos as observed for the mature animals.

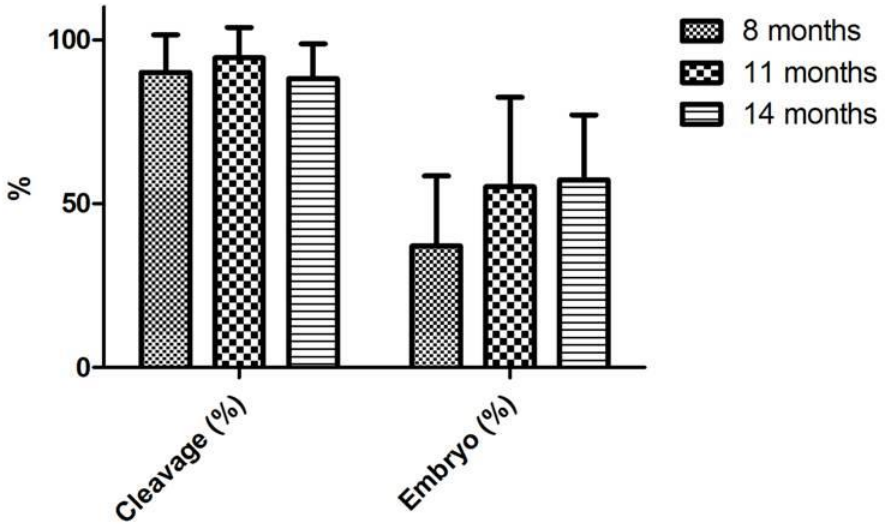

Figure 2.3 Effect of donor age on the ability to cleave and develop to the blastocyst stage. It is possible to see that cleavage rates were not significantly affected by age, whereas embryo percentage has a tendency to be lower for peri-pubertal animals.

2.5.2 Microarray analysis

Embryos from four animals were used for microarray analysis based on two age-related contrasts (8 vs 14 months and 11 vs 14 months), 14 months being the reference condition. The EmbryoGENE bovine microarray slide (EMBV3) contains a total of 42,242 probes, 31,138 of which are associated with reference genes, novel transcribed regions, alternatively spliced exons, and 3' untranslated region variants (Robert et al., 2011). At first, the transcriptome profile of the samples was assessed by hierarchical cluster analysis (Fig. 2.4), using Morpheus software (Broad Institute - https://software.broadinstitute.org/morpheus/). It was then possible to visualize how the animals grouped according to the different age conditions. The cluster analysis shows the 8 months group to be different from the 11 months and the 14 months groups.

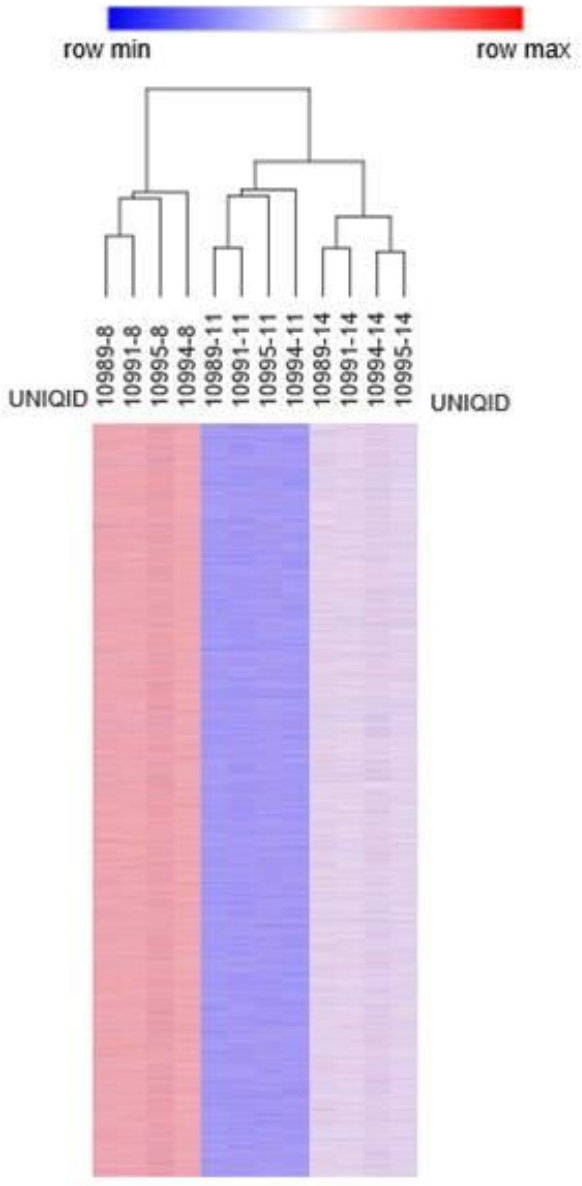

Figure 2.4 Hierarchical cluster analysis of the transcriptomic profile for each age condition. Hierarchical clustering distinguishes the 8 months group from the 11 months and the 14 months groups.

As mentioned above, differentially expressed probes were identified if they met the 1.5 fold-change and $p < 0.05$ cut-offs. Accordingly, age-related contrasts allowed to identify 242 differentially expressed genes for the 8 vs 14 months contrast and 296 for the 11 vs 14 months comparison (Fig. 2.5). One hundred and nineteen genes were up-regulated in the 8 vs 14 months contrast, and 158 in the 11 vs 14 months contrast. On the other hand, 123 genes were down-regulated in the first contrast and 138 in the

second. Twenty-two differentially expressed probes were common to both conditions studied and were affected in the same direction (either up- or down-regulated in both groups). Of these 22 probes, 9 were up-regulated and 13 were down-regulated in both conditions.

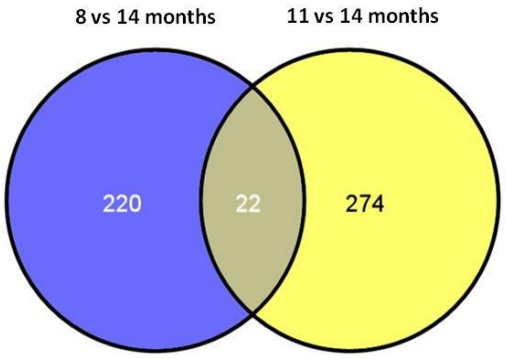

Figure 2.5 Number of differentially expressed probes for each contrast. A probe was considered as differentially expressed if its fold-change was > 1.5 or < -1.5 and its p value was < 0.05.

Eleven out of the 22 differentially expressed probes were associated to known genes and are presented in Table 2.3. Among these, ACSS1 (acyl-CoA synthetase short-chain family member 1), INPPL1 (inositol polyphosphate phosphatase-like 1) and EIF4E2 (eukaryotic translation initiation factor 4E family member 2) are important in embryo and organism development; their Gene Ontology (GO) terms referring to functions such as in utero embryonic development, translation regulation activity, structure, metabolism, and immune system development (Gene Ontology, Ensembl).

Gene	Status (in both conditions)
Acyl-CoA synthetase short-chain family member 1 (ACSS1)	Up-regulated
Inositol Polyphosphate Phosphatase-Like 1 (INPPL1)	Up-regulated
Beta-2-microglobulin (B2M)	Up-regulated
DNA-damage regulated autophagy modulator 1 (DRAM1)	Up-regulated
Cathepsin L2 (CTSL2)	Up-regulated

Heterogeneous Nuclear Ribonucleoprotein A1-like 2 (HNRNPA1L2)	Up-regulated
Chromosome 5 Open Reading Frame 45 (C5H12orf45)	Down-regulated
Hypothetical protein MGC134093	Down-regulated
Eukaryotic translation initiation factor 4E family member 2 (EIF4E2)	Down-regulated
SIX Homeobox 3 (SIX3)	Down-regulated
Chemokine (C-X-C motif) ligand 14 (CXCL14)	Down-regulated

Table 2.3 Status of 11 known genes differentially expressed in embryos from oocytes collected at 8 and 11 months compared to 14 months. Six genes were up-regulated while five were down-regulated in both contrasts.

An upstream regulator approach with IPA allowed analysis of the complete set of microarray data highlighting the importance of age-affected gene expression networks (Table 2.4). Networks such as the mTOR and PPAR signaling pathways, as well as the NRF2-mediated oxidative stress response pathway were of particular interest, all three being linked to metabolism functions. The p values of all the presented pathways were significant. Additionally, the upstream analysis pointed to HNF4α (hepatocyte nuclear factor 4α), TP53 (cellular tumor antigen p53), beta-oestradiol, ESR1 (estrogen receptor 1), TGFβ1 (transforming growth factor β1), and MYC (Myc proto-oncogene protein) as being molecules of interest (Table 2.5). Functions downstream of β-oestradiol (chemical - endogenous mammalian) and MYC (transcription regulator) had a tendency towards activation in both contrasts; while TP53 (transcription regulator) and TGFβ1 (growth factor) -associated functions were slightly inhibited in both contrasts (TP53 functions were considered as *inhibited* in the 11 vs 14 months contrast). HNF4α (transcription regulator) influences functions that had a tendency towards activation in the first contrast, and towards inhibition in the second. Finally, ESR1 (ligand-dependent nuclear receptor) presented the opposite pattern: its downstream functions were negatively affected in the 8 vs 14 months contrast, and activated in the 11 vs 14 months contrast.

Pathway	8 vs 14 months				11 vs 14 months			
	Status	z score	Enrichment ratio	P value	Status	z-score	Enrichment ratio	P value
mTOR signaling	Inhibition*	-1,236	43,3%	1,51 x 10^{-}	Activation	0,156	32,0%	3,15 x 10^{-}

				9				5
EIF2 signaling	Inhibition*	-0,152	43,6%	$1,55 \times 10^{-9}$	Inhibition*	-1,48	34,0%	$2,36 \times 10^{-6}$
NRF2-mediated oxidative stress response	Inhibition*	-0,493	43,3%	$5,48 \times 10^{-9}$	Inhibition*	-1,257	38,3%	$4,67 \times 10^{-9}$
Integrin signaling	Inhibition*	-1,606	39,8%	$3,01 \times 10^{-7}$	Activation*	0,602	36,3%	$2,47 \times 10^{-8}$
PPAR signaling	Inhibition*	-0,926	44,7%	$6,80 \times 10^{-6}$	Inhibition	-0,962	28,7%	$2,20 \times 10^{-2}$
Signaling by Rho Family GTPases	Activation	0,453	38,6%	$2,65 \times 10^{-7}$	Activation*	0,5	34,3%	$7,8 \times 10^{-8}$
Androgen signaling	Inhibition	-1,043	40,4%	$6,20 \times 10^{-5}$	Inhibition*	-1,213	38,6%	$2,19 \times 10^{-6}$

Table 2.4 Canonical pathways affected by age. IPA analysis identified the major gene pathways affected in each contrast. The status (activated or inhibited) was determined from the z score, while the p value confirmed significance. The enrichment ratio refers to the overlap of the observed versus the predicted regulated genes or, more precisely, to the number of genes that enrich each indicated pathway. Asterisks mark the five pathways that were the most affected in each condition. mTOR Signaling, EIF2 Signaling, NRF2-mediated oxidative stress response, Integrin Signaling, and PPAR Signaling were the dominant pathways affected in the 8 vs 14 months contrast and were all inhibited. EIF2 Signaling, NRF2-mediated oxidative stress response, Integrin Signaling, Signaling by Rho Family GTPases, and Androgen Signaling were the dominant pathways affected in the 11 vs 14 months contrast. Integrin Signaling and Signaling by Rho Family GTPases were activated in this second contrast.

Upstream regulator	8 vs 14 months		11 vs 14 months	
	Activation z score	P value	Activation z score	P value
HNF4α	0,780	$1,42 \times 10^{-54}$	-0,119	$4,64 \times 10^{-47}$
TP53	-0,263	$4,49 \times 10^{-37}$	-2,170	$1,77 \times 10^{-31}$
β-oestradiol	0,455	$1,73 \times 10^{-25}$	1,383	$1,75 \times 10^{-30}$
ESR1	-1,347	$2,90 \times 10^{-21}$	2,321	$1,46 \times 10^{-20}$
TGFβ1	-0,232	$4,48 \times 10^{-21}$	-0,214	$3,96 \times 10^{-17}$
MYC	0,784	$6,45 \times 10^{-19}$	1,984	$6,37 \times 10^{-28}$

Table 2.5 Upstream analysis to identify regulator molecules. IPA analysis identified genes and molecules that influenced pathways and functions in each contrast. The status was determined from the z score, while the p value confirmed significance. An arbitrary z score cutoff was established by the IPA software: the downstream network associated with a regulator was therefore considered as activated if $z > 2$ and inhibited if $z < -2$.

2.5.3 Quantitative RT-qPCR validation

Microarray data were validated using quantitative RT-qPCR. Seven candidate genes were selected according to the difference in their expression level compared to the reference condition and their relevance in embryo development processes: ACSS1, PABPC1, NANOG, EIF4E2, SUV420H1, RALY, and PRPF6 (Table 2.7 - Supplemental data). Four replicates were tested for each age condition (8, 11 and 14 months), for each gene. Validation was done in two steps and allowed to compare microarray and qPCR results in two groups of animals: the same animals that were used for microarray analysis (Boviteq IDs 10989, 10991, 10994 and 10995) constituted the first group; while the second group included four different individuals (Boviteq IDs 10990, 10992, 10993, and 10996). While there were no significant differences between the two peri-pubertal age conditions and the reference age condition, it is possible to rely on the observed trends and relative abundance to confirm the microarray. Synthesized results of the qPCR validation, where each candidate gene confirmed the microarray data in one or more conditions, are available in *Supplemental data* (Table 2.7).

From the validation data obtained, it is possible to conclude that microarray and qPCR observations slightly differ, whether they were compared using the same individuals or different animals. This might be due to the small number of animals in each group and variability in embryonic development between individuals.

2.6 Discussion and conclusion

This study is the first to demonstrate the effect of donor age on bovine embryo quality by transcriptomic analysis using peri-pubertal commercial stimulated Holstein subjects. The use of the same animals at different ages is a quite powerful design to extract fine differences in the observed gene expression data. The transcriptomic microarray hybridization technique, following amplification of nucleic acids, is now a particularly reliable, sensitive, and efficient method to be used on samples containing low amounts of biological material (Shojaei Saadi et al., 2014). This technique is also well adapted to study the bovine as the *Bos taurus* genome is now accessible. Our

group has contributed to the development of the EmbryoGENE transcriptomic platform, which has previously been used to assess the influence of environment on embryo development (Gad et al., 2012; Robert et al., 2011). Transcriptomic analysis of gene expression is also helpful in the identification of molecular mechanisms which influence biological processes and cellular functions. Here, we show that gene pathways involved in embryonic development are affected by age. The blastocyst stage was chosen to assess embryo quality mainly because the embryo, at that time, reflects the sum of all stresses and influences caused by *in vitro* culture.

2.6.1 Reproductive performance of the animals

Throughout the study, the only parameter to vary was *age* while all other parameters such as environment, feeding, ovarian stimulation protocols, semen origin, and embryo culture conditions were kept constant for all animals. It is therefore possible to say that the differences observed between the groups were essentially associated with donor age.

In the present study, the reproductive performance of the animals was consistent with previous findings. In fact, even if follicle number data from this study were not statistically significant, the tendency presented in Figure 2.1 agrees with the literature data showing that young donors produce more follicles than sexually mature subjects (Lonergan, 1990; Moreno et al., 1992; Presicce et al., 1997). Results are also coherent with Kauffold et al. (2005), who reported that follicles > 8 mm in diameter contain oocytes that are more competent to develop to the blastocyst stage.

While more follicles are produced, embryos derived from oocytes from young donors are different and less competent than the ones obtained from mature animals (Armstrong, 2001; Khatir et al., 1996; Khatir et al., 1998a; Palma et al., 2001; Revel et al., 1995). Our data supports this impaired developmental competence associated with young donors. Young heifers produced lower numbers of embryos (morula and blastocysts) which is also in agreement with previous findings (Bols et al., 1999; Looney et al., 1995; Revel et al., 1995; Torres et al., 2015). It was recently demonstrated by our team, on a large dataset, that the lower developmental

competence of oocytes obtained from young donors reduces the benefits of their use in IVF (Landry et al., 2016). However, it is important to note the high variability of the observations in most groups. This variability was previously reported by Revel et al. (1995), Taneja et al. (2000), and Majerus et al. (1999), and is thought to be partly caused by the number of follicles recovered per individual, ranging from 5 to 32 in the present study.

While more follicles could be obtained from young donors following ovarian stimulation, the cleavage percentage following *in vitro* fertilization was similar to that observed for adult subjects. Presicce et al. (1997) reported that animals younger than 8 months had poorer cleavage and blastocyst rates compared to sexually mature cows. At 11 months, however, the rates for these two parameters were similar to the rates for adult females (Presicce et al., 1997). In our study, however, cleavage rates were not significantly affected by age, whereas embryo percentage was lower for pre-pubertal animals. This trend was also observed in a study by Ax et al. (2005), where animals younger than 10 months produced embryos that were of poorer quality and not viable for transfer.

2.6.2 Microarray analysis - genes of interest

The importance of examining several thousands of genes at the same time was highlighted by the results of microarray analysis. The characteristics and functions of the most differentially expressed genes (DEGs) in each condition (8 vs 14 months and 11 vs 14 months) were investigated using Ingenuity Pathway Analysis (IPA). As mentioned previously, the bovine microarray includes a total of 42,242 probes, 31,138 of which are associated with reference genes, novel transcribed regions, alternatively spliced exons, and 3' untranslated region variants (Robert et al., 2011). Of this number, we found that 242 and 296 genes were differentially expressed in the 8 vs 14 months contrast and in the 11 vs 14 months contrast, respectively (Fig. 5). Therefore, a very small proportion of the targets of interest were differentially expressed in at least one contrast (0.78% for the 8 vs 14 months contrast and 0.95% for the 11 vs 14 months contrast). Moreover, the difference in intensity level of the majority of these differentially expressed probes was close to the detection cut-off

used (fold-changes (FC) ranging from -2.7587 to 2.0625 ($p < 0.05$) in the first contrast, and from -2.5204 to 2.1703 ($p < 0.05$) in the second contrast), suggesting that the effect of age on embryo quality may be subtle, although slight differences in gene expression can have a significant impact at the blastocyst stage. Additionally, some genes were differentially expressed in both conditions: the 22 DEGs reported in Figure 4. Amongst these genes, 11 were known sequences that are associated with a biological function (Table 2.3). While their function may not be particularly relevant in the given context, the fact that the expression of these 11 genes varied in a similar way in both conditions has caught our attention. In fact, for each gene, the abundance of the transcript was affected in the same direction and at similar intensity amplitude in both groups. This finding contributes to support our hypothesis that there is an observable age effect.

Three genes from this group were selected for discussion as their functions are pertinent to metabolism and embryonic development. Luong et al. (Luong et al., 2000) demonstrated that the bovine *Acyl-CoA synthetase short-chain family member 1* (ACSS1) gene encodes for an enzyme that is involved in fatty acid activation and plays a key role in the metabolism of acetate. Fujino et al. (Fujino et al., 2001) also reported that bovine ACSS1 activates more ^{14}C-acetate to CO_2 than it does for lipids, suggesting that the acetyl-CoA for the oxidative pathway is produced by ACSS1. Furthermore, a previous study from our team highlighted the fact that exposure of pre-implantation embryos to oxidative stress is associated with the up-regulation of several metabolic markers (Cagnone and Sirard, 2013). In this same study, supplementation of the *in vitro* culture medium with BSO (0.4 mM buthionine sulfoximine), a pro-oxidant agent inhibiting the synthesis of glutathione, resulted in the up-regulation of the ACSS1 transcript (adjusted p value < 0.02 (Cagnone and Sirard, 2013)). The fact that ACSS1 was up-regulated in both age contrasts in this study suggests that embryos from younger donors struggle with oxidative stress-like conditions and that there is a need for the activation of acetate and, consequently, of the oxidative stress response pathway.

The inositol polyphosphate phosphatase-like 1 (INPPL1) gene is also involved in metabolism and encodes for SH2-domain-containing inositol polyphosphate 5-phosphatase 2 (SHIP2) (Pesesse et al., 1997). It was reported that the *in vitro* over-expression of SHIP2 reduced insulin sensitivity in human and rodents (Sasaoka et al., 2001; Wada et al., 2001), and negatively affected glucose tolerance in mice (Fukui et al., 2005; Kagawa et al., 2008). Its expression was enhanced when cells were exposed to growth factors and insulin (Habib et al., 1998). In our data sets, there is a tendency for *Insulin-like growth factor* (IGF1) to be slightly up-regulated in both contrasts, although not significantly. Up-regulation of INPPL1 may indicate an increased risk of insulin resistance, therefore increased susceptibility to the development of metabolic syndrome-associated hypertension, obesity and Type II diabetes.

Finally, EIF4E2 is part of the Eukaryotic translation initiation factor 4E (EIF4E) family and is mainly known for its role in translation initiation (Sonenberg and Gingras, 1998; Uniacke et al., 2012; Yi et al., 2013). The down-regulation of this gene in blastocysts from younger animals may therefore indicate less efficient protein synthesis which may affect embryo development. However, Long et al. (2001) suggested that the low level of EIF4E mRNA in prepubertal tissues compared to pubertal ones, might be the result of changes in the hormonal environment linked to the onset of puberty.

Amongst the other microarray DEGs, some are known for their implication in embryonic development, in metabolic processes, and/or in gene expression regulation (Table 2.6).

In the 8 vs 14 months contrast, RALY and PRPF6 were significantly more abundant in embryos from younger donors, whereas MYST4, PABPC1 and SUV420H1 were significantly down-regulated in the 8 months condition (Table 2.6). The RALY Heterogeneous Nuclear Ribonucleoprotein protein is involved in mRNA processing and preimplantation embryo development regulation and is closely linked to the *agouti* (*a*) gene on mouse chromosome 2. Two phenotypes can be observed in mice carrying the A^y allele: at first, the "yellow agouti obese mouse" syndrome (heterozygote for the A^y allele) results in the ectopic over-expression of the agouti

gene (Michaud et al., 1994; Wolff et al., 1999) and is associated with dominant pleiotropic effects such as hyperinsulinemia (Hellerstrom and Hellman, 1963), obesity, and susceptibility to the development of tumors (Michaud et al., 1993). RALY is essential for embryonic development as its deletion, resulting from the homozygous A^y lethal yellow mutation, causes embryonic lethality (Duhl et al., 1994; Michaud et al., 1993) mainly at the blastocyst stage just before implantation (Granholm and Johnson, 1978; Pedersen, 1974), probably due to defects in the trophectoderm and the inner cell mass (Barsh et al., 1990; Papaioannou and Gardner, 1979; Papaioannou, 1988). Hence, RALY being differentially up-regulated in the 8 vs 14 months contrast (FC = 2.062; $p < 0.05$) may highlight favorable conditions for the development of dominant pleiotropic effects, including obesity and hyperinsulinemia, in the progeny of younger donors.

Pre-mRNA-processing factor 6 (PRPF6) is a member of the small ribonucleoprotein (tri-snRNP) spliceosome complex and is associated with mitochondrial function and oxidative phosphorylation efficiency (Devin et al., 1996; Shinohara et al., 1993) in the human skeletal muscle (Lane et al., 1998). An increase in the abundance of PRPF6 transcripts may enhance ATP production, favor electron flow through the respiratory chain (Lane et al., 1998), and decrease susceptibility to insulin resistance (Hofman et al., 1997). On the other hand, knowing the involvement of this gene in some diseases, its up-regulation in our 8 vs 14 months contrast may indicate an unbalance and an increased susceptibility to the development of non-metabolic syndromes.

The down regulation of MYST4, PABPC1, and SUV420H1 may point to sub-optimal growth conditions, potentially affecting gametogenesis and embryo development. In the case of MYST4, which is present in oocytes and in *in vitro* produced embryos (McGraw et al., 2007), down-regulation may affect downstream genes and their functions and, ultimately, impair histone acetylation activity. This may limit the accessibility of underlying genes and, therefore, gene activation (Berger, 2002). MYST4 (or MORF) binds to a unique epigenetic regulator, *Bromodomain- and PHD finger-containing protein 1* (BRPF1), which stimulates acetyltransferase activity and

gene expression (Doyon et al., 2006; Lalonde et al., 2013; Ullah et al., 2008). Our data, showing down-regulation of MYST4 and of BRPF1, suggest impaired gametogenesis and embryo development in younger animals. Down-regulation of *poly(A)-binding protein, cytoplasmic 1* (PABPC1) may contribute to impaired oocyte maturation and early embryo development, as it plays an important role in the translation regulation of stored maternal mRNAs (Ozturk et al., 2014). Down-regulation of SUV420H1 would also have a negative impact on embryo development, considering its involvement in gene expression regulation (Schotta et al., 2004; Souza et al., 2009).

In the 11 vs 14 months contrast, the NANOG, PLAU, and TET3 genes were significantly up-regulated in blastocysts from the younger animals, while SMAD6 and COX8B were significantly down-regulated (Table 2.6). The homeobox transcription factor NANOG is known for its transcription regulation function in the *Bos taurus* genome. The expression of this gene was detectable mainly in the mouse embryo ICM and in embryonic stem cells (Mitsui et al., 2003). Also, the bovine protein encoded by NANOG is ICM specific at the blastocyst stage (Kuijk et al., 2008). NANOG also plays a key role in the pluripotency status of embryonic stem cells, as well as in their proliferation and renewal (Chambers et al., 2003). Our team has recently documented the *in vitro* effect on the bovine ICM and TE tissues and has reported that NANOG was more expressed in the TE of the *in vitro* embryos, in comparison to the *in vivo* ones. The contrary is observed in the case of the ICM, where NANOG is found to be more expressed in the *in vivo* embryos (Hosseini et al., 2015). The data from this study then suggest that up-regulation of NANOG may result in reduced repression of the trophoblast, which could affect its differentiation, causing implantation failure. The up-regulation of PLAU, on the other hand, may favor implantation, particularly the invasion process as reported by Aflalo et al. (2004) and by Sappino et al. (1989) in mice. This contradiction between NANOG and PLAU expression and their function may therefore suggest non-optimal implantation for embryos from 11 month-old donors.

Gene	Gene symbol	Status	Known functions
RALY Heterogeneous Nuclear Ribonucleoprotein	**RALY**	Up-regulated in 8 vs 14 mo contrast (FC 2,06; p<0,05)	- Involved in mRNA metabolism (splicing, stability, nuclear export) in the preimplantation embryo (Carpenter et al., 2006; Han et al., 2010; Krecic and Swanson, 1999; Ostareck-Lederer and Ostareck, 2004; Weighardt et al., 1996) - Associated with lethal yellow mutation in the mouse *agouti (a)* locus (Michaud et al., 1993) - Essential in mouse preimplantation development (Duhl et al., 1994) - May act as transcriptional cofactor for genes involved in cholesterol biosynthesis in mice (Sallam et al., 2016)
Pre-mRNA-processing factor 6	**PRPF6**	Up-regulated in 8 vs 14 mo contrast (FC 1,93; p<0,05)	- Pre-mRNA splicing (Zhao et al., 2002) - Essential for cell viability and vertebrate development (Bujakowska et al., 2009; Graziotto et al., 2008; Urushiyama et al., 1997) - Involved in human autosomal-dominant Retinitis pigmentosa (Tanackovic et al., 2011) - Favors cancer proliferation by preferential splicing of growth regulation genes (Adler et al., 2014) - Androgen receptor co-activation (Zhao et al., 2002)
Histone (K-lysine) acetyltransferase	**MYST4**	Down-regulated in 8 vs 14 mo contrast (FC -1,65; p<0,05)	- Acetyl transferase activity (Champagne et al., 2001) - Early mammalian gametogenesis (McGraw et al., 2007) - Involved in acetylation events in gamete and embryo development (McGraw et al., 2007) - Involved in leukemogenesis (Carapeti et al., 1998; Murati et al., 2004; Panagopoulos et al., 2001)
Poly(A) binding protein, cytoplasmic 1	**PABPC1**	Down-regulated in 8 vs 14 mo contrast (FC -1,77; p<0,05)	- Translational regulation of stored maternal mRNAs (Ozturk et al., 2014) - Prevention of deadenylation (Guzeloglu-Kayisli et al., 2008)
Histone methyltransferase (H4-K20 specific)	**SUV420H1**	Down-regulated in 8 vs 14 mo contrast (FC -1,83; p<0,05)	- Gene expression regulation by trimethylation of histone H4 (H4K20) (Schotta et al., 2004; Souza et al., 2009) - Catalysis of H4K20me2 in vivo (Tsang et al., 2010) - Regulation of the ERK cascade in human cancer (Vougiouklakis et al., 2015)
Homeobox protein NANOG	**NANOG**	Up-regulated in 11 vs 14 mo contrast (FC 2,17; p<0,05)	- Transcription factor (Mitsui et al., 2003) - Inner cell mass and embryonic stem cells proliferation and self-renewal (Chambers et al., 2003) - Acquisition of embryonic stem cell pluripotency and prevention the differentiation into extraembryonic endoderm and trophectoderm (Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998; Reik, 2007) - Blocks embryonic stem cells bone morphogenetic protein-induced mesoderm differentiation by binding to SMAD1 (Suzuki et al., 2006)
Plasminogen activator, urokinase	**PLAU**	Up-regulated in 11 vs 14 mo contrast (FC 1,86; p<0,05)	- Cleavage of the zymogen plasminogen, forming the active plasmin enzyme (Ellis et al., 1991) - Involved in implantation process in human (Khamsi et al., 1996), mouse (Sappino et al., 1989) and rat embryos (Aflalo et al., 2004) (1,36) - Linked to Quebec platelet disorder (PBD) (Paterson et al., 2010) - Cumulus cell expansion (Leyens et al., 2004)

			- Signal transduction (Tang et al., 1998)
TET methylcytosine dioxygenase 3	**TET3**	Up-regulated in 11 vs 14 mo contrast (FC 1,75; p<0,05)	- Methylcytosine dioxygenase activity (Tahiliani et al., 2009) - DNA demethylation in mouse (Gu et al., 2011) and in bovine (Page-Lariviere and Sirard, 2014) -Activation of somatic Oct4 gene in mouse (Gu et al., 2011) - Histone H3-K4 trimethylation (Deplus et al., 2013) - Embryonic stem cell maintenance and inner cell mass cell specification (Ito et al., 2010)
Cytochrome c oxidase subunit 8B	**COX8B**	Down-regulated in 11 vs 14 mo contrast (FC -2,20; p<0,05)	- Lipid metabolism (Seale et al., 2007) - Brown-fat specific marker of differentiation (Garcia et al., 2016; Seale et al., 2007) - Cytochrome c oxydase activity/mitochondrial oxydative phosphorylation (Puigserver and Spiegelman, 2003)
SMAD family member 6	**SMAD6**	Down-regulated in the 11 vs 14 mo contrast (FC -2,52; p<0,05)	- Transcription co-repressor of BMP signaling (Adelman et al., 2002; Bai et al., 2000) - Inhibition of TGFβ family signaling (Imamura et al., 1997; Kawabata et al., 1999; Massague, 1998) - Promotion of neuronal differentiation through inhibition of the Wnt/β-catenin pathway (Xie et al., 2011) - Inhibition of erythropoiesis in human cord blood hematopoietic (Kang et al., 2012)
Acyl-CoA synthetase short-chain family member 1	**ACSS1**	Up-regulated in both contrasts ($FC_{8\text{-}14mo}$ 1,66; p<0,05 $FC_{11\text{-}14\ mo}$ 1,55; p<0,05)	- Acetate metabolism and acetyl-CoA biosynthesis in mitochondria (Fujino et al., 2001) - Fatty acid oxidation, increased insulin sensitivity and muscle strength (Gu et al., 2009; Sahlin and Harris, 2008) - Involved in acetate-dependent lipid synthesis for the survival of cancer cells with a low-glycolysis phenotype (Yun et al., 2009)
Inositol polyphosphate phosphatase-like 1	**INPPL1**	Up-regulated in both contrasts ($FC_{8\text{-}14mo}$ 1,66; p<0,05 $FC_{11\text{-}14\ mo}$ 1,55; p<0,05)	- Cell adhesion and spreading (Prasad et al., 2001) - Glucose homeostasis and negative regulation of insulin signalling *in vivo* and *in vitro* (Blero et al., 2001; Dyson et al., 2005; Ishihara et al., 1999; Pesesse et al., 1997) -Associated with the human metabolic syndrome (Hyvonen et al., 2012) and in human and rodent Type II diabetes (Marion et al., 2002) - Lipid phosphatase activity (Pesesse et al., 1997) - Regulation of gene expression in mouse embryo (Rohrschneider et al., 2005)
Eukaryotic translation initiation factor 4E family member 2	**EIF4E2**	Down-regulated in both contrasts ($FC_{8\text{-}14mo}$ -1,54; p<0,05 $FC_{11\text{-}14\ mo}$ -2,10; p<0,05)	- Cap-dependent translation initiation activity in eukaryotic cells (Sonenberg and Gingras, 1998; Uniacke et al., 2012; Yi et al., 2013) - Growth regulation (Flynn and Proud, 1996) - Bovine mammary gland development (Long et al., 2001) - Translation of hypoxia response proteins, which drives human cancer tumor progression (Uniacke et al., 2014; Yi et al., 2013)

Table 2.6 Genes of interest and their known functions

2.6.3 Microarray analysis - upstream analysis

While individual gene variation is interesting to investigate, the strength of Ingenuity Pathway Analysis resides in the possibility to investigate how the different conditions

affect how the genes interact with each other, and how they affect signaling pathways and biological functions. In this study, the bioinformatics upstream analysis highlighted that age will affect the mTOR and PPAR signaling pathways, as well as the NRF2-mediated oxidative stress response pathway. The p values associated with these pathways confirmed significance (p values ranging from $p < 1.51 \times 10^{-9}$ to $p < 2.20 \times 10^{-2}$), which reinforces our suggestion that a metabolic cause potentially explains the differences that are observed between embryos from peri-pubertal and adult subjects.

The mTOR (mammalian target of rapamycin) pathway (inhibited in the 8 vs 14 months contrast - z -1.236; $p < 1.51 \times 10^{-9}$) is associated with cell survival and proliferation, and its malfunction may result in the development of some metabolic diseases (Gingras et al., 2001; Huang and Houghton, 2003; Shi et al., 2003). Moreover, Murakami et al. (2004) reported that mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. This pathway is mediated by hormonal (cytokines, insulin, growth factors) and nutritional (amino acids) stimuli (Shah et al., 2000; van Sluijters et al., 2000) and is involved in processes such as myogenesis (Erbay and Chen, 2001; Shu et al., 2002), adipogenesis (Bell et al., 2000; Gagnon et al., 2001; Yeh et al., 1995), insulin signaling regulation, and pancreas β cells insulin secretion (Xu et al., 1998a; Xu et al., 1998b).

The peroxysome proliferator-activated receptor (PPAR) signaling pathway is mainly associated with lipid metabolism, adipogenesis, and inflammation (Barish et al., 2006; Varga et al., 2011) and was also inhibited in the 8 vs 14 months contrast (z -0.926; $p < 6.80 \times 10^{-6}$). In fact, PPARs are activated in the presence of fatty acid ligands and are therefore involved in the regulation of lipid metabolism gene expression. It was also reported that PPARδ plays a role in fatty acid oxidation in the heart, liver, and muscle (Barish et al., 2006; Poulsen et al., 2012). On the other hand, PPARϒ is important for adipogenesis, insulin sensivity (Tontonoz and Spiegelman, 2008), trophoblast differentiation, and metabolism, as well as in cardiac, placental, and adipose tissue development (Barak et al., 1999).

Finally, the NRF2-mediated oxidative stress response pathway is associated with embryonic survival under oxidative stress conditions, NRF2 (nuclear factor (erythroid derived) like 2) being the central gene that regulates the activity of antioxidant, detoxifying, and stress response enzymes. Reactive intermediates, such as free radicals and peroxides can affect cell functions and constituents. Oxidative stress also impairs mitochondrial function, which can ultimately cause apoptosis or altered embryonic development (Kadenbach et al., 2004), and is thought to be caused by endogenous and exogenous factors during the IVC of embryos (Guerin et al., 2001). Consistent with this finding, Gad et al. (2012) also reported that embryos cultured *in vitro* before the embryonic genome activation were more sensitive to oxidative stress, and that the NRF2-mediated oxidative stress response was the most important pathway activated as a result of culture. In the current study, the NRF2 mediated oxidative stress response pathway was globally inhibited in both age conditions compared to the reference condition ($z = -0.493$, $p < 5.48 \times 10^{-9}$ for the 8 vs 14 months contrast and $z = -1.257$, $p < 4.67 \times 10^{-9}$ for the 11 vs 14 months contrast). The inhibition of this pathway potentially indicates a better capacity to survive antioxidant reduction, allowing survival of the embryos with a quieter metabolism, a strategy known as the Warburg effect (Krisher and Prather, 2012; Le et al., 2010; Vander Heiden et al., 2009). The Warburg phenomenon was first described in 1924 by Otto Warburg (Vander Heiden et al., 2009) and refers to the process whereby malignant cancer cells undergo anaerobic glycolysis (resulting in lactate production) instead of oxidative phosphorylation in order to metabolize glucose (Cairns et al., 2011). The embryo is thought to behave in a similar way when exposed to sub-optimal conditions (Krisher and Prather, 2012). Cagnone and Sirard (2013) reported a similar situation when bovine embryos were exposed to high glucose concentrations *in vitro*: the blastocysts responded with increased anaerobic glycolysis to compensate for the impairment of energy metabolism, or to protect mitochondria from ROS production.

Considering the influence of these three pathways on mitochondral function (Figure 2.6), it would be safe to say that their inhibition can impair metabolic functions and embryo development.

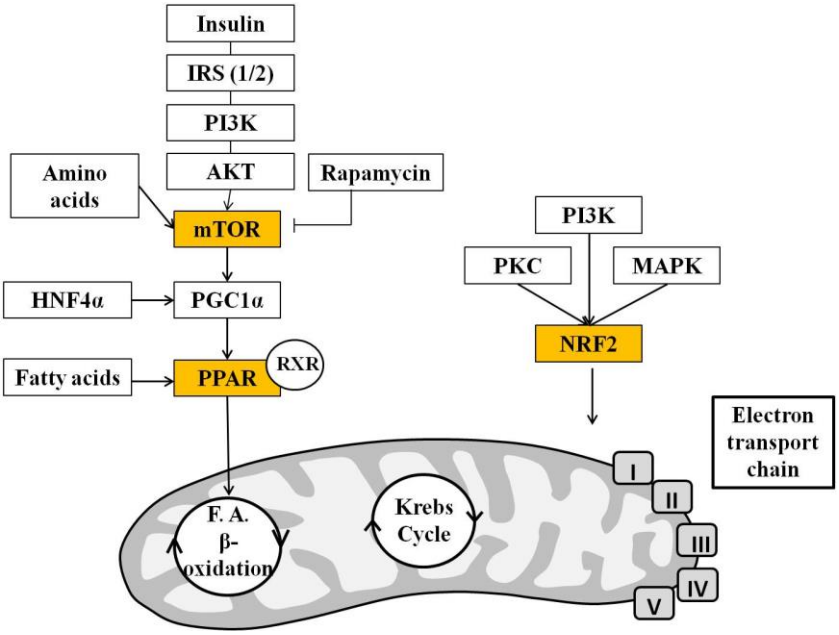

Figure 2.6 Implication of gene expression pathways in mitochondria function (adapted from Boland et al. (2013) and Scarpulla et al. (2012))

The upstream regulator analysis allowed the identification of transcripts that may have initiated specific biological processes early in development that are important later at the blastocyst stage, but these transcripts may no longer be present at that stage. The main upstream regulators that were identified in both contrasts are presented in Table 2,5. One of them was identified as major upstream regulator in both conditions: *Hepatocyte nuclear factor 4 alpha* (HNF4α), which is involved in hepatocyte differentiation and therefore controls the expression of many hepatic genes (Battle et al., 2006; Bolotin et al., 2010). This transcription factor also plays a role in lipid and glucose metabolism (Hayhurst et al., 2001) , as well as in insulin secretion by pancreatic β-cells (Byrne et al., 1995; Yamagata et al., 1996). Furthermore, HNF4α is expressed in the embryo and is involved in ATP production and mitochondrial enzyme activity (Odom et al., 2004; Wang et al., 2000). Our team recently suggested that an early expression of HNF4α, at the morula stage, would be linked to the transcriptomic modifications that were observed in blastocysts that had been exposed to stress conditions and that are associated with the Warburg effect (Cagnone and Sirard, 2013). HNF4α also controls PPARα and HIF1A which are key factors in the Warburg effect (Cairns et al., 2011).

In our study, functions downstream of HNF4α were significantly activated in the 8 vs 14 months contrast (z score = 0.780; $p < 1.42 \times 10^{-54}$), while they were significantly inhibited in the 11 vs 14 months contrast (z score = -0.119; $p < 4.64 \times 10^{-47}$). Our data are therefore consistent with previous reports from our team: Cagnone and Sirard (2013) suggested that the expression of HNF4α in the early embryo, followed by its loss at the blastocyst stage may be critical for the embryo to adapt to the metabolic stress induced by high glucose exposure. Embryos that fail to activate HNF4α, and therefore the Warburg metabolism, may not be able to develop past the morula stage. On the other hand, the activation of the Warburg metabolism, which favours lactate production and lipid accumulation, was associated with low quality *in vitro*-produced embryos (Cagnone and Sirard, 2013).

In conclusion, it seems that three metabolism pathways were affected in embryos from the younger animals, leading us to believe that their nutrition and/or the *in vitro* embryo culture conditions might not be optimal for oocytes/embryos from pre-pubertal females. The capacity of the embryo to develop in a certain environment does not necessarily imply that this environment is adequate - it rather highlights the capacity of the embryo to tolerate and adapt to non-optimal conditions. Hence, we believe that the main difference between embryos produced at peri-pubertal and at adult ages are related to metabolic conditions leading to a higher impact of *in vitro* conditions on blastocyts from younger heifers. Although this study showed that the effect of age is marginal and does not significantly impair embryo quality, the results suggest that modifications to the heifer nutritional program and to embryo culture conditions should be investigated in order to palliate the age effect.

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2.7 Supplemental Data

Gene	8 vs 14 months		11 vs 14 months	
	Same animals	Different animals	Same animals	Different animals
ACSS1		X		X
PABPC1	X		X	X
NANOG			X	
EIF4E2		X	X	
SUV420H1	X			
RALY			X	X
PRPF6			X	

Table 2.7 qPCR validation. Validation was done in two steps and compared microarray and qPCR results in two groups of animals: the exact same animals that were used for microarray analysis (Boviteq IDs 10989, 10991, 10994 and 10995) constituted the first group (*same animals*), while the second group (*different animals*) corresponded to four different individuals (Boviteq IDs 10990, 10992, 10993, 10996). Each candidate gene is shown to confirm the microarray data in one or more conditions.

CHAPITRE 3: Epigenetic evaluation of bovine blastocysts obtained from peri-pubertal heifers at 8, 11 and 14 months using the same adult bull and *in vitro* fertilization.

3.1 Résumé

Ce projet vise à documenter l'effet de l'âge sur la qualité de l'embryon bovin. Pour ce faire, dix jeunes femelles Holstein ont subi trois cycles de stimulation ovarienne, permettant trois collectes d'ovocytes (8, 11, 14 mois). Ces ovocytes ont ensuite été fécondés *in vitro*, puis la plateforme EmbryoGENE a permis d'évaluer les patrons de méthylation de l'ADN au stade blastocyste. L'analyse de contraste selon l'âge (8 vs 14 mois et 11 vs 14 mois) a donc mis en évidence 5787 régions différentiellement méthylées (DMRs) pour le premier contraste et 3658 pour le deuxième, alors que les résultats présentent une distribution non-aléatoire des DMRs au sein des différentes régions du génome. On observe une tendance à l'hyperméthylation chez les embryons des donneuses de 8 mois, alors que l'inverse est noté chez les embryons de celles de 11 mois. Finalement, une analyse *upstream* a permis d'isoler certains gènes qui influenceraient l'expression de cibles sensibles à la méthylation.

3.2 Abstract

In the dairy industry, the high selection pressure combined with the increased efficiency of assisted reproduction technologies (ART) are leading towards the use of younger females for reproduction purpose, which is known to reduce the interval between generations. This situation could impair embryo quality, decreasing the success rate of the ART procedures and the values of resulting offspring. This work complements a previous study by our team on transcriptomic signature associated with age and aims to document the effects of oocyte donor age on embryo DNA methylation, in order to caracterize the epigenetic consequences of using young females for reproduction purpose.

Young Holstein heifers (n=10) were used at three different ages for ovarian stimulation protocols and oocyte collections (at 8, 11 and 14 months). All of the oocytes were fertilized *in vitro* with the semen of one adult bull, generating three lots of embryos per animal. Each animal was its own control for the evaluation of the effects of age. The EmbryoGENE plateform was used for the assessment of DNA methylation status at the blastocyst stage. Contrasts were set with animals at 8 vs 14 months and at 11 vs 14 months and used for microarray hybridization.

Age-related contrast analysis (8 vs 14 mo and 11 vs 14 mo) identified 5787 differentially methylated regions (DMRs) for the first contrast, and 3658 for the second. In both conditions, the results display a non-random distribution of the DMRs across the different regions. In fact, the promoter, distal promoter as well as in the intronic regions contain high proportions of the DMRs in both contrasts. Embryos from 8-month-old donors appear to show more hypermethylation, while embryos from 11 month-old donors display a less different phenotype. The upstream regulator genes TP53, TGFβ1, TNF and HNF4α were found to influence the expression of methylation sensitive targets, which are shown to be more hypermethylated in embryos from younger donors.

3.3 Introduction

In the dairy industry, the use of young animals for reproduction purpose has become possible through the development of ovum pick up (OPU) methods and the development of *in vitro* embryo production (IVM, IVF, IVC) and embryo transfer (ET), which are combined to the increased efficiency of genomic selection. Since it is possible to identify the best animals early in life, the possibility of retrieving oocytes from hormonally stimulated pre-pubertal and peri-pubertal heifers using ultrasound (Brogliatti and Adams, 1996; Fry et al., 1998; Khatir et al., 1998b; Majerus et al., 1999; Presicce et al., 1997; Rick et al., 1996) is appealing. The procedure does not generate subsequent consequences on the health and production potential of the donors (Ax et al., 2005; Majerus et al., 1999; Presicce et al., 1997) and the genetic gain of dairy herds is accelerated (Doormaal, 2012; Lohuis, 1995a).

Many research groups have studied the characteristics of the oocytes and the embryos derived from pre- and peri-pubertal bovine donors, which are considered less competent compared to gametes from mature cows (> 14 months of age) (Armstrong, 2001; Damiani et al., 1996; Duby et al., 1996; Khatir et al., 1996; Presicce et al., 1997; Revel et al., 1995; Seidel et al., 1971). This situation is highlighted by a reduced oocyte developmental competence and efficiency to undergo maturation (Gandolfi et al., 1998; Khatir et al., 1998a; Salamone et al., 2001), reduced oocyte protein expression (Gandolfi et al., 1998; Levesque and Sirard, 1994), and embryo rate (Landry et al., 2016), higher rates of embryonic loss after embryo transfer(Zaraza et al., 2010) and a lower live and healthy offspring rates (Ax et al., 2005; Khatir et al., 1998b; Majerus et al., 1999; Palma et al., 2001; Revel et al., 1995)

The lower quality of oocyte and embryos can be associated with the oocyte legacy as demonstrated by the transcriptomic analysis (Morin-Dore et al., 2017) but aslo by the epigenetic modifications either coming from the oocyte DNA itself or resulting from the different early embryo physiology caused by a different oocyte maternal RNA contribution. The litterature now supports that some epigenetic modifications are heritable and can occur in the first week post-fertilization, when the embryo is particularily vulnerable (Wang et al., 2014). During that period in mammals, changes

in the DNA methylation are known to influence the development and the health of the offspring and, eventually, of its own progeny (Evertts et al., 2010; Turek-Plewa and Jagodzinski, 2005).

DNA methylation is thought to influence embryo development as the most important epigenetic mechanism involved in gene expression regulation. DNA methyltransferases (DNMTs) are responsible for the transfer of a methyl group to the cytosine residue of CpG dinucleotides, which results in the DNA sequence being less accessible to the transcription machinery and, thus, reducing or inhibiting gene transcription (Bird and Wolffe, 1999). In mammals, the epigenetic marks are acquired during gametogenesis and early embryo development, where two important demethylation and methylation waves establish the epigenome (Denis et al., 2011; Wu and Zhang, 2012). The DNA methylation pattern changes in a sex and stage dependent manner during bovine preimplantation embryo development (Dobbs et al., 2013). After fertilization, the pattern of DNA methylation changes in a dynamic way through demethylation and re-methylation process. Wang and collaborators (Wang et al., 2014) have recently demonstrated using different lines of mice that the paternal genome, along with an important portion of the maternal genome, undergoes active demethylation following fertilization. This group has also pointed to the presence of 5C-hydroxyl-methyl groups in both genomes, which seem to be replaced by non-methylated cytosines during embryonic development. Along with the active demethylation process, the maternal genome is also subjected to passive demethylation, through the exclusion of DNMT1, preventing the addition of methyl groups to newly synthesized DNA (Bestor, 2000). In mammals, DNA methylation is essential for normal embryonic development, X chromosome inactivation and genomic imprinting (Bird, 2002).

It is known that embryos are sensible and respond to their environment (Cagnone and Sirard, 2013; Ginther, 2016; Nachreiner and Ginther, 1969) and that stress occurring during early embryonic development may impact tissue structure and functions, which may predispose the individual and its progeny to diseases. It is also well documented that embryos produced *in vitro* show greater susceptibility to develop

epigenetic-related diseases as well as metabolic and imprinting disorders (Maher et al., 2003). *In vitro* produced embryos display transcriptomic profiles that show alterations when compared to *in vivo* produced embryos (Gad et al., 2012). Using the same EmbryoGENE plateform, the effects associated with ART were linked with a time-dependant increase in modification of DNA methylation (DMRs) (Salilew-Wondim et al., 2015)). This group has reported higher DNA methylation dysregulation in *in vitro* produced blastocysts, when compared with embryos at zygote, 4-cell and 16-cell stages. Furthermore, it has been previously reported by our group that embryos from young donors show greater sensibility to the *in vitro* conditions, compared to embryos from adult donors (Morin-Dore et al., 2017). In fact, a transcriptomic analysis has highlighted key elements supporting the idea of a metabolic cause to explain the differences that are observed between blastocysts from peri-pubertal and adult subjects. The new dataset presented in this paper described a non-random modification of methylation distribution in blastocysts associated with the age of the oocyte donor as an additional possible explanation of the embryonic or post-embryonic phenotypes.

3.4 Material and methods

3.4.1 Chemicals

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

3.4.2 Animals

This work is complementary to a study recently published by our team (Morin-Dore et al., 2017) and therefore displays similarities in the experimental design. Briefly, prepubertal *Bos taurus* Holstein heifers (n=10) were used for this experiment. Each heifer underwent three ovarian stimulation cycles, resulting in three gamete collections, at the ages of 8, 11 (average 10.8) and 14 (average 13.7) months. The gametes were fertilized *in vitro* with spermatozoa from one adult male, producing three embryo lots per animal. Each heifer was its own control for the measurement of age effect. This animal phase of the study was performed in an industrial IVF setting

at Boviteq (Saint-Hyacinthe, QC, Canada), a specialized center for bovine embryo transfer and other assisted reproduction technologies involved in research and development.

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 our routine commercial activities. The study did not require handling animals on university premises.

3.4.3 Ovarian stimulation and gamete collection

Each heifer was first treated with progesterone (CIDR) during the luteal phase in order to repress dominant follicles to reduce the risk of spontaneous ovulation. The dominant follicle was aspirated 36 h prior to administration of hormones. The ovarian stimulation program consisted of six injections of 30 mg FSH (Folltropin-V, Bioniche Animal Health, Belleville, ON, Canada) administered at 12 h intervals. Ovum pick up was performed 43 h after the last FSH injection, the optimal *coasting* (FSH starvation) period (Nivet et al., 2012). Using transvaginal ultrasonography, follicular diameters were measured and cumulus-oocyte complexes (COCs) were collected by OPU under epidural (COOK Medical, Bloomington, IN, USA). COCs were collected in warm HEPES-buffered Tyrode's medium (TLH) containing Hepalean (10 µL/mL) and transferred to the laboratory for IVM. The gamete collection procedures took place in the time period between October 2014 and May 2015. *In vitro* maturation, *in vitro* fertilization and *in vitro* culture was performed by Boviteq, following the protocol previously described (Morin-Dore et al., 2017). Blastocyst development was assessed using industrial criteria for embryo transfer as recommended by the International Embryo Transfer Society at days 7 and 8 post-fertilization (Nivet et al.,

2012). The embryos were finally snap-frozen in liquid nitrogen and stored at -80°C until DNA extraction.

3.4.4 Genomic DNA isolation and DNA fragmentation

Epigenetic analysis was performed on blastocysts from four heifers (Boviteq IDs 10989, 10991, 10994 and 10995) using microarray hybridization. Five embryos per heifer were selected according to developmental stage and quality, and pooled. DNA methylation can be assessed by microarray analysis, which allows the comparison of age related contrasts and differentially methylated regions (DMRs) identification in embryos. Our group contributed to the development of the EmbryoGENE epigenetic platform, which has previously been used to assess the influence of the environment on embryo development.

As described by Salilew-Wondim et al. (2015), genomic DNA was isolated from all blastocysts pools (5 embryos per pool) using the *Allprep DNA/RNA micro kit* (Qiagen) according to the manufacturer's protocol, following the *Parallel gDNA and total RNA extraction* protocol and finally eluted in 30 µl elution buffer. Following the EmbryoGENE *DNA fragmentation and amplification* protocol, the genome DNA was then fragmented using the MseI enzyme in the presence of 0.5 µl bovine serum albumin (100x), 10 µl NEB buffer 4 (10x) and spike-in controls at 37 ^{O}C for 16 h followed by further incubation at 65 ^{O}C for 20 min. After the end of incubation period, gDNA was precipitated using linear acrylamide (Ambion) and sodium acetate (3M, pH 5.2) and ethanol. After repeated washing in 70% ethanol, the pellets were dissolved in 5 µl nuclease free water (Salilew-Wondim et al., 2015).

3.4.5 Adapter ligation and methyl sensitive enzymes digestion

The protocols regarding adapter/linker ligation and methyl sensitive enzyme cleavage suitable for EmbryoGENE DNA Methylation Array (EDMA) platform have been described previously in detail (Shojaei Saadi et al., 2014). Briefly, MseLig12 (100 µM) and MseLig21 (100 µM) adapters were ligated at the fragmented gDNA sites in the presence of 10× One-Phor-All (OPA) Buffer PLUS (Pharmacia Biotech) and nuclease free water. The reaction was incubated at 65 ^{O}C for 20 min to initiate

annealing and the temperature was decreased to 15 ^{O}C at a rate of 1 ^{O}C /min. Following this, ATP (10mM) and T4 DNA Ligase (5U/μL) was added to the reaction and incubated for 16 hrs at 15 ^{O}C. At the end of reaction, unmethylated genomic sites within the MseI-MseI region of the fragmented were cleaved by DNA FastDigest™ methyl-sensitive restriction endonucleases enzymes, HpaII, Hinp1I and AciI (Salilew-Wondim et al., 2015).

3.4.6 Confirmation of methyl sensitive cleavage

Cleavage by methyl sensitive enzyme was confirmed by analyzing the spike-in controls added during gDNA fragmentation step. For this, qPCR was performed using primers targeting HpaII, HinP1I or AciI sites. Undigested spike-in (1/1000 dilution) was used as a positive control and non-template controls were also added as negative control. The amplification plot and the dissociation curve were evaluated for each of the HpaII, AciI or HinP1I primer sets.

3.4.7 Genomic DNA amplification

After methyl sensitive enzyme cleave, the gDNA was subjected to ethanol precipitation and the resulting gDNA pellets were dissolved in nuclease free water. The gDNA was then subjected to two round of ligation-mediated polymerase chain reaction (LM-PCR) as indicated previously (Klein et al., 1999; Shojaei Saadi et al., 2014). After PCR amplification, a sample of the PCR product was loaded onto 3% ethidium bromide stained agarose gel electrophoresis to confirm the distribution of the digested gDNA. Once the presence of several bands were observed, further amplification was performed. Amplification reaction was performed in 4 steps: at first, the samples were incubated at 94 ^{O}C for 60 min, 65 ^{O}C for 30 s and 72 ^{O}C for 2 min, followed by 14 cycles at 94 ^{O}C for 40 s, 65 ^{O}C for 30 s and 72 ^{O}C for 90 s. The reaction then continued for 9 cycles at 94 ^{O}C for 40 s, 65 ^{O}C for 30 s and 72 ^{O}C for 2 min and finally terminated after incubating at 72 ^{O}C for 5 min.

The PCR products were then purified using *PCR purification kit* (Qiagen) and finally eluted in 30 μl of 1/10 buffer elution buffer. Following this, the gDNA was incubated

in the presence of buffer 4 NEB (10X), BSA (100x), Msel (10 u/µl) and water at 37 ^{O}C followed by incubation at 65 ^{O}C for 20 min. At the end of reaction, the samples were purified using *PCR purification kit* (Qiagen) and finally eluted in 30 µl of 1/10 Buffer elution buffer. The quantity of amplified digested gDNA after linker removal was measured using NanoDrop 1000 spectrophotometer.

3.4.8 Dye labeling and hybridization

Two micrograms of gDNA was labelled with either Cy-3 or Cy-5 dyes using *ULS Fluorescent gDNA labelling kit* (Kreatech Biotechnology) at 85 ^{O}C for 30 min. The samples were then purified using the Qiagen *PCR purification kit* to remove uncoupled dyes. The dye incorporation and DNA concentration were measured using NanoDrop 1000 spectrophotometer. Hybridizations were performed by mixing 1 µg of labeled gDNA sample of the treatment group (8 or 11 months), 1 µg of labeled gDNA of the control blastocyst group (14 months), 25 µl of bovine Cot-1 DNA (1.0 mg/mL, Applied Genetics Laboratories), 2.6 µl of Agilent 100x Blocking Agent and 130 µl of Agilent 2x HI-RPM Hybridization Buffer (Agilent Technologies). The samples were incubated at 95°C for 3 min, at 37°C for 30 min and then 65 µl of Agilent-CGH Block was added to each of the samples. The samples were loaded onto the microarray and hybridization was performed in a hybridization oven (Shel Lab) for 40 hrs at 65°C and 20 rpm. The hybridizations were performed according to the following design: for each heifer, the two peri-pubertal age periods were individually compared to the control age period (i.e., 8 months vs 14 months; 11 months vs 14 months) for a total of two comparisons per animal. Overall, eight hybridizations, corresponding to the four biological replicates and two comparisons, were performed in EmbryoGENE DNA Methylation Array (EDMA). The slides were scanned using the Tecan PowerScanner microarray scanner (Tecan Group Ltd, Mðnnerdorf, Switzerland) and features were extracted using ArrayPro 6.4 (Media Cybernetics, Bethesda, MD) (Labrecque et al., 2013).

3.4.9 Array data analysis

The array data analysis methods and downstream analysis pipelines are described in details (Shojaei Saadi et al., 2014). Briefly, after Loess normalization, quantile inter-array scale normalization was performed and fitted to a linear model and thus, Bayesian statistics of differential expression were obtained. Differentially methylated probes were then identified from the full set of probes using linear models for microarray data (limma) [27]. Probes which showed significant ($p < 0.05$) differences with absolute log2 (fold-change) 1.5 between the treatment and the reference sample were considered as differentially methylated regions (DMRs). Therefore, probes which showed a significant increase in signal intensity by 1.5 folds and higher in 8- and 11-months groups compared to the control group were considered as hypermethylated probes while probes which displayed a reduced signal intensity by 1.5 folds and higher in 8 and 11 months groups compared to the 14 months group were considered as hypomethylated probes. Enrichment analysis for genome-scale DNA methylation data was performed using string of integrated scripts that sorts the genome data into CpG island density, CpG island length, CpG island distance, genomic location and types of repetitive elements. Moreover, the gene ontology enrichment analysis was performed for genes that were associated with differentially methylated regions. Probes were associated with the GO terms of a gene provided the probe fragment falls within the introns, exons or promoter of a particular gene. For comparative analysis of methylation profile and gene expression data, the genes absolute log2 (-fold-change) 1.5 and p value < 0.05 were selected from our previous data (Morin-Doré et al. 2016).

The gene lists were then analyzed with Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com). All statistically significant genes ($P < 0.05$; fold change > 1.5 or <-1.5) were uploaded to the application and each identifier was mapped to its corresponding object in the Ingenuity database. It is clear that the fold change used in epigenomic analysis is not comparable to the one used in transcriptomic analysis, but Ingenuity's function is to identify networks that link genes that are known to work together or upstream/downstream of each other. The

analysis obtained indicates that the changes observed in methylation are not random as they target groups of genes more than others. The p value is extracted by comparison to randomized distribution. Also, the direction of the change in methylation may be opposite to the RNA level, but this inversion does not change the IPA interpretation if we exclude the directional effect. The functional analysis identified the upstream regulators that were the most influent to the molecules in the dataset (Labrecque et al., 2014). Network Analyst web application allowed the identification of dominant gene networks (www.networkanalyst.com).

3.4.10 Validation of differentially methylated regions

A validation step is required to confirm our data.

3.5 Results

3.5.1 Identification of the number of probes above background

Embryos from four animals were used for array analysis based on two age-related contrasts (8 vs 14 months and 11 vs 14 months), 14 months being considered as the reference condition. The use of the same animals at different ages fertilized by the same bull ejaculate is a quite powerful design to extract fine differences in the epigenetic status, as this intra-individual comparison minimizes bias. The EmbryoGENE DNA Methylation Array (EDMA) consists of 414 565 probes; of this number, 20 355 correspond to genes, while 34 378 are associated to CpG islands (Shojaei Saadi et al., 2014). The repartition of the probes in gene regions, in CpG content and neighborhood context and in repetitive element classes is well detailed by Shojaei Saadi et al. (2014).

After the gDNA extraction, restriction enzymes fragmentation, adapter ligation, methyl-sensitive restriction enzymes digestion (HpaII, AciI and Hinp1L), digestion quality control, ligation-mediated PCR amplification, labelling and hybridization steps, the probes whose intensity signal surpassed background signal were evaluated for downstream analysis. As mentioned, the probes of interest were identified as such

if their intensity level exceeded the mean fluorescence intensity plus four times the standard deviation of the non-specific negative control probes.

Figure 3.1 shows the number of probes that were expressed above the background in each age condition, in each contrast. In both cases, the blue circle includes the probes expressed above the background in all four arrays for the control group (14 months) while the red circle corresponds to the probes expressed above the background in all four arrays for the treatment groups (8 months or 11 months). Finally, the yellow circle highlights the number of probes that are differentially methylated, but which are not necessarily expressed above background in all four arrays of the two given conditions.

It is therefore possible to observe that, for the first contrast, 171 414 probes were expressed above the background in all arrays in the 14 months group, while 204 414 were expressed above background in all arrays of the 8-month group. 839 probes were expressed in all arrays of the control condition, but not in all arrays of the 8-month condition, while 1 425 were expressed in all arrays of the 8-month group only, but not in all arrays of the control condition. Interestingly, 159 062 probes were detected in all arrays and both conditions, generating 2 735 DMRs (1,72%). Comparatively, 225 387 probes were expressed above the background in all arrays of the control condition of the second contrast, while 205 457 were expressed above the background in all arrays of the 11-month group. It is possible to observe that 815 probes were unique of the 14 months condition, whereas 527 were unique of the 11 months condition. Finally, 191 803 probes were detected in both conditions, 1 972 (1,03%) of these being considered as differentially methylated. The residual 788 DMRs in 8 vs 14 months contrast and the 344 in 11 vs 14 months contrast were significant but not present in all arrays. False discovery rate (FDR) selection was not applied as the conditions being compared, the effect of age on the same animals, does not generate sufficient differences to require a background reduction by the FDR procedure.

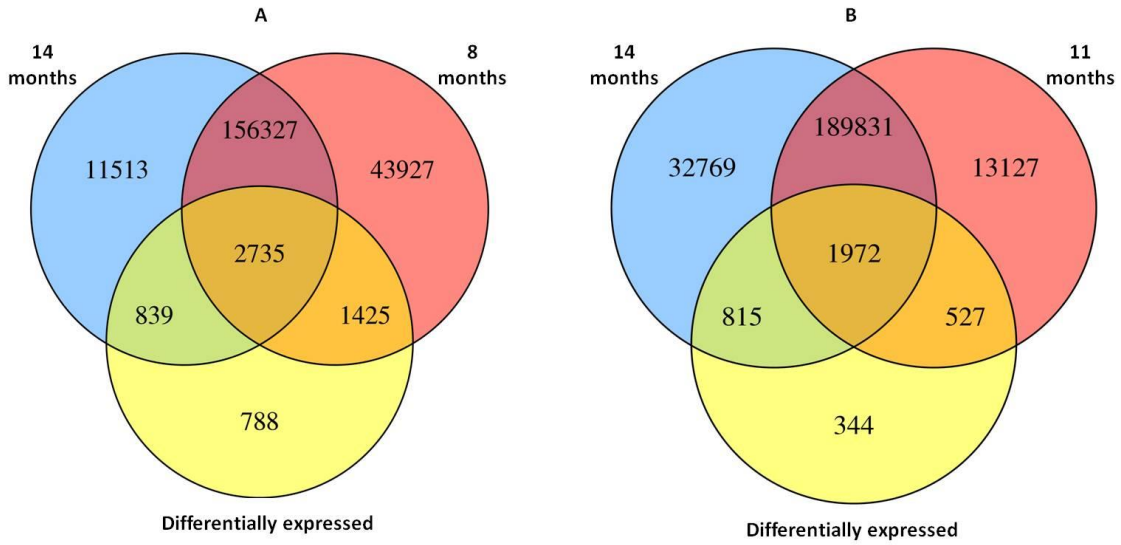

Figure 3.1. Probes expressed above background in all arrays of a given condition. a) 8 versus 14 months contrast; b) 11 versus 14 months contrast.

3.5.2 Identification of the differentially methylated regions (DMRs)

As mentioned above, differentially methylated probes were identified if they met the 1.5 fold-change and $p < 0.05$ cut-offs. Accordingly, the analysis of the differentially methylated regions (DMRs) has allowed to identify 5787 regions of interest in the first contrast and 3658 in the second. It is therefore possible to witness more methylation dys-regulation in the embryos obtained from the youngest donors. Of these numbers, 3068 probes (53%) were associated with more methylation in the 8 vs 14 months contrast. On the other hand, 42% of the DMRs (1544 probes) were hypermethylated in the 11 vs 14 months contrast. Embryos from 8 month-old animals are therefore seems to have slightly more methylated DMRs (11% more) than those obtained from adult animals, while the blastocysts from 11 month-old heifers display less demethylation, with respect to the reference condition.

The DMRs were analyzed and some were found in coding gene regions. This is the case for 1848 DMRs in the 8 vs 14 months contrast and for 1212 DMRs in the 11 vs 14 months contrast. Amongst these, 54% (1000 probes) of these regions were associated with hypermethylation in the first contrast and 46% (559 probes) displayed more methylation in the second contrast. This balanced distribution has also been

previously observed in embryos produced *in vitro* compared to *in vivo* (Salilew-Wondim et al., 2015).

We compared the two lists of DMRs and were able to identify 109 DMR-associated probes that were common to both conditions studied (Fig. 3.2). A total of 37 probes were found to be located in the intronic regions of known genes. Amongst these, the probes found in the introns of the genes ZC3H7B (Zinc Finger CCCH-Type Containing 7B), SLC6A4 (Sodium-dependent serotonin transporter), CLYBL (Citrate lyase beta like), DOK5 (Docking protein 5), NPAS3 (Neuronal PAS Domain Protein 3), FARS2 (Phenylalanyl-tRNA synthetase 2, mitochondrial), CLEC16A (C-type lectin domain family 16 member A) and SFRP1 (Secreted frizzled related protein 1) were more methylated in both contrast, while the ones that are potentially influencing INSIG1 (Insulin induced gene 1), DDX23 (DEAD-Box Helicase 23), KLHL12 (Kelch-like family member 12), CCDC60 (Coiled-Coil Domain Containing 60), GTF3C1 (General Transcription Factor IIIC Subunit 1), ANK3 (Ankyrin 3) were less methylated in both contrasts. Among these, INSIG1, DDX23 and KLHL12 are important for organism development and lipid metabolism. The promoter regions of the WNT10A (Wnt family member 10), RASL11B (RAS like family 11 member B), GRK6 (G protein-coupled receptor kinase 6), ABHD13 (Abhydrolase domain containing 13), CSK (C-src tyrosine kinase), TGFBR2 (Transforming growth factor beta receptor 2) genes were also found to be affected by DNA methylation.

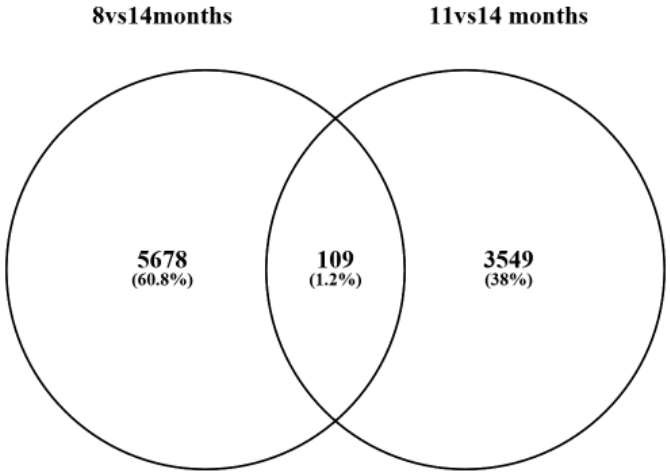

Figure 3.2. Number of differentially methylated regions in each contrast. A probe was considered as differentially methylated if its fold-change was > 1.5 or < -1.5 and its p value was < 0.05. 5787 probes were differentially methylated in the first contrast, while 3658 probes were differentially methylated in the second contrast. 109 DMR-associated probes were found to be common to both datasets.

3.5.3 Localization of the DMRs

EDMA analysis pipeline has allowed to localize the differentially methylated regions in the genome. We have therefore investigated the repartition of the DMRs through (1) gene regions, (2) CpG islands content and neighborhood context and (3) repetitive element classes.

3.5.3.1 DMRs in gene regions

Gene regions are targeted by 71% of the EDMA probes and include *intronic*, *exonic* and *promoter* regions, to which correspond 34% (n= 140 919), 13% (n=54 660) and 24% (n=99 516) of the total EDMA probes, respectively. The *promoter* region itself is divided into three groups of promoters: proximal promoter (first 1 kbp; 3% of the probes; n=11 947), intermediate promoter (first 5 kbp; 5% of the probes; n=22 334) and distal promoter (first 50 kbp; 16% of the probes; n=65 235) (Shojaei Saadi et al., 2014). From our data, we were able to determine that the intronic region contained the larger proportion of DMRs with 38,8% and 39,8% of the differentially methylated probes in the 8 vs 14 and 11 vs 14 months contrasts, respectively. This corresponds to 0,54% and 0,35% of the total EDMA probe set (Table 3.1). It is possible to observe more hypermethylation in the intronic regions of embryos from 8 months old donors in the first contrast, whereas there is more hypermethylation in embryos from adult animals in the second contrast (Fig. 3.3).

The promoter region also contains an important proportion of the DMRs, with 20,6% and 21,9% of the DMRs in the 8 vs 14 months and the 11 vs 14 months contrasts, respectively. In both contrasts, the distal promoter regions contain the highest proportion of DMRs, followed by the intermediate promoter and the proximal promoter regions. It is possible to observe that the proximal and intermediate

promoters are hypomethylated in embryos from 8 months old donors, while the distal promoter is more hypermethylated in this same age condition. On the other hand, all three promoter regions are hypomethylated in embryos from 11 months old heifers.

Finally, the exonic regions hold a smaller proportion of the DMRs, with 9,4% and 9,6% for the 8 months and 11 months contrasts, respectively. Even if less probes are associated with this region, it is possible to observe a clear hypermethylation in embryos from 8 and 11 month-old animals, in comparison to the embryos from adult animals.

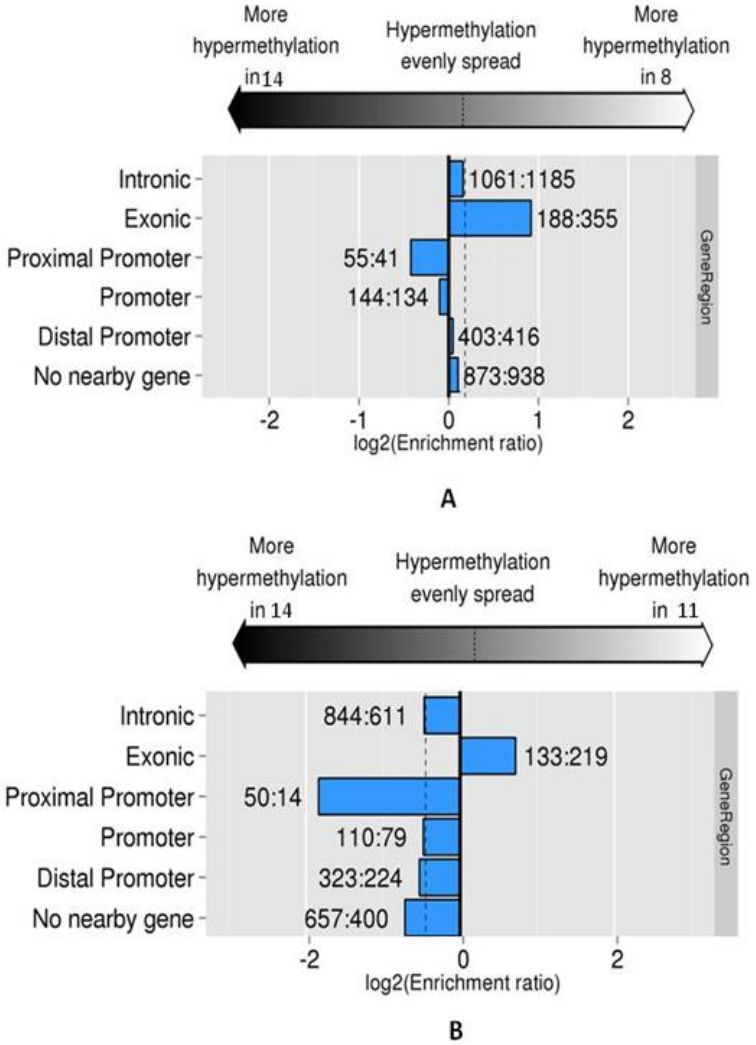

Figure 3.3. Differentially methylated gene regions in a) 8 versus 14 months contrast; b) 11 versus 14 months contrast.

Gene regions	Proportion of the DMRs		Proportion of total EDMA probes in each region
	8 vs 14 months	11 vs 14 months	
Intronic	38,8%	39,8%	34%
Exonic	9,4%	9,6%	13%
Promoter	20,6%	21,8%	24%
Proximal promoter	1,65%	1,75%	3%
Intermediate promoter	4,8%	5,2%	5%
Distal promoter	14,2%	14,9%	16%
Intergenic	31,3%	28,9%	29%

Table 3.1. Proportion of the DMRs and proportion of the DMRs in total EDMA probe set in gene regions.

3.5.3.2 DMRs in CpG islands and neighborhood context

CpG islands and neighborhood context regions include the *CpG islands themselves*, the *CpG shore*, the *CpG shelf* and *open sea* regions, to which correspond 13% (n= 52 228), 7% (n=31 385), 25% (n=102 642) and 55% (n=227 968) of the total EDMA probes, respectively. The non-CpG island DMRs were found to be in CpG shore, CpG shelf and open sea categories if the associated probe was located 1–2 kbp, 2–4 kbp and > 4 kbp from the nearest CpG island, respectively. The *CpG island* region itself is divided into two enrichment categories: *length* (small - 2,5%, n=10 411; intermediate - 8%, n=31 373; long - 2,5%, n=10 444) and *density* (low - 2,5%, n=10 862; intermediate - 8%, n=31 134; high - 2,5%, n=10 232) (Shojaei Saadi et al., 2014). In both contrasts, although CpG islands themselves only represent 2,6% of the DMRs, it is possible to witness the importance of hypermethylation in the embryos from younger animals.

For the first contrast, our data shows more hypermethylation in embryos from 8 month-old donors in all CpG regions, regardless of length or density (Fig. 3.4). It is

possible to see that *small* CpG islands are more hypermethylated in the embryos from 8 month-old donors, followed by *intermediate* and *long* CpG islands. For this same group of animals, it is also possible to observe more hypermethylation in high density CpG islands, followed by low density and intermediate density regions. A different pattern is observed in the 11 vs 14 month contrast, where our data shows more hypermethylation in embryos from 11 month-old animals, whereas it is possible to observe a very slight methylation difference between the groups in CpG shore region and more hypermethylation in the 14 month-old animals for CpG shelf and Open sea regions. It is possible to see that small CpG islands are more hypermethylated in embryos from 11 months animals, followed by intermediate and long CpG islands. With respect to density, we noticed that intermediate density CpG islands were more hypermethylated in the 11 months animals, followed by high density and low density CpG islands.

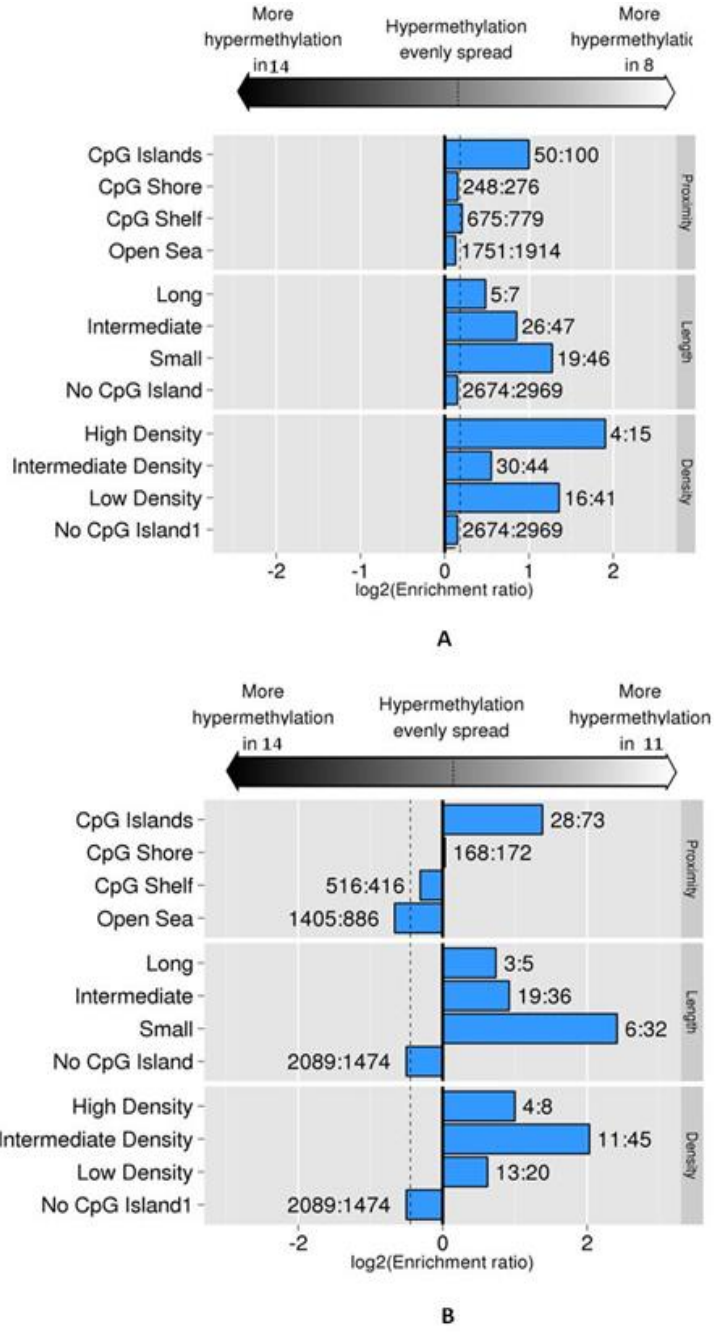

Figure 3.4. Differentially methylated CpG regions in a) 8 versus 14 months contrast; b) 11 versus 14 months contrast.

CpG content and neighborhood context	Proportion of the DMRs		Proportion of total EDMA probes in each region
	8 vs 14 months	11 vs 14 months	

CpG islands	2,6%	2,8%	13%
Long	0,2%	0,2%	2,5%
Intermediate	1,26%	1,5%	8%
Small	1,12%	1,03%	2,5%
High density	0,3%	0,3%	2,5%
Intermediate density	1,3%	1,5%	8%
Low density	1,0%	0,9%	2,5%
CpG shore	9,05%	9,3%	7%
CpG shelf	25,1%	25,5%	25%
Open sea	63,3%	62,6%	55%
No CpG	97,5%	97,4%	87%

Table 3.2. Proportion of the DMRs and proportion of the DMRs in total EDMA probe set in CpG content and neighborhood context.

3.5.3.3 DMRs in repetitive element classes

Finally, the repetitive element classes include short-interspersed repetitive elements (SINEs), long-interspersed repetitive elements (LINEs), simple repeats, long terminal repeats (LTR), low complexity repetitive elements and DNA regions, to which correspond 23% (n= 111 503), 14% (n=67 122), 10% (n=46 415), 6% (n=27 431), 2% (n=11 527) and 4% (n=17 507) of the total EDMA probes, respectively (Shojaei Saadi et al., 2014). Our data shows that all repetitive element classes show more hypermethylation in embryos from 8-month old donors, whereas more hypomethylation is observed in embryos from 11 months animals, except for the simple repeat class (Fig. 3.5, Table 3.3).

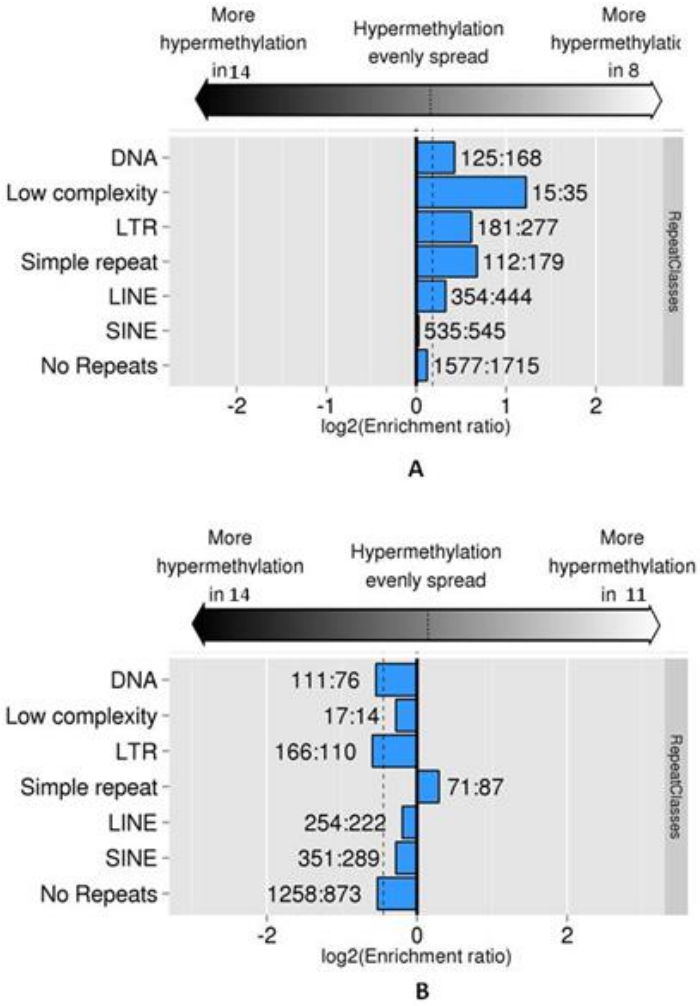

Figure 3.5. Differentially methylated repetitive element classes in a) 8 versus 14 months contrast; b) 11 versus 14 months contrast.

Repetitive Element Classes	Proportion of the DMRs		Proportion of total EDMA probes in each region
	8 vs 14 months	11 vs 14 months	
DNA	5,1%	5,1%	4%
Low complexity	0,9%	0,8%	2%
LTR	7,9%	7,5%	6%
Simple repeat	5,0%	4,3%	10%
LINE	13,8%	13,0%	14%
SINE	18,7%	17,5%	23%

No Repeats	56,9%	58,3%	41%

Table 3.3. Proportion of the DMRs and proportion of the DMRs in total EDMA probe set in repetitive element classes.

3.5.4 Chromosomal distribution of the DMRs

At first, whole-genome DNA methylation profile was assessed and represented by a circular graph, for each contrast (Figures 3.6 and 3.7). It is possible to notice a more global hypomethylation of most chromosomes associated with the embryos from 11-month-old donors (Fig. 3.7).

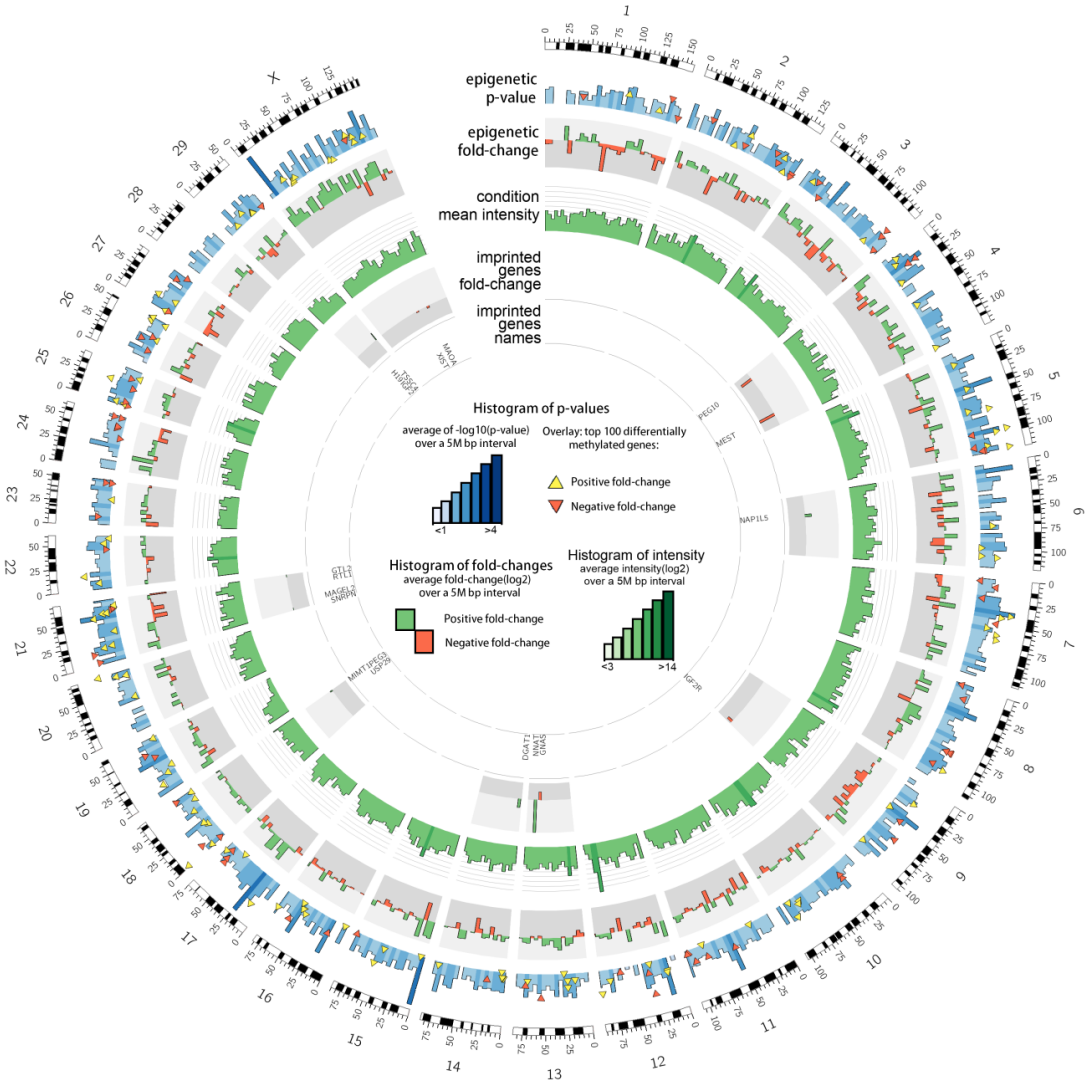

Figure 3.6. Circular graph presenting the overall methylation levels in the embryos from 8-month old donors (8 vs 14 months contrast). All layers display values over a window of 5000 bases, while the overlay of the p-value represents the location of the 100 most differentially methylated probes. Positive and negative fold-changes represent the level of hypomethylation and hypermethylation in blastocysts from younger donors.

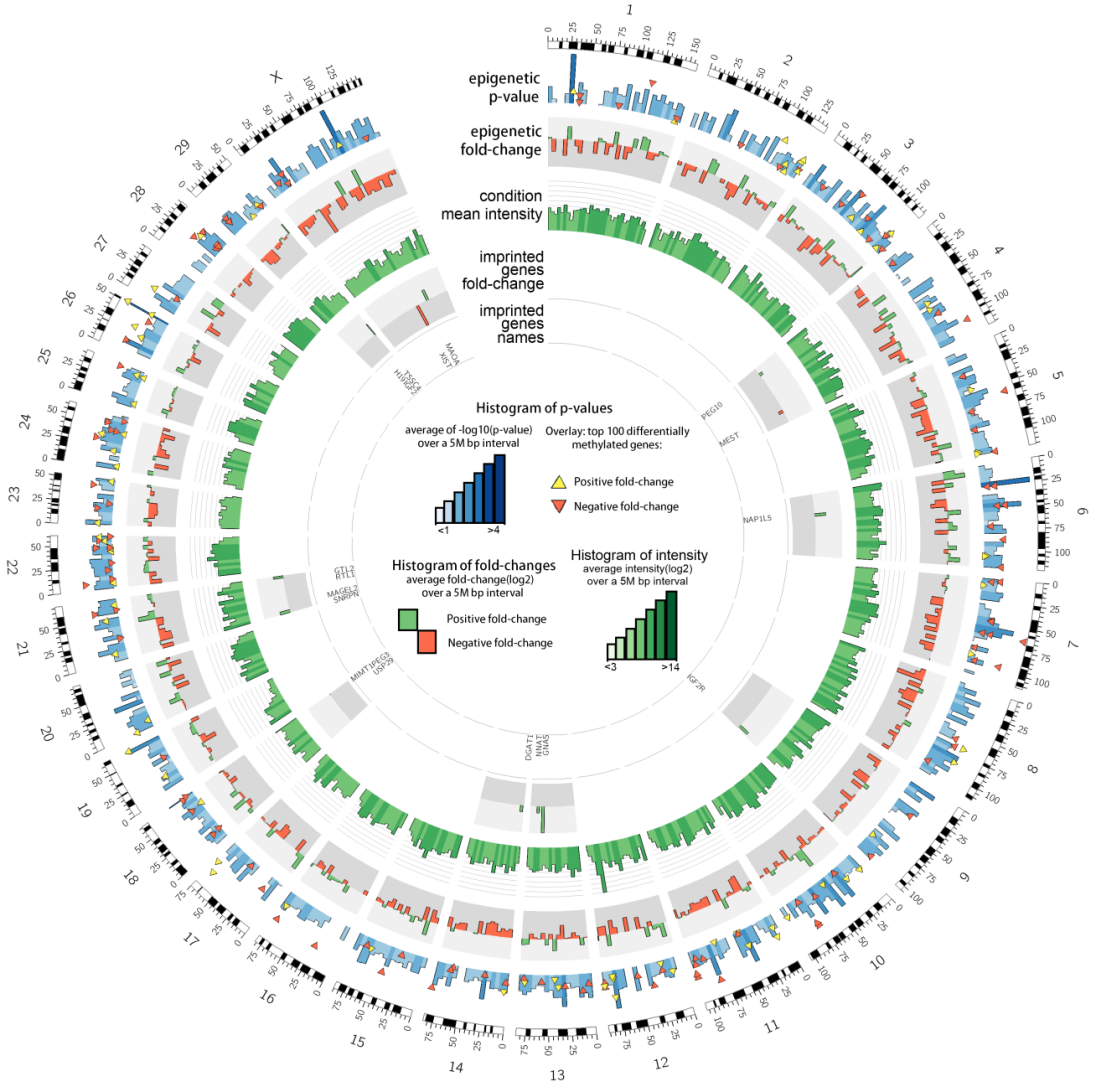

Figure 3.7. Circular graph presenting the overall methylation levels in the embryos from 11-month old donors (11 vs 14 months contrast). All layers display values over a window of 5000 bases, while the overlay of the p-value represents the location of the 100 most differentially methylated probes. Positive and negative fold-changes

represent the level of hypomethylation and hypermethylation in blastocysts from younger donors.

We have also analysed the distribution of the DMRs in the chromosomes, as a percentage of the total EDMA probes associated with each of the 29 autosomes and the X chromosome (Table 3.4). Slightly higher DMR representation as observed in chromosomes 8, 13, 16, 27 and 28 for the first contrast and in chromosomes 14, 15, 22 and X for the second contrast.

Chromosome	EDMA probes	8 vs 14 months		11 vs 14 months	
		DMRs	% EDMA probes	DMRs	% EDMA probes
1	16 413	226	1,38 %	148	0,90 %
2	17 594	239	1,36 %	159	0,90 %
3	18 808	256	1,36 %	169	0,89 %
4	15 559	175	1,12 %	140	0,90 %
5	19 019	258	1,36 %	162	0,85 %
6	12 768	163	1,28 %	90	0,70 %
7	18 538	267	1,44 %	146	0,79 %
8	15 076	237	1,57 %	145	0,96 %
9	12 452	159	1,28 %	93	0,75 %
10	16 322	247	1,51 %	156	0,96 %
11	19 433	297	1,53 %	175	0,90 %
12	11 584	122	1,05 %	115	0,99 %
13	16 717	268	1,60 %	165	0,99 %
14	11 533	171	1,48 %	124	1,08 %
15	12 314	190	1,54 %	123	1,00 %
16	14 007	218	1,56 %	135	0,96 %
17	12 898	171	1,33 %	94	0,73 %
18	18 245	261	1,43 %	142	0,78 %
19	13 070	154	1,18 %	112	0,86 %
20	9 325	139	1,49 %	71	0,76 %
21	12 761	193	1,51 %	122	0,96 %
22	13 153	202	1,54 %	134	1,02 %

23	11 861	162	1,37 %	96	0,81 %
24	10 515	136	1,29 %	95	0,90 %
25	14 363	203	1,41 %	116	0,81 %
26	9 629	146	1,52 %	90	0,93 %
27	6 868	111	1,62 %	61	0,89 %
28	6 638	112	1,69 %	64	0,96 %
29	10 531	132	1,25 %	86	0,82 %
X	11 797	170	1,44 %	130	1,10 %

Table 3.4. Distribution of the DMRs as a percentage of the total EDMA probes associated with each of the 29 autosomes and the X chromosome.

3.5.5 Upstream and network analysis

An upstream regulator approach with Ingenuity Pathway Analysis (IPA) allowed the analysis of the complete set of microarray data, highlighting the importance of genes and molecules of influence (see the *Methods* section for the explanation of how IPA is used on methylation data compared to transcriptomic data). This analysis pointed to molecules of interest, which are presented in Table 3.5.

Upstream regulator	8 vs 14 months			11 vs 14 months		
	Activation z score	P value	Differentially methylated targets	Activation z score	P value	Differentially methylated targets
Cellular tumor antigen p53 (TP53)	**-0,116**	**1.90×10^{-12}**	**153**	**-0,867**	**9.13×10^{-10}**	**105**
Erb-b2 receptor tyrosine kinase 2 (ERBB2)	**-0,334**	**2.86×10^{-8}**	**77**	-0,070	1.72×10^{-5}	49
Transforming growth factor β1 (TGFβ1)	**-0,833**	**4.18×10^{-7}**	**151**	**-0,299**	**5.98×10^{-13}**	**126**
L-dopa	**-0,791**	**6.34×10^{-6}**	**67**	0,196	$9,11 \times 10^{-7}$	55
Catenin β1 (CTNNB1)	**0.800**	**2.43×10^{-5}**	**71**	1,189	$8,17 \times 10^{-4}$	48
Cyclic AMP-responsive element-	**-2,613**	**3.03×10^{-5}**	**54**	0,648	$7,08 \times 10^{-3}$	33

binding protein 1 (CREB1)						
β-oestradiol	0,367	3.06×10^{-4}	148	**0,420**	**2.13×10^{-8}**	**121**
Tumor necrosis factor (TNF)	0,167	$6,76 \times 10^{-4}$	133	**1,339**	**$3,26 \times 10^{-7}$**	**110**
ERG transcription factor	1,500	$4,86 \times 10^{-5}$	27	**-0,258**	**$5,00 \times 10^{-7}$**	**24**
Myc proto-oncogene protein (MYC)	0,035	1.90×10^{-4}	93	-0,035	1.28×10^{-6}	71
Hepatocyte nuclear factor 4α (HNF4α)	1,332	1.44×10^{-3}	154	0,000	1.83×10^{-4}	117

Table 3.5. Upstream analysis to identify regulator molecules. IPA analysis identified genes and molecules that influenced pathways and functions in each contrast. Bold characters indicate the most important upstream regulators in each condition. The status was determined from the z score (from fold-change values of the target genes), while the p value confirmed significance. An arbitrary z score cutoff was established by the IPA software: the downstream network associated with a regulator was therefore considered as activated if $z > 2$ and inhibited if $z < -2$. For each upstream regulator molecule, the number of targets affected by methylation is mentioned.

Functions downstream of CTNNB1 (transcription regulator), β-oestradiol (chemical - endogenous mammalian), TNF (cytokine) and HNF4α (transcription regulator) were associated with a positive z-score in both contrasts while TP53 (transcription regulator), ERBB2 (kinase) and TGFβ1 (growth factor)-associated z-score were negative in both contrasts. ERG and MYC (transcription regulators) influence functions that had a tendency towards activation in the first contrast, and towards inhibition in the second. Finally, L-dopa (chemical - endogenous mammalian) and CREB1 (transcription regulator) presented the opposite pattern.

Network Analyst web application allowed analysis of the complete set of microarray data and identification of age-affected gene networks (Table 3.6). Networks involving structure and lipid metabolism were found to be dominant. The p values of all the presented pathways were significant.

Network	Number of genes (hits)	P value
Structural molecule activity	85	2.07×10^{-5}
Phospholipid binding	56	4.24×10^{-2}
Regulation of transcription from RNA polymerase II promoter	52	5.03×10^{-7}
Nuclease activity	44	1.04×10^{-5}
Lipid biosynthetic process	41	2.53×10^{-6}
Integrin binding	37	1.22×10^{-2}
Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amidines	33	1.34×10^{-2}
Fatty acid oxidation	29	4.17×10^{-4}
Steroid dehydrogenase activity	18	4.73×10^{-2}
Anatomical structure morphogenesis	15	4.88×10^{-5}

Table 3.6. Identification of age-affected gene networks using Network Analyst.

3.6 Discussion and conclusions

This study is the first to demonstrate the effect of donor age on bovine embryo quality by epigenetic analysis using peri-pubertal commercial stimulated Holstein subjects. It has been previously reported by our group that embryos from young donors show subtle but greater sensibility to the *in vitro* conditions, compared to embryos from adult donors (Morin-Dore et al., 2017). In fact, the transcriptomic analysis of the same embryos has highlighted key elements supporting the idea of a metabolic cause to explain the differences that are observed between blastocysts from peri-pubertal and adult subjects. Complementary to our previous study, the new dataset presented in this paper describes a non-random modification of methylation distribution in blastocysts associated with the age of the oocyte donor as an additional explanation of the embryonic and post-embryonic phenotypes. The use of the same animals at different ages is a quite powerful design to extract fine differences in the observed DNA methylation status, as this intra-individual comparison minimizes

bias. Throughout the study, the only parameter to vary was *age* while all other parameters such as environment, feeding, ovarian stimulation protocols, semen origin, and embryo culture conditions were kept constant for all animals. It is therefore possible to say that the differences observed between the groups were essentially associated with donor age.

3.6.1 Identification, localization and analysis of the DMRs

As mentioned, the probes of interest were first identified if their fluorescence intensity was found to exceed the background fluorescence intensity (Figure 3.1). We were then able to identify the differentially methylated probes in both contrasts, which were considered as such if their intensity level met the 1.5 fold-change and $p < 0.05$ cut-offs. This has allowed to highlight 5787 DMRs in the 8 vs 14 months contrast and 3658 DMRs in the 11 vs 14 months contrast (Figure 3.2), showing subtle, but greater methylation dys-regulation in embryos from younger donors. Of this number, 1848 and 1212 DMRs are found in known gene sequences in the first and second contrasts, respectively. Considering this, a very small proportion of the targets of interest were differentially methylated in at least one contrast (1,39% for the 8 vs 14 months contrast and 0.88% for the 11 vs 14 months contrast). Moreover, the difference in intensity level of the majority of these differentially methylated probes was close to the detection cut-off used (fold-changes ranging from -3.4993 to -1.5 and from 1.5 to 3.5299 ($p < 0.05$) in the first contrast, and from -3.3502 to -1.5 and from 1.5 to 3.1276 ($p < 0.05$) in the second contrast), suggesting that the effect of age on embryo quality may be subtle, although it is known that slight differences in DNA methylation can have a significant impact at the blastocyst stage.

The EmbryoGENE DNA methylation analysis pipeline allowed to assess the localization of the DMRs in the genome, showing their distribution in gene regions, CpG islands and surroundings as well as in repetitive element classes. In both contrasts, the results display a non-random distribution of the DMRs across the different regions (Figures 3.3, 3.4 and 3.5). In fact, the promoter as well as in the intronic regions contain high proportions of the DMRs in both contrasts. The investigation of dysregulated methylation sites may bring attention into the genomic

regions that could be methylated or demethylated in the different blastocyst groups, with respect to age of the donor.

Lev Maor et al. (2015) have recently reported that DNA methylation in a gene's promoter region was mainly associated with repression of gene transcription while methylation occuring in the gene body did not limit the expression of this gene; it was further reported that the influence of DNA methylation in gene body could impact the fine-tuning of gene expression through the alternative splicing mechanism. Our data show that the promoter regions are slightly hypomethylated in embryos from younger donors. However, compared to the embryos from adult donors, these regions display more hypermethylation in embryos from 8 month-old donors than in embryos from 11 month-old animals. Furthermore, the proximal promoter regions of 41 known genes were found to be hypermethylated in the embryos from younger donors. It is also the case for 134 gene promoter regions (Fig. 3.3). Network Analyst web application allowed the analysis of the interactions between these specific genes. We therefore observe that biological processes such as regulation of transcription (44 hits; $p < 7{,}78 \times 10^{-17}$), lipid biosynthesis (36 hits; $p < 5{,}17 \times 10^{-15}$), regulation of intracellular transport (23 hits; $p < 5{,}33 \times 10^{-14}$) and positive regulation of cell differentiation (11 hits; $p < 5{,}60 \times 10^{-11}$) could be affected by the hypermethylation of key genes' promoters. Other networks involving structure and immune response were also found to be important. Distal promoter regions were also found to hold high proportions of the DMRs in both contrasts, displaying more hypomethylation in embryos from 11-month old donors. In fact, 14,2% and 14,9% of the probes were found in the distal promoter region in the first and second contrasts, respectively. Of this number, 416 probes were hypermethylated in embryos from 8-month old donors, while 224 probes were hypermethylated in blastocysts from 11-month old animals. Distal promoter methylation has been documented and is also thought to influence gene expression: Blattler et al. (2014) have recently reported that DNA methylation could be important in the silencing of distal regulatory elements.

In complement, intronic regions of genes display a greater proportion of DMRs, showing more hypermethylation in the embryos from 8-month old donors in the first

contrast and less demethylation in embryos from 11-month old animals in the second contrast (Fig. 3.3). Exonic regions are also found to be more hypermethylated in blastocysts from younger donors. Methylation of the first exon is known to cause gene silencing (Brenet et al., 2011). We have therefore further investigated DMR repartition and found hypermethylated probes in the first exon of 47 genes in embryos from 8 month-old donors, and in the first exon of 29 genes in embryos from 11 month-old donors. Potential silencing of those genes would influence G2/M transition of mitotic cell cycle (7 hits; $p < 1{,}15 \times 10^{-9}$), chromatin remodeling (6 hits; $p < 3{,}21 \times 10^{-9}$) and macromolecule biosynthesis processes (9 hits; $p < 1{,}48 \times 10^{-8}$) (Network Analyst).

CpG islands are DNA fragments that are often found in 5' regions of genes (including promoter regions), where they can influence the transcription process, and that present high concentrations (60 to 70%) of cytosine and guanine nucleotides (Evertts et al., 2010; Turek-Plewa and Jagodzinski, 2005). CpG islands, which are normally associated with nucleosome-free DNA sequences (Choi, 2010), are known to positively influence transcription by improving DNA accessibility (Moore et al., 2013). The high concentrations of cytosine bases make the CpG island regions sensible to DNA methylation; it therefore goes without saying that methylation in the CpG islands potentially results in the repression of gene expression (Moore et al., 2013). It is known that CpG dinucleotides display aberrant methylation patterns in the case human cancer cells, establishing regional hypermethylation in CpG islands and surroundings, despite a global DNA hypomethylation (Feinberg et al., 1988; Feinberg and Vogelstein, 1983; Gama-Sosa et al., 1983). Also, a correlation between an altered DNA methylation profile at CpG island shores and corresponding gene expression was reported (Ji et al., 2010) and methylation dys-regulation in CpG island shores may affect cell differentiation (Doi et al., 2009). In our data set, the analysis of the distribution of the DMRs in CpG content and neighborhood context shows more methylation in embryos obtained from younger donors, in comparison with the embryos from the control animals. It is also possible to observe a global hypermethylation of all CpG regions, length and densities of the 8-month-old donors' embryos. The methylation status and potential repression of the transcription of key

genes and pathways observed in the embryos from younger donors is thought to be a sign of the embryo's adaptation response to a sub-optimal environment.

Repetitive elements consist of repeated patterns of interspaced DNA sequences, that account for 46,5% of the bovine genome (Adelson et al., 2009). They include low complexity repetitive elements, long terminal repeats (LTR), simple repeats, long-interspersed repetitive elements (LINEs) and short-interspersed repetitive elements (SINEs). Repetitive elements help with the understanding of the genome body structures subjected to aberrant methylation patterns. It has been previously reported that hypomethylation of these sequences may be linked to human cancer and other diseases (Chalitchagorn et al., 2004; Florl et al., 2004; Kimura et al., 2003). Our data present that repetitive elements were also subject to methylation regulation and every group of repetitive elements studied were found to be hypermethylated in the embryos from 8-month-old donors, while almost completely hypomethylated in embryos from 11-month-old donors.

A previous study by a collaborating group has investigated the stage specific effect of *in vitro* culture environment on the DNA methylation response of the resulting blastocysts, where embryos were cultured *in vitro* up to different growth stages, before being transferred into recipients, and were compared to *in vivo* developed blastocysts (Salilew-Wondim et al., 2015). The main conclusion was that longer exposure of early preimplantation embryo to the *in vitro* culture condition was associated with higher dysregulation of the DNA methylation pattern in the resulting embryos, with changes observed with both hypermethylation and hypomethylation. It has caught our attention that the methylation patterns observed with our data display similarities with the completely *in vitro* produced blastocysts from this previous study. Among others, Salilew-Wondim et al. (2015) have reported differentially methylated CpG islands and significant number of DMRs in blastocysts derived from embryos cultured completely under *in vitro* conditions. They have also pointed to a significant number of promoter and gene body categories being affected by the DMRs, in comparison with the control group. The epigenome is now seen as an "environmental memory", which is associated with capacity of the genome to adapt to

changing conditions (Jablonka and Raz, 2009). Taking into consideration that the completely *in vitro* produced embryo is considered by Salilew-Wondim et al. (2015) as the most altered phenotype in response to culture conditions, the fact that we obtain a similar pattern in blastocysts from our younger donors may highlight a higher impact of the *in vitro* conditions on the blastocysts from younger animals and the capacity of the embryos to adapt to suboptimal nutrition or culture conditions.

3.6.2 Chromosomal distribution and imprinted genes

Whole-genome DNA methylation profile was assessed for each contrast and allowed to notice a global hypermethylation of the 8-month-old donors' embryos and a less demethylation in most chromosomes associated with the embryos from 11-month-old donors (Figures 3.6 and 3.7). We have also observed a tendency for hypermethylation in telomeric regions of genes in both contrasts. This situation is not unexpected as these regions hold high concentrations of CpG islands, which are shown, in our dataset, to be hypermethylated in embryos from younger donors.

The methylation status of imprinted genes has also been investigated. Imprinted genes are essential for normal embryo development (Peters, 2014) and their dysfunction is known to be associated with diseases (Lim and Maher, 2010). They have a mono-allelic type of expression and are mostly found in clusters in different chromosomic regions, forming the *imprinting control regions* (ICRs). At this moment, about 150 imprinted genes have been identified in mammals (Bartolomei and Ferguson-Smith, 2011), while about 30 are known in the bovine (Tian, 2014). DNA methylation plays a crucial role in the establishment of genomic imprinting, influencing the expression of the alleles. For example, a paternal imprinted gene will then promote the expression of the paternal allele, while the maternal allele will be inactivated by DNA methylation (Turek-Plewa and Jagodzinski, 2005). Our findings highlight the imprinted neuronatin (NNAT), diacylglycerol O-acyltransferase 1 (DGAT1) and paternally expressed gene 1 (PEG3) genes as being the significantly hypermethylated and insulin-like growth factor 2 receptor (IGF2R), significantly hypomethylated, in the embryos of 8-month old donors. Some of these genes are known to be methylated at the very last step of oogenesis in the mouse and this could

be an indication of a slower/incomplete imprinting process prior to puberty (Gahurova et al., 2017). On the other hand, only DGAT1 was significantly hypermethylated in embryos from 11-month old donors. Methylation status aberrations and dysfunction of these genes may result in pathology considering their involvement in growth, cell proliferation and p53 mediated apoptosis (Jiang et al., 2010), brain development (Joseph et al., 1995) and triglyceride synthesis (Grisart et al., 2002). Furthermore, promoter hypermethylation has been shown to decrease *PEG3* mRNA expression in established cancer cell lines due to promoter hypermethylation (Dowdy et al., 2005; Maegawa et al., 2001).

3.6.3 Upstream and comparative analysis

While individual gene variation is interesting to investigate, the strength of pathway analysis resides in the possibility to investigate how the different conditions affect how the genes interact with each other, and how they affect signaling pathways and biological functions. Network Analyst web application allowed analysis of the complete set of microarray data and identification of age-affected gene networks (Table 6). Networks involving structure, lipid metabolism and regulation of transcription were highlighted. The p values of all the presented pathways were significant. Also, the Ingenuity Pathway Analysis (IPA) upstream regulator analysis allowed the identification of genes and molecules that may have initiated specific biological processes early in development that are important later at the blastocyst stage, but these transcripts may no longer be present at that stage. The main upstream regulators that were identified in both contrasts are presented in Table 5. Although IPA is not as efficient for the analysis of methylation data as it is for transcriptomic data (see *Methods* section), the upstream analysis is relevant as it allows mainly to link together genes that are dependent of the selected upstream regulators. In this study, considering the importance of the highlighted upstream regulators in embryo development, the analysis of their downstream targets in search of methylation irregularities revealed itself to be interesting. For each gene and molecule, we have been able to identify the targets that were potentially sensible to methylation. We have further investigated the importance of TP53, TGFβ1, TNF and HNF4α genes, as

they are known to influence embryo development and as they influence a greater number of differentially methylated targets in our data set. These upstream regulators were found to have an influence on targets that are hypermethylated in the embryos obtained from the younger group of donors. In our previous study, transcriptomic data upstream analysis pointed to HNF4α (hepatocyte nuclear factor 4α), TP53 (cellular tumor antigen p53), beta-oestradiol, ESR1 (estrogen receptor 1), TGFβ1 (transforming growth factor β1), and MYC (Myc proto-oncogene protein) as being molecules of interest (Morin-Dore et al., 2017).

Cellular tumor antigen p53 (TP53) is associated with tumor suppressing, and is known to induce growth arrest or apoptosis (Ko and Prives, 1996). It was demonstrated that its activation is triggered in the presence of intracellular reactive oxygen species (Kruse and Gu, 2009; Vousden and Ryan, 2009). This makes TP53 a potential modulator of damage responses, favoring successful preimplantation (Toyoshima, 2009) and foetal development (Torchinsky and Toder, 2010). In fact, Schmid et al. (Schmid et al., 1991) have reported that the high abundance of TP53 is associated with regular cellular differentiation during embryogenesis in mouse (Schmid et al., 1991). TP53 has also been shown to suppress Nanog expression in embryonic stem cells after DNA damage (Han et al., 2008; Lin et al., 2005). In the present study, functions downstream of TP53 have a tendency towards inhibition in both contrasts, based on the fold-change values associated with the downstream targets which may be inverted for methylation compared to gene expression. From an epigenetic point of view, it is possible to observe that 153 and 105 differentially methylated targets are associated with an upstream regulation by TP53 in the 8 vs 14 months contrast and in the 11 vs 14 months contrast, respectively. 56% of the target genes are hypermethylated in the first contrast, while 42% are hypermethylated in the second contrast. The fact that more downstream targets of TP53 bear methylation marks in the 8 vs 14 months contrast is thought to influence biological processes such as lipid biosynthetic process, inflammatory response and fatty acid oxidation and perhaps point out to a reduced capacity of the embryo to develop normally.

Transforming growth factor β1 (TGFβ1) is known to be involved in cellular growth and proliferation as well as in the regulation of growth, differentiation and function of the placenta (Munson et al., 1996; Pickup et al., 2013; Siegel and Massague, 2003). Like TP53, this growth factor is an oxidative stress marker. Functions downstream of TGFβ also have a tendency towards inhibition in both contrasts. In the present study, 151 and 126 differentially methylated targets are under the influence of TGFβ1 in the 8 vs 14 months contrast and in the 11 vs 14 months contrast, respectively. 54% of the target genes are hypermethylated in the first contrast, while 48% are hypermethylated in the second contrast. Methylation dysregulation in downstream targets of TGFβ1 is also thought to influence lipid biosynthetic process, fatty acid oxidation as well as regulation of transcription. Smads were reported to act as intracellular modulators of the TGFβ such that a disruption of the TGFβ/Smad signaling pathway can be linked to cancer and other disorders (Massague et al., 2000). SMAD6 gene is found to be significantly hypermethylated in blastocysts from 8-month old donors.

Tumor necrosis factor (TNF) is a cytokine known to provoke cell proliferation, inflammation and, eventually, apoptosis in response to stress (Liu, 2005). It has been reported that TNF is among the most important modulators of apoptosis in the mouse and human cultured granulosa cells (Matsubara et al., 2000; Quirk et al., 1998) as well as in rat ovarian cells (Kaipia et al., 1996). Dysfunction of this gene and its downtream targets could impact embryo development. Functions downstream of TNF also have a tendency towards inhibition in both contrasts, with 133 and 110 differentially methylated targets under the influence of this regulator in the 8 vs 14 months contrast and in the 11 vs 14 months contrast, respectively. 57% of the target genes are hypermethylated in the first contrast, while 46% are hypermethylated in the second contrast.

HNF4α is expressed in the embryo and is involved in mitochondrial enzyme activity (Odom et al., 2004; Wang et al., 2000). It is involved in hepatocyte differentiation and controls the expression many hepatic genes (Battle et al., 2006; Bolotin et al., 2010). This gene is also known for its role in lipid and glucose metabolism (Hayhurst et al., 2001), as well as in insulin secretion by pancreatic β-cells (Byrne et al., 1995;

Yamagata et al., 1996). In the present study, 154 and 117 differentially methylated targets are under the influence of HNF4α in the 8 vs 14 months contrast and in the 11 vs 14 months contrast, respectively. 51% of the target genes are hypermethylated in the first contrast, while 44% are hypermethylated in the second contrast. Our team has recently reported that the early expression of HNF4α, at the morula stage, would be linked to the transcriptomic modifications that were observed in blastocysts that had been exposed to stress conditions and that are associated with the Warburg effect (Cagnone and Sirard, 2013). HNF4α was also previously identified by transcriptomic analysis as a major upstream regulator in the same context as presented in this study (Morin-Dore et al., 2017); functions downstream of HNF4α were significantly activated in the 8 vs 14 months contrast, while they were significantly inhibited in the 11 vs 14 months contrast. These findings were found to be consistent with a study by Cagnone et al. (Cagnone and Sirard, 2013), in which the expression of HNF4α in the early embryo, followed by its loss at the blastocyst stage was demonstrated to be critical for the embryo to adapt to the metabolic stress induced by high glucose exposure. Embryos that fail to activate HNF4α, and therefore the Warburg metabolism, may not be able to develop past the morula stage. On the other hand, the activation of the Warburg metabolism, which favours lactate production and lipid accumulation, was associated with low quality *in vitro*-produced embryos (Cagnone and Sirard, 2013).

Transcriptomic analysis has highlighted key elements supporting the idea of a metabolic cause to propose an explanation for the differences that are observed between blastocysts from peri-pubertal and adult subjects, leading to believe that the nutrition and/or the *in vitro* embryo culture conditions might not be optimal for oocytes/embryos from pre-pubertal females. (Morin-Dore et al., 2017). In complement, we here report a non-random modification of methylation distribution in blastocysts associated with the age of the oocyte donor as an additional explanation of the embryonic and post-embryonic phenotypes. Embryos from 8-month-old donors appear to show slightly more methylation dys-regulation, while embryos from 11-month-old donors display a less different phenotype. The upstream regulator genes TP53, TGFβ1, TNF and HNF4α were found to potentially influence the expression of

methylation sensitive targets, which are shown to be more hypermethylated in embryos from younger donors. Globally, this study shows that the effect of age is marginal and does not significantly impair embryo quality.

3.7 References

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Conclusion générale

En réponse à l'hypothèse de départ, soit que *les embryons obtenus à partir d'ovocytes de donneuses péri-pubères sont différents de ceux provenant de vaches adultes*, l'analyse du transcriptome et de l'épigénome au stade blastocyste présentent des différences entre les embryons de jeunes donneuses et ceux des animaux adultes, affectant potentiellement la compétence embryonnaire.

L'analyse transcriptomique globale a d'abord permis de dénombrer 242 gènes différentiellement exprimés pour le premier contraste (8 vs 14 mois) et 296 pour le deuxième (11 vs 14 mois). Les constats suggèrent une cause métabolique pour expliquer les différences observées entre les sujets péri-pubères et adultes, trahissant un impact plus grand des conditions *in vitro* sur les blastocystes produits par les plus jeunes donneuses que chez les adultes. Les embryons semblent être marginalement affectés par l'âge de la donneuse et la qualité s'avère très bonne dès l'âge de 8 mois. Pour sa part, l'analyse épigénétique des blastocystes a permis d'observer une distribution non-aléatoire des marques épigénétiques au sein du génome des embryons. De plus, les génomes des embryons provenant de donneuses de 8 mois présentent une hyperméthylation globale plus marquée que ceux provenant de donneuses de 11 mois. En complément, l'analyse *upstream* a permis d'identifier certains gènes d'intérêt qui influencent l'expression de cibles sensibles à la méthylation; celles-ci sont davantage hyperméthylées chez les embryons des plus jeunes donneuses.

Cette étude est la première à analyser l'effet de l'âge sur la qualité de l'embryon bovin par analyse du transcriptome et de l'épigénome. Dans un contexte d'élevage intensif, il importe de bien comprendre les paramètres influençant la productivité, le potentiel génétique et les performances de reproduction des animaux. Les constats soutiennent que l'effet de l'âge sur la qualité embryonnaire est subtile et que la qualité demeure très bonne dès l'âge de 8 mois. À court terme, les résultats permettent d'explorer les conditions de nutrition des génisses ainsi que les conditions de culture des embryons afin de pallier aux différences observées.