UNIVERSITÉ LAVAL

Role and Modulation of Maternal Transcripts During the First Cleavage Divisions in Bovine Embryos

Thèse

Ernesto Orozco-Lucero

Doctorat en sciences animales
Philosophiæ doctor (Ph.D.)

Québec, Canada

Résumé

Ce travail porte sur l'identification, la fonction et la régulation des molécules maternelles d'ARNm qui dirigent la compétence développementale juste après la fécondation chez les bovins.

Tout d'abord, en utilisant le modèle du temps écoulé jusqu'au premier clivage zygotique et à travers l'évaluation du transcriptome des embryons à 2-cellules, il fut possible de déterminer la signature moléculaire des niveaux extrêmes de compétence au développement et sélectionner des molécules candidates pour des études postérieures. Les résultats ont montré que les embryons de capacité développementale variable diffèrent dans certaines fonctions comme la réparation de l'ADN, le traitement de l'ARN, la synthèse de protéines et l'expression génique définies par des ARNm synthétisés par l'ovocyte.

Pour obtenir une confirmation fonctionnelle, une paire de transcrits maternels (l'un détecté dans notre sondage précédent et l'autre étant une molécule reliée) ont été inhibés par « knock-down » dans des ovocytes. Les effets du knock-down de ces facteurs de transcription sont apparus avant la formation des blastocystes dû à une diminution de la capacité au clivage et celle à progresser après le stage de 8-cellules. L'analyse moléculaire des embryons knock-down survivants suggère qu'un de ces facteurs de transcription est un contrôleur crucial de l'activation du génome embryonnaire, qui représente une fenêtre développementale dans l'embryogenèse précoce.

Dans la dernièr étude, nous avons testé si les facteurs de transcription d'intérêt sont modulés au niveau traductionnel. Des ARNm rapporteurs couplés à la GFP (Protéine fluorescente) contenant soit la version courte ou la version longue de la séquence 3'-UTR des deux molécules furent injectées dans des zygotes pour évaluer leur dynamique traductionnelle. Les résultats ont montré que les éléments *cis*-régulateurs localisés dans les 3'-UTRs contrôlent leur synchronisation traductionnelle et suggèrent une association entre la compétence développementale et la capacité de synthèse de ces protéines. Ceci conduit à

l’idée que ces facteurs de transcription cruciaux sont aussi contrôlés au niveau traductionnel chez les embryons précoces.

Les connaissances acquises ont joué un rôle essentiel pour définir le contrôle potentiel des molécules maternelles sur les embryons au début de leur développement. Cette étude nous montre aussi une utilisation potentielle de cette information ainsi que les nouveaux défis présents dans le secteur des technologies reproductives.

Abstract

This work explores the identity, the function, and the regulation of maternal mRNA molecules that drive developmental competence shortly after fertilization in cattle.

First of all, by using the model of the time of first zygotic cleavage and assessing the transcriptome of 2-cell embryos, it was possible to determine the molecular fingerprint of extreme levels of developmental competence and select candidate molecules for further monitoring. Data implied that early embryos of variable developmental capacity differ in functions including DNA repair, RNA processing, protein synthesis, and gene expression that are dictated by oocyte-synthesized mRNA.

To obtain a functional confirmation, a pair of maternal transcripts (one detected in our previous survey and other related molecule) were knocked-down in oocytes that were further cultured. The effects of ablating these transcription factors were evident before blastocyst formation due to a decrease in cleavage capacity, as well as progression past the 8-cell stage. The molecular analysis of surviving knocked-down embryos suggested that one of these transcription factors is a pivotal orchestrator of the activation of the embryonic genome, a critical developmental window in early embryogenesis.

In the last survey, we asked whether the transcription factors of interest are modulated at the translational level. Reporter mRNAs containing either short or long versions of the 3'-UTR sequences of both molecules were injected in zygotes to look at their translational dynamics. Results showed that *cis*-acting elements located in the 3'-UTRs govern their timely translation and suggested an association between developmental competence and protein synthesis capacity. This led to the notion that these crucial transcription factors are also controlled at the translational level in early embryos.

The acquired knowledge was instrumental to define the possible control operated by maternal molecules on embryos at the onset of their development, as well as some of the challenges and potential use of this information in the field of reproductive technologies.

Contents

List of Tables

List of Figures

List of Abbreviations and Symbols

(E)	Embryonic day
[Ca2+]i	Intracellular calcium
%	Percentage
A-	Deadenylated
A+	Polyadenylated
AARE	Amino acid response element
AAUAAA	Hexanucleotide; nuclear polyadenylation signal
ACTB	Beta-actin
ANOVA	Analysis of variance
ANXA	Annexin
APC	Adenomatous Polyposis Coli
APC/C	Anaphase-promoter complex
ARE	A/U-rich element
ARNT	Aryl hydrocarbon receptor nuclear translocator
ART	Applied reproductive technology
ATF	Activating Transcription Factor
ATF1L	GFP-Long ATF1_UTR3 construct
ATF1mo	ATF1 morpholino
ATF1s	GFP-short ATF1_UTR3 construct
ATF2L	GFP-Long ATF2_UTR3 construct
ATF2mo	ATF2 morpholino
ATF2s	GFP-short ATF2_UTR3 construct
ATM	Ataxia Telangiectasia Mutated
ATP1A1	ATPase, Na^+/K^+ Transporting, Alpha 1 Polypeptide
ATR	Ataxia-telangiectasia and Rad3-related
AUG	Start codon
AURKAIP1	Aurora kinase A-interacting protein 1
BCB	Brilliant cresyl blue
bp	Pair of bases
BRCA	Breast cancer early onset
BTAF1	BTFIID transcription factor-associated, 170-kDa Mot1 hom. (*S. cerevisiae*)
BTC	Betacellulin
bZIP	Basic leucine zipper domain
cAMP	AMP, cyclic
CBP	CREB-binding protein
CCNA	Cyclin A
CCND2	Cyclin D2
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CENPF	Centromere protein E
CHUK	Conserved helix-loop-helix ubiquitous kinase
CHX	Cycloheximide
CKS1B	CDC28 Protein Kinase Regulatory Subunit 1B
COC	Cumulus-oocyte complex
CPE	Cytoplasmic polyadenylation element

CPEB	Cytoplasmic polyadenylation element-binding protein
CPSF	Cleavage and polyadenylation specific factor
CRE	cAMP-response element
CREB	cAMP-response element-binding protein
CREBBP	CREB-binding protein
CREM	cAMP-response element modulator
CTNNB1	Beta-catenin-1
d	Day
DAZL	Deleted in azoospermia-like
DBF4	DBF4 homolog (*S. cerevisiae*)
DNAJA1	DnaJ (Hsp40) Homolog, Subfamily A, Member 1
DRE	DAZL-recognition element
DSB	DNA double-strand break
dsRNA	Double-stranded RNA
dTXr	Dextran Texas red
DUSP6	Dual Specificity Phosphatase 6
DYNC1I1	Dynein, Cytoplasmic 1, Intermediate Chain 1
eCPE	Embryonic cytoplasmic polyadenylation element
ED	External diameter
EDEN	Embryo deadenylation element
EEF1A1	Eukaryotic Translation Elongation Factor 1 Alpha 1
EGA	Embryonic genome activation
EGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
EIF	Eukaryotic Translation Initiation Factor
ELAVL1	ELAV Like RNA Binding Protein 1
ENY2	Enhancer Of Yellow 2 Homolog (*Drosophila*)
EPAB	Embryonic poly(A)-binding protein
EXOSC10	Exosome component
FC	Fold change
FIGLA	Factor in the germline, alpha
G6PDH	Glucose-6-phosphate dehydrogenase
GFP	Green Fluorescent Protein
GFPA-	GFP mRNA deadenylated
GFPA+	GFP mRNA polyadenylated
GFPA30	GFP mRNA 30 adenine residues
GO	Gene ontology
GRB2	Growth factor receptor-bound protein 2
GV	Germinal vesicle
GVBD	Germinal vesicle-breakdown
h	Hours
HAS	Hyaluronan synthase
HEX	Hexanucleotide, nuclear polyadenylation signal
HKG	Housekeeping gene
hpi	Hours post-insemination
HSP70	Heat shock 70kDa protein
ID	Internal diameter

IF	Immunofluorescence
IFN	Interferon
IGF2	Insulin-like growth factor 2
INHB	Inhibin, beta
IVC	*In vitro* culture
IVD	*In vitro*-derived
IVM	*In vitro* maturation
IVP	*In vitro* produced / *in vitro* embryo production
KCNJ8	Potassium Channel, Inwardly Rectifying Subfamily J, Member 8
KD	Knockdown
kDa	Kilodaltons
KLF4	Kruppel-like factor 4 (gut)
KO	Knockout
L	Long
ldsRNA	Long double-stranded RNA
MAPS	Motif Associated with Polyadenylation Signal
MATER	Maternal Antigen That Embryos Require
Mb	Mega-bases
MBE	Musashi-binding element
MET	Maternal-embryonic transition
MII	Metaphase II
miRNA	Micro-RNA
MO	Morpholino oligonucleotides
MRE11A	Meiotic recombination 11 homologA (*S. cerevisiae*)
mRNA	Messenger RNA
MSH6	MutS homolog 6 (*Escherichia coli*)
MSX1	Msh Homeobox 1
MSY2	(YBX2, Y Box Binding Protein 2)
MuERV-L	Murine endogenous retrovirus
MW	Molecular weight
MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
MZT	Maternal-zygotic transition
NASP	Nuclear Autoantigenic Sperm Protein (Histone-Binding)
NDFIP1	Nedd4 Family Interacting Protein 1
NDUFS	NADH Dehydrogenase (Ubiquinone) Fe-S Protein
NPS	Nuclear polyadenylation signal
nt	Nucleotides
NUSAP1	Nucleolar And Spindle Associated Protein 1
OCT4	Octamer-Binding Protein 4
p-Zy	Presumptive zygote
P38-MAPK	P38 Mitogen-Activated Kinase (MAPK14)
PABP	Poly(A)-binding protein
PABPC1	Poly(A)-binding protein, cytoplasmic 1
PABPN1	Poly(A)-binding protein, nuclear 1
PABPNL1	Poly(A)-binding protein, nuclear like-1
Paf	1-o-alkyl-2-acetyl-sn-gylcero-3- phosphocholine
PAIP1	Poly(A)-interacting protein 1

PAP	Poly(A)-polymerase
PARN	Poly(A)-Specific Ribonuclease
PB	Polar body
PBE	Pumilio-binding element
PCBP1	Poly(RC) Binding Protein 1
PCNA	Proliferating cell nuclear antigen
PKA	Protein kinase, cAMP-dependent
pL	Picoliters
PN	Pronucleus
poly(A)	Poly-adenine
POU5F1	POU Class 5 Homeobox 1
PRDX1	Peroxiredoxin 1
PRE	Polyadenylation response element
PSMB2	Proteasome (Prosome, Macropain) Subunit, Beta Type, 2
PTTG1	Pituitary Tumor-Transforming 1 (securin)
Pum	Pumilio
RAD51	RAD51 Recombinase
Rb	Retinoblastoma protein
RGS16	Regulator Of G-Protein Signaling 16
RISC	RNA-induced silencing complex
RNAi	RNA-interference
RPL	Ribosomal protein L
rRNA	Ribosomal RNA
RT-qPCR	Reverse Transcriptase-quantitative PCR
s	Short
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SIAH2	Siah E3 Ubiquitin Protein Ligase 2
siRNA	Small-interference RNA
SKIIP	SKI-Interacting Protein (SNW1, SNW Domain Containing 1)
SKP1	S-Phase Kinase-Associated Protein 1
SLBP	Stem-loop binding protein
SOD	Superoxide dismutase
SP1	Sp1 transcription factor
SRFS3	Serine/Arginine-Rich Splicing Factor 3 (SRSF3)
STDmo	Standard control morpholino
SUPT4H1	Suppressor of Ty 4 homolog 1 (*S. cerevisiae*)
TACC3	Transforming, Acidic Coiled-Coil Containing Protein 3
TAE	Translation activating element
TAF	RNApolymerase II, TATA box binding protein associated factor, 150 kDa.
TF	Transcription factor
TFBS	Transcription factor-binding site
TNFAIP6	Tumor Necrosis Factor alpha–Induced Protein 6
TP53	Tumor protein P53
TRAPPC3	Trafficking Protein Particle Complex 3
TRC	Transcription-requiring complex
TRE	Translation repressing element
UBE2K	Ubiquitin-conjugating enzyme E2K

uninj	Uninjected
UPP	Ubiquitin-proteasome pathway
UTR	Untranslated region
WB	Western blot
YWHAG	Tyr. 3-monooxygenase/tryptophan 5-monooxygenase activat. prot. gamma
YY1	Ying-yang 1
ZGA	Zygotic genome activation
ZP	Zona pellucida
Zscan4	Zinc Finger And SCAN Domain Containing 4

"Heard melodies are sweet, but those unheard are sweeter" (J.K).

"All we need to do is make sure we keep talking" (S.H).

Acknowledgements

Through the years in Québec I had the opportunity to meet wonderful people. Now, is time to express how grateful I am for their unique help and the experiences we shared. It is not possible to mention all these nice people, but they should be sure I always remember them.

First of all, I would want to express my deepest gratitude to my advisor Marc-André Sirard. After having the honor of being accepted as his student, I received not only an opportunity of education within a top-class work group, but also to learn from his discipline, responsibility, unbeatable energy, problem-solving mind, innovation, as well as passion for science. Thanks, so much for your patience and daily encouragement to improve. You're with no doubt one of the main role models in my life.

The benefits of my stay at ULaval would have been far from complete without my co-supervisor Claude Robert. Thanks for your example and critical spirit, your indispensable support to my project and to the group, in addition to the incomparable quality of your classes and molecular expertise, as well as your sharp reasoning. I'm also in profound debt with my other professors and members of the former CRBR, particularly Janice Bailey, Pierre Leclerc, Jacques Tremblay, and François Richard for their support even before my arrival to ULaval, for sharing their expertise and contributing to the consolidation of an amazing research center. As for François R and Jacques, together with Jon LaMarre from Guelph, besides their indispensable support during the years and now the final feedback by serving on my PhD committee, they have been exceptional examples in research.

I'm also thankful to other professors and members of SAN and FSAA for their crucial help: Dany Cinq-Mars, Robert Chartrand, Jean-Paul Laforest, Lyne Fortier, Janny Bérubé, and Jean-Claude Dufour. On the lab bench and next to it, a research student requires the irreplaceable assistance of our technicians, professionals, and managers: Isabelle Dufort, Isabelle Laflamme, Christine G, Alexandre Bastien, Dominic G, Eric Fournier, Isabelle Gilbert, Julie N, Nancy B, Micheline G, Serge Daigle, and Richard Prince. Thanks a lot for teaching me all the necessary, driving me through the labs, and of course, your analysis and for making our projects possible. I was also lucky to meet and learn from post doc fellows,

former students, and visitor researchers, as Daulat, Nicolas Guillemin, Maëlla G, Béatrice de M, Dessie Salilew-Wondim, Morteza, Julieta, Christian Vigneault, and Annick Bergeron. I show my appreciation for your unique example, teaching, company, and support. In addition, last year I had the chance to stay at UOGuelph for a pivotal part of my project at the lab of Allan King, Elizabeth St-John, Pavneesh Madan, Jon LaMarre, Monica Antenos, Ed, Fazl, Gyu-Jin and N-Y Rho, Ryoung-Hoon, Tamas, and Leslie. I'm quite grateful for your welcoming, support, and friendship, as well as that of Nihad, Cristina, Sharon, and Harold during that unforgettable summer. Moreover, I extend my gratitude to all singular students I was lucky to met at CRBR, including Sara, Angus (both that starting me to show what is living in Québec and Canada, and who survived to me at my arrival), Habib, Akintayo, O. D'Amours, Luis, Audrey Bunel, Florence, Annie Girard, Gaël, Rémi L, Marie C, and Anne-Laure… « et aussi à la nouvelle gang composée de » Léoni, David, Patricia, Simon, Rachèle et Chloé. Thanks, so much for your friendship and help.

Besides the lab mates, I met a bunch of nice people with whom to share some free time: "los compadres" Arturo Duarte y Maria Garcia, Aude Popek, Mahder, Vishal, Rafael IB, Ana Sofia, Emperador, Yhosvanni, Mylene A, Patrick M, Luc Pelletier, Alma D, Dr. Pinedo, Pascal D, Donatien, Analisse, José Luis M, and Stéphanie Robert. I always remember those who helped me in Mexico shortly before my initial and last departures: Alejandro Martinez (my other great role model), Dora F and Ángel D, Moisés B and Karla, F. Plenge, N. Lobo, Pepe Nuñez, Boni, as well as Beto Grado. In my very personal circle in Quebec I'm indebted to all the community of Saint-Thomas-D'Aquin, especially Alexandre Julien, Martin Lagacé, and Marie-Josée G: You don't know how much you helped me! Of course, I incur everything to my loving parents, as well as to my siblings and their families, to whom I owe my life and my whole current being. They were always available in spite of any circumstance… in addition to the little lady that has recast all my life, together with her family: Lupita Férnandez. She brought me back and took incomparable care of me. I have no words to thank my beloved ones for everything: All the honor and love that you deserve!

Now, I'm going back to my México, which I missed. I'm quite sure I'll miss Québec so much: My life completely changed here… Thanks!

Foreword

Chapters 2 and 6 consist on published papers. Chapters 3 and 4 will be soon submitted for publication.

Chapter 2:
Orozco-Lucero E, Dufort I, Robert C, Sirard MA. 2014. Rapidly cleaving bovine two-cell embryos have better developmental potential and a distinctive mRNA pattern. *Mol Reprod Dev* **81:** 31-41.

In this chapter Ernesto Orozco-Lucero is responsible of all experiments, data analysis, and writing the paper. Isabelle Dufort designed and collaborated in the experiments, as well as data analysis, and reviewed the paper. Claude Robert participated in the project elaboration, interpretation of results, and paper revision. Marc-André Sirard conceived the project, interpreted the analysis, and reviewed the manuscript. All coauthors approved the final version of the manuscript.

Chapter 3:
The knockdown of ATF1 and ATF2 transcripts in germinal vesicle oocytes reveals their crucial roles in bovine early development.

Chapter 4:
Regulation of ATF1 and ATF2 transcripts by sequences in their 3'-untranslated region in cleavage-stage cattle embryos.

In these two chapters Ernesto Orozco-Lucero collaborated in all experiments, data analysis, and writing the paper. Isabelle Dufort designed and collaborated in the experiments, as well as data analysis, and reviewed the paper. Claude Robert participated in the project elaboration, interpretation of results, and paper revision. Marc-André Sirard conceived the project, interpreted the analysis, and reviewed the manuscript.

Chapter 6:

Orozco-Lucero E, Sirard MA. 2014. Molecular markers of fertility in cattle oocytes and embryos: Progress and challenges. *Anim Reprod* **11:** 183-94.

In this chapter Ernesto Orozco-Lucero is responsible of obtaining data and writing the manuscript. Marc-André Sirard obtained information, conceptualized the paper, and reviewed the manuscript. The coauthors approved the final version of the manuscript.

1. Introduction to Early Development

1.1. The Oocyte and Its Origin

The oocyte is a highly specialized cell capable of supporting fertilization and early embryogenesis in metazoans once it completes its differentiation. It provides half of the genetic material (haploid complement, 1N) to the embryo by the moment of fertilization, while the sperm collaborates with the other part, to finally restore the diploid chromosomal complement (2N). In addition, the female gamete contributes with transcripts and proteins to allow developmental progression throughout the first stages of embryogenesis during a transcriptionally silenced period, also termed the *maternal* or *embryonic program*, before the activation of the genome from embryonic source. The egg is a totipotent cell as it possesses the capacity to generate all the cell types of the future embryo and the adult organism. Furthermore, the oocyte is able to reprogram the genetic material of a somatic and differentiated cell following the transfer of the nucleus of the later to a recipient ooplasm and then constitute a viable embryo (rev. Song and Wessel 2005; Macaulay et al. 2011; Von Stetina and Orr-Weaver 2011), in an analogous manner to that of the remodeling of the sperm nuclear material by the oocyte cytoplasm upon fertilization (McLay and Clarke 2003). To better understand the complex biology of the egg, it is important to focus on the origin of the germ cell lineage during early embryonic life and the molecular and cellular events that will lead to the rise of female gametes.

Germ cells (GCs) in both male and female embryos are derived from undifferentiated precursors called primordial germ cells (PGCs) in early development. During their lifespan, PGCs pass through the phases of establishment of the germ cell lineage, migration, colonization of the primordial gonad, and proliferation (Vanderhyden 2002). In turn, the ancestors of the PGCs are located in the proximal (posterior) epiblast, structure originated from the inner cell mass (ICM) of the blastocyst. In such place these ancestor cells are induced to become GCs by BMP4 and BMP8b signalization produced by the adjacent extra-embryonic ectoderm. This precursor cell population expresses *Pou5f1*, *Dppa3* (*Stella*) and *Ifitm3* (*Fragilis*) markers of GCs (Saitou et al. 2002; Saitou et al. 2005). However, these three factors are not exclusive of the germinal lineage, since they are also expressed in early embryos. In addition, these ancestor cells can produce both companion somatic cells and actually specified GCs. Consequently, it is not until the expression of *Blimp1* (*Prdm1*)

in a small subpopulation of around six cells by embryonic day (E) 6.25 in the mouse epiblast (Ohinata et al. 2005) that the precursor cells are first committed to a germinal fate, given the repression of somatic cell-specific *Hox* genes by *Blimp1* (McLaren and Lawson 2005). Subsequently, the founder population of approximately 45 PGCs moves to the base of the allantois by (E)7.25 in mice. PGCs show tissue-non-specific alkaline phosphatase (TNAP) activity, which has been used to help in localization of this kind of cells, but is not exclusive of GCs (De Felici et al. 2004). On the other side, Wrobel and Suss (1998) reported that in the bovine embryo the putative PGCs population is first detected by (E)18, and later incorporated close to the mesonephros by (E)23-25. As it can be noticed, PGCs are originally located in extragonadal compartments in distinct species.

In order to colonize the developing gonad, the PGCs (which in the cow have a diameter of 30 μm) start a migration from their place of origin next to the primitive ectoderm, passing through the embryonic posterior gut by using amoeboid movements and pseudopodia (Aerts and Bols 2010a). Durcova-Hills et al. (2003) reported that in the mouse this migration takes place from (E)9.5 to (E)11. By contrast, in bovines it occurs from (E)30 to (E)64 when all PGCs finally reach the developing gonad. It is strongly suggested that throughout the migratory pathway, the PGCs are driven to the gonad by chemotactic signals produced by the genital ridge. These molecules are apparently also important for PGCs survival and proliferation during the displacement period and include Kit Ligand (KL, SCF), bFGF, TNF-alpha, LIF, CNTF, oncostatin-M, SDF-1, BMP4, TGF β 1, activin, Gas6, neuregulin- β , and PACAP (Donovan et al. 2001; De Felici et al. 2004). For example, KL is expressed in the surface of the somatic cells in the migratory pathway, while its receptor, KIT, is produced by PGCs (Vanderhyden 2002) creating a regulation system kept later on until the oocyte-cumulus cells interaction. Moreover, KL possibly assures PGCs survival by inhibiting the apoptotic molecular system that can be induced by BAX (Krysko et al. 2008). Concerning attachment, the adhesion properties offered by the substrate to the PGCs during their migration appear fundamental, as it has been observed by the interaction of this group of cells with collagen IV, fibronectin, and laminin, to which these cells connect through integrins (Garcia-Castro et al. 1997). It must be noted that as PGCs progress towards the gonadal territory their population experiences an important

initial proliferation. In mice the founder population of around 40-45 PGCs increases to more than 3,000 cells by (E)11, moment when they reach the gonadal crests in this species (Morrish and Sinclair 2002).

During migration, PGCs behavior and phenotype are identical in embryos of both sexes (McLaren 2001). Nevertheless, the molecular events that determine the phenotypic sex of the GCs start by the end of the PGCs displacement, and in such processes the gonadal somatic cells are directly involved. In female embryos, the triggering factor of GCs sex specification is *Dax1* (Morrish and Sinclair 2002). This gene is expressed in the bipotential gonad but diminishes its transcript levels in male embryos by (E)12.5, the time of gonad differentiation in the mouse, while it remains highly expressed by somatic cells in the future ovary. Thus, it constitutes a possible antagonist of the *Sry* masculinizing factor (Morrish and Sinclair 2002). In the bovine, the differentiation of the gonad occurs by (E)40 (Wrobel and Suss 1998), implying that the first PGCs arriving to the developing gonads (still genital crests) in this species by (E)30-35 do so when such structure is about to exhibit sexual dimorphism. When PGCs are located in the already differentiated gonad, the female GCs are now termed *oogonia*. This cellular population is mitotically very active and continues into the embryonic ovary the proliferation that had shown during their previous displacement. In the cow, the maximum number of these cells is estimated in 2.1×10^6 (Aerts and Bols 2010a). At this moment the GCs will be prepared to switch from mitotic divisions to meiosis before forming primordial follicles.

The induction of the first meiotic division occurs in the cow by (E)70 (Magre and Vigier 2001) and there is evidence that the timing of this process is dictated by the surrounding somatic cells. The triggering mechanism for meiosis seems to involve the induction of retinoic acid (RA) of the expression of the meiotic markers *Stra8*, *Sycp3*, and *Dmc1*, given the high levels of RA in the embryonic female gonad in comparison to the testis, in which the male GCs do not enter meiosis until puberty (Swain 2006; Bowles and Koopman 2007). Once the oogonia start meiotic divisions they are called *oocytes*. In all mammals meiosis stops at prophase I and this occurs in the cow by (E)90, a concomitant event with the first complete assembly of primordial follicles (Aerts and Bols 2010a), which according to the

observations of Nilsson and Skinner (2009) start to form by (E)80. This initial blockage of meiosis can persist during months or years, depending on the species, and such period is termed *dictyate*. According to Zheng and Dean (2007), mouse oocytes stop their first meiotic division specifically at diplotene, while there is no clear consensus of the exact time point at which it occurs at the end of prophase I in the bovine, but it is considered to take place in a moment between pachytene and diplotene stages. The oocytes meiotically arrested in prophase I contain a nucleus called *germinal vesicle* (GV). Meiosis restarts and rupture of GV will take place later during development induced by the gonadotropin surge (Edwards 1965).

1.2. Oocyte Growth and Folliculogenesis

The growth of the oocyte in the follicle takes place from the establishment of primordial follicles up to just before the moment of final maturation. In contrast, the follicle grows until the moment of ovulation and passes through the stages of primary, secondary (pre-antral), tertiary, and pre-ovulatory follicle (antral).

1.2.1. Pre-antral follicular development

Contrary to rodents, which do not form primordial follicles until birth, folliculogenesis in bovines starts by (E)90 with the presence of fully enclosed oocytes in primordial follicles. These follicles are located in the ovary cortex and their gametes (primary oocytes) measure around 30 μm in diameter (Braw-Tal and Yossefi 1997). Nilsson and Skinner (2009) have proved that progesterone regulates primordial follicle assembly since decreasing levels of this hormone are correlated with high primordial follicle formation at the end of gestation in cattle. Previously, the mitotically active oogonia form GCs clusters, which establish a syncytium, surrounded by somatic cells derived from sex cords. After inception of meiosis, the nurse cells extend cytoplasmic projections into the interconnected oogonia to divide the clusters and start surrounding individual oocytes, constituting a single layer of flat pre-granulosa cells around each gamete (Fig. 1-1). In turn, this cellular monolayer is enclosed by a basal lamina (van Wezel and Rodgers 1996; Aerts and Bols 2010a).

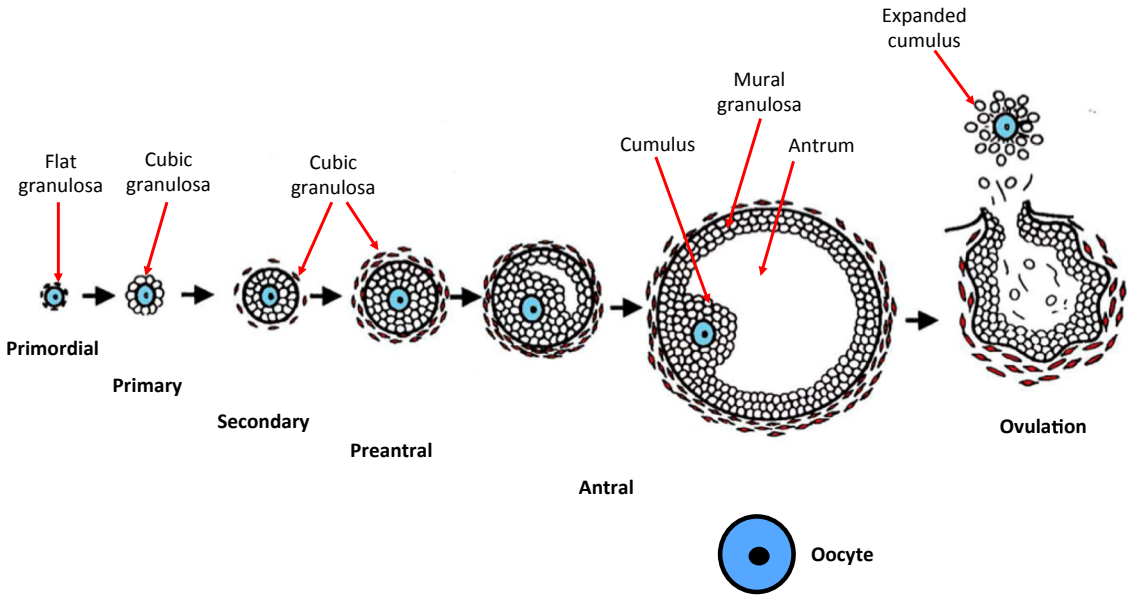

Figure 1-1 Folliculogenesis (Orisaka et al. 2009)
The main features of the disctint developmental stages throughout folliculogenesis are illustrated. Copyright-free scheme reproduced and adapted from BioMed Central © 2009.

A first selection of oocytes occurs by the moment of meiosis entry since many of these gametes suffer attrition, basically occurring through apoptotic mechanisms (Krysko et al. 2008). This is reflected by the number of primordial follicles at the moment of birth, which in a calf is approximately 1.1 x 10^6, in contrast to the peak of GCs during embryogenesis. However, bovine fetuses show an average of 130,000 primordial follicles in such moment, while in most domestic species the number of this type of follicles ranges from 100,000 to 400,000 per newborn (Senbon et al. 2003; Aerts and Bols 2010a). These primordial follicles constitute the ovary complement from which oocytes will be recruited for ovulation during all the female reproductive lifespan without *de novo* replenishment (Senbon et al. 2003; Aerts and Bols 2010a). In recent years, the reports of the laboratory of J.L. Tilly (Johnson et al. 2004; Johnson et al. 2005) opened the discussion concerning the possible renewal of GCs stock during mammalian female adulthood from undifferentiated cells, which could be generated from bone marrow stem cell populations. Nevertheless, this presumptive biological mechanism is still highly controversial and needs further demonstration (Telfer et al. 2005; Vogel et al. 2005). As it has been mentioned, folliculogenesis begins with primordial follicles and these will enter the growing pools of follicles that will be recruited

until they enlarge and form primary and secondary follicles. This phase is called *follicle growth initiation* or *follicle activation*, and it is an irreversible process. To get into the growing wave, primordial follicles must leave the resting pool where quiescent follicles remain and account for the vast majority of the follicular population in the ovary. This is probably induced by KL signaling from the neighbor granulosa cells (Aerts and Bols 2010a). Moreover, Yang and Fortune (2008) have demonstrated that in the cow the meiotic arrest must be completed in prophase I, with high ovarian expression of *YBX2* (*MSY2*) mRNA before activation of primordial follicles.

Although there is no tangible difference in oocyte diameter between primordial and primary follicles, the later ones are already committed to growth, and the developmental progress to the primary follicular stage is marked by one of the first major changes during folliculogenesis: The transformation of flat pre-granulosa cells to cuboidal granulosa cells, as well as the proliferation of the same (Aerts and Bols 2010a). In spite that most of the growth waves occur after birth in cattle, a few primordial follicles are activated during fetal life and the earlier primary and secondary follicles are found by (E)140 and (E)210, respectively (Yang and Fortune 2008). Besides the KIT/KL communication system (also present in primordial follicles) between the gamete and its nurturing follicular cells, modulation control exists between both cell types through gap junctions (heterologous channels), as well as among the granulosa cell population (homologous channels). The integrity of the heterologous channels is kept even when the *Zona Pellucida* (ZP) is synthetised at a later stage. Furthermore, the oocyte-secreted factors, GDF9 and BMP15, have been shown to be crucial for follicular development as they are mitogens for granulosa cells (de Matos et al. 1997; Carabatsos et al. 2000; Knight and Glister 2003; Senbon et al. 2003; Knight and Glister 2006). By all these molecular interchange mechanisms the distinct types of cells inside a follicle are interconnected and a bidirectional communication is established between the oocyte and granulosa cells. Through this system the granulosa cells provide nutrients, maturation and meiotic regulating molecules (ribonucleosides), and elements used upon fertilization (e.g. cysteine, precursor of glutathione) to the gamete, while the oocyte orchestrates the proliferation, differentiation, and some functions (glycolysis, steroidogenesis) of the granulosa cells, and later the cumulus expansion (de

Matos et al. 1997; Carabatsos et al. 2000; Knight and Glister 2003; Senbon et al. 2003; Knight and Glister 2006).

In agreement with the data of Braw-Tal and Yossefi (1997) the initial deposition of the glycoprotein-containing ZP matrix, fundamental for fertilization, over the bovine oocyte's plasma membrane takes place in the early secondary follicular stage, and the first appearance of fully-enclosed oocytes by ZP occurs by the late secondary stage. Similarly, another important developmental change in the secondary follicle is the conformation of multiple layers of granulosa cells around the oocyte, in addition to an important growth of the gamete, reaching a diameter of around 70 μm by the late secondary stage (Braw-Tal and Yossefi 1997). Finally, the same group reported that the inner theca begins to form around the granulosa cells by the early secondary follicular stage in cattle, and subsequently the basal lamina is totally surrounded by theca cells in the late secondary follicle. The theca accounts for the vascularization of the ovarian follicle, contrary to the avascular internal portion beyond the basal lamina, constituted by granulosa cells and the oocyte (Braw-Tal and Yossefi 1997).

1.2.2. Antral follicular phase

The tertiary follicle (also called *antral* or *Graafian*) is characterized by the presence of a fluid-filled cavity (*antrum*) in the middle of the granulosa cell population, ensuing in this way the differentiation of such cells in two subpopulations: 1) The most peripheral one includes those cells termed *mural* granulosa; 2) those in direct contact with the gamete (*corona radiata*) together with the ones comprising the cell layers separating it from the antral fluid and the mural granulosa cells constitute the *cumulus*. The antral space contains a complex liquid mixture of proteins, hormones, and ions involved in endocrine/paracrine regulation (Senbon et al. 2003; Huang et al. 2006; Adams et al. 2008). It must be remarked that in contrast to rodents, whose oocytes reach their maximum size by the moment of follicular antrum appearance, oocytes of antral follicles in domestic species continue with a remarkable growth during this developmental stage (Motlik et al. 1984). Hence, the early (small) antral follicle in the cow that attains a size of 250-500 μm harbours an oocyte with an average diameter of 93 μm and on whose plasma membrane the deposition of the ZP still

increases (Braw-Tal and Yossefi 1997). Thereafter, the preovulatory (mature Graafian) includes an oocyte with the biggest size: Its diameter reaches or can slightly surpass 130 µm in cattle (Otoi et al. 1997; Fair 2003). If this gamete has restarted meiosis after the prophase-I arrest it is then termed secondary oocyte. Nonetheless, in monovulatory species typically only a gamete is ovulated at the end of each estrous cycle. Before that, the ovulating follicle must become dominant (Fig. 1-2).

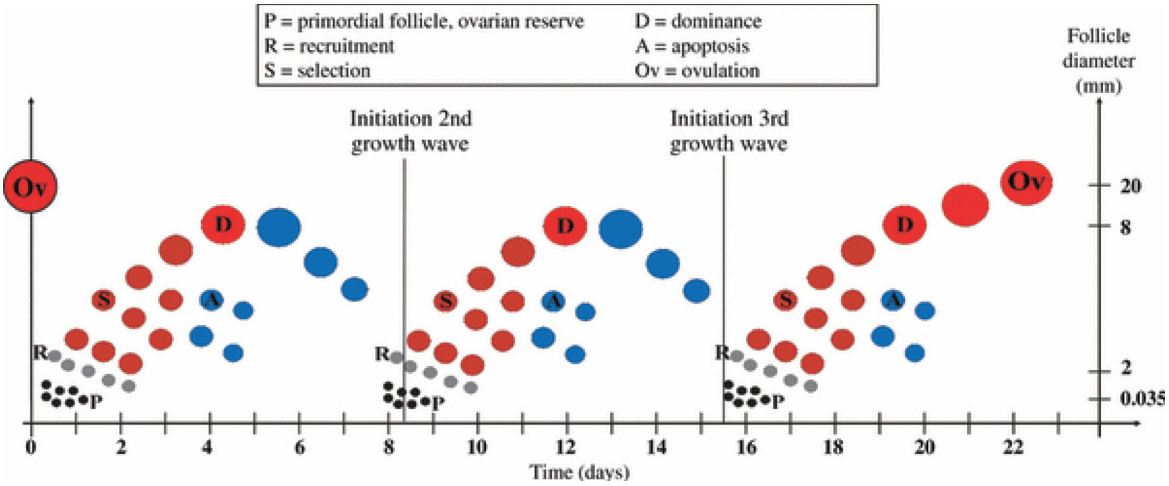

Figure 1-2 Follicular dynamics (Aerts and Bols 2010b)
Representation of the distinct follicular phases and their timing during a three-follicular wave estrous in the cow. Scheme reproduced with permission of Wiley © 2010.

As in most domesticated animals and humans, the estrous cycle in cattle presents follicular growth waves, and in this last species it commonly manifests in 2-3 waves per cycle. In general, only one follicle per wave becomes dominant. Only the *dominant follicle* (DF) of the last wave in the estrus cycle ovulates. The length of the estrous cycle in bovines has an average of 21 or 23 days for cycles with two or three waves, respectively (Adams et al. 2008; Aerts and Bols 2010b). In comparison with the pre-antral stage, the antral follicular phase represents the shortest period of the follicular development. In cows, the antral growth has an average length of 42 out of the 180 days of the total folliculogenesis. Therefore, a complete antral phase typically needs the extent of two estrous cycles to be finished. Each growth wave consists of *recruitment*, *selection*, and *dominance* periods (Adams et al. 2008; Aerts and Bols 2010b). In the first phase a cohort of 5-10 antral

follicles avoid atresia due to the presence of high levels of circulating FSH, which performs a recruitment function for the new cohort of tertiary growing follicles at the beginning of each wave. The selection takes place when the largest follicle reaches around 8.5 mm in diameter. By this moment, a follicle commits to become the DF (generally the largest and/or the most developed one), while the rest turn into *subordinate follicles* (SF) and start regression: The dominance stage starts. The DF then becomes the major suppressor (by synthesizing inhibin and estradiol) of FSH secretion (thus, of SFs growth and emergence of new follicular cohorts), since this follicle is by this moment capable to survive and continue its growth even in basal levels of FSH (Adams et al. 2008; Aerts and Bols 2010b). This is achieved by the DF by switching from FSH to LH dependence. If the DF rises when a *corpus luteum* (CL) is still present, the progesterone secreted will inhibit LH pulse frequency, preventing the DF from obtaining sufficient amounts of this hormone to complete its growth and thus the DF will enter atresia (Fig. 1-2). On the contrary, when the DF emerges during follicular phase (absence of CL) the increased LH pulsatility will allow it to finish growth until ovulation (Adams et al. 2008; Aerts and Bols 2010b). However, in order to accomplish developmental viability, the to-be-ovulated gamete must attain maturation during the final stages of folliculogenesis.

1.3. Oocyte Maturation

Although the oocyte experiences maturation to some extent and an extensive growth during the earlier phases of oogenesis, a unique form of final maturation is provoked in the *in vivo* environment by the gonadotrophic discharge of LH at the last stages of the peri-ovulatory follicle evolution. Altogether with the previous development, this oocyte terminal maturation is crucial to confer the developing egg with the capacity to be successfully fertilized and produce a viable embryo and a healthy offspring. It can be considered that this process has three different subtypes of maturation: *Nuclear* (meiotic), *cytoplasmic*, and *molecular* (Sirard et al. 2006; Mermillod et al. 2008).

1.3.1. Nuclear maturation

Meiosis is a cellular division exclusive to GCs of both genders in which, by contrast to mitosis, GCs first duplicate their nuclear DNA and subsequently are subjected to a double

cell division with the same number of chromosomal complement partition. In this way, both oocyte and sperm assure that their genetic material is haploid by the moment of their final differentiation in order to restore the diploid chromosomal state upon fertilization (Brunet and Maro 2005; Richard 2007). Moreover, the meiotic division results in a high genetic recombination following chromosomal synapses. In the oocyte, *meiotic* or *nuclear maturation* refers to the meiotic re-activation after nuclear arrest at the late prophase-I stage in mammals. As other maturation events, meiotic resumption is prompted *in vivo* by the LH surge, or by the oocyte retrieval from its follicle (Brunet and Maro 2005; Richard 2007). The ability of the female gamete to reinitiate meiosis is associated with its size in a progressively acquired process. Fair et al. (1995) demonstrated in cattle that although the oocytes with a diameter slightly smaller than 100 μm are able to progress to MI-phase *in vitro*, the number of those reaching MII is low, while only oocytes surpassing a 110 μm size are able to achieve the final steps of meiosis. The later diameter is accomplished in bovine oocytes from follicles of 2-<3 mm (Fair et al. 1995). Under the microscope the first mark of meiotic restart is the disappearance of the GV membrane, or *germinal vesicle breakdown* (GVBD), followed by chromosomal condensation and alignment into the first metaphase plate and extrusion of the first polar body (PB) to eliminate the duplicated genetic material and keep a transitory 2N chromosomal number. Meiosis then progresses until MII stage when a new blockage takes place (Fig. 1-3). Finally, the meiotic division is again resumed upon sperm activation with the consequent second PB expulsion (Massicotte 2006; Ferreira et al. 2009).

At the molecular level, the activity of the maturation promoting factor (MPF) controls the entry into and exit from M-phase during meiotic progression. MPF that is in turn regulated by the cytostatic factor (CSF), in which Mos (c-Mos) is a component, exerts a kinase activity rendered by p34/cdc2 (CDK1), which forms a complex with the regulatory subunit cyclin B1 (Brunet and Maro 2005; Malcuit and Fissore 2007). As in the mouse (Brandeis et al. 1998), CycB2 (CCNB2) exists in the bovine, although only CycB1 (CCNB1) is the limiting factor for meiosis restart in the latter species: CycB1 is one (Levesque and Sirard 1996; Sirard et al. 1998; Sirard 2001) of the newly synthesized proteins during the GV-GVBD transition and is required for such developmental progression (Coenen et al. 2004;

Massicotte et al. 2006). It must be noted that CycB1 in cattle has two alternative isoforms whose translation is regulated through polyadenylation (Tremblay et al. 2005). Concerning the molecular dynamics of CycB1 and MPF activity through bovine oocyte maturation, it has been observed that CycB1 protein appears after 3 h of initiated IVM (Levesque and Sirard 1996) and 4 h in advance to GVBD (Coenen et al. 2004). Subsequently, as demonstrated by the studies of Wu et al. (1997) a peak of MPF activity allows chromosome condensation (MI) by 6-12 h of maturation, while MPF activity level decreases at the AI/TI transition after 16-18 h of culture (the *Bos taurus* protein bears the classic biphasic activity pattern of CycB1), then leading to 1st PB extrusion by 18-20 h with an increase in MPF activity with a plateau by 20-24 h IVM (MII). Finally, MPF in conjunction with CSF brings the oocyte into an arrested state at MII (Russo et al. 2009).

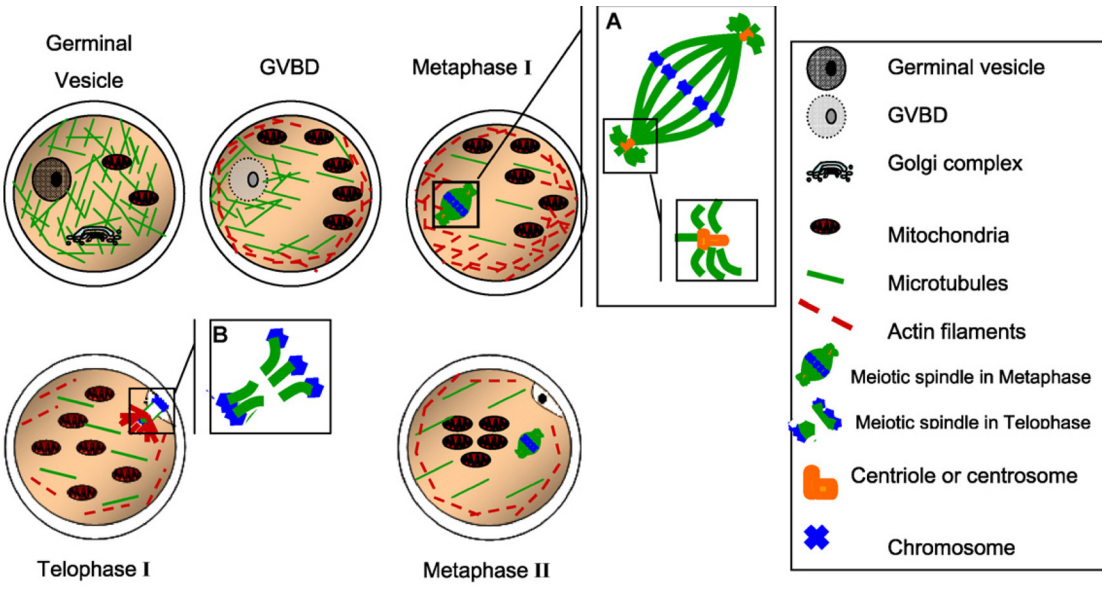

Figure 1-3 Nuclear maturation (Ferreira et al. 2009)
A) and B) insets of the meiotic spindle. GVBD, germinal vesicle breakdown. Scheme reproduced with permission of Elsevier © 2009.

1.3.2. Cytoplasmic maturation

This series of events occurs in the ooplasm and although cytoplasmic maturation begins before LH surge, it is simultaneous at some points with meiosis resumption since cytoplasmic modifications are not finished until the final capacitation of the egg. According to Sirard (2001) and Ferreira et al. (2009), cytoplasmic maturation consists in both the

repositioning of organelles and cortical granules (CGs) developed during oocyte growth, as well as cytoskeletal dynamic changes (Fig. 1-4). Frequently, molecular maturation is considered as part of the cytoplasmic process, but the first will be discussed later.

Organelle redistribution directly depends on the appropriate function of cytoskeletal components like microtubules and microfilaments. According to the cell's requirements mitochondria are transferred to subcellular compartments of high- energy demand during oocyte maturation. Hence, such organelles migrate from a more peripheral distribution before LH discharge *in vivo* (GV-oocyte) to a more homogeneous allocation by 15 h after LH stimulation (MI), and to a centrally clustered localization closely before or after ovulation, around 24 h following the LH peak (MII). The same reallocation occurs for lipid droplets that increase in number and size. The movement of mitochondria and lipid droplets follows a similar pattern during IVM (Wang et al. 2009). Simultaneously, Golgi complexes gradually decrease in number. Other structures are subjected to dynamic changes towards the end of maturation: The GCs notably proliferate in comparison to their first appearance by the secondary follicle stage, and are placed directly below the plasma membrane in preparation to block polyspermy through exocytosis of their content (Soloy et al. 1997; Ferreira et al. 2009). Such redistribution of CGs (Fig. 1-4) is apparently associated with oocyte size in the bovine since Otoi et al. (1997) observed that those gametes reaching a diameter of 115-120 μm are less susceptible to polyspermy. In addition, ribosomes regroup around chromosomes. All these changes reflect a basal metabolic level in the overall cell and the aim of the oocyte to save energy in preparation for fertilization, and the readiness of energy sources (lipid compartments associated to mitochondria) for the earlier embryonic cleavages (Hyttel et al. 1997). Alternatively, it is known that the oocyte loses its attachments to the cumulus cells through gap junctions by the end of maturation as a pre-requisite for cumulus expansion. This communication has been demonstrated as indispensable during maturation as the gamete utilizes lactate, pyruvate, and even alanine, malate, aspartate, and oxalacetate as energy molecules taken from the cumulus cells and then processed in the ooplasm (Cetica et al. 2003), in addition to their participation in meiotic modulation (Ali and Sirard 2005; Atef et al. 2005).

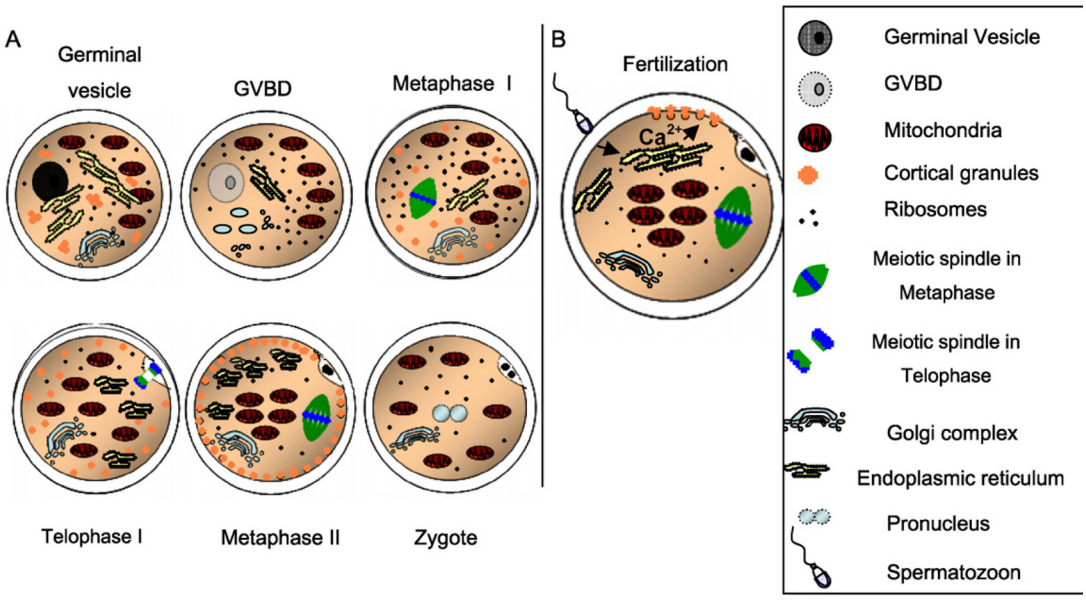

Figure 1-4 Cytoplasmic maturation (Ferreira et al. 2009)
A) Stages from germinal vesicle to zygote; B) intracellular calcium (Ca^{2+}) release upon sperm entry. GVBD, germinal vesicle breakdown. Scheme reproduced with permission of Elsevier © 2009.

1.3.3. Molecular maturation

Typically, molecular maturation has been considered as part of the cytoplasmic maturation, since the first occurs in the cytoplasm of the developing egg (or at least the vast majority of such process does). However, the complexity and the undeniable importance of the molecular maturation for oocyte development has frequently prompted discussion as a separate biological event. According to Sirard et al. (2006) and Ferreira et al. (2009), the molecular maturation consists of the transcription, stocking, and processing of transcripts in the oocyte during the growth of the gamete before the transcriptionally silenced period that marks the final maturation of the oocyte up to the activation of the embryonic genome. These events are concomitant at some points with the nuclear and cytoplasmic maturation, and also include the translation and storage of some of the codified proteins by the transcribed/stored mRNAs. Conversely, a wide diversity and amount of the molecular messages will only be translated at the appropriate moment when their codified proteins become necessary for the female gamete. For this reason, an adequate mechanism for the deposition of stabilized transcripts must be accomplished in the ooplasm. Moreover, such a system must also guarantee the safety of the mRNAs to avoid their degradation (Sirard et

al. 2006; Ferreira et al. 2009). Some details of the mechanisms involved in molecular maturation are discussed next.

1.4. Molecular Regulation of the Maternal Stockpile

1.4.1. Oocyte transcription activation and silence

In comparison to the oocyte in mice, the female gamete in cattle is transcriptionally inactive both during the primordial and primary follicle stages (granular nucleoli lacking of fibrillar centers), until the formation of the secondary follicle. At such moment, transcription is then instigated in the bovine oocyte, accompanied by the transformation of the nucleolus to a conformation capable of producing ribosomes (migration of fibrillar centers towards nucleoli). Subsequently, synthesis of mRNAs importantly increases up to a maximum level at the early antral phase, when the oocyte reaches approximately 110 μm in diameter closely before ceasing to grow up (Fair et al. 1997; Hyttel et al. 1997; Hyttel et al. 2001). Since that moment the oocyte decreases mRNA production rates. When it measures 120 μm in diameter (still at GV-phase, just before the onset of the final maturation), the cow's gamete is practically transcriptionally quiescent, as remarked by Lodde et al. (2007) and Lodde et al. (2008), who observed a correlation of this condition with an electron-dense fibrillar nucleolus and condensed chromatin (non-permissive conformation for transcriptional machinery). This transcriptionally inert form is common to distinct species and will be kept in the oocyte during all maturation steps, as well as throughout the first embryonic cleavages until *embryonic genome activation*, EGA (rev. De Sousa et al. 1998; Sirard 2010; Macaulay et al. 2011; Clarke 2012). Of note is the fact that Mamo et al. (2011) determined by microarray analysis that the abundance level of 589 transcripts (~28% of the evaluated pool) increased upon IVM in bovine oocytes and this trend was verified by RT-qPCR of selected candidates, where seven transcripts corroborated such behavior even when random primers were employed. Such findings could be explained by the very low transcription rate existent at the very end of the growth phase, just prior transcription it is completely suppressed at the onset of meiosis resumption (rev. Labrecque and Sirard 2014). Thus, the female gamete must produce and store enough molecular messages and proteins during the growth phase in order to support development in the course of transcriptional inactivity (rev. De Sousa et al. 1998; Sirard 2010; Macaulay et al. 2011; Clarke 2012).

1.4.2. Oocyte global transcriptome profile during maturation

Several studies on the analysis of the differences in mRNA levels during maturation of cattle oocytes will be described in this part of the review. There are strong evidences suggesting that a crucial part of the processes conferring the bovine oocyte with the molecular machinery to be developmentally competent take place during oocyte growth (Blondin et al. 1997a; Blondin et al. 1997b; Sirard et al. 1999; Blondin et al. 2002), before the final evolution from GV to MII. As a brief note, Sirard et al. (2006) defined *developmental competence* as the capacity of an oocyte to produce a healthy offspring. A widely accepted simplified measure of developmental competence is the ability of an egg to produce a blastocyst. For a thorough review of molecular markers of competence in oocytes from mouse, human, and cow see Wrenzycki et al. (2007) and Labrecque and Sirard (2014). A number of models used to discern the developmental competence level of oocytes in cattle include: Follicle size (Robert et al. 2000; Donnison and Pfeffer 2004; Mourot et al. 2006; Racedo et al. 2008; Caixeta et al. 2009), *in vivo* or *in vitro* maturation (Lonergan et al. 2003; Katz-Jaffe et al. 2009), age of donor (Patel et al. 2007; Dorji et al. 2012), follicular stage (Ghanem et al. 2007; Lingenfelter et al. 2007), electrophoretic migration (Dessie et al. 2007), FSH coasting duration (Labrecque et al. 2013), BCB staining (Ghanem et al. 2007; Opiela et al. 2008; Torner et al. 2008; Opiela et al. 2010), and chromatin configuration (Lodde et al. 2007; Lodde et al. 2008; Labrecque et al. 2015).

Oocyte maturation represents the first developmental phase following the start of the transcription blockage subsequent to the female gamete growth. Therefore, the study of the transcriptome at this stage can help understanding both the molecular regulation aimed at favoring an adequate maturation, as well as the subsequent modulation of the progressing steps through the first embryonic cleavages until EGA. In agreement with Fair et al. (2007), there is a difference in the mRNA levels of several genes at distinct moments during maturation of cattle oocytes, even considering the transcriptional quiescent period encompassing GV-MII. Discrepancies in transcripts levels pre- and post-maturation have also been reported in mouse (Cui et al. 2007) and human oocytes (Assou et al. 2006). Nevertheless, the variation of the amount of specific mRNA species is likely influenced by their poly(A) elongation and/or shortening, and selective degradation or translation, rather

than transcription occurrence (Fair et al. 2007). Overall, it is considered that throughout the transition from MI to MII about 30% of mRNA stockpiles are selectively degraded (Conti 2011).

Dalbies-Tran and Mermillod (2003) compared the transcript profile of cattle GV- and MII oocytes and found differential abundance in the mRNA of 70 genes during nuclear maturation, which were ontogenetically grouped in cell cycle control, apoptosis, and DNA transcription. Later on Fair et al. (2007) indicated that transcripts like *GDF9*, *ZP2*, *ZP3*, and *NALP5* (*MATER*) are overrepresented in GV- in comparison to MII- bovine oocytes. In such studies it was concluded that the major presence in immature gametes of mRNAs with preferential-, *GDF9* and *NALP5* (Pennetier et al. 2004), and specific presence, *ZP2* and *ZP3* (Topper et al. 1997), in cattle oocytes might indicate an important and time-specific action of such genes prior to nuclear maturation (Fair et al. 2007). In the last survey more than 800 transcripts displayed variable levels during meiotic maturation. By comparing these results with the findings of Misirlioglu et al. (2006), Fair's team found that common transcripts with higher abundance at MII mainly belonged to gene ontology (GO) categories as metabolism, transport, and cell death regulation. Thus, it was concluded that mRNAs involved in DNA regulation, metabolism, and internal/external signaling appear to be overrepresented at MII in bovine oocytes (Fair et al. 2007). This could appear contradictory due to the general regard of oocyte maturation as a period when mRNA synthesis is practically null. However, in agreement with Memili et al. (1998) and Pennetier et al. (2005) transcriptional activity is present in bovine oocytes during a short time window at the beginning of IVM (before GVBD). In a more recent survey, Mamo et al. (2011) detected that the top networks enriched in transcripts with differential abundance levels between GV and MII cattle oocytes corresponded to cellular assembly and organization, protein trafficking, translation, post-translational modification, and cell to cell signaling. On the other side, special care should be taken when considering a given transcript as preferentially represented in MII- in comparison to GV-oocytes as such difference might rather obey a major detection of the mRNA species due to poly(A) tail length variation and not to an increase in the presence of the transcript (Thelie et al. 2009). Such mechanism will be further discussed in section 1.4.3. Other possible source for the increase in the levels of

specific transcripts during maturation could be a proposed novel model of RNA transfer in the follicle, given that it was determined migration of mRNA and non-coding RNA from cumulus cells to the oocyte during maturation in the cow (Macaulay et al. 2014; Macaulay et al. 2015).

By using cross hybridization it has been possible to identify oocyte-specific (or preferential) genes conserved in distinct species. Vallee et al. (2005) and Vallee et al. (2006) confirmed that the transcripts *GDF9*, *BMP15*, *ZP*, and *Spindlin* with a widely known presence in oocytes, together with several with no reported role, as *MLF1IP*, *BTG4*, and *xPTB* are located only in the oocyte of mice (GV-), cattle (GV-), and *X. laevis* (immature, stage IV-V). Interestingly, a total of 208 mRNAs, including *ZAR1*, were found as maternal transcripts shared in all the species involved, suggesting a wide conservation of regulatory molecular mechanisms in oocytes. Furthermore, gene ontology (GO) analyses revealed that the top biological processes were RNA metabolism and cell cycle, while the main cellular component and the major molecular function were RNP complex and RNA binding, respectively (Vallee et al. 2008). Even if not a complete picture of the evolutionarily conserved oocyte maturation mechanisms was obtained since only immature oocytes were processed, results provided relevant insights of common molecular networks conserved in three phylogenetically distant species at the onset of female gamete maturation (Vallee et al. 2008).

1.4.3. Polyadenylation and deadenylation during maturation

In addition to the oocyte mRNA control by the masking system described for mRNP bodies, the 3'-polyadenylation status of the transcripts is a basic system used by the egg to modulate the recruitment of a given mRNA for translation (to suppress or to induce it). In somatic cells most nascent transcripts receive a poly(A) tail in the nucleus and bind to ribosomes for protein synthesis shortly after they are exported to the cytoplasm. Conversely, some mRNAs produced during oocyte growth (and several of them remaining up to embryonic cleavage stage) are translationally repressed by cytoplasmic deadenylation (Bachvarova et al. 1985; Paynton et al. 1988). At this point deadenylation precedes masking and storage. Thus, Eichenlaub-Ritter and Peschke (2002) remark that transcripts with a

short poly(A) tail (<90 nt) are stable (translationally quiescent), while those molecules with a length of 150 adenosine (A) residues or more are prompted for immediate use in protein synthesis. In oocytes the poly(A) tail length of the dormant transcripts is modified in response to physiological stimuli (e.g. follicular growth phase environment, molecular signals for maturation and ovulation). The trend points to a global change in the polyadenylation patterns of cattle oocytes with specific and simultaneous shortening or elongation of the poly(A) tail in distinct mRNA species (Eichenlaub-Ritter and Peschke 2002). Those transcripts that experienced deadenylation apparently were not recruited for degradation in one study (Brevini et al. 2002), but Thelie et al. (2007) found that particular molecules were degraded to some extent. These observations support the notion that those mRNAs experiencing deadenylation are either degraded or conserved in the ooplasm for ulterior translation according the oocyte requirements, while several mRNAs are selected to be polyadenylated and used rapidly.

In the cow, the oocyte-produced *CycB1* mRNA constitutes a clear example of an mRNA species subjected to polyadenylation for early translation (Lequarre et al. 2004), and the appearance of its poly(A)$^+$ transcript corresponds to that of its protein (Tremblay et al. 2005). Continuing in line with the biological relevance of transcript polyadenylation, there is evidence that a strictly regulated change of poly(A) length pattern is correlated to oocyte quality, as implied by Brevini et al. (2002) who characterized the polyadenylation state of transcripts like *CX32*, *CX43*, *OCT4*, *PLAKO*, *TPA*, *PAP*, *HSP70*, and *Glut1*, and observed variation in the poly(A) patterns between 2-cell embryo groups (and their originating oocytes) regarding their level of competence. Furthermore, the crucial character of the poly(A) tail status of maternal transcripts in early development has been validated in other domestic species: The culture of pig oocytes with the polyadenylation suppressor cordycepin resulted in remarkable low cleavage and blastocyst rates following IVF/IVP (Zhang et al. 2009). Since the mechanisms controlling deadenylation and polyadenylation status of maternal transcripts are not only employed during maturation but also crucial during early cleavage, the regulating processes relying on *cis*-sequences and *trans*-acting factors will be discussed in section 1.7.6.

1.4.4. Masking and storage of maternal transcripts

The oocyte stocks transcripts in its cytoplasm during the growth phase. The indispensable nature of the stored mRNAs relies on the need of the female gamete to synthesize proteins during a period when transcription activity is very low, if not considered as inexistent at specific periods (Lodde et al. 2007). Therefore, such molecules have been demonstrated as crucial in female gametes in all species studied: For example, *Mos*-directed RNA interference (RNAi) prevented meiosis entry in *Xenopus* oocytes (Sagata et al. 1988), while maturation is suppressed in cattle oocytes when they are cultured with alpha-amanitin, inhibitor of RNA polymerases II and III (Kastrop et al. 1991). Moreover, the accretion of transcripts in the oocyte cytoplasm remains fundamental during subsequent early development because it has been demonstrated by experiments focused on a group of oocyte-specific genes, for which directed genetic ablation (knock-out, KO) in the mouse causes early embryonic arrest. Some of them are *Nalp5*, *Zar1*, *Npm2*, and *Dppa3*. Due to their origin (egg) and highly deleterious phenotype for embryogenesis upon KO are called *maternal effect genes*. For review see Zheng and Dean (2007).

As will be discussed in the next section, the oocyte selectively modifies the polyadenylation status of the transcripts throughout maturation (Brevini et al. 2002). The length of the poly(A) tail is an important determinant of the mRNA fate because there is an association between recruitment for translation or degradation with high or low/null polyadenylation, respectively (Richter 1999). Thus, to accomplish the vital cytoplasmic pileup of mRNAs, the oocyte must use a mechanism to ensure that the deadenylated molecules are protected against degradation by ribonucleases, but at the same time keep them isolated from the translational machinery in order to produce their codified protein only at the moment required by the female gamete or the future embryo (rev. Macaulay et al. 2011; Clarke 2012). Anderson and Kedersha (2006) reported that the apparently universal mechanism used by developing eggs for transcript masking (translational repression) consists in maternal ribonucleoprotein (mRNP) particles, also called *germ cell granules* (GCGs), thus constituting a cytoplasmic RNA silencing/protective conserved system. In oocytes from *Xenopus* up to 80% of the inactive mRNAs are attached to mRNP complexes by mean of FRGY2, a GC-specific Y-box protein that links to mRNA molecules when these are not

coupled to polyribosomes (Anderson and Kedersha 2006). In vertebrates, Y-box proteins are the main components of mRNP particles and such proteins bind RNA molecules apparently in an unspecific way in frogs (Sommerville and Ladomery 1996). Concerning mammals, the Y-box proteins MSY1, MSY2, and MSY4 have been identified in mouse oocytes and early embryos (Bettegowda and Smith 2007).

MSY2 (*YBX2*) is the mouse homologue of the frog *FRGY2* protein. This molecule has also been reported in humans (Tekur et al. 1999) and cattle (Vigneault et al. 2004; Vigneault et al. 2009b). MSY2 maintains the GC-specific expression pattern of FRGY2, and its protein is present in high amounts in mice spermatogenic cells where it participates in protamine translational blockage by binding to a consensus sequence in the 3'-UTR of the *Prm1* mRNA (Giorgini et al. 2001). In the female, MSY2 is expressed in oocytes from primordial follicles in the mouse (Gu et al. 1998) and pigs (Shi et al. 2007) in conformity to its function in masking of transcripts since the growth phase. Through its binding to mRNAs, which is generally considered as unspecific for sequences in the oocyte but the data of Giorgini et al. (2001) and Yang et al. (2005) in male mouse GCs suggest a specific mRNA targeting also in female gametes. MSY2 mask such molecules, repressing their translation. Contrastingly, this protein can evoke transcription of those genes bearing Y-box motifs (a protein-DNA interaction). Although the detailed process of MSY2 binding to mRNAs is not known yet, it is possible that the protein accomplishes such task by joining nascent transcripts inside the nucleus to render them packaged since (or before) the first moment they reach the cytoplasmic compartment (rev. Macaulay et al. 2011; Clarke 2012). The shuttling of this protein into the nucleus seems to be in accordance with the finding of two nuclear localization signals of the human homologue (Tekur et al. 1999). A widespread distribution of MSY2 in the oocyte, and therefore its importance, is reflected by its estimated presence of up to 2% in the total protein pool of mice oocytes. In spite of a logical notion of the cytoplasmic location of MSY2 due to its attachment to mRNP particles, it was found that cytoskeleton (including cortex) accounts for 75% of the concentration of MSY2 in the female gamete. Such discovery not only strongly suggests a physical interaction of mRNPs and cytoskeletal components, as could be suggested by the

cortical distribution of the RNA-binding protein Staufen (Brevini et al. 2007), but also questions the actual localization of the stocked mRNAs in the oocyte (Yu et al. 2001).

The magnitude of the MSY2 role has been validated by functional assays. The physical stabilization of the maternal frog's protein with mRNA has been validated *in vitro* (Matsumoto et al. 2003). Moreover, Yu et al. (2004) observed that the RNAi of *Msy2* produces a remarkable deprivation of the overall mRNA species in oocytes accompanied of significant perturbation on both meiotic maturation structural hallmarks and associated protein synthesis, rendering severe infertility in mice (low ovulation and failure at fertilization). Similarly, the *Msy2* KO in mice affects oocyte maturation leading to female infertility likely due to overall decrease of stored mRNA levels (poly- and deadenylated) and aberrantly high transcription activity at MII, in addition to disruption of the cumulus cells connection to mutant oocytes (Medvedev et al. 2011). Therefore, MSY2 is a master switch of maternal transcripts regulation in mammallian oocytes through its mRNA stabilizing role. Another maternal factor that is probably involved in mRNA stabilization is Pcbp1, although its functions have not yet been clearly elucidated. Its mRNA is required for proper transcriptional silencing between meiosis and ZGA in mice. Therefore, it may be necessary for correct MZT (Xia et al. 2012).

1.4.5. Protein synthesis during maturation

As it has been reviewed, the tight regulation of gene expression at the post-transcriptional level in oocytes (masking and polyadenylation/deadenylation of transcripts) reflects the crucial nature of an appropriate protein synthesis during a period that also includes the final steps of intrafollicular development. Subsequently, the occurrence of translation during oocyte maturation is with no doubt fundamental since it has been validated in several species: Induction of GVBD of *Xenopus* oocytes requires translation of Mos and a Cdc2-associated protein (Ferrell 1999). Similarly, resumption of the first meiosis relies on protein synthesis in domestic animals: The inhibitor of translation, cycloheximide (CHX) blocks the progress towards GVBD in swine (Fulka et al. 1986), while the same drug has been repeatedly demonstrated to prevent maturation in bovine oocytes (Kastrop et al. 1991; Levesque and Sirard 1996; Lonergan et al. 1998; Sirard et al. 1989). However, translation is

a hallmark of the maturation process in cattle oocytes since synthesis of numerous polypeptides begins at GV-phase and extends up to MII stage, as reported by Sirard et al. (1989), Tomek et al. (2002), Coenen et al. (2004), and Massicotte et al. (2006). In addition to translation, maturation of oocytes in cattle is accompanied by posttranslational changes of the proteins present during such phase, as is the phosphorylation status (Vigneron et al. 2004).

To verify the timely translation of CycB1 in bovine oocytes Levesque and Sirard (1996) and Tremblay et al. (2005) detected the protein after the onset of maturation (just after GV-phase). This occurred in parallel with the appearance of the polyadenylated transcript (Tremblay et al. 2005), confirming the results from Robert et al. (2002) who did not find the protein form in GV-arrested cattle oocytes even when the *CycB1* deadenylated transcript was present. All this corroborated the CycB1 protein as a triggering agent of meiosis I resumption in cattle oocytes (Robert et al. 2002; Tremblay et al. 2005). Concerning the nature of the newly synthesized proteins during maturation, assays of 2-D electrophoresis allowed the identification of individual polypeptides from oocyte extracts. The proteins included HSC71, HSP70, CypA, UCH-L1, GSTM5, Cct5, E-FABP, 2,3-BPGM, E2D3, and beta-actin. Given that such proteins were present throughout oocyte maturation and up to 8-cell stage they were considered as maternal housekeeping proteins (MHKPs) with a putative function in both bovine oocyte and early embryo (Massicotte et al. 2006). These late findings provide additional evidence supporting the notion that molecular maturation in the oocyte provides elements that will be crucial for the early embryo (Sirard et al. 2006). Thus, the female gamete is at this developmental point ready for fertilization.

1.5. Ovulation and Fertilization

In mammals, the oocyte is ovulated in response to the LH surge and the subsequent final maturation of the egg. In the cow, ovulation takes place around 29-31 h after the gonadotropic stimulation (Driancourt et al. 2001). At the moment of fertilization the oocyte is still at the MII-arrest stage accomplished by the end of meiotic maturation. In cattle, Chian and Sirard (1996) have observed that the first signs of fertilization occur *in vitro* by 4

h post-insemination (hpi), while a maximum of sperm penetration evidence (98%) is noticed at 8 hpi. Thus, an average period when IVF takes place in this species is 6-12 hpi (for review, Labrecque and Sirard 2010). By employing murine models it has been established that the fertilizing sperm induces oocyte activation through delivery of PLC-Z in the egg cytoplasm, leading to the generation of IP3 in the ooplasm to finally produce intracellular calcium ($[Ca^{2+}]i$) oscillations (Larman et al. 2004) when Ca^{2+} pools are released from endoplasmic reticulum. Malcuit and Fissore (2007) described oocyte activation as a series of molecular cascades induced by the boost of $[Ca^{2+}]i$ levels leading to the next steps: 1) Exocytosis of CGs content in order to modify the oolemma and ZP, blocking polyspermy; 2) resumption of meiosis II and release of the 2nd PB following degradation of CycB simultaneously with inactivation of M-phase associated kinases; and 3) constitution of both pronuclei. It must be remarked that the molecular agents responsible for oocyte activation cascade were made and compiled in the oocyte during maturation. These are composed of metaphase-associated kinases and Ca^{2+}-sensitive elements (Malcuit and Fissore 2007).

To achieve male pronucleus (PN) generation, after sperm nuclear rupture the egg must decondense the paternal chromosomes by cleaving their disulphide bonds and substitute their protamines by histones therein enclosing male DNA into nucleosomes and producing the more relaxed and transcriptional machinery-accessible conformation of chromatin. Like the molecular factors used during oocyte activation, this protamine-substituting machinery is produced and accumulated during oocyte maturation (McLay and Clarke 2003). In addition, the ooplasm provides vesicles derived from the endoplasmic reticulum to surround the remodeled paternal genetic material by a new nuclear envelope. DNA from both pronuclei is duplicated before mixing up during the first mitotic cycle, thus generating a diploid 2-cell embryo (McLay and Clarke 2003). Previously, the completion of MII rendered a female PN owning a 1N chromosomal complement that together with that of the male PN forms a 2N nucleus in the late zygote that is the first stage of embryogenesis. Even though it constitutes a new organism, the embryo is still tightly attached to maternal developmental regulation during the first cellular divisions.

1.6. Embryonic Cleavage and First Cell Cycles

Early embryogenesis is characterized by a series of extremely fast mitotic divisions, termed *cleavages*, aimed at dividing the large volume of the initial zygote in two daughter cells, which in turn will generate four cells and so forth. The cells formed from these symmetric divisions are called *blastomeres* and are totipotent. In comparison to other vertebrates, cleavage divisions in mammals can be asynchronous. Beyond the cleavage stage, which extent is particular to every species, the mitotic divisions notably decrease in speed and this appears to be a hallmark in all species studied (Gilbert 2006). Subsequently, compaction occurs. This event is observed around the 32-64 cell stage in bovines (rev. Vigneault 2008) when the embryo is known as *morula* and some cell differentiation takes place in preparation for blastocyst formation (Johnson and Ziomek 1981; Abe et al. 1999). Compaction is followed by the appearance of an aqueous cavity, *blastocoel*, that will gradually expand until accomplishing the first major differentiation of embryonic life consisting in the rise of the trophectoderm towards the periphery and the pluripotent internal cell mass (ICM) lineages, while the blastocyst increases its diameter (the first enlargement of external size during embryogenesis). Shortly after, the ZP is hatched and the blastocyst is released. All these events occur in cattle within one week after fertilization (rev. Badr et al. 2007).

In relation to the timing of cleavages, it is slow by far in mammals in comparison to the quick division of embryos of other vertebrates. For instance, the end of the cleavage stage in *Xenopus* occurs by the 12^{th} cell cycle when the embryo reaches a content of around 4,000 cells in just 8 h post-insemination (hpi). Then, the ensuing cell divisions in the frog are characterized by a gradual decrease in speed (Newport and Kirschner 1982a; Newport and Kirschner 1982b). Alternatively, some authors consider that the cleavage rate in cattle embryos shows approximately one cell division per day (Ushijima et al. 2008; Ushijima et al. 2009). More in detail, bovine embryos achieve the 4- and 8-cell stage by 36-50 and 56-64 hpi in average, respectively with a duration of 13 h for the second and 14 h for the third cell cycle, whereas cleavage to the 16-cell phase is accomplished by 80-86 hpi (Fig. 1-5), corresponding to a 21-30 h length for the fourth cell cycle (Barnes and Eyestone 1990). Thus, it could be noticed that the rate of cell division progressively slows down as the

embryo approaches the end of the cleavage stage, which is presented at the 8-cell phase in bovines followed by a remarkable lengthening of the fourth cell cycle (Barnes and Eyestone 1990).

At the molecular level, multiple factors intervene in cell cycle activity and regulation either in somatic lineages, gametes, or early embryonic cells. However, in the early embryo, particularities exist in cell cycle regulation that must be highlighted. Firstly, the cleavage stage consists of a rapid succession of mitotic divisions dominated by the presence of S- and M-stages, lacking (or just with a short representation in some cell cycles) the gap phases (G1 and G2), where most of the transcript synthesis normally takes place (Barnes and Eyestone 1990). The absence of cell cycle periods where mRNA synthesis occurs reflects the fact that the early embryo initially experiences a transcriptionally inactive period when all its biological processes depend on transcripts and proteins from maternal origin. In addition, several lines of evidence suggest that the transition from the fast divisions during cleavage to a slower rate of mitosis is apparently correlated with the takeover of developmental control by the embryonic genome in different species (Barnes and Eyestone 1990).

As stated by Gilbert (2006), the transition from the end of meiosis II in the fertilized egg to the first mitotic cell cycle in the zygote depends on the inactivation of MPF and destruction of the CycB1 protein, in a similar way to the resumption of meiosis in the oocyte. The fast cell cycles in the course of the early cleavage period rely only on the shift of M and S mitotic stages with are driven in such moment by the activity of Cdc2/CycB1. MPF action is highest during mitosis, and as soon as it finishes, CycB1 is degraded. Cdc2, the kinase regulated by this cyclin, activates mitosis through phosphorylation of targets like the regulatory subunit of cytoplasmic myosin, histones, and the nuclear membrane protein lamin, thus triggering the organization of the mitotic spindle, chromatin condensation, and nuclear envelope disassembly, respectively (Gilbert 2006). CycB1 has not only been corroborated as the central switch of meiosis entrance in bovine oocytes by the finding of its protein form (Levesque and Sirard 1996), but its maternal transcript has also been found throughout cleavage with high levels remaining by the first embryonic division. This

implies the crucial CycB1 role during early embryogenesis in this species (Tremblay et al. 2005).

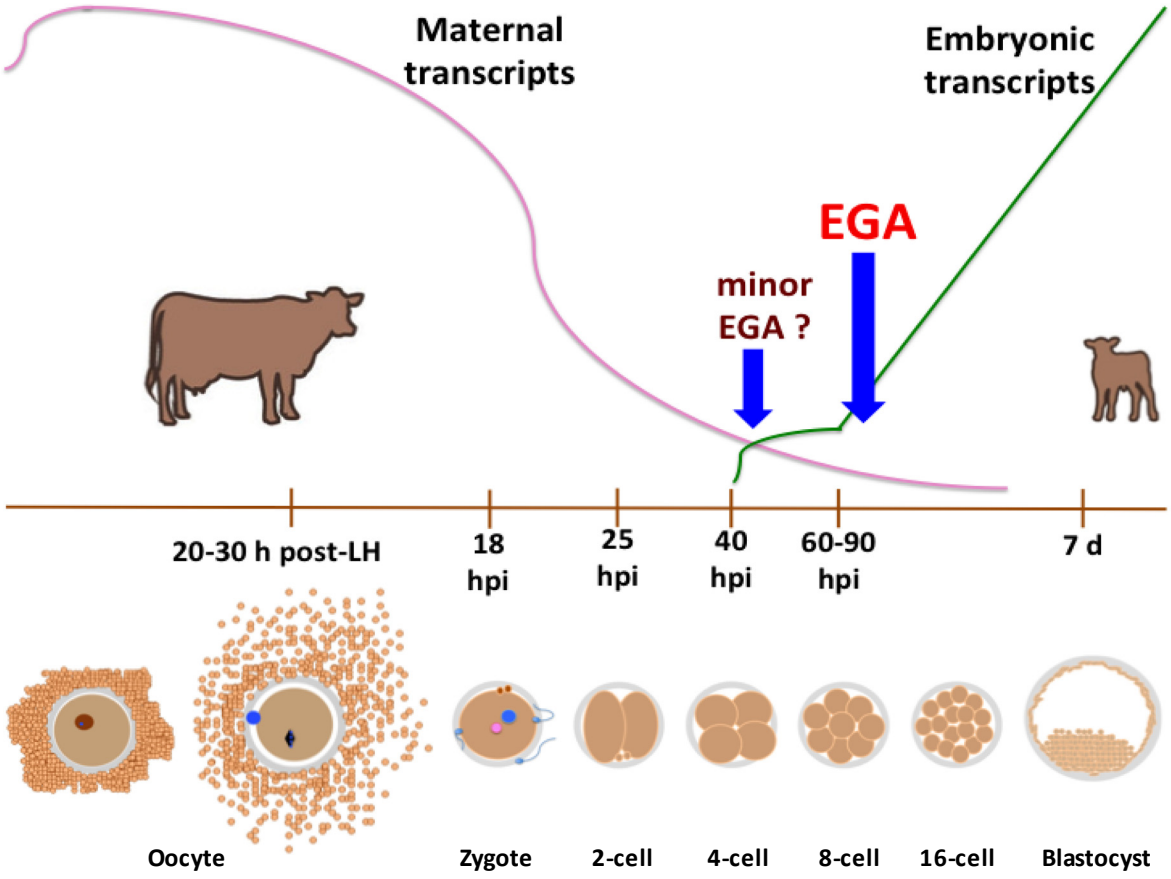

Figure 1-5 Dynamics of early development in cattle and embryonic genome activation
h, hours; LH, luteinizing hormone; hpi, hours post-insemination; EGA, embryonic genome activation; d, days. Scheme adapted from Kasinathan et al. (2015). Copyright-free (Macmillan Publishers Ltd. © 2015).

One of the first events of the mitotic division is the formation of kinetochores that will associate with spindle microtubules to segregate chromosomes. In agreement with Toralova et al. (2009), CENPF (mitosin) is one of the earliest proteins that associate to pre-kinetochores and is a basic instrument throughout all the mitotic division, being rapidly destroyed after this finishes. The vital function of CENPF has been confirmed in early bovine embryos since the maternal transcript is expressed but diminishes by the early 8-cell stage (Kanka et al. 2009). However, it is not completely degraded but rather complemented

by the protein of embryonic origin, reflecting the importance of CENPF both during pre-MET period and after EGA (Toralova et al. 2009). The reappearance of CENPF expression at high levels is similar to the expression of a related transcript, *CENPE*, by the moment of the major burst of *de novo* transcription from the bovine embryonic genome (Vigneault et al. 2009a). The RNAi of *CENPF* in cattle zygotes notably impairs development after the 8-cell stage, concurrently with EGA in this species, denoting its crucial function at the cleavage stage (Toralova et al. 2009).

By the end of the M-phase the cell must ensure passage to subsequent phases in order to complete the cell cycle. One of the modulating mechanisms to exit from M-stage consists of the proteolysis of PTTG1 (securin) and CycB1 by proteasome-dependent degradation. This mechanism is driven by the APC/C ligase, thus allowing sister chromatids separation (previously blocked by PTTG1) in coordination with exit from mitosis (inhibited by CycB1, whose activity and that of separase are mutually obstructed) and the consequent advance to the next phase of mitosis, anaphase (Wolf et al. 2007). These events are simultaneous with additional Cdc2 inactivation upon dephosphorylation bringing to a permissive state several proteins that were before restrained by the kinase activity of Cdc2. By interfering with Cdc2 performance the activity of several phosphatases prepares the ulterior progression of the cell cycle towards cytokinesis (Wolf et al. 2007). Prior to the initiation of anaphase, the cell has to assure the correct DNA segregation for the future daughter cells. This is performed by the *spindle assembly checkpoint* (SAC) complex that also includes APC/C ligase. The initial action of SAC hampers the premature transition from M-stage to anaphase until all chromosomes are adequately assembled with the bipolar spindle, but after PTTG1 and CycB1 ubiquitination it allows separase (ESPL1) to segregate sister chromatids (Wei et al. 2011). It has been demonstrated that both the RNAi-mediated ablation of genes integrating the mitotic checkpoint complex (MCC), a subset of SAC, as well as nocodazole spindle depolymerization accelerate the M-anaphase progression in the course of the 1st cleavage in mouse embryos. In contrast, the overexpression of SAC proteins hindered the same developmental transition, suggesting SAC as fundamental for mitosis progression during the murine cleavage period (Wei et al. 2011). Hence, if anaphase, telophase, and cytokinesis were successfully performed, the early blastomeres

would be ready to evolve into another M-phase. However, subsequent work from Sebestova et al. (2012) demonstrated that a fully functional SAC is present in mouse oocytes in spite of being obtained from aged females or hybrids. It was determined that a normal SAC system on its own is unable to halt anaphase-promoting complex activation, even when multiple kinetochores are misaligned with the consequent aberrant chromosome congression, and eggs enter anaphase without delay. Notably, Sebestova's results appear related with recent findings of McCoy et al. (2015), where the main cause of aneuploidy in human embryos was anaphase lagging (that leads to chromosome loss). According to Sebestova et al. (2012), the final outcome of errors of chromosome alignment is high incidence of embryonic aneuploidy. Therefore, it was proposed that oocytes, in contrast to somatic cells, lack a mechanism responsible for correcting chromosome misalignment as an intermediary between SAC and APC functions in spite of normality of these last two systems (Sebestova et al. 2012).

The advancement of first embryonic mitotic divisions to the end of the cleavage stage starts gradually adding gap phases to the cell cycle. The appearance of a marked G2 phase (of a duration of up to 6 h) in the 3rd cell cycle in some bovine blastomeres has been reported (Barnes and Eyestone 1990). The transition of mitosis in the early embryos means that new factors join the cell cycle due to the association of gap periods. Some of them are cyclins A2, D1, D2, D3, E1 and E2, and Cdk4 and -6. The relative importance of these cell cycle regulators has been tested and the results indicate redundancy among cyclins and Cdk members in their respective groups, given that only double genetic ablation of several of them generates lethality in the mouse. Nonetheless, the crucial role of Cdc2, CycB1, and CycA2 has been confirmed by the death of homozygous null mutants (Artus et al. 2006).

The paramount importance of the accuracy in cell cycle execution during the cleavage stage is manifest when considering the major effects that it exerts over embryo viability since one of the most fundamental factors during such period is the maintenance of a correct ploidy (2N) of blastomeres. In this way the genome integrity should be guaranteed at the chromosome level, but single nucleotide abnormalities should not be ruled out (Hyttel et al. 2001; Leidenfrost et al. 2011; Pers-Kamczyc et al. 2012). Specifically, in the bovine species

there is a high incidence of failures from the first to the third cleavage divisions which may generate an irreversible cell cycle blockage leading to death of single blastomeres with consequent developmental anomalies, or in the most extreme cases (but not rarely) an immediate embryo demise. Such cell division errors importantly account for the developmental heterogeneity observed in groups of cattle embryos both in *in vitro* and *in vivo* models, although with a higher presence in the former ones (Hyttel et al. 2001; Leidenfrost et al. 2011; Pers-Kamczyc et al. 2012).

Concerning the nature of the cytogenetic phenotype originated by the cell division failures, an increase of mixoploidy frequency has been observed in cattle embryos from day 2 to 5 in culture. However, during the period immediately following IVF there is a substantial incidence of pure polyploid embryos (the slowest embryos being the most affected by polyploidy), which gradually decreases until its disappearance in the batch of 9-16 cell embryos analyzed (Viuff et al. 2000). From such study it could be inferred that the origin of polyploidy can be traced back to gametogenesis and/or IVF with a consequent elimination of polyploid embryos, while the inception of mixoploidy is apparently imposed by the moment of IVF or in the course of embryo culture (Hyttel et al. 2001). Nevertheless, it remains to be established how noxious is the death or arrest of an individual early blastomere for subsequent bovine embryo development since it was frequently noticed that the third and fourth cleavages produced less than 8 and 16 viable cells, respectively, which continued dividing through further embryogenesis given their normal genotype/epigenotype (Leidenfrost et al. 2011). The same group concluded that the type of cell demise of early blastomeres points out to a mitotic cell death rather than apoptotic processes due to morphology assessment, negative TUNEL results, and low amount of apoptotic triggering transcripts from oocyte to the first cleavage stages (Leidenfrost et al. 2011). Although it should not be ignored that some polyploidy could originate from paternal contribution, available evidence suggests that those chromosomally abnormal gametes are mostly oocytes: Up to 14.6% in comparison with a maximum of 7% anomalies in spermatocytes (Lechniak et al. 1996). All this also underscores the vital nature of meiosis, as well as the molecular factors stored during oocyte maturation due to the fact that the molecular machinery responsible for cell cycle progression (e.g. induction of meiosis

resumption/mitosis) and the processes circumventing chromosomal abnormalities (centrosome and spindle assembly modulation, cell surveillance/DNA repair, and cell cycle checkpoints) during the early cleavage is from maternal origin (Leidenfrost et al. 2011).

1.7. Maternal-Embryonic Transition

The maternally-inherited gene products (mRNA, protein) become gradually exhausted through the first embryonic cell divisions in the absence of additional transcriptional activity (Memili and First, 2000; Gilbert et al. 2009). Moreover, the embryo as a new individual that will produce the differentiating cell lineages that give raise an adult organism needs a distinct group of mRNAs and proteins, coming from its own genome, rather than those provided by the egg (Sirard 2010). This is the rationale for the *maternal-to-embryonic transition* (MET), defined as the developmental time point where the transcripts and proteins produced and stored by the oocyte are replaced by those synthesized by the newly formed embryo. This switch in the origin of mRNAs and proteins during embryogenesis is performed by the gradual activation of the genome of embryonic origin (Sirard 2010). In agreement with Schultz (2002) and Minami et al. (2007), the MET can be understood as a series of events comprising three major phases: 1) Degradation/use of mRNAs and proteins inherited from the egg, which are not subsequently expressed because they might be unnecessary or even prejudicial for the embryo; 2) substitution of maternal transcripts by a small subset of messages generated by the embryo to mainly cover housekeeping functions when such oocyte mRNAs are already consumed; and 3) transcription of novel mRNAs by modifying the gene expression pattern (appearance of transcripts from embryonically-activated genes), to finally accomplish EGA and the capacity of the new organism to perform functions proper for the embryo.

Given that the chronology of MET and EGA is particular to the species, in this review both terms will be employed since they may apply to all groups of metazoans. On the other hand, the concepts *maternal-zygotic transition* (MZT) and *zygote genome activation* (ZGA) are frequently used to refer to these biological events independently of the animal species, although because a major activation of the transcriptional activity occurring just after the

zygote stage is specific to mice the concepts MZT and ZGA will not be generalized here (Sirard 2010).

1.7.1. Embryonic genome activation

The tightly regulated MET comprises several sub-phases in preparation for the takeover of developmental gene control by the embryo or EGA. This can be considered as a sub-step of MET (Sirard 2010). The burst of transcriptional activity from the embryonic genome is preceded by the transcriptionally dormant period after oocyte maturation and common to all species studied. The activation of embryonic genes occurs by the end of the embryonic cleavage stage at variable time points depending on the species. Such a milestone is achieved after multiple cell divisions in invertebrates, but in a very short time. For instance, *Drosophila* embryos turn on their genome after 14 cell cycles and just around 2.5 h (Tadros and Lipshitz 2009). Conversely, mammals accomplish EGA during the first few cleavages but in the order of days. The culture in presence of alpha-amanitin has demonstrated (due to embryonic arrest and differential mRNA or protein fingerprints) the occurrence of EGA at 2-cell in mice (Schultz 1993; Aoki et al. 1997), 4 to 6-cell in pigs (Anderson et al. 2001), 4 to 8-cell in humans (Braude et al. 1988), 8 to 16-cell in rabbits (Henrion et al. 1997) and bovines (Barnes and First 1991; Memili and First 1998; Memili and First 1999), and 16-cell stage in sheep (Crosby et al. 1988). Notably, this developmental blockage beyond MET due to drug inhibition is similar to that frequently observed in IVP embryos in cattle and in other species (Memili and First 2000). This strongly suggests major implications of potential EGA failure in IVP-embryos when compared against their IVD counterparts (Gad et al. 2012; Sirard 2012). At this point it must be underscored the apparent relationship between onset of EGA and [Ca2+]i transients occurring after fertilization (Larman et al. 2004). Jin and O'Neill (2010) established that [Ca2+]i transients, whose major amplitude occurs in mid 2-cell embryos in the mouse and are mediated by PI3K signaling, are essential for preimplantation development progress. In agreement, oocyte-derived PI3K cascade is crucial for ZGA in mice, as demonstrated by Zheng et al. (2010) with the genetic ablation of *Pdk1* in mouse embryos that failed to properly activate their genome and arrested at the 2-cell stage.

Regarding the nature of genes transcribed by the time of EGA, in zebrafish striking function differences were observed between maternally- and zygotically-transcribed genes, corresponding with RNA regulation (codifying for ribosomal and spliceosome component proteins) and DNA control (DNA-binding proteins, histones, chromatin modifiers), respectively. Nevertheless, a minority (3%) of the transcripts identified were produced by both the maternal and the zygotic genomes and were thus termed *maternal-zygotic transcripts*. It was suggested that only a select group of genes is newly expressed after activation of the embryonic genome due to the shared importance of their encoded products for both pre- and post-EGA stages (Heyn et al. 2014). Furthermore, it was determined that the first embryonically-synthesized transcripts are generally intron-poor and around four times shorter than maternal transcripts, presumptively due to the short time (~15 min) of the first cell cycles in the zebrafish embryo (Heyn et al. 2014). In relation to mammals, it has been demonstrated that cattle embryos strongly activate rRNA genes at the fourth cell cycle. This reflects the need for a massive and accurate start in the expression of genes aimed at sustaining cellular functions (Hyttel et al. 2000). Similarly, Vallee et al. (2009) reported that the *LDHB* mRNA experiences important decrease from the cattle GV- to the 8-cell phase *in vivo*, with a marked outbreak of transcription afterwards until blastocyst formation. This can be explained by the fact that the oocyte uses pyruvate and lactate, metabolized by the LDHB enzyme as its main oxidative substrates (Cetica et al. 2003), which are still used by the blastocyst, although at less important rates than the egg/cleavage-stage embryo (Lane and Gardner 2000).

On the other side, Zeng et al. (2004) observed that EGA in mice mostly induces transcription of genes involved in RNA processing and transcription in addition to genes related to cell cycle. In cattle, the gene circuits instigated by EGA were revealed by comparing the transcriptomic profile of (control) late 8-cell embryos (8-12 cells) against that of embryos at the same stage but cultured with alpha-amanitin. Initially, in addition to factors related to protein biosynthesis, RNA processing, and gene transcription, GO analyses uncovered genes involved in pluripotency regulation as overrepresented in control embryos, including *ZFP42*, *KLF4*, *TPT1*, and *GABPA*. These results are in line with the events supposed to have primacy after MET, as they are transcriptional activity, protein

supply, and regulation of the pluripotent state in preparation for cell lineage segregation during subsequent embryogenesis (Vigneault et al. 2009a). More recent characterization of data from the same work, although employing a more powerful GO platform (IPA, INGENUITY®), confirmed and enriched the examination when it found processes like transcription regulation, protein biosynthesis, RNA processing, DNA replication/nucleus biosynthesis, and protein degradation/modification as overrepresented at EGA stage in the bovine (Sirard 2010). Consistently, in the survey of Misirlioglu et al. (2006) it was reported that the newly transcribed genes of 8-cell cattle embryos preferably represent among other GO terms, transcription and chromatin structure. These two reports in the bovine are in line with the exacerbated biological functions found by Zeng *et al.* (2004) in mice. This last group concluded, like Hamatani et al. (2004), Sirard (2010), and Toralova et al. (2012) that EGA induces molecular factors in a step-wise and selective fashion, rather than global, according to the embryo's more immediate needs. This was shown in the microarray study in mice of Hamatani et al. (2004), where it was suggested that ZGA is succeeded by a mid-preimplantation gene activation (MGA), where gene expression switches to support the physiological and morphological modifications during the morula to blastocyst transition. Nonetheless, it must be underscored that recent results by Abe et al. (2015) demonstrated pervasive transcription at the zygote stage in mice, although the authors suggested that such activity rendered non-functional transcripts (with quite inefficient splicing) apparently to prime chromatin in preparation to synthesize transcripts actually functional (Abe et al. 2015). Therefore, by correctly activating its genome the embryo assures its preparation for ulterior development.

One of the earliest needs of the developing embryo is the synthesis of rRNA. Jakobsen et al. (2006) established that bovine embryos are apparently able to start transcription of rRNA at the late 4-cell stage (G2-phase of the third cell cycle). This putative onset of rRNA synthesis in the rising nucleoli appeared delayed in IVP embryos in comparison to those produced *in vivo* and was suggested as a possible reason for the increased competence of IVD-embryos (Jakobsen et al. 2006). Nevertheless, this assumption needs further reconsideration since in the report by Fulka and Langerova (2014) the lack of nucleoli did not impede rRNA transcription during murine ZGA (at least partially accomplished). In

contrast, nucleoli seemed crucial for further development. After experimental excision of nucleoli, fertilized eggs progressed through development but soon arrested at the 2-cell stage. Toralova et al. (2012) stated that the phosphoprotein nucleophosmin (NPM1) localization during preimplantation development in bovines implicated a possible function during nucleologenesis since its distribution reflected vacuolized nucleoli complete formation around the time of major EGA. The functional NPM1 protein involved in nucleoli production at the onset of the major burst of embryonic transcription appears to correspond to that translated from the maternal transcript. This is due to the fact that *de novo*-synthesized *NPM1* mRNA is first detected at the 8-cell stage. Although RNAi of *NPM1* did not cause any conspicuous effects on cytoskeleton and nuclear structure either, acquisition of somatic-like nucleophosmin localization outside of the nucleoplasm after translocation inside of nucleoli, which normally occurs at the 8-cell stage, was delayed one cell cycle (Toralova et al. 2012). This could denote a partial/temporal impact of NPM1 on EGA. Moreover, ablation of maternal *NPM1* generated a moderate but significant decrease in blastocyst rate, suggesting that although the stock of the maternal NPM1 protein might have not decreased sufficiently to create a more deleterious phenotype, NPM1 plays a key role during MET in bovines (Toralova et al. 2012).

A crucial role during EGA might also be played by nuclear chaperones. Bouleau et al. (2014) described a rich maternal accumulation of *npm2* transcripts in zebrafish. This mRNA was proved to be oocyte-specific and it was not *de novo* synthesized from the embryonic genome. It was observed a gradual degradation/use of the *npm2* transcript, with a marked decrease around MBT (a typical profile of a maternal factor). Npm2 protein had nuclear localization and it was abundant even in unfertilized eggs and kept strong levels during embryogenesis, when the protein continued to be synthesized from the maternal transcript stock. Ablation of *npm2* in zygotes showed that the newly-translated protein is indispensable for developmental progress up to the initial phase of gastrulation in teleosts (Bouleau et al. 2014). Furthermore, *dusp1*, *her5*, and *dact1* expression levels were dramatically decreased. Since these three factors are markers of the first wave of embryonic transcription, this observation proved the crucial role of Npm2 in zebrafish EGA. This transcriptional impairment preceded embryonic arrest. Given that the impact of *Npm2*

knockdown occurred in spite of sustained high levels of the protein synthesized before fertilization, it could be hypothesized different roles for the maternally-inherited protein (e.g. sperm chromatin decondensation) and the *de novo* translated protein (e.g. modulation of EGA) following fertilization (Bouleau et al. 2014). The observation that the maternal NPM2 is dynamically regulated during MET in cattle (Lingenfelter et al. 2011) reflects the crucial character of nuclear chaperones in early development that might be shared with phylogenetically-distant vertebrate groups.

1.7.2. Potential existence of minor embryonic genome activation

Redundant evidence suggests the existence of a small wave of transcriptional activity before the main burst of mRNA synthesis from the embryonic genome in cattle, as well as in other species. This was addressed in rabbit zygotes by *LacZ* transgene expression (Delouis et al. 1992). In the mouse, Latham et al. (1991) registered the synthesis of the 70 kDa-transcription requiring complex (TRC), marker of MZT, demonstrating that the 1-cell embryo's cytoplasm is both permissive and inductor of transcription of a heterologous nucleus. Subsequently, transcriptional activity was localized by BrUTP incorporation into the paternal PN at the late zygote- (Bouniol et al. 1995) and mid-S phase (Aoki et al. 1997) in mice, while the activation of transgenes in zygotes has been demonstrated by the transcription of an exogenous *Hsp70.1* gene (Christians et al. 1995). The previous works also denote an earlier onset of ZGA in the male PN in comparison to the female one (Beaujean et al. 2010). In spite of the fact that alpha-amanitin-sensitive BrUTP incorporation has been detected, adding more data supporting the presence of transcription in mouse zygotes, no transcripts modulated by such drug have been identified by microarrays during that phase (Zeng and Schultz 2005). Therefore, *MuERV-L* (Svoboda et al. 2004) and one mRNA reported by Hamatani et al. (2004) are still the only *de novo* transcripts from the minor EGA (1-cell) identified so far in mice. Even when transcription has been described in the murine zygote, translation of embryonic messages does not seem to occur until the 2-cell stage (Davis and Schultz 1997).

In the cow, since the report of Kopecny (1989) it has been considered that EGA occurs in this species by the 8-16 cell phase. Nevertheless, it has been observed that a small subset of

proteins at the 4-cell phase enlarges in number by 8-cell (Barnes and First 1991), suggesting that a small and gradual transcription also occurs in cattle before previously thought. [3H]uridine (Hyttel et al. 1996; Viuff et al. 1996) and [35S]UTP (Memili et al. 1998) assays proved RNA synthesis in 2-cell embryos, whereas Memili and First (1998) demonstrated that transcription in the 1- to 2-cell embryo is needed for progression beyond the 8-16 cell stage. In addition, a slight level of [3H]uridine incorporation was detected as early as the 1-cell stage, accompanied by protein synthesis from the zygote, implying that the initial transcripts and protein synthesis of embryonic origin are coupled in bovines, contrary to the mouse. The authors suggested that MET in cattle comprehends a small transcriptional activity ("minor wave") between 1- and 4-cell stages, followed by a major activation in the 8-16 cell embryo (Memili and First 1999). However, similar to the murine model, the identities of the messages appearing during the potential minor EGA in cattle are not well elucidated. In the report of Kanka et al. (2009) a sole mRNA sensitive to alpha-amanitin, *SRFS3*, was found as transcribed in the 4-cell bovine embryo, while Mondou et al. (2012) showed microRNA synthesis at the 2-cell stage and confirmed Kanka's results for *SRFS3*.

So far, there is a profound lack of identification of mRNAs generated during the putative minor EGA and inhibited by alpha-amanitin both in murine and bovine models. In relation to this, it has been proposed that in spite of the elevated BrUTP incorporation in mouse zygotes, the lack of identification of such transcripts might result from incomplete transcription, and failures in nuclear export and mRNA stability, rendering mRNAs lacking poly(A) tail and thus undetectable with methods based on targeting such region. Moreover, RNA polymerase II (RNPII) participates in the production of some snRNA and snoRNAs that are not polyadenylated. If these RNAs accounted for the observed BrUTP incorporation in the zygote, they could collaborate with the preparation of major ZGA (Zeng and Schultz 2005). Alternatively, the contribution (in some extent) to the alpha-amanitin-resistant pre-EGA transcription in cattle and rodent zygotes by another type of RNA polymerase could be worth of investigation since it has been reported that an isoform of a mitochondrial RNA polymerase is able to produce mRNAs in human and rodent cultured cell lines. This enzyme localized to the nucleus and was shown to be unaffected by

alpha-amanitin (Kravchenko et al. 2005). This could be proved by the activation of reporters coupled to embryo-specific promoters (Sirard 2010), besides working under developmentally non-arrested conditions. For instance, Gagne et al. (1995) observed transcription more than 50 hpi in cell division-arrested bovine zygotes, showing that time and not cell cycle would allow transcription from such reporters (Gagne et al. 1995). A more recent report addressing the issue of minor EGA in cattle arose from E. Wolf's group where in addition to data supporting the occurrence of major EGA at the 8-cell stage, it was detected a minor wave of *de novo* transcription in 4-cell embryos (Fig. 1-5). Remarkably, the main processes found in the presumptive minor EGA corresponded not only to transport but also to mRNA processing and translation, which agrees with the functions suggested above as crucial to surpass this developmental milestone. Moreover, the transcription factor *KLF17* is first transcribed at the 4-cell stage in their dataset (Graf et al. 2014). KLF17 belongs to the same family as KLF4 and KLF5. KLF4 is known for being a master regulator of pluripotency and used for nuclear reprogramming (Takahashi and Yamanaka 2006). KLF proteins can modulate *Oct4*, *Nanog*, and *Cdx2* (rev. Suzuki et al. 2015). In spite of the fact that samples from Graf et al. (2014) were not processed with any spike-in RNA external control and the existence of minor EGA may still remain controversial, this report contains comprehensive data to help to understand this critical developmental window in bovines. Similarly, in the transcriptome fingerprint of cattle *in vivo* eggs and early embryos by Jiang et al. (2014) they detected occurrence of bovine EGA at the 4 to 8 cell transition, as in Kues et al. (2008).

1.7.3. Nuclear organization

Nuclear spatial configuration also accounts for a crucial regulation of gene expression. This notion has shed light to the theory of *nuclear regionalization*, inferring that gene location is regulated in function to their expression status (Beaujean et al. 2010). Given that the interior of the nucleus lacks membrane-bounded divisions, the segregation of the nuclear components has to be done through dynamic compartments consisting of stable proteins, filaments, and distinct chromatin arrangements/positions during interphase (*chromosome territory*). In this way, genes are placed according to their transcriptional state (rev. Beaujean et al. 2010). This is done by the heterogeneous localization of proteins inside the

nucleus in which specific DNA-protein interactions allow the formation of chromosomal loops, and by this chromatin bending, genome compaction is regulated, chromosomes are segregated in territories (compartments of the order of Mb), and gene sequences are exposed to different proteinaceous environments (e.g. distinct concentrations of transcription regulators) affecting their expression. It is known that gene-sparce and repressed regions tend to be embedded to the nuclear periphery close to heterochromatin, while more active and gene dense sections generally pack towards more internal places (Meldi and Brickner 2011).

On the other side, the association of chromosomes with major nuclear structures, like the lamina, affects transcription and high order chromatin folding. This is possible because of the existence of large chromatin regions called *lamin-associated domains*, LADs (Bielanski et al. 1993) and these are commonly suppressed. However, LAD interactions are dynamic and change through cell differentiation. Moreover, the existence of a nuclear matrix, whose scaffold of fibro-granular RNP filaments is used by chromosomes to fold and modify transcription has been proposed. Nuclear matrix proteins attach to particular DNA sequences, the scaffold/matrix-associated regions (S/MARs), which tend to be non-coding and interweaved, to help in folding, finally constraining loci positioning (subnuclear gene localization) and gene expression due to distinct accessibility of the transcriptional machinery (Meldi and Brickner 2011). It has been observed that several genes are located at the periphery during their dormant state, but subsequently migrate through looping to more internal positions when transcriptionally active. Another strategy to control gene expression is to place clustered or functionally related genes in transcriptional foci next to nuclear pores to facilitate mRNA export. By these systems, the nucleus can provide preferential arrangements to optimize specific gene expression (Meldi and Brickner 2011). These mechanisms underscore the importance of proteins that bend chromosomes during interphase, including those of the nuclear matrix. An example of the importance of nuclear matrix proteins during early development comes after the work of Vigneault (2008), who demonstrated that depletion of *MATRIN3* in cattle oocytes dramatically decreases blastocyst rates upon IVP protocol application, extending the insights of nuclear scaffold functioning to early development in domestic species. In relation to other nuclear compartments, it has

been noticed that centromeric and pericentromeric sequences acquire a radial configuration around nucleolar precursor bodies (NPBs) at the beginning of the 2-cell stage in mice (Casanova et al. 2013). Such conformation is completely lost in the 8-cell embryo and is completely dependent on transcription at major satellite regions, but is independent of DNA replication. Furthermore, this large change in heterochromatin configuration and major satellite transcription are regulated by reverse single-stranded pericentric lncRNAs (Casanova et al. 2013). Remarkably, transcription of satellites must also be tightly regulated. De La Fuente et al. (2015) found that maternal ATRX is necessary to avoid noxious mitotic recombination at minor satellite sequences (centromeric). Moreover, ATRX, in conjunction with H3K9me3 and H4K20me3 repressive marks, was shown to silence transcription of major satellite sequences (pericentromeric) in the maternal pronucleus. Analogously to the report of Casanova et al. (2013) in mice, Pichugin et al. (2010) reported attainment of chromocenter-like structures in 8-cell bovine embryos. Since in both models the formation of such arrangements is concomitant with EGA, this suggests their correlation with *de novo* transcription across species (Casanova et al. 2013). In spite of the questions concerning the function of nuclear organization, increasing evidence supports its crucial role to fine-tune transcription (rev. Beaujean et al. 2010; Meldi and Brickner 2011), which includes the possibility for the embryo to adequately seize gene expression control and overcome a major developmental barrier.

A detailed analysis of the potential relationship between sub-nuclear structure dynamic rearrangement and the onset of transcription is provided by Aguirre-Lavin et al. (2012) in the mouse, where differences between maternal and paternal chromatin shortly after fertilization were found by tridimensional analysis of nuclear compartments. In monitored zygotes pericentromeric heterochromatin arrangement is temporally delayed in male pronuclei. Pericentromeric heterochromatin becomes strongly decondensed by the end of the first cell cycle in both pronuclei, suggesting a relationship between decondensed heterochromatin and transcriptional activation at minor ZGA. Subsequently, separation of pericentromeric heterochromatin from NPBs (or nucleoli) is initiated at the 2-cell stage and ends up in 4-cell embryos and is in this last moment when the NPBs fuse. This transition at the 4-cell stage might correspond to the fact that RNPI apparatus is functionally assembled

from this moment on (Aguirre-Lavin et al. 2012). In relation to chromocenters, these are clearly conspicuous in 4- and 8-cell mouse embryos. Nevertheless, chromocenters start forming from the 2-cell stage concomitantly with major ZGA and embryonic genome reprogramming, denoting a potential functional association (Probst et al. 2010). Moreover, the initial transcription of rRNAs takes place in mid/late 2-cell embryos, which was preceded by the assembly of rRNA synthesis machinery during the early second cell cycle (Aguirre-Lavin et al. 2012). Concerning some of the possible involved factors orchestrating remodeling of heterochromatin, it could be mentioned small centromeric RNAs, as well as asymmetric DNA and histone marks (Bouzinba-Segard et al. 2006; Aguirre-Lavin et al. 2012). Aguirre-Lavin et al. (2012) established that reorganization of pericentromeric heterochromatin could contribute to the acquisition of the totipotent status and may be a requirement of synthesis of satellite transcripts observed by Probst et al. (2010) who also reported that such satellite transcripts are necessary for further development. The possible role of all this chromatin reorganization could be to separate or allow specific genome portions to approach with the purpose of suppressing or activating transcription of particular genes (Aguirre-Lavin et al. 2012). Noteworthy, the deep changes in heterochromatin that take place during MET also seem to occur in parallel with striking modifications of chromosome territories (Koehler et al. 2009), nuclear envelope, and lamina (Popken et al. 2015), as it has been reported in cattle embryos.

1.7.4. DNA damage repair

The influence of DNA integrity on developmental competence is a major characteristic in early embryos conserved across species. For instance, it has been described that mRNAs encoding DNA repair proteins are present in oocytes and preimplantation embryos in mice (Titus et al. 2013), pigs (Bohrer et al. 2015), cattle (Henrique Barreta et al. 2012), humans (Menezo et al. 2007; Titus et al. 2013), which could imply that these factors are necessary throughout all stages of preimplantation development (rev. Bohrer et al. 2015). Levels of transcripts coding for proteins involved in DNA repair have been observed to vary between fast- and slow-cleaving embryos in pigs (Bohrer et al. 2015) and cattle (Henrique-Barreta et al 2012). According to Lechniak et al. (2008) this characteristic seems correlated with developmental competence since embryos that attain cleavage divisions early have been

shown to be more apt to reach the blastocyst stage throughout species, as hamsters (Gonzales et al. 1995), mice (Warner et al. 1993), humans (Fenwick et al. 2002; Ivec et al. 2011), pigs (Dang-Nguyen et al. 2010), goats (Villamediana et al. 2001), water buffaloes (Rajhans et al. 2010), and cattle (Van Soom et al. 1997; Dinnyes et al. 1999). In bovines, Henrique Barreta et al. (2012) pinpointed that *RAD52* and *53BP1* (both involved in homologous recombination, HR, and DNA repair) mRNAs were at higher levels in the medium/low competence group, but only around 36 hpi (thus, likely from maternal mRNA contribution). The same group of embryos also displayed higher incidence of phosphorylated H2A.X (H2A.Xph), histone marker of DNA damage. By exposing embryos to UV irradiation at 72 hpi *53BP1*, *RAD51*, and *KU70* levels were decreased, but when irradiated at 168 hpi the same three transcripts were upregulated. This suggested that embryos at or shortly after EGA can translate proteins involved in both HR and non-homologous end-joining (NHEJ) DNA repair pathways, although they are still unable to replace their mRNAs since the transcription is still incipient, meaning that close to EGA time bovine embryos are not yet completely competent to respond to DNA damage. In contrast, at the blastocyst stage transcription is already high and able to synthesize DNA damage-related mRNAs to be readily translated (Henrique Barreta et al. 2012).

In swine, it was determined that the less competent late-cleaving embryos are endowed with higher levels of transcripts coding for proteins involved in both HR (e.g. *RAD51-52*, *MRE11A*, *ATM*, *ATR*) and NHEJ (e.g. *PRKDC*, *XRCC4-6*, *LIG4*) pathways at (E)3 and (E)5. Given the cell number of the monitored low-competence embryos (less than 4 cells at day 3), the increase in such transcripts might have occurred before EGA (Bohrer et al. 2015), which seems associated with the previous observation that cattle embryos with reduced competence have signs of premature transcriptional activity-associated chromatin remodeling prior to EGA (Bastos et al. 2008). Moreover, slow pig embryos had an enhanced incidence of RAD51-positive- and RAD51-negative H2A.Xph foci, representing HR and NHEJ processes, respectively. These results denote the presence of both DNA repair pathways in pig preimplantation embryos. Furthermore, the finding that embryos with poor competence contained higher levels of *CHK1-2* mRNAs suggests that reduced cleavage speed and cell proliferation obey the induction of cell cycle checkpoints by

dsDNA damage (Bohrer et al. 2015). Consequently, it was concluded that presence of DNA damage events in preimplantation embryos is a central factor defining developmental competence and cleavage kinetics, where diminished speed to cleave could indicate that the embryo is preventing further blastomere proliferation until DNA is repaired to avoid segregation of ruptured dsDNA. In comparison to already-inflicted DNA offenses, defective capacity to repair DNA was considered a secondary factor (but not discarded) for reduced competence in preimplantation embryos (Bohrer et al. 2015).

1.7.5. Cell cycle and the first transcription products

DNA replication appears to have important but not always full effects on EGA throughout species. In *Xenopus*, Collart et al. (2013) demonstrated with an elegant experiment the influence of the abundance of the DNA replication factors *Cut5*, *RecQ4*, *Treslin*, and *Drf1* on MBT (and thus EGA). It was shown that the increase in nucleus:cytoplasm ratio that is concomitant with progressing embryonic divisions (as development approaches to MBT), decreases DNA synthesis rate. This was accompanied by diminished levels of the four limiting DNA replication factors. Furthermore, it was proved that over-expression of the replication factors delays the onset of transcription from the embryonic genome. Noteworthy, not all transcripts of embryonic origin were affected by the titration of the four DNA replication factors, which strongly suggested that other regulatory mechanisms exist to complement control of EGA in frogs (Collart et al. 2013). In the mouse, a punctual transcriptional repressive state is established in late 2-cell embryos in some genes that were shown to be previously expressed in zygotes and early 2-cell embryos (Hamamoto et al. 2014). Due to the observation that transcriptional silencing of constructions containing viral promoters is released in 2-cell mouse embryos by the use of DNA replication inhibitors (aphidicolin), it was implied that the transcriptional regulatory mechanism (activating or repressive) depends mostly on the second round of DNA duplication (Forlani et al. 1998). Nonetheless, since Hamamoto et al. (2014) detected that deletion of a short (56 bp) sequence of the *Tktl1* promoter, containing a TATA- and a GC box, rendered precocious transcriptional suppression of *Tktl1* in zygotes, which normally occurs in late 2-cell embryos, it was proposed that the transcriptional repression to which some particular genes are subjected at or before ZGA in mice (at least for *Tktl1*), is set in two consecutive steps

shortly after fertilization in the mouse and would be due to changes in chromatin (loose/tight) state. The initial step would be at the zygote stage and settled by chromatin modifications during the first mitosis, while the last one would rely on chromatin rearrangement throughout the second cell cycle (Hamamoto et al. 2014). With a similar strategy of pharmacological suppression of DNA replication Sonehara et al. (2008) established that the first and second rounds of replication of genomic material are selectively activating or inhibitory for transcription from certain genes during the first burst of transcription in mouse embryos, including *Eif1a*, *Hsp70.1*, *Zscan4*, and the retrotransposon *MuERV-L*. Given that *MuERV-L* is transcribed very early during minor ZGA (early/mid S-phase of the first cell cycle, at 8 hpi), whereas transcription of the other three markers appears to be normally delayed until major ZGA, the authors implicated that the distinct effect of DNA synthesis on transcription of the four monitored genes obeys to their intrinsic chronology (Sonehara et al. 2008).

Retrotransposons, like the ones synthesized at the onset of fertilization, are necessary for early embryogenesis in mammals. The existence of a Dicer isoform exclusively present in the oocyte was confirmed to be functional to drive the endogenous oocyte RNAi system and that originates from a promoter based on a retrotransposing element. Disruption of such promoter leaded to mouse infertility and meiotic defects (Flemr et al. 2013) previously observed in *Dicer* knockout (Murchison et al. 2007; Tang et al. 2007). Kigami et al. (2003) reported that *MuERV-L* transcription was independent from DNA replication and likely driven by the time elapsed after fertilization, the so-termed *zygotic clock*. The importance of *MuERV-L* for early embryogenesis was determined by a temporal but significant delay to attain the 4-cell stage after *MuERV-L* ablation (Kigami et al. 2003). Another transposon, *LINE-1*, has a crucial role in embryonic progression since its knockdown causes developmental arrest at the 2-4 cell stages. These deleterious effects on embryogenesis could be explained by the fact that a panel of five genes displayed misregulated transcript levels in 2-cell embryos as a consequence of *LINE-1* suppression (Beraldi et al. 2006). In relation to a possible impact of ARTs use on expression of transposable elements, Liang et al. (2013) unveiled that methylation of murine *LINE-1* in blastocysts is affected by superovulation with PMSG. Expression of retrotransposons at the onset of embryonic

transcription appears to be a conserved mechanism in mammals since Bui et al. (2009) confirmed that a considerable number of retrotransposons are also expressed post-fertilization in cattle.

Among the earliest transcripts reported in mammalian embryos are *Hsp70*, (part of TRC) and *Zscan4*. *Hsp70.1* (*Hspa1b*) is transcribed at the early 2-cell stage in mice (Christians et al. 1995; Bevilacqua et al. 2000; Fiorenza et al. 2004; Le Masson and Christians 2011). Edwards et al. (1997) determined that a 68 kDa-isoform of HSP70.1 (also termed HSPA1A in cattle) is translated upon heat shock induction as early as the 2-cell stage. However, HSP70.1 protein synthesis in 2-cell embryos was shown to be alpha-amanitin-resistant, whereas this initial translation of HSP70.1 turned to be pharmacologically inhibited by the 4-cell stage. Therefore, the authors concluded that challenging environmental conditions are able to trigger premature embryonic transcription before the commonly known major burst of EGA at the 8-cell stage in cow embryos (Edwards et al. 1997). A subsequent investigation by the same group added evidence of *HSP70.1* appearance at the transcript level in bovine 2-cell embryos, which was modest when thermal stress was not applied and much higher when embryos were heat-shocked (Chandolia et al. 1999). However, some doubt that the presence of *HSP70.1* mRNA in 2-cell embryos in the absence of thermal stress is due to actual embryonic transcription comes from the fact that besides its effect as inhibitor of RNA elongation, actinomycin D has also been suggested to impact mRNA stability (Park and Murphy 1996). Thus, the assumption of transcription of *HSP70.1* starting at the early 2-cell stage in cattle must be taken carefully.

Zscan4 is a putative transcription factor exclusively expressed in 2-cell embryos and ESC in mice. *Zscan4d* is the predominant paralog transcribed in embryos and restricted to the late 2-cell stage and already degraded in 3-cell embryos, whereas it is scarce or absent in ESC. RNAi of *Zscan4* leads to a 24 hr-delay in development in 2-4 cell embryos that were able to form blastocysts (Falco et al. 2007). Nonetheless, such blastocysts failed to expand and hatch, whereas implantation upon transfer was inhibited. The observation that blastocysts generated after *Zscan4d* overexpression were unable to implant corroborated the pivotal role of a transcript restricted to a short window during ZGA (and that needs to be

immediately degraded) for subsequent development in mice (Falco et al. 2007). Noteworthy, it was demonstrated that mouse ESC subjected to transient *Zscan4* overexpression rejuvenate and ameliorate their proliferation and pluripotency potential (Amano et al. 2013). Results from Amano et al. (2013) are in line with the findings of Hirata et al. (2012) who observed that the forced expression of *Zscan4* in mouse embryonic fibroblasts, in conjunction with three (*Sox2*, *Oct4*, and *Klf4*) out four Yamanaka factors (Takahashi and Yamanaka 2006), permits the generation of induced pluripotent stem cells (iPSC). Notably, Zscan4 drives fibroblast reprogramming toward pluripotency through a pathway distinct to that observed with the use of more traditional iPSC-generating molecules since Zscan4 was discovered as the first factor able to trigger somatic cell reprogramming by inducing expression of genes preferentially identified in oocytes and preimplantation embryos (Hirata et al. 2012). In relation to bovines, although Graf et al. (2014) did not include *ZSCAN4* in the list of first detected embryonic mRNAs, their survey showed that this transcript was dramatically increased at the 8-cell stage in comparison to 4-cell embryos. Therefore, it could be plausible that *ZSCAN4* has interesting roles still to explore during EGA in cattle.

1.7.6. Post-transcriptional and post-translational processing of maternal gene products

There is no doubt that the first transcripts synthesized from the embryonic genome are crucial for further developmental progress. Nevertheless, gene products already stored in the egg are crucial to trigger EGA (Wang and Latham 2000; Sirard 2010). Maternal mRNA and proteins are subjected to a number of changes following transcription and translation. This processing includes: splicing (not discussed here); masking; polyadenylation/deadenylation and translational recruitment of mRNAs (see 1.7.6.3); post-translational modifications, as well as simple degradation. Such mechanisms aim to regulate the availability of a given mRNA species to be translated soon or stored for later protein synthesis, its degradation when it becomes no longer useful, in addition to the presence and/or activity of a particular protein (rev. Brook et al. 2009). As was mentioned, such post-transcriptional and post-translational alterations occur at important levels during oocyte maturation. Nevertheless, this regulation is also pivotal for post-fertilization steps.

Notably, in a similar way as described during oocyte maturation, transcript modulation involves *cis*-elements mostly located at the *3'-untranslated region* (3'-UTR), although some of them are occasionally found within the open reading frame and the 5'-UTR. These sequences are targeted by *trans*-acting factors that consist of small RNAs or proteins (rev. Brook et al. 2009).

1.7.6.1. Protein degradation and posttranslational modifications

Consistent with the aforementioned, besides mRNA, oocyte-generated proteins are driven to degradation during MET. It is well known the mechanism of degradation of maternal proteins is required for meiosis resumption but that hampers embryonic development (Bettegowda and Smith 2007; Stitzel and Seydoux 2007). For instance, Sagata et al. (1989) have clearly documented the disappearance of the Mos polypeptide in *Xenopus*. The *ubiquitin-proteasome pathway* (UPP) has a preponderant role in maternal protein destruction in the early embryo and is essential for MET (Josefsberg et al. 2000; Brunet and Maro 2005). In this way it has been documented how CycB protein is destroyed by the UPP system in *Xenopus* (Glotzer et al. 1991). Accordingly, it has been found that UPP degradation circuits represent a large component of the fully-grown GV oocyte transcriptome in mice (Evsikov et al. 2006). Pang et al. (2011) indicated that the microinjection of a miR-135a inhibitor in mouse zygotes decreases cleavage. In addition, it was demonstrated that miR-135a modulates expression of the E3 ubiquitin ligase *Siah1* both in cultured human cells and murine zygotes. This finding appears correlated with the fact that abundance of miR-135a temporarily increased in zygotes, whereas *Siah1* mRNA levels started to drop since such stage. The effects of miR-135a on murine preimplantation development were shown to be partially mediated by Siah1a and its proteolytic degradation role (Pang et al. 2011). Cullin 1 (CUL1) is part of ubiquitin ligase complexes. In bovines Kepkova et al. (2011) found that *CUL1* mRNA is increased in 4- and 8-cell IVP embryos in comparison to their IVD counterparts. Besides UPP-mediated maternal protein degradation autophagy is also crucial for maternal-zygotic transition, as demonstrated by Tsukamoto et al. (2008) in mice. Autophagy is shortly induced after fertilization in murine embryos (Yamamoto et al. 2014). Granzyme G is part of the granzyme/perforin pathway (rev. Grossman et al. 2003). *Gzmg* codes for a serine-protease whose ablation by MOs leaded to

impaired RNA synthesis (shown by BrUTP incorporation assay), as well as inhibition of blastocyst generation. Therefore, it was proposed that granzyme G plays a role in murine MZT through its involvement in protein degradation (Tsai et al. 2010).

In bovines, a persuasive indication of maternal protein elimination during early development comes from the gradual decrease in protein amounts during the pre-MET period from the oocyte stage to the 8-cell embryo (Gilbert et al. 2009). In the same species MSY2 is a maternally-stored protein that is eliminated at the 16-cell stage (Vigneault et al. 2009b). Such observation is in conformity with the maternal expression pattern of such protein in mice and its consequent elimination in 2-cell embryos (Yu et al. 2001). MSY2 protein degradation is coincident with EGA in both species. Thus, the tendency of MSY2 to diminish after oocyte maturation until its disappearance by, or shortly after EGA in mammals denotes how maternal transcripts are protected from degradation until the moment they have to be unmasked for their translation or destruction (Vigneault et al. 2004). Consequently, the correct clearance of oocyte-derived factors that might otherwise interfere with the embryonic genetic program helps in MET occurrence.

Among the factors stocked in the ooplasm already in protein form, some will experience post-translational modifications that will permit them to act before EGA. For instance, Poueymirou and Schultz (1989) found that suppression of PKA activity in murine 1-cell embryos hampers the translation of embryonic proteins by the 2-cell stage, including TRC, and it perturbs the ability to proceed to first cleavage depending on the inhibitor used, likely because of different rates of protein phosphorylation. This is presumptively due to the lack of activation of transcriptional machinery of maternal origin by PKA, thus impeding its participation in EGA. This is in line with the disappearance of *Hsp70* transcript after abating PKA activity, but not upon CHX administration in mouse zygotes (Manejwala et al. 1991). Furthermore, the role of the maternally produced CycA2 protein at 1-cell in phosphorylating other proteins helps to induce transcription (Hara et al. 2005), as that mediated by Sp1 (Fojas de Borja et al. 2001), while the kinase activity of CycA2 over Rb diminishes the action of this later one, increasing transcription through E2F proteins, whereas Rb modulation over the SWI/SNF and HAT/HDAC circuits ceases, changing the

conformation of histones to a more permissive state (Harbour and Dean 2000). Such findings corroborate that maternal protein phosphorylation is necessary for ZGA (Bellier et al. 1997). Alternatively, the *C*-terminal domain (CTD) of RNPII experiences dephosphorylation and its concomitant binding to gene promoters and subsequent activation just before EGA both in murine and rabbit embryos at 2- and 8 to 16-cell stages, respectively. This mechanism is needed for developmental progression (Bellier et al. 1997). Similarly, Memili and First (1998) observed a burst in the hypophosphorylated form of RNPII at the 2- to 4-cell stages in cattle, in agreement with the increase in early transcription at such phases and probably in preparation to the major onset of EGA in 8 to 16-cell embryos (Memili and First 1998). All these data support post-translational modifications of maternal proteins as indispensable for EGA initiation in several species.

1.7.6.2. Turnover of maternal transcripts

Orchestration of the events allowing a correct MET also includes timely and selective elimination of maternal transcripts. The maternal mRNA/protein clearance program and the appearance of new proteins of maternal origin, as well as the transcription and translation from the embryonic genome must not be chronologically regarded in a straight-forward way since they can highly overlap at distinct time points. Deadenylation is widely recognized as one of the hallmarks of mRNAs prone to translational repression and subsequent degradation (Goldstrohm and Wickens 2008; Cooke et al. 2010) when they are not subjected to such process for temporal masking (translational repression) and storage, as it happens in the egg (Bettegowda and Smith 2007; Bettegowda et al. 2008). Although the processes that trigger deadenylation are not totally elucidated, some authors suggest that (A) residue loss may be related to CPE presence *per se*, or is regulated by other signals located at the 3'-UTR (de Moor et al. 2005). However, results from *Xenopus* oocytes indicate a putative negative feedback after a first wave of cytoplasmic polyadenylation that could induce subsequent deadenylation (Belloc and Mendez 2008). As mentioned, it is widely accepted that the 3'-UTR of mRNAs plays a key role in determining which molecules are deprived of their poly(A) tail to be destabilized. In this context, RNA-binding proteins (RBPs) and miRNAs attach to specific sequences in the 3'-UTR to form

complexes that will recruit deadenylases to remove 3'-end (A) residues (Schier 2007; Cooke et al. 2010).

Several of the multiple deadenylases very well characterized in *Xenopus* and mammals are PARN and those integrating a cytoplasmic deadenylation complex CCR4-CAF1-NOT. This complex links RBPs and miRNAs to eliminate (A) residues from the bound mRNA (Goldstrohm and Wickens 2008; Cooke et al. 2010). Among the RBPs leading to deadenylation are PABP and NANOS2 in mice (Fabian et al. 2009), and CUG-BP in humans (Moraes et al. 2006). In relation to the identity of the targeted sequences, Alizadeh et al. (2005) validated that in a group of maternal mRNAs rapidly degraded after fertilization in mice (*Gdf9*, *H1foo*, *Mos*, and *tPA*) their 3'-UTRs lacked of sequences as AREs or EDEN-like. Conversely, it was found that these transcripts contained consensus CPEs always proximal (54 nt of distance) to a nuclear polyadenylation sequence (NPS) defined by the hexanucleotide (HEX) AAUAAA (Alizadeh et al. 2005). When the same group compared the results of the global transcriptomic analysis from Hamatani et al. (2004) experiments found similar 3'-UTR conformations in mRNAs subjected to a fast elimination following egg activation. Conversely, in *CycA2* and *Hprt* transcripts, which are not quickly destroyed upon fertilization, the CPEs are located at ~120 nt from AAUAAA (Alizadeh et al. 2005). In cattle, it has been demonstrated that during *in vivo* pre-implantation development the oocyte-specific transcripts *ZP2*, *ZP3*, *ZP4*, *NALP9*, and *FIGLA* experience a quick decay after fertilization, while *CycB1*, *HMGN2*, *PTTG1*, and *BTG4*, follow a progressive degradation from oocyte to blastocyst stages (Vallee et al. 2009). Thus, the surplus of $[Ca^{2+}]i$ triggered by oocyte activation induces a massive wave of degradation of all transcripts stored in the egg and it is more extensive in comparison with the mRNA turnover pattern started in meiotic maturation (Alizadeh et al. 2005). Other sequence involved in mRNA deadenylation corresponds to *Pum-binding element* (PBE), which is bound by Pumilio (Pum) protein. Pum has been observed to bind CPEB and to be recruited towards CPE-containing repressed 3'-UTRs. By this mechanism, Pum blocks *CycB1* translation and delays oocyte maturation in *Xenopus* (Nakahata et al. 2003). A detailed analysis by Pique et al. (2008) shed light on the existence of a combinatorial code in which the relative localization of NPS, CPE, and PBE sequences delineates if a transcript

is destined to translational suppression or recruitment, as well as the timing of such processes during maturation of frog eggs. It was suggested that such 3'-UTR combinatorial molecular language could be extrapolated to other vertebrate groups (Pique et al. 2008).

In animal cells miRNAs are transcribed by RNPII to produce a primary miRNA and are then processed in the nucleus by the nuclease Drosha and in the cytoplasm by Dicer, generating a precursor-miRNA and a mature miRNA of ~22 nt in length. The final miRNA is assembled into RNA-induced silencing complex (RISC), which consists of RNPs collaborating with the miRNA targeting of transcripts (Pillai 2005; Plasterk 2006). The miRNA attaches to single or multiple partially complementary sequences located at the 3'-UTR of the mRNA, therefore impeding its translation, probably by blocking the association eIF4E/5'-cap (Humphreys et al. 2005) and/or prompting it for deadenylation/degradation (Fabian et al. 2009). Another possible mode of action of miRNA-associated mRNA degradation might be the inclusion of the targeted transcript in miRNA-containing RNPs (miRNPs), followed by translocation into P-bodies (Pillai 2005). The to-be-cleared transcript could then possibly lose the 5'-cap and be digested by 5'-3'-exonuclease activity inside P-bodies, under a translational repressed state (Parker and Song 2004; Teixeira et al. 2005). The research interest on miRNA implication in mammalian embryogenesis started with the finding that *Dicer* KO mice embryos die as soon as (E)8.5 and manifest abnormalities from (E)7.5 (Bernstein et al. 2003). Nevertheless, the discovery of the general disappearance of P-bodies, stockpile of miRNA components, during mouse oocyte growth towards maturation (Ma et al. 2010) questioned the imperative role of miRNAs in the fully matured oocyte and early embryo. Likewise, two works surprisingly remarked that the highly deleterious effects on mouse embryonic development upon *Dicer* depletion are rather mediated (or at least the earliest ones) by endogenous small interfering RNAi (siRNA) pathways and not by miRNAs (the mature form of both types of molecules is processed by Dicer), even when miRNAs are also fairly present in oocytes (Ma et al. 2010; Suh et al. 2010). Therefore, it may be concluded that the restraint of miRNA networks (but perhaps not totally) could be a key aspect accomplished by the murine mature egg to facilitate the overall gene reprogramming after fertilization to form totipotent blastomeres (Svoboda 2010). Despite the disproval of miRNAs as one of the main molecular effectors

in murine MZT, interesting indications of their potential association with early development control were obtained in the cow. The results of Tesfaye et al. (2009) strongly suggested a role of miRNAs in the control of target transcripts just before fertilization in the bovine egg and during all the pre-implantation period. Subsequently, Lingenfelter et al. (2011) suggested a direct regulation of *NPM2* by miR-181a in cattle oocytes and early embryos, while Tripurani et al. (2011b) alluded for a similar control of *NOBOX* by miR-196a. These last two reports implied the modulation of oocyte-specific genes (thus, maternal mRNA) by miRNAs before bovine MET.

Among the reasons for the discrepancies in the role of miRNAs during pre-MET development of the two species above, firstly it could be plausible that abrogation of miRNA pathways by the period encompassing the final oocyte maturation to MET does not happen in the same way in the cow as it was substantiated in mice due to the distinct timing of MET in both species (8 to 16- and 2-cell stage in bovines and mice, respectively). This may imply that the need of a miRNA inhibiting system acting rapidly after fertilization to allow an immediate DNA reprogramming (mouse model), as proposed by Svoboda (2010), is not required in cattle since another molecular buffering mechanism might exist during the comparably long time of MET arrival in the latter species, being permissive for a correct gene resetting in the course of bovine cleavage stage even in presence of functional miRNA networks, contrary to the model inferred in mice. Another possibility consists of a putative non-promiscuous inhibition of miRNA systems in mammals before MET as implied by the deep suppression of *Let-7* in rodent oocytes concomitant with the conservation of a fairly high level of miR-30 in the report of Ma et al. (2010). Thus, it might be inferred that some miRNAs are specifically repressed before mammalian MET while others remain active. Moreover, a high impact of miRNAs during early development must not be excluded, as it is possible that miRNAs gradually become more influential during embryogenesis after EGA (Suh and Blelloch 2011). Hence, a crucial role of miRNAs in maternal molecules elimination is apparently variable in time throughout early development between bovines and rodents.

Analysis of six different miRNAs during preimplantation development in bovines revealed maternal abundance patterns since their levels were high before and shortly after fertilization, followed by an abrupt decrease from the moment of EGA. The authors inferred that this event might reflect that miRNAs synthesized in the egg are involved in regulation of MET in cattle (Abd El Naby et al. 2013). Specifically, it was reported reciprocally-inversed abundance patterns between miRNAs and their predicted target transcript during bovine preimplantation development: miR-205 and *PLCB1*; -96 and *ITPR1*; -146a and *IRAK1*. From this pattern a putative maternal mRNA decay mechanism driven by miRNAs in this species could be inferred. Nevertheless, it was suggested that targeting of a particular mRNA may not always result in turnover of this later molecule but instead in a translational inhibition (Abd El Naby et al. 2013). Another potential case of mRNA regulation by miRNA during early development in cattle is constituted by *FIGLA* and miR-212. In this species FIGLA A that miR-212 induces *FIGLA* decay during MET in bovines since both RNAs showed inversely correlated abundance patterns during development, whereas the mimic triggered ablation of FIGLA protein in embryos. Moreover, miR-212 suppresses both activity of a reporter construct containing the 3'-UTR of FIGLA and the bovine protein expression in cultured cells (Tripurani et al. 2013). Tripurani et al. (2011a) indicated that *NOBOX* mRNA was still abundant in zygotes, although almost completely degraded in morulae since it was not synthesized at EGA. Remarkably, turnover of *NOBOX* seemed dynamically controlled by miRNAs because results from Tripurani et al. (2011b) implied that miR-196a drives the negative regulation of *NOBOX* mRNA throughout MET in bovine embryos. On the other side, *NOBOX* RNAi injection in bovine fertilized eggs impaired blastocyst production rate and cell allocation, whereas transcripts synthesized from the embryonic genome and critical for early development (involved in transcriptional control, cell cycle, signaling, pluripotency) were affected (Tripurani et al. 2011a).

Other component of the systems aimed at transcript silencing and degradation consists in siRNAs. These molecules are segments of ~21-23 nt in length produced from diverse long double-stranded RNA (ldsRNAs) substrates, which like the miRNAs are processed by Dicer. This ribonuclease cuts the original molecule into numerous distinct siRNAs that are

wrapped into a RISC, complex integrated by Ago proteins (Suh and Blelloch 2011). As with miRNAs, the RISC is loaded with a single-stranded siRNA, which drives recognition of cognate mRNAs while the passenger chain is removed and destroyed. When base-pairing between the small RNA and the target transcript is perfect, as in the case of siRNAs, the mRNA is sliced by Ago2 (Suh and Blelloch 2011). The transcript fragments generated by this endonucleolytic activity are then directed to the general cellular mRNA degradation machinery, where a 5'-3' exonuclease mechanism likely occurs by Xrn1 and exosome action (Valencia-Sanchez et al. 2006), resulting in an RNAi mechanism.

It is generally considered that somatic cells in vertebrates are devoid of endogenous siRNAs (endo-siRNAs), which is in striking contrast with the ubiquitous presence of miRNAs in somatic tissues (Suh and Blelloch 2011). In addition, it is widely known that administration of exogenous dsRNA triggers the interferon (IFN) pathway, which consists in a promiscuous cytokine response in somatic mammalian cells caused when dsRNA activates PKR, blocking translation by phosphorylating eIF-alpha. Furthermore, the IFN reaction prompts general RNA degradation with a highly noxious effect for the cell (de Veer et al. 2005). Conversely, oocytes and cells of early embryos of mammals have been demonstrated not only to harbor miRNAs, but also multiple endo-siRNAs (Tam et al. 2008). This property seems to be related to the lack of IFN pathway induction by experimental introduction of dsRNA in rodent oocytes and early embryonic cells (Svoboda et al. 2000; Stein et al. 2005). However, the biological relevance of the IFN reaction idle in oocytes has not been elucidated (Svoboda 2008). Another enigma is the exact biological function of RNAi systems in oocytes and early embryos of mammals, but the main putative function of endo-siRNAs in mammalian oocytes/embryos is post-transcriptional gene regulation of transposable elements/pseudogenes (Tam et al. 2008; Watanabe et al. 2008). The relevance of siRNAs for mammalian early embryogenesis was functionally demonstrated by the arrest at the 2-cell phase when *Ago2* was knocked-down in mice (Lykke-Andersen et al. 2008). This phenotype was similar to that reported by Tang et al. (2007) upon KO of *Dicer* where it was also observed spindle dynamics and chromosome segregation failure. In both cases, multiple mRNAs were misregulated upon gene ablation. This is in agreement with the description of numerous dsRNA-producing pseudogenes

whose gene targets are related to microtubule and spindle processes (Tam et al. 2008). The severe phenotypes observed either after *Dicer* or *Ago2* deletions independent of miRNA networks given that suppression of *Dgcr8* (involved in miRNA processing) does not impair oocyte mRNA expression, while development is not affected in great extent (Suh et al. 2010). Thus, endo-siRNAs are essential for early development since meiosis and up to MET, at least in the mouse (Svoboda and Flemr 2010; Suh and Blelloch 2011). Therefore, consistently with the two waves of mRNA degradation machinery during MET described by Sirard (2010), one provided by the oocyte and the second consisting of factors from the embryonic genome, endo-siRNAs appear to be the major oocyte-synthesized component for messages clearance before EGA, while miRNAs would constitute the main actors in the course of posterior embryogenesis (Suh and Blelloch 2011). Besides targeting of 3'-end to regulate mRNA deadenylation/turnover during preimplantation development, other mechanism to trigger destruction of maternal transcripts are induced by 5'-decapping. In this context, through ablation assays it was determined maternal *DCP1A* and *DCP2* mRNAs are necessary for proper MZT in mice (Ma et al. 2013).

1.7.6.3. Recruitment for translation

Translation prior to MET is necessary for the activation of *de novo* transcription, as proved with the decrease in transcripts of embryonic origin after inhibition of maternal protein synthesis by administering CHX in the mouse 1- (Hamatani et al. 2004) and 2-cell embryo (Wang and Latham 1997). Moreover, translation inhibition and abatement of mRNA mobilization (suppression of polyadenylation with 3'-deoxyadenosine) from maternal sources abrogates overall activation of transcriptional activity in murine embryos from the 1-cell stage (Aoki et al. 2003). Similarly, the appearance of new proteins is mandatory for MET in cattle given that CHX abates progress through such stage (rev. Sirard 2001). Moreover, synthesis of MHKPs between the late GV and 8-cell phases in bovines, which are likely involved in crucial metabolic support but not directly in EGA, is accompanied by the appearance of new maternal proteins in the 8-cell embryo when compared with previous stages, but especially with the egg. These other maternal proteins might represent some of the factors necessary for MET allowance (Massicotte et al. 2006), but they have to be characterized further. In this way, it is plausible that the novel maternal polypeptides are

aimed at transcriptional regulation leading to a correct EGA, as suggested by Hamatani et al. (2004) and Sirard (2010). Correspondingly, a CHX-sensitive rise of the TBP and SP1 proteins of maternal origin in the mouse zygote and 2-cell (coincident with ZGA) stages has been reported (Worrad et al. 1994). In agreement with the importance explained for embryonic translation above, synthesis of mRNAs corresponding to ribosomal proteins at the time of EGA is a common characteristic of diverse species, including mouse, rabbit, and cattle (rev. Bui et al. 2009). Therefore, the importance of sustaining a controlled protein translation since the very moment when the embryo seizes control of gene expression can be proposed (Bui et al. 2009). The early embryo is able to translate an important level of maternal transcripts even before MET, both in model (Tadros and Lipshitz 2009; Wang and Latham 1997) and domestic species (Barnes and First 1991). Although protein synthesis is present since oocyte maturation (Sirard et al. 1989; Levesque and Sirard 1996; Coenen et al. 2004), a distinct subset of maternal mRNAs is prompted for translation by $[Ca^{2+}]i$ transients upon fertilization, like *CycA2* and *Spindlin*, as observed in the mouse. Similar to the recruitment of transcripts during oocyte maturation, the *cis* elements that direct selection of specific transcripts for translation upon fertilization are located in the 3'-UTR. Nevertheless, these sequences seem to differ from those used during maturation (Ducibella et al. 2006). For example, a potential CPE consisting of a dodeca-uridine tract (also termed *embryonic CPE*) at ~1.1 kb from the hexanucleotide AAUAAA has been found in the *Spindlin* transcript (Oh et al. 2000).

mRNA needs to contain a CPE at a distance of less than 100 nt from the hexanucleotide AAUAAA at 3'-UTR to be subjected to polyadenylation (Fox et al. 1989; McGrew et al. 1989; Simon and Richter 1994; Stebbins-Boaz et al. 1996). Poly(A) tail elongation is a complex process; at least seven proteins have been traditionally known to be involved. One of the best schemes of translational recruitment corresponds to the *closed-loop model* (Fig. 1-6). According to Tome and Wollenhaupt (2012) the CPE sequence is first bound by CPE-binding protein (CPEB) normally with the help of a mediator, cleavage and polyadenylation specificity factor (CPSF), to cleave a pre-mRNA. Consequently, CPSF and poly(A)-polymerase (PAP) are in charge of (A) residues attachment. As soon as the poly(A) 3'-term has begun to be elongated, poly(A)-binding protein (PABP) joins the complex to associate

with PAP to allow this last enzyme to continue polymerization of the chain's extreme up to the point it reaches a length of 200-300 (A) residues, when the polymerization becomes limited by an ionic unbalance between PABP and the growing transcript. Finally, translation is initiated by the action of initiator factors eIF4G and EIF4E with PABP. On the other hand, maskin impedes translation by interacting with CPEB and competing with EIF4G for EIF4E binding, thus blocking the association of the 40S ribosomal subunit to avoid translation. When protein synthesis is about to start, the phosphorylation of CPEB allows it to split from maskin (Fig. 1-6), leading to a translational-permissive conformation of the complex (rev. Tome and Wollenhaupt 2012). As discussed by Flemr et al. (2010), CPEB is part of the mRNP particles and other novel related structures that contain quiescent mRNAs in mouse oocytes. Hence, the interaction of CPEB and MSY2 should be considered too.

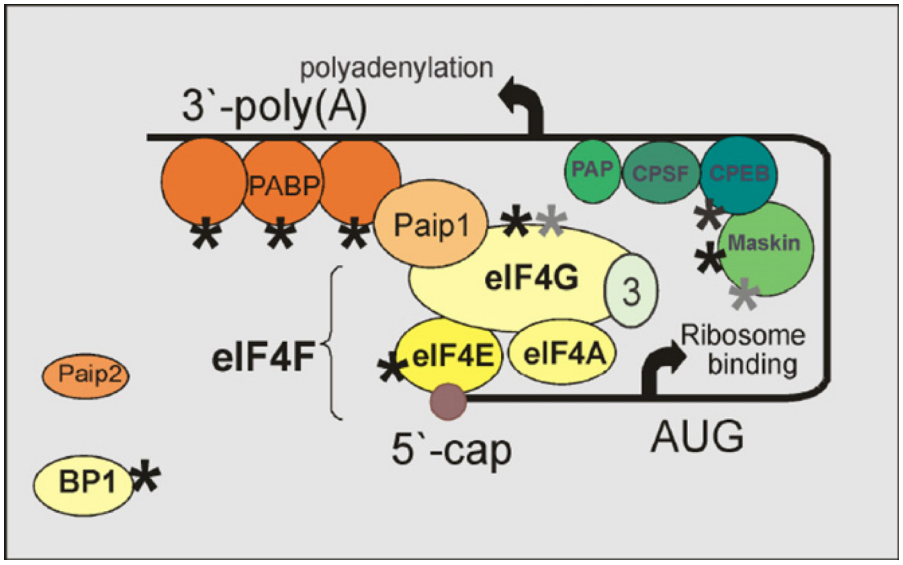

Figure 1-6 Closed-loop model of mRNA recruitment (Tomek and Wollenhaupt 2012)
PABP, poly(A)-binding protein; Paip1, poly(A)-binding protein interacting protein 1; PAP, poly(A)-polymerase; CPSF, cleavage and polyadenylation specificity factor; CPEB, cytoplasmic polyadenylation element-binding protein; AUG, translation initiation codon; eIF-, eukaryotic translation initiation factor; BP1, eukaryotic translation initiation factor-binding protein (EIF4EBP1, 4EBP1); *, phosphorylation site. Scheme reproduced with permission of Elsevier © 2012.

Polysomes examination by Potireddy et al. (2010) revealed valuable information about the mechanism of maternal mRNA translational recruitment in mice. Assessment between MII

and zygote stages indicated that mRNA translation is regulated by a complex mixture of positive and negative stimuli driven by opposing *cis*-sequences and transcript secondary structure, where the translationally suppressed state seems to be released after fertilization. Multiple modulatory motifs were localized in the 3-UTR, including the polyadenylation response element (PRE) and novel CPEs and the most outstanding sequences were termed main *translation activating element* (TAE) and *translation repressing element* (TRE). Furthermore, a number of secondary configurations adopted by the translating mRNAs were identified (Potireddy et al. 2010). Remarkably, the motifs identified in such studies differ from those known to modulate translation during maturation either in frogs or mammals and such translational regulation stopped immediately after the zygote stage (Potireddy et al. 2010). Additional complexity was unveiled when Chen et al. (2011) determined profiles of transcript recruitment that correlate with cell cycle and whose translating mRNAs contained DAZL-binding motifs. Moreover, it was reported that translation recruitment of the maternal *Dazl* is modulated by CPEB1. DAZL is indispensable for meiosis completion (spindle conformation and function) and development up to MZT since CPEB and DAZL work together in a sequential way during such developmental window in mice by allowing translation of transcripts that control cell cycle and thus allow progression through cleavage stage (Chen et al. 2011). In pigs, Dobbs et al. (2010) did not observe direct association between consensus CPEs, poly(A) tail length, and mRNA levels in pigs prior to EGA. Furthermore, parthenotes displayed discrepancies in transcript abundance respective to IVF-embryos. Thus, the authors concluded that CPEs do not seem to be the unique factor controlling transcript stability during swine early development, which denotes a complexity also denoted in murine models of translational recruitment (Dobbs et al. 2010).

In cattle, analysis of transcript populations according to their polyadenylation length during oocyte maturation also pointed out to the importance of *cis*-sequences localized in the 3'-UTR. A number of candidate mRNAs were verified, including *ATF1*, *ATF2*, and *ELAVL2*, and their 3'-UTR identified and correlated with polyadenylation status (Gohin et al. 2014). In this way, Gohin et al. (2014) found that CPE (U_5AU), NPS, DAZL recognition element (U_3CU_3), ARE (AU_3A), and a novel sequence called *motif associated with polyadenylation*

signal, MAPS (U_5CU_2), as well as a MAPS-like (U_4CU_3) regulate cytoplasmic polyadenylation and deadenylation levels of maternal transcripts during oocyte maturation in cattle. It was proposed that such 3'-UTR motifs could control translational recruitment later in development (Gohin et al. 2014). Similarly, Reyes et al. (2015) corroborated the positive influence of NPS and CPE presence on cytoplasmic polyadenylation during oocyte maturation in cattle.

In some examples of the significance of cytoplasmic polyadenylation of maternal mRNA, *DCP1A* and *DCP2* transcripts are translationally induced during maturation through CPEs. Their codified proteins are modulated by CDC2A-mediated phosphorylation and they are necessary for both mRNA degradation following meiotic reactivation and MZT (Ma et al. 2013). The decrease of the mRNA of the circadian deadenylase *Nocturnin* (*Ccr4nl*) between fertilization and the 2-cell stage accompanied by stable levels of the CCR4NL protein suggests translation of the maternal transcript in preparation for MZT in mice. The timing to reach developmental hallmarks (up to the blastocyst stage) was much longer upon KD of *Nocturnin*, whereas its overexpression lead to almost null blastocyst production. The authors suggested a key role for Nocturnin during murine MZT (Nishikawa et al. 2013). Concerning the factors that might be directly associated with cytoplasmic polyadenylation, the knockout of the embryonic PABP (*Epab*) generated morphologically abnormal oocytes in mice whose chromatin configuration and transcriptional silencing during maturation were disrupted due to failures in acquisition of meiotic competence and translational activation (Lowther and Mehlmann 2015). Moreover, alternative splicing differentially regulates *EPAB* expression pre- and post-8 cell stage in human embryos, which experience EGA at the 4-cell stage. This post-transcriptional regulation of *EPAB* in human embryos contrasts with the translational regulation of the EPAB in frogs and mice (Guzeloglu-Kayisli et al. 2014). Interestingly, Ozturk et al. (2014) observed that ovarian superstimulation disrupted *Epab* and *Pabpc1* mRNA levels in mouse oocytes and embryos. In the cow, *PABPNL1* is expressed in oocytes and might be correlated with developmental quality at the MII stage. The potential significance of PABPNL1 might be related to the importance of PABP family members in regulating translation recruitment (Biase et al. 2010).

1.7.7. Transcriptional machinery

This section will discuss the function of transcriptional regulators originated from translation of maternal mRNA. RNPII protein must be recruited to gene core promoters by general transcription factors (GTFs). These include TFIIA, -B, -D, -E, -F, and -H. Typically, GTFs assemble together with RNPII on the gene core promoter to form a pre-initiation complex (PIC). Phosphorylation of CTD of RNPII by TFIIH enables initiation of transcription with subsequent elongation of mRNA, snRNA, or miRNA (Egloff and Murphy 2008). Besides the basal transcriptional machinery, other transcription factors (up-regulators or down-regulators) are important, like the different components of mediator complexes, and other co-activators of transcription, whose expression may be ubiquitous, or tissue/cell type-specific and/or depend on developmental time points, ensuring the proper expression of a given gene in time and space. The role of these other proteins is to increase or decrease the affinity of RNPII to the core promoter. This is accomplished through binding to sequences around the promoter to diminish the space between the core promoter and the PIC when the distance between them is short or medium (co-activators), or very large (as the mediators bound to enhancers). For this purpose, transcription factors and coactivators bend or condense the distant DNA sequences (rev. Krishnamurthy and Hampsey 2009). For instance, Sp1, which probably recruits TBP, increases in the nucleus of mouse zygotes, favoring MZT (Worrad et al. 1994). Comparably, Vigneault et al. (2004) indicated that the mRNA level of *TBP*, component of the PIC, in addition to that of the important transcription factors *YY1*, *CREB*, *OCT4*, *TEAD2*, *ATF1*, and the co-activators *P300* and *YAP65*, followed expression patterns of maternal type with high levels in bovine GV oocytes, decreasing until a minimum at 8-cell stage and recovering elevated amounts in blastocysts. This tendency suggested that such transcriptional regulators are translated before 8-cell to incorporate the components allowing EGA and then their mRNA is synthesized again by the pre-implantation embryo (Vigneault et al. 2004). Subsequent studies confirmed this hypothesis at the protein level with the increased expression of TBP from the 8-16 cell stage onwards in cattle embryos and the nuclear localization of the polypeptide, in agreement with its role as GTF (Vigneault et al. 2009b). On the contrary, gene expression from the embryonic genome has been demonstrated to produce mRNA of transcription factors, as *ZFP42*, *GABPA*, and *KLF4* during the onset of EGA in cattle. This

study corroborated the role of transcription modulation components synthesized shortly after EGA to drive the embryo further in pre-implantation development (Vigneault et al. 2009a).

At the late 2-cell stage in mice, there is a suppression of the activity of TATA-containing promoters. Transcription from these is substituted by the concerted action of enhancers and TATA-less promoters during the rest of the preimplantation phase. Active TATA-less promoters are commonly found in HKGs to favor rapid cleavage during early embryogenesis (rev. Kageyama et al. 2007). After transcriptomic profiling Kageyama et al. (2007) indicated that a major difference in gene expression exists between mouse zygotes and 2-cell embryos. The comparison of immature oocytes against 1-cell embryos was smaller, probably due to the fact that transcriptional activity in zygotes is still low, which makes plausible the notion that an important fraction of the transcriptional regulators responsible for ZGA induction is already present before fertilization, either still as mRNA or already as protein. Referring to translation specifically, it is possible to infer that most of the mRNA to be used during preimplantation development is translated only after fertilization (Kageyama et al. 2007), a process that involves transcript recruitment (Potireddy et al. 2010; Chen et al. 2011). The abundance of different genes expressed in 2-cell embryos could be attributed to the striking increase in mRNA species encoding transcription factors between zygote and 2-cell stages. This may be required to switch from enhancer-independent to enhancer-dependent transcriptional control, as well as to change the cellular context from a highly differentiated oocyte to totipotency (Kageyama et al. 2007). Moreover, mRNAs of constitutive TFs are present during the transition from 1- to 2-cell, including *RNPI*, *RNPII*, *NFY-b*, and *Sp1*, whereas a strong transcription of TFs containing Ets and RHR domains starts in 2-cell embryos. This suggests that, in addition to supporting future transcription due to the synthesis of basic TFs, the 2-cell stage appears crucial since TFs that regulate cell proliferation dependent on cytokines are actively transcribed. It must be underscored that Ets proteins are inductors of TATA-less promoters, which agrees with the use of this mechanism during the preimplantation period (Kageyama et al. 2007). Finally, the abrupt enrichment of TFs with functions in cancer cells seems to be helpful in keeping totipotency and for cell proliferation in the early embryo given that

the list of these molecules transcribed at the 2-cell stage included members of Ets, Rel, and Myb families (Kageyama et al. 2007).

1.8. Activating Transcription Factors

Results from Vigneault et al. (2004) and Vigneault et al. (2009) suggested that ATFs could be involved in regulation of EGA in cattle. The ATF/CREB family of transcription factors is included in the group of proteins that contain a bZIP domain, but have the particularity to bind to the sequence TGACGTCA, which is the consensus cAMP-response element (CRE), in response to environmental stimuli in order to keep cellular homeostasis. Remarkably, diverse members of the family have the capacity to regulate cell survival in a positive and/or negative way (rev. Hai and Hartman 2001; Vlahopoulos et al. 2008). cAMP response element-binding protein (CREB/CREB1) is probably the best known member of the ATF/CREB family (rev. Hai and Hartman 2001; Shanware et al. 2010). CREB has generated a major interest in transcription regulation studies due to the multiple genes that it could potentially control. For example, work with human tissues demonstrated that CREB is able to bind up to 4,084 promoter sites genome-wide according to the DNA methylation status around the targeted CRE (Zhang et al. 2005). The ATF/CREB family also contains activating transcription factor (ATF) 1 and cAMP-responsive element modulator (CREM). As for CREB, both ATF1 and CREM proteins can either homodimerize or heterodimerize with other family members to form the functional dimeric transcription factor (rev. O'Neill et al. 2012). Dimerization appears to allow functional redundancy between proteins composing the duplex, which provides plasticity for transcriptional control in distinct cell scenarios, as it has been the case of histone H4 regulation by ATF1 and CREB (Guo et al. 1997). In an embryonic context, Bleckmann et al. (2002) found that CREB and ATF1 (but not CREM) are robustly co-expressed throughout murine preimplantation development. Homodimers or heterodimers of ATF1 and CREB are capable to target CREs in order to induce them. This observation, together with the great extent of functional similarity motivated the functional studies of both transcription factors by lack-of-function models (rev. Jin and O'Neill 2007; Jin and O'Neill 2010). The knockout of both alleles ($^{-/-}$) of either *Atf1* or *Creb* performed by Bleckmann et al. (2002) did not produce any significant phenotype in mice embryos, suggesting that the individual ablation of each factor could be

compensated by the presence of the two alleles of the other one due to an apparent functional redundancy. In contrast, the double knockout of both transcription factors (*Atf1*$^{-/-}$, *Creb*$^{-/-}$) lead to embryonic death produced by high apoptotic incidence in developmentally delayed morula around (E)3.5, whereas wild type embryos normally reached the blastocyst stage by that time (Bleckmann et al. 2002). Remarkably, the presence of only one functional *Atf1* allele in the complete absence of *Creb* (*Atf1*$^{+/-}$, *Creb*$^{-/-}$) was permissive to developmental progression to ZP hatching, although embryos were unable to form a proper ICM and died around (E)9.5, denoting a partial compensation of the complete lack of *Creb* when a single *Aft1* allele is functional. Consequently, it was concluded that the resulting phenotype of *Atf1* and/or *Creb* mutant embryos is sensitive to the present allele(s) and relies on gene dosage since both proteins can compensate each other (due to functional redundancy), although not in all cases given that they are not fully equivalent (Bleckmann et al. 2002). Thus, it was proposed that the concerted action of ATF1 and CREB transcription factors is essential for the transcriptional regulation of cell survival in early embryos (Bleckmann et al. 2002; Jin and O'Neill 2010).

Concerning the regulation of ATF/CREB members, either CREB or ATF proteins can be activated following similar stimuli. For instance, Shanware et al. (2010) indicated that both CREB and ATF1 are phosphorylated by the ATM kinase in response to genotoxic stress and DNA damage in cultured somatic cells, whereas the same conclusions were drawn for ATF2, another family member (Bhoumik et al. 2005). In an early developmental context, the observation that a CRE-reporter vector in immature mouse oocytes remained suppressed, while expression from the same reporter was positive in aphidicolin-arrested zygotes chronologically at the 2-cell stage (Schwartz and Schultz 1992) hints that CRE-activating factors are either synthesized or activated in preparation to ZGA. This points out to ATF/CREB proteins as candidates to be involved in this process (rev. Jin and O'Neill 2010). During the last decade, studies mostly by X.L. Jin and C. O'Neill have produced detailed data about the modulation of ATF1 and CREB proteins at the onset of preimplantation murine development. It was determined that the first important increase in CREB nuclear presence takes place at the mid 2-cell stage, which occurs in parallel with the appearance of the phosphorylated form of CREB (Jin and O'Neill 2007). CREB function

and nuclear localization are apparently induced around the 2-cell stage in mouse embryos via phosphorylation by two different pathways: 1) cAMP and PKA (rev. Jin and O'Neill 2007); 2) calmodulin-dependent kinase (CaMK) activity triggered by Ca^{2+} transients, which can be induced by the embryotrophin Paf (Jin and O'Neill 2010). Therefore, because of the dependence of CREB activity on phosphorylation, the function of CREB is associated with the cellular metabolic state (Jin and O'Neill 2007). Subsequently, the already phosphorylated CREB recruits the co-activator CBP (CREBBP). This in turn activates the function of the transcriptional machinery (rev. Jin and O'Neill 2007; Jin and O'Neill 2011). On the other hand, appearance of nuclear ATF1 started in mid/late zygotes. Nuclear distribution of the bulk of ATF1 depends on P38-MAPK (MAPK14) signaling only, whereas the maximum level of phosphorylation (and activation) of ATF1 at the 2-cell stage relies on the concerted action of both P38-MAPK and CaMK pathways. Moreover, concentration of ATF1 in the nucleus was insensitive to alpha-amanitin but depended on protein synthesis, implying that nuclear translocation of ATF1 is independent of newly transcribed factors but requires translational recruitment of maternal mRNAs in mice (Jin and O'Neill 2014).

In relation to the possible significance of the nuclear localization and activation of CREB and ATF1 during early preimplantation development, Jin and O'Neill (2014) reported that the individual pharmacological ablation of CaMK or P38-MAPK pathways rendered developmental block of 8-cell mouse embryos. In contrast, inhibition of both signaling pathways lead to arrest at the 2-cell stage. Thus, considering the less severe effects of suppressing CaMK or P38-MAPK signaling separately, in contrast with the more deleterious effects when simultaneously inhibited, it is possible to infer that these pathways are functionally redundant but that their combined action is crucial for developmental progress beyond the 2-cell stage, coincident with ZGA. In this way, given that calmodulin and P38-MAPK are required for the nuclear accumulation and activation of ATF1 and CREB at the 2-cell stage, it could be suggested that the cooperative function of both ATF1 and CREB might be essential for embryonic first transcription (Jin and O'Neill 2014) and even more when considering that relative epigenetic permissiveness exists in 2-cell murine embryos (rev. Jin and O'Neill 2010). In addition, the joint action of ATF1 and CREB as

heterodimers, together with the vast number of potential targets of the CREB transcription factor in mammalian genomes, and the fact that CRE-containing promoters are frequently involved in transcriptional activity regulating cell survival, proliferation, and differentiation makes the simultaneous action of ATF1 and CREB at the time of ZGA an appealing candidate mechanism to trigger transcription not only of primary target genes, but also of secondary molecules crucial for early development (rev. Jin and O'Neill 2007; Jin and O'Neill 2010; Jin and O'Neill 2011; O'Neill et al. 2012). However, it must be mentioned that CaMK and P38-MAPK pathways not only regulate CREB and ATF1 but also multiple proteins. Consequently, other transcription factors might be involved in the control of ZGA (rev. Jin and O'Neill 2014). For instance, TEAD2 and Sp1 are functional at the 2-cell stage in mice. Moreover, it is not yet known whether the transcriptome generated by the combined action of CREB and ATF1 is the same as that produced by each transcription factor separately. Therefore, further work is required (rev. Jin and O'Neill 2010).

ATF2 also accounts for important regulation of homeostasis and cell fate decisions. This transcription factor can act as either oncogenic or anti-oncogenic factor in multiple tumor types according to the molecular and cellular context (rev. Vlahopoulos et al. 2008; Gozdecka and Breitwieser 2012). Disruption of the DNA-binding domain of Atf2 lead to death of neonatal mice due to severe respiratory distress (meconium aspiration syndrome). Respiratory failure originates from altered cytotrophoblast functions that produce a drop in oxygen supply during gestation. Placental dysfunction is in turn correlated with down-regulation of PDGFRα (Maekawa et al. 1999). Neuronal-specific *Atf2* knockout impairs brain development as revealed by the analysis of (E)18.5 null-mice. With such results it was concluded that ATF2 is in charge of survival signaling of somatic and visceral motor neurons and it can regulate the phosphorylation state of JNK and P38-MAPK (Ackermann et al. 2011).

Similar to other ATF/CREB members, ATF2 is capable of homodimerization or selective heterodimerization with other proteins from its family or from the Fos/Jun family. This characteristic offers great versatility to ATF2 function so it can target not only CRE sequences, but also AP-1 elements, stress-response elements (StRE), UV-responsive

elements (URE), as well as the proximal element of the IFN-tau promoter (rev. Vlahopoulos et al. 2008) and the amino acid response element, AARE (Bruhat et al. 2007). Regulation of ATF2 appears as complex as that of other members of its own family. For example, it can form heterodimers with P300 (EP300/KAT3B), a transcriptional co-activator, and in this way modulate transcription of c-Jun that codes for another binding partner of ATF2 (rev. Gozdecka and Breitwieser 2012). ATF2 can be phosphorylated by P38-MAPK (Ventura et al. 2003) and PKC to become activated as part of the final steps of stress response (rev. Gozdecka and Breitwieser 2012). However, absence of ATF2 can lead to altered P38-MAPK activity (Ackerman et al. 2011). At the gene expression level, it is possible that Atf2 is modulated by Sp1 and ATF/CREB proteins since its promoter contains binding elements for these transcription factors (rev. Vlahopoulos et al. 2008).

Work from Kawasaki et al. (2000) in HeLa cells demonstrated that ATF2 possesses intrinsic histone acetyl-transferase capacity with high specificity for H2B and H4, whereas Hinkley and Perry (1992) reported that ATF2 was involved in the transcriptional control of the *H2b* gene in frog embryos. It seems that histone promoter modulation by ATF2 can lead to chromatin structure and gene expression alterations. This mechanism was shown to be mediated by AARE-bound ATF2 in mouse fibroblasts by using an amino acid starvation model that leaded to acetylation of both histones H2B and H4 (Bruhat et al. 2007). Interestingly, new evidence on the importance of histone H2B for preimplantation development recently arose from a study by Ooga et al. (2015), where the RNAi of *Rnf20* diminished the levels of the mono-ubiquitinated H2B (H2Bub1) and caused death of mouse morulae. Besides transcriptional regulation, ATF2 appears to have major roles in cell cycle control. For instance, ATF2 exerts positive regulation on the expression of central actors in cell cycle modulation, like CycA and CycD1, thus impacting on the outcome of proliferative/antiproliferative decisions (rev. Vlahopoulos et al. 2008; Gozdecka and Breitwieser 2012). Noteworthy, the above-mentioned involvement of ATF2 in regulation of cell cycle and oncogenesis appears shared with other ATF/CREB members. ATF1 was found by Yoshizumi et al. (1995) as essential to trigger transcription from *CycA* promoter in growing bovine aortic endothelial cells (BAEC), whereas it had negative effects on *CycA* expression in the same cell strain under confluent conditions. On the other hand, binding of

CDK3 potentiates the transcriptional activity of ATF1 in human tumor cells (Zheng et al. 2008).

Concerning the presence and potential roles of ATF1 and ATF2 gene products during early development in species other than the mouse, both mRNA and protein of ATF2 were observed by Villarreal and Richter (1995) as developmentally regulated during the span between egg and blastula in *Xenopus* and started an abrupt decline at this last stage. The remaining transcript and protein were preferentially distributed towards the animal pole of blastulae, suggesting an important role of ATF2 in gene expression control during early development in frogs (Villarreal and Richter 1995). In domestic animals, *ATF1* transcript levels appear to be regulated during cattle preimplantation development, where apparently the most important changes in its abundance pattern occur just before EGA (Vigneault et al. 2004). Furthermore, ATF2 protein was observed by Vigneault et al. (2009b) to translocate from cytoplasm to nuclei just prior to the 8-cell stage, the moment of EGA in bovines. Given such information it is plausible to infer the need of ATF2 protein inside the nucleus in preparation of EGA in cattle. Held et al. (2012) found that *ATF1* transcript levels are higher in the sister blastomere of the most competent 2-cell embryos in cattle, which suggests a putative developmental advantage of embryos with a surplus of *ATF1* mRNA.

1.9. Knock-Down of Specific Transcripts

Transcript ablation or gene knock-down encompasses multiple gene silencing techniques aimed at targeting a specific RNA species with a complementary anti-sense strand of oligonucleotides in order to induce either degradation of the transcript by the cell's intrinsic molecular machinery, or steric blockage of its translation in case of mRNA. Due to the variable gene-silencing strategies based on anti-sense oligonucleotides complex classification systems exist according to target molecule, triggered biological pathway, structure, or chemical modifications. For the purposes of the current review, two main gene knock-down systems are mentioned: 1) RNAi; and 2) anti-sense oligonucleotides (rev. Dias and Stein 2002; Kurreck 2003; Summerton 2007; Deleavey and Damha 2012; Jain et al. 2012).

1.9.1. Different types of systems for knock-down assays

RNAi is an endogenous and robust sequence-specific cell defense mechanism aimed at silencing viral nucleic acids (rev. Kurreck 2003). In animals, the naturally-occurring RNAi phenomenon was first described in the nematode *C. elegans* by Fire et al. (1998), where dsRNA suppressed the expression of both endogenous and heterologous genes (Fire et al. 1998). A long-lasting controversy corresponds to the debate of whether or not an intrinsic and completely functional RNAi system is actually present in mammalian somatic cells. This is because several arguments against, but mostly the notion that RNAi should be considered redundant as an antiviral system since the interferon (IFN) response (a cytokine-mediated signaling elicited by the presence of dsRNA, where the intervention of PKR and 2'-5' OAS can lead to apoptosis) is present in such cells. Nevertheless, new evidence supporting the occurrence of an endogenous RNAi system in somatic mammalian cells was published during the last few years (Svoboda 2014). Li et al. (2013) and Maillard et al. (2013) demonstrated that rodent kidney fibroblasts and ESC, respectively, contain an RNAi system originating from ancient viral sequences. After the original observations from Fire et al. (1998) in nematodes it was discovered that a 21 nt-long dsRNA sufficed to elicit RNAi-driven gene silencing in cultured human cells (Elbashir et al. 2001). Since then and due to its high efficiency at silencing gene expression, RNAi has been frequently employed to perform transcript ablation for experimental purposes and as a potential therapeutic agent (rev. Kurreck et al. 2003; Schellander et al. 2007; Deleavey and Damha 2012; Jain et al. 2012).

Overall, the RNAi mechanism relies on the loading of one of the strands of a 21-24 bp dsRNA molecule (with 3' overhangs of 2 nt) onto the RNA-induced silencing complex (RISC) upon processing by the RNase III enzyme Dicer in the cytoplasm. Dicer commonly works in association with the dsRNA-binding protein TRBP. The strand that is loaded on RISC corresponds to the *guide*, whereas the *passenger* strand is discarded. The guide strand is complementary or antisense to the targeted transcript to which it hybridizes after RISC finds an accurate target. When the complementarity of the guide strand is partial with the target transcript, it is said that the original 21-24 bp dsRNA molecule is a miRNA (rev. Campbell and Choy 2005; Deleavey and Damha 2012). In contrast, when complementarity

occurs throughout the full-length of the guide strand, the former dsRNA is termed a small-interfering RNA (siRNA). In this last case RISC cleaves the target transcript whose pieces will be further degraded. Currently, experimental siRNA by directly using molecules already of a 21-24 bp-length is probably the most popular siRNA method. Nevertheless, other variants of the RNAi system are long double-stranded RNA (ldsRNA), Dicer substrate siRNA (DsiRNA), circular RNA, short-hairpin RNA (shRNA), and small internally-segmented interfering RNA, sisiRNA (rev. Campbell and Choy 2005; Deleavey and Damha 2012). ldsRNA molecules experimentally introduced into cells commonly have a length of up to 300-400 bp (Paradis et al. 2005; Tesfaye et al. 2010). ldsRNA must be cleaved by Dicer up to a shorter siRNA before loading the molecule onto RISC (rev. Campbell and Choy 2005). DsiRNA is a subtype of the siRNA method with high transcript ablation power, where a 25-30 bp-long dsRNA molecule is used with the purpose of being easily cleaved by Dicer to increase the silencing effect in comparison with common 21-24 bp siRNAs (Kim et al. 2005; Caballero et al. 2015). Finally, shRNA are generally loop-forming RNAs generally expressed with the introduction of a vector in the cells whose gene expression is sought to be silenced (rev. Deleavey and Damha 2012).

Another knockdown system, the antisense oligonucleotides (AS-ONs), consists of single-stranded chains that typically range from 13 to 25 nt in length. Throughout their history, AS-ONs have varied from either DNA or RNA nucleotide derivatives, to the newest chemically-modified oligonucleotides with variations in either their backbone, nucleotide base, or both. AS-ONs function by either two main mechanisms: 1) forming an antisense-transcript duplex that blocks ribosomal binding by sterical hindrance and preventing translation; or 2) recruitment of RNase H for transcript cleavage. However, some AS-ONs have been used to prevent polyadenylation or affect alternative splicing. The sequence of single-stranded AS-ONs is unique to a specific target sequence (rev. Deleavey and Damha 2012; Jain et al. 2012). The phosphorothioate oligonucleotides used at the beginning of AS-ONs technologies were plagued with non-specific off-targeting effects due to the negative charge of phosphorothioates that are bound by proteins recognizing polyanions. This led to the design of AS-ONs with new chemistries in order to increase specificity. Currently, the most used AS-ONs include: 2'-deoxy-2'-fluoro-beta-D-arabino nucleic acid (FANA);

locked nucleic acid (LNA); chimeric LNA.DNA oligonucleotide; unlocked nucleic acid (UNA); cyclohexene nucleic acid (CeNA); tricycle-DNA (tcDNA); N3'-P5' phosphoroamidate (NP); peptide nucleic acid (PNA); and MO (rev. Dias and Stein 2002; Kurreck 2003). The so-called *third generation* of AS-ONs encompasses PNAs, LNAs, and MOs. These AS-ONs do not efficiently activate RNase H. Instead, their mechanism of action relies on steric interactions that prevent translation or hybridization of their target transcripts with their respective target sequences. Off-target effects of AS-ONs of the third generation are diminished to a minimum due to the neutral nature of their backbone (rev. Jain et al. 2012). LNAs show a rigid backbone that allows a remarkable duplex stability and strong binding after hybridizing to their target sequences. LNAs can be used as anti-miRNAs or to knockdown other transcripts (rev. Deleavey and Damha 2012). LNA. DNA chimeric gapmers combine the high binding and stability strength of LNAs with the ability of DNA molecules to recruit RNase H with a consequent cleavage of the target transcripts. LNA.DNA gapmers have been widely and successfully tested as anti-miRNA agents (rev. Jain et al. 2012).

MOs are AS-ONs where the five-member ring of furanose (from DNA or RNA) is substituted by a six-member morpholino ring, while the phosphate bonds are substituted by non-ionic phosphorodiamidate linkages that provide a neutral nature (rev. Kurreck 2003; Summerton 2007; Deleavey and Damha 2012). According to J.E. Summerton, owner and manager of Gene Tools LLC, the company that commercially provides MOs (Summerton 2007), the structural characteristics of MOs signify several advantages in gene-silencing technology namely: 1) Putative full stability and very diminished degradation even in complex biological systems, as developing embryos; 2) increased binding strength for complementary RNA sequences. This efficiency allows to invade most secondary structures of target transcripts; 3) highly reduced off-targeting effects due to their apparent absence of interaction with extracellular/cellular proteins or other macromolecular complexes (e.g. heparin), as well as absence of degradation products; 4) MOs virtually do not activate IFN response or any other immune system activity and are non-toxic to cells (Summerton 2007). Characteristics like the exquisite specificity (extremely reduced off-targeting) and strong affinity of MOs, have also been recognized by other authors (Dias and Stein 2002; Kurreck

2003; Deleavey and Damha 2012). MOs have been successfully employed to block translation (Siddall et al. 2002; Foygel et al. 2008; Kashiwagi et al. 2010; Tsai et al. 2010; Vogt et al. 2012; Yue et al. 2013; Garg et al. 2015), as anti-miRNA agents (Yu et al. 2011); to inhibit polyadenylation (Wada et al. 2012); or to affect alternative splicing (Garg et al. 2015). In case of translational suppression applications, MOs are designed to target exactly the AUG initiation codon or just a few nucleotides upstream or downstream of such sequence for maximal inhibitory effect. Typically, MOs aimed at translation inhibition are around 25-nt long (rev. Dias and Stein 2002; Kurreck 2003; Summerton 2007). Given the neutral backbone of MOs, these are unable to form complexes with cationic lipids (rev. Dias and Stein 2002) and thus alternative methods have been designed as an option to substitute transfection to deliver MOs to living cells or embryos. In this case, an example of a suitable commercial method consists of the Endo-Porter system that consists on a peptide-based agent able to drive endocytosis to produce cytosolic delivery of MOs (Summerton 2005). On the other hand, as mentioned in the next section, MOs have also been successfully delivered through microinjection.

1.9.2. Knock-down technology in early developmental studies

In non-mammalian laboratory models, MOs have been accurately microinjected in zebrafish (Wada et al. 2012; Yue et al. 2013) and *Xenopus* (Kashiwagi et al. 2010) zygotes and 2-cell embryos. The use of *in ovo* electroporation has allowed the delivering of MOs and dsRNA in chickens (Krull 2004), whereas a number of reports show the feasibility of using these AS-ON in mice through transfection (Siddall et al. 2002) and microinjection (Foygel et al. 2008; Tsai et al. 2010; Vogt et al. 2012) in zygotes, 2-, and 8-cell embryos. In domestic mammals, Huang et al. (2015) applied MOs to pig MII oocytes through microinjection. In cattle, O'Meara et al. (2011) obtained promising results of transfection of zygotes with siRNA to target *E-cadherin* (*CDH1*/*CDHE*). Although the authors remarked that such technique still required refinement in order to improve subsequent developmental rates, transfection was proposed as a potential alternative to microinjection (O'Meara et al. 2011). In order to transfect oocytes or zygotes, the liposomes containing the silencing molecule must pass through the oolemma. Thus, any remaining layer of cumulus cells should be excised before transfection. Since bovine oocytes require the presence of the

cumulus in order to mature properly (Fukui and Sakuma 1980; Sirard et al. 1988), the use of transfection in this species is limited to developmental stages after maturation. In addition, transfection requires removal of ZP, a process that adds manipulation to the oocytes/zygotes. O'Meara et al. (2011) indicated a significant deleterious effect of ZP removal on posterior development of zygotes. It is plausible that this procedure would prove even more aggressive in other developmental stages, like the highly physical damage-prone immature oocytes (Paradis et al. 2005). To the best of our knowledge, besides the report from Yang et al. (2014), MOs have not yet been reported in another knock-down studies during preimplantation development in cattle. Nevertheless, due to the feasibility of MOs use during early development in other mammals (e.g. mouse), as well as the success of microinjection in bovine oocytes and zygotes as shown in Table 1-1 to perform multiple RNAi assays, it appears plausible to use microinjection to analyze the effects of gene knock-down through MOs in cattle early development.

Table 1-1 Reports of gene knockdown in cattle pre-implantation development

Target	Interf. molecule	Stage	Results	Reference
CCNB1	ldsRNA	GV, MII [cytoch. B]	Failure to arrest at MII; spontaneous oocyte activation	Paradis et al. (2005)
CDH1 (*CDHE*)	dsRNA	p-Zy	Reduced blastocyst rate	Nganvongpanit et al. (2006a)
POU5F1	dsRNA	p-Zy	Not significant	Nganvongpanit et al. (2006a)
C-MOS	dsRNA	GV	Increase of PB extrusion; spontaneous oocyte activation	Nganvongpanit et al. (2006b)
p66Shc	shRNA	GV	Reduced cleavage and blastocyst rates; decrease in permanent embryo arrest (at 2-4 cell)	Favetta et al. (2007)
BIRC5 (*Survivn*)	dsRNA	p-Zy	Reduced blastocyst rate and quality; increased apoptosis in blastocysts	Park et al. (2007)
CDH1	dsRNA	p-Zy	Not significant	Tesfaye et al. (2007)
CX43	dsRNA	p-Zy	Diminished blastocyst quality	Tesfaye et al. (2007)
HMGN1	siRNA	GV	Not significant	Vigneault (2008)
HMGN2	siRNA	GV	Not significant	Vigneault (2008)
MATRIN3	siRNA	GV	Dramatic decrease in blastocyst rate	Vigneault (2008)
FS	siRNA	p-Zy	Reduced 8-16 cell and blastocyst rates; diminished blastocyst quality	Lee et al. (2009)
KPNA7	siRNA	p-Zy	Reduced 8-16 cell and blastocyst rates	Tejomurtula et al. (2009)

Interf, interference; ldsRNA, long double-stranded RNA; dsRNA, double-stranded RNA; shRNA, short-hairpin RNA; siRNA, small-interference RNA; GV, germinal vesicle-oocyte; MII, metaphase II-oocyte; p-Zy, presumptive zygote; PB, polar body.

Table 1-1 (continued) Reports of gene knockdown in cattle pre-implantation development

Target	Interf. Molecule	Stage	Results	Reference
CENPF	dsRNA	p-Zy	Developmental arrest at 8-cell; decrease of 16-cell and morula rates	Toralova et al. (2009)
BIRC6 (*Apollon*)	ldsRNA, shRNA	p-Zy	Reduced 8-cell and blastocyst rates; increased caspase activity, apoptosis; increased *BAX*/*BCL2* ratio, *SMAC* and *CASP9* mRNAs in blastocysts	Salilew-Wondin et al. (2010)
MSX1	ldsRNA, siRNA	GV, p-Zy	Delayed PB extrusion; reduced 8-cell and blastocyst rates; impaired mRNA levels of *BMP15*, *ZNF43*, *ZNF85*, *ZNF91*, *RIOK3*, *ALF*, *AURKA*, *PTTG1*, *EEF1A1*, *MNS1*, *LGALS3*, *INF-tau*, and *RPL23*	Tesfaye et al. (2010)
CDH1	siRNA	p-Zy (M,T)	Reduced blastocyst rate	O'Meara et al. (2011)
NOBOX	siRNA	p-Zy	Failure in the transcription of the embryonic *KLF5*, *PITX2*, *OCT4*, *NANOG*, *WEE1*, *CCNE2*, *JAG1*, *FZD8*; reduced blastocyst; disruption of cell lineage determination	Tripurani et al. (2011)
DNMT1	siRNA	p-Zy (SCNT)	Decreased hypermethylation of the satellite I region; increase of cleavage and blastocyst rates	Yamanaka et al. (2011)
JMJD3 (*KDM6B*)	siRNA	MII (parth.)	Blockage of the H3K27me3 ablation during preimplantation development; reduced blastocyst rate	Canovas et al. (2012)
DPPA3 (*STELLA*)	siRNA	GV	Abrogation of the asymmetry of 5hmC levels between both pronuclei	Bakhtari et al. (2014)
JY-1	siRNA	GV	Reduced cumulus expansion, maturation, fast-cleaving embryos, 8-16 cell; null blastocyst formation	Lee et al. (2014b)

Interf, interference; ldsRNA, long double-stranded RNA; dsRNA, double-stranded RNA; shRNA, short-hairpin RNA; siRNA, small-interference RNA; GV, germinal vesicle-oocyte; MII, metaphase II-oocyte; p-Zy, presumptive zygote; SCNT, somatic cell nuclear transfer; parth, parthenotes; PB, polar body; M, microinjection; T, transfection.

Table 1-1 (continued) Reports of gene knockdown in cattle pre-implantation development

Target	Interf. Species	Stage	Results	Reference
SMAD4	siRNA	p-ZY	Impairment of EGA and transcription of *DSC2*, *CRABP1*, *SLC38A2*, and *CXCL6*; decrease in fast-cleaving embryo, 8-16 cell, and blastocyst rates	Lee et al. (2014c)
CDC20	MO	(den.) MI, MII [cytoch. B]	Meiosis I: Reduced PB1 extrusion; arrest at/before MI; abnormal spindle formation. Meiosis II: Not significant	Yang et al. (2014)
H1FOO	siRNA	(den.) GV-MI [cytoch. B]	Meiosis impairment: PB extrusion decrease	Yun et al. (2015)
lncRNA1	DsiRNA	(den.) MII	Accelerated embryonic kinetics; increased blastocyst quality; 179 differential transcripts; 3706 DNA regions differentially methylated; profiling of *CXADR*, *AVIL*, *CD9*, and *PLAU* consistent for both the transcriptomic and DNA methylation arrays	Caballero et al. (2015)
USF1	siRNA	GV, p-Zy	GV-oo injection: Reduced *GDF9*, *FST*; enhanced *JY-1*, *TWIST2* mRNAs in MII-oo. p-Zy: Decreased 8-16 cell and blastocyst rates	Datta et al. (2015)
ROCK1	siRNA	GV	Meiosis impairment: Inhibition of cell cycle progression and PB extrusion; abatement of actin, phospho-cofilin, and phospho-MLC abundance	Lee et al. (2015)
SMAD2/3	siRNA	p-Zy	Decreased 8-16 cell and blastocyst rates; reduced CTGF mRNA in blastocysts (in *SMAD2* RNAi)	Zhang et al. (2015)

Interf, interference; siRNA, small-interference RNA; lncRNA, long non-coding RNA; DsiRNA, Dicer-substrate small-interfering RNA; GV, germinal vesicle-oocyte; MI, metaphase I; MII, metaphase II-oocyte; p-Zy, presumptive zygote; PB, polar body; PB1, first polar body.

1.10. Hypothesis and Objectives

Our general hypothesis is that bovine oocytes and their derived embryos with distinct levels of developmental competence display differential abundance levels of specific maternal transcripts. The key importance of this molecular signature is reflected by the fact that a deficit in the presence of a maternal mRNA with crucial developmental roles significantly impacts the progress of embryogenesis.

Our first objective was to obtain the global molecular profile of 2-cell embryos of extreme levels of developmental competence (high and low) in order to identify the mRNAs preferentially accumulated in the most competent embryos that reflect their maternal molecular inheritance

Our second objective was to assess the effects of the suppression of specific maternal mRNAs on the developmental outcome of bovine preimplantation embryos.

Our third objective was to investigate the putative impact of polyadenylation on translation dynamics of *specific transcripts* with distinct 3'-UTR sequences (short or long) during the first cleavage divisions in bovine embryos. After *in vitro* culture, the dynamics of eGFP translation of every construct was compared by using epifluorescence microscopy at 24, 36, 48, 60, and 80 hpi. The performance of deadenylated constructs was contrasted with that of their respective polyadenylated control, as well as that of mRNA containing only the sequence of eGFP.

1.11. References

Abd El Naby WS, Hagos TH, Hossain MM, Salilew-Wondim D, Gad AY, Rings F, Cinar MU, Tholen E, Looft C, Schellander K, Hoelker M, Tesfaye D. 2013. Expression analysis of regulatory microRNAs in bovine cumulus oocyte complex and preimplantation embryos. *Zygote* **21:** 31-51.

Abe H, Yamashita S, Itoh T, Satoh T, Hoshi H. 1999. Ultrastructure of bovine embryos developed from in vitro-matured and -fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. *Mol Reprod Dev* **53:** 325-35.

Abe K, Yamamoto R, Franke V, Cao M, Suzuki Y, Suzuki MG, Vlahovicek K, Svoboda P, Schultz RM, Aoki F. 2015. The first murine zygotic transcription is promiscuous and uncoupled from splicing and 3' processing. *EMBO J* **34:** 1523-37.

Ackermann J, Ashton G, Lyons S, James D, Hornung JP, Jones N, Breitwieser W. 2011. Loss of ATF2 function leads to cranial motoneuron degeneration during embryonic mouse development. *PLoS One* **6:** e19090.

Adams GP, Jaiswal R, Singh J, Malhi P. 2008. Progress in understanding ovarian follicular dynamics in cattle. *Theriogenology* **69**: 72-80.

Aerts JM, Bols PE. 2010a. Ovarian follicular dynamics: a review with emphasis on the bovine species. Part I: Folliculogenesis and pre-antral follicle development. *Reprod Domest Anim* **45**: 171-179.

Aerts JM, Bols PE. 2010b. Ovarian follicular dynamics. A review with emphasis on the bovine species. Part II: Antral development, exogenous influence and future prospects. *Reprod Domest Anim* **45**: 180-187.

Aguirre-Lavin T, Adenot P, Bonnet-Garnier A, Lehmann G, Fleurot R, Boulesteix C, Debey P, Beaujean N. 2012. 3D-FISH analysis of embryonic nuclei in mouse highlights several abrupt changes of nuclear organization during preimplantation development. *BMC Dev Biol* **12:** 30.

Ali A, Sirard MA. 2005. Protein kinases influence bovine oocyte competence during short-term treatment with recombinant human follicle stimulating hormone. *Reproduction* **130**: 303-310.

Alizadeh Z, Kageyama S, Aoki F. 2005. Degradation of maternal mRNA in mouse embryos: selective degradation of specific mRNAs after fertilization. *Mol Reprod Dev* **72:** 281-290.

Amano T, Hirata T, Falco G, Monti M, Sharova LV, Amano M, Sheer S, Hoang HG, Piao Y, Stagg CA, Yamamizu K, Akiyama T, Ko MS. 2013. Zscan4 restores the developmental potency of embryonic stem cells. *Nat Commun* **4:** 1966.

Anderson JE, Matteri RL, Abeydeera LR, Day BN, Prather RS. 2001. Degradation of maternal cdc25c during the maternal to zygotic transition is dependent upon embryonic transcription. *Mol Reprod Dev* **60:** 181-188.

Anderson P, Kedersha N. 2006. RNA granules. *J Cell Biol* **172:** 803-808.

Aoki F, Hara KT, Schultz RM. 2003. Acquisition of transcriptional competence in the 1-cell mouse embryo: requirement for recruitment of maternal mRNAs. *Mol Reprod Dev* **64:** 270-274.

Aoki F, Worrad DM, Schultz RM. 1997. Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. *Dev Biol* **181:** 296-307.

Artus J, Babinet C, Cohen-Tannoudji M. 2006. The cell cycle of early mammalian embryos: lessons from genetic mouse models. *Cell Cycle* **5:** 499-502.

Assou S, Anahory T, Pantesco V, Le Carrour T, Pellestor F, Klein B, Reyftmann L, Dechaud H, De Vos J, Hamamah S. 2006. The human cumulus--oocyte complex gene-expression profile. *Hum Reprod* **21**: 1705-1719.

Atef A, Francois P, Christian V, Marc-Andre S. 2005. The potential role of gap junction communication between cumulus cells and bovine oocytes during in vitro maturation. *Mol Reprod Dev* **71**: 358-367.

Bachvarova R, De Leon V, Johnson A, Kaplan G, Paynton BV. 1985. Changes in total RNA, polyadenylated RNA, and actin mRNA during meiotic maturation of mouse oocytes. *Dev Biol* **108:** 325-331.

Badr H, Bongioni G, Abdoon AS, Kandil O, Puglisi R. 2007. Gene expression in the in vitro-produced preimplantation bovine embryos. *Zygote* **15:** 355-367.

Balboula AZ, Stein P, Schultz RM, Schindler K. 2013. Knockdown of RBBP7 unveils a requirement of histone deacetylation for CPC function in mouse oocytes. *Cell Cycle* **13:** 600-611.

Barnes FL, Eyestone NL. 1990. Early cleavage and the maternal zygotic transition in bovine embryos. *Theriogenology* **33:** 141-152

Barnes FL, First NL. 1991. Embryonic transcription in in vitro cultured bovine embryos. *Mol Reprod Dev* **29:** 117-123.

Bastos GM, Goncalves PB, Bordignon V. 2008. Immunolocalization of the high-mobility group N2 protein and acetylated histone H3K14 in early developing parthenogenetic bovine embryos derived from oocytes of high and low developmental competence. *Mol Reprod Dev* **75:** 282-290.

Beaujean N, Mason K, Bonnet-Garnier A, Salvaing J, Debey P. 2010. [Embryonic genome organization after fertilization in mammals]. *Biol Aujourdhui* **204:** 205-213.

Belloc E, Mendez R. 2008. A deadenylation negative feedback mechanism governs meiotic metaphase arrest. *Nature* **452:** 1017-21.

Bellier S, Chastant S, Adenot P, Vincent M, Renard JP, Bensaude O. 1997. Nuclear translocation and carboxyl-terminal domain phosphorylation of RNA polymerase II delineate the two phases of zygotic gene activation in mammalian embryos. *EMBO J* **16:** 6250-6262.

Beraldi R, Pittoggi C, Sciamanna I, Mattei E, Spadafora C. 2006. Expression of LINE-1 retroposons is essential for murine preimplantation development. *Mol Reprod Dev* **73:** 279-287.

Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. 2003. Dicer is essential for mouse development. *Nat Genet* **35:** 215-217.

Bettegowda A, Lee KB, Smith GW. 2008 Cytoplasmic and nuclear determinants of the maternal-to-embryonic transition. *Reprod Fertil Dev* **20:** 45-53.

Bettegowda A, Smith GW. 2007 Mechanisms of maternal mRNA regulation: implications for mammalian early embryonic development. *Front Biosci* **12:** 3713-3726.

Bevilacqua A, Fiorenza MT, Mangia F. 2000. A developmentally regulated GAGA box-binding factor and Sp1 are required for transcription of the hsp70.1 gene at the onset of mouse zygotic genome activation. *Development* **127:** 1541-1551.

Bhoumik A, Takahashi S, Breitweiser W, Shiloh Y, Jones N, Ronai Z. 2005. ATM-dependent phosphorylation of ATF2 is required for the DNA damage response. *Mol Cell* **18:** 577-587.

Biase FH, Martelli L, Puga R, Giuliatti S, Santos-Biase WK, Fonseca Merighe GK, Meirelles FV. 2010. Messenger RNA expression of Pabpnl1 and Mbd3l2 genes in oocytes and cleavage embryos. *Fertil Steril* **93:** 2507-2512.

Bielanski A, Loewen KS, Del Campo MR, Sirard MA, Willadsen S. 1993. Isolation of bovine herpesvirus-1 (BHV-1) and bovine viral diarrhea virus (BVDV) in association with the in vitro production of bovine embryos. *Theriogenology* **40**: 531-538.

Bleckmann SC, Blendy JA, Rudolph D, Monaghan AP, Schmid W, Schutz G. 2002. Activating transcription factor 1 and CREB are important for cell survival during early mouse development. *Mol Cell Biol* **22:** 1919-1925.

Blondin P, Bousquet D, Twagiramungu H, Barnes F, Sirard MA. 2002. Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biol Reprod* **66**: 38-43.

Blondin P, Coenen K, Guilbault LA, Sirard MA. 1997a. In vitro production of bovine embryos: developmental competence is acquired before maturation. *Theriogenology* **47**: 1061-1075.

Blondin P, Guilbault LA, Sirard MA. 1997b. The time interval between FSH-P administration and slaughter can influence the developmental competence of beef heifer oocytes. *Theriogenology* **48**: 803-813.

Bohrer RC, Coutinho AR, Duggavathi R, Bordignon V. 2015. The Incidence of DNA Double-Strand Breaks Is Higher in Late-Cleaving and Less Developmentally Competent Porcine Embryos. *Biol Reprod* **[in press]**.

Bouleau A, Desvignes T, Traverso JM, Nguyen T, Chesnel F, Fauvel C, Bobe J. 2014. Maternally inherited npm2 mRNA is crucial for egg developmental competence in zebrafish. *Biol Reprod* **91:** 43.

Bouniol C, Nguyen E, Debey P. 1995. Endogenous transcription occurs at the 1-cell stage in the mouse embryo. *Exp Cell Res* **218:** 57-62.

Bouzinba-Segard H, Guais A, Francastel C. 2006. Accumulation of small murine minor satellite transcripts leads to impaired centromeric architecture and function. *Proc Natl Acad Sci U S A* **103:** 8709-8714.

Bowles J, Koopman P. 2007. Retinoic acid, meiosis and germ cell fate in mammals. *Development* **134**: 3401-3411.

Brandeis M, Rosewell I, Carrington M, Crompton T, Jacobs MA, Kirk J, Gannon J, Hunt T. 1998. Cyclin B2-null mice develop normally and are fertile whereas cyclin B1-null mice die in utero. *Proc Natl Acad Sci U S A* **95**: 4344-4349.

Braude P, Bolton V, Moore S. 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* **332:** 459-461.

Braw-Tal R, Yossefi S. 1997. Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. *J Reprod Fertil* **109**: 165-171.

Brevini TA, Cillo F, Antonini S, Gandolfi F. 2007. Cytoplasmic remodelling and the acquisition of developmental competence in pig oocytes. *Anim Reprod Sci* **98:** 23-38.

Brevini TA, Lonergan P, Cillo F, Francisci C, Favetta LA, Fair T, Gandolfi F. 2002. Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. *Mol Reprod Dev* **63:** 510-517.

Brook M, Smith JW, Gray NK. 2009. The DAZL and PABP families: RNA-binding proteins with interrelated roles in translational control in oocytes. *Reproduction* **137:** 595-617.

Bruhat A, Cherasse Y, Maurin AC, Breitwieser W, Parry L, Deval C, Jones N, Jousse C, Fafournoux P. 2007. ATF2 is required for amino acid-regulated transcription by orchestrating specific histone acetylation. *Nucleic Acids Res* **35:** 1312-1321.

Brunet S, Maro B. 2005. Cytoskeleton and cell cycle control during meiotic maturation of the mouse oocyte: integrating time and space. *Reproduction* **130**: 801-811.

Bui LC, Evsikov AV, Khan DR, Archilla C, Peynot N, Hénaut A, Le Bourhis D, Vignon X, Renard JP, Duranthon V. 2009. Retrotransposon expression as a defining event of genome reprogramming in fertilized and cloned bovine embryos. *Reproduction* **138:** 289-299.

Caballero J, Gilbert I, Fournier E, Gagne D, Scantland S, Macaulay A, Robert C. 2014. Exploring the function of long non-coding RNA in the development of bovine early embryos. *Reprod Fertil Dev* **27**: 40-52.

Caixeta ES, Ripamonte P, Franco MM, Junior JB, Dode MA. 2009. Effect of follicle size on mRNA expression in cumulus cells and oocytes of Bos indicus: an approach to identify marker genes for developmental competence. *Reprod Fertil Dev* **21:** 655-664.

Campbell TN, Choy FY. 2005. RNA interference: past, present and future. *Curr Issues Mol Biol* **7:** 1-6.

Carabatsos MJ, Sellitto C, Goodenough DA, Albertini DF. 2000. Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Dev Biol* **226**: 167-179.

Casanova M, Pasternak M, El Marjou F, Le Baccon P, Probst AV, Almouzni G. 2013. Heterochromatin reorganization during early mouse development requires a single-stranded noncoding transcript. *Cell Rep* **4(6):** 1156-1167.

Cetica P, Pintos L, Dalvit G, Beconi M. 2003. Involvement of enzymes of amino acid metabolism and tricarboxylic acid cycle in bovine oocyte maturation in vitro. *Reproduction* **126:** 753-763.

Chandolia RK, Peltier MR, Tian W, Hansen PJ. 1999. Transcriptional control of development, protein synthesis, and heat-induced heat shock protein 70 synthesis in 2-cell bovine embryos. *Biol Reprod* **61:** 1644-1648.

Chen J, Melton C, Suh N, Oh JS, Horner K, Xie F, Sette C, Blelloch R, Conti M. 2011. Genome-wide analysis of translation reveals a critical role for deleted in azoospermia-like (Dazl) at the oocyte-to-zygote transition. *Genes Dev* **25:** 755-766.

Chian RC, Sirard MA. 1996. Protein synthesis is not required for male pronuclear formation in bovine zygotes. *Zygote* **4:** 41-48.

Christians E, Campion E, Thompson EM, Renard JP. 1995. Expression of the HSP 70.1 gene, a landmark of early zygotic activity in the mouse embryo, is restricted to the first burst of transcription. *Development* **121:** 113-122.

Clarke HJ. 2012. Post-transcriptional control of gene expression during mouse oogenesis. *Results Probl Cell Differ* **55:** 1-21.

Coenen K, Massicotte L, Sirard MA. 2004. Study of newly synthesized proteins during bovine oocyte maturation in vitro using image analysis of two-dimensional gel electrophoresis. *Mol Reprod Dev* **67**: 313-322.

Collart C, Allen GE, Bradshaw CR, Smith JC, Zegerman P. 2013. Titration of four replication factors is essential for the Xenopus laevis midblastula transition. *Science* **341:** 893-896.

Conti M. 2011. When an egg is not an egg: compromised maternal mRNA storage and stabilization. *Biol Reprod* **85**: 429-430.

Cooke A, Prigge A, Wickens M. 2010. Translational repression by deadenylases. *J Biol Chem* **285:** 28506-28513.

Crosby IM, Gandolfi F, Moor RM. 1988. Control of protein synthesis during early cleavage of sheep embryos. *J Reprod Fertil* **82:** 769-775.

Cui XS, Li XY, Yin XJ, Kong IK, Kang JJ, Kim NH. 2007. Maternal gene transcription in mouse oocytes: genes implicated in oocyte maturation and fertilization. *J Reprod Dev* **53**: 405-418.

Dalbies-Tran R, Mermillod P. 2003. Use of heterologous complementary DNA array screening to analyze bovine oocyte transcriptome and its evolution during in vitro maturation. *Biol Reprod* **68**: 252-261.

Dang-Nguyen TQ, Kikuchi K, Somfai T, Ozawa M, Nakai M, Maedomari N, Viet-Linh N, Kanai Y, Nguyen BX, Nagai T. 2010. Evaluation of developmental competence of in vitro-produced porcine embryos based on the timing, pattern and evenness of the first cleavage and onset of the second cleavage. *J Reprod Dev* **56:** 593-600.

Datta TK, Rajput SK, Wee G, Lee K, Folger JK, Smith GW. 2015. Requirement of the transcription factor USF1 in bovine oocyte and early embryonic development. *Reproduction* **149:** 203-212.

Davis W Jr, Schultz RM. 1997. Role of the first round of DNA replication in reprogramming gene expression in the preimplantation mouse embryo. *Mol Reprod Dev* **47(4):** 430-434.

De Felici M, Scaldaferri ML, Lobascio M, Iona S, Nazzicone V, Klinger FG, Farini D. 2004. Experimental approaches to the study of primordial germ cell lineage and proliferation. *Hum Reprod Update* **10**: 197-206.

De La Fuente R, Baumann C, Viveiros MM. 2015. ATRX contributes to epigenetic asymmetry and silencing of major satellite transcripts in the maternal genome of the mouse embryo. *Development* **142:** 1806-1817.

de Matos DG, Furnus CC, Moses DF. 1997. Glutathione synthesis during in vitro maturation of bovine oocytes: role of cumulus cells. *Biol Reprod* **57**: 1420-1425.

de Moor CH, Meijer H, Lissenden S. 2005. Mechanisms of translational control by the 3' UTR in development and differentiation. *Semin Cell Dev Biol* **16:** 49-58.

De Sousa PA, Westhusin ME, Watson AJ. 1998. Analysis of variation in relative mRNA abundance for specific gene transcripts in single bovine oocytes and early embryos. *Mol Reprod Dev* **49**: 119-130.

de Veer MJ, Sledz CA, Williams BR. 2005. Detection of foreign RNA: implications for RNAi. *Immunol Cell Biol* **83:** 224-228.

Deleavey GF, Damha MJ. 2012. Designing chemically modified oligonucleotides for targeted gene silencing. *Chem Biol* **19:** 937-954.

Delouis C, Bonnerot C, Vernet M, Nicolas JF. 1992. Expression of microinjected DNA and RNA in early rabbit embryos: changes in permissiveness for expression and transcriptional selectivity. *Exp Cell Res* **201:** 284-291.

Dessie SW, Rings F, Holker M, Gilles M, Jennen D, Tholen E, Havlicek V, Besenfelder U, Sukhorukov VL, Zimmermann U et al. 2007. Dielectrophoretic behavior of in vitro-derived bovine metaphase II oocytes and zygotes and its relation to in vitro embryonic developmental competence and mRNA expression pattern. *Reproduction* **133**: 931-946.

Deuve JL, Bonnet-Garnier A, Beaujean N, Avner P, Morey C. 2015. Antagonist Xist and Tsix co-transcription during mouse oogenesis and maternal Xist expression during pre-implantation development calls into question the nature of the maternal imprint on the X chromosome. *Epigenetics* **[in press]**.

Dias N, Stein CA. 2002. Antisense oligonucleotides: basic concepts and mechanisms. *Mol Cancer Ther* **1:** 347-355.

Dinnyes A, Lonergan P, Fair T, Boland MP, Yang X. 1999. Timing of the first cleavage post-insemination affects cryosurvival of in vitro-produced bovine blastocysts. *Mol Reprod Dev* **53:** 318-324.

Dobbs KB, Spollen WG, Springer G, Prather RS. 2010. The role of cytoplasmic polyadenylation element sequence on mRNA abundance during porcine embryogenesis and parthenogenetic development. *Mol Reprod Dev* **77:** 699-709.

Donnison M, Pfeffer PL. 2004. Isolation of genes associated with developmentally competent bovine oocytes and quantitation of their levels during development. *Biol Reprod* **71:** 1813-1821.

Donovan PJ, De Miguel MP, Hirano MP, Parsons MS, Lincoln AJ. 2001. Germ cell biology--from generation to generation. *Int J Dev Biol* **45**: 523-531.

Dorji, Ohkubo Y, Miyoshi K, Yoshida M. 2012. Gene expression profile differences in embryos derived from prepubertal and adult Japanese Black cattle during in vitro development. *Reprod Fertil Dev* **24:** 370-381.

Driancourt MA, Reynaud K, Smitz J. 2001. Differences in follicular function of 3-month-old calves and mature cows. *Reproduction* **121:** 463-474.

Ducibella T, Schultz RM, Ozil JP. 2006. Role of calcium signals in early development. *Semin Cell Dev Biol* **17:** 324-332.

Durcova-Hills G, Wianny F, Merriman J, Zernicka-Goetz M, McLaren A. 2003. Developmental fate of embryonic germ cells (EGCs), in vivo and in vitro. *Differentiation* **71**: 135-141.

Edwards JL, Ealy AD, Monterroso VH, Hansen PJ. 1997. Ontogeny of temperature-regulated heat shock protein 70 synthesis in preimplantation bovine embryos. *Mol Reprod Dev* **48:** 25-33.

Edwards RG. 1965. Maturation in vitro of human ovarian oocytes. *Lancet* **2:** 926-929.

Egloff S, Murphy S. 2008. Role of the C-terminal domain of RNA polymerase II in expression of small nuclear RNA genes. *Biochem Soc Trans* **36:** 537-539.

Eichenlaub-Ritter U, Peschke M. 2002. Expression in in-vivo and in-vitro growing and maturing oocytes: focus on regulation of expression at the translational level. *Hum Reprod Update* **8:** 21-41.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411:** 494-498.

Evsikov AV, Graber JH, Brockman JM, Hampl A, Holbrook AE, Singh P, Eppig JJ, Solter D, Knowles BB. 2006. Cracking the egg: molecular dynamics and evolutionary aspects of the transition from the fully grown oocyte to embryo. *Genes Dev* **20:** 2713-2727.

Fabian MR, Mathonnet G, Sundermeier T, Mathys H, Zipprich JT, Svitkin YV, Rivas F, Jinek M, Wohlschlegel J, Doudna JA, Chen CY, Shyu AB, Yates JR 3rd, Hannon GJ, Filipowicz W, Duchaine TF, Sonenberg N. 2009. Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. *Mol Cell* **235:** 868-880.

Fair T. 2003. Follicular oocyte growth and acquisition of developmental competence. *Anim Reprod Sci* **78**: 203-216.

Fair T, Carter F, Park S, Evans AC, Lonergan P. 2007. Global gene expression analysis during bovine oocyte in vitro maturation. *Theriogenology* **68 Suppl 1**: S91-97.

Fair T, Hulshof SC, Hyttel P, Greve T, Boland M. 1997. Nucleus ultrastructure and transcriptional activity of bovine oocytes in preantral and early antral follicles. *Mol Reprod Dev* **46**: 208-215.

Fair T, Hyttel P, Greve T. 1995. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Mol Reprod Dev* **42**: 437-442.

Falco G, Lee SL, Stanghellini I, Bassey UC, Hamatani T, Ko MS. 2007. Zscan4: a novel gene expressed exclusively in late 2-cell embryos and embryonic stem cells. *Dev Biol* **307:** 539-550.

Favetta LA, Madan P, Mastromonaco GF, St John EJ, King WA, Betts DH. 2007. The oxidative stress adaptor p66Shc is required for permanent embryo arrest in vitro. *BMC Dev Biol* **7:** 132.

Fenwick J, Platteau P, Murdoch AP, Herbert M. 2002. Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos in vitro. *Hum Reprod* **17:** 407-412.

Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA, Navarro PA. 2009. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology* **71**: 836-848.

Ferrell JE Jr. 1999. Xenopus oocyte maturation: new lessons from a good egg. *Bioessays* **21:** 833-842.

Fiorenza MT, Bevilacqua A, Canterini S, Torcia S, Pontecorvi M, Mangia F. 2004. Early transcriptional activation of the hsp70.1 gene by osmotic stress in one-cell embryos of the mouse. *Biol Reprod* **70:** 1606-1613.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391:** 806-811.

Flemr M, Ma J, Schultz RM, Svoboda P. 2010. P-body loss is concomitant with formation of a messenger RNA storage domain in mouse oocytes. *Biol Reprod* **82:** 1008-1017.

Flemr M, Malik R, Franke V, Nejepinska J, Sedlacek R, Vlahovicek K, Svoboda P. 2013. A retrotransposon-driven dicer isoform directs endogenous small interfering RNA production in mouse oocytes. *Cell* **155:** 807-816.

Flemr M, Moravec M, Libova V, Sedlacek R, Svoboda P. 2014. Lin28a is dormant, functional, and dispensable during mouse oocyte-to-embryo transition. *Biol Reprod* **90:** 131.

Fojas de Borja P, Collins NK, Du P, Azizkhan-Clifford J, Mudryj M. 2001. Cyclin A-CDK phosphorylates Sp1 and enhances Sp1-mediated transcription. *EMBO J* **20:** 5737-5747.

Forlani S, Bonnerot C, Capgras S, Nicolas JF. 1998. Relief of a repressed gene expression state in the mouse 1-cell embryo requires DNA replication. *Development* **125:** 3153-3166.

Fox CA, Sheets MD, Wickens MP. 1989. Poly(A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by the sequence UUUUUAU. *Genes Dev* **3:** 2151-2162.

Foygel K, Choi B, Jun S, Leong DE, Lee A, Wong CC, Zuo E, Eckart M, Reijo Pera RA, Wong WH, Yao MW. 2008. A novel and critical role for Oct4 as a regulator of the maternal-embryonic transition. *PLoS One* **3:** e4109.

Fukui Y, Sakuma Y. 1980. Maturation of bovine oocytes cultured in vitro: relation to ovarian activity, follicular size and the presence or absence of cumulus cells. *Biol Reprod* **22:** 669-673.

Fulka H, Langerova A. 2014. The maternal nucleolus plays a key role in centromere satellite maintenance during the oocyte to embryo transition. *Development* **141:** 1694-1704.

Fulka J Jr, Motlik J, Fulka J, Jilek F. 1986. Effect of cycloheximide on nuclear maturation of pig and mouse oocytes. *J Reprod Fertil* **77:** 281-285.

Gad A, Hoelker M, Besenfelder U, Havlicek V, Cinar U, Rings F, Held E, Dufort I, Sirard MA, Schellander K, Tesfaye D. 2012. Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. *Biol Reprod* **87:** 100.

Garcia-Castro MI, Anderson R, Heasman J, Wylie C. 1997. Interactions between germ cells and extracellular matrix glycoproteins during migration and gonad assembly in the mouse embryo. *J Cell Biol* **138**: 471-480.

Garg A, Wesolowski D, Alonso D, Deitsch KW, Ben Mamoun C, Altman S. 2015. Targeting protein translation, RNA splicing, and degradation by morpholino-based conjugates in Plasmodium falciparum. *Proc Natl Acad Sci U S A* **112:** 11935-11940.

Ghanem N, Holker M, Rings F, Jennen D, Tholen E, Sirard MA, Torner H, Kanitz W, Schellander K, Tesfaye D. 2007. Alterations in transcript abundance of bovine oocytes recovered at growth and dominance phases of the first follicular wave. *BMC Dev Biol* **7**: 90.

Gilbert I, Scantland S, Sylvestre EL, Gravel C, Laflamme I, Sirard MA, Robert C. 2009. The dynamics of gene products fluctuation during bovine pre-hatching development. *Mol Reprod Dev* **76**: 762-772.

Gilbert SF. 2006. Developmental Biology. 8th ed. Sinauer Associates. Sunderland, MA. 785 pp.

Giorgini F, Davies HG, Braun RE. 2001. MSY2 and MSY4 bind a conserved sequence in the 3' untranslated region of protamine 1 mRNA in vitro and in vivo. *Mol Cell Biol* **21:** 7010-7019.

Glotzer M, Murray AW, Kirschner MW. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature* **349:** 132-138.

Gohin M, Fournier E, Dufort I, Sirard MA. 2014. Discovery, identification and sequence analysis of RNAs selected for very short or long poly A tail in immature bovine oocytes. *Mol Hum Reprod* **20:** 127-138.

Goldstrohm AC, Wickens M. 2008. Multifunctional deadenylase complexes diversify mRNA control. *Nat Rev Mol Cell Biol* **9:** 337-344.

Gonzales DS, Pinheiro JC, Bavister BD. 1995. Prediction of the developmental potential of hamster embryos in vitro by precise timing of the third cell cycle. *J Reprod Fertil* **105:** 1-8.

Gozdecka M, Breitwieser W. 2012. The roles of ATF2 (activating transcription factor 2) in tumorigenesis. *Biochem Soc Trans* **40:** 230-234.

Graf A, Krebs S, Zakhartchenko V, Schwalb B, Blum H, Wolf E. 2014. Fine mapping of genome activation in bovine embryos by RNA sequencing. *Proc Natl Acad Sci U S A* **111:** 4139-4144.

Grossman WJ, Revell PA, Lu ZH, Johnson H, Bredemeyer AJ, Ley TJ. 2003. The orphan granzymes of humans and mice. *Curr Opin Immunol* **15:** 544-552.

Gu W, Tekur S, Reinbold R, Eppig JJ, Choi YC, Zheng JZ, Murray MT, Hecht NB. 1998. Mammalian male and female germ cells express a germ cell-specific Y-Box protein, MSY2. *Biol Reprod* **59:** 1266-1274.

Guo B, Stein JL, van Wijnen AJ, Stein GS. 1997. ATF1 and CREB trans-activate a cell cycle regulated histone H4 gene at a distal nuclear matrix associated promoter element. *Biochemistry* **36:** 14447-14455.

Guzeloglu-Kayisli O, Lalioti MD, Babayev E, Torrealday S, Karakaya C, Seli E. 2014. Human embryonic poly(A)-binding protein (EPAB) alternative splicing is differentially regulated in human oocytes and embryos. *Mol Hum Reprod* **20:** 59-65.

Hai T, Hartman MG. 2001 The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* **273:** 1-11.

Hamamoto G, Suzuki T, Suzuki MG, Aoki F. 2014. Regulation of transketolase like 1 gene expression in the murine one-cell stage embryos. *PLoS One* **9:** e82087.

Hamatani T, Carter MG, Sharov AA, Ko MS. 2004. Dynamics of global gene expression changes during mouse preimplantation development. *Dev Cell* **6:** 117-131.

Hara KT, Oda S, Naito K, Nagata M, Schultz RM, Aoki F. 2005. Cyclin A2-CDK2 regulates embryonic gene activation in 1-cell mouse embryos. *Dev Biol* **286:** 102-113.

Harbour JW, Dean DC. 2000. Chromatin remodeling and Rb activity. *Curr Opin Cell Biol* **12:** 685-689.

Held E, Salilew-Wondim D, Linke M, Zechner U, Rings F, Tesfaye D, Schellander K, Hoelker M. 2012. Transcriptome fingerprint of bovine 2-cell stage blastomeres is directly correlated with the individual developmental competence of the corresponding sister blastomere. *Biol Reprod* **87:** 154.

Henrion G, Brunet A, Renard JP, Duranthon V. 1997. Identification of maternal transcripts that progressively disappear during the cleavage period of rabbit embryos. *Mol Reprod Dev* **47:** 353-362.

Henrique Barreta M, Garziera Gasperin B, Braga Rissi V, de Cesaro MP, Ferreira R, de Oliveira JF, Gonçalves PB, Bordignon V. 2012. Homologous recombination and non-homologous end-joining repair pathways in bovine embryos with different developmental competence. *Exp Cell Res* **318:** 2049-2058.

Heyn P, Kircher M, Dahl A, Kelso J, Tomancak P, Kalinka AT, Neugebauer KM. 2014. The earliest transcribed zygotic genes are short, newly evolved, and different across species. *Cell Rep* **6:** 285-292.

Hinkley C, Perry M. 1992. Histone H2B gene transcription during Xenopus early development requires functional cooperation between proteins bound to the CCAAT and octamer motifs. *Mol Cell Biol* **12:** 4400-4411.

Hirata T, Amano T, Nakatake Y, Amano M, Piao Y, Hoang HG, Ko MS. 2012. Zscan4 transiently reactivates early embryonic genes during the generation of induced pluripotent stem cells. *Sci Rep* **2:** 208.

Huang HF, He RH, Sun CC, Zhang Y, Meng QX, Ma YY. 2006. Function of aquaporins in female and male reproductive systems. *Hum Reprod Update* **12**: 785-795.

Huang J, Zhang H, Wang X, Dobbs KB, Yao J, Qin G, Whitworth K, Walters EM, Prather RS, Zhao J. 2015. Impairment of preimplantation porcine embryo development by histone demethylase KDM5B knockdown through disturbance of bivalent H3K4me3-H3K27me3 modifications. *Biol Reprod* **92:** 72.

Humphreys DT, Westman BJ, Martin DI, Preiss T. 2005. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. *Proc Natl Acad Sci U S A* **102:** 16961-16966.

Hyttel P, Fair T, Callesen H, Greve T. 1997. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology* **47:** 23–32

Hyttel P, Viuff D, Avery B, Laurincik J, Greve T. 1996. Transcription and cell cycle-dependent development of intranuclear bodies and granules in two-cell bovine embryos. *J Reprod Fertil* **108:** 263-270.

Hyttel P, Viuff D, Fair T, Laurincik J, Thomsen PD, Callesen H, Vos PL, Hendriksen PJ, Dieleman SJ, Schellander K et al. 2001. Ribosomal RNA gene expression and chromosome aberrations in bovine oocytes and preimplantation embryos. *Reproduction* **122**: 21-30.

Ivec M, Kovacic B, Vlaisavljevic V. 2011. Prediction of human blastocyst development from morulas with delayed and/or incomplete compaction. *Fertil Steril* **96:** 1473-1478.

Jain ML, Bruice PY, Szabo IE, Bruice TC. 2012. Incorporation of positively charged linkages into DNA and RNA backbones: a novel strategy for antigene and antisense agents. *Chem Rev* **112:** 1284-1309.

Jakobsen AS, Avery B, Dieleman SJ, Knijn HM, Vos PL, Thomsen PD. 2006. Transcription of ribosomal RNA genes is initiated in the third cell cycle of bovine embryos. *Mol Reprod Dev* **73:** 196-205.

Jiang Z, Sun J, Dong H, Luo O, Zheng X, Obergfell C, Tang Y, Bi J, O'Neilll R, Ruan Y, Chen J, Tian XC. 2014. Transcriptional profiles of bovine in vivo pre-implantation development. *BMC Genomics* **15:** 756.

Jin XL, O'Neill C. 2007. cAMP-responsive element-binding protein expression and regulation in the mouse preimplantation embryo. *Reproduction* **134:** 667-675.

Jin XL, O'Neill C. 2010. The presence and activation of two essential transcription factors (cAMP response element-binding protein and cAMP-dependent transcription factor ATF1) in the two-cell mouse embryo. *Biol Reprod* **82:** 459-468.

Jin XL, O'Neilll C. 2011. Regulation of the expression of proto-oncogenes by autocrine embryotropins in the early mouse embryo. *Biol Reprod* **84:** 1216-1224.

Jin XL, O'Neill C. 2014. The regulation of the expression and activation of the essential ATF1 transcription factor in the mouse preimplantation embryo. *Reproduction* **148:** 147-157.

Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT, Tilly JL. 2005. Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* **122**: 303-315.

Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. 2004. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* **428**: 145-150.

Johnson MH, Ziomek CA. 1981. The foundation of two distinct cell lineages within the mouse morula. Cell **24:** 71-80.

Josefsberg LB, Galiani D, Dantes A, Amsterdam A, Dekel N. 2000. The proteasome is involved in the first metaphase-to-anaphase transition of meiosis in rat oocytes. *Biol Reprod* **62:** 1270-1277.

Kageyama S, Gunji W, Nakasato M, Murakami Y, Nagata M, Aoki F. 2007. Analysis of transcription factor expression during oogenesis and preimplantation development in mice. *Zygote* **15:** 117-128.

Kanka J, Kepkova K, Nemcova L. 2009. Gene expression during minor genome activation in preimplantation bovine development. *Theriogenology* **72:** 572-583.

Kashiwagi K, Kashiwagi A, Kurabayashi A, Hanada H, Nakajima K, Okada M, Takase M, Yaoita Y. 2010. Xenopus tropicalis: an ideal experimental animal in amphibia. *Exp Anim* **59:** 395-405.

Kasinathan P, Wei H, Xiang T, Molina JA, Metzger J, Broek D, Kasinathan S, Faber DC, Allan MF. 2015. Acceleration of genetic gain in cattle by reduction of generation interval. *Sci Rep* **5:** 8674.

Kastrop PM, Hulshof SC, Bevers MM, Destrée OH, Kruip TA. 1991. The effects of alpha-amanitin and cycloheximide on nuclear progression, protein synthesis, and phosphorylation during bovine oocyte maturation in vitro. *Mol Reprod Dev* **28:** 249-254.

Katz-Jaffe MG, McCallie BR, Preis KA, Filipovits J, Gardner DK. 2009. Transcriptome analysis of in vivo and in vitro matured bovine MII oocytes. *Theriogenology* **71:** 939-946.

Kawasaki H, Schiltz L, Chiu R, Itakura K, Taira K, Nakatani Y, Yokoyama KK. 2000. ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation. *Nature* **405:** 195-200.

Kepkova KV, Vodicka P, Toralova T, Lopatarova M, Cech S, Dolezel R, Havlicek V, Besenfelder U, Kuzmany A, Sirard MA, Laurincik J, Kanka J. 2011. Transcriptomic analysis of in vivo and in vitro produced bovine embryos revealed a developmental change in cullin 1 expression during maternal-to-embryonic transition. *Theriogenology* **75:** 1582-1595.

Kigami D, Minami N, Takayama H, Imai H. 2003. MuERV-L is one of the earliest transcribed genes in mouse one-cell embryos. *Biol Reprod* **68:** 651-654.

Kim DH, Behlke MA, Rose SD, Chang MS, Choi S, Rossi JJ. 2005. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol* **23:** 222-226.

Knight PG, Glister C. 2003. Local roles of TGF-beta superfamily members in the control of ovarian follicle development. *Anim Reprod Sci* **78**: 165-183.

Knight PG, Glister C. 2006. TGF-beta superfamily members and ovarian follicle development. *Reproduction* **132**: 191-206.

Koehler D, Zakhartchenko V, Froenicke L, Stone G, Stanyon R, Wolf E, Cremer T, Brero A. 2009. Changes of higher order chromatin arrangements during major genome activation in bovine preimplantation embryos. *Exp Cell Res* **315:** 2053-2063.

Kopecny V. 1989. High-resolution autoradiographic studies of comparative nucleologenesis and genome reactivation during early embryogenesis in pig, man and cattle. *Reprod Nutr Dev* **29:** 589-600.

Kravchenko JE, Rogozin IB, Koonin EV, Chumakov PM. 2005. Transcription of mammalian messenger RNAs by a nuclear RNA polymerase of mitochondrial origin. *Nature* **436:** 735-739.

Krishnamurthy S, Hampsey M. 2009. Eukaryotic transcription initiation. *Curr Biol* **19:** R153-156.

Krull CE. 2004. A primer on using in ovo electroporation to analyze gene function. *Dev Dyn* **229:** 433-439.

Krysko DV, Diez-Fraile A, Criel G, Svistunov AA, Vandenabeele P, D'Herde K. 2008. Life and death of female gametes during oogenesis and folliculogenesis. *Apoptosis* **13**: 1065-1087.

Kues WA, Sudheer S, Herrmann D, Carnwath JW, Havlicek V, Besenfelder U, Lehrach H, Adjaye J, Niemann H. 2008. Genome-wide expression profiling reveals distinct

clusters of transcriptional regulation during bovine preimplantation development in vivo. Proc Natl Acad Sci U S A **105:** 19768-19773.

Kurreck J. 2003. Antisense technologies. Improvement through novel chemical modifications. *Eur J Biochem* **270:** 1628-1644.

Labrecque R, Lodde V, Dieci C, Tessaro I, Luciano AM, Sirard MA. 2015. Chromatin remodelling and histone m RNA accumulation in bovine germinal vesicle oocytes. *Mol Reprod Dev* **82:** 450-462.

Labrecque R, Sirard MA. 2014. The study of mammalian oocyte competence by transcriptome analysis: progress and challenges. *Mol Hum Reprod* **20:** 103-116.

Labrecque R, Vigneault C, Blondin P, Sirard MA. 2013. Gene expression analysis of bovine oocytes with high developmental competence obtained from FSH-stimulated animals. *Mol Reprod Dev* **80:** 428-440.

Lane M, Gardner DK. 2000. Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. *Biol Reprod* **62:** 16-22.

Larman MG, Saunders CM, Carroll J, Lai FA, Swann K. 2004. Cell cycle-dependent Ca2+ oscillations in mouse embryos are regulated by nuclear targeting of PLCzeta. *J Cell Sci* **117:** 2513-2521.

Latham KE, Solter D, Schultz RM. 1991. Activation of a two-cell stage-specific gene following transfer of heterologous nuclei into enucleated mouse embryos. *Mol Reprod Dev* **30:** 182-186.

Le Masson F, Christians E. 2011. HSFs and regulation of Hsp70.1 (Hspa1b) in oocytes and preimplantation embryos: new insights brought by transgenic and knockout mouse models. *Cell Stress Chaperones* **16:** 275-285

Leandri RD, Archilla C, Bui LC, Peynot N, Liu Z, Cabau C, Chastellier A, Renard JP, Duranthon V. 2009. Revealing the dynamics of gene expression during embryonic genome activation and first differentiation in the rabbit embryo with a dedicated array screening. *Physiol Genomics* **36:** 98-113.

Lechniak D, Pers-Kamczyc E, Pawlak P. 2008. Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos. *Reprod Biol* **8:** 23-42.

Lechniak D, Switonski M, Sosnowski M. 1996. The incidence of bovine diploid oocytes matured in vitro. *Theriogenology* **46:** 267-277.

Lee KB, Bettegowda A, Wee G, Ireland JJ, Smith GW. 2009. Molecular determinants of oocyte competence: potential functional role for maternal (oocyte-derived) follistatin in promoting bovine early embryogenesis. *Endocrinology* **150:** 2463-2471.

Lee KB, Wee G, Zhang K, Folger JK, Knott JG, Smith GW. 2014b. Functional role of the bovine oocyte-specific protein JY-1 in meiotic maturation, cumulus expansion, and subsequent embryonic development. *Biol Reprod* **90:** 69.

Lee KB, Zhang K, Folger JK, Knott JG, Smith GW. 2014c. Evidence supporting a functional requirement of SMAD4 for bovine preimplantation embryonic development: a potential link to embryotrophic actions of follistatin. *Biol Reprod* **91:** 62.

Lee SR, Xu YN, Jo YJ, Namgoong S, Kim NH. 2015. The Rho-GTPase effector ROCK regulates meiotic maturation of the bovine oocyte via myosin light chain phosphorylation and cofilin phosphorylation. *Mol Reprod Dev* **[in press]**.

Leidenfrost S, Boelhauve M, Reichenbach M, Gungor T, Reichenbach HD, Sinowatz F, Wolf E, Habermann FA. 2011. Cell arrest and cell death in mammalian preimplantation development: lessons from the bovine model. *PLoS One* **6:** e22121.

Lequarre AS, Traverso JM, Marchandise J, Donnay I 2004. Poly(A) RNA is reduced by half during bovine oocyte maturation but increases when meiotic arrest is maintained with CDK inhibitors. Biol Reprod **71:** 425-431.

Levesque JT, Sirard MA. 1996. Resumption of meiosis is initiated by the accumulation of cyclin B in bovine oocytes. *Biol Reprod* **55**: 1427-1436.

Li Y, Lu J, Han Y, Fan X, Ding SW. 2013. RNA interference functions as an antiviral immunity mechanism in mammals. *Science* **342:** 231-234.

Liang XW, Cui XS, Sun SC, Jin YX, Heo YT, Namgoong S, Kim NH. 2013. Superovulation induces defective methylation in line-1 retrotransposon elements in blastocyst. *Reprod Biol Endocrinol* **11:** 69.

Lingenfelter BM, Dailey RA, Inskeep EK, Vernon MW, Poole DH, Rhinehart JD, Yao J. 2007. Changes of maternal transcripts in oocytes from persistent follicles in cattle. *Mol Reprod Dev* **74:** 265-272.

Lingenfelter BM, Tripurani SK, Tejomurtula J, Smith GW, Yao J. 2011. Molecular cloning and expression of bovine nucleoplasmin 2 (NPM2): a maternal effect gene regulated by miR-181a. *Reprod Biol Endocrinol* **9:** 40.

Lodde V, Modina S, Galbusera C, Franciosi F, Luciano AM. 2007. Large-scale chromatin remodeling in germinal vesicle bovine oocytes: interplay with gap junction functionality and developmental competence. *Mol Reprod Dev* **74**: 740-749.

Lodde V, Modina S, Maddox-Hyttel P, Franciosi F, Lauria A, Luciano AM. 2008. Oocyte morphology and transcriptional silencing in relation to chromatin remodeling during the final phases of bovine oocyte growth. *Mol Reprod Dev* **75**: 915-924.

Lonergan P, Fair T, Khatir H, Cesaroni G, Mermillod P. 1998. Effect of protein synthesis inhibition before or during in vitro maturation on subsequent development of bovine oocytes. *Theriogenology* **50:** 417-431.

Lonergan P, Gutierrez-Adan A, Rizos D, Pintado B, de la Fuente J, Boland M. 2003. Relative messenger RNA abundance in bovine oocytes collected in vitro or in vivo before and 20 h after the preovulatory luteinizing hormone surge. *Mol Reprod Dev* **66:** 297-305.

Lowther KM, Mehlmann LM. 2015. Embryonic Poly(A) Binding Protein (EPAB) Is Required During Early Stages of Mouse Oocyte Development for Chromatin Organization, Transcriptional Silencing, and Meiotic Competence. *Biol Reprod* **[in press]**.

Lykke-Andersen K, Gilchrist MJ, Grabarek JB, Das P, Miska E, Zernicka-Goetz M. 2008. Maternal Argonaute 2 is essential for early mouse development at the maternal-zygotic transition. *Mol Biol Cell* **19:** 4383-4392.

Ma J, Flemr M, Stein P, Berninger P, Malik R, Zavolan M, Svoboda P, Schultz RM. 2010. MicroRNA activity is suppressed in mouse oocytes. *Curr Biol* **20:** 265-270.

Ma J, Flemr M, Strnad H, Svoboda P, Schultz RM. 2013. Maternally recruited DCP1A and DCP2 contribute to messenger RNA degradation during oocyte maturation and genome activation in mouse. *Biol Reprod* **88:** 11.

Macaulay AD, Gilbert I, Caballero J, Barreto R, Fournier E, Tossou P, Sirard MA, Clarke HJ, Khandjian EW, Richard FJ, Hyttel P, Robert C. 2014. The gametic synapse: RNA transfer to the bovine oocyte. *Biol Reprod* **91:** 90.

Macaulay AD, Gilbert I, Scantland S, Fournier E, Ashkar F, Bastien A, Shojaei Saadi HA, Gagne D, Sirard MA, Khandjian EW, Richard FJ, Hyttel P, Robert C. 2015. Cumulus cell transcripts transit to the bovine oocyte in preparation for maturation. Biol Reprod **[in press]**.

Macaulay A, Scantland S, Robert C. 2011. RNA Processing During Early Embryogenesis: Managing Storage, Utilisation and Destruction, RNA Processing, Prof. Paula Grabowski (Ed.), ISBN: 978-953-307-557-0, InTech, **DOI: 10.5772/20375**.

Maekawa T, Bernier F, Sato M, Nomura S, Singh M, Inoue Y, Tokunaga T, Imai H, Yokoyama M, Reimold A, Glimcher LH, Ishii S. 1999. Mouse ATF-2 null mutants display features of a severe type of meconium aspiration syndrome. *J Biol Chem* **274:** 17813-17819.

Maillard PV, Ciaudo C, Marchais A, Li Y, Jay F, Ding SW, Voinnet O. 2013. Antiviral RNA interference in mammalian cells. *Science* **342:** 235-238.

Malcuit C, Fissore RA. 2007. Activation of fertilized and nuclear transfer eggs. *Adv Exp Med Biol* **591:** 117-131.

Mamo S, Carter F, Lonergan P, Leal CL, Al Naib A, McGettigan P, Mehta JP, Evans AC, Fair T. 2011. Sequential analysis of global gene expression profiles in immature and in vitro matured bovine oocytes: potential molecular markers of oocyte maturation. *BMC Genomics* **12:** 151.

Manejwala FM, Logan CY, Schultz RM. 1991. Regulation of hsp70 mRNA levels during oocyte maturation and zygotic gene activation in the mouse. *Dev Biol* **144:** 301-308.

Maserati M, Dai X, Walentuk M, Mager J. 2014. Identification of four genes required for mammalian blastocyst formation. *Zygote* **22:** 331-339.

Massicotte L. 2006. *Traduction des ARNM maternels d'ovocytes bovins impliqués dans le développement embryonnaire*. In: *Collection Mémoires et thèses électroniques*, **157 p**. Université Laval, Québec.

Massicotte L, Coenen K, Mourot M, Sirard MA. 2006. Maternal housekeeping proteins translated during bovine oocyte maturation and early embryo development. *Proteomics* **6**: 3811-3820.

Matsumoto K, Tanaka KJ, Aoki K, Sameshima M, Tsujimoto M. 2003. Visualization of the reconstituted FRGY2-mRNA complexes by electron microscopy. *Biochem Biophys Res Commun* **306:** 53-58.

McCoy RC, Demko Z, Ryan A, Banjevic M, Hill M, Sigurjonsson S, Rabinowitz M, Fraser HB, Petrov DA. 2015. Common variants spanning PLK4 are associated with mitotic-origin aneuploidy in human embryos. *Science* **348:** 235-238.

McGrew LL, Dworkin-Rastl E, Dworkin MB, Richter JD. 1989. Poly(A) elongation during Xenopus oocyte maturation is required for translational recruitment and is mediated by a short sequence element. *Genes Dev* **3:** 803-815.

McLaren A. 2001. Mammalian germ cells: birth, sex, and immortality. *Cell Struct Funct* **26**: 119-122.

McLaren A, Lawson KA. 2005. How is the mouse germ-cell lineage established? *Differentiation* **73**: 435-437.

McLay DW, Clarke HJ. 2003. Remodelling the paternal chromatin at fertilization in mammals. *Reproduction* **125**: 625-633.

Medvedev S, Pan H, Schultz RM. 2011. Absence of MSY2 in mouse oocytes perturbs oocyte growth and maturation, RNA stability, and the transcriptome. *Biol Reprod* **85:** 575-583.

Meldi L, Brickner JH. 2011. Compartmentalization of the nucleus. *Trends Cell Biol* **21:** 701-708.

Memili E, Dominko T, First NL. 1998. Onset of transcription in bovine oocytes and preimplantation embryos. *Mol Reprod Dev* **51:** 36-41.

Memili E, First NL. 1998. Developmental changes in RNA polymerase II in bovine oocytes, early embryos, and effect of alpha-amanitin on embryo development. *Mol Reprod Dev* **51**: 381-389.

Memili E, First NL. 1999. Control of gene expression at the onset of bovine embryonic development. *Biol Reprod* **61:** 1198-1207.

Memili E, First NL. 2000. Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. *Zygote* **8:** 87-96.

Menezo Y Jr, Russo G, Tosti E, El Mouatassim S, Benkhalifa M. 2007. Expression profile of genes coding for DNA repair in human oocytes using pangenomic microarrays, with a special focus on ROS linked decays. *J Assist Reprod Genet* **24:** 513-520.

Mermillod P, Dalbies-Tran R, Uzbekova S, Thelie A, Traverso JM, Perreau C, Papillier P, Monget P. 2008. Factors affecting oocyte quality: who is driving the follicle? *Reprod Domest Anim* **43 Suppl 2**: 393-400.

Minami N, Suzuki T, Tsukamoto S. 2007. Zygotic gene activation and maternal factors in mammals. *J Reprod Dev* **53:** 707-715.

Misirlioglu M, Page GP, Sagirkaya H, Kaya A, Parrish JJ, First NL, Memili E. 2006. Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos. *Proc Natl Acad Sci U S A* **103:** 18905-18910.

Mondou E, Dufort I, Gohin M, Fournier E, Sirard MA. 2012. Analysis of microRNAs and their precursors in bovine early embryonic development. *Mol Hum Reprod* **18:** 425-434.

Moraes KC, Wilusz CJ, Wilusz J. 2006. CUG-BP binds to RNA substrates and recruits PARN deadenylase. RNA **12:** 1084-1091.

Morrish BC, Sinclair AH. 2002. Vertebrate sex determination: many means to an end. *Reproduction* **124**: 447-457.

Motlik J, Crozet N, Fulka J. 1984. Meiotic competence in vitro of pig oocytes isolated from early antral follicles. *J Reprod Fertil* **72**: 323-328.

Mourot M, Dufort I, Gravel C, Algriany O, Dieleman S, Sirard MA. 2006. The influence of follicle size, FSH-enriched maturation medium, and early cleavage on bovine oocyte maternal mRNA levels. *Mol Reprod Dev* **73**: 1367-1379.

Murchison EP, Stein P, Xuan Z, Pan H, Zhang MQ, Schultz RM, Hannon GJ. 2007. Critical roles for Dicer in the female germline. *Genes Dev* **21:** 682-693.

Nakahata S, Kotani T, Mita K, Kawasaki T, Katsu Y, Nagahama Y, Yamashita M. 2003. Involvement of Xenopus Pumilio in the translational regulation that is specific to cyclin B1 mRNA during oocyte maturation. *Mech Dev* **120:** 865-880.

Nganvongpanit K, Muller H, Rings F, Gilles M, Jennen D, Holker M, Tholen E, Schellander K, Tesfaye D. 2006a Targeted suppression of E-cadherin gene expression in bovine preimplantation embryo by RNA interference technology using double-stranded RNA. *Mol Reprod Dev* **73:** 153-163.

Nganvongpanit K, Muller H, Rings F, Hoelker M, Jennen D, Tholen E, Havlicek V, Besenfelder U, Schellander K, Tesfaye D. 2006b Selective degradation of maternal and embryonic transcripts in in vitro produced bovine oocytes and embryos using sequence specific double-stranded RNA. *Reproduction* **131:** 861-874.

Nilsson EE, Skinner MK. 2009. Progesterone regulation of primordial follicle assembly in bovine fetal ovaries. *Mol Cell Endocrinol* **313**: 9-16.

Nishikawa S, Hatanaka Y, Tokoro M, Shin SW, Shimizu N, Nishihara T, Kato R, Takemoto A, Amano T, Anzai M, Kishigami S, Hosoi Y, Matsumoto K. 2013. Functional analysis of nocturnin, a circadian deadenylase, at maternal-to-zygotic transition in mice. *J Reprod Dev* **59:** 258-265.

Newport J, Kirschner M. 1982a. A major developmental transition in early Xenopus embryos: I. characterization and timing of cellular changes at the midblastula stage. *Cell* **30:** 675-686.

Newport J, Kirschner M. 1982. A major developmental transition in early Xenopus embryos: II. Control of the onset of transcription. *Cell* **30:** 687-696.

Oh B, Hwang S, McLaughlin J, Solter D, Knowles BB. 2000. Timely translation during the mouse oocyte-to-embryo transition. *Development* **127:** 3795-3803.

Ohinata Y, Payer B, O'Carroll D, Ancelin K, Ono Y, Sano M, Barton SC, Obukhanych T, Nussenzweig M, Tarakhovsky A et al. 2005. Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* **436**: 207-213.

O'Meara CM, Murray JD, Mamo S, Gallagher E, Roche J, Lonergan P. 2011. Gene silencing in bovine zygotes: siRNA transfection versus microinjection. *Reprod Fertil Dev* **23:** 534-543.

O'Neill C, Li Y, Jin XL. 2012. Survival signaling in the preimplantation embryo. *Theriogenology* **77:** 773-784.

Ooga M, Suzuki MG, Aoki F. 2015. Involvement of histone H2B monoubiquitination in the regulation of mouse preimplantation development. *J Reprod Dev* **61:** 179-184.

Opiela J, Katska-Ksiazkiewicz L, Lipinski D, Slomski R, Bzowska M, Rynska B. 2008. Interactions among activity of glucose-6-phosphate dehydrogenase in immature oocytes, expression of apoptosis-related genes Bcl-2 and Bax, and developmental competence following IVP in cattle. *Theriogenology* **69**: 546-555.

Opiela J, Lipinski D, Slomski R, Katska-Ksiazkiewicz L. 2010. Transcript expression of mitochondria related genes is correlated with bovine oocyte selection by BCB test. *Anim Reprod Sci* **118:** 188-193.

Orisaka M, Tajima K, Tsang BK, Kotsuji F. 2009. Oocyte-granulosa-theca cell interactions during preantral follicular development. *J Ovarian Res* **2:** 9.

Otoi T, Yamamoto K, Koyama N, Tachikawa S, Suzuki T. 1997. Bovine oocyte diameter in relation to developmental competence. *Theriogenology* **48**: 769-774.

Ozturk S, Yaba-Ucar A, Sozen B, Mutlu D, Demir N. 2014. Superovulation alters embryonic poly(A)-binding protein (Epab) and poly(A)-binding protein, cytoplasmic 1 (Pabpc1) gene expression in mouse oocytes and early embryos. *Reprod Fertil Dev* **[in press]**.

Pang RT, Liu WM, Leung CO, Ye TM, Kwan PC, Lee KF, Yeung WS. 2011. miR-135A regulates preimplantation embryo development through down-regulation of E3 Ubiquitin Ligase Seven In Absentia Homolog 1A (SIAH1A) expression. *PLoS One* **6:** e27878.

Paradis F, Vigneault C, Robert C, Sirard MA. 2005. RNA interference as a tool to study gene function in bovine oocytes. *Mol Reprod Dev* **70**: 111-121.

Park SK, Murphy S. 1996. Nitric oxide synthase type II mRNA stability is translation- and transcription-dependent. *J Neurochem* **67:** 1766-1769.

Parker R, Song H. 2004. The enzymes and control of eukaryotic mRNA turnover. Nat *Struct Mol Biol* **11:** 121-127.

Paynton BV, Rempel R, Bachvarova R. 1988. Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. *Dev Biol* **129:** 304-314.

Patel OV, Bettegowda A, Ireland JJ, Coussens PM, Lonergan P, Smith GW. 2007. Functional genomics studies of oocyte competence: evidence that reduced transcript

abundance for follistatin is associated with poor developmental competence of bovine oocytes. *Reproduction* **133**: 95-106.

Pennetier S, Uzbekova S, Guyader-Joly C, Humblot P, Mermillod P, Dalbies-Tran R. 2005. Genes preferentially expressed in bovine oocytes revealed by subtractive and suppressive hybridization. *Biol Reprod* **73:** 713-720.

Pennetier S, Uzbekova S, Perreau C, Papillier P, Mermillod P, Dalbies-Tran R. 2004. Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15, and VASA in adult bovine tissues, oocytes, and preimplantation embryos. *Biol Reprod* **71**: 1359-1366.

Pers-Kamczyc E, Pawlak P, Rubes J, Lechniak D. 2012. Early cleaved bovine embryos show reduced incidence of chromosomal aberrations and higher developmental potential on day 4.5 post-insemination. *Reprod Domest Anim* **47:** 899-906.

Pichugin A, Le Bourhis D, Adenot P, Lehmann G, Audouard C, Renard JP, Vignon X, Beaujean N. 2010. Dynamics of constitutive heterochromatin: two contrasted kinetics of genome restructuring in early cloned bovine embryos. *Reproduction* **139:** 129-137.

Pillai RS. 2005. MicroRNA function: multiple mechanisms for a tiny RNA? *RNA* **11:** 1753-1761.

Pique M, Lopez JM, Foissac S, Guigo R, Mendez R. 2008. A combinatorial code for CPE-mediated translational control. *Cell* **132:** 434-448.

Plasterk RH. 2006. Micro RNAs in animal development. *Cell* **124:** 877-881.

Popken J, Graf A, Krebs S, Blum H, Schmid VJ, Strauss A, Guengoer T, Zakhartchenko V, Wolf E, Cremer T. 2015. Remodeling of the Nuclear Envelope and Lamina during Bovine Preimplantation Development and Its Functional Implications. *PLoS One* **10:** e0124619.

Potireddy S, Midic U, Liang CG, Obradovic Z, Latham KE. 2010. Positive and negative cis-regulatory elements directing postfertilization maternal mRNA translational control in mouse embryos. *Am J Physiol Cell Physiol* **299:** C818-27.

Poueymirou WT, Schultz RM. 1989. Regulation of mouse preimplantation development: inhibition of synthesis of proteins in the two-cell embryo that require transcription by inhibitors of cAMP-dependent protein kinase. *Dev Biol* **133:** 588-599.

Probst AV, Okamoto I, Casanova M, El Marjou F, Le Baccon P, Almouzni G. 2010. A strand-specific burst in transcription of pericentric satellites is required for chromocenter formation and early mouse development. *Dev Cell* **19:** 625-638.

Racedo SE, Wrenzycki C, Herrmann D, Salamone D, Niemann H. 2008. Effects of follicle size and stages of maturation on mRNA expression in bovine in vitro matured oocytes. *Mol Reprod Dev* **75:** 17-25.

Rajhans R, Kumar GS, Dubey PK, Sharma GT. 2010. Effect of timing of development on total cell number and expression profile of HSP-70.1 and GLUT-1 in buffalo (Bubalus bubalis) oocytes and preimplantation embryos produced in vitro. *Cell Biol Int* **34:** 463-468.

Reyes JM, Chitwood JL, Ross PJ. 2015. RNA-Seq profiling of single bovine oocyte transcript abundance and its modulation by cytoplasmic polyadenylation. *Mol Reprod Dev* **82:** 103-114.

Richard FJ. 2007. Regulation of meiotic maturation. *J Anim Sci* **85**: E4-6.

Richter JD. 1999. Cytoplasmic polyadenylation in development and beyond. *Microbiol Mol Biol Rev* **63:** 446-456.

Robert C, Barnes FL, Hue I, Sirard MA. 2000. Subtractive hybridization used to identify mRNA associated with the maturation of bovine oocytes. *Mol Reprod Dev* **57:** 167-175.

Robert C, Hue I, McGraw S, Gagne D, Sirard MA. 2002. Quantification of cyclin B1 and p34(cdc2) in bovine cumulus-oocyte complexes and expression mapping of genes involved in the cell cycle by complementary DNA macroarrays. *Biol Reprod* **67:** 1456-1464.

Russo GL, Bilotto S, Ciarcia G, Tosti E. 2009. Phylogenetic conservation of cytostatic factor related genes in the ascidian Ciona intestinalis. *Gene* **429:** 104-111.

Sagata N, Oskarsson M, Copeland T, Brumbaugh J, Vande Woude GF. 1988. Function of c-mos proto-oncogene product in meiotic maturation in Xenopus oocytes. *Nature* **335:** 519-525.

Sagata N, Watanabe N, Vande Woude GF, Ikawa Y. 1989. The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature* **342:** 512-518.

Saitou M, Barton SC, Surani MA. 2002. A molecular programme for the specification of germ cell fate in mice. *Nature* **418**: 293-300.

Saitou M, Payer B, O'Carroll D, Ohinata Y, Surani MA. 2005. Blimp1 and the emergence of the germ line during development in the mouse. *Cell Cycle* **4**: 1736-1740.

Salilew-Wondim D, Holker M, Rings F, Phatsara C, Mohammadi-Sangcheshmeh A, Tholen E, Schellander K, Tesfaye D. 2010 Depletion of BIRC6 leads to retarded

bovine early embryonic development and blastocyst formation in vitro. *Reprod Fertil Dev* **22:** 564-579.

Sebestova J, Danylevska A, Novakova L, Kubelka M, Anger M. 2012. Lack of response to unaligned chromosomes in mammalian female gametes. *Cell Cycle* **11:** 3011-3018.

Schellander K, Hoelker M, Tesfaye D. 2007. Selective degradation of transcripts in mammalian oocytes and embryos. *Theriogenology* **68 Suppl 1:** S107-115.

Schier AF. 2007. The maternal-zygotic transition: death and birth of RNAs. *Science* **316:** 406-407.

Schultz RM. 1993. Regulation of zygotic gene activation in the mouse. *Bioessays* **15:** 531-538.

Schultz RM. 2002. The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. *Hum Reprod Update* **8:** 323-331.

Schwartz DA, Schultz RM. 1992. Zygotic gene activation in the mouse embryo: involvement of cyclic adenosine monophosphate-dependent protein kinase and appearance of an AP-1-like activity. *Mol Reprod Dev* **32:** 209-216.

Senbon S, Hirao Y, Miyano T. 2003. Interactions between the oocyte and surrounding somatic cells in follicular development: lessons from in vitro culture. *J Reprod Dev* **49**: 259-269.

Shanware NP, Zhan L, Hutchinson JA, Kim SH, Williams LM, Tibbetts RS. 2010. Conserved and distinct modes of CREB/ATF transcription factor regulation by PP2A/B56gamma and genotoxic stress. *PLoS One* **5:** e12173.

Shi K, Yuan X, Du L, Pan D, Zhang Y, Zhao Y, Deng X, Hu X, Wu C, Li N. 2007. Advanced methods of isolation and identification of porcine primordial follicles. *Anim Reprod Sci* **101:** 163-171.

Siddall LS, Barcroft LC, Watson AJ. 2002. Targeting gene expression in the preimplantation mouse embryo using morpholino antisense oligonucleotides. *Mol Reprod Dev* **63:** 413-421.

Simon R, Richter JD. 1994. Further analysis of cytoplasmic polyadenylation in Xenopus embryos and identification of embryonic cytoplasmic polyadenylation element-binding proteins. *Mol Cell Biol* **14:** 7867-7875.

Sirard MA. 2001. Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology* **55**: 1241-1254.

Sirard MA. 2010. Activation of the embryonic genome. *Soc Reprod Fertil Suppl* **67:** 145-158.

Sirard MA. 2012. Factors affecting oocyte and embryo transcriptomes. *Reprod Domest Anim* **47:** 148-155.

Sirard MA, Florman HM, Leibfried-Rutledge ML, Barnes FL, Sims ML, First NL. 1989. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. *Biol Reprod* **40:** 1257-1263.

Sirard MA, Parrish JJ, Ware CB, Leibfried-Rutledge ML, First NL. 1988. The culture of bovine oocytes to obtain developmentally competent embryos. *Biol Reprod* **39:** 546-552.

Sirard MA, Picard L, Dery M, Coenen K, Blondin P. 1999. The time interval between FSH administration and ovarian aspiration influences the development of cattle oocytes. *Theriogenology* **51**: 699-708.

Sirard MA, Richard F, Blondin P, Robert C. 2006. Contribution of the oocyte to embryo quality. *Theriogenology* **65**: 126-136.

Sirard MA, Richard F, Mayes M. 1998. Controlling meiotic resumption in bovine oocytes: a review. *Theriogenology* **49**: 483-497.

Soloy E, Srsen V, Pavlok A, Hyttel P, Thomsen PD, Smith SD, Prochazka R, Kubelka M, Hoier R, Booth P, Motlík J, Greve T. 1997. Establishment of the block against sperm penetration in parthenogenetically activated bovine oocytes matured in vitro. *J Reprod Fertil* **111:** 151-157.

Sommerville J, Ladomery M. 1996. Transcription and masking of mRNA in germ cells: involvement of Y-box proteins. *Chromosoma* **104:** 469-478.

Son DS, Arai KY, Roby KF, Terranova PF. 2004. Tumor necrosis factor alpha (TNF) increases granulosa cell proliferation: dependence on c-Jun and TNF receptor type 1. *Endocrinology* **145:** 1218-1226.

Sonehara H, Nagata M, Aoki F. 2008. Roles of the first and second round of DNA replication in the regulation of zygotic gene activation in mice. *J Reprod Dev* **54:** 381-384

Song JL, Wessel GM. 2005. How to make an egg: transcriptional regulation in oocytes. *Differentiation* **73**: 1-17.

Stebbins-Boaz B, Hake LE, Richter JD. 1996. CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and c-mos mRNAs and is necessary for oocyte maturation in Xenopus. *EMBO J* **15:** 2582-2592.

Stein P, Zeng F, Pan H, Schultz RM. 2005. Absence of non-specific effects of RNA interference triggered by long double-stranded RNA in mouse oocytes. *Dev Biol* **286:** 464-471.

Suh N, Baehner L, Moltzahn F, Melton C, Shenoy A, Chen J, Blelloch R. 2010. MicroRNA function is globally suppressed in mouse oocytes and early embryos. *Curr Biol* **20:** 271-277.

Suh N, Blelloch R. 2011. Small RNAs in early mammalian development: from gametes to gastrulation. *Development* **138:** 1653-1661.

Summerton JE. 2005. Endo-Porter: a novel reagent for safe, effective delivery of substances into cells. *Ann N Y Acad Sci* **1058:** 62-75.

Summerton JE. 2007. Morpholino, siRNA, and S-DNA compared: impact of structure and mechanism of action on off-target effects and sequence specificity. *Curr Top Med Chem* **7:** 651-660.

Suzuki S, Nozawa Y, Tsukamoto S, Kaneko T, Manabe I, Imai H, Minami N. 2015. CHD1 acts via the Hmgpi pathway to regulate mouse early embryogenesis. *Development* **142:** 2375-2384.

Svarcova O, Laurincik J, Avery B, Mlyncek M, Niemann H, Maddox-Hyttel P. 2007. Nucleolar development and allocation of key nucleolar proteins require de novo transcription in bovine embryos. *Mol Reprod Dev* **74:** 1428-1435.

Svoboda P. 2008. RNA silencing in mammalian oocytes and early embryos. *Curr Top Microbiol Immunol* **320:** 225-256.

Svoboda P. 2010. Why mouse oocytes and early embryos ignore miRNAs? *RNA Biol* **7:** 559-563.

Svoboda P. 2014. Renaissance of mammalian endogenous RNAi. *FEBS Lett* **588:** 2550-2556.

Svoboda P, Flemr M. 2010. The role of miRNAs and endogenous siRNAs in maternal-to-zygotic reprogramming and the establishment of pluripotency. *EMBO Rep* **11** 590-597.

Svoboda P, Stein P, Anger M, Bernstein E, Hannon GJ, Schultz RM. 2004. RNAi and expression of retrotransposons MuERV-L and IAP in preimplantation mouse embryos. *Dev Biol* **269:** 276-285.

Svoboda P, Stein P, Hayashi H, Schultz RM. 2000. Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development* **127:** 4147-4156.

Swain A. 2006. Sex determination: time for meiosis? The gonad decides. *Curr Biol* **16**: R507-509.

Tadros W, Lipshitz HD. 2009. The maternal-to-zygotic transition: a play in two acts. *Development* **136:** 3033-3042.

Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126:** 663-676.

Tam OH, Aravin AA, Stein P, Girard A, Murchison EP, Cheloufi S, Hodges E, Anger M, Sachidanandam R, Schultz RM, Hannon GJ. 2008. Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. *Nature* **453:** 534-538.

Tang F, Kaneda M, O'Carroll D, Hajkova P, Barton SC, Sun YA, Lee C, Tarakhovsky A, Lao K, Surani MA. 2007. Maternal microRNAs are essential for mouse zygotic development. *Genes Dev* **21:** 644-648.

Teixeira D, Sheth U, Valencia-Sanchez MA, Brengues M, Parker R. 2005. Processing bodies require RNA for assembly and contain nontranslating mRNAs. *RNA* **11:** 371-382.

Tejomurtula J, Lee KB, Tripurani SK, Smith GW, Yao J. 2009. Role of importin alpha8, a new member of the importin alpha family of nuclear transport proteins, in early embryonic development in cattle. *Biol Reprod* **81:** 333-342.

Tekur S, Pawlak A, Guellaen G, Hecht NB. 1999. Contrin, the human homologue of a germ-cell Y-box-binding protein: cloning, expression, and chromosomal localization. *J Androl* **20:** 135-144.

Telfer EE, Gosden RG, Byskov AG, Spears N, Albertini D, Andersen CY, Anderson R, Braw-Tal R, Clarke H, Gougeon A, McLaughlin E, McLaren A, McNatty K, Schatten G, Silber S, Tsafriri A. 2005. On regenerating the ovary and generating controversy. *Cell* **122**: 821-822.

Tesfaye D, Lonergan P, Hoelker M, Rings F, Nganvongpanit K, Havlicek V, Besenfelder U, Jennen D, Tholen E, Schellander K. 2007. Suppression of connexin 43 and E-cadherin transcripts in in vitro derived bovine embryos following culture in vitro or in vivo in the homologous bovine oviduct. *Mol Reprod Dev* **74:** 978-988.

Tesfaye D, Regassa A, Rings F, Ghanem N, Phatsara C, Tholen E, Herwig R, Un C, Schellander K, Hoelker M. 2010. Suppression of the transcription factor MSX1 gene delays bovine preimplantation embryo development in vitro. *Reproduction* **139**: 857-870.

Tesfaye D, Worku D, Rings F, Phatsara C, Tholen E, Schellander K, Hoelker M. 2009. Identification and expression profiling of microRNAs during bovine oocyte maturation using heterologous approach. *Mol Reprod Dev* **76:** 665-677.

Thelie A, Papillier P, Pennetier S, Perreau C, Traverso JM, Uzbekova S, Mermillod P, Joly C, Humblot P, Dalbies-Tran R. 2007. Differential regulation of abundance and

deadenylation of maternal transcripts during bovine oocyte maturation in vitro and in vivo. *BMC Dev Biol* **7:** 125.

Thelie A, Papillier P, Perreau C, Uzbekova S, Hennequet-Antier C, Dalbies-Tran R. 2009. Regulation of bovine oocyte-specific transcripts during in vitro oocyte maturation and after maternal-embryonic transition analyzed using a transcriptomic approach. *Mol Reprod Dev* **76:** 773-82.

Titus S, Li F, Stobezki R, Akula K, Unsal E, Jeong K, Dickler M, Robson M, Moy F, Goswami S, Oktay K. 2013. Impairment of BRCA1-related DNA double-strand break repair leads to ovarian aging in mice and humans. *Sci Transl Med* **5:** 172ra21.

Tomek W, Torner H, Kanitz W. 2002. Comparative analysis of protein synthesis, transcription and cytoplasmic polyadenylation of mRNA during maturation of bovine oocytes in vitro. *Reprod Domest Anim* **37:** 86-91.

Tomek W, Wollenhaupt K. 2012. The "closed loop model" in controlling mRNA translation during development. *Anim Reprod Sci* **134:** 2-8.

Topper EK, Kruijt L, Calvete J, Mann K, Topfer-Petersen E, Woelders H. 1997. Identification of bovine zona pellucida glycoproteins. *Mol Reprod Dev* **46**: 344-350.

Toralova T, Benesova V, Kepkova KV, Vodicka P, Susor A, Kanka J. 2012. Bovine preimplantation embryos with silenced nucleophosmin mRNA are able to develop until the blastocyst stage. *Reproduction* **144:** 349-359.

Toralova T, Susor A, Nemcova L, Kepkova K, Kanka J. 2009. Silencing CENPF in bovine preimplantation embryo induces arrest at 8-cell stage. *Reproduction* **138:** 783-791.

Torner H, Ghanem N, Ambros C, Holker M, Tomek W, Phatsara C, Alm H, Sirard MA, Kanitz W, Schellander K, Tesfaye D. 2008. Molecular and subcellular characterisation of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity. *Reproduction **135:*** 197-212.

Tremblay K, Vigneault C, McGraw S, Sirard MA. 2005. Expression of cyclin B1 messenger RNA isoforms and initiation of cytoplasmic polyadenylation in the bovine oocyte. *Biol Reprod* **72**: 1037-1044.

Tripurani SK, Lee KB, Wang L, Wee G, Smith GW, Lee YS, Latham KE, Yao J. 2011a. A novel functional role for the oocyte-specific transcription factor newborn ovary homeobox (NOBOX) during early embryonic development in cattle. *Endocrinology* **152**: 1013-1023.

Tripurani SK, Lee KB, Wee G, Smith GW, Yao J. 2011b. MicroRNA-196a regulates bovine newborn ovary homeobox gene (NOBOX) expression during early embryogenesis. *BMC Dev Biol* **11:** 25.

Tripurani SK, Wee G, Lee KB, Smith GW, Wang L, Jianboyao. 2013. MicroRNA-212 post-transcriptionally regulates oocyte-specific basic-helix-loop-helix transcription factor, factor in the germline alpha (FIGLA), during bovine early embryogenesis. *PLoS One* **8:** e76114.

Tsai TC, Lin W, Yang SH, Cheng WT, Cheng EH, Lee MS, Chong KY, Chen CM. 2010. Granzyme G is expressed in the two-cell stage mouse embryo and is required for the maternal-zygotic transition. *BMC Dev Biol* **10:** 88.

Tsukamoto S, Kuma A, Mizushima N. 2008. The role of autophagy during the oocyte-to-embryo transition. *Autophagy* **4:** 1076-1078.

Ushijima H, Akiyama K, Tajima T. 2008. Transition of cell numbers in bovine preimplantation embryos: in vivo collected and in vitro produced embryos. *J Reprod Dev* **54:** 239-243.

Ushijima H, Akiyama K, Tajima T. 2009. Classification of morphological changes based on the number of cleavage divisions in bovine embryos. *J Reprod Dev* **55:** 83-87.

Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. 2006. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev* **20:** 515-524.

Vallee M, Aiba K, Piao Y, Palin MF, Ko MS, Sirard MA. 2008. Comparative analysis of oocyte transcript profiles reveals a high degree of conservation among species. *Reproduction* **135:** 439-448.

Vallee M, Dufort I, Desrosiers S, Labbe A, Gravel C, Gilbert I, Robert C, Sirard MA. 2009. Revealing the bovine embryo transcript profiles during early in vivo embryonic development. *Reproduction* **138**: 95-105.

Vallee M, Gravel C, Palin MF, Reghenas H, Stothard P, Wishart DS, Sirard MA. 2005. Identification of novel and known oocyte-specific genes using complementary DNA subtraction and microarray analysis in three different species. *Biol Reprod* **73:** 63-71.

Vallee M, Robert C, Methot S, Palin MF, Sirard MA. 2006. Cross-species hybridizations on a multi-species cDNA microarray to identify evolutionarily conserved genes expressed in oocytes. *BMC Genomics* **7:** 113.

Van Soom A, Ysebaert MT, de Kruif A. 1997. Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos. *Mol Reprod Dev* **47:** 47-56.

van Wezel IL, Rodgers RJ. 1996. Morphological characterization of bovine primordial follicles and their environment in vivo. *Biol Reprod* **55**: 1003-1011.

Vanderhyden B. 2002. Molecular basis of ovarian development and function. *Front Biosci* **7:** d2006-2022.

Ventura JJ, Kennedy NJ, Lamb JA, Flavell RA, Davis RJ. 2003. c-Jun NH(2)-terminal kinase is essential for the regulation of AP-1 by tumor necrosis factor. *Mol Cell Biol* **23:** 2871-2882.

Vigneault C. 2008. *Étude de l'activatioin de la transcription chez le jeune embryon bovin*. In: *Collection Mémoires et thèses électroniques*, **240 p**. Université Laval, Québec.

Vigneault C, Gravel C, Vallee M, McGraw S, Sirard MA. 2009a. Unveiling the bovine embryo transcriptome during the maternal-to-embryonic transition. *Reproduction* **137:** 1245-1257.

Vigneault C, McGraw S, Massicotte L, Sirard MA. 2004. Transcription factor expression patterns in bovine in vitro-derived embryos prior to maternal-zygotic transition. *Biol Reprod* **70:** 1701-1709.

Vigneault C, McGraw S, Sirard MA. 2009b. Spatiotemporal expression of transcriptional regulators in concert with the maternal-to-embryonic transition during bovine in vitro embryogenesis. *Reproduction* **137:** 13-21.

Vigneron C, Perreau C, Dalbies-Tran R, Joly C, Humblot P, Uzbekova S, Mermillod P. 2004. Protein synthesis and mRNA storage in cattle oocytes maintained under meiotic block by roscovitine inhibition of MPF activity. *Mol Reprod Dev* **69:** 457-465.

Viuff D, Greve T, Avery B, Hyttel P, Brockhoff PB, Thomsen PD. 2000. Chromosome aberrations in in vitro-produced bovine embryos at days 2-5 post-insemination. *Biol Reprod* **63:** 1143-1148.

Villamediana P, Vidal F, Paramio MT. 2001. Cytogenetic analysis of caprine 2- to 4-cell embryos produced in vitro. *Zygote* **9:** 193-199.

Villarreal XC, Richter JD. 1995. Analysis of ATF2 gene expression during early Xenopus laevis development. *Gene* **153:** 225-229.

Viuff D, Avery B, Greve T, King WA, Hyttel P. 1996. Transcriptional activity in in vitro produced bovine two- and four-cell embryos. *Mol Reprod Dev* **43:** 171-179.

Vlahopoulos SA, Logotheti S, Mikas D, Giarika A, Gorgoulis V, Zoumpourlis V. 2008. The role of ATF-2 in oncogenesis. *Bioessays* **30:** 314-327.

Vogel G. 2005. Reproductive biology. Controversial study finds an unexpected source of oocytes. *Science* **309**: 678-679.

Vogt EJ, Meglicki M, Hartung KI, Borsuk E, Behr R. 2012. Importance of the pluripotency factor LIN28 in the mammalian nucleolus during early embryonic development. *Development* **139:** 4514-4523.

Von Stetina JR, Orr-Weaver TL. 2011. Developmental control of oocyte maturation and egg activation in metazoan models. *Cold Spring Harb Perspect Biol* **3**: a005553.

Wada T, Hara M, Taneda T, Qingfu C, Takata R, Moro K, Takeda K, Kishimoto T, Handa H. 2012. Antisense morpholino targeting just upstream from a poly(A) tail junction of maternal mRNA removes the tail and inhibits translation. *Nucleic Acids Res* **40:** e173.

Wang LY, Wang DH, Zou XY, Xu CM. 2009. Mitochondrial functions on oocytes and preimplantation embryos. *J Zhejiang Univ Sci B* **10**: 483-492.

Wang Q, Latham KE. 1997. Requirement for protein synthesis during embryonic genome activation in mice. *Mol Reprod Dev* **47:** 265-270.

Wang Q, Latham KE. 2000. Translation of maternal messenger ribonucleic acids encoding transcription factors during genome activation in early mouse embryos. *Biol Reprod* **62:** 969-978.

Warner CM, Panda P, Almquist CD, Xu Y. 1993. Preferential survival of mice expressing the Qa-2 antigen. *J Reprod Fertil* **99:** 145-147.

Watanabe T, Imai H, Minami N. 2008. Identification and expression analysis of small RNAs during development. *Methods Mol Biol* **442:** 173-185.

Wei Y, Multi S, Yang CR, Ma J, Zhang QH, Wang ZB, Li M, Wei L, Ge ZJ, Zhang CH, Ouyang YC, Hou Y, Schatten H, Sun QY. 2011. Spindle assembly checkpoint regulates mitotic cell cycle progression during preimplantation embryo development. *PLoS One* **6:** e21557.

Wolf F, Sigl R, Geley S. 2007. '... The end of the beginning': cdk1 thresholds and exit from mitosis. *Cell Cycle* **6:** 1408-1411.

Worrad DM, Ram PT, Schultz RM. 1994. Regulation of gene expression in the mouse oocyte and early preimplantation embryo: developmental changes in Sp1 and TATA box-binding protein, TBP. *Development* **120:** 2347-2357.

Wrenzycki C, Herrmann D, Niemann H. 2007. Messenger RNA in oocytes and embryos in relation to embryo viability. *Theriogenology* **68 Suppl 1**: S77-83.

Wrobel KH, Suss F. 1998. Identification and temporospatial distribution of bovine primordial germ cells prior to gonadal sexual differentiation. *Anat Embryol (Berl)* **197**: 451-467.

Wu B, Ignotz G, Currie WB, Yang X. 1997. Dynamics of maturation-promoting factor and its constituent proteins during in vitro maturation of bovine oocytes. *Biol Reprod* **56**: 253-259.

Xia M, He H, Wang Y, Liu M, Zhou T, Lin M, Zhou Z, Huo R, Zhou Q, Sha J. 2012. PCBP1 is required for maintenance of the transcriptionally silent state in fully grown mouse oocytes. *Cell Cycle* **11:** 2833-2842.

Yamamoto A, Mizushima N, Tsukamoto S. 2014. Fertilization-induced autophagy in mouse embryos is independent of mTORC1. *Biol Reprod* **91:** 7.

Yamanaka K, Sakatani M, Kubota K, Balboula AZ, Sawai K, Takahashi M. 2011. Effects of downregulating DNA methyltransferase 1 transcript by RNA interference on DNA methylation status of the satellite I region and in vitro development of bovine somatic cell nuclear transfer embryos. *J Reprod Dev* **57:** 393-402.

Yang J, Medvedev S, Reddi PP, Schultz RM, Hecht NB. 2005. The DNA/RNA-binding protein MSY2 marks specific transcripts for cytoplasmic storage in mouse male germ cells. *Proc Natl Acad Sci U S A* **102:** 1513-1518.

Yang MY, Fortune JE. 2008. The capacity of primordial follicles in fetal bovine ovaries to initiate growth in vitro develops during mid-gestation and is associated with meiotic arrest of oocytes. *Biol Reprod* **78**: 1153-1161.

Yang WL, Li J, An P, Lei AM. 2014. CDC20 downregulation impairs spindle morphology and causes reduced first polar body emission during bovine oocyte maturation. *Theriogenology* **81:** 535-544.

Yoshizumi M, Hsieh CM, Zhou F, Tsai JC, Patterson C, Perrella MA, Lee ME. 1995. The ATF site mediates downregulation of the cyclin A gene during contact inhibition in vascular endothelial cells. *Mol Cell Biol* **15:** 3266-3272.

Yu J, Deng M, Medvedev S, Yang J, Hecht NB, Schultz RM. 2004. Transgenic RNAi-mediated reduction of MSY2 in mouse oocytes results in reduced fertility. *Dev Biol* **268:** 195-206.

Yu J, Hecht NB, Schultz RM. 2001. Expression of MSY2 in mouse oocytes and preimplantation embryos. *Biol Reprod* **65:** 1260-1270.

Yu YM, Gibbs KM, Davila J, Campbell N, Sung S, Todorova TI, Otsuka S, Sabaawy HE, Hart RP, Schachner M. 2011. MicroRNA miR-133b is essential for functional recovery after spinal cord injury in adult zebrafish. *Eur J Neurosci* **33:** 1587-1597.

Yue HM, Li Z, Wu N, Liu Z, Wang Y, Gui JF. 2013. Oocyte-specific H2A variant H2af1o is required for cell synchrony before midblastula transition in early zebrafish embryos. *Biol Reprod* **89:** 82.

Yun Y, An P, Ning J, Zhao GM, Yang WL, Lei AM. 2015. H1foo is essential for in vitro meiotic maturation of bovine oocytes. *Zygote* **23:** 416-425.

Zeng F, Baldwin DA, Schultz RM. 2004. Transcript profiling during preimplantation mouse development. *Dev Biol* **272:** 483-496.

Zeng F, Schultz RM. 2005. RNA transcript profiling during zygotic gene activation in the preimplantation mouse embryo. *Dev Biol* **283:** 40-57.

Zhang DX, Cui XS, Kim NH. 2009. Involvement of polyadenylation status on maternal gene expression during in vitro maturation of porcine oocytes. *Mol Reprod Dev* **76:** 881-889.

Zhang K, Rajput SK, Lee KB, Wang D, Huang J, Folger JK, Knott JG, Zhang J, Smith GW. 2015. Evidence Supporting a Role for SMAD2/3 in Bovine Early Embryonic Development: Potential Implications for Embryotropic Actions of Follistatin. *Biol Reprod* **[in press]**.

Zhang X, Odom DT, Koo SH, Conkright MD, C anettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen E, Kadam S, Ecker JR, Emerson B, Hogenesch JB, Unterman T, Young RA, Montminy M. 2005. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proc Natl Acad Sci U S A* **102:** 4459-4464.

Zheng D, Cho YY, Lau AT, Zhang J, Ma WY, Bode AM, Dong Z. 2008. Cyclin-dependent kinase 3-mediated activating transcription factor 1 phosphorylation enhances cell transformation. *Cancer Res* **68:** 7650-7660.

Zheng P, Dean J. 2007. Oocyte-specific genes affect folliculogenesis, fertilization, and early development. *Semin Reprod Med* **25**: 243-251.

Zheng W, Gorre N, Shen Y, Noda T, Ogawa W, Lundin E, Liu K. 2010. Maternal phosphatidylinositol 3-kinase signalling is crucial for embryonic genome activation and preimplantation embryogenesis. *EMBO Rep* **11:** 890-895.

2. Rapidly Cleaving Bovine Two-cell Embryos Have Better Developmental Potential and a Distinctive mRNA Pattern

Ernesto Orozco-Lucero,[1] Isabelle Dufort,[1] Claude Robert[1] and Marc-André Sirard[1,*]

[1]Centre de Recherche en Biologie de la Reproduction (CRBR), Faculté des Sciences de l'Agriculture et de l'Alimentation, Département des Sciences Animales, Pavillon INAF, Université Laval, Québec, Qc, Canada

This paper has been published in the journal "Molecular Reproduction and Development".

Reference:

Orozco-Lucero E, Dufort I, Robert C, Sirard MA. 2014. Rapidly cleaving bovine two-cell embryos have better developmental potential and a distinctive mRNA pattern. *Mol Reprod Dev* **81:** 31-41.

2.1. Résumé

Les embryons de mammifères qui arrivent rapidement au stade de deux cellules en culture ont une probabilité plus élevée de produire des blastocystes viables. Notre objectif était de séparer des embryons bovins selon leur temps requis pour le premier clivage zygotique et d'évaluer leur niveau global de l'ARNm. Lors de la fecondation *in vitro*, tous les embryons qui ont clivé à 29.5 hpi (précoces) furent cultivés séparément de ceux qui ont clivé à 46 hpi (tardifs). Le taux de blastocystes étaient 46.1 ± 3.7% et 6.1 ± 3.4% pour des embryons précoces et tardifs, respectivement ($P<0.01$). Sept réplicats d'embryons sélectionnés à deux cellules ont été récupérés à chaque moment afin d'être caractériser par micropuce (n = 4) et d'effectuer un RT-PCR (n = 3) ; le reste a été laissé en culture pour que les blastocystes soient évalués. Un total de 774 et 594 transcripts ont été identifiés comme différemment exprimés dans les embryons précoces et tardifs, respectivement (fold change ± 1.5, $P <0.05$), avec des différences importantes reliées au niveau du cycle cellulaire, de l'expression génique, du traitement de l'ARN et de la dégradation protéique. Un total de 12 transcrits ont été évalués par PCR quantitative et parmi lesquels *ATM*, *ATR*, *CTNNB1*, *MSH6*, *MRE11A*, *PCNA*, *APC*, *CENPE* et *GRB2* se sont révélés être en accord avec les résultats d'hybridation. En raison du fait que ces molécules sont directement ou indirectement associées à la régulation du cycle cellulaire, à la réponse au dommage de l'ADN et au contrôle de la transcription, nos résultats suggèrent fortement des rôles clés pour ces fonctions biologiques pendant le développement préimplantatoire chez les mammifères.

2.2. Abstract

Mammalian embryos that rapidly reach the two-cell stage in culture have a higher probability of becoming viable blastocysts. Our goal was to separate two-cell bovine embryos based on their zygotic cleavage timing, and to assess their global mRNA levels. Following *in vitro* fertilization, all embryos that cleaved by 29.5 hpi (early) were cultured separately from those that divided at 46 hpi (late). The blastocyst rates were 46.1 ± 3.7% and 6.1 ± 3.4% for early- and late-cleavers, respectively ($P < 0.01$). Seven replicates of selected two-cell embryos were collected at each time point for microarray characterization ($n = 4$) and quantitative reverse-transcriptase PCR ($n = 3$); the rest were left in culture for blastocyst evaluation. A total of 774 and 594 probes were preferentially present in early- and late-cleaving embryos, respectively (fold change ± 1.5, $P < 0.05$), with important contrasts related to cell cycle, gene expression, RNA processing, and protein degradation functions. A total of 12 transcripts were assessed by quantitative PCR, of which *ATM*, *ATR*, *CTNNB1*, *MSH6*, *MRE11A*, *PCNA*, *APC*, *CENPE*, and *GRB2* were in agreement with the hybridization results. Since most of these molecules are directly or indirectly associated with cell-cycle regulation, DNA damage response, and transcription control, our results strongly suggest key roles for those biological functions in mammalian preimplantation development.

2.3. Introduction

In large mammals, female gametes possess variable levels of developmental capacity. This property is one of the contributing factors to the relatively low success rates of assisted reproductive technologies. In the last decades, numerous studies have explored the underlying causes of the variable competence in mammalian eggs and have consequently classified oocytes, or the early embryos derived from them, based on their quality using several strategies, including embryonic cell division timing. Quantifiable traits such as blastocyst rate, embryo survival after cryopreservation, and pregnancy following embryo transfer support the use of early embryonic cleavage kinetics as a predictor of developmental capacity (Lechniak et al. 2008). For example, earlier-cleaving embryos produced *in vitro* have been suggested to resemble *in vivo* embryos based on the shorter cleavage kinetics of *in vivo*-derived embryos compared to those produced *in vitro* (Holm et al. 2002) The higher competency of early-dividing embryos has been corroborated in multiple species, including mice (Warner et al. 1993), hamsters (Gonzales et al. 1995), humans (Fenwick et al. 2002; Ivec et al. 2011), pigs (Dang-Nguyen et al. 2010), goats (Villamediana et al. 2001), water buffaloes (Rajhans et al. 2010), and cows (VanSoom et al. 1997; Dinnyes et al. 1999). Nonetheless, the underlying relationship between fast embryonic cleavage and elevated developmental competence is not well understood. It has been proposed that this variability could be associated with the culture environment (Lane and Gardner, 1997; Peippo et al. 2001), as well as the quality of the fertilizing spermatozoon (Ward et al. 2001; Menezo, 2006; Alomar et al. 2008; Berger et al. 2011), the embryo's sex (Yadav et al. 1993; Lonergan et al. 1999; Rizos et al. 2008; Pers-Kamczyc et al. 2012), and its genetic background - for example, expression of the fast allele of the Ped gene in rodents (Wu et al. 1999). Intrinsic attributes of the maternal gamete are presently considered the major elements governing the capacity of an embryo to undergo fast cleavage (Shoukir et al. 1997; Van Soom et al. 2007). Lechniak et al. (2008) stated that the time to first zygotic cleavage in bovine ranges from 24 to 48 hr post-insemination (hpi), establishing the population of early cleavers as those individuals that reach the two-cell stage no later than 30 hpi. Among the possible sources of faster embryonic cleavage are steady completion of nuclear maturation (Dominko and First, 1997), an increased number of mitochondria, lack of chromosomal aberrations (Lechniak et al. 2008), the attainment of

full size by the gamete (Vandaele et al. 2007), and the mRNA content inherited by the cleavage-stage embryo from the egg. Notably, in the instant after first cleavage, the embryo still contains a substantial proportion of the egg's stockpiled mRNA, which is subjected to translation in subsequent cell cycles (Gilbert et al. 2009) before embryonic genome activation (EGA). Previous studies have analyzed specific mRNA accumulation in early-cleaving bovine embryos. For example, SRY-box (*SOX*) was elevated in early early-cleaving embryos (Gutierrez-Adan et al. 2004), and an overrepresentation of mRNAs related to oxidation-reduction regulation networks was observed in the most competent two-cell embryos (Held et al. 2012). The mRNA content must also be evaluated according to poly-adenine (poly(A)) tail length. Brevini et al. (2002) observed variable polyadenylation patterns of specific transcripts between fast and slow-developing embryos, including poly(A) polymerase (*PAP*) and octamer-binding protein 4 (*OCT4*). Other groups have reported that fast two-cell embryos contain higher mRNA levels of cyclin B1 (*CCNB1*), histone H3A (*H3A*; Fair et al. 2004), cyclin B2 (*CCNB2*), pituitary tumor-transforming 1 (*PTTG1*; Mourot et al. 2006), histone H2A (*H2A*), and YY1 and E4TF1-associated factor 1 (*YEAF1*; Dode et al. 2006) transcripts that correspond to cell cycle progress modulators, histones, and transcription factors. Accordingly, the functions of cell cycle and gene expression also appear to be critical during the maternal to embryonic transition in mammals (Sirard, 2010). As oocytes of variable quality contain varying amounts of certain transcripts and because two groups of derived embryos with different competence can be obtained by assessing their time to first zygotic cleavage, the transcriptomes of these two classes of embryos were compared. Analysis of RNA was accomplished with a new transcriptome platform that includes thousands of 3'-untranslated-region (3'-UTR) isoforms to identify candidate genes whose expression is related to developmental potential.

2.4. Results

2.4.1. Relationship between zygotic cleavage timing and blastocyst rate

The time taken for bovine zygotes to reach the two-cell stage *in vitro* clearly influenced ($P = 0.0016$) the blastocyst rate in seven independent replicates. Specifically, fast embryos

showed a markedly increased number of blastocysts produced at Day 7 ($P < 0.01$), with an average difference of 40% when compared to their slow-cleaving counterparts (Fig. 2-1).

2.4.2. Large-scale transcriptome analysis

From a total of 37,238 targeted gene transcripts included on the microarray slide, 11,390 and 11,647 were detected above background level in fast- and slow-cleaving embryos, respectively. Of those, 620 were exclusively present in fast-cleaving and 871 in slow-cleaving embryos, while 10,630 were shared by both populations; when considering all the transcripts in early and late embryos, this totalled 12,121 targets (Fig. 2-2A). Statistical analysis showed that in the group of targets with a differential abundance of ≥1.5-fold change between both conditions, 774 targets were higher in fast-cleaving versus 594 increased in slow-cleaving two-cell embryos, for a total of 1,368 variable molecules when considering $P < 0.05$ (Fig. 2-2B) and target signal intensity, expressed as mean across conditions, above a value of 7.0. All the supplemental data from this work can be accessed online (http://onlinelibrary.wiley.com).

2.4.3. Reverse transcriptase-quantitative PCR validation

The transcript levels of 12 genes were measured by reverse-transcriptase quantitative PCR. These included the DNA damage response proteins and cell-cycle regulators *ATM*, *ATR*, *MSH6*, *MRE11A*, *RPA2*, *PCNA*, *APC*, and *CENPE*; the signalling molecules *CTNNB1* and *GRB2*; the transcription factor *TAF2*; and the mRNA modulator *DHX9*. From this list, 83.3% (10/12) showed the same tendency as the microarray results (all but *DHX9* and *RPA2*), and 75% (9/12) were in agreement with the hybridization results with at least $P < 0.05$ (Fig. 2-3; Table 2-1).

2.4.4. Functional characterization

Gene ontology (GO) analysis by Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com) showed that the main molecular and cellular (biological) functions containing targets with distinct differences between early and late-cleaving two-cell embryos consisted of (A) cell cycle; (B) RNA post-transcriptional modification; (C) cellular assembly and organization; and (D) gene expression (Table 2-2). The top molecular

networks highlighted by the same software are consistent with the mechanisms revealed by the interconnected molecules, such as RNA post-transcriptional modification, cell cycle, cellular assembly and organization, post-translational modification, DNA replication, DNA recombination and repair, and nucleic acid metabolism. Interestingly, when IPA was further interrogated about the disparities between the two-cell embryo classes, the results were comparable to the processes observed in the differentially regulated biological functions and molecular networks. This is due to how varying molecules mapped to top canonical pathways, such as P53 signaling, protein ubiquitination, DNA double-strand-break repair by non-homologous end joining, and the role of BRCA1 in DNA damage response (Table 2-3). The list of all the canonical pathways and their integrating genes is shown in Supplemental Table S3. Finally, the three most significant upstream regulators predicted by IPA (Fig. 2-5) were CDKN1A (inhibited), TP53 (inhibited), and ionomycin (activated).

2.5. Discussion

This article reveals the complete mRNA picture associated with rapidly cleaving bovine two-cell embryos and identifies some key factors and pathways associated with their unique developmental capacity. The discovery that DNA repair is important in early embryos is intriguing, opening up a new direction for expanding research on developmental competence.

2.5.1. Accuracy of the zygotic cleavage timing model

The main goal of this embryonic kinetics experiment was to separate populations of two-cell embryos based on the time to first zygotic cleavage, thus generating two groups with contrasting developmental capacity to be characterized by transcriptomics. The results validated that cleavage timing indeed had an impact on developmental outcome, as the two extreme populations produced significantly different blastocyst percentages. The early-cleaving (29.5 hpi) two-cell embryo subset accounted for the vast majority of blastocysts in our seven *in vitro*-production cycles (Fig. 2-1). It can be assumed that our experimental system allowed us to distinguish the mRNA content from two-cell embryos of distinct quality on the basis of the time to first zygotic cleavage.

2.5.2. Transcriptome analysis

2.5.2.1. Cell cycle

A preponderant function distinguishing the two-cell embryos of differing competence was cell-cycle modulation. In particular, Centromere protein E (*CENPE*), which was elevated in fast-cleaving two-cell embryos, can be considered of major relevance for subsequent cleavages. This motor centromeric protein is necessary for accurate chromosome alignment through its kinetochore association with spindle microtubules (Zhu et al. 2005), and is a spindle assembly checkpoint (SAC)-related protein, based on its involvement in potentiating the signal of this checkpoint (Polanski, 2013). Although Yao et al. (2000) observed that a faulty CENPE markedly lengthens the cell cycle and activates SAC in somatic cells, Homer (2011) and Polanski (2013) indicate that failure of CENPE in oocytes would instead allow miscarried meiotic progression, leading to aneuploidy, which is a major cause of blastomere loss (Viuff et al. 2000; Pers-Kamczyc et al. 2012). This fact appears to be tightly correlated with the findings of a decline in *Cenpe* mRNA decline in aging oocytes reported by Pan et al. (2008). An association between CENPE and Centromere protein F (CENPF) has also been observed (Yao et al. 2000), thus linking these data to the observation by Toralova et al. (2009) that RNA interference of *CENPF* leads to arrest by the 8-cell stage in the bovine. CENPE arrives to the midbody before S-phase kinase associated protein 1 (SKP1) during mitosis. The interaction of both proteins is crucial for cytokinesis to occur since SKP1 likely targets CENPE for degradation, thereby allowing cytokinesis to proceed (Liu et al. 2006). Interestingly, *SKP1* abundance is higher in fast-cleaving two-cell embryos (Supplemental Table S1), therefore, both CENPE and SKP1 could be considered key factors for developmental competence. DNA damage. The time to first zygotic cleavage revealed a crucial role for DNA damage response. ATM and ATR kinases are of particular relevance since they assess the presence of single- and double-strand breaks (DSBs), allowing cell cycle progression during mitosis and meiosis or stalling it through the recruitment of DNA damage response factors, such as BRCA1 and BRCA2, and homologous recombination repair proteins in the case of DSB (Shechter et al. 2004; Lange et al. 2011), which involve the direct action of both MSH6 and MRE11A. The notion that the DSB-repair pathway is crucial for early development is consistent with the report from Titus et al. (2013) that showed a decrease of *ATM*, *MRE11A*, and *BRCA1*

transcripts with age in both human and rodent eggs, which also correlated with oocyte death and impaired fertility. Similarly, multiple DNA-damage-response and related factors, including *MSH6*, *MRE11A*, *PCNA*, and *ATR*, were prevalent at the mRNA level in human oocytes and were reduced in older eggs (Menezo et al. 2007; Albertini, 2013). On the other hand, ATM is also able to control CTNNB1 function (Morkel et al. 2003). Modina et al. (2007) reported that the proper dynamic distribution of the CTNNB1 protein during the bovine preimplantation period is correlated with fast embryo kinetics and increased blastocyst rate. This might be related to the association of CTNNB1 and adenomatous polyposis coli (APC) proteins, which excludes CTNNB1 from the nucleus and interferes with its role in promoting apoptosis (Henderson, 2000). APC also interacts with PCNA (Brocardo et al. 2011), a fundamental partner of DNA polymerases delta and epsilon and thus a master component of DNA duplication and excision repair (Moldovan et al. 2007). PCNA is considered to be involved in developmental competence in pig oocytes (Kim et al. 2010) and cattle embryos (Markkula et al. 2001). It should be noted that this proliferation marker acts downstream of the DNA replication checkpoint. Together, the relationship of these molecular cascades agrees with the abundance of their transcripts in the most competent, bovine two-cell embryos (Fig. 2-4). Subsequent protein analysis would be required to assess if the excess mRNA is stored for later use (after the two-cell stage) or is translated earlier.

2.5.2.2. Transcription control

In the context of transcriptional silence, maternally stored molecules related to gene expression control may define an embryo's fate. This has been one of our major hypotheses in the quest for oocyte competence over the years (Sirard, 2010), and results from this study support the notion that this biological process could be considered a central module of the events impacting early developmental outcome. In addition to its role in DNA duplication, PCNA can modify chromatin by inducing a transcriptionally obstructive conformation (Prosperi, 2006; Liang et al. 2011). The role of PCNA in transcriptional regulation in the embryo, however, is still unclear. Transcription factors of high importance were over-represented in fast-cleaving embryos. For example, mRNA encoding BTFIID transcription factor-associated, 170-kDa Mot1 homolog, (*Saccharomyces cerevisiae*; *BTAF1*) and *TAF2*,

both integrators of RNA polymerase II complexes, were elevated in this population. These transcription factors could regulate EGA in bovines, either positively to start *de novo* transcription, or to maintain a controlled transcriptional shutdown before EGA. Again, protein validation would be helpful to clarify this issue.

2.5.2.3. Upstream regulators

One approach to understand how identified factors encoded by the mRNA signatures participate in the observed differential phenotype relies on the predicted activation or inhibition of upstream regulators by GO software. TP53, for example was inferred by IPA to be inhibited due to the clustered relative level of mRNA of its downstream targets. Consistent with its role as transcription factor, multiple differential mRNAs in our data correspond to genes regulated by TP53. Most of them are involved in cell cycle function, DNA damage response, protein ubiquitination, mRNA processing, and transcription regulation (Fig. 2-5A). Although TP53 itself has not been noted in our laboratory to differ in germinal vesicle-stage oocytes in relation to their quality, higher transcript abundance of its modulator tumor protein p53 inducible nuclear protein 1 (*TP53INP1*; Jiang et al. 2006) has been observed in bovine oocytes of increased competence, both in a coasting model (Labrecque et al. 2013) and in relation to follicle size (Labrecque, unpublished information). Curiously, *CDKN1A*, a gene regulated by TP53, was also inhibited in the upstream regulators prediction (Fig. 2-5B). This last node also contained the functions of cell cycle, DNA damage response, and mRNA management. Additionally, the differential signatures of molecules involved in transcription, mRNA processing, and protein ubiquitination identified ionomycin as a putative, activated upstream regulator (Fig. 2-5C). Since this ionophore is associated with an increase in intracellular free calcium (Ca^{2+}), this prediction may point to Ca^{2+} entry into the cytoplasm upon egg activation, which triggers a notable degradation of maternal mRNA in mouse eggs, accompanied by a distinct translation compared to that before activation (Ducibella et al. 2002; Knott et al. 2006; Ducibella and Fissore, 2008; Horner and Wolfner, 2008); such transcript degradation has yet to be clearly demonstrated in cattle, however. It is also known that the protein synthesis profile is modified in bovine oocytes upon fertilization, and varies rapidly up to the eight-cell stage (Chian and Sirard, 1996; Massicotte et al. 2006). Given that recruitment of oocyte

mRNAs for translation largely depends on the presence of a long poly(A) tail (Brevini et al. 2002; Eichenlaub-Ritter and Peschke, 2002; Bettegowda and Smith, 2007), part of the differences between fast- and slow-cleaving embryos could be due to their capacity to elicit appropriate Ca^{2+} oscillations after fertilization, which would modify poly(A)-tail length of specific transcripts. The molecular origins of such mechanism are still controversial, though, and would require further exploration regarding how it participated in the establishment of bovine developmental competency.

In summary, the current results indicate that the most competent early embryos in cattle are better endowed with a series of specific mRNAs encoding cell-cycle regulation and DNA-damage-response factors. These functions are pivotal in early mammalian development because of their respective roles in the avoidance of aneuploidy (cell cycle checkpoint) and the molecular reaction against DNA breaks (DNA damage response). Moreover, gene expression regulation was identified as a predominant molecular module for bovine EGA, implying a close relationship between cell cycle and transcription control in the establishment of developmental competence in mammals. The contrasting abundance reported here for a single or a few maternal molecules probably does not account for the observed differences in developmental performance. Instead, the combination of variations in a complete subset of key maternal transcripts may result in more uniform cleavage rates and EGA timing, as expected for the most competent embryos.

2.6. Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

2.6.1. *In vitro* production of bovine blastocysts

Oocyte collection was performed from slaughterhouse cow ovaries, followed by *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) according to standard techniques in our laboratory (Cagnone et al. 2012; Cagnone et al. 2013).

2.6.2. Separation of fast- and slow-cleaving 2-cell embryos and survey of the developmental outcome

Assessment of speed to the first zygotic cleavage: During each of seven independent IVP cycles (biological replicates), 2-cell embryos were differentiated based on their timing of zygotic cleavage. Briefly, presumptive embryos were observed at 29.5, 35, and 46 hpi to distinguish early, middle, and late cleavers, respectively. At each time point, 5 to 10 embryos at the 2-cell stage were harvested from the dish, completely stripped of any remaining cumulus cell with a thin glass pipette, washed 3 times in PBS, pooled into 0.5-ml microtubes in a minimum volume of PBS, and stored at -80°C until RNA extraction. In parallel, all other cleaved embryos at each time point were transferred to new droplets based on their cleavage timing category, in order to separate three embryo subpopulations, and the rest of the culture was performed as described above.

2.6.2.1. Developmental competence estimation: The blastocyst rate was calculated by dividing the total number of blastocysts by the total number of 2-cell embryos at collection. The effect of cleavage timing on blastocyst rate was analyzed through Kruskal-Wallis test, whereas differences between embryo groups were considered significant when $P < 0.05$ after pgirmess analysis (R package).

2.6.3. Determination of differential transcript levels in 2-cell embryos

Microarray analysis: Total RNA from fast- or slow-cleaving 2-cell embryos taken from four independent IVP cycles (5-10 embryos/replicate) was extracted and purified using Arcturus PicoPure RNA Isolation Kit (Life Technologies, Burlington, ON, Canada) according to the manufacturer's instructions. Purified RNA was amplified by T7 *in vitro* transcription, subsequently labelled with Cy3 and Cy5 as previously described (Cagnone et al. 2012; Cagnone et al. 2013; Labrecque et al. 2013), and the resulting aRNA (825 ng/replicate) was hybridized onto Agilent-manufactured EmbryoGENE bovine slides (Robert et al. 2011) in a two-color dye swap design for a total of 8 arrays. Slides were scanned with the PowerScanner (Tecan, Mannedorf, Switzerland) and features were extracted with ArrayPro 6.3 (MediaCybernetics, Rockville, MD). Finally, the intensity files were analyzed by FlexArray 1.6.1 (http://genomequebec.mcgill.ca/FlexArray), where raw data correction

consisted of a background subtraction with a subsequent normalization within (Loess) and between (quantile) arrays. The statistical comparison of early vs. late embryos used the Limma algorithm and targets were considered to have differential levels when the FC reached ±1.5 with $P<0.05$. Data were deposited into the NCBI Gene Expression Omnibus: GEO series accession number GSE50633 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50633).

2.6.3.1. RT-qPCR

Total RNA from 3 new biological replicates (5-10 embryos/replicate) was reverse-transcribed with oligo-dT and qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. Specific primers for each selected gene were designed with Primer Quest (Integrated DNA Technologies, Coralville, IA) and qPCRs were performed by using LC Fast Start DNA Master SYBR Green I and the LightCycler 2.0 (Roche, Laval, QC, Canada), with 125 nM of each primer and the equivalent of 0.25 embryo per 20 µl-reaction. Subsequently, the PCR template was quantified with a standard curve of five points containing a PCR amplicon input from 0.2 pg to 0.02 fg per reaction. Finally, data were normalized with GeNORM (Vandesompele et al. 2002) by using the values of three reference genes (ACTB, CHUK, and SDHA) whose mRNAs were shown to be stable between our fast- and slow-cleaving 2-cell embryos. Primer sequences, product sizes, annealing temperatures, and accession numbers are shown in Table 2-4.

2.6.4. Functional analysis of differential mRNA levels profile in 2-cell embryos

Network generation, canonical pathway analysis, and upstream regulator prediction: Identifiers from targets with distinct signal magnitudes between early and late embryos, called network eligible molecules (NEMs), were exported from Flex Array to IPA, where such information was analyzed to compile both molecular networks (gene product interactions) and canonical pathways with the differences between fast- and slow-cleaving embryos. IPA was used to build schematic representations of crucial molecular pathways variably regulated in 2-cell embryos according to their cleavage speed. Each identifier was mapped to its corresponding object in IPA's database. NEMs were algorithmically assessed (right-tailed Fisher's exact test) to generate molecular networks based on the functional

connectivity of the molecules they contained, as well as the main molecular and cellular (biological) functions of the targets with varying levels between both embryo groups. The significance of the association between the input dataset and the canonical pathways included in IPA's library was measured as follows: 1) A ratio of the number of targets from the dataset that map to the pathway divided by the total number of molecules found in the canonical pathway; 2) Fisher's exact test. Similar to the biological functions and the molecular networks, red and green symbols in the canonical pathways represent genes whose mRNA was respectively increased or decreased in fast- versus slow-cleaving embryos. Grey symbols represent genes whose transcript level was higher than background intensity but showed no difference between conditions, whereas white nodes represent genes absent in the microarray survey or below the background level. IPA inferred the activation or inhibition state of putative upstream regulators in the input dataset according to an overlap p-value (Fisher's Exact Test), followed by the calculation of z-score. This last algorithm is a statistical approach aiming to quantitatively define whether an upstream regulator is significantly predicted to be "activated" ($z > 0$) or "inhibited" ($z < 0$).

2.7. Declaration of Interest

The authors declare that there is no conflict of interest.

2.8. Acknowledgements

We thank Isabelle Laflamme (Universite Laval, Québec, Canada) for her technical assistance in the *in vitro* production of bovine embryos, Dr. Marc-Andre Laniel for revising the manuscript, as well as the NSERC EmbryoGENE network for the slides and the platform access. EOL is supported by a FRQNT studenship from the Government of Quebec and the EmbryoGENE network.

2.9. References

Albertini DF. 2013. Vanishing returns on the investment that is the ovarian reserve. J *Assist Reprod Genet* **30:** 283-284.

Alomar M, Tasiaux H, Remacle S, George F, Paul D, Donnay I. 2008. Kinetics of fertilization and development, and sex ratio of bovine embryos produced using the semen of different bulls. *Anim Reprod Sci* **107:** 48-61.

Berger DS, AbdelHafez F, Russell H, Goldfarb J, Desai N. 2011. Severe teratozoospermia and its influence on pronuclear morphology, embryonic cleavage and compaction. *Reprod Biol Endocrinol* **9:** 37.

Bettegowda A, Smith GW. 2007. Mechanisms of maternal mRNA regulation: implications for mammalian early embryonic development. *Front Biosci* **12:** 3713-3726.

Blondin P, Sirard MA. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol Reprod Dev* **41:** 54-62.

Brand M, Yamamoto K, Staub A, Tora L. 1999. Identification of TATA-binding protein-free TAFII-containing complex subunits suggests a role in nucleosome acetylation and signal transduction. *J Biol Chem* **274:** 18285-18289.

Brevini TA, Lonergan P, Cillo F, Francisci C, Favetta LA, Fair T, Gandolfi F. 2002. Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. *Mol Reprod Dev* **63:** 510-517.

Brocardo MG, Borowiec JA, Henderson BR. 2011. Adenomatous polyposis coli protein regulates the cellular response to DNA replication stress. *Int J Biochem Cell Biol* **43:** 1354-1364.

Cagnone GL, Dufort I, Vigneault C, Sirard MA. 2012. Differential gene expression profile in bovine blastocysts resulting from hyperglycemia exposure during early cleavage stages. *Biol Reprod* **86:** 50.

Cagnone GL, Sirard MA. 2013. Transcriptomic signature to oxidative stress exposure at the time of embryonic genome activation in bovine blastocysts. *Mol Reprod Dev* **80:** 297-314.

Chian RC, Sirard MA. 1996. Protein synthesis is not required for male pronuclear formation in bovine zygotes. *Zygote* **4:** 41-48.

Dang-Nguyen TQ, Kikuchi K, Somfai T, Ozawa M, Nakai M, Maedomari N, Viet-Linh N, Kanai Y, Nguyen BX, Nagai T. 2010. Evaluation of developmental competence of in

vitro-produced porcine embryos based on the timing, pattern and evenness of the first cleavage and onset of the second cleavage. *J Reprod Dev* **56:** 593-600.

Dikovskaya D, Newton IP, Nathke IS. 2004. The adenomatous polyposis coli protein is required for the formation of robust spindles formed in CSF Xenopus extracts. *Mol Biol Cell* **15:** 2978-2991.

Dinnyes A, Lonergan P, Fair T, Boland MP, Yang X. 1999. Timing of the first cleavage post-insemination affects cryosurvival of in vitro-produced bovine blastocysts. *Mol Reprod Dev* **53:** 318-324.

Dode MA, Dufort I, Massicotte L, Sirard MA. 2006. Quantitative expression of candidate genes for developmental competence in bovine two-cell embryos. *Mol Reprod Dev* **73:** 288-297.

Dominko T, First NL. 1997. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. *Mol Reprod Dev* **47:** 456-467.

Du J, Chen L, Shen J. 2009. Identification of FANCA as a protein interacting with centromere-associated protein E. *Acta Biochim Biophys Sin (Shanghai)* **41:** 816-821.

Ducibella T, Fissore R. 2008. The roles of Ca2+, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. *Dev Biol* **315:** 257-279.

Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, Kopf GS, Fissore R, Madoux S, Ozil JP. 2002. Egg-to-embryo transition is driven by differential responses to Ca(2+) oscillation number. *Dev Biol* **250:** 280-291.

Eichenlaub-Ritter U, Peschke M. 2002. Expression in in-vivo and in-vitro growing and maturing oocytes: focus on regulation of expression at the translational level. *Hum Reprod Update* **8:** 21-41.

Fair T, Murphy M, Rizos D, Moss C, Martin F, Boland MP, Lonergan P. 2004. Analysis of differential maternal mRNA expression in developmentally competent and incompetent bovine two-cell embryos. *Mol Reprod Dev* **67:** 136-144.

Fenwick J, Platteau P, Murdoch AP, Herbert M. 2002. Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos in vitro. *Hum Reprod* **17:** 407-412.

Gilbert I, Scantland S, Sylvestre EL, Gravel C, Laflamme I, Sirard MA, Robert C. 2009. The dynamics of gene products fluctuation during bovine pre-hatching development. *Mol Reprod Dev* **76:** 762-772.

Gonzales DS, Pinheiro JC, Bavister BD. 1995. Prediction of the developmental potential of hamster embryos in vitro by precise timing of the third cell cycle. *J Reprod Fertil* **105:** 1-8.

Gosden R, Lee B. 2010. Portrait of an oocyte: our obscure origin. *J Clin Invest* **120:** 973-983.

Guermah M, Ge K, Chiang CM, Roeder RG. 2003. The TBN protein, which is essential for early embryonic mouse development, is an inducible TAFII implicated in adipogenesis. *Mol Cell* **12:** 991-1001.

Gutierrez-Adan A, Rizos D, Fair T, Moreira PN, Pintado B, de la Fuente J, Boland MP, Lonergan P. 2004. Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. *Mol Reprod Dev* **68:** 441-448.

Hansen PJ, Fear JM. 2011. Cheating death at the dawn of life: developmental control of apoptotic repression in the preimplantation embryo. *Biochem Biophys Res Commun* **413:** 155-158.

Held E, Salilew-Wondim D, Linke M, Zechner U, Rings F, Tesfaye D, Schellander K, Hoelker M. 2012. Transcriptome fingerprint of bovine 2-cell stage blastomeres is directly correlated with the individual developmental competence of the corresponding sister blastomere. *Biol Reprod* **87:** 154.

Henderson BR. 2000. Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. *Nat Cell Biol* **2:** 653-660.

Holm P, Booth PJ, Callesen H. 2002. Kinetics of early in vitro development of bovine in vivo- and in vitro-derived zygotes produced and/or cultured in chemically defined or serum-containing media. *Reproduction* **123:** 553-565.

Homer H. 2011. New insights into the genetic regulation of homologue disjunction in mammalian oocytes. *Cytogenet Genome Res* **133:** 209-222.

Horner VL, Wolfner MF. 2008. Transitioning from egg to embryo: triggers and mechanisms of egg activation. *Dev Dyn* **237:** 527-544.

Ivec M, Kovacic B, Vlaisavljevic V. 2011. Prediction of human blastocyst development from morulas with delayed and/or incomplete compaction. *Fertil Steril* **96:** 1473-1478.

Jiang PH, Motoo Y, Garcia S, Iovanna JL, Pebusque MJ, Sawabu N. 2006. Down-expression of tumor protein p53-induced nuclear protein 1 in human gastric cancer. *World J Gastroenterol* **12:** 691-696.

Kim J, You J, Hyun SH, Lee G, Lim J, Lee E. 2010. Developmental competence of morphologically poor oocytes in relation to follicular size and oocyte diameter in the pig. *Mol Reprod Dev* **77:** 330-339.

Klejman MP, Pereira LA, van Zeeburg HJ, Gilfillan S, Meisterernst M, Timmers HT. 2004. NC2alpha interacts with BTAF1 and stimulates its ATP-dependent association with TATA-binding protein. *Mol Cell Biol* **24:** 10072-10082.

Knott JG, Gardner AJ, Madgwick S, Jones KT, Williams CJ, Schultz RM. 2006. Calmodulin-dependent protein kinase II triggers mouse egg activation and embryo development in the absence of Ca2+ oscillations. *Dev Biol* **296:** 388-395.

Labrecque R, Vigneault C, Blondin P, Sirard MA. 2013. Gene expression analysis of bovine oocytes with high developmental competence obtained from FSH-stimulated animals. *Mol Reprod Dev* **80:** 428-440.

Lane M, Gardner DK. 1997. Nonessential amino acids and glutamine decrease the time of the first three cleavage divisions and increase compaction of mouse zygotes in vitro. *J Assist Reprod Genet* **14:** 398-403.

Lange J, Pan J, Cole F, Thelen MP, Jasin M & Keeney S. 2011. ATM controls meiotic double-strand-break formation. *Nature* **479:** 237-240.

Lechniak D, Pers-Kamczyc E, Pawlak P. 2008. Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos. *Reprod Biol* **8:** 23-42.

Liang Z, Diamond M, Smith JA, Schnell M, Daniel R. 2011. Proliferating cell nuclear antigen is required for loading of the SMCX/KMD5C histone demethylase onto chromatin. *Epigenetics Chromatin* **4:** 18.

Liu D, Zhang N, Du J, Cai X, Zhu M, Jin C, Dou Z, Feng C, Yang Y, Liu L, Takeyasu K, Xie W, Yao X. 2006. Interaction of Skp1 with CENP-E at the midbody is essential for cytokinesis. *Biochem Biophys Res Commun* **345:** 394-402.

Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P, Boland MP. 1999. Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *J Reprod Fertil* **117:** 159-167.

Markkula M, Raty M, Jauhiainen L, Paranko J, Raula J, Makarevich A. 2001. Ratio of proliferating cell nuclear antigen-positive nuclei to total cell number is higher in day 7 than in day 8 vitrified in vitro-produced bovine embryos. *Biol Reprod* **65:** 52-59.

Massicotte L, Coenen K, Mourot M, Sirard MA. 2006. Maternal housekeeping proteins translated during bovine oocyte maturation and early embryo development. *Proteomics* **6:** 3811-3820.

Menezo YJ. 2006. Paternal and maternal factors in preimplantation embryogenesis: interaction with the biochemical environment. *Reprod Biomed Online* **12:** 616-621.

Menezo Y Jr, Russo G, Tosti E, El Mouatassim S, Benkhalifa M. 2007. Expression profile of genes coding for DNA repair in human oocytes using pangenomic microarrays, with a special focus on ROS linked decays. *J Assist Reprod Genet* **24:** 513-520.

Modina S, Abbate F, Germana GP, Lauria A, Luciano AM. 2007. Beta-catenin localization and timing of early development of bovine embryos obtained from oocytes matured in the presence of follicle stimulating hormone. *Anim Reprod Sci* **100:** 264-279.

Moldovan GL, Pfander B, Jentsch S. 2007. PCNA, the maestro of the replication fork. *Cell* **129:** 665-679.

Morkel M, Huelsken J, Wakamiya M, Ding J, van de Wetering M, Clevers H, Taketo MM, Behringer RR, Shen MM, Birchmeier W. 2003. Beta-catenin regulates Cripto- and Wnt3-dependent gene expression programs in mouse axis and mesoderm formation. *Development **130:*** 6283-6294.

Mourot M, Dufort I, Gravel C, Algriany O, Dieleman S, Sirard MA. 2006. The influence of follicle size, FSH-enriched maturation medium, and early cleavage on bovine oocyte maternal mRNA levels. *Mol Reprod Dev* **73:** 1367-1379.

Oliveros JC. 2007. VENNY. An interactive tool for comparing lists with Venn Diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html.

Ozil JP, Banrezes B, Toth S, Pan H, Schultz RM. 2006. Ca2+ oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. *Dev Biol* **300:** 534-544.

Pan H, Ma P, Zhu W, Schultz RM. 2008. Age-associated increase in aneuploidy and changes in gene expression in mouse eggs. *Dev Biol* **316:** 397-407.

Peippo J, Kurkilahti M, Bredbacka P. 2001. Developmental kinetics of in vitro produced bovine embryos: the effect of sex, glucose and exposure to time-lapse environment. *Zygote* **9:** 105-113.

Pers-Kamczyc E, Pawlak P, Rubes J, Lechniak D. 2012. Early cleaved bovine embryos show reduced incidence of chromosomal aberrations and higher developmental potential on day 4.5 post-insemination. *Reprod Domest Anim* **47:** 899-906.

Polanski Z. 2013. Spindle assembly checkpoint regulation of chromosome segregation in mammalian oocytes. *Reprod Fertil Dev* **25:** 472-483.

Prosperi E. 2006. The fellowship of the rings: distinct pools of proliferating cell nuclear antigen trimer at work. *FASEB J* **20:** 833-837.

Rajhans R, Kumar GS, Dubey PK, Sharma GT. 2010. Effect of timing of development on total cell number and expression profile of HSP-70.1 and GLUT-1 in buffalo (Bubalus bubalis) oocytes and preimplantation embryos produced in vitro. *Cell Biol Int* **34:** 463-468.

Rizos D, Bermejo-Alvarez P, Gutierrez-Adan A, Lonergan P. 2008. Effect of duration of oocyte maturation on the kinetics of cleavage, embryo yield and sex ratio in cattle. *Reprod Fertil Dev* **20:** 734-740.

Robert C, Nieminen J, Dufort I, Gagne D, Grant JR, Cagnone G, Plourde D, Nivet AL, Fournier E, Paquet E, Blazejczyk M, Rigault P, Juge N, Sirard MA. 2011. Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays. *Mol Reprod Dev* **78:** 651-664.

Shechter D, Costanzo V, Gautier J. 2004. Regulation of DNA replication by ATR: signaling in response to DNA intermediates. *DNA Repair (Amst)* **3:** 901-908.

Shoukir Y, Campana A, Farley T, Sakkas D. 1997. Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. *Hum Reprod* **12:** 1531-1536.

Sirard MA. 2010. Activation of the embryonic genome. *Soc Reprod Fertil Suppl* **67:** 145-158.

Titus S, Li F, Stobezki R, Akula K, Unsal E, Jeong K, Dickler M, Robson M, Moy F, Goswami S, Oktay K. 2013. Impairment of BRCA1-related DNA double-strand break repair leads to ovarian aging in mice and humans. *Sci Transl Med* **5:** 172ra21.

Toralova T, Susor A, Nemcova L, Kepkova K, Kanka J. 2009. Silencing CENPF in bovine preimplantation embryo induces arrest at 8-cell stage. *Reproduction* **138:** 783-791.

Van Soom A, Vandaele L, Goossens K, de Kruif A, Peelman L. 2007. Gamete origin in relation to early embryo development. *Theriogenology* **68:** S131-137.

Van Soom A, Ysebaert MT, de Kruif A. 1997. Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos. *Mol Reprod Dev* **47:** 47-56.

Vandaele L, Mateusen B, Maes DG, de Kruif A, Van Soom A. 2007. Temporal detection of caspase-3 and -7 in bovine in vitro produced embryos of different developmental capacity. *Reproduction* **133:** 709-718.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real□time quantitative RT□PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3:** RESEARCH0034.

Villamediana P, Vidal F, Paramio MT. 2001. Cytogenetic analysis of caprine 2- to 4-cell embryos produced in vitro. *Zygote* **9:** 193-199.

Viuff D, Greve T, Avery B, Hyttel P, Brockhoff PB, Thomsen PD. 2000 Chromosome aberrations in in vitro-produced bovine embryos at days 2-5 post-insemination. *Biol Reprod* **63:** 1143-1148.

Wansleeben C, van Gurp L, de Graaf P, Mousson F, Marc Timmers HT, Meijlink F. 2011. An ENU-induced point mutation in the mouse Btaf1 gene causes post-gastrulation embryonic lethality and protein instability. *Mech Dev* **128:** 279-288.

Ward F, Rizos D, Corridan D, Quinn K, Boland M, Lonergan P. 2001. Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development in vitro and fertility in vivo. *Mol Reprod Dev* **60:** 47-55.

Warner CM, Panda P, Almquist CD, Xu Y. 1993. Preferential survival of mice expressing the Qa-2 antigen. *J Reprod Fertil* **99:** 145-147.

Wu L, Feng H, Warner CM. 1999. Identification of two major histocompatibility complex class Ib genes, Q7 and Q9, as the Ped gene in the mouse. *Biol Reprod* **60:** 1114-1119.

Yadav BR, King WA, Betteridge KJ. 1993. Relationships between the completion of first cleavage and the chromosomal complement, sex, and developmental rates of bovine embryos generated in vitro. *Mol Reprod Dev* **36:** 434-439.

Yao X, Abrieu A, Zheng Y, Sullivan KF, Cleveland DW. 2000. CENP-E forms a link between attachment of spindle microtubules to kinetochores and the mitotic checkpoint. *Nat Cell Biol* **2:** 484-491.

Zhu C, Zhao J, Bibikova M, Leverson JD, Bossy-Wetzel E, Fan JB, Abraham RT, Jiang W. 2005. Functional analysis of human microtubule-based motor proteins, the kinesins and dyneins, in mitosis/cytokinesis using RNA interference. *Mol Biol Cell* **16:** 3187-3199.

2.10. Tables and Figures

Table 2-1 Symmetrical raw fold changes and p-values of microarray analysis and RT-qPCR validation of fast- compared to slow 2-cell embryos

Molecule	Microarray, fold change	Microarray, p-value	RT-qPCR, fold change	RT-qPCR, p-value
ATM	2.0703	0.0232	2.3482	0.0144
ATR	2.5648	0.0124	1.5022	0.0795
CTNNB1	1.7981	0.0230	1.7547	0.0095
MSH6	2.3183	0.0059	2.8176	0.001
MRE11A	1.8318	0.0191	1.5828	0.0406
PCNA	2.0954	0.0102	1.8970	0.0062
APC	4.1845	0.0030	3.9560	0.0015
CENPE	2.6071	0.0034	1.9509	0.0098
TAF2	2.0653	0.0110	2.0345	0.022
GRB2	-1.5802	0.0443	-1.8567	0.0399

Table 2-2 Top molecular and cellular functions (biological functions)

Name	P-value	Number of molecules
Cell cycle	$4.89E^{-9}$ to $2.02E^{-2}$	136
RNA post-transcriptional modification	$5.5E^{-7}$ to $2.16E^{-3}$	31
Cellular assembly and organization	$4.07E^{-6}$ to $2.02E^{-2}$	140
Gene expression	$5.67E^{-6}$ to $1.9E^{-2}$	153
Cellular function and maintenance	$5.86E^{-6}$ to $2.02E^{-2}$	137

Table 2-3 Top canonical pathways

Name	P-value	Ratio
P53 signalling	$5.04E^{-7}$	16/96 (0.167)
Protein ubiquitination pathway	$9.1E^{-6}$	26/268 (0.097)
DNA double-strand break repair by non-homologous end joining	$1.28E^{-4}$	5/19 (0.263)
Polyamine regulation in colon cancer	$1.89E^{-4}$	6/29 (0.207)
Role of BRCA1 in DNA damage response	$4.67E^{-4}$	9/65 (0.138)

Table 2-4 Details of the RT-qPCR in bovine 2-cell embryos

Gene symbol	Fw-primer sequence (5'-3')	Rv-primer sequence (5'-3')	Tm (°C)	Product (bp)
ATM	ATTCCAGCAGACCAGC CCATTA	AGAACGCCACTTCGCT GAGAAA	59	363
ATR	TGTCGTTCCACCGCAGT TATGT	GTTCCCATCGGACCCA TTCC	59	212
CTNNB1	TTGTACTGGAGCCCTTC ACATCCTA	TCAGCTCAACCGAAA GCCGTTTC	59	320
MSH6	GCATCGCAGTGTTGGA TGTGT	TTCTGTCTGAGGCACC AAGTCT	59	393
MRE11A	AAGAGCAGGCACTAGT CTGGAGAT	TCTGGGACATGGGTTT GCTTGATGA	59	257
RPA2	AACACTGTGGTCCCTCC AGAAACA	TATTCCCACCGAAGTT CCCAGCTT	60	244
PCNA	TTTGGCTCCCAAGATCG AGGAT	CAAATGAAGGCACTG TCCTGT	56	383
APC	TGAACAAGTTTACCCA GCCTGCTT	GCAGCCATCTCACCTC AAATACC	60	276
CENPE	AAGGAGTCGCCGAAAT CTTGGT	TCCTAGTGGCAAAGT GGGAACT	58	331
TAF2	TCAACTCCAGGGCTCTC CAAAT	TTCTTCTTGTGCTCGT GGTGGT	59	266
GRB2	TCAATGGGAAAGACGG CTTCATCC	TGCCGCTGTTTGCTGA GCATTT	60	114
DHX9	ATGCTGAACACAATCC GCCAGA	ACCACCACCTCCAAC ATAGCTT	59	294
ACTB	ATCGTCCACCGCAAAT GCTTCT	GCCATGCCAATCTCAT CTCGTT	60	101
CHUK	TGATGGAATCTCTGGA ACAGCG	TGCTTACAGCCCAACA ACTTGC	57	180
SDHA	TGACGAGTACGATTAC TCCAAGCC	TTGATGTCAACGTAGG AGAGCGTG	57	96

ACTB, Actin, beta; ATM, Ataxia-telangiectasia mutated; *ATR*, Ataxia-telangiectasia and Rad3-related; *APC*, Adenomatous polyposis coli; *CENPE*, Centromere protein E; *CHUK*, Conserved helix-loop-helix ubiquitous kinase; *CTNNB1*, Beta-catenin-1; *DHX9*, DEAH (Asp-Glu-Ala-His) Box Helicase 9 ; *GRB2*, Growth factor receptor-bound protein 2; *MRE11A*, Meiotic recombination 11 homolog A (*S. cerevisiae*); *MSH6*, MutS homolog 6 (E. coli); *PCNA*, Proliferating cell nuclear antigen; *RPA2*, Replication Protein A2, 32kDa; *SDHA*, Succinate dehydrogenase complex, subunit A; *TAF2*, RNA polymerase II, TATA box binding protein (TBP)-associated factor, 150 kDa.

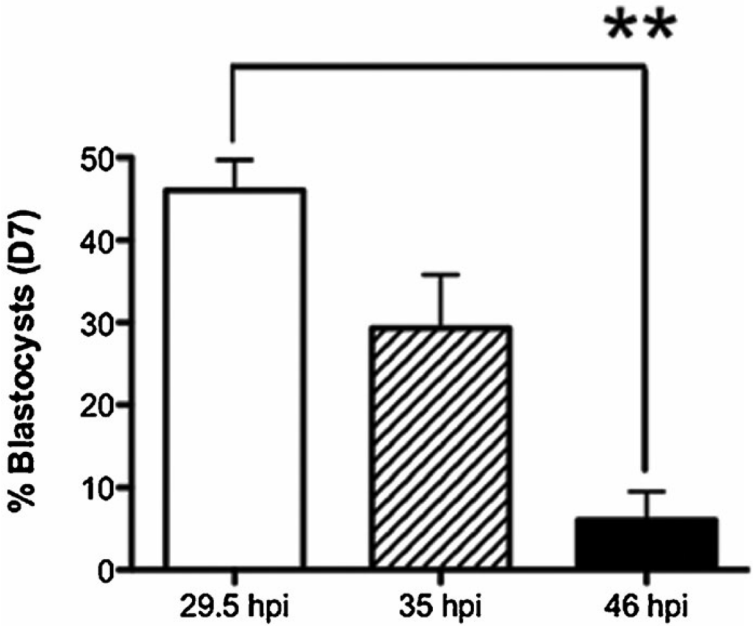

Figure 2-1 Impact of cleavage timing on blastocyst production
Mean ± SEM of seven independent experiments. Effect of time on blastocyst rate was detected by Kruskal-Wallis test (P=0.0016). Significant differences between groups (pgirmess in R package): P<0.01 (**). SEM, standard error of the mean; D7, day 7; hpi, hours post-insemination.

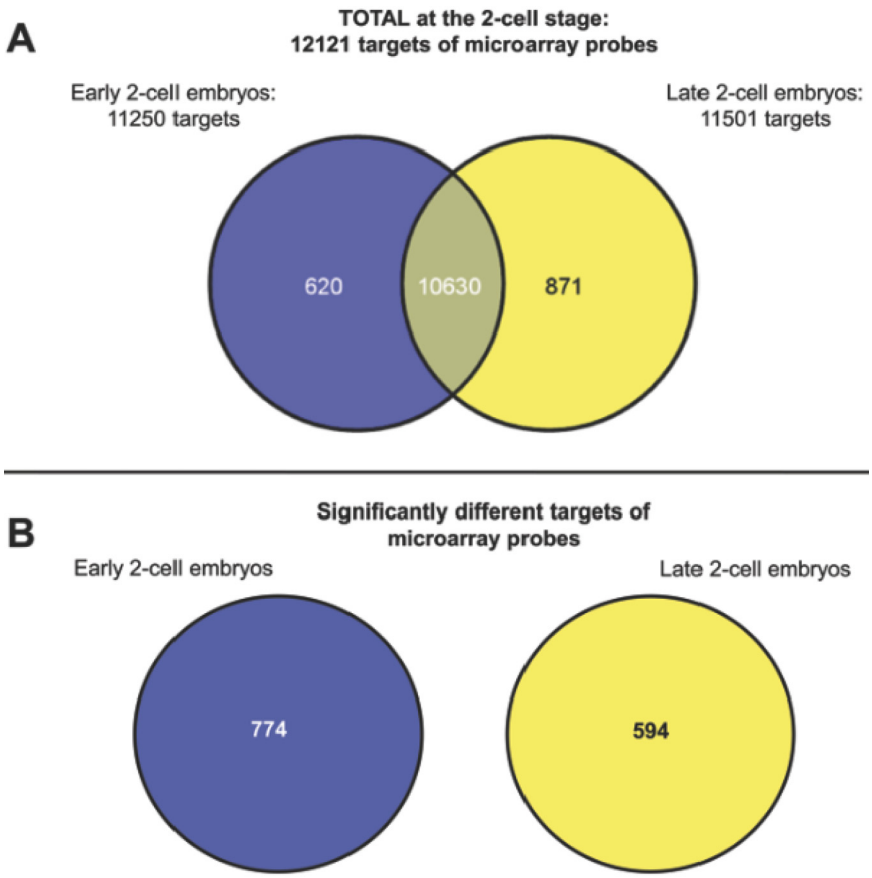

Figure 2-2 Overall comparison by microarray of gene transcript targets present at the 2-cell stage

A) 12,121 targets had an intensity over the background level when considering an intensity >150.0 in both embryo subpopulations, from which 10,630 were shared between fast- and slow-cleaving 2-cell embryos, while 620 were exclusive to early embryos and 871 were present in late embryos only. B) Target abundance level was considered significantly different between early and late 2-cell embryos when FC ≥1.5 fold, P <0.05, and mean across conditions ≥7.0. In the early group, 774 targets were higher, whereas 594 were increased in late 2-cell embryos. Diagram constructed with VENNY tool (Oliveros 2007).

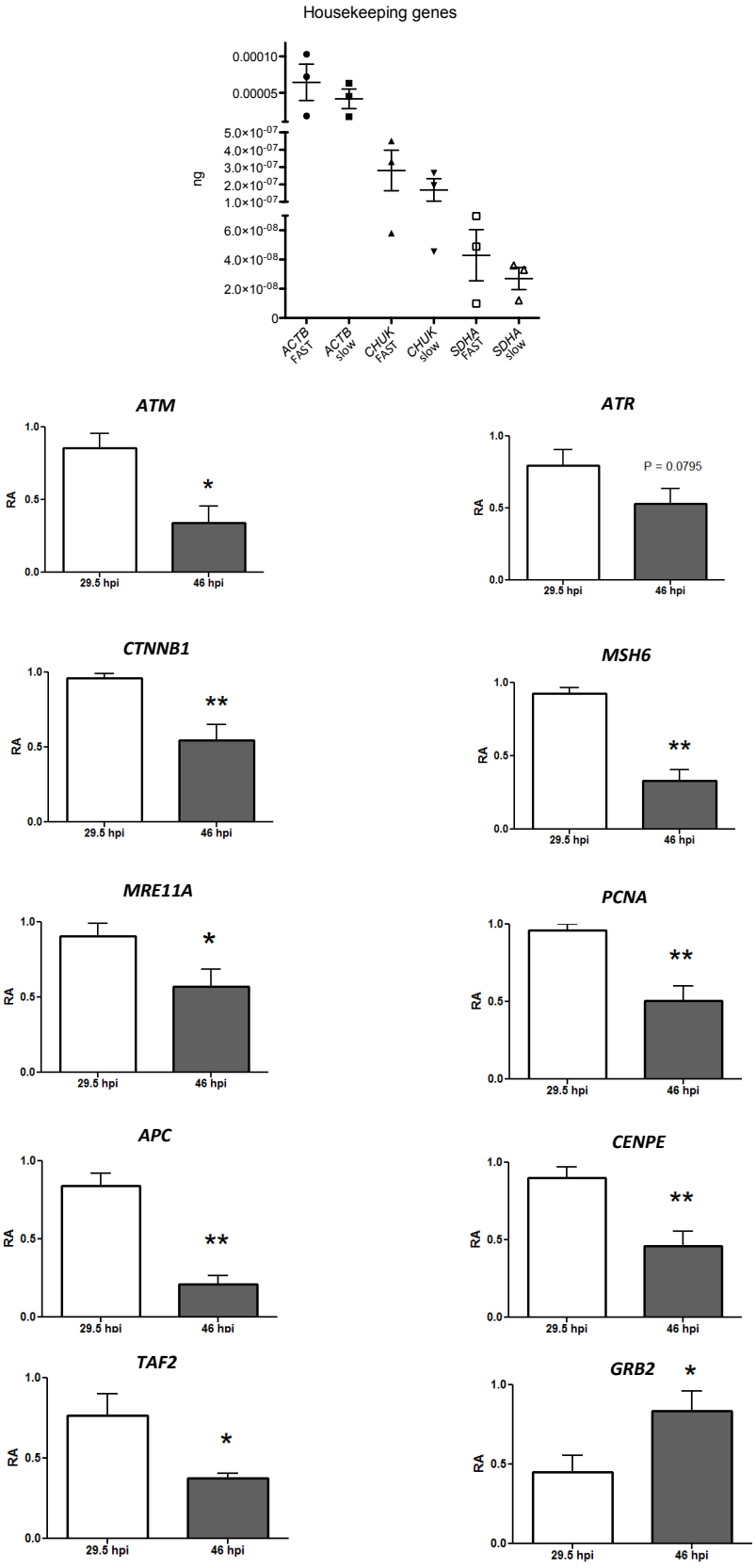
Housekeeping genes
ng
0.00010
0.00005
5.0×10-07
4.0×10-07
3.0×10-07
2.0×10-07
1.0×10-07
6.0×10-08
4.0×10-08
2.0×10-08
0
ACTB FAST
ACTB slow
CHUK FAST
CHUK slow
SDHA FAST
SDHA slow
ATM
ATR
P = 0.0795
CTNNB1
MSH6
MRE11A
PCNA
APC
CENPE
TAF2
GRB2
RA
1.0
0.5
0.0
29.5 hpi
46 hpi
*
**

Figure 2-3 RT-qPCR validation of transcript abundance in fast- (29.5 hpi) and slow-cleaving (46 hpi) 2-cell embryos

Mean ± standard error of the mean (SEM) of three independent replicates of 5-10 2-cell embryos. Differences were considered to be significant when $P < 0.05$ (unpaired one-tailed t–test). RA, relative abundance; hpi, hours post-insemination. *ACTB*, Actin, beta; *ATM*, Ataxia-telangiectasia mutated; *ATR*, Ataxia-telangiectasia and Rad3-related; *APC*, Adenomatous polyposis coli; *CENPE*, Centromere protein E; *CHUK*, Conserved helix-loop-helix ubiquitous kinase; *CTNNB1*, Beta-catenin-1; *GRB2*, Growth factor receptor-bound protein 2; *MRE11A*, Meiotic recombination 11 homolog A (*S. cerevisiae*); *MSH6*, MutS homolog 6 (*E. coli*); *PCNA*, Proliferating cell nuclear antigen; *SDHA*, Succinate dehydrogenase complex, subunit A; *TAF2*, RNA polymerase II, TATA box binding protein (TBP)-associated factor, 150 kDa.

.

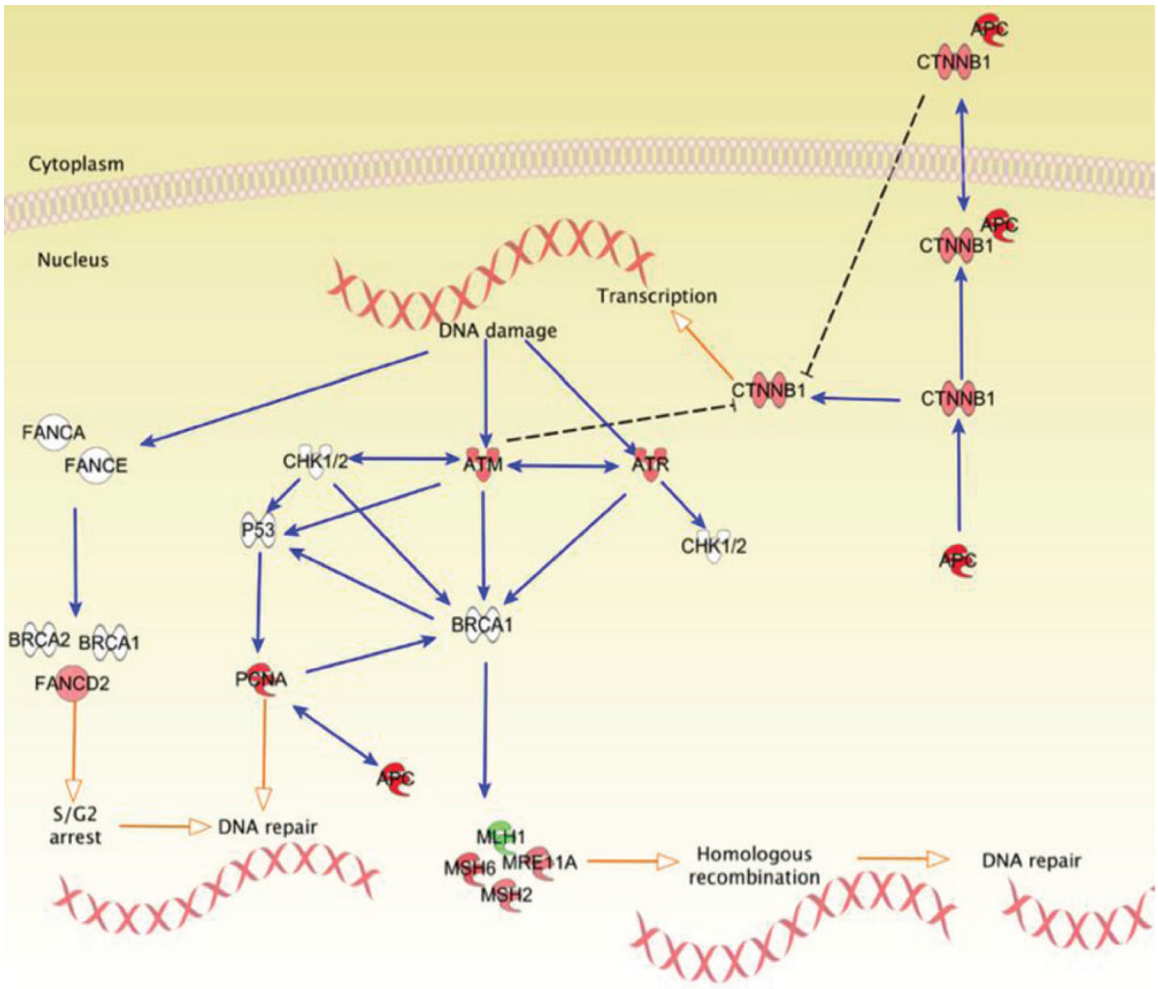

Figure 2-4 ATM-related pathway
Molecular relationships based on regulation between gene products. Red and green symbols show genes whose mRNAs were respectively higher and lower in fast-cleaving 2-cell embryos. Pink symbols correspond to molecules with a tendency to be increased in early embryos. White symbols are molecules not significantly different. ATM, Ataxia-telangiectasia mutated; ATR, Ataxia-telangiectasia and Rad3-related; APC, Adenomatous polyposis coli; BRCA1/2, Breast cancer early onset 1/2; CHK1/2, Checkpoint kinase 1/2; CTNNB1, Beta-catenin-1; FANCA/D2/E, Fanconi anemia, complementation group A/D2/E; MLH1, MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli); MRE11A, Meiotic recombination 11 homolog A (*S. cerevisiae*); MSH2, MutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli); MSH6, MutS homolog 6 (E. coli); P53, Tumor protein P53 (TP53); PCNA, Proliferating cell nuclear antigen; PRKDC, Protein kinase, DNA-activated, catalytic polypeptide (DNA-PK). Diagram constructed with IPA software.

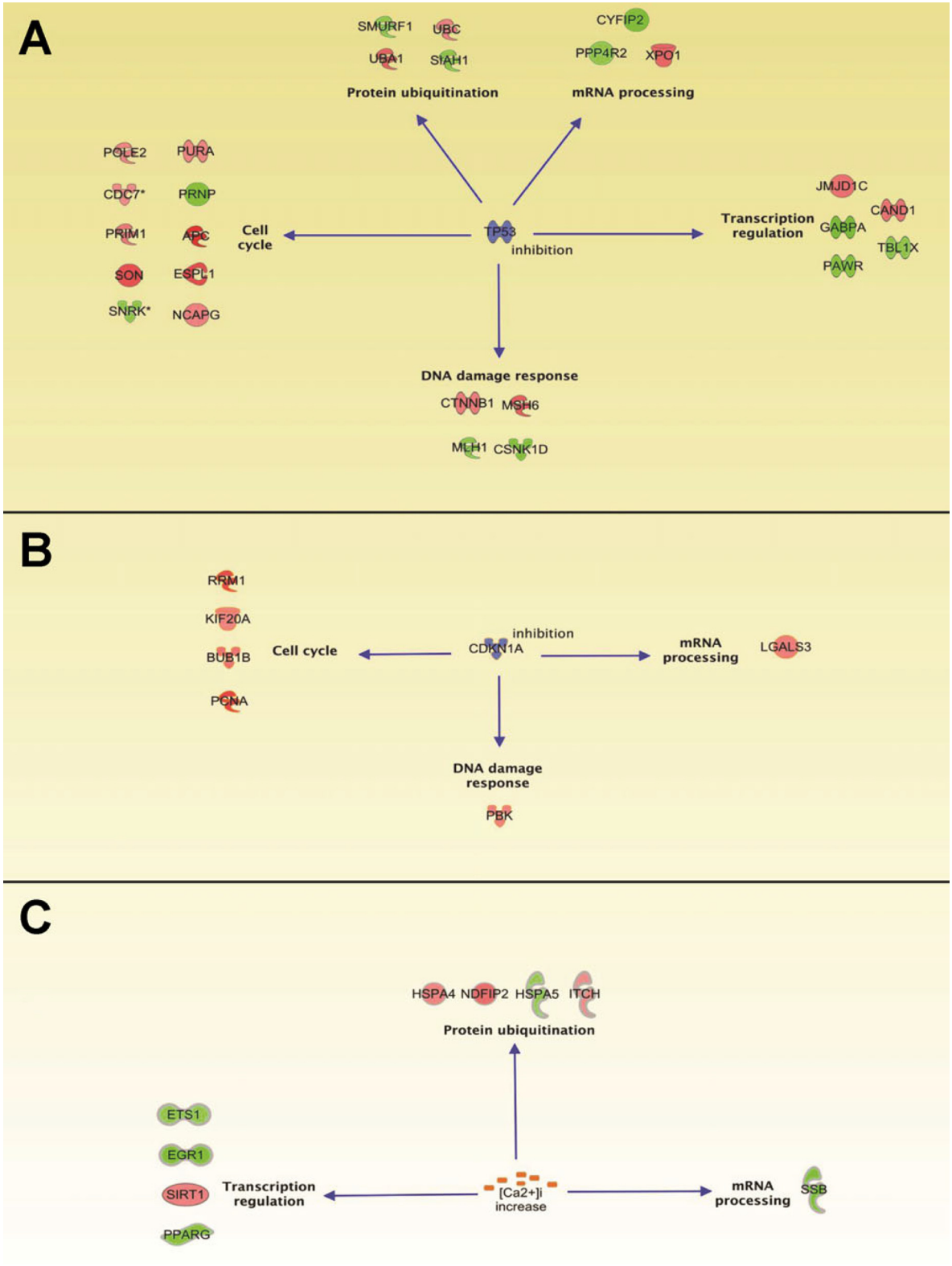
A
SMURF1
UBC
UBA1
SIAH1
Protein ubiquitination
CYFIP2
PPP4R2
XPO1
mRNA processing
POLE2
PURA
CDC7*
PRNP
PRIM1
APC
SON
ESPL1
SNRK*
NCAPG
Cell cycle
TP53
inhibition
Transcription regulation
JMJD1C
CAND1
GABPA
TBL1X
PAWR
DNA damage response
CTNNB1
MSH6
MLH1
CSNK1D
B
RRM1
KIF20A
BUB1B
PCNA
Cell cycle
inhibition
CDKN1A
mRNA processing
LGALS3
DNA damage response
PBK
C
HSPA4
NDFIP2
HSPA5
ITCH
Protein ubiquitination
ETS1
EGR1
SIRT1
PPARG
Transcription regulation
[Ca2+]i increase
mRNA processing
SSB

Figure 2-5 Upstream regulator prediction
A) TP53 inhibition. B) CDKN1A inhibition. C) $[Ca^{2+}]i$ increase. Blue and orange symbols denote inhibited and activated regulators, respectively. Red and green symbols show genes whose mRNAs were respectively higher and lower in fast-cleaving 2-cell embryos. APC, Adenomatous polyposis coli; BUB1B, BUB1 mitotic checkpoint serine/threonine kinase B; $[Ca^{2+}]i$, intracellular calcium ion concentration; CAND1, Cullin-associated and neddylation-dissociated 1; CDC7, Cell division cycle 7; CDKN1A, cyclin-dependent kinase inhibitor 1A (p21, Cip1); CSNK1D, Casein kinase 1, delta; CTNNB1, Beta-catenin-1; CYFIP2, Cytoplasmic FMR1-interacting protein 2; EGR1, Early growth response 1; ESPL1, Extra spindle pole bodies homolog 1 (*S. cerevisiae*); ETS1, V-ets erythroblastosis virus E26 oncogene homolog 1 (avian); GABPA, GA binding protein transcription factor, alpha subunit 60 kDa; HSPA4, Heat shock 70 kDa protein 4; HSPA5, Heat shock protein 5 (78 kDa); ITCH, Itchy E3 ubiquitin protein ligase; JMJD1C, Jumonji domain-containing 1C; KIF20A, Kinesin family member 20A; LGALS3, Lectin, galactoside-binding, soluble, 3; MLH1, MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli); MSH6, MutS homolog 6 (E. coli); NCAPG, Non-SMC condensin complex, subunit G; NDFIP2, Nedd4 family-interacting protein 2; PAWR, PRKC, apoptosis, WT1, regulator; PBK, PDZ-binding kinase; PCNA, Proliferating cell nuclear antigen; POLE2, Polymerase (DNA directed, epsilon 2, accessory subunit); PPARG, Peroxisome proliferator-activated receptor gamma; PPP4R2, Protein phosphatase 4, regulatory subunit 2; PRIM1, Primase, DNA, polypeptide 1 (49 kDa); PRNP, Prion protein; PURA, Purine-rich element binding protein A; RRM1, Ribonucleotide reductase M1; SIAH1, Siah E3 ubiquitin protein ligase 1; SIRT1, Sirtuin 1; SMURF, SMAD-specific E3 ubiquitin protein ligase 2; SNRK, SNF-related kinase; SON, SON DNA binding protein; SSB, Sjogren syndrome antigen B (autoantigen La); TBL1X, Transducin (beta)-like 1X-linked; TP53, Tumor protein P53; UBA1, Ubiquitin-like modifier activating enzyme 1; UBC, Ubiquitin C; XPO1, Exportin 1 (CRM1 homolog, yeast). Diagram constructed with IPA software.

3. The Knockdown of ATF1 and ATF2 Transcripts in Germinal Vesicle Oocytes Reveals their Crucial Roles in Bovine Early Development

Ernesto Orozco-Lucero,[1] Isabelle Dufort,[1] Claude Robert[1] and Marc-André Sirard[1]

[1]Centre de Recherche en Biologie de la Reproduction (CRBR), Faculté des Sciences de l'Agriculture et de l'Alimentation, Département des Sciences Animales, Pavillon INAF, Université Laval, Québec, Qc, Canada

3.1. Résumé

L'association entre la qualité de l'ovocyte et la capacité développementale est bien démontrée chez les grands mammifères. Plus spécifiquement, nous savons que l'avenir de l'embryon précoce est directement relié aux transcrits stockés dans le cytoplasme de l'ovocyte. Dans une étude précédante, nous avons identifié des molécules candidates associées avec des embryons bovins qui clivent précocement et avec une haute compétence développementale. Ces molécules incluent ATF1, un membre de la famille CREB. L'objectif de cette étude a été d'évaluer les effets du « knock-down » des ARNm de *ATF1* et *ATF2* sur l'embryogenèse précoce chez le bovin. Des ovocytes au stade GV furent micro-injectés avec des morpholino oligonucléotides ciblant soit *ATF1* (ATF1mo), *ATF2* (ATF2mo) ou sans capacité de liaison (STDmo). Lors de IVF/IVP standard, le taux de clivage a diminué dans le groupe ATF2mo ($P < 0.05$). Le taux de blastocyste/8-16 cellules était plus bas après le knockdown de *ATF1* ($P < 0.01$) et le taux général de blastocyste a aussi été réduit avec ATF1mo ($P < 0.05$) et ATF2mo ($P > 0.05$). Une baisse de l'expression nucléaire de ATF1a été détectée par immunofluorescence dans des embryons à 8-cellules ($P < 0.05$) de même que les niveaux totaux de ATF1 détectés par Western blot (WB) qui ont chuté pareillement chez les embryons ATF1mo ($P = 0.1467$) et ATF2mo ($P = 0.0171$) au même stade. Par contre, ATF1mo a augmenté le signal de ATF2 en WB ($P = 0.0502$). Les niveaux d'ARNm de *ATF1* ($P = 0.0403$), *ATF3* ($P = 0.0563$), *KLF4* ($P = 0.0134$) et *SP1* ($P = 0.0145$) ont été réduits dans les embryons à 8-cellules suivies à la réduction de ATF1. Ces résultats suggèrent que le blocage de la traduction de ATF1 et ATF2 à partir du stage GV altère le développement préimplantatoire chez les bovins et supporte l'hypothèse que les embryons avec une quantité accrue de ces facteurs de transcription sont plus compétents, possiblement à travers de leur capacité de moduler l'activation du génome embryonnaire.

3.2. Abstract

The association between oocyte quality and developmental capacity is well demonstrated in large mammals and more precisely, the outcome of early embryos is directly linked to transcripts stored in the ooplasm. Previous work identified candidate molecules linked with early cleaving-bovine embryos with higher developmental competence, including ATF1 (CREB family). The objective was to assess the effects of knocking-down *ATF1* and *ATF2* mRNAs on early cattle embryogenesis. GV-oocytes were microinjected with morpholinos targeting either *ATF1* (ATF1mo), *ATF2* (ATF2mo), or with no binding activity (STDmo). Upon standard IVF/IVP, the cleavage rate diminished in the ATF2mo group ($P < 0.05$). The blastocyst/8-16 cell percentage decreased after *ATF1* knockdown ($P < 0.01$), while the overall blastocyst rate also diminished with ATF1mo ($P < 0.05$), and ATF2mo ($P > 0.05$). A decrease in ATF1 nuclear protein was detected by immunofluorescence in ATF1mo 8-cell embryos ($P < 0.05$), whereas the total ATF1 levels detected by Western blot also diminished in ATF1mo ($P = 0.1467$) and ATF2mo ($P = 0.0171$) embryos at the same stage. Contrastingly, ATF1mo raised the WB signal of ATF2 ($P = 0.0502$). The mRNA levels of *ATF1* ($P = 0.0403$), *ATF3* ($P = 0.0563$), *KLF4* ($P = 0.0134$), and *SP1* ($P = 0.0145$) were diminished in 8-cell embryos upon ATF1 reduction. These results suggest that blocking the translation of ATF1/2 from the GV-stage impairs preimplantation development in bovines and support the hypothesis that embryos with higher amounts of these transcription factors are more competent, possibly through their capacity to modulate embryonic genome activation.

3.3. Introduction

Oocyte quality remains one of the most important challenges in human and animal reproductive technologies (ART). Despite years of effort, the understanding of what makes a good egg and results in a healthy embryo is still incomplete. Concerning the maternal inheritance driving the developmental capacity of oocytes and cleavage-stage embryos before activation of their genome, proteins and transcripts originated in the ooplasm are considered to be crucial for the embryo's fate (rev. Krisher, 2004; Orozco-Lucero and Sirard, 2014; Sirard 2010). Multiple reports in both model and domestic animals indicates variable abundance levels of specific mRNAs in oocytes or embryos, according to the competence status discerned through distinct criteria (rev. Wrenzycki et al. 2007; Labrecque and Sirard 2014), including cleavage timing (Orozco-Lucero and Sirard 2014). It has been previously determined that the mRNA levels of the c-AMP Response Element Binding protein (CREB) family member, Activating Transcription Factor 1 (*ATF1*), are higher in the most competent 2-cell bovine embryos according to their cleavage timing (Orozco-Lucero et al. 2014). Moreover, Held et al. (2012) found that *ATF1* transcripts are higher in the sister blastomere of the most competent 2-cell embryos in cattle. Both reports might suggest a putative developmental advantage for those cleavage-stage embryos of this species with a surplus of *ATF1* mRNA. Moreover, the transcript levels of *ATF1* appear to be regulated during bovine preimplantation development, where apparently the most prominent change in its abundance pattern occurs just before embryonic genome activation, EGA (Vigneault et al. 2004). On the other side, the protein of another constituent of the CREB family of transcription factors (TFs), ATF2, was observed by Vigneault et al. (2009) to translocate from cytoplasm to nuclei just prior to the 8-cell stage in cattle, the moment when EGA takes place in this species. Given such information it is plausible to imply the need for ATF2 protein inside the nucleus in preparation of EGA in cattle. Therefore, all these findings bring attention to the potential roles of ATF1 and ATF2, as well as those of the molecular class that they belong to, during early development.

ATF/CREB family TFs are a large group of basic leucine zipper (bZIP) proteins that in spite of their variable physiological functions have the ability to respond to environmental stimuli, as growth factors and cAMP, in order to sustain cellular homeostasis through

transcriptional regulation. Furthermore, ATF proteins are frequently implicated in developmental mechanisms, including carcinogenesis and embryonic processes (rev. Vigneault et al. 2004; Vlahopoulos et al. 2008), which appears to be in agreement with the notion of the potential determinant roles of ATF1 and ATF2 during early development. More specifically, ATF1 has been demonstrated to be required for embryo survival in rodents given that it works closely in concert with the central transcription modulator CREB (Bleckmann et al. 2002). Subsequently, evidence of the preponderant gene regulatory actions of ATF1 spanning to the embryonic context arose from Jin and O'Neill's reports, where it was corroborated that ATF1 is necessary for zygotic genome activation, ZGA (Jin and O'Neill 2010) and tightly modulated in mouse early embryos (Jin and O'Neill 2014). In a different way, another member of the CREB family, ATF2, might not only function in gene regulation but also indirectly exert a major impact on cell cycle control since it modulates the transcription of *CCND1* and *CCNA*, which have preponderant roles during G1 and S-phases of the cell cycle, respectively (rev. Vlahopoulos et al. 2008). Consequently, it might be plausible that the combined action of both ATF1 and ATF2 pathways are instrumental for the control of EGA and the first cleavages in mammalian embryos before this crucial developmental time point. Therefore, it could be postulated that oocytes and cleaving embryos with higher capacity to readily translate either ATF1 or ATF2, due to an enhanced abundance of their respective mRNAs from maternal origin, are better endowed with developmental competence. To test this hypothesis, immature oocytes from cattle were subjected to either *ATF1* or *ATF2* knockdown and the developmental phenotype and molecular signature of the resulting embryos were assessed. The current results suggest pivotal roles of both TFs during the preimplantation period in mammals.

3.4. Materials and Methods

All chemicals were from Sigma-Aldrich (St. Louis MO), unless otherwise stated.

3.4.1. *In vitro* production of bovine blastocysts

Oocyte collection was performed from slaughterhouse cow ovaries, followed by *in vitro* maturation (IVM), fertilization (IVF), and culture (IVC) according to standard techniques (Ashkar et al. 2010), although steer serum was used at 10%.

3.4.2. Microinjection of oocytes

Morpholino oligonucleotides (MOs) targeting the 5'-UTR of either *ATF1* (ATF1mo) or *ATF2* (ATF2mo) from cattle, or with no binding activity (Standard [negative] Control oligo, STDmo) were purchased from Gene Tools, LLC. (Philomath OR). The MOs sequences (5' to 3') were: ATF1mo, AGAATCTTCCATAATCAACTGTGGC; ATF2mo, CATAAGCTGAACAACTTATCACGTC; and CCTCTTACCTCAGTTACAATTTATA for STDmo. Immediately before microinjection MOs were individually prepared to a final concentration of 0.966 mM mixed with 4 mg/mL dextran Texas red (dTXr), 10,000 MW (Mol. Probes, Burlington ON). Water was used to resuspend both MOs and dTXr. Borosilicate micropipettes were prepared with a P-97 puller (Sutter Instr., Novato CA) and a microforge (Narishige, East Meadow NY). The holding pipette was burn-polished and had an external diameter (ED) of 105-120 μm and an internal diameter of 60-85 μm. This needle was coupled to a hydraulic control (Narishige) filled with silicone oil. The injection pipette had a maximal ED of 1-1.5 μm. This needle was coupled to an air pneumatic control (Narishige). Both kind of pipettes had a 25-30° taper angle. Immediately following ovarian aspiration, full cumulus-enclosed GV-oocytes were placed in maintenance medium (same for standard IVM but omitting hormones and including 3.5 IU heparin) at 38.5°C/5%CO_2 when awaiting microinjection. Microinjection medium was modified from the method of Christian Vigneault (Boviteq, St-Hyacinthe, QC) and contained Medium 199 Hanks-Hepes (Gibco, Burlington ON) adjusted at 273 mOsm and supplemented with 2.5 mM NaOH, 10% (v/v) steer serum, as well as 0.2 mM pyruvate and 0.1 mM cysteamine. For every 20-50 COCs 150 μL-droplets of microinjection medium were prepared on a lid of a 60 mm-Petri dish covered with 9 mL of silicone oil and placed on the microscope stage at 37°C. The COCs of a single treatment were microinjected at a time, whereas the rest of the groups remained at 37°C/5%CO_2 to diminish exposure to a detrimental environment. Uninjected COCs were left outside of the incubator at the same time as a microinjected group. The order of manipulation of the groups was alternated between experiments. For microinjection COCs were fixed through the cumulus to the opening of the holding pipette (Paradis et al. 2005; Favetta et al. 2007), whereas the injection needle was broken close to the tip on the same droplet immediately before starting the run. Every oocyte was injected with an estimated volume of 4-8 pL. To corroborate accuracy of microinjection,

epifluorescence was used at a submaximal intensity and only for 1-3 seconds to avoid damage due to UV light exposure (Fig. 3-1). At the end of microinjection each group of COCs was washed twice and kept in maintenance medium inside the incubator until all groups were injected. At that time all groups were washed four times and cultured in standard IVM with hormones and without heparin.

3.4.3. Estimation of the effect on phenotype

Cleavage, 8-16 cell (both assessed at 94 hpi), and day 8-blastocyst (192 hpi) rates were calculated by dividing the total number of either cleaved, 8-16 cell embryos, or day 8-blastocysts by the total number of viable presumptive zygotes in every treatment by the end of IVF. The blastocyst/8-16 cell embryo rate was calculated by dividing the number of day 8-blastocysts by the amount of 8-16 cell embryos per group. Hatching rate was the result of dividing hatched day-8 blastocysts by either viable zygotes or 8-16 cell embryos. The effect of MO microinjection was analyzed through one-way ANOVA and Newman-Keuls *post-hoc* test.

3.4.4. Western blot

SDS-PAGE was performed according to standard procedures (Laemmli 1970). 150 cumulus-denuded oocytes or 2-cell embryos, as well as 50 4-cell embryos were loaded per electrophoresis lane for protein profiling throughout stages. For knockdown evaluation, embryos composed of 6-8 cells at 94 hpi were considered early 8-cell embryos and used for the current study, whereas embryos of 10 cells and more were regarded as late 8-cell embryos and excluded from the experiments. Three independent replicates of 35 embryos at the early 8-cell stage were used. Western blotting was carried out as in Scantland et al. (2011). The primary antibodies for ATF1 and ATF2 were AF4370 (R & D Systems, Minneapolis MN) and sc-6233 (Santa Cruz Botech., Dallas TX), respectively, both diluted at 1:100. For localization of the housekeeping protein the 4967 B-actin antibody was used (Cell Signaling Tech., Whitby ON) at a 1:500 dilution. Secondary antibodies were a goat anti-mouse (for ATF1), donkey anti-rabbit (for ATF2 and beta-actin) HRP-conjugates (Mol. Probes) diluted at 40 ng/mL. Blots were processed for ATF1 and beta-actin localization first after which they were mildly stripped (Dostaler-Touchette et al. 2009).

Absence of any remaining signal was assessed and then blots were re-blocked and re-hybridized for ATF2 detection. Statistical analysis performed through one-tailed unpaired T-test.

3.4.5. Immunofluorescence

Protein assessment through immunofluorescence (IF) was carried out as described by Pagé-Larivière and Sirard (2014). The primary antibody consisted on sc-243 (Santa Cruz Biotech.) diluted at 1:300, whereas the secondary antibody was a goat anti-mouse AF488-conjugate (Mol. Probes) diluted at 2 µg/mL. All nuclei of three (Uninjected, STDmo) or five (ATF1mo) different embryos were analyzed. All pictures were taken at 12% laser power and 7.5 gain. For densitometry assessment ImageJ software was used to estimate the nuclear/cytoplasmic ratio of ATF1 signal. One-way ANOVA and Newman-Keuls *post-hoc* test were employed for statistical analysis.

3.4.6. Determination of differential transcript levels in 8-cell embryos

To collect 8-cell embryos for RT-qPCR it was employed the same criterion to grade developmental stage as previously in IF and WB. Total RNA from three biological replicates (5-10 early 8-cell embryos/replicate) was reverse-transcribed with oligo-dT as previously described (Orozco-Lucero et al. 2014). Every 20 µl-reaction contained the equivalent of 0.3 embryo. Data were normalized with the values of the exogenous control, GFP spike-in (Vigneault et al. 2004). Primer sequences, product sizes, and annealing temperatures are shown in Table 3-1.

3.4.7. Transcription factor binding sites prediction

The Vertebrata algorithm at a relative profile score threshold of 80-98% of the JASPAR software (http://jaspar.genereg.net) was employed to detect putative ATF1, ATF2, and SP1 transcription factor binding sites (TFBS) in the selected promoters. To obtain the input promoter sequences the EmbryoGENE Genome Browser (http://emb-bioinfo.fsaa.ulaval.ca) was interrogated by requesting 1000 bases of promoter/upstream in the option "Genomic Sequence".

3.5. Results

3.5.1. ATF1 and ATF2 protein expression through different stages in bovines

When performing a knockdown of a protein-coding transcript with the purpose of abating the levels of the respective protein and impeding its function, either through exogenous small-interfering RNAs (siRNA) that drive the target mRNA to degradation, or by using morpholino oligonucleotides (MO), which block translation from the mRNA, it is crucial to determine the absence or presence (and its relative abundance levels) of the target protein. In order to know when the protein form of either ATF1 or ATF2 is present prior to fertilization and during cleavage stages, WB analysis of cattle germinal vesicle (GV)-oocytes, 2-, and 4-cell embryos was performed. The results indicated that ATF1 is absent in immature oocytes but is readily translated at important levels at or before the 2-cell stage, as demonstrated by the presence of a band of approximately 70 kDa, also detected in 4-cell embryos (Fig. 3-2A). In contrast, eggs prior to maturation were found to already contain the translated ATF2, corresponding to a single band with an estimated size of 70-75 kDa. This molecule remained at the protein level up to the 4-cell stage, when it started to diminish its expression (Fig. 3-2B).

3.5.2. Effects of morpholino oligonucleotides against ATF1 and ATF2 on development

The effects of ATF1 and ATF2 knockdown by MO on cattle early development were assessed through the analysis of developmental rates. While the microinjection of MO targeting ATF2 significantly diminished cleavage rate in comparison to all other groups ($P < 0.05$; Fig. 3-3A) the microinjection did not produce significant differences in the percentage of 8-16 cell embryos between groups (Fig. 3-3B). On the other side, MO microinjection showed an impact over the overall blastocyst rates when considering the number of non-lysed presumptive zygotes ($P = 0.0023$; Fig. 3-3C), or the total amount of embryos that reached the 8-cell stage ($P = 0.0058$; Fig. 3-3D). A significant decrease in blastocyst rates was noticed when MO against ATF1 were microinjected and this was true based on total non-lysed presumptive zygotes ($P < 0.05$) or the total amount of embryos that reached/surpassed the 8-cell stage ($P < 0.01$). In relation to the knockdown of ATF2, the overall blastocyst rate tended to decline when ATF2mo was compared against STDmo (Fig. 3-3C; $P > 0.05$) or uninjected ($P < 0.05$). Hatching rates of day-8 blastocysts did not differ

between treatments (Fig. 3-3E, 3-3F). Concerning general morphology, no differences were readily detected between treatment groups at the blastocyst (Fig. 3-4) stage of embryos that survived microinjection.

3.5.3. Western blot of microinjected 8-cell embryos

After observing the impact on cattle preimplantation development upon microinjection of MO against either *ATF1* or *ATF2*, we tried to determine if such effect was due to changes in the levels of the protein in 8-cell embryos. Given the hypothetical limit to accurately validate the efficient knockdown of the target mRNA by RT-qPCR upon use of MO (rev. Deleavey and Damha 2012), it was rather measured by the relative abundance levels of the codified protein by WB (Fig. 3-5A). ATF1mo ($P = 0.1467$) and ATF2mo ($P = 0.0171$) diminished the ATF1 protein levels in 8-cell bovine embryos (Fig. 3-5B). As for ATF2 a significant increase ($P = 0.0502$) of ATF2 protein abundance in 8-cell embryos from the ATF1mo group (Fig. 3-5C) was observed.

3.5.4. Immunofluorescence of microinjected 8-cell embryos

To confirm WB results, the protein expression of ATF1 was compared by immunofluorescence in nuclei of early 8-cell embryos microinjected with MO (Fig. 3-6A). The signal of ATF1 was significantly lower ($P < 0.05$) in embryos produced after specifically targeting ATF1 with MO (Fig. 3-6B).

3.5.5. Transcription factor binding sites prediction

The *in silico* analysis of putative TFBS generated results are shown in Table 3-2. Briefly, the promoters of *Bos taurus ARNT*, *ATF1*, *ATF4*, *DBF4*, *ELAVL1*, *SP1,* and *TP53*, contained binding sites for ATF1; ATF2 was predicted to target *ARNT*, *ATF1*, *CCNA2*, *DBF4*, *GADD45A*, *SP1*, *TP53*, promoters; whereas SP1 had putative TFBS in *DAZL*, *HSPA1A*, *MYC*, and *OCT4* promoters.

3.5.6. RT-qPCR analysis of microinjected 8-cell embryos

With the purpose of validating the molecular effects of either ATF1 or ATF2 ablation by MO microinjection, the mRNA abundance levels of a group of downstream candidate target

genes was quantified (Fig. 3-7). An exogenous control *GFP* spike-in was used for both corroborating reproducibility among treatments at the end of the RT-qPCR process, and for normalizing the abundance levels of the tested endogenous transcripts instead of using housekeeping genes (HKGs) as normalizing factors. This was due to the difficulty to find HKGs whose transcripts show stability around the period of EGA in cattle (Vigneault et al. 2007; Gilbert et al. 2009; Robert 2008; Ross et al. 2010), which corresponds to the scope of the RT-qPCR analysis in this work since 8-cell embryos were assessed for transcriptomic profiling. The exogenous control *GFP* spike-in showed no significant differences among the four groups of 8-cell embryos originated after microinjection, which accounts for the technical accuracy of the RT-qPCR between treatments. On the other hand, the mRNA levels of the housekeeping genes (HKGs) *ACTB*, *UBE2K* and *YWHAG*, which were quantified here without purposes of being used as factors for normalization but rather to demonstrate the absence of side effects of the MO, did not vary significantly either. Furthermore, the transcript abundance of a number of downstream candidate targets of ATF1 and ATF2, namely *SUPT4H1*, *ARNT*, and *TP53* did not differ between embryo groups, whereas both *ATF3* and *DBF4* (uninjected vs. ATF1mo, $p = 0.0563$ and $p = 0.0903$, respectively) mRNAs showed tendency to diminish upon ATF1 ablation. Moreover, *KLF4* (ATF1mo vs. ATF2mo, $p = 0.0134$) and *SP1* (uninjected vs. ATF1mo, $p = 0.0145$; ATF1mo vs. ATF2mo, $p = 0.0128$; STDmo vs. ATF1mo, $p = 0.059$) transcripts decreased in embryos injected with the MO targeting ATF1. Regarding the effects of ATF1 and ATF2 depletion on the levels of their own transcripts at the 8-cell stage, there were no changes in *ATF2* mRNA abundance, whereas the *ATF1* transcript significantly decreased in embryos microinjected with MO against sequences within its own 5'-UTR (Uninjected vs. ATF1mo, $P = 0.0403$).

3.6. Discussion

The current work highlights the importance of ATF1 and ATF2 proteins in early embryogenesis in bovines and reveals a potential explanation for their respective roles. The putative functions of both molecules imply their importance in the modulation of cell division during the early embryonic cleavage stage, as well as their involvement in the control of the preponderant developmental step of EGA. First, the significant decrease in

cleavage rate as well as the trend to diminish the amount of embryos that progress beyond EGA when ATF2 was targeted suggests a permissive role of this protein in early cell cycle regulation. This biological function has been suggested to be pivotal for progression during preimplantation development in mammals (Sirard 2010; Pers-Kamczyc et al. 2012; Orozco-Lucero et al. 2014; Orozco-Lucero and Sirard 2014). Moreover, this notion has been confirmed by multiple reports with the ablation, pharmacological inhibition, and overexpression of factors involved in cell cycle regulation throughout species, including mice (Buffone et al. 2009; Schindler et al. 2012; Wang et al. 2013; De La Fuente et al. 2015) and cattle (Nganvongpanit et al. 2006; Paradis et al. 2005; Toralova et al. 2009; Yang et al. 2014; Lee et al. 2015), and even in distant vertebrate taxa as in the survey from Collart et al. (2013) in frog embryos.

The possible involvement of ATF2 in cell cycle control is suggested by the report by Maekawa et al. (2008) where ATF2 activated *Gadd45a* transcription in mouse breast tumour. GADD45 protein has been found to bind to PCNA in human cultured cells, suggesting GADD45 involvement in DNA replication and/or repair (Smith et al. 1994). Moreover, ATF2 targets *PTEN* promoter in human endothelial cells (Shen et al. 2006). PTEN has been recently implicated in follicle activation in bovine ovaries that involves cell cycle regulation potentially through CDC20 and MAP16 (Yang and Fortune 2015). On the other side, *CCND1* and *CCNA* might also be regulated by ATF2. *Ccnd1* transcription is induced by P38-ATF2 signalling in murine melanoma cells (Recio and Merlino 2002), and *Ccnd1* expression was raised in mouse chondrocytes due to direct promoter targeting by ATF2 (Beier et al. 1999). Finally, Breitwieser et al. (2007) demonstrated that ATF2 triggers a negative feedback loop leading to P38-MAPK dephosphorylation and inactivation in mouse liver. P38-MAPK has been implicated in cell cycle control (regulation of Cdk4, Ccnd2 mRNAs) in mouse heart (del Barco Barrantes et al. 2011), whereas Suhail et al. (2015) discovered that P38-MAPK is involved together with TACC3, P53, and P21 in stress-induced signalling leading to G1-arrest in cultured human cells. Although *CDK4*, *CCND2*, or *CCNA2* would have made interesting targets to measure, none of the above-mentioned cell cycle control genes were assessed here due to the lack of data concerning

their transcript in cattle embryos, or given the low levels of their mRNA around the time of EGA time in cattle (Graf et al. 2014).

In our results, whereas no direct morpholino effects were observed on its own transcript it was surprising to find that ATF1 protein decreased in ATF2mo embryos, suggesting that ATF2 might influence ATF1 directly at the protein level without modifying transcription incidence. This scenario appears to point out to either translation regulation or to mRNA stability of ATF1. As previously mentioned, ATF2 may inactivate P38-MAPK (Breitwieser et al. 2007). If this is also true in the preimplantation context, it could affect *ATF1* mRNA stability. Chen et al. (2010) indicated that inhibition of P38-MAPK during the differentiation of mouse ESCs toward cardiac cells downregulated *Nkx2.5* (*Csx1*, *S. pombe*) mRNA, while Csx1 plays a role in *Atf1* transcript turnover in yeasts (Rodriguez-Gabriel et al. 2003). Furthermore, Day and Veal (2010) observed that Sty1 (homologue of the mammalian P38-MAPK) regulates *Atf1* mRNA stability. Thus, the role of P38-MAPK in a feedback loop modulating ATF1 transcript turnover/translation rate could be an interesting possibility to investigate in mammals.

In relation to the nature of the *ATF2* transcript targeted in our study, some insight may come from studies focused on the timing of the appearance of the embryonic mRNA. Graf et al. (2014) detected the synthesis of the embryonic *ATF2* from the 8-cell stage. Thus, it seems reasonable that the impact of the ablation of ATF2 by MOs was mostly due to targeting of the mRNA of maternal origin since the collected embryos for RT-qPCR analysis corresponded to the early 8-cell stage. Moreover, in our model microinjection was performed in GV-oocytes, which provided an earlier developmental window for MOs to suppress maternal transcripts. However, a small contribution of a potential abatement of the embryonic *ATF2* mRNA cannot be completely excluded considering the relatively long lifespan of MOs in the cytosol (Siddall et al. 2002; Summerton 2007)**.** To further explore the potential consequence of the reduction of *ATF2* transcripts, a number of putative ATF2-regulated genes were chosen for the current study according to known regulation information in diverse tissues. Nonetheless, the absence of impact on mRNA levels of these ATF2 downstream genes upon ATF2mo microinjection (as well as those of ATF1 and

ATF2 themselves), together with the modest decrease of blastocyst rate in the ATF2mo group could suggest that the lack of strong effects was due to the already high protein levels prior to IVM. This was determined in our study in agreement with observations from Vigneault et al. (2009). Such a scenario appeared as reflected by the inability of the ATF2mo treatment to diminish ATF2 protein expression. In addition, no measurable detection of ATF2 was achieved with IF and thus WB results could not be confirmed. Alternatively, the battery of putative ATF2-downstream genes tested in the current study was limited and we do not discard any potential effect of ATF2mo microinjection on the mRNA levels of other candidate genes subordinate to ATF2 and involved in cell cycle modulation. In spite of the lack of selected direct target measurable significant effect, the modest deleterious effects upon ablation of ATF2, together with previous observations of ATF2 translocation to nuclei at the time of EGA in cattle embryos (Vigneault et al. 2009) support the notion of the crucial role of this TF during the cleavage-stage period in bovines.

The importance of ATF1 for bovine preimplantation development was supported here by the phenotype after culture to the blastocyst stage, as well as the molecular signature of 8-cell embryos upon *ATF1* ablation. Moreover, a dramatic decrease of the blastocyst/8-16 cell rate in ATF1mo embryos was observed apparently implying that ablation of *ATF1* leaded to failure at the moment of EGA since only a minority of the ATF1mo embryos that reached or surpassed the 8-cell stage (~10%) were able to progress further through development and form blastocysts. This parameter in ATF1mo embryos was in striking contrast with the ATF2 group, where > 60% of the embryos that arrived to the 8-cell stage were capable of reaching the blastocyst stage at levels similar to the control groups. In contrast, the lack of effects in hatching occurrence in surviving ATF1mo-blastocysts might suggest that once the embryos survive the negative effects of knockdown by MOs at EGA they can develop to the blastocyst stage in a similar way to controls.

Two possibilities could explain the down-regulation of developmental competence upon ATF1mo treatment: 1) A relatively major importance of ATF1 for early embryogenesis in cattle in comparison to ATF2; and 2) a more effective knockdown in terms of the protein. Results from both WB and IF assays indicated that ATF1mo effectively reduced the

abundance of the ATF1 protein. These observations agree with the second option above. Attainment of lower ATF1 levels in the current model by MOs microinjection seems to be facilitated by the late translation of ATF1 during development when compared against ATF2 (ATF1 was detected here at the 2-cell stage, although the possibility of being translated at the M-II or zygote stages should not be ruled out).

In order to explore the potential impact of ATF1 on gene regulation during early embryogenesis a number of putative ATF1-regulated genes were chosen according to literature: Direct target, *TP53*; and indirect targets, *ATF3*, *DBF4*, and *KLF4* given that bovine *ELAVL1* (*HuR*) and *DBF4* promoters were predicted to be bound by ATF1 (Table 3-2), while downregulation of the RNA-binding protein ELAVL1 diminishes *ATF3* transcript (Pan et al. 2005). Likewise, ATF1 binds *ATF4* promoter (Kobayashi et al. 1997). ATF3 acts either an activator or repressor of gene expression in response to cellular stress and has been implicated with carcinogenesis (rev. Thompson et al. 2009; Hai et al. 2010). Furthermore, ATF3 activates *TP53* transcription and decreases the degradation of its protein (Yan et al. 2005). Thus, ATF3 might be of developmental importance for the early embryo (Fig. 3-8). Since ATF1mo 8-cell embryos showed a trend of diminished *ATF3* mRNA, it could be suggested that *ATF1* knockdown only had a slight effect on *ATF3* transcript abundance. *DBF4* mRNA levels in knockdown embryos displayed a similar profile to *ATF3*. If this were caused by ATF1mo the expected regulation of ATF1 on *DBF4* transcription would be modest. It must be added that Vigneault et al. (2009) determined that *DBF4* is one of the embryonic transcripts synthesized at the late 8-cell stage in cattle and it is known for being a master switch in chromatin assembly and DNA duplication (Matthews and Guarne 2013; Jeffery et al. 2015), key functions for subsequent molecular regulation and developmental changes (Fig. 3-8) during early embryogenesis (rev. Sirard 2010; Bogliotti and Ross 2015).

Since *KLF4* was diminished in *ATF1* knockdown embryos it remains possible that ATF1 is required for *KLF4* synthesis at the onset of EGA, although no ATF1 binding sites were found in the bovine *KLF4* promoter, suggesting indirect regulation. As mentioned above, one potential mechanism through which ATF1 could affect *KLF4* mRNA levels could be

via ATF4 (Kobayashi et al. 1997; Harding et al. 2003), but such a scenario needs to be evaluated in an early embryo context. It is compelling to note that Graf et al. (2014) detected the first appearance of the embryonic *KLF4* mRNA in bovine 8-cell embryos, which implies that *KLF4* transcript and protein are required early after EGA in cattle. Widely known implications of KLF4 in development are its roles as one of the master regulators of pluripotency and cell reprogramming (Takahashi and Yamanaka 2006; Dutta 2013). In a developmental competence context, Henderson et al. (2014) established that *KLF4* transcript is precociously synthesized around EGA time in rabbit embryos produced *in vitro* when compared against their *in vivo*-produced counterparts. Thus, the implications of the putative causal link between ATF1 and KLF4 molecular module around EGA time in cattle should be explored during developmental progression from 8-cell embryos to blastocysts (Fig. 3-8).

SP1 mRNA levels are also negatively impacted in ATF1mo embryos. The notion that ATF1 could activate transcription of *SP1* is supported by the fact that bovine *SP1* is predicted as a target of ATF1 (Table 3-2). Graf et al. (2014) discovered a sizable increase of *SP1* mRNA in 8-cell embryos in comparison to their 4-cell counterparts. This molecular fingerprint of *Sp1* seemed similar in rodents. Wang and Latham (2000) reported that *Sp1* is first transcribed from the embryonic genome soon after fertilization (mid 2-cell), concurrent with the first transcriptional waves in that species, and its levels were progressively amplified throughout the preimplantation period. Consequently, it was suggested that Sp1 could be one of the proteins involved in maternal-zygotic transition (Wang and Latham 2000). SP1 is a zinc-finger TF related to KLF4. SP1 binds GC-rich motifs in hundreds of mammalian promoters and it can act either as an activator or repressor. SP1 is a central modulator of multiple processes including housekeeping, cell proliferation and growth, apoptosis, DNA damage response, hypoxia, carcinogenesis, and chromatin remodelling (rev. Worrad and Schultz 1997; Cummins and Taylor 2005; Lomberk and Urrutia 2005; Li and Davie 2010).

In early development, Bevilacqua et al. (2000) demonstrated that SP1 is part of the molecular machinery responsible for *Hsp70.1* (a marker of EGA across species) expression

in mouse 2-cell embryos, whereas Zhao and Meng (2005) indicated that members of the Sp1 family play pivotal roles in multiple developmental mechanisms and tissues in embryos across vertebrates. Another appealing gene subordinated to SP1 is *DAZL*. It is known that SP1 binds the promoter of *DAZL* and drives its gene expression in germinal cells in pigs (Linher et al. 2009), whereas Chen et al. (2011) demonstrated that the ablation of *DAZL* generated a 2-cell stage blockage, suggesting that DAZL is essential for murine ZGA through its RNA-binding functions. Concerning pluripotency-related and reprogramming factors, comparison of the promoters of the human, murine, and bovine *Oct4* gene showed the existence of a conserved SP1-binding site for the three species (Nordhoff et al. 2001). A functional demonstration of the control of *OCT4* expression by SP1 arose from the report of Yang et al. (2005), where it was shown that SP1 regulates the promoter of *OCT4* in humans, both positively and negatively. However, establishment of stemness of ICM might not be regulated in the same way in the cow as in other mammals, since OCT4 has not been correlated to the specification of the pluripotent cell lineage in the bovine embryo (Khan et al. 2012). The Myc oncoprotein has been used as reprogramming factor towards pluripotency (Takahashi and Yamanaka 2006; Dutta 2013), whereas SP1 has been associated with expression of c-Myc in *Xenopus* oocytes (Modak et al. 1993). In relation to fertility and developmental competence, biopsies from cow embryos that did not produce pregnancy were enriched in *EEF1A1* mRNA (El-Sayed et al. 2006), whereas Johnson and Krieg (1995) found that SP1 is crucial in the regulation of the promoter activity of the somatic form of *EEF1A* in *Xenopus*. Moreover, it appears plausible that SP1 could regulate expression of these developmentally important genes in *Bos taurus* because SP1-binding sites were localized in the promoters of *HSP70* (*HSPA1A*), *DAZL*, *MYC*, and *EEF1A1* in our *in silico* analysis (Table 3-2). Likewise, the potential implication of MYC and EEF1A1 in development from or shortly after EGA in cattle could be posited due to the finding that the onset of their synthesis occurs in 8-cell embryos (Graf et al. 2014). Finally, a potential self-regulatory loop between both ATFs and SP1 (Fig. 3-8) could be an interesting possibility to explore given that the promoter of *ATF2* contains Sp1-elements (rev. Vlahopoulos et al. 2008), and it was observed here that the ATF1mo-embryos had abated levels of *SP1*, whereas the same knockdown-embryos displayed diminished ATF2 protein.

Given that MOs are supposed to hamper translation of their target mRNAs instead of driving their degradation, the decline in *ATF1* transcript levels in ATF1mo embryos might hint that the maternal stock of this TF is able to modulate synthesis of its own mRNA at the moment of EGA. Accordingly, ATF1 was predicted to bind its own promoter (Table 3-2). In addition, ATF1 has been suggested as one of the first mRNAs to be synthesized after EGA in cattle (Vigneault et al. 2004) and was later confirmed by Graf et al. (2014) to be first transcribed in 8-cell cattle embryos. Therefore, as in the case of *ATF2* it seems more plausible that the knockdown of *ATF1* with MOs mainly targets the maternal mRNA rather than the embryonic transcripts. Similar to ATF2mo, ATF1mo targets all isoforms (constitutive, UTR3alt, or splice variant) of their target transcript. Therefore, it is not possible to know with the current results the specific contribution of the ablation of every *ATF1* mRNA isoform to the observed effects on both downstream target genes expression and culture phenotype. *ATF1* has been previously suggested to be a developmentally regulated transcript during initial cleavage in cattle embryos (Vigneault et al. 2004) and it has been implicated as a potential marker of competence at the 2-cell stage in the same species (Held et al. 2012; Orozco-Lucero et al. 2014). In mice, the double knockout of *Atf1* and its heterodimerization partner, *Creb* leaded to embryonic death produced by high apoptosis in developmentally delayed morula (Bleckmann et al. 2002). Therefore, the current results in cattle, alluding that ATF1 is necessary before (maternal ATF1 regulating the onset of *de novo* transcription; Fig. 3-8) and after EGA (embryonic ATF1 required for further development), as well as those in literature from rodents seem concordant with the notion that ATF1 performs indispensable functions during preimplantation development and EGA that are conserved across mammals.

In summary, the results described here are in agreement with the notion that both ATF1 and ATF2 are crucial for preimplantation development in mammals, and that a higher amount of these TFs at the mRNA or the protein level makes embryos more competent. Moreover, this work identifies a putative mechanism by which ATF1 and ATF2 could exert their molecular effects favoring early embryogenesis and notably mediating important events during the activation of *de novo* transcription from the embryonic genome, where the more feasible pathways inferred from this work are the regulation of the embryonic *ATF1*, *KLF4*,

and *SP1* by the maternal ATF1. Finally, given the pivotal role of both molecules in early development, potential upstream pathways should be investigated to enhance relative levels of both TFs to test the effects of such rise on developmental capacity.

3.7. Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

3.8. Funding

This work was supported by the Natural Sciences and Engineering Research Council of Canada. E. Orozco-Lucero held bourses from Consejo Nacional de Ciencia y Tecnología (México) and Fonds de recherche du Québec – Nature et technologies (Québec).

3.9. Acknowledgements

We thank Elizabeth St-John, Monica Antenos, W. Allan King, Pavneesh Madan, and Jonathan LaMarre (University of Guelph); Isabelle Laflamme and Alexandre Bastien (Université Laval); Christian Vigneault (L'Alliance Boviteq, LAB).

3.10. References

Ainbinder E, Amir-Zilberstein L, Yamaguchi Y, Handa H, Dikstein R. 2004. Elongation inhibition by DRB sensitivity-inducing factor is regulated by the A20 promoter via a novel negative element and NF-kappaB. *Mol Cell Biol* **24:** 2444-2454.

Aqil M, Mallik S, Bandyopadhyay S, Maulik U, Jameel S. 2015. Transcriptomic Analysis of mRNAs in Human Monocytic Cells Expressing the HIV-1 Nef Protein and Their Exosomes. *Biomed Res Int* **492395**.

Ashkar FA, Semple E, Schmidt CH, St John E, Bartlewski PM, King WA. 2010. Thyroid hormone supplementation improves bovine embryo development in vitro. *Hum Reprod* **25:** 334-344.

Beier F, Lee RJ, Taylor AC, Pestell RG, LuValle P. 1999. Identification of the cyclin D1 gene as a target of activating transcription factor 2 in chondrocytes. *Proc Natl Acad Sci U S A* **96:** 1433-1438.

Bevilacqua A, Fiorenza MT, Mangia F. 2000. A developmentally regulated GAGA box-binding factor and Sp1 are required for transcription of the hsp70.1 gene at the onset of mouse zygotic genome activation. *Development* **127:** 1541-1551.

Bleckmann SC, Blendy JA, Rudolph D, Monaghan AP, Schmid W, Schutz G. 2002. Activating transcription factor 1 and CREB are important for cell survival during early mouse development. *Mol Cell Biol* **22:** 1919-1925.

Bogliotti YS, Ross PJ. 2015. Molecular mechanisms of transcriptional and chromatin remodeling around embryonic genome activation. *Anim Reprod* **12:** 52-61

Breitwieser W, Lyons S, Flenniken AM, Ashton G, Bruder G, Willington M, Lacaud G, Kouskoff V, Jones N. 2007. Feedback regulation of p38 activity via ATF2 is essential for survival of embryonic liver cells. *Genes Dev* **21:** 2069-2082.

Buffone MG, Schindler K, Schultz RM. 2009. Overexpression of CDC14B causes mitotic arrest and inhibits zygotic genome activation in mouse preimplantation embryos. *Cell Cycle* **8:** 3904-3913.

Chen J, Melton C, Suh N, Oh JS, Horner K, Xie F, Sette C, Blelloch R, Conti M. 2011. Genome-wide analysis of translation reveals a critical role for deleted in azoospermia-like (Dazl) at the oocyte-to-zygote transition. *Genes Dev* **25:** 755-766.

Chen M, Lin YQ, Xie SL, Wang JF. 2010. Mitogen-activated protein kinase in endothelin-1-induced cardiac differentiation of mouse embryonic stem cells. *J Cell Biochem* **111:** 1619-1628.

Collart C, Allen GE, Bradshaw CR, Smith JC, Zegerman P. 2013. Titration of four replication factors is essential for the Xenopus laevis midblastula transition. *Science* **341:** 893-896.

Cummins EP, Taylor CT. 2005. Hypoxia-responsive transcription factors. *Pflugers Arch* **450:** 363-371.

Day AM, Veal EA. 2010. Hydrogen peroxide-sensitive cysteines in the Sty1 MAPK regulate the transcriptional response to oxidative stress. *J Biol Chem* **285:** 7505-7516.

De La Fuente R, Baumann C, Viveiros MM. 2015. ATRX contributes to epigenetic asymmetry and silencing of major satellite transcripts in the maternal genome of the mouse embryo. Development **142:** 1806-1817.

del Barco Barrantes I, Coya JM, Maina F, Arthur JS, Nebreda AR. 2011. Genetic analysis of specific and redundant roles for p38alpha and p38beta MAPKs during mouse development. *Proc Natl Acad Sci U S A* **108:** 12764-12769.

Deleavey GF, Damha MJ. 2012. Designing chemically modified oligonucleotides for targeted gene silencing. *Chem Biol* **19:** 937-954.

Dostaler-Touchette V, Bedard F, Guillemette C, Pothier F, Chouinard PY, Richard FJ. 2009. Cyclic adenosine monophosphate (cAMP)-specific phosphodiesterase is functional in bovine mammary gland. *J Dairy Sci* **92:** 3757-3765.

Dutta D. 2013. Signaling pathways dictating pluripotency in embryonic stem cells. *Int J Dev Biol* **57:** 667-675.

El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, Sirard MA, Schellander K, Tesfaye D. 2006. Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiol Genomics* **28:** 84-96.

Favetta LA, Madan P, Mastromonaco GF, St John EJ, King WA, Betts DH. 2007. The oxidative stress adaptor p66Shc is required for permanent embryo arrest in vitro. *BMC Dev Biol* **7:** 132.

Gilbert I, Scantland S, Sylvestre EL, Gravel C, Laflamme I, Sirard MA, Robert C. 2009. The dynamics of gene products fluctuation during bovine pre-hatching development. *Mol Reprod Dev* **76:** 762-772.

Graf A, Krebs S, Zakhartchenko V, Schwalb B, Blum H, Wolf E. 2014. Fine mapping of genome activation in bovine embryos by RNA sequencing. *Proc Natl Acad Sci U S A* **111:** 4139-4144.

Ha HC, Hester LD, Snyder SH. 2002. Poly(ADP-ribose) polymerase-1 dependence of stress-induced transcription factors and associated gene expression in glia. *Proc Natl Acad Sci U S A* **99:** 3270-3275.

Hai T, Wolford CC, Chang YS. 2010. ATF3, a hub of the cellular adaptive-response network, in the pathogenesis of diseases: is modulation of inflammation a unifying component? *Gene Expr* **15:** 1-11.

Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D. 2003. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* **11:** 619-633.

Held E, Salilew-Wondim D, Linke M, Zechner U, Rings F, Tesfaye D, Schellander K, Hoelker M. 2012. Transcriptome fingerprint of bovine 2-cell stage blastomeres is directly correlated with the individual developmental competence of the corresponding sister blastomere. *Biol Reprod* **87:** 154.

Henderson GR, Brahmasani SR, Yelisetti UM, Konijeti S, Katari VC, Sisinthy S. 2014. Candidate gene expression patterns in rabbit preimplantation embryos developed in vivo and in vitro. *J Assist Reprod Genet* **31:** 899-911.

Huang D, Shipman-Appasamy PM, Orten DJ, Hinrichs SH, Prystowsky MB. 1994. Promoter activity of the proliferating-cell nuclear antigen gene is associated with inducible CRE-binding proteins in interleukin 2-stimulated T lymphocytes. *Mol Cell Biol* **14:** 4233-4243.

Jeffery DC, Kakusho N, You Z, Gharib M, Wyse B, Drury E, Weinreich M, Thibault P, Verreault A, Masai H, Yankulov K. 2015. CDC28 phosphorylates Cac1p and regulates the association of chromatin assembly factor I with chromatin. *Cell Cycle* **14:** 74-85.

Jin XL, O'Neill C. 2010. The presence and activation of two essential transcription factors (cAMP response element-binding protein and cAMP-dependent transcription factor ATF1) in the two-cell mouse embryo. *Biol Reprod* **82:** 459-468.

Jin XL, O'Neill C. 2014. The regulation of the expression and activation of the essential ATF1 transcription factor in the mouse preimplantation embryo. *Reproduction* **148:** 147-157.

Johnson AD, Krieg PA. 1995. A Xenopus laevis gene encoding EF-1 alpha S, the somatic form of elongation factor 1 alpha: sequence, structure, and identification of regulatory elements required for embryonic transcription. *Dev Genet* **17:** 280-290.

Keembiyehetty CN, Candelaria RP, Majumdar G, Raghow R, Martinez-Hernandez A, Solomon SS. 2002. Paradoxical regulation of Sp1 transcription factor by glucagon. *Endocrinology* **143(4):** 1512-1520.

Khan DR, Dube D, Gall L, Peynot N, Ruffini S, Laffont L, Le Bourhis D, Degrelle S, Jouneau A, Duranthon V. 2012. *PLoS One* **7:** e34110.

Kobayashi M, Shimomura A, Hagiwara M, Kawakami K. 1997. Phosphorylation of ATF-1 enhances its DNA binding and transcription of the Na,K-ATPase alpha 1 subunit gene promoter. *Nucleic Acids Res* **25:** 877-882.

Krisher RL. 2004. The effect of oocyte quality on development. *J Anim Sci* **82:** 14-23.

Labrecque R, Sirard MA. 2014. The study of mammalian oocyte competence by transcriptome analysis: progress and challenges. *Mol Hum Reprod* **20:** 103-116.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Lee SH, Bahn JH, Whitlock NC, Baek SJ. 2010. Activating transcription factor 2 (ATF2) controls tolfenamic acid-induced ATF3 expression via MAP kinase pathways. *Oncogene* **29:** 5182-5192.

Lee SR, Xu YN, Jo YJ, Namgoong S, Kim NH. 2015. The Rho-GTPase effector ROCK regulates meiotic maturation of the bovine oocyte via myosin light chain phosphorylation and cofilin phosphorylation. *Mol Reprod Dev* **[in press]**.

Li L, Davie JR. 2010. The role of Sp1 and Sp3 in normal and cancer cell biology. *Ann Anat* **192:** 275-283.

Linher K, Cheung Q, Baker P, Bedecarrats G, Shiota K, Li J. 2009. An epigenetic mechanism regulates germ cell-specific expression of the porcine Deleted in Azoospermia-Like (DAZL) gene. *Differentiation* **77:** 335-349.

Lomberk G, Urrutia R. 2005. The family feud: turning off Sp1 by Sp1-like KLF proteins. Biochem J **392:** 1-11.

Madeja ZE, Sosnowski J, Hryniewicz K, Warzych E, Pawlak P, Rozwadowska N, Plusa B, Lechniak D. 2013. Changes in sub-cellular localisation of trophoblast and inner cell mass specific transcription factors during bovine preimplantation development. *BMC Dev Biol* **13:** 32.

Maekawa T, Sano Y, Shinagawa T, Rahman Z, Sakuma T, Nomura S, Licht JD, Ishii S. 2008. ATF-2 controls transcription of Maspin and GADD45 alpha genes independently from p53 to suppress mammary tumors. *Oncogene* **27:** 1045-1054.

Maekawa T, Shinagawa T, Sano Y, Sakuma T, Nomura S, Nagasaki K, Miki Y, Saito-Ohara F, Inazawa J, Kohno T, Yokota J, Ishii S. 2007. Reduced levels of ATF-2 predispose mice to mammary tumors. *Mol Cell Biol* **27:** 1730-1744.

Matthews LA, Guarne A. 2013. Dbf4: the whole is greater than the sum of its parts. *Cell Cycle* **12:** 1180-1188.

Matsushima-Nishiu M, Unoki M, Ono K, Tsunoda T, Minaguchi T, Kuramoto H, Nishida M, Satoh T, Tanaka T, Nakamura Y. 2001. Growth and gene expression profile analyses of endometrial cancer cells expressing exogenous PTEN. *Cancer Res* **61:** 3741-3749.

Mayer SI, Dexheimer V, Nishida E, Kitajima S, Thiel G. 2008. Expression of the transcriptional repressor ATF3 in gonadotrophs is regulated by Egr-1, CREB, and ATF2 after gonadotropin-releasing hormone receptor stimulation. *Endocrinology* **149:** 6311-6325.

Modak SP, Principaud E, Spohr G. 1993. Regulation of Xenopus c-myc promoter activity in oocytes and embryos. *Oncogene* **8:** 645-654.

Mondou E, Dufort I, Gohin M, Fournier E, Sirard MA. 2012. Analysis of microRNAs and their precursors in bovine early embryonic development. *Mol Hum Reprod* **18:** 425-434.

Nganvongpanit K, Müller H, Rings F, Hoelker M, Jennen D, Tholen E, Havlicek V, Besenfelder U, Schellander K, Tesfaye D. 2006. Selective degradation of maternal and embryonic transcripts in in vitro produced bovine oocytes and embryos using sequence specific double-stranded RNA. *Reproduction* **131:** 861-874.

Nordhoff V, Hubner K, Bauer A, Orlova I, Malapetsa A, Scholer HR. 2001. Comparative analysis of human, bovine, and murine Oct-4 upstream promoter sequences. *Mamm Genome* **12:** 309-317.

Orozco-Lucero E, Dufort I, Robert C, Sirard MA. 2014. Rapidly cleaving bovine two-cell embryos have better developmental potential and a distinctive mRNA pattern. *Mol Reprod Dev* **81:** 31-41.

Orozco-Lucero E, Sirard MA. 2014. Molecular markers of fertility in cattle oocytes and embryos: Progress and challenges. *Anim Reprod* **11:** 183-194.

Page-Lariviere F, Sirard MA. 2014. Spatiotemporal expression of DNA demethylation enzymes and histone demethylases in bovine embryos. *Cell Reprogram* **16:** 40-53.

Pan YX, Chen H, Kilberg MS. 2005. Interaction of RNA-binding proteins HuR and AUF1 with the human ATF3 mRNA 3'-untranslated region regulates its amino acid limitation-induced stabilization. *J Biol Chem* **280(41):** 34609-34616.

Papassava P, Gorgoulis VG, Papaevangeliou D, Vlahopoulos S, van Dam H, Zoumpourlis V. 2004. Overexpression of activating transcription factor-2 is required for tumor growth and progression in mouse skin tumors. *Cancer Res* **64:** 8573-8584.

Paradis F, Vigneault C, Robert C, Sirard MA. 2005. RNA interference as a tool to study gene function in bovine oocytes. *Mol Reprod Dev* **70:** 111-121.

Pers-Kamczyc E, Pawlak P, Rubes J, Lechniak D. 2012. Early cleaved bovine embryos show reduced incidence of chromosomal aberrations and higher developmental potential on day 4.5 post-insemination. *Reprod Domest Anim* **47:** 899-906.

Recio JA, Merlino G. 2002. Hepatocyte growth factor/scatter factor activates proliferation in melanoma cells through p38 MAPK, ATF-2 and cyclin D1. *Oncogene* **21:** 1000-1008.

Robert C. 2008. Challenges of functional genomics applied to farm animal gametes and pre-hatching embryos. *Theriogenology* **70:** 1277-1287.

Robert C, McGraw S, Massicotte L, Pravetoni M, Gandolfi F, Sirard MA. 2002 Quantification of housekeeping transcript levels during the development of bovine preimplantation embryos. *Biol Reprod* **67:** 1465-1472.

Rodriguez-Gabriel MA, Burns G, McDonald WH, Martin V, Yates JR 3rd, Bahler J, Russell P. 2003. RNA-binding protein Csx1 mediates global control of gene expression in response to oxidative stress. *EMBO J* **22:** 6256-6266.

Ross PJ, Wang K, Kocabas A, Cibelli JB. 2010. Housekeeping gene transcript abundance in bovine fertilized and cloned embryos. *Cell Reprogram* **12:** 709-717.

Scantland S, Grenon JP, Desrochers MH, Sirard MA, Khandjian EW, Robert C. 2011. Method to isolate polyribosomal mRNA from scarce samples such as mammalian oocytes and early embryos. *BMC Dev Biol* **11:** 8.

Schmeier S, MacPherson CR, Essack M, Kaur M, Schaefer U, Suzuki H, Hayashizaki Y, Bajic VB. 2009. Deciphering the transcriptional circuitry of microRNA genes expressed during human monocytic differentiation. *BMC Genomics* **10:** 595.

Shen YH, Zhang L, Gan Y, Wang X, Wang J, LeMaire SA, Coselli JS, Wang XL. 2006. Up-regulation of PTEN (phosphatase and tensin homolog deleted on chromosome ten) mediates p38 MAPK stress signal-induced inhibition of insulin signaling. A cross-talk between stress signaling and insulin signaling in resistin-treated human endothelial cells. *J Biol Chem* **281:** 7727-7736.

Siddall LS, Barcroft LC, Watson AJ. 2002. Targeting gene expression in the preimplantation mouse embryo using morpholino antisense oligonucleotides. *Mol Reprod Dev* **63:** 413-421.

Sirard MA. 2010. Activation of the embryonic genome. *Soc Reprod Fertil Suppl* **67:** 145-158.

Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Connor PM, Fornace AJ Jr. 1994. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science* **266:** 1376-1380.

Suhail TV, Singh P, Manna TK. 2015. Suppression of centrosome protein TACC3 induces G1 arrest and cell death through activation of p38-p53-p21 stress signaling pathway. *Eur J Cell Biol* **94:** 90-100.

Summerton JE. 2007. Morpholino, siRNA, and S-DNA compared: impact of structure and mechanism of action on off-target effects and sequence specificity. *Curr Top Med Chem* **7:** 651-660.

Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126:** 663-676.

Thompson MR, Xu D, Williams BR. 2009. ATF3 transcription factor and its emerging roles in immunity and cancer. *J Mol Med (Berl)* **87:** 1053-1060.

Toralova T, Susor A, Nemcova L, Kepkova K, Kanka J. 2009. Silencing CENPF in bovine preimplantation embryo induces arrest at 8-cell stage. *Reproduction* **138:** 783-791.

Tsai EY, Yie J, Thanos D, Goldfeld AE. 1996. Cell-type-specific regulation of the human tumor necrosis factor alpha gene in B cells and T cells by NFATp and ATF-2/JUN. *Mol Cell Biol* **16:** 5232-5244.

Vigneault C, Gilbert I, Sirard MA, Robert C 2007. Using the histone H2a transcript as an endogenous standard to study relative transcript abundance during bovine early development. *Mol* Reprod *Dev* **74:** 703-715.

Vigneault C, McGraw S, Massicotte L, Sirard MA. 2004. Transcription factor expression patterns in bovine in vitro-derived embryos prior to maternal-zygotic transition. *Biol Reprod* **70:** 1701-1709.

Vigneault C, McGraw S, Sirard MA. 2009. Spatiotemporal expression of transcriptional regulators in concert with the maternal-to-embryonic transition during bovine in vitro embryogenesis. *Reproduction* **137:** 13-21.

Vlahopoulos SA, Logotheti S, Mikas D, Giarika A, Gorgoulis V, Zoumpourlis V. 2008. The role of ATF-2 in oncogenesis. *Bioessays* **30:** 314-327.

Wang L, Lu A, Zhou HX, Sun R, Zhao J, Zhou CJ, Shen JP, Wu SN, Liang CG. 2013. Casein kinase 1 alpha regulates chromosome congression and separation during mouse oocyte meiotic maturation and early embryo development. *PLoS One* **8:** e63173.

Wang Q, Latham KE. 2000. Translation of maternal messenger ribonucleic acids encoding transcription factors during genome activation in early mouse embryos. *Biol Reprod* **62:** 969-978.

Worrad DM, Schultz RM. 1997. Regulation of gene expression in the preimplantation mouse embryo: temporal and spatial patterns of expression of the transcription factor Sp1. *Mol Reprod Dev* **46:** 268-277.

Wrenzycki C, Herrmann D, Niemann H. 2007. Messenger RNA in oocytes and embryos in relation to embryo viability. *Theriogenology* **68 Suppl 1:** S77-83.

Yan C, Lu D, Hai T, Boyd DD. 2005. Activating transcription factor 3, a stress sensor, activates p53 by blocking its ubiquitination. *EMBO J* **24:** 2425-2435.

Yang HM, Do HJ, Oh JH, Kim JH, Choi SY, Cha KY, Chung HM, Kim JH. 2005. Characterization of putative cis-regulatory elements that control the transcriptional activity of the human Oct4 promoter. *J Cell Biochem* **96:** 821-830.

Yang KT, Lin CY, Huang HL, Liou JS, Chien CY, Wu CP, Huang CW, Ou BR, Chen CF, Lee YP, Lin EC, Tang PC, Lee WC, Ding ST, Cheng WT, Huang MC. 2008. Expressed transcripts associated with high rates of egg production in chicken ovarian follicles. *Mol Cell Probes* **22:** 47-54.

Yang MY, Fortune JE. 2015. Changes in the Transcriptome of Bovine Ovarian Cortex During Follicle Activation in Vitro. *Physiol Genomics* **[in press]**.

Yang WL, Li J, An P, Lei AM. 2014. CDC20 downregulation impairs spindle morphology and causes reduced first polar body emission during bovine oocyte maturation. *Theriogenology* **81:** 535-544.

Zhao C, Meng A. 2005. Sp1-like transcription factors are regulators of embryonic development in vertebrates. *Dev Growth Differ* **47:** 201-211.

3.11. Tables and Figures

Table 3-1 Details of RT-qPCR in 8-cell embryos

Accession	Gene Symbol	Fw-primer (5'-3')	Rv-primer (5'-3')	An. T° (°C)	Product (bp)
NM_ 173993	*ARNT*	GCAACACATTC CCTCCTAC	AAGCTGCCTAC ACCAAAC	53	250
NM_ 001075289	*ATF1*	AATACTTGTGC CCAGCAACC	TGTAAGGGTCA CGGGAGATG	58	133
NM _001081584	*ATF2*	TGCAGTGCGGC AGCTTTAGATA CT	CATCAACTGCT GCTACACCAAC	60	153
NM_ 001046193	*ATF3*	TCAGGACTCGC ACCATTA	CGGCACAAACA TCAGTAGAG	53	250
NM_00107 5476	*DBF4*	GCCCATTTGAT GCAGAGA	GCATGTCCCTTT CGTACTC	53	285
NM_00110 5385	*KLF4*	TGAACTGACCA GGCACTA	ACTTGTTGGGA ACTTGACC	53	250
NM_00107 8027	*SP1*	GGAGGAAGGAG ACCATTCT	AGGGTTGAGTC AGGGAAA	53	246
NM_17420 1	*TP53*	TCGGGAGAGGT CAGAATGTGTT CC	CTTTGGCACTG AGGTTCACCAA GG	60	409
NM_00103 4792	*SUPT4H1*	GGAGCTAAATG GGCAGAAC	AGGGAAAGAGG GCAAGAA	53	287

Fw, forward; Rv, reverse; An. T°, annealing temperature; bp, basis pairs; *ARNT*, Aryl hydrocarbon receptor nuclear translocator; *ATF1/2/3*, Activating Transcription Factor 1/2/3; *KLF4*, Kruppel-like factor 4 (gut); *SP1*, SP1 transcription factor; *TP53*, tumor protein 53; *SUPT4H1*, Suppressor of Ty 4 homolog 1 (*S. cerevisiae*).

Table 3-1 (continued) Details of RT-qPCR in 8-cell embryos

Accession	Gene Symbol	Fw-primer (5'-3')	Rv-primer (5'-3')	An. T° (°C)	Product (bp)
U73901	*GFP*	GCAGAAGAACG GCATCAAGGTG AA	TGGGTGCTCAG GTAGTGGTTGT	59	143
NM_173979	*ACTB*	ATCGTCCACCG CAAATGCTTCT	GCCATGCCAAT CTCATCTCGTT	60	101
NM_174080	*UBE2K*	GATCCACAAGA TGCAGTAGTAG	GTTGATGCTCCT CCAAGAAG	53	291
NM_174793	*YWHAG*	GCCACTGTCCA ATGAAGAA	TGCTCTCATACT GGGTCTC	53	254

Fw, forward; Rv, reverse; An. T°, annealing temperature; bp, basis pairs; *GFP*, Green Fluorescent Protein; *ACTB*, actin, beta; *UBE2K*, ubiquitin-conjugating enzyme E2K; *YWHAG*, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma.

Table 3-2 Transcription factor binding sites prediction

TF	Target	# B. sites	Relative score (range)
ATF1	*ARNT*	3	0.8031 – 0.87744
ATF1	*ATF1*	1	0.81166
ATF1	*ATF2*	0	---
ATF1	*ATF3*	0	---
ATF1	*ATF4*	13	0.80244 – 0.9555
ATF1	*DBF4*	1	0.80797
ATF1	*ELAVL1*	8	0.80438 – 0.99467
ATF1	*KLF4*	0	---
ATF1	*SP1*	1	0.8193
ATF1	*SUPT4H1*	0	---
ATF1	*TP53*	1	0.86911
ATF2	*ARNT*	1	0.8031
ATF2	*ATF1*	1	0.81166
ATF2	*ATF2*	0	---
ATF2	*ATF3*	0	---
ATF2	*CCNA2*	2	0.80648 – 0.8739
ATF2	*CCND*	0	---
ATF2	*DBF4*	1	0.80797
ATF2	*GADD45A*	2	0.80827 – 0.83064
ATF2	*KLF4*	0	---
ATF2	*PTEN*	0	---
ATF2	*RBBP6*	0	---
ATF2	*SP1*	1	0.8193
ATF2	*SUPT4H1*	0	---
ATF2	*TP53*	1	0.86911
SP1	*DAZL*	≥ 4	0.9843 – 1.00
SP1	*HSPA1A (HSP70)*	58	0.80756 – 0.94348
SP1	*MYC*	≥ 3	0.9523 – 0.9773
SP1	*OCT4*	≥ 9	0.9018 – 1.00

A relative score closer to 1.0 denotes higher prediction stringence. JASPAR database (http://jaspar.genereg.net). TF, transcription factor; B. sites, binding sites *ATF1/2/3*, Activating Transcription Factor 1/2/3/4; *SP1*, Sp1 transcription factor; *ARNT*, aryl hydrocarbon receptor nuclear translocator; *DBF4*, DBF4 homolog (*S. cerevisiae*); *ELAVL1*, ELAV-like RNA-binding protein 1; *KLF4*, Kruppel-like factor 4 (gut); *SUPT4H1*, suppressor of Ty 4 homolog 1 (*S. cerevisiae*); *TP53*, tumor protein 53; *CCNA2*, cyclin A2; *CCND*, cyclin D; *GADD45A*, Growth Arrest and DNA-Damage-inducible, alpha; *PTEN*, Phosphatase and Tensin Homolog; *RBBP6*, Retinoblastoma-Binding Protein 6; *DAZL*, Deleted in Azoospermia-like; *HSPA1A*, Heat Shock 70 kDa Protein 1A; *MYC*, v-Myc Avian Myelocytomatosis Viral Oncogene Homolog; OCT4, Octamer-binding protein 4.

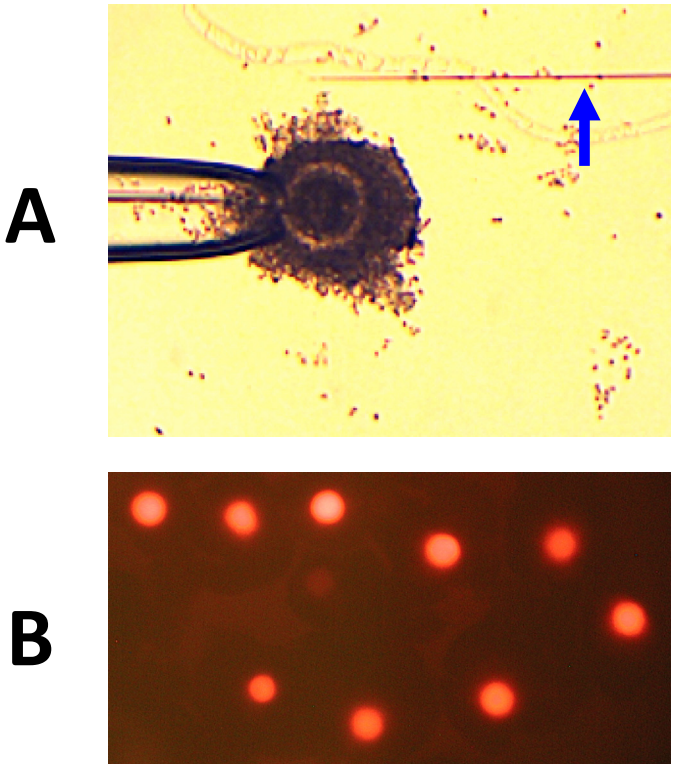

Figure 3-1 Microinjection process
A) microinjection of cumulus-enclosed immature oocytes. Microinjection needle containing dextran Texas Red and MO (arrow). Bright field, 100X original magnification. B) accurate microinjection was verified by red fluorescence in the ooplasm before transferring COCs to maturation media. Epifluorescence, 40X original magnification.

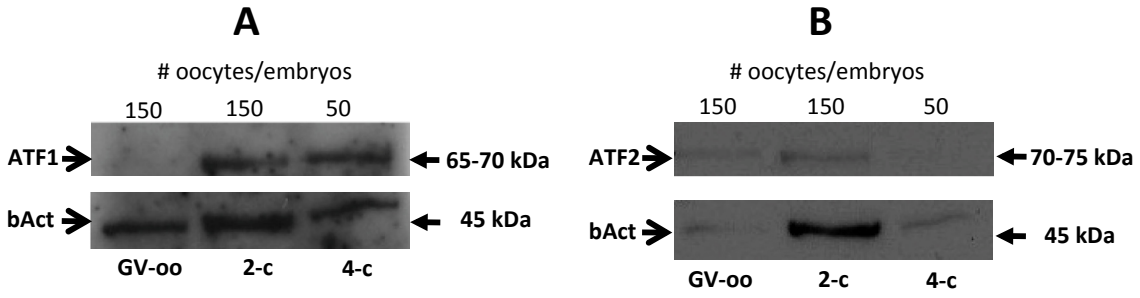

Figure 3-2 ATF1 and ATF2 protein expression through different stages in bovines
A) ATF1 immunolocalization. B) ATF2 immunolocalization. Images show a single Western blot. ATF1, Activating Transcription Factor 1; ATF2, Activating Transcription Factor 2; bAct, beta-actin; GV-oo, GV-oocytes; 2-c, 2-cell embryos; 4-c, 4-cell embryos; kDa, kilodaltons.

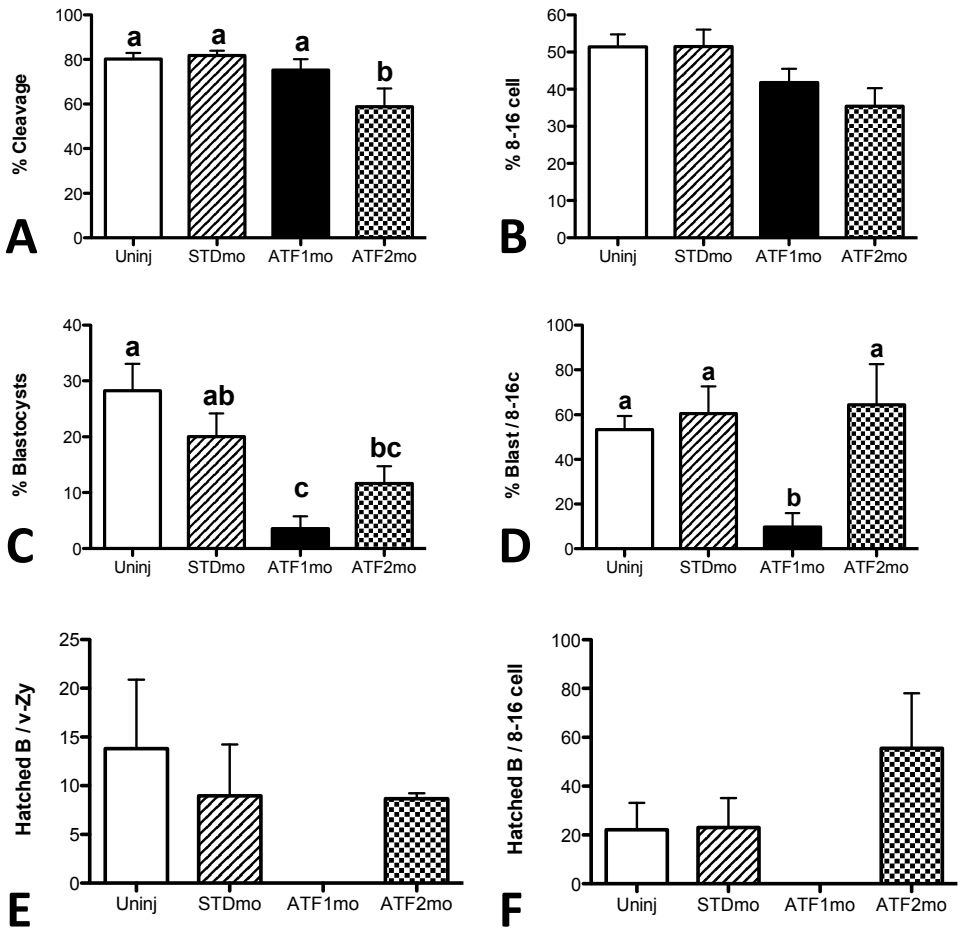

Figure 3-3 Effects of morpholino oligonucleotides (MO) on development
A) MO treatment significantly impacted cleavage (one-way ANOVA), P = 0.0018 (**). Newman-Keuls *post hoc* test, ATF2mo vs STDmo, (*); ATF2mo vs Uninj, (*); ATF2mo vs ATF1mo, (*); ATF1mo vs. STDmo, (ns); ATF1mo vs Uninj, (ns); Uninj vs STDmo, (ns); n = 9. B) MO treatment significantly impacted 8-16 cell rate, P = 0.0273 (*); n = 9. C) MO treatment significantly impacted blastocyst rate, P = 0.0023 (**); ATF1mo vs Uninj, (**); ATF1mo vs STDmo, (*); ATF1mo vs ATF2mo, (ns); ATF2mo vs. Uninj, (*); ATF2mo vs STDmo, (ns); STDmo vs. Uninj, (ns); Uninj, STDmo, ATF1mo, n = 5; ATF2mo, n = 3. D) MO treatment significantly impacted % blastocyst/8-16 cell, P = 0.0058 (**); ATF1mo vs Uninj, (**); ATF1mo vs STDmo, (**); ATF1mo vs ATF2mo, (*); ATF2mo vs. Uninj, (ns); ATF2mo vs STDmo, (ns); STDmo vs. Uninj, (ns); Uninj, STDmo, ATF1mo, n = 5; ATF2mo, n = 3. E) MO treatment did not significantly impact % Hatched B / v-Zy (ns); Uninj, STDmo, ATF1mo, n = 5; ATF2mo, n = 3. F) MO treatment did not significantly impact % Hatched B / 8-16 cell (ns); Uninj, STDmo, ATF1mo, n = 5; ATF2mo, n = 3. Different superscripts denote significant differences (at least P < 0.05). N, embryos: A and B (Uninj, 176; STDmo, 175; ATF1mo, 174; ATF2mo, 155); C and E (Uninj, 98; STDmo, 102; ATF1mo, 92; ATF2mo, 73); D and F (Uninj, 54; STDmo, 39; ATF1mo, 35; ATF2mo, 19). Blast, blastocysts; 8-16c, 8-16 cell embryos; Hatched B, hatched blastocysts; v-Zy, viable presumptive zygotes; Uninj, uninjected; STDmo, standard control morpholino; ATF1mo, ATF1 morpholino; ATF2mo, ATF2 morpholino.

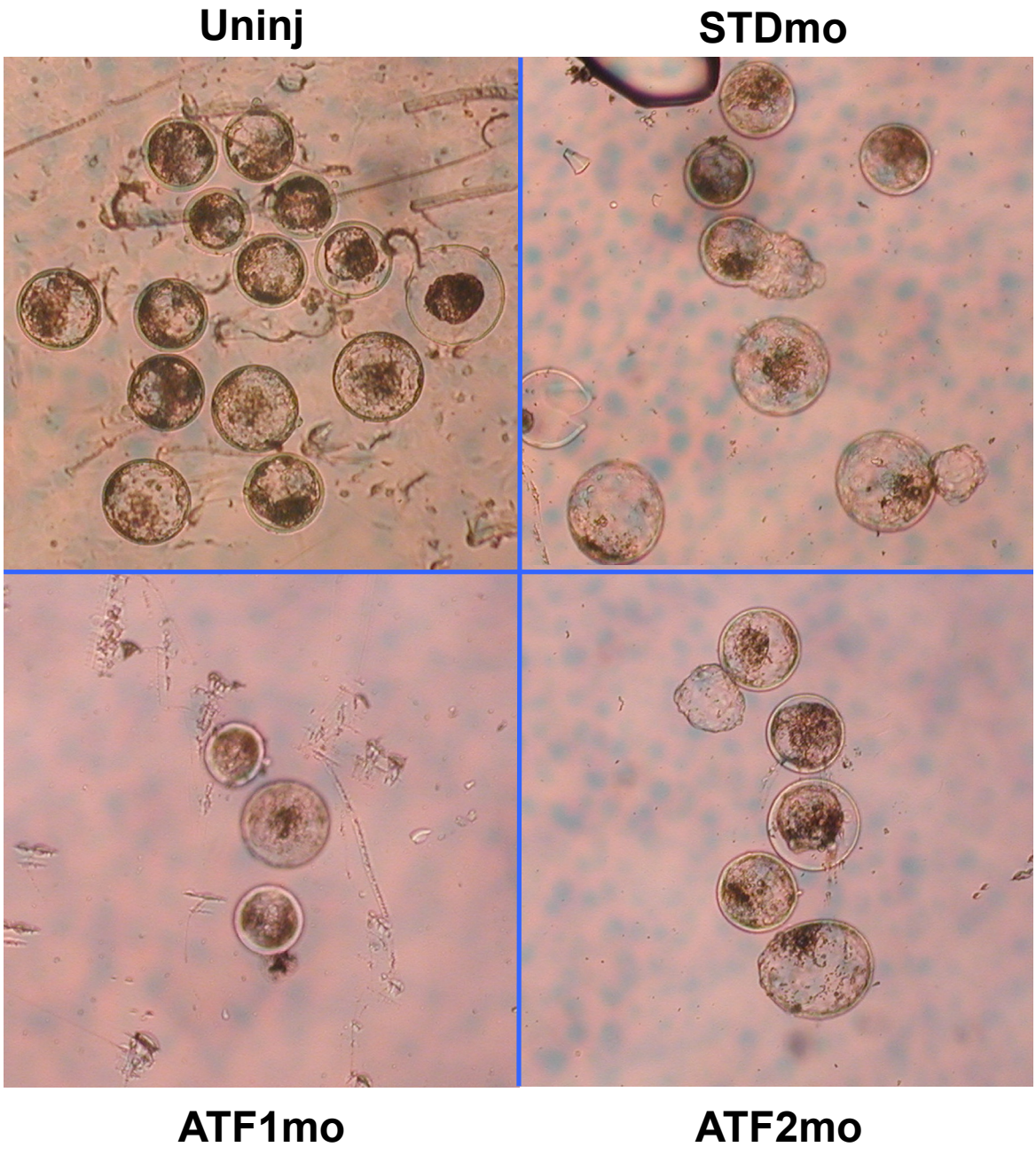

Figure 3-4 Embryos produced after morpholino oligonucleotides (MO) microinjection A) 8-cell embryos. B) Day-8 blastocysts. 100X original magnification. Pictures are representative of nine replicates in A and of five (Uninj, STDmo, and ATF1mo) and three (ATF2mo) replicates in B. Uninj, uninjected; STDmo, standard control morpholino; ATF1mo, ATF1-morpholino; ATF2mo, ATF2-morpholino.

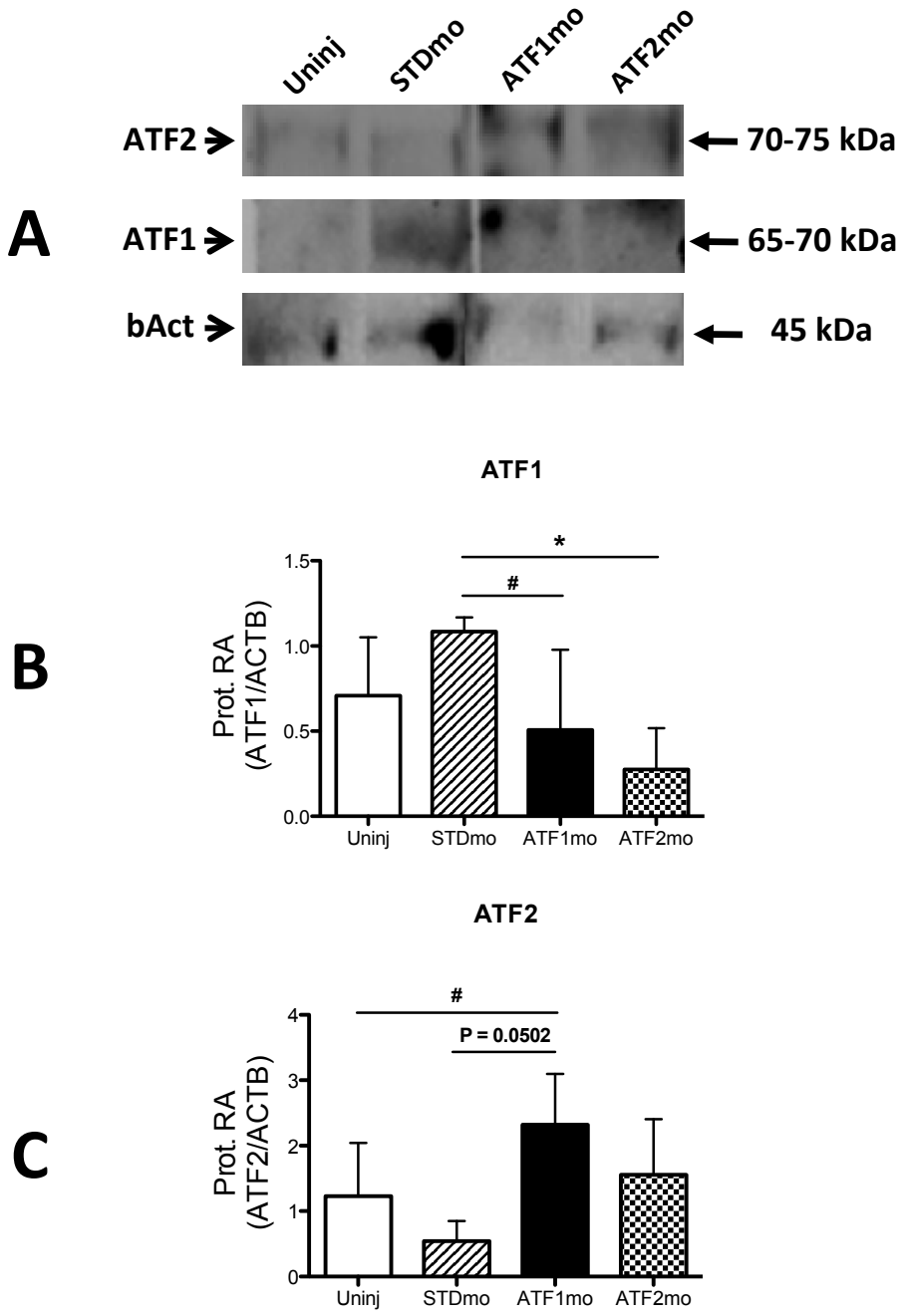

Figure 3-5 Western blot of microinjected 8-cell embryos

A) ATF1, ATF2, and bAct immunoblots; pictures are representative of three independent replicates. B) ATF1 protein expression; STDmo vs. ATF1mo, P = 0.1467 (#); STDmo vs. ATF2mo, P = 0.0171 (*). C) ATF2 protein expression; Uninj vs. ATF1mo, P = 0.1936 (#); STDmo vs. ATF1mo, P = 0.0502 (#). ATF1 and ATF2 were normalized relative to beta-actin (ACTB) expression. Plots were estimated from the three independent replicates represented in A). bAct, beta-actin; Uninj, uninjected; STDmo, standard control morpholino; ATF1mo, ATF1-morpholino; ATF2mo, ATF2-morpholino; kDa, kilodaltons; Prot, protein; RA, relative abundance.

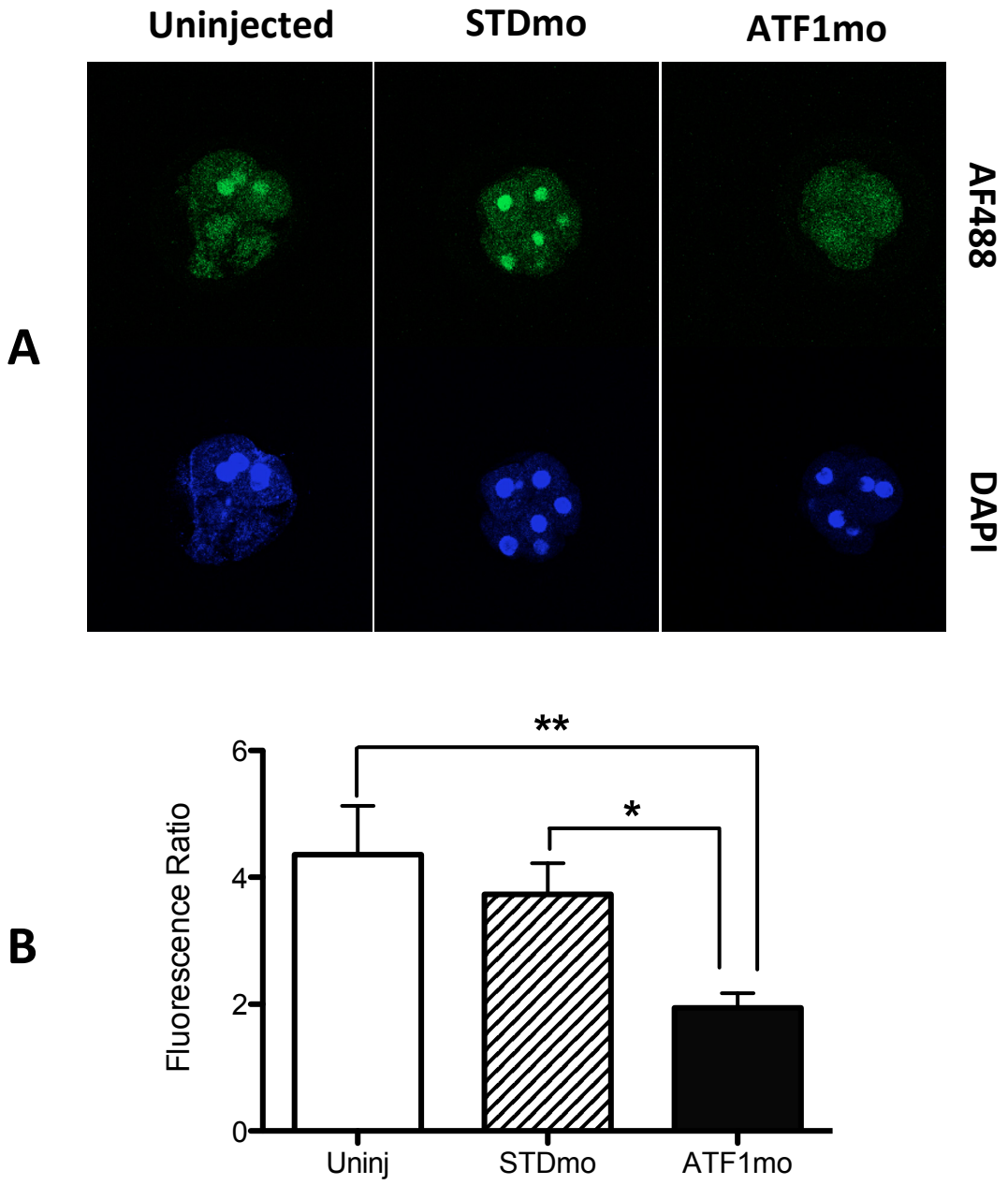

Figure 3-6 Immunofluorescence of microinjected 8-cell embryos
A) Confocal microscopy pictures representative of the analyzed embryos; 400X original magnification. B) MO treatment significantly impacted the nuclear / cytoplasmic fluorescence ratio of ATF1 protein, $P = 0.0051$ (**). Significant differences between groups when $P < 0.05$ (*), $P < 0.01$ (**). Plots were estimated from embryos represented in A). Uninj, three embryos, 10 nuclei; STDmo, three embryos, 13 nuclei; ATF1mo, five embryos, 13 nuclei. Uninj, uninjected; STDmo, standard control-morpholino; ATF1mo, ATF1-morpholino; AF488, Alexa Fluor 488; DAPI, 4', 6-diamidino-2-phenylindole.

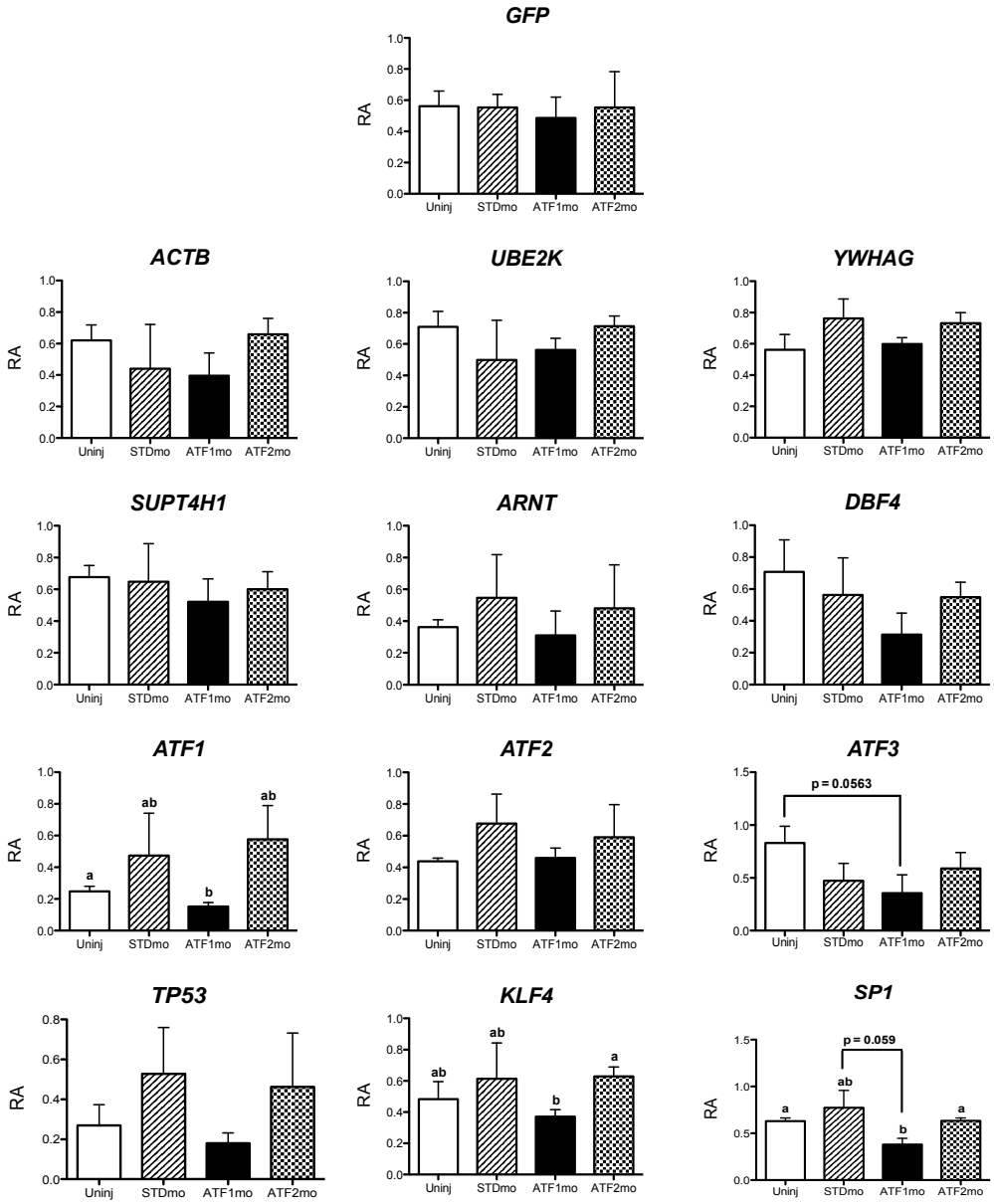

Figure 3-7 Determination of differential transcript levels in 8-cell embryos by RT-qPCR

Transcripts of all genes were normalized relative to *GFP* spike-in. Bars represent means ± standard error of three independent replicates. Distinct superscripts denote significant differences, $P < 0.05$. RA, relative abundance of mRNA; Uninj, uninjected; STDmo, standard control-morpholino, ATF1mo, ATF1-morpholino; ATF2mo, ATF2-morpholino; *GFP*, Green fluorescent protein; *ACTB*, beta-actin; *UBE2K*, ubiquitin-conjugating enzyme E2K; *YWHAG*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma; *SUPT4H1*, suppressor of Ty 4 homolog 1 (*S. cerevisiae*); *ARNT*, aryl hydrocarbon receptor nuclear translocator; *DBF4*, DBF4 homolog (*S. cerevisiae*); *ATF1*, Activating Transcription Factor 1; *ATF2*, Activating Transcription Factor 2; *ATF3*, Activating Transcription Factor 3; *TP53*, tumor protein 53; *KLF4*, Kruppel-like factor 4 (gut); *SP1*, Sp1 transcription factor.

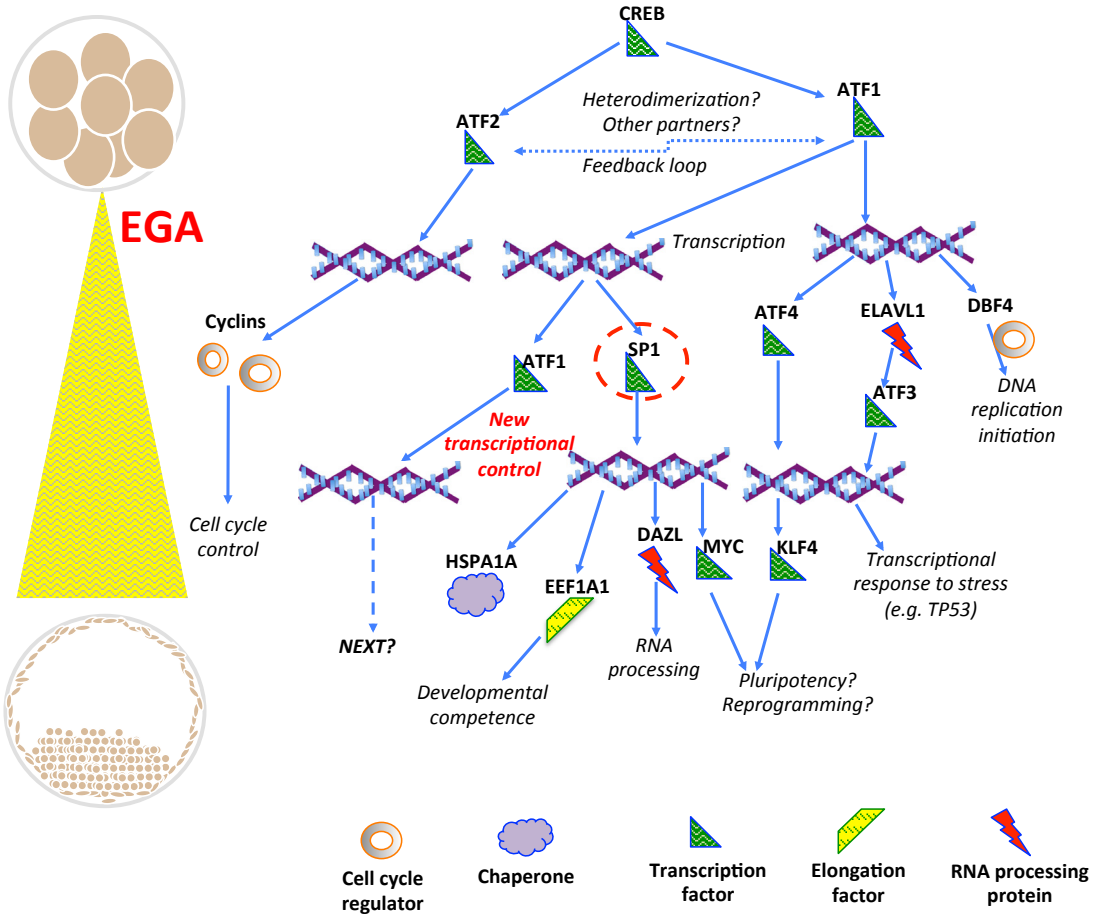

Figure 3-8 Proposed model of ATF1 and ATF2 regulation at time of embryonic genome activation (EGA)

CREB, c-AMP response element binding protein; ATF1, Activating Transcription Factor 1; ATF2, Activating Transcription Factor 2; ATF3, Activating Transcription Factor 3; ATF4, Activating Transcription Factor 4; miR-21, micro-RNA 21; SP1, transcription factor SP1; ELAVL1, ELAV-like RNA binding protein 1; DBF4, DBF4 homolog (*S. cerevisiae*); HSPA1A (HSP70.1), heat shock 70 kDa protein 1A; EEF1A1, eukaryotic translation elongation factor 1 alpha 1; DAZL, deleted in azoospermia-like; MYC (c-MYC), v-Myc avian myelocytomatosis viral oncogene homolog; KLF4, Kruppel-like factor 4 (gut); TP53, tumor protein 53.

4. Regulation of ATF1 and ATF2 Transcripts by Sequences in their 3'-Untranslated Region in Cleavage-stage Cattle Embryos

Ernesto Orozco-Lucero,[1] Isabelle Dufort,[1] Claude Robert[1] and Marc-André Sirard[1]

[1]Centre de Recherche en Biologie de la Reproduction (CRBR), Faculté des Sciences de l'Agriculture et de l'Alimentation, Département des Sciences Animales, Pavillon INAF, Université Laval, Québec, Qc, Canada

4.1. Résumé

La composition de la séquence de la queue 3'-UTR des ARN messagers dans les ovocytes et les embryons chez les mammifères dirige le minutage de leur polyadénylation et de leur traduction. Les objectifs de cette étude visaient à démontrer l'influence des éléments *cis*-régulateurs dans la queue 3'-UTR des transcrits importants pour le développement, ATF1 et ATF2, sur leur traduction synchronisée pendant les premiers clivages chez des embryons bovins. Huit ARNm rapporteurs différents (*GFP* liée à la 3'-UTR des isoformes courts ou longs de *ATF1*/*2* bovin, soit polyadenylés ou deadenylés), ainsi que le contrôle *GFP* ARNm, furent microinjectés séparément dans des zygotes potentiels de vache à 18 hpi. La traduction de la GFP fut évaluée entre 24 et 80 hpi par épifluorescence. Les résultats indiquent que la polyadenylation, ou la présence d'une séquence 3'-UTR dans des constructions deadenylées, est requise pour la traduction de la GFP. Tous les ARNm exogènes ont été traduits aussitôt qu'à 24 hpi avec l'exception de long-deadenylée *ATF2*-UTR, dont la traduction a commencé à 36 hpi. Globalement, la GFP a été plus visiblement traduite dans des embryons compétents (clivés), particulièrement celle des constructions longues-*ATF1*/*2*. Ces résultats montrent une traduction de GFP orchestrée selon les séquences dans le 3'-UTR de ATF1/2 et indiquent une différence entre les isoformes courts et longs. D'ailleurs, les embryons avec capacité de clivage ont montré une capacité traductionnelle augmentée des constructions évaluées. L'identification des séquences *cis*-régulatrices localisées dans la 3'-UTR de *ATF1*/*2* contribuera à comprendre le contrôle de la traduction de l'ARNm maternel pendant le développement précoce chez le bovin.

4.2. Abstract

In mammalian oocytes and early embryos sequence composition of the 3'-UTR of mRNA governs its timely polyadenylation and translation. The objectives of this study were to demonstrate the influence of *cis*-elements in the 3'-UTR of the developmentally important *ATF1* and *ATF2* transcripts on their timely translation during first cleavages in bovine embryos. Eight different reporter mRNAs (*GFP* fused to the 3'-UTR of short or long isoforms of cattle *ATF1*/*2*, either polyadenylated or deadenylated), as well as a control *GFP* mRNA were microinjected separately in cow presumptive zygotes at 18 hpi. GFP translation was assessed between 24 and 80 hpi by epifluorescence. Results indicate that presence of either polyadenine, or 3'-UTR sequence in deadenylated constructs is required for GFP translation (implying polyadenylation). All exogenous mRNAs were translated as soon as 24 hpi, except for long-deadenylated *ATF2*-UTR, whose main translation began at 36 hpi. Overall, GFP was more visibly translated in competent (cleaving) embryos, particularly in long *ATF1*/*2* constructs. The current data show a timely GFP translation in bovine embryos depending on sequences in the 3'-UTR of *ATF1*/*2* and indicate a difference between short and long isoforms. In addition, cleaving embryos displayed an increased translational capacity of the tested constructs. Identification of the *cis*-sequences in the 3'-UTR of *ATF1*/*2* will contribute to the understanding of maternal mRNA translation regulation during cattle early development.

4.3. Introduction

Early developmental progression largely depends on maternal transcripts synthesized and stored in the ooplasm before the embryo initiates its own transcription. The identity and functionality of these molecules has been explored during recent years in mice (Hamatani et al. 2004; Zeng et al. 2004), humans (Kocabas et al. 2006), pigs (Toms et al. 2014), rabbits (Leandri et al. 2009), and cattle (Misirlioglu et al. 2006; Fair et al. 2007; Labrecque et al. 2015). However, in eggs and early embryos the presence of the mRNA does not directly correlate with protein levels (Gilbert et al. 2009; Smits et al. 2014). This is due to the higher stability of their mRNA through deadenylation (Bachvarova et al. 1985; Paynton et al. 1988) and storage (rev. Anderson and Kedersha 2006) until *de novo* polyadenylation and recruitment for translation (Paris et al. 1988; Vassalli et al. 1989; Brevini-Gandolfi 1999). In cows for instance, Krischek and Meinecke (2002), Tomek et al. (2002), Lequarre et al. (2004), Traverso et al. (2005), and Tremblay et al. (2005) arrived at similar conclusions in relation to the requirement of polyadenylation of maternal transcripts and synthesis of their codified proteins for progression of oocyte maturation. In addition, polyadenylation status of maternal mRNA has been related to developmental competence (Brevini-Gandolfi et al. 1999; Brevini et al. 2002).

The occurrence of translation of particular maternal mRNA species during the preimplantation period has been shown in mammals through assessment of the polyribosomal fraction (Potireddy et al. 2006; Scantland et al. 2011). Mobilization of mRNA towards translation must be strictly orchestrated to fulfill the cell's physiological needs obeying a highly determined timing (Wang and Latham 2000; Potireddy et al. 2006; Chen et al. 2011). Work from Pique et al. (2008) unveiled a molecular code where the combination, number, and relative distance of the *cis*-elements nuclear polyadenylation signal (NPS), Pumilio-binding element (PBE), and cytoplasmic polyadenylation element (CPE) in the 3'-untranslated region (UTR) of mRNAs of frog oocytes governs the balance between translational inhibition (through deadenylation) and activation (by polyadenylation) of maternally-stored transcripts during maturation. A similar mechanism, in which sequence motifs in the 3'-UTR of maternal mRNA drive positive or negative signals, has also been demonstrated in rodents (Potireddy et al. 2006; Potireddy et al. 2010;

Chen et al. 2011). While McGraw et al. (2006) indicated the existence of an NPS and one A/U-rich element (ARE) in the 3'-UTR of the bovine oocyte-specific histone *H1FOO*, Tremblay et al. (2005) and Thelie et al. (2007) established that the polyadenylation/deadenylation profile of specific transcripts during IVM in bovine oocytes is correlated with the presence of putative CPE sequences. Nevertheless, Dobbs et al. (2010) did not observe direct association between putative CPEs and poly(A) tail length in pigs prior to embryonic genome activation (EGA). Thus, the authors concluded that CPEs do not seem to be the unique factor controlling transcript stability during swine early development and this could be possible for other mammalian species. In agreement, Gohin et al. (2014) discovered that, besides the widely known NPS and CPE sequences, a fraction of transcripts subjected to adenylation/deadenylation control throughout maturation of cattle eggs contain additional 3'-UTR motifs potentially involved in poly(A) tail length modulation.

It is interesting to note that the mRNA of the Activating Transcription Factor (*ATF*) *1* and *ATF2* contain short poly(A) tails in cow oocytes (Gohin et al. 2014). Both transcription factors are potentially crucial for early cattle embryogenesis given that Held et al. (2012) found that the *ATF1* mRNA is enriched in the sister blastomere of the most competent bovine 2-cell embryos, whereas Vigneault et al. (2009) observed the nuclear translocation of ATF2 protein by the time of EGA in the same species. Thus, this information implies elongation of the poly(A) tail of maternal *ATF1* and *ATF2* following fertilization in cows. In the survey of Tremblay et al. (2005) it was demonstrated the existence of *cyclin B1* short and long isoforms in cow oocytes and this last isoform contains a putative CPE that apparently triggers its elongation during IVM. In a similar fashion, *ATF1* and *ATF2* displayed short (s) and long (L) isoforms (due to 3'-UTR tiling) in the transcriptomic catalogue of cattle early developmental stages produced by our group (Robert et al. 2011). Nonetheless, a functional confirmation of the translational regulation of *ATF1*/*2* due to *cis*-elements in their 3'-UTR has not yet been observed. Due to the aforementioned observations it is plausible that specific *cis*-elements located in the 3'-UTR of *ATF1* and *ATF2* drive their timely translation when their proteins are required during early embryogenesis in the cow. Therefore, in order to better comprehend the control of

repression and activation of maternal mRNA during cattle early development by specific *cis*-elements, this study explored the dynamics of GFP translation driven by the 3'-UTR of either short or long isoforms of *ATF1* and *ATF2* transcripts in bovine zygotes and cleavage-stage embryos. Important translational patterns were discovered in terms of time, cleavage capacity, and specific modulatory 3'-UTR motifs. The proposed orchestrating mechanism appears as a potential link between key *cis*-elements in maternal mRNA, management of stored transcripts, developmental competence, and upstream regulatory molecular signaling.

4.4. Results

In order to assess the impatct of poly(A) tail length on the translation of the protein encoded by specific mRNAs, reporter constructs containing specific UTRs with varying poly(A) tail length were prepared.

4.4.1. Effect of poly(A) tail presence and time on reporter expression

Firstly, the presence of a poly A tail is essential for translation as the injection of a GFP mRNA with no adenylation (A-) did not produce any protein and hence fluorescence (Fig. 4-1). With a long (>150 A) poly(A) tail (A+) *GFP* was expressed at nearly maximal levels as early as 24 hpi. However, presence of GFP was significantly enhanced from 36 hpi. No differences in GFP expression upon microinjection of *GFP* with a small tail (*GFPA30*) mRNA were observed (Fig. 4-2). No differences were found when comparing *GFPA+* against *GFPA30* at the same time points (Fig. 4-3). When testing short (s) and long (L) candidate 3'-UTRs significant differences throughout time were not detected in *ATF1sA+*, *ATF1sA-*, *ATF1LA+*, *ATF1LA-*, *ATF2sA+*, *ATF2sA-*, and *ATF2LA+* either, while GFP was expressed at submaximal levels upon microinjection of all seven constructs at 24 hpi. Nevertheless, GFP expression was barely detected at 24 hpi in the group of *ATF2LA-*, followed by a dramatic increase in GFP translation from 36 hpi with an steady increase throughout cleavage development (Fig. 4-2). There were no differences when contrasting *ATF1sA+* and *ATF1sA-* or *ATF2sA+* against *ATF2sA-* (Fig. 4-3). In contrast, microinjection of the *ATF1LA+* construct produced higher GFP expression at all time points except 36 hpi, while the only difference between *ATF2LA+* and *ATF2LA-* was at 24 hpi, when GFP

translation in the later group was still low or absent but recovered dramatically as soon as 36 hpi (Fig. 4-2; Fig. 4-3; Fig. 4-4).

4.4.2. Impact of cleaving capacity on GFP expression

Concerning the effects of cleavage on fluorescence of the reporter, GFP abundance was higher in presumptive zygotes than cleaved embryos at 24 hpi, while significant differences with the opposite pattern were present at all other time points. A similar behavior was observed in *GFPA30* at all time points except at 36 hpi, where no differences were detected (Fig. 4-5). In *ATF1sA+* and *ATF1sA-* GFP expression was higher in the absence of cleavage at both 24- and 36 hpi or only at 24 hpi, respectively and any difference was lost thereafter (Fig. 4-6). Levels of GFP were increased in *ATF1LA+* zygotes contrasted with their cleaved counterparts at 24 hpi. This difference was lost at the second timed assessment, while fluorescence was significantly amplified in cleaved embryos across 48-, 60-, and 80 hpi. As for *ATF1LA-*, putative 1-cell embryos showed significantly more fluorescent signal at 24 hpi and any significant difference was lost subsequently, although a trend to higher GFP in cleaved embryos was noted. Interestingly, a striking contrast between *ATF1sA+* and *ATF1LA+* was observed, in which GFP expression was higher in absence of embryonic division upon microinjection of the short isoform. In contrast, translation was significantly higher in *ATF1LA+* cleaved embryos compared to zygotes. Therefore, non-cleaved eggs are less likely to translate either *ATF1LA+* or *ATF1LA-* (Fig. 4-6). While no differences were observed in *ATF2sA+* across time, *ATF2sA-* zygotes displayed increased GFP only at 24 hpi. Besides the higher GFP translation in the absence of cleavage at 24 hpi in *ATF2LA+*, the fluorescence analysis uncovered that cleaved embryos treated with either this construction or *ATF2LA-* exhibited higher GFP signal during further cleavage development in a similar way as long *ATF1* constructs. In summary, the improved GFP expression in cleaved embryos compared to putative zygotes upon microinjection of the long ATF2 construct contrasting with higher translation in *ATF2s* zygotes than in ATF1 constructions since such profile was observed for both *ATF2sA+* vs. *ATF2LA+* and *ATF2sA-* vs. *ATF2LA-* (Fig. 4-7).

4.4.3. 3'-Untranslated region sequences

The total number of specific *cis*-elements localized in the four distinct types of 3'-UTR analyzed in our study (corresponding to *ATF1s*, *ATF1L*, *ATF2s*, and *ATF2L*) is shown in Table 4-1 (data for the long 3'-UTR isoform is the addition of the sequences from the short 3'-UTR and those of the long isoform). The complete sequences of the constructs and positions of all *cis*-elements mapped in this study are available in Supplemental File 4-1. It is remarkable that *ATF1s* displays only two consensus and potential CPE. *ATF1L* in contrast contains 10 of these motifs, whereas the number of PBE is 1 and 13 for *ATF1s* and *ATF1L*, respectively (Table 4-1; Fig. 4-8). A different scenario is seen for *ATF2* as one and four NPS, 10 and 16 consensus/potential CPE, one and two embryonic CPE (Oh et al. 2000), nine and 23 PBE, four and seven ARE, as well as two and five motif associated with polyadenylation signal (MAPS)/MAPS-like sequences (Gohin et al. 2014) are located in *ATF2s* and *ATF2L*, respectively. Considering the latter, it is only *ATF2L* that includes the only two consensus MAPS found. In addition, two DAZL-recognition elements (DRE) are shared by *ATF2s* and *ATF2L*. Finally, one and two embryo deadenylation element (EDEN)-like sequences (Uzbekova et al. 2006) are mapped in *ATF1s* and *ATF1L*, respectively, whereas two polyadenylation response element (PRE)-like stretches (Charlesworth et al. 2004) are observed in *ATF1L* (Table 4-1; Fig. 4-8). With respect to distances between important motifs in 3-UTR of *ATF1* and *ATF2*, it is of note that *ATF1L* contains consensus/potential CPEs at -81 and -45 nt of its NPS, whereas in *ATF2L* the CPEs are separated by more than 70 nt. In this last 3'-UTR, 1st CPE and 3rd PBE separated by 6 nt; the 4th CPE and 5th PBE are located right next to each other; the 6th PBE and 1st NPS have a distance of just 2 nt in between; the 5th CPE and 1st NPS are separated by 51 nt; and the 6th CPE and 3rd NPS separated by 31 nt.

4.5. Discussion

The current work provides functional evidence of the influence of 3'-UTR sequences on translational dynamics of two important transcription activators, ATF1 and -2 during mammalian early embryogenesis. This was achieved by the analysis of expression of an exogenous reporter protein with time following fertilization and the capacity of embryos to cleave. In addition, *cis*-acting elements were localized in the tested constructs in an effort to

explain such translational control mechanisms, as well as to infer the possible upstream molecular regulators.

4.5.1. Presence of poly(A) tail or 3'-untranslated region is necessary for GFP translation

The presence of either a poly(A) tail synthesized *in vitro* before microinjection or the fusion of *GFP* with a 3'-UTR of any of the tested constructions was necessary to observe GFP translation. This was clear considering the absence of fluorescence upon *GFPA*-microinjection in contrast to reporter translation in deadenylated constructs. Moreover, a short to medium-size poly(A) tail was sufficient to drive GFP translation, as demonstrated by the microinjection of *GFP* mRNA that included the stretch of 30 adenine residues, although with a non-significantly lower performance at the onset of the cleavage period in comparison to *GFPA+*, whose poly(A) tail length was estimated to have an average of 150 adenine residues (data not shown). In agreement with the observations upon microinjection of *GFP* mRNAs it is the fact that the requirement of polyadenylation of an mRNA to be recruited for translation is widely accepted (rev. Tomek and Wollenhaupt 2012; Charlesworth et al. 2013). This notion suggests that the GFP expression generated following microinjection of deadenylated *GFP-ATF* 3'-UTR constructs was due to polyadenylation driven by the corresponding 3'-UTR and specifically by *cis*-elements included in these sequences of all tested *GFP-ATF* 3'-UTR transcripts. The identity of these motifs will be discussed below.

4.5.2. Elapsed time from fertilization affects GFP expression

Initiation of GFP expression of all constructions with a 3'UTR, in spite of being microinjected in a deadenylated or polyadenylated form, took place from 24 and 36 hpi and appears independent of cleavage occurrence. Thus, such behavior is in agreement with the concept of the "zygotic clock", which refers to the elapsed time from fertilization (Wiekowski et al. 1991; Schultz 1993). Control of the onset of transcription by a zygotic clock was described in fertilized eggs with gene reporters in cattle (Gagne et al. 1995), as well as expression of endogenous genes in the murine model (Kigami et al. 2003). Christians et al. (1995) and Qiu et al. (2003) reported that translation of HSP70.1 codified

by mRNA synthesized at zygotic genome activation (ZGA) is also initially governed by the elapsed time after fertilization. Although time appears to modulate protein synthesis from the current *GFP-ATF 3'-UTR* reporters, one cannot discard the possibility that translation could be regulated by other factors, like early embryonic division (and the underlying DNA duplication), as it has been previously indicated for both transcription and translation in mice (Wiekowski et al. 1991; Christians et al. 1995; Sonehara et al. 2008).

As mentioned before, all constructs except *ATF2LA-*, produce fluorescence soon after microinjection but this last construct required an additional period of 12 hr to induce synthesis of the exogenous protein independently of the presence or absence of cleavage. Given that GFP synthesis in the *ATF2LA-* group was delayed in contrast with *ATF2LA+* it can be suggested that the major polyadenylation of *ATF2LA-* occurs around 36 hpi. Moreover, because the deadenylated short *ATF2* rendered translation from 24 hpi it could be inferred that the lagged GFP translation from *ATF2LA-* relies on temporary repression of polyadenylation (or alternatively late induction) by sequence motifs located specifically in the long *ATF2* 3'-UTR. The nature of the candidate *cis*-elements involved in this mechanism and contained in *ATF2LA-* will be discussed in the following sections.

4.5.3. Increased cleaving capacity improves translation of long constructs

The observation that translation appeared enhanced in competent (cleaving) embryos both in long *GFP-ATF* constructs, as well as in *GFP* mRNA without any 3'-UTR (but with a poly(A) tail) suggests that developmental competence might be associated with intrinsic endowment of translational capacity. Because of the bias of GFP expression at 24 hpi it can be proposed that this could reflect the number of zygotes that will actually divide soon, whereas an important portion of cleaved embryos with positive GFP signal from 36 hpi onwards originated from the zygotes that fluoresced by 24 hpi.

4.5.4. Potential Motifs in the 3'-untranslated region and possible regulatory upstream factors

The multiple additional sequence motifs localized in long 3'-UTRs of *ATF1/2* with respect to their short isoforms offers a conundrum concerning putative regulatory mechanisms of

translation activation and repression. This *cis*-originated potential modulatory system could explain the distinct profiles of GFP expression between constructs containing short and long 3'-UTR. The increased capacity of the most competent embryos to translate an already polyadenylated transcript from long constructs might denote that cleaving embryos are enriched with *trans*-acting factors involved in circularization of mRNA or translation initiation when such molecules already contain a poly(A) tail of adequate size, as it was for *ATF1s*/*LA*+ and *ATF2s*/*LA*+ mRNAs. Interestingly, Labrecque et al. (2013) indicated that the mRNAs of seven translation initiator factors (*EIF3G*, *EIF1AD*, *EIF3C*, *EIF4E1B*, *EIF5A*, *EIF4A3*, *EIF4A2*) and a translation initiator factor kinase (*EIF2AK1*) as well as those of two poly(A)-binding proteins (*PABPN1*, *PABPC1L*) have differential abundance levels in GV-oocytes according to their quality from cows subjected to FSH superstimulation and withdrawal protocol, while *EIF4A1* was affected in cattle following FSH-coasting/cetrotide treatment (Labrecque et al. 2014). Furthermore, transcripts of *EIF5A2* and *EIF2AK4* were found at higher levels in the most viable 2-cell bovine embryos (Orozco-Lucero et al. 2014). Therefore, it appears plausible that factors enriched in the most competent oocytes, or embryos shortly after fertilization, allow a better translational capacity that could account for increased protein synthesis from determined mRNAs during cleavage stages. In such a model, the possible targeted sequences in the long 3'-UTRs of *ATF1*/*2* with a long 3'-UTR that would make such isoform (already polyadenylated) translated at higher levels in competent embryos could correspond to ARE motifs, which are more abundant in the extended 3'-UTR of both *ATF1* and *ATF2* than their short isoforms (Fig. 4-8). Besides the known role of AREs in deadenylation pathways leading to translational inhibition (Voeltz and Steitz 1998), Sladic et al. (2004) established that human PABPs are able not only to bind poly(A) tail but also ARE sequences. Moreover, PABP1 can also function as a translation activator when binding to mRNA sites other than poly(A) tail (Burgess and Gray 2012; Smith et al. 2014) and for this reason PABP has even been qualified as a "translation elongation factor" by some authors (Kahvejian et al. 2005). A possible test for the role of quite specific *cis*-elements could be the preparation of trimmed or site-mutated transcripts and for evaluation of the resulting translational dynamics through microinjection. Due to the molecular anatomy of *ATF1L*, where the only ARE

exclusive for such isoform is located in the middle of the 3'-UTR, the method of choice would be site-directed mutagenesis.

The most obvious examples of candidate sequences regulating the distinct translational kinetics due to polyadenylation are the constituted by PBE, NPS, and CPE interactions. Pique et al. (2008) detected a combinatorial code of number and relative position of these elements in *Xenopus* eggs that determines activation or inhibition. Since a standard parameter for polyadenylation activation can be considered a distance of around 60 nt between CPE (either upstream or downstream) and NPS (rev. Radford et al. 2008), it can be inferred that the first two CPEs and the NPS of *ATF1L* could define an activating mechanism, but not the next CPEs as they are too far from the NPS. It is accepted that NPS is bound by the protein cleavage and polyadenylation specificity factor (CPSF) in order to drive polyadenylation (Tomek and Wollenhaupt 2012). In this regard, it is remarkable that bovine oocytes from cows under optimal and suboptimal regimes of FSH-coasting differed in the transcript levels of *CPSF1*, *CPSF2*, and *CPSF3L* (Labrecque et al. 2013), which points out again the possibility that the molecular signal triggered by FSH starvation before oocyte maturation is instrumental for the elevated translational capacity of competent embryos (Fig. 4-9). The importance of the mRNA-processing protein DAZL for translational dynamics was underscored by the observations that polysomal transcripts at or shortly before ZGA are enriched with DRE motifs (Chen et al. 2011). Thus, the DRE-sites shared by both isoforms of *ATF2* deserve consideration for monitoring through elimination of DRE(s).

The late translation recruitment that was observed for *ATF2LA*- is an intriguing observation that posits the existence of *cis*-elements in its 3'-UTR that exert a temporal negative effect on polyadenylation. Gohin et al. (2014) performed *in silico* analysis of *Xenopus* and zebrafish sequences and found that the MAPS motif appears present in transcripts containing a CPE. Remarkably, in the same work it was detected that consensus MAPS sites are enriched in deadenylated mRNAs in cow oocytes and their analysis of the molecular anatomy of 3'-UTR of *ATF1* and *ATF2* showed that only the second transcript bears a consensus MAPS (Gohin et al. 2014). Such indication seems to support the notion

of a *cis*-based mechanism that retarded GFP translation from *ATF2LA*-. Therefore, it could be plausible that MAPS constitutes a regulatory mechanism of endogenous *ATF2* with a long 3'-UTR in bovine early development to be translated at the opportune moment. Upon data mining Gohin et al. (2014) established that a candidate GO-term associated with MAPS is constituted by RNA binding (GO:0003723, amigo.geneontology.org). Analysis of this accession retrieves EXOSC7 from *Canis lupus*. EXOSC7 is an exosome component. The exosome is a structure that is mainly known by its transcript degradation function, although it is also involved in transcript retention (rev. Fasken and Corbett 2009). Since Labrecque et al. (2013) detected altered levels of *EXOSC5*, *EXOSC8*, and *EXOSC10* mRNA in bovine oocytes according to their competence level, it might be plausible that MAPS constitutes a temporal retardation system for translation of specific transcripts during early development. Alternatively, another candidate which could interact with MAPS is the initiation factor EIF2G, given that the GO:0003723 (Gohin et al. 2014) leads to access EIF2G (EIF2S3) of rhesus monkey (XP_001112836.1, NCBI). Also, cow oocytes of the highest quality contain increased levels of *EIF2G* (Labrecque et al. 2013), advocating that this elongation factor could be a regulatory mechanism for translation of developmentally competent oocytes or cleavage-stage embryos (Fig. 4-9).

An upstream regulator analysis was performed (IPA, Ingenuity Systems, www.ingenuity.com) by using the candidate proteins that potentially bind the *cis*-elements detected in the present work. Such *in silico* assessment placed CDKN1A as a putative upstream regulator (data not shown). This cyclin-dependent kinase inhibitor was previously detected as a candidate upstream regulator in fast-cleaving 2-cell bovine embryos (Orozco-Lucero et al. 2014), further supporting that the potential translational control proposed here is associated with developmental competence. CDKN1A is in turn modulated by TP53. Considering that FSHR signaling leads to p38 MAPK regulation via PKA (Ali and Sirard 2005; Khan et al. 2015) and that such MAPK has an effect on TP53 (rev. Xu et al. 2014), this could point to a central regulatory pathway of developmental competence.

In conclusion, the results from the current work suggest modulation of the translational dynamics of *ATF1* and *ATF2* due to *cis*-elements located in their 3'-UTR. Timing of the

expression of *GFP-ATF* 3'-UTR constructions upon microinjection in putative zygotes can give an idea of the synchrony of translation the endogenous *ATF1*/*2* according to their 3'-UTR (long or short) once their respective mRNA is free in the ooplasm and available to the translational machinery. Our observations of *ATF1*/*2* regulation through 3'-UTR indicates that expression of both transcription factors is not only controlled at the transcriptional level (Vigneault et al. 2004; Held et al. 2012; Orozco-Lucero et al. 2014), but also at the translational level, as previously implied by data from Vigneault et al. (2009), which highlights the importance of a tight control of these transcription factors for early embryogenesis outcome. Assessment of the localized *cis*-elements lead to the discovery of the potential proteins that modulate timing of ATF1/2 protein synthesis. Such analysis suggested a link between translational control and developmental competence.

4.6. Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

4.6.1. Preparation of mRNAs

The full coding sequences of enhanced *GFP* (*eGFP*), as well as the short or long 3'-UTR of the bovine *ATF1* and *ATF2* were cloned (details in Supplemental File 4-2) to generate the following transcripts: *GFP*, *GFP*-short *ATF1*_UTR3 (*ATF1s*), *GFP*-Long *ATF1*_UTR3 (*ATF1L*), *GFP*-short *ATF2*_UTR3 (*ATF2s*), *GFP*-Long *ATF2*_UTR3 (*ATF2L*). mRNAs were synthesized and 5'-capped with the mMESSAGE mMACHINE T7 Ultra Kit (Ambion, Foster City, CA). The length of the mRNAs (excluding the poly-adenine tail) was the following: *GFP*, 720; *ATF1*_short, 2,092; *ATF1*_Long, 2,858; *ATF2*_short, 2,209; and *ATF2*_Long, 4,013 nt. For polyadenylated mRNAs it was estimated an average of 150 adenine residues additional to the length of the transcripts above. A total of 11 mRNAs were ready for microinjection: *GFPA+*, *GFPA30*, *GFPA-*, *ATF1sA+*, *ATF1sA-*, *ATF1LA+*, *ATF1LA-*, *ATF2sA+*, *ATF2sA-*, *ATF2LA+*, and *ATF2LA-*.

4.6.2. *In vitro* production of bovine embryos

Standard techniques from our laboratory (Cagnone et al. 2012; Cagnone and Sirard 2013) were followed to produce embryos after *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) using cow ovaries from a local slaughterhouse.

4.6.3. Microinjection of presumptive zygotes

Just prior to microinjection mRNA aliquots were allowed to thaw and each mRNA was prepared to a final concentration of 747 nM mixed with 4 mg/mL dextran Texas red (dTXr), 10,000 MW (Mol. Probes, Burlington, ON, Canada). Borosilicate micropipettes (with filament) were manufactured with a P-87 puller (Sutter Instr., Novato CA) and a microforge (Narishige, East Meadow NY). The holding micropipette was burn-polished to leave an internal diameter of 30-40 μm and an external diameter (ED) of around 110 μm that was coupled to a hydraulic control (Narishige) filled with mineral oil, whereas the injection micropipette had an ED of 1.5-2.0 μm. This needle was loaded by capillarity with the mRNA/dTXr mixture and coupled to an air pneumatic control (Narishige). A 25-30° taper angle was added to both types of needles. Microinjection of cumulus-denuded presumptive zygotes was performed at 18 hpi. Embryos already cleaved at such time point were discarded from the experiment. Microinjection medium consisted on SOF1 supplemented with 25 mM HEPES. Groups of 10-20 putative zygotes were placed in droplets of 150 μL microinjection medium placed on the microscope stage at 37°C. Zygotes of a single treatment were microinjected at once, whereas the other groups remained at 38.5°C/5% CO_2 to diminish exposure to a detrimental environment and the order in which groups were injected was alternated between experiments. The injection needle was broken close to the tip just before starting the experiment. Every presumptive zygote was injected with an estimated volume of 6-10 pL. To corroborate mRNA delivery in the cytoplasm, dTXr-epifluorescence was used at a submaximal intensity for 1-3 seconds to diminish UV light-driven damage (Fig. 4-10). At the end of microinjection each group of zygotes was washed four times and returned to SOF1 38.5°C/5% O_2/6.5% CO_2/88.5% N_2.

4.6.4. Fluorescence assessment

Presence of GFP fluorescence was monitored in zygotes or cleaved embryos at 24, 36, 48, 60, and 80 hpi by using epifluorescence at a submaximal intensity. Pictures were taken with an Axiocam ERc 5s camera (Zeiss, Oberkochen, Germany) and the number of GFP-positive embryos was recorded.

4.6.5. Statistical analysis

After evaluation of GFP translation in putative zygotes/cleaved embryos the effect of time on GFP expression was calculated through one-way ANOVA and Newman Keuls *post-hoc* test, whereas unpaired one-tailed T-test was used to assess the effect of cleavage on GFP translation. Differences were considered when $P < 0.05$.

4.6.6. Localization of 3'-UTR motifs

For *in silico* study 3'-UTR sequences of the tested constructs were analyzed with the megablast algorithm from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and motifs were then mapped.

4.7. Acknowledgements

We thank Isabelle Laflamme and Isabelle Gilbert (Université Laval, Québec, Canada) for their technical assistance in the *in vitro* production of bovine embryos and critical comments. EOL was supported by a FRQNT studentship from the Government of Quebec and a CONACYT boursary from Mexico.

4.8. References

Ahringer J, Rosenquist TA, Lawson DN, Kimble J. 1992. The Caenorhabditis elegans sex determining gene fem-3 is regulated post-transcriptionally. *EMBO J* **11:** 2303-2310.

Ali A, Sirard MA. 2005. Protein kinases influence bovine oocyte competence during short-term treatment with recombinant human follicle stimulating hormone. *Reproduction* **130:** 303-310.

Anderson P, Kedersha N. RNA granules. 2006. *J Cell Biol* **172:** 803-808.

Arumugam K, Macnicol MC, Macnicol AM. 2012. Autoregulation of Musashi1 mRNA translation during Xenopus oocyte maturation. *Mol Reprod Dev* **79:** 553-563.

Bachvarova R, De Leon V, Johnson A, Kaplan G, Paynton BV. 1985. Changes in total RNA, polyadenylated RNA, and actin mRNA during meiotic maturation of mouse oocytes. *Dev Biol* **108:** 325-331.

Brevini TA, Lonergan P, Cillo F, Francisci C, Favetta LA, Fair T, Gandolfi F. 2002. Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. *Mol Reprod Dev* **63:** 510-517.

Brevini-Gandolfi TA, Favetta LA, Mauri L, Luciano AM, Cillo F, Gandolfi F. 1999. Changes in poly(A) tail length of maternal transcripts during in vitro maturation of bovine oocytes and their relation with developmental competence. *Mol Reprod Dev* **52:** 427-433.

Burgess HM, Gray NK. 2012. An integrated model for the nucleo-cytoplasmic transport of cytoplasmic poly(A)-binding proteins. *Commun Integr Biol* **5:** 243-247.

Cagnone GL, Dufort I, Vigneault C, Sirard MA. 2012. Differential gene expression profile in bovine blastocysts resulting from hyperglycemia exposure during early cleavage stages. *Biol Reprod* **86:**50.

Cagnone GL, Sirard MA. 2013. Transcriptomic signature to oxidative stress exposure at the time of embryonic genome activation in bovine blastocysts. *Mol Reprod Dev* **80:** 297-314.

Charlesworth A, Cox LL, MacNicol AM. 2004. Cytoplasmic polyadenylation element (CPE)- and CPE-binding protein (CPEB)-independent mechanisms regulate early class maternal mRNA translational activation in Xenopus oocytes. *J Biol Chem* **279:** 17650-17659.

Charlesworth A, Meijer HA, de Moor CH. 2013. Specificity factors in cytoplasmic polyadenylation. *Wiley Interdiscip Rev RNA* **4:** 437-461.

Chen HH, Welling M, Bloch DB, Munoz J, Mientjes E, Chen X, Tramp C, Wu J, Yabuuchi A, Chou YF, Buecker C, Krainer A, Willemsen R, Heck AJ, Geijsen N. 2014. DAZL limits pluripotency, differentiation, and apoptosis in developing primordial germ cells. *Stem Cell Reports* **3:** 892-904.

Chen J, Melton C, Suh N, Oh JS, Horner K, Xie F, Sette C, Blelloch R, Conti M. 2011. Genome-wide analysis of translation reveals a critical role for deleted in azoospermia-like (Dazl) at the oocyte-to-zygote transition. *Genes Dev* **25:** 755-766.

Christians E, Campion E, Thompson EM, Renard JP. 1995. Expression of the HSP 70.1 gene, a landmark of early zygotic activity in the mouse embryo, is restricted to the first burst of transcription. *Development* **121:** 113-122.

Cragle C, MacNicol AM. 2014. Musashi protein-directed translational activation of target mRNAs is mediated by the poly(A) polymerase, germ line development defective-2. *J Biol Chem* **289:**14239-14251.

Cui J, Sartain CV, Pleiss JA, Wolfner MF. 2013. Cytoplasmic polyadenylation is a major mRNA regulator during oogenesis and egg activation in Drosophila. *Dev Biol* **383:**121-131.

Dobbs KB, Spollen WG, Springer G, Prather RS. 2010. The role of cytoplasmic polyadenylation element sequence on mRNA abundance during porcine embryogenesis and parthenogenetic development. *Mol Reprod Dev* **77:** 699-709.

Evsikov AV, Marin de Evsikova C. 2009. Gene expression during the oocyte-to-embryo transition in mammals. *Mol Reprod Dev* **76:** 805-818.

Fair T, Carter F, Park S, Evans AC, Lonergan P. 2007. Global gene expression analysis during bovine oocyte in vitro maturation. *Theriogenology* **68 Suppl 1:** S91-97.

Fasken MB, Corbett AH. 2009. Mechanisms of nuclear mRNA quality control. *RNA Biol* **6:** 237-241.

Fox CA, Sheets MD, Wickens MP. 1989. Poly(A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by the sequence UUUUUAU. *Genes Dev* **3:** 2151-2162.

Gagne M, Pothier F, Sirard MA. 1995. Effect of microinjection time during postfertilization S-phase on bovine embryonic development**.** *Mol Reprod Dev* **41:** 184-94.

Gilbert I, Scantland S, Sylvestre EL, Gravel C, Laflamme I, Sirard MA, Robert C. 2009. The dynamics of gene products fluctuation during bovine pre-hatching development. *Mol Reprod Dev* **76:** 762-772.

Gohin M, Fournier E, Dufort I, Sirard MA. 2014. Discovery, identification and sequence analysis of RNAs selected for very short or long poly A tail in immature bovine oocytes. *Mol Hum Reprod* **20:** 127-138.

Hamatani T, Carter MG, Sharov AA, Ko MS. 2004 Dynamics of global gene expression changes during mouse preimplantation development. *Dev Cell* **6:** 117-131.

Held E, Salilew-Wondim D, Linke M, Zechner U, Rings F, Tesfaye D, Schellander K, Hoelker M. 2012. Transcriptome fingerprint of bovine 2-cell stage blastomeres is directly correlated with the individual developmental competence of the corresponding sister blastomere. *Biol Reprod* **87:** 154.

Kahvejian A, Svitkin YV, Sukarieh R, M'Boutchou MN, Sonenberg N. 2005. Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. *Genes Dev* **19:** 104-113.

Kigami D, Minami N, Takayama H, Imai H. 2003. MuERV-L is one of the earliest transcribed genes in mouse one-cell embryos. *Biol Reprod* **68:** 651-654.

Khan DR, Guillemette C, Sirard MA, Richard FJ. 2015. Characterization of FSH signalling networks in bovine cumulus cells: a perspective on oocyte competence acquisition. *Mol Hum Reprod* **21:** 688-701.

Kocabas AM, Crosby J, Ross PJ, Otu HH, Beyhan Z, Can H, Tam WL, Rosa GJ, Halgren RG, Lim B, Fernandez E, Cibelli JB. 2006. The transcriptome of human oocytes. *Proc Natl Acad Sci U S A* **103:** 14027-14032.

Krischek C, Meinecke B. 2002. In vitro maturation of bovine oocytes requires polyadenylation of mRNAs coding proteins for chromatin condensation, spindle assembly, MPF and MAP kinase activation. *Anim Reprod Sci* **73:** 129-140.

Labrecque R, Lodde V, Dieci C, Tessaro I, Luciano AM, Sirard MA. 2015. Chromatin remodelling and histone m RNA accumulation in bovine germinal vesicle oocytes. *Mol Reprod Dev* **82:** 450-462.

Labrecque R, Vigneault C, Blondin P, Sirard MA. 2013. Gene expression analysis of bovine oocytes with high developmental competence obtained from FSH-stimulated animals. *Mol Reprod Dev* **80:** 428-440.

Labrecque R, Vigneault C, Blondin P, Sirard MA. 2014. Gene expression analysis of bovine oocytes at optimal coasting time combined with GnRH antagonist during the no-FSH period. *Theriogenology* **81:** 1092-1100.

Leandri RD, Archilla C, Bui LC, Peynot N, Liu Z, Cabau C, Chastellier A, Renard JP, Duranthon V. 2009. Revealing the dynamics of gene expression during embryonic genome activation and first differentiation in the rabbit embryo with a dedicated array screening. *Physiol Genomics* **36:** 98-113.

Lequarre AS, Traverso JM, Marchandise J, Donnay I. 2004. Poly(A) RNA is reduced by half during bovine oocyte maturation but increases when meiotic arrest is maintained with CDK inhibitors. *Biol Reprod* **71:** 425-431.

McGraw S, Vigneault C, Tremblay K, Sirard MA. 2006. Characterization of linker histone H1FOO during bovine in vitro embryo development. *Mol Reprod Dev* **73:** 692-699.

Misirlioglu M, Page GP, Sagirkaya H, Kaya A, Parrish JJ, First NL, Memili E. 2006. Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos. *Proc Natl Acad Sci U S A* **103:** 18905-18910.

Nakahata S, Kotani T, Mita K, Kawasaki T, Katsu Y, Nagahama Y, Yamashita M. 2003. Involvement of Xenopus Pumilio in the translational regulation that is specific to cyclin B1 mRNA during oocyte maturation. *Mech Dev* **120:** 865-880.

Oh B, Hwang S, McLaughlin J, Solter D, Knowles BB. 2000. Timely translation during the mouse oocyte-to-embryo transition. *Development* **127:** 3795-3803.

Orozco-Lucero E, Dufort I, Robert C, Sirard MA. 2014. Rapidly cleaving bovine two-cell embryos have better developmental potential and a distinctive mRNA pattern. *Mol Reprod Dev* **81:** 31-41.

Paris J, Osborne HB, Couturier A, Le Guellec R, Philippe M. 1988. Changes in the polyadenylation of specific stable RNA during the early development of Xenopus laevis. *Gene* **72:** 169-176.

Paynton BV, Rempel R, Bachvarova R. 1988. Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. *Dev Biol* **129:** 304-314.

Pique M, Lopez JM, Foissac S, Guigo R, Mendez R. 2008. A combinatorial code for CPE-mediated translational control. *Cell* **132:** 434-448.

Potireddy S, Midic U, Liang CG, Obradovic Z, Latham KE. 2010. Positive and negative cis-regulatory elements directing postfertilization maternal mRNA translational control in mouse embryos. *Am J Physiol Cell Physiol* **299:** C818-827.

Potireddy S, Vassena R, Patel BG, Latham KE. 2006. Analysis of polysomal mRNA populations of mouse oocytes and zygotes: dynamic changes in maternal mRNA utilization and function. *Dev Biol* **298:** 155-166.

Qiu JJ, Zhang WW, Wu ZL, Wang YH, Qian M, Li YP. 2003. Delay of ZGA initiation occurred in 2-cell blocked mouse embryos. *Cell Res* **13:** 179-185.

Radford HE, Meijer HA, de Moor CH. 2008. Translational control by cytoplasmic polyadenylation in Xenopus oocytes. *Biochim Biophys Acta* **1779:** 217-229.

Robert C, Nieminen J, Dufort I, Gagne D, Grant JR, Cagnone G, Plourde D, Nivet AL, Fournier E, Paquet E, Blazejczyk M, Rigault P, Juge N, Sirard MA. 2011. Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays. *Mol Reprod Dev* **78:** 651-664.

Rosenthal ET, Tansey TR, Ruderman JV. 1983. Sequence-specific adenylations and deadenylations accompany changes in the translation of maternal messenger RNA after fertilization of Spisula oocytes. *J Mol Biol* **166:** 309-327.

Scantland S, Grenon JP, Desrochers MH, Sirard MA, Khandjian EW, Robert C. 2011. Method to isolate polyribosomal mRNA from scarce samples such as mammalian oocytes and early embryos. *BMC Dev Biol* **11:** 8.

Schultz RM. 1993. Regulation of zygotic gene activation in the mouse. *Bioessays* **15:** 531-538.

Sladic RT, Lagnado CA, Bagley CJ, Goodall GJ. 2004. Human PABP binds AU-rich RNA via RNA-binding domains 3 and 4. *Eur J Biochem* **271:** 450-457.

Smith RW, Blee TK, Gray NK. 2014. Poly(A)-binding proteins are required for diverse biological processes in metazoans. *Biochem Soc Trans* **42:** 1229-1237.

Smits AH, Lindeboom RG, Perino M, van Heeringen SJ, Veenstra GJ, Vermeulen M. 2014. Global absolute quantification reveals tight regulation of protein expression in single Xenopus eggs. *Nucleic Acids Res* **42:** 9880-9891.

Sonehara H, Nagata M, Aoki F. 2008. Roles of the first and second round of DNA replication in the regulation of zygotic gene activation in mice. *J Reprod Dev* **54:** 381-384.

Thelie A, Papillier P, Pennetier S, Perreau C, Traverso JM, Uzbekova S, Mermillod P, Joly C, Humblot P, Dalbies-Tran R. 2007. Differential regulation of abundance and deadenylation of maternal transcripts during bovine oocyte maturation in vitro and in vivo. *BMC Dev Biol* **7:** 125.

Tomek W, Torner H, Kanitz W. 2002. Comparative analysis of protein synthesis, transcription and cytoplasmic polyadenylation of mRNA during maturation of bovine oocytes in vitro. *Reprod Domest Anim* **37:** 86-91.

Tomek W, Wollenhaupt K. 2012. The "closed loop model" in controlling mRNA translation during development. *Anim Reprod Sci* **134:** 2-8.

Toms D, Tsoi S, Dobrinsky J, Dyck MK, Li J. 2014. The effects of glial cell line-derived neurotrophic factor on the in vitro matured porcine oocyte transcriptome. *Mol Reprod Dev* **81:** 217-229.

Traverso JM, Donnay I, Lequarre AS. 2005. Effects of polyadenylation inhibition on meiosis progression in relation to the polyadenylation status of cyclins A2 and B1 during in vitro maturation of bovine oocytes. *Mol Reprod Dev* **71:** 107-114.

Tremblay K, Vigneault C, McGraw S, Sirard MA. 2005. Expression of cyclin B1 messenger RNA isoforms and initiation of cytoplasmic polyadenylation in the bovine oocyte. *Biol Reprod* **72:** 1037-1044.

Uzbekova S, Roy-Sabau M, Dalbies-Tran R, Perreau C, Papillier P, Mompart F, Thelie A, Pennetier S, Cognie J, Cadoret V, Royere D, Monget P, Mermillod P. 2006. Zygote arrest 1 gene in pig, cattle and human: evidence of different transcript variants in male and female germ cells. *Reprod Biol Endocrinol* **4:** 12.

Vassalli JD, Huarte J, Belin D, Gubler P, Vassalli A, O'Connell ML, Parton LA, Rickles RJ, Strickland S. 1989. Regulated polyadenylation controls mRNA translation during meiotic maturation of mouse oocytes. *Genes Dev* **3:** 2163-2171.

Vigneault C, McGraw S, Massicotte L, Sirard MA. 2004. Transcription factor expression patterns in bovine in vitro-derived embryos prior to maternal-zygotic transition. *Biol Reprod* **70:** 1701-1709.

Vigneault C, McGraw S, Sirard MA. 2009. Spatiotemporal expression of transcriptional regulators in concert with the maternal-to-embryonic transition during bovine in vitro embryogenesis. *Reproduction* **137:** 13-21.

Voeltz GK, Steitz JA. 1998. AUUUA sequences direct mRNA deadenylation uncoupled from decay during Xenopus early development. *Mol Cell Biol* **18:** 7537-7545.

Wang Q, Latham KE. 2000. Translation of maternal messenger ribonucleic acids encoding transcription factors during genome activation in early mouse embryos. *Biol Reprod* **62:** 969-978.

Wickens M, Stephenson P. 1984. Role of the conserved AAUAAA sequence: four AAUAAA point mutants prevent messenger RNA 3' end formation. *Science* **226:** 1045-1051.

Wiekowski M, Miranda M, DePamphilis ML. 1991. Regulation of gene expression in preimplantation mouse embryos: effects of the zygotic clock and the first mitosis on promoter and enhancer activities. *Dev Biol* **147:** 403-414.

Xu Y, Li N, Xiang R, Sun P. 2014. Emerging roles of the p38 MAPK and PI3K/AKT/mTOR pathways in oncogene-induced senescence. *Trends Biochem Sci* **39:** 268-276.

Zeng F, Baldwin DA, Schultz RM. 2004. Transcript profiling during preimplantation mouse development. *Dev Biol* **272:** 483-496.

4.9. Tables and Figures

Table 4-1 Number of 3'-UTR motifs localized in bovine ATF1 and ATF2 mRNA

Motif	*ATF 1s*	*ATF 1L*	*ATF 2s*	*ATF 2L*	Sp.	Reference
NPS, HEX (A_2UA_3)	2	3	1	4	X, Mm	Vassalli et al. (1989); Wickens and Stephenson (1984)
Consensus CPE (U_4AU)	1	6	3	8	X, Mm	Fox et al. (1989); McGrew et al. (1989); Oh et al. (2000)
Potential CPE	1	4	7	8	Mm, Bt	Oh et al. (2000); Thelie et al. (2007); Gohin et al. (2014)
Embryonic CPE (eCPE), dodecauridine (U_{12})	0	0	1	2	Mm	Oh et al. (2000)
MBE (AUAGU)	1	2	3	3	X	Arumugam et al. (2012)
PBE (UGUA)	1	13	9	23	X	Nakahata et al. (2003)
ARE (AU_3A)	2	3	4	7	X, Bt	Voeltz and Steitz (1998); McGraw et al. (2006)
DRE (U_3GU_4)	0	0	2	2	Mm, Bt	Chen et al. (2014); Gohin et al. (2014)
MAPS (U_5CU_2)	0	0	0	2	Bt	Gohin et al. (2014)
MAPS-like	1	1	2	3	Bt	Gohin et al. (2014)
EDEN-like	1	2	0	0	Ss	Uzbekova et al. (2006)
PRE-like	0	2	0	0	X, Mm	Charlesworth et al. (2004) Potireddy et al. (2006)

ATF1s, *GFP*-short *ATF1*_UTR3 construct; *ATF1L*, *GFP*-Long *ATF1*_UTR3 construct; *ATF2s*, *GFP*-short *ATF2*_UTR3 construct; *ATF2L*, *GFP*-Long *ATF2*_UTR3 construct; Sp, species; X, *Xenopus*; Mm, *Mus musculus*; Bt, *Bos taurus*; Ss, *Sus scrofa*; HEX, hexanucleotide; NPS, nuclear polyadenylation sequence; CPE, cytoplasmic polyadenylation element; MBE, Musashi-binding element; PBE, Pumilio-binding element; ARE, A/U-rich element; DRE, DAZL-recognition element; MAPS, motif associated with polyadenylation signal; EDEN, embryo deadenylation element; PRE, polyadenylation response element.

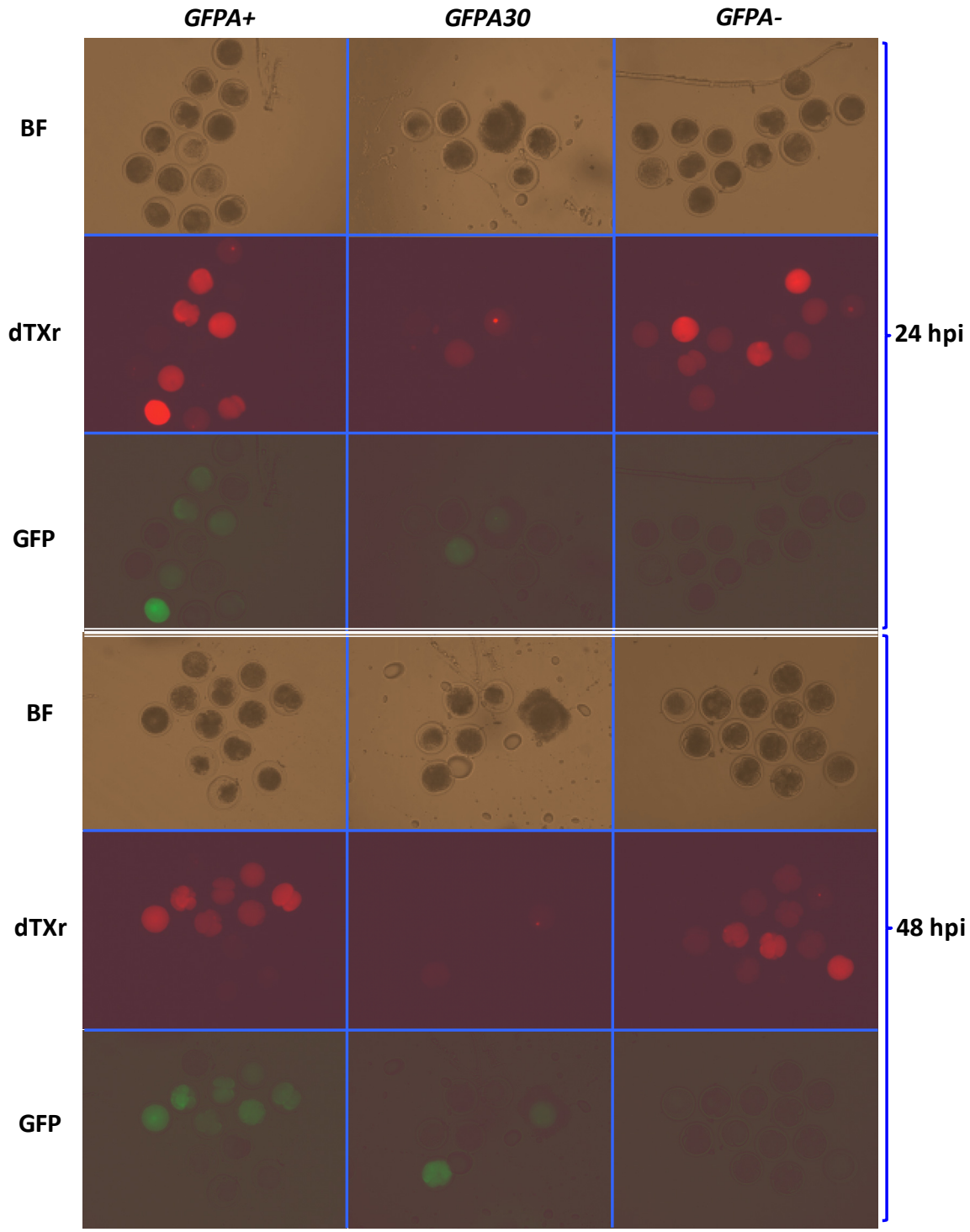

Figure 4-1 Epifluorescence micrographies of presumptive zygotes/cleaved embryos microinjected with *GFP* mRNA

Pictures were taken at 24 and 48 hours post-insemination (hpi). 100X original magnification. BF, bright field; dTXr, dextran Texas red; GFP, Green Fluorescent Protein; *GFPA+*, *GFP* mRNA polyadenylated; *GFPA30*, *GFP* mRNA 30 adenine residues; *GFPA-*, *GFP* mRNA deadenylated.

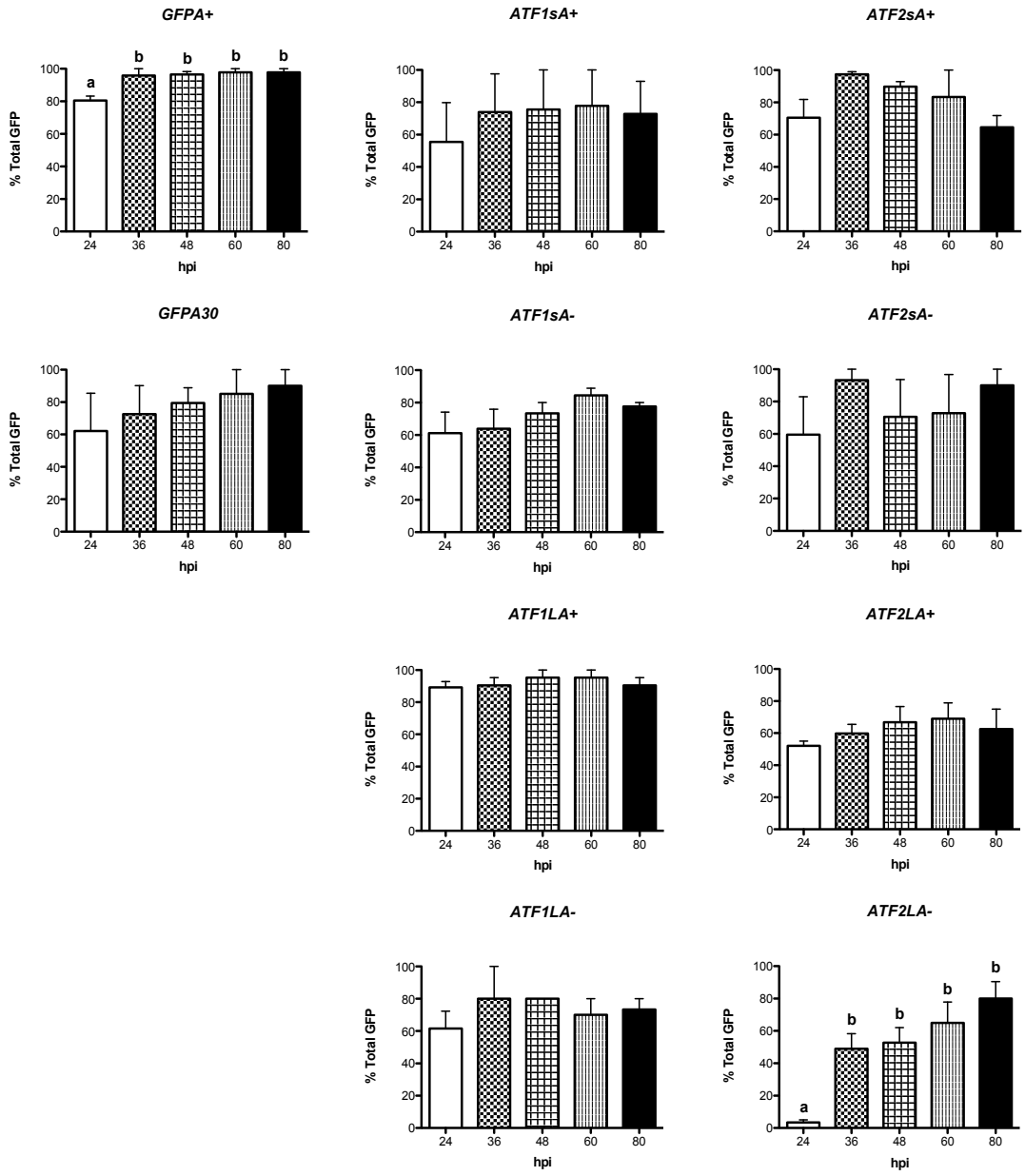

Figure 4-2 Effects of time on GFP expression of different constructs microinjected in presumptive zygotes

Distinct superscripts denote significant differences (at least $P < 0.05$). % Total GFP, number of presumptive zygotes or cleaved embryos positive to GFP/total individuals; hpi, hours post-insemination; *GFPA+*, GFP mRNA polyadenylated (N = 52); *GFPA30*, *GFP* mRNA 30 adenine residues (N = 21); *GFPA-*, *GFP* mRNA deadenylated (N = 53); *ATF1sA+*, *GFP*-short *ATF1*_UTR3 construct polyadenylated (N = 39); *ATF1sA-*, *GFP*-short *ATF1*_UTR3 construct deadenylated (N = 31); *ATF1LA+*, *GFP*-Long *ATF1*_UTR3 construct polyadenylated (N = 29); *ATF1LA-*, *GFP*-Long *ATF1*_UTR3 construct deadenylated (N = 27); *ATF2sA+*, *GFP*-short *ATF2*_UTR3 construct polyadenylated (N = 54); *ATF2sA-*, *GFP*-short *ATF2*_UTR3 construct deadenylated (N = 27); *ATF2LA+*, *GFP*-Long *ATF2*_UTR3 construct polyadenylated (N = 86); *ATF2LA-*, *GFP*-Long *ATF2*_UTR3 construct deadenylated (N = 165).

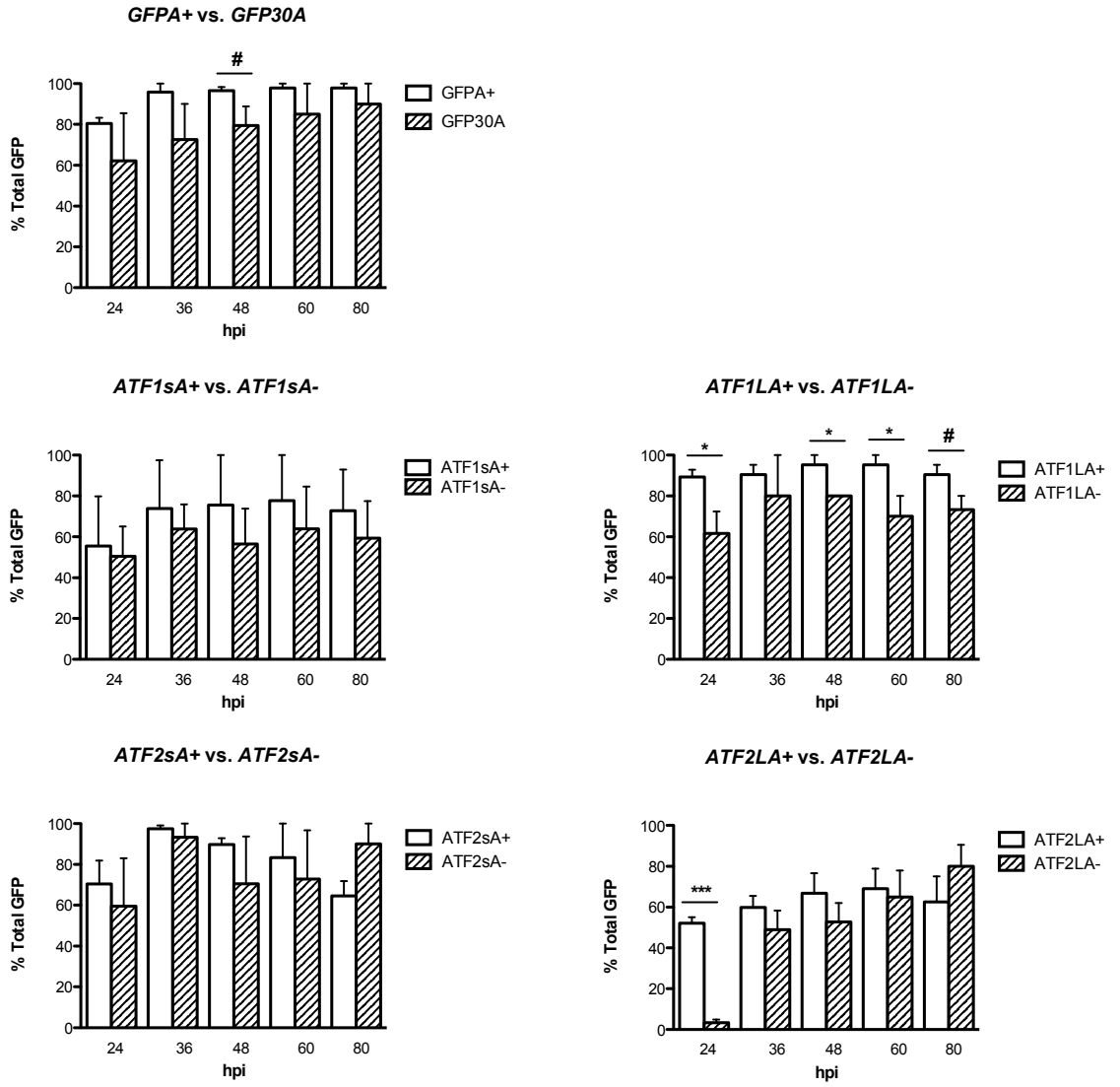

Figure 4-3 Comparison of results from the same mRNA with distinct polyadenylation status upon microinjection in presumptive zygotes

Distinct superscripts denote significant differences (at least $P < 0.05$). % Total GFP, number of presumptive zygotes or cleaved embryos positive to GFP/total individuals; hpi, hours post-insemination; *GFPA+*, *GFP* mRNA polyadenylated (N = 52); *GFPA30*, *GFP* mRNA 30 adenine residues (N = 21); *GFPA-*, *GFP* mRNA deadenylated (N = 53); *ATF1sA+*, *GFP*-short *ATF1*_UTR3 construct polyadenylated (N = 39); *ATF1sA-*, *GFP*-short *ATF1*_UTR3 construct deadenylated (N = 31); *ATF1LA+*, GFP-Long *ATF1*_UTR3 construct polyadenylated (N = 29); *ATF1LA-*, *GFP*-Long *ATF1*_UTR3 construct deadenylated (N = 27); *ATF2sA+*, *GFP*-short ATF2_UTR3 construct polyadenylated (N = 54); *ATF2sA-*, *GFP*-short *ATF2*_UTR3 construct deadenylated (N = 27); *ATF2LA+*, *GFP*-Long *ATF2*_UTR3 construct polyadenylated (N = 86); *ATF2LA-*, *GFP*-Long *ATF2*_UTR3 construct deadenylated (N = 165).

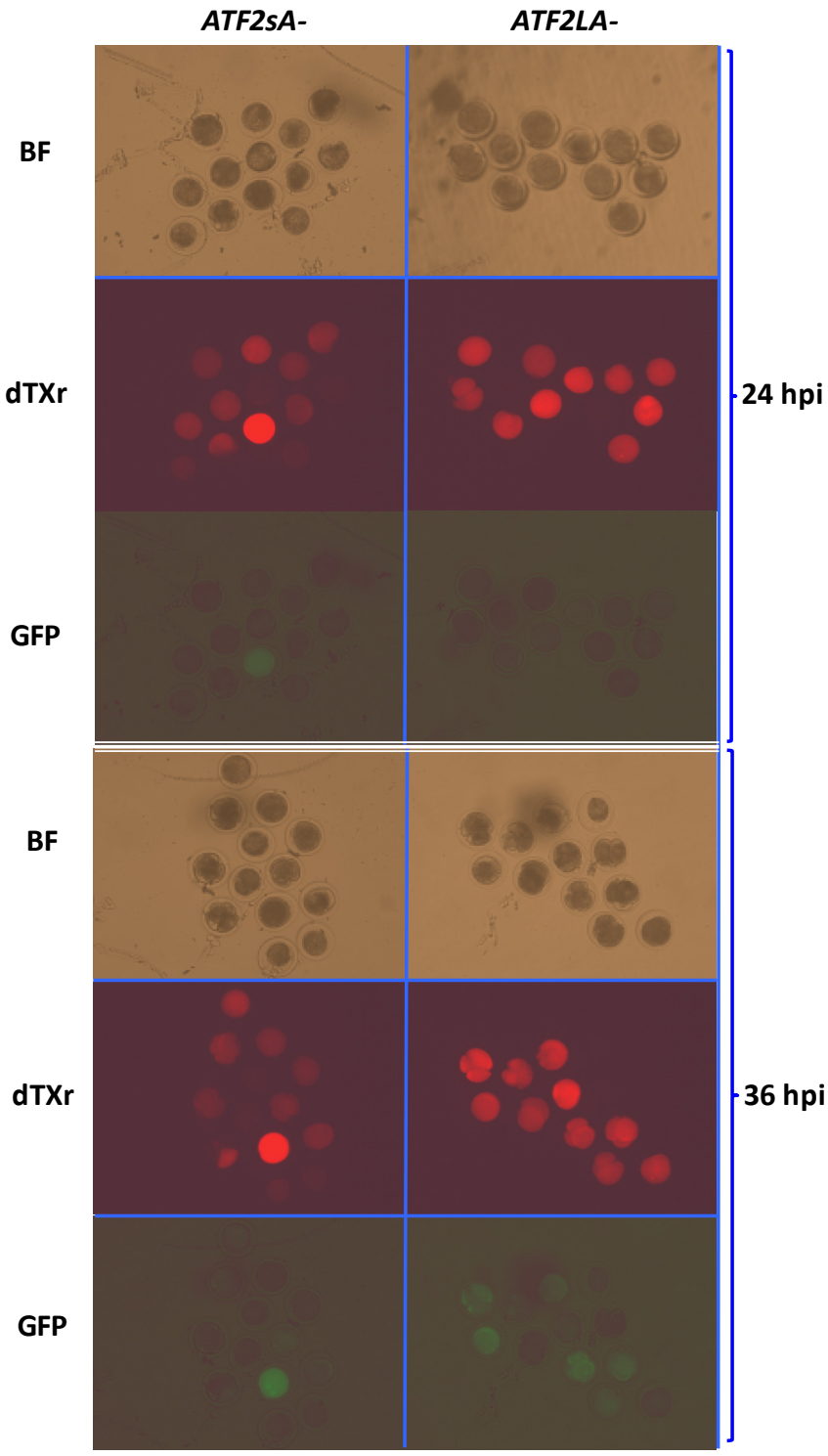

Figure 4-4 Epifluorescence micrographies of presumptive zygotes/cleaved embryos microinjected with either *ATF2sA*- or *ATF2LA*- mRNA

Pictures were taken at 24 and 36 hours post-insemination (hpi). 100X original magnification. BF, bright field; dTXr, dextran Texas red; *ATF2sA*-, *GFP*-short *ATF2*_UTR3 construct deadenylated; *ATF2LA*-, *GFP*-Long *ATF2*_UTR3 construct deadenylated.

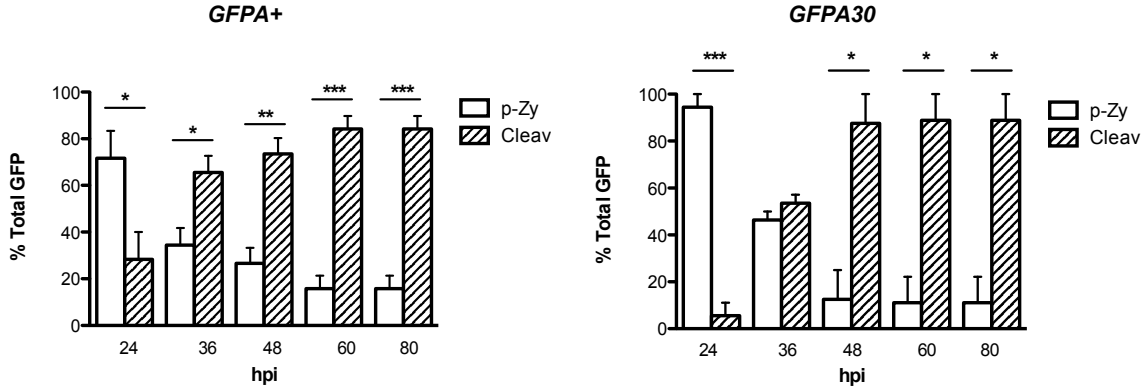

Figure 4-5 Effects of cleavage on GFP expression of different *GFP* mRNAs microinjected in presumptive zygotes

Significant differences were considered when $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***). % Total GFP, number of presumptive zygotes or cleaved embryos positive to GFP/total individuals; hpi, hours post-insemination; p-Zy, presumptive zygote; cleav, cleavage; *GFPA+ GFP* mRNA polyadenylated (N = 52); *GFPA30*, *GFP* mRNA 30 adenine residues (N = 21); *GFPA-*, *GFP* mRNA deadenylated (N = 53); *ATF1sA+*, *GFP*-short *ATF1*_UTR3 construct polyadenylated (N = 39).

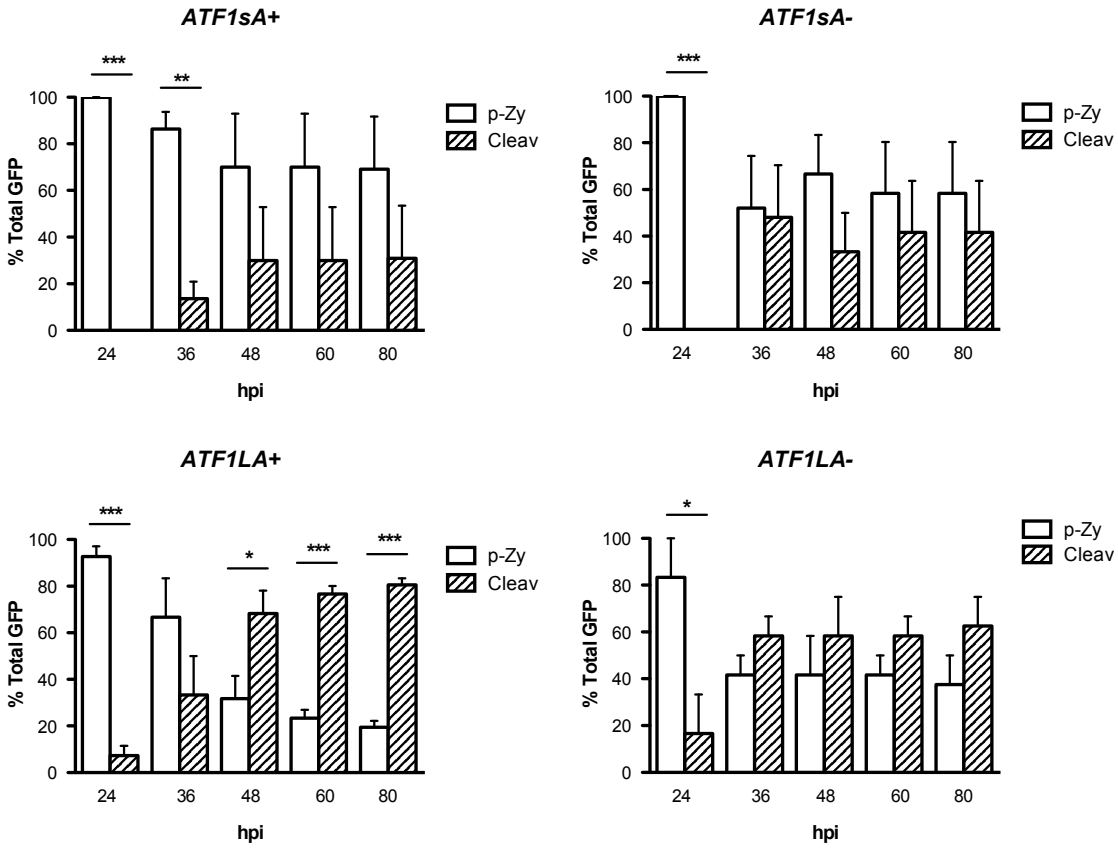

Figure 4-6 Effects of cleavage on GFP expression of different *GFP-ATF1*_UTR3 constructs microinjected in presumptive zygotes

Significant differences were considered when $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***). % Total GFP, number of presumptive zygotes or cleaved embryos positive to GFP/total individuals; hpi, hours post-insemination; p-Zy, presumptive zygote; cleav, cleavage; *ATF1sA+*, *GFP*-short *ATF1*_UTR3 construct polyadenylated (N = 39); *ATF1sA-*, *GFP*-short *ATF1*_UTR3 construct deadenylated (N = 31); *ATF1LA+*, *GFP*-Long *ATF1*_UTR3 construct polyadenylated (N = 29); *ATF1LA-*, *GFP*-Long *ATF1*_UTR3 construct deadenylated (N = 27).

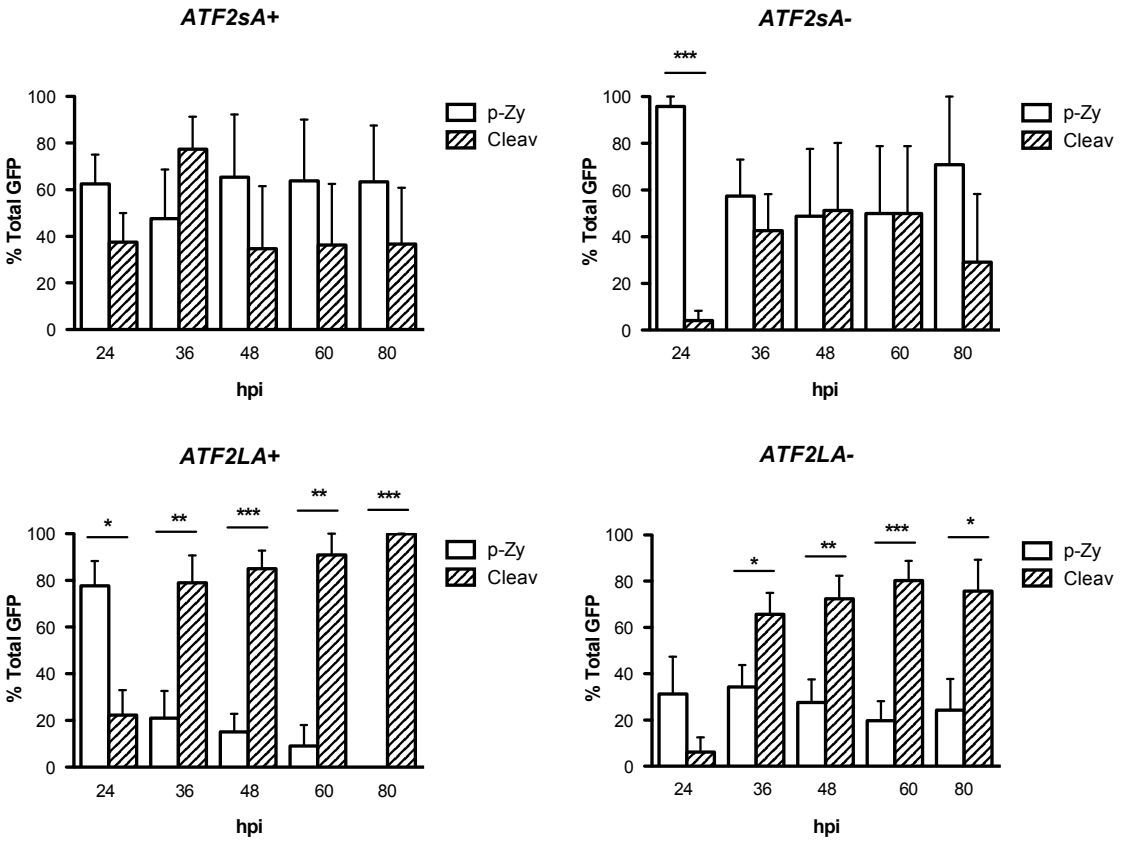

Figure 4-7 Effects of cleavage on GFP expression of different *GFP-ATF2*_UTR3 constructs microinjected in presumptive zygotes

Significant differences were considered when $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***). % Total GFP, number of presumptive zygotes or cleaved embryos positive to GFP/total individuals; hpi, hours post-insemination; p-Zy, presumptive zygote; cleav, cleavage; *ATF2sA+*, *GFP*-short *ATF2*_UTR3 construct polyadenylated (N = 54); *ATF2sA-*, *GFP*-short *ATF2*_UTR3 construct deadenylated (N = 27); *ATF2LA+*, *GFP*-Long *ATF2*_UTR3 construct polyadenylated (N = 86); *ATF2LA-*, *GFP*-Long *ATF2*_UTR3 construct deadenylated (N = 165).

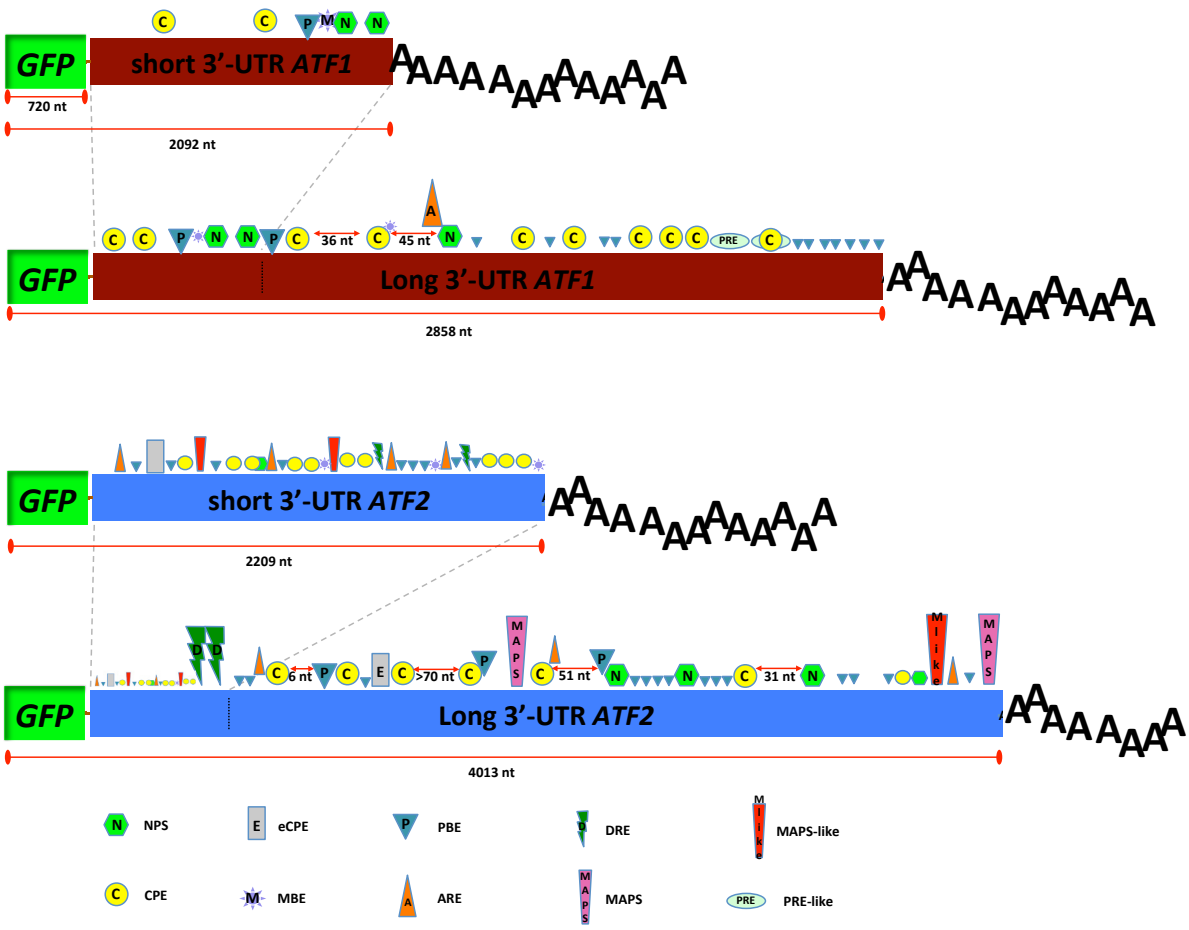

Figure 4-8 Constructs used in this study

GFP, Green Fluorescent Protein; *ATF1*, Activating Transcription Factor 1; *ATF2*, Activating Transcription Factor 2; UTR, untranslated region; nt, nucleotides; A, adenine; NPS, nuclear polyadenylation sequence; CPE, cytoplasmic polyadenylation element; eCPE, embryonic cytoplasmic polyadenylation element; MBE, Musashi-binding element; PBE, Pumilio-binding element; ARE, A/U-rich element; DRE, DAZL-recognition element; MAPS, motif associated with polyadenylation signal; MAPS-like, motif associated with polyadenylation signal-like; PRE, polyadenylation response element.

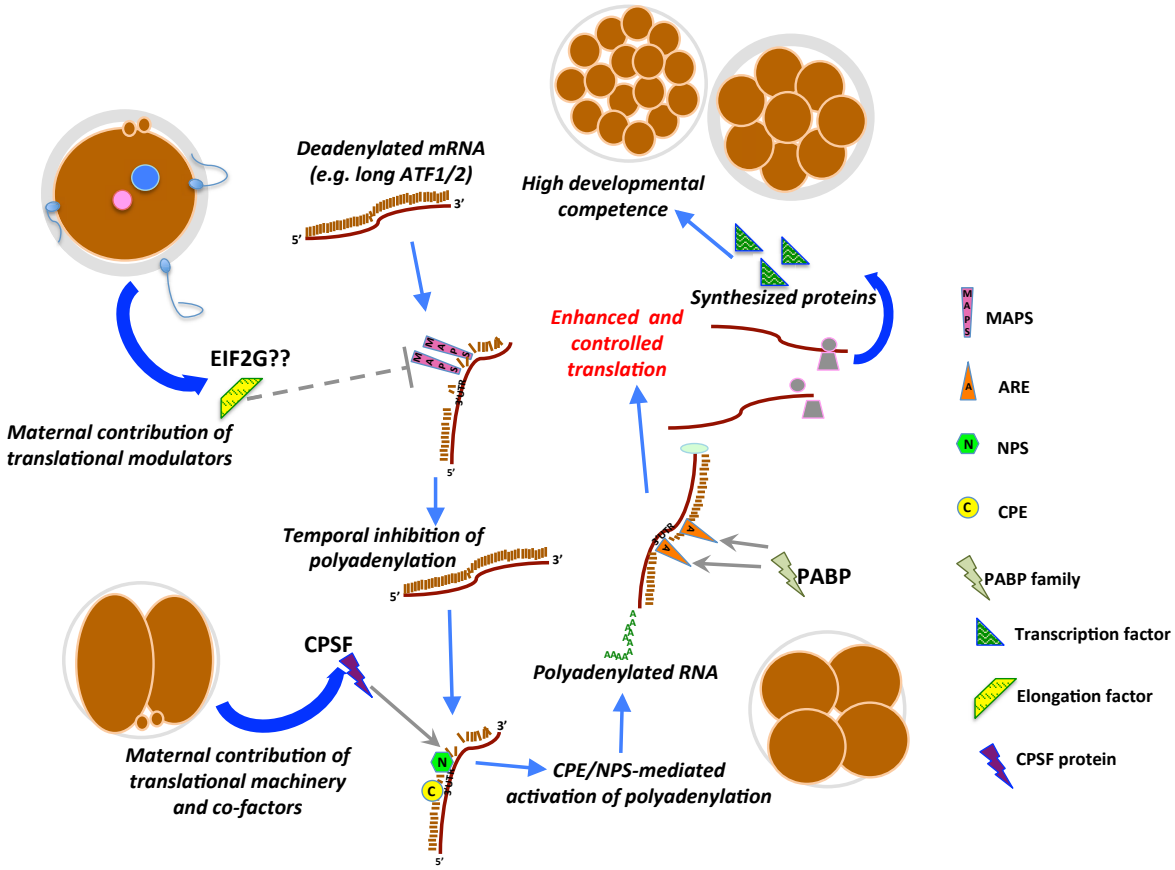

Figure 4-9 Proposed model of *ATF1* and *ATF2* translational regulation through sequence motifs in their 3'-UTR

EIF2G (EIF2S3), eukaryotic elongation factor 2-gamma; CPSF, cleavage and polyadenylation specific factor; ATF1, Activating Transcription Factor 1; ATF2, Activating Transcription Factor 2; PABP, poly(A)-binding protein; CPE, cytoplasmic polyadenylation element; NPS, nuclear polyadenylation sequence; ARE, A/U-rich element; MAPS, motif associated with polyadenylation signal.

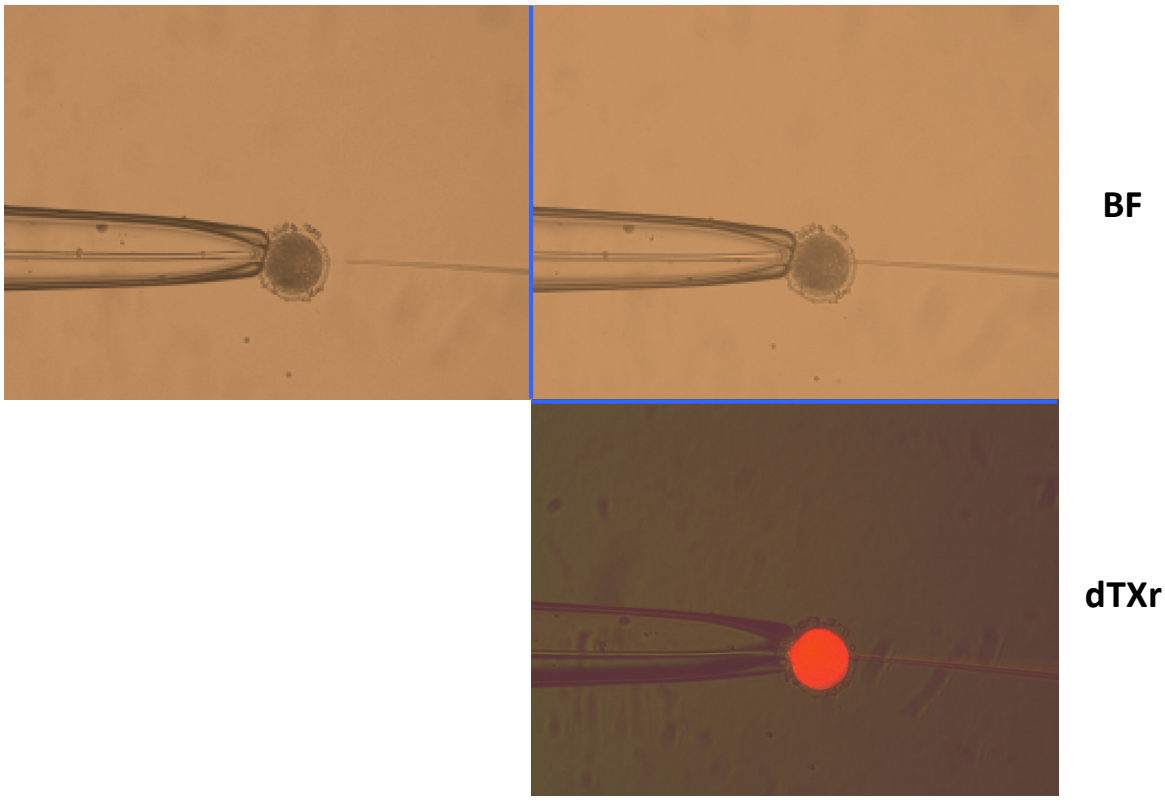

Figure 4-10 Presumptive zygote microinjection
Micrographies showing the microinjection of a presumptive zygote with a mixture of GFP mRNA and dTXr. 100X original magnification. BF, bright field; dTXr, dextran Texas red; GFP, Green Fluorescent Protein.

Supplemental File 4-1

Complete mRNA sequences

*ATF1*_short

cctcaatctggttccacagttcAGGCAGCTCATATTTCTCATATTGCTCAACAGGTATCATCTT
TATCAGAGAGTGAGGAGTCTCAGGACTCATCTGACAGCATAGGCTCCTCACAG
AAAACCCACGGGATCCTAGCACGGCGCCCATCTTACAGAAAGATTTTGAAAGA
CCTATCTTCTGAAGATATACGGGGCAGAAAAGGAGACGGAGAAAACCCTGGAG
TCTCTGCTGTCACTTCTATGTCTGTTCCAACTCCCATCTATCAGACCAGCACCGG
ACAGTACATTGCCATTGCCCCAAATGGAGCCTTACAGCTGGCCAGTCCAGGCAC
AGATGGAGTACAGGGCCTTCAGACATTAACCATGACAAATTCAGGCAGTACTC
AGCAAGGTACAACAATTCTCCAGTACGCACAGACTTCTGATGGACAACAAATA
CTTGTGCCCAGCAACCAGGTTGTTGTACAAACTGCGTCAGGAGATATGCAGAC
ATATCAGATCCGTACTACACCTTCAGCTACTTCACTACCACAGACTGTGGTGAT
GACATCTCCCGTGACCCTTACATCTCAGACATCCAAGACAGATGACCCCCAACT
GAAAAGAGAAATAAGGTTGATGAAAAACAGAGAAGCTGCTCGAGAATGTCGC
AGAAAGAAGAAAGAATATGTGAAATGCCTGGAAAATCGAGTCGCAGTTCTGGA
AAATCAAAACAAAACTCTAATAGAAGAGTTAAAAACTTTGAAGGATCTTTATT
CTAATAAAAGTGTTTGATTCTTAAGAAAGAAAATATTTTTGTGGACTTGCCTAA
AAATTAGATGGATTTCTTTTTAGTGGAGTTTTATGAATTAAAAGGTCAAAAATG
AAGCTTTTTATTTAGGTTTTTACAACTCAAAGAGAAATACCTTACATGAGAGAT
CTGGTGACAGAGGATAAAGTGGAAAAAGACCTTCAAGGAAGTTACTGGCACAA
CTGGAAGCTCTGTAAAAATTAAACATACTCAAGAAACATTTGAAATGAACTTTC
ACCAATTCAAATTTTCTAAATAACAATAGTTGCCAATATTCCAGAAATGGTAGA
TAAGATGAAATTTGGTAAATCAAATCTTTACAAATAAATCAGTTTACCGTTACA
GGTTTGCATTTCTGTTTCCTAAAATTTATCTTTCTTCAATTGTGTGTGTGTGTTCT
GTTTCTGCCAATAAATTCTACATTGCAAATAGAAAAGAAAAGCTAATACATAA
CTAAATATATAAATTATGTATCTTGATTATATATACTTgttccgttgtcaggtctcaaatgg

NPS consensus CPE potential CPE
embryonic (eCPE) U12 MBE PBE ARE DRE
MAPS MAPS-like
EDEN-like
cctcaatctggttccacagttc = Fw-primer
gttccgttgtcaggtctcaaatgg = Rv-primer

***ATF1*_Long**

GTTTTTAAAAGTCTGTTCGTTGGACTCAGGAGGGGTTTTGAGACTAGGTTAACA
ATTTTTGAGGCCTTGTCCTAAAAGACATCTAAGGTACATGAATGGAGTATGGTG
ATTTTGTAACATTTTTTATCAGAAAGGAAAAAGAATTGTTTAAAAGTTTGATAC
TTTTAAATAGTTTTTTGGTTACTCTGGGAATGGTGATTTTCTACCAATATTTAAT
AAAATTGTTTTTTGATTCTATATTCTGTATGCAGTTGAATATACCTTACTTACTCT
GTTGTGCTTTAATAGAATGGAATGTTTACAGGCCCTTAAGATAGAGTATTTTGA
AAACCTTCTGAAGATGCATACCAAAGTTTTCCAAGAGGATTTTATAATCAGTTT
AATGTAAGGTTTATCAGATTCTAAATACAGTTACTAAGGCAATTTTATGTTAGA
GACTATTTTGTAATGTAGTGAATGGTACATTTATAAGAAAAGTGACTGCCAATA
TATTTTTATAGCTAATCTTTATAAATTCTAAAGTTAAGTTTTTAATGATTATTTT
AAATGTTTATATAGTTTGTTAGTAAAAAATATTTTGCATCTCAAAGTATCATTT
TTATATTATGGGAAGCAAAGTTTCCAGATTGGCTAATATTTGAATTGTAAGTTT
TGTATGCAGTTTATCCGAAGTCAAGAAATGCTGTCAGCACACCAGTGTTCAACC
TCTGTATTCCAACTTGTATACACTTTGAAATTGTACTGCAAAACTATTGTGCGCT
TCTTATACAATATgtatcatattgttgtgtatgaaattaaagg

NPS consensus CPE potential CPE
embryonic (eCPE) U12 MBE PBE ARE DRE
MAPS MAPS-like
EDEN-like PRE-like
gtatcatattgttgtgtatgaaattaaagg = Rv-primer

***ATF2*_short**

cctgcagtgcggcagctttagatacTCATTAGTGACTTCAAAGGGAAATCAAGGAAAGACCAG
TTTCCATTTATGCGAAATCTGTGGTTGTAAATTTTTTTTTTTTTTACTTGAAATTAA
ATTTGACTCTAAAGTTGGTGTAGCAGCAGTTGATGATCAGACTGAACAAGTTTT
TAGTCTCTGGAAAAAGACTGATTTTGCTTTTTTTATAAATATTGTTAGATTTATT
AATTTTTTCTGTGCTCAATGTGTAAATTGTATTATAATTCATTGTGGTTTATTTCA
CTTTTAATTTGGGGGTGTTTTAATAAATGGGGGGTGTTACTGAATCTTTCTTCCTA
CTTCCACTTCTTTTGACCACCTCTTAACCCTCAACTATGATGGTAGTTTTGTTAT
ATCATTTATACCAAAGTTCTGCATAGTCCCTATTGACTTTGTAATGTTAACAAG
GTCATAAAGCACTAGCAAGTGAAAGAAATTTGCTTTTAATCTTTTTGCCTTTTAT
TTTGCACATTATGCAAAAGGAAGAACATTAGCGAACACTTTTTAAGTGAGTGA
AATGTGGTAAAAGACATACAGTGCTTTTAGGCACACTCTTAATGAAGGTCAGTA
ATAGTGCATTTTCTTTAAGATTTTAAGTAAGCATGAATATTGGAATCCTTTATCA
TTTCAATGACGACTGTTTTTCAAGTCTTAAATTCAGTATTTTAAATCCTATGTTT
TGAGGCTAACAATATGAAATTATATAAAGTACAATACAGGGTTATCGGATACC
TAATAATTTTTTGATATTAGATTTTTGGTTTTGTTTATTTTTTACAGATTTCAGGT
TTTAAACTGAGAAGTTTATGCATAATCAAGTAAGGTATGGTTGCCAGAAAAGC
CTAAAAATTACTACTTAGAAAATTTAAGACTGTTTACCCCCATTGTCTTGTACTT
GCAAGCTAACTTGTACTTATTCTTGTGGAAGCACTGTCATCTTTTAGTAGCCAAT
TTTGATAACGTTTCTCGTGGGAAAAAATCAGTATCTATCTTTAGAACAATGTAA
CTATAATGTGGGACACAAGAGTGAGTGAGAGAGAGTGAGTATGTGTGTATGTG
TGTGTCTTTCAATAGTTTATGCCAGCAATCTTTGCTTGAATGTTTAACGATGCCT
TCAGTGTGATGCTGGCCAATAGATGATTGCAATTTAAGATGTCATTACTGTGCA
GGCTTGGATAACTAACATTCCATGATGTAGTTTGTTTCTGATGAGATGATTGTA
GGTACACTTTTCTCATTATCCAATCATCTGTGGGATACTGAGTTTTCTAATGTGC
CATTATCTATTTTTATTCTGCAGTTATGTTCAAAATACAGTACAATATTTTAAAA
TAGACAAAATTGTTAAAAATAAAGTAGTAGATGTGTGTAAAAAAACTTTGTAA
AATAGttatgagtcctccccagtagcgacctctgg

NPS consensus CPE potential CPE
embryonic (eCPE) U12 MBE PBE ARE DRE
MAPS MAPS-like

cctgcagtgcggcagctttagatac = Fw-primer
gttatgagtcctccccagtagcgacctctgg = Rv-primer

***ATF2*_Long**

CATTCAAGCAGGATTCCATTATGTAAATATCTGTAACACATTTATAATAAGTTG
TGTGGTTTGTTCTGCATCCATACTACACTATTTGCTAAAGTCTCAGTGCCATCTC
CTAATGAGACTGACATTTTAAAAACCTGTATGGAATATCCTTGGCAATTCAAGA
AAATACCCCACCTTGCCTAAGTTCCGAACTGGGAAACATTCAGATTGTACAAAT
GACATTTCAGGACTTTCAAGTATGAAGATAATAGGAATTTTATTGTTTGGCTTA
TAAAAAATGAGAGCATTTTTGGTTGATAATTGTACTTCTAAAGTTTTTTTTTTTT
AACTGATTTTTATAATTTTCAGGTACTCAATTTCTAATCATGACTTTGCCGTAGTT
ATGCTAAGGAGTTGATCTCAAAGGCACAAAATATGAATTCTGCAAGAAGGCTG
TTTTTTTATTGTAGTTTGGATGGGTTAGGAAAAGCCTCCGTTTTTCATTCTCCTAG
GTCAGTCAGTGCATTTTTCTTAAATTTTATTATTTATGCAAGTTAAAGTTCTTTG
GTAACAGGAATTCTTGCAACTGTAAAATAAAACTACATAGATGTAAGAAGTCA
TGTAAACGGTTAATGAGCTTATTACCAAGGTTAGCAAAACTCATTGTAAATCAG
TCTGTACTCAGCAAATAAAAATCATTATTAGTTGTATAAACACAAATTCCATTT
TGACTTTCAGGATGTCACACTACTTCTGTACCTAGCATTTTGAGTCCTTATATTT
GCAATGTTACACAAACTGTACTATTTTCTTTTATGTGCAGTTTGCATGAGTAAAC
CATCAAAGAATAAATTCTATCTTTAAATTATGTTCATAATTTGCTTGTTCTTATC
TGTCTTTCCTAAAATAAGATTACTTGATCATGATGTTTTTCTCCTTCAGAATGTT
TATTACCTGAGAAGAGCCTAATATTCTGTTTCCTGTGATAAAATTAATTTCTTCT
CAAAGACCTTTCAGCTGCAAGGTCACTCAGGCCTCTTTCTTCTTCTCTGCATTCA
CTTTAATTTATGTCTCCAGCCCAGAATATTCCCTTACCCACACACACAACTACTA
CACACAGATGCACCAGCCTTTGGTTTATCTTGGTAACTGCCTGCCAAAACAACT
CTTATCTTTCACTCTGAACTGTATAATCTTCCCTTACCCTGCCTTCAAACACACT
GCTCCCATAATGTTTCATAAAGCAATTGATTACCACACCATCCTTCCAGTTGCT
GAGGTCAGAACAACCTTGTTCTCATCGTTGATTCCTCACTTCTCACATCCAACA
CATCAAGAAATGCTGTTACTTTACCTTCAGAATAATACAGAATTCAGCCACTCA
TCATTTCCAGTGCTATCACCCTGATCTGTGCCCTCGTTGTTCCTCACCCAGAGTA
CTGAATACCTTACACCCACCTCCTTTCTTTCACCCTGCCTGCTACAGCCCATCCT
CAATACAGAAGCCCCACTGAAGCTTTCCAAACATGCAACGGGTTAGGGCACTC
CTCTGCTCGAGTTCTAGAGCAGCTCCTCATTACATTCAGAGCTGGAGCCAGCCC
TTACAGGGGCCTACAAGGCCTGTTCCCCCAACTGCATCCCATTAGATCTCTGAC
CTCATCTCCTACCACACTGTGAGCACTGCTGGGTGTCCGTTTTTCTTCTAGGCAC
TGGGTATACACTAACAGCCAAAACACAAAAACTCCTGCTTTGGGGAAGCTTAC
ATTCTAAGTGGGGAGAGAGGTTTTAGGGAAACACAACACAgtatcagtacatcatcttactgtg

Primer NPS consensus CPE potential CPE embryonic (eCPE) U12 MBE PBE ARE DRE MAPS MAPS-like

gtatcagtacatcatcttactgtg = Rv-primer

ATF1, Activating Transcription Factor 1; *ATF2*, Activating Transcription Factor 2; NPS, nuclear polyadenylation sequence; CPE, cytoplasmic polyadenylation element; eCPE, embryonic cytoplasmic polyadenylation element MBE, Musashi-binding element; PBE, Pumilio-binding element; ARE, A/U-rich element; DRE, DAZL-recognition element; MAPS, motif associated with polyadenylation signal; EDEN, embryo deadenylation element; PRE, polyadenylation response element; Fw, forward; Rv, reverse.

Supplemental File 4-2

Preparation of mRNAs

All primer sequences and annealing temperatures are shown in Table 4-2 and Table 4-3. *eGFP* containing *Hind*III sites at both termini was amplified from the pCMS-*eGFP* vector (Clontech, Mountain View, CA). Such amplicon, as well as the pCR3.1 plasmid were digested with HindIII (New England Biolabs, Ipswich, MA) and then ligated with T4 DNA Ligase (Roche, Laval, QC, Canada) to generate the pCR3.1/*eGFP* construction. *ATF1*_shortUTR3 and *ATF2*_shortUTR3 including *Eco*RI restriction sites at both ends were amplified from bovine granulosa cells. The amplicons and the pCR3.1/*eGFP* vector were restricted with *Eco*RI (New England) and ligated with T4 DNA Ligase to produce the pCR3.1/*eGFP*/*ATF1*_shortUTR3 and pCR3.1/*eGFP*/*ATF2*_shortUTR3 constructions. *ATF1*_LongUTR3 was obtained from the same tissue and the amplicon was TA-cloned in the pCR3.1 vector (Invitrogen, Burlington ON, Canada) to render the preliminary pCR3.1/*eGFP*/*ATF1*_LongUTR3 construct that was digested with *Eco*RI in the same way as pCR3.1/eGFP. The resulting *Eco*RI-*ATF1*_LongUTR3-*Eco*RI fragment and the linealized pCR3.1/*eGFP* were exposed to T4 DNA Ligase, resulting in pCR3.1/*eGFP*/*ATF1*_LongUTR3 construction. The amplicon *Bam*HI-*ATF2*_LongUTR3-*Bam*HI was amplified from granulosa cells and restricted with *Bam*HI (New England) and then ligated to pCR3.1/*eGFP* to originate the construct pCR3.1/*eGFP*/*ATF2*_LongUTR3. Upon generation of the above-mentioned five constructions, the 11 mRNAs (*GFPA*+, *GFPA30*, *GFPA*-, *ATF1sA*+, *ATF1sA*-, *ATF1LA*+, *ATF1LA*-, *ATF2sA*+, *ATF2sA*-, *ATF2LA*+, and *ATF2LA*-) were obtained by combining the T7 promoter-*GFP*-Fw primer and the corresponding Rv-primer followed by processing with the mMESSAGE mMACHINE T7 Ultra Kit (Ambion, Foster City, CA) according to manufacturer's instructions.

Supplemental Table 4-2 Details of PCRs for mRNA preparation

Fragment	Fw-primer	Rv-primer	Annealing T° (°C)
*Hind*III-*GFP*-*Hind*III	*Hind*III-*GFP*-Fw	*Hind*III-*GFP*-Rv	55
*Eco*RI-*ATF1*_shortUTR3-*Eco*RI	*Eco*RI-*ATF1*_shortUTR3-Fw	*Eco*RI-*ATF1*_shortUTR3-Rv	57
*ATF1*_LongUTR3	*ATF1*_LongUTR3-Fw	*ATF1*_LongUTR3-Rv	57
*Eco*RI-*ATF2*_shortUTR3-*Eco*RI	*Eco*RI-*ATF2*_shortUTR3-Fw	*Eco*RI-*ATF2*_shortUTR3-Rv	57
*Bam*HI-*ATF2*_LongUTR3-*Bam*HI	*Bam*HI-*ATF2*_LongUTR3-Fw	*Bam*HI-*ATF2*_LongUTR3-Rv	57
T7 promoter/*GFP*	T7 promoter/*GFP*-Fw	*GFP*-Rv	55
T7 promoter/*GFPA30*	T7 promoter/*GFP*-Fw	*GFPA30*-Rv	55
T7 promoter/*GFP*-*ATF1*_shortUTR3	T7 promoter/*GFP*-Fw	*ATF1*_shortUTR3-Rv	55
T7 promoter/*GFP*-*ATF1*_LongUTR3	T7 promoter/*GFP*-Fw	*ATF1*_LongUTR3-Rv	55
T7 promoter/*GFP*-*ATF2*_shortUTR3	T7 promoter/*GFP*-Fw	*ATF2*_shortUTR3-Rv	55
T7 promoter/*GFP*-*ATF2*_LongUTR3	T7 promoter/*GFP*-Fw	*ATF2*_LongUTR3-Rv	55

T°, temperature; *ATF1*, Activating Transcription Factor 1; *ATF2*, Activating Transcription Factor 1; *GFP*, Green Fluorescent Protein; *GFPA30*, Green Fluorescent Protein 30 adenine residues; *Hind*III, *Hind*III endonuclease; *Eco*RI, *Eco*RI endonuclease; *Bam*HI, *Bam*HI endonuclease; Fw, forward; Rv, reverse.

Supplemental Table 4-3 Primer sequences

Primer	Sequence (5'-3')
*Hind*III-*GFP*-Fw	GGGaagcttCCATGGTGAGCAAGGGCGAGGAGC
*Hind*III-*GFP*-Rv	GGGaagcttTTACTTGTACAGCTCGTCC
*Eco*RI-*ATF1*_shortUTR3-Fw	gaattcCCTCAATCTGGTTCCACAGTTC
*Eco*RI-*ATF1*_shortUTR3-Rv	gaattcCCATTTGAGACCTGACAACGGAAC
*Eco*RI-*ATF2*_shortUTR3-Fw	gaattcCCTGCAGTGCGGCAGCTTTAGATAC
*Eco*RI-*ATF2*_shortUTR3-Rv	gaattcCCAGAAATCGCTACTGGGGAGGACTCATAAC
*Bam*HI-*ATF2*_LongUTR3-Fw	ggatccCCTGCAGTGCGGCAGCTTTAGATAC
*Bam*HI-*ATF2*_LongUTR3-Rv	ggatccCACAGTTAAGATGATGTACTGA
T7 promoter/*GFP*-Fw	TAATACGACTCACTATAGGGCCGCGGCGCCACCATGGTGAGCAAGGGCGAGGAGC
GFP-Rv	ATGGTGAGCAAGGGCGAGGAGC
GFPA30-Rv	TTTTTTTTTTTTTTTTTTTTTTTTTTTTCCCGGGTTACTTGTACAGCTCGTCC
*ATF1*_UTR3-Fw	CCTCAATCTGGTTCCACAGTTC
*ATF1*_shortUTR3-Rv	CCATTTGAGACCTGACAACGGAAC
*ATF1*_LongUTR3-Rv	CCTTTAATTTCATACACAACAATATGATAC
*ATF2*_UTR3-Fw	CCTGCAGTGCGGCAGCTTTAGATAC
*ATF2*_shortUTR3-Rv	CCAGAGGTCGCTACTGGGGAGGACTCATAAC
*ATF2*_LongUTR3-Rv	CACAGTAAGATGATGTACTGATAC

ATF1, Activating Transcription Factor 1; *ATF2*, Activating Transcription Factor 1; *GFP*, Green Fluorescent Protein; *GFPA30*, Green Fluorescent Protein 30 adenine residues; *Hind*III, *Hind*III endonuclease; *Eco*RI, *Eco*RI endonuclease; *Bam*HI, *Bam*HI endonuclease; Fw, forward; Rv, reverse.

5. General Conclusion

With the advent of a broader use of reproductive technologies, both in human medicine and animal science, there is a compelling requirement not only to improve our understanding of the first days of embryonic development, but also that of the oocyte, from which the early embryo takes most of its inheritance. In addition, to better comprehend the complex physiology of both entities, we must gain insight into how the egg is influenced by the ovarian environment during its formation and final maturation. For such purposes, the study of maternal transcripts is a powerful approach to reveal the molecular physiology of the oocyte. The stored mRNA molecules may reflect the intra-follicular events upstream from the egg, but are also instrumental to project towards the molecular control of the early embryo. The picture of maternal transcripts is still far from being completed, although encouraging progress has been obtained in the last decades and through this work.

In the first part of this project we aimed to determine the overall fingerprint of the maternal transcriptome related to variable levels of developmental competence. Transcriptomic results were useful at defining the biological functions of DNA damage response, in agreement with results from Henrique Barreta et al. (2012) and Bohrer et al. (2015); gene expression; RNA management; and protein synthesis and turnover, as impacted by variable levels of competence. The identity of hundreds of molecules potentially involved in regulation of developmental capacity defined by oocyte-driven events was elucidated, and with it the opportunity to select candidate molecules for functional testing, as it was the case of *ATF1*, in a similar way than Held et al. (2012). Data obtained from our transcriptomic survey is instrumental to explore upstream and downstream molecular regulation through the use of transversal modeling with other developmental stages in an effort to explain the molecular profile observed at the 2-cell stage, as well as how this could influence the embryo's outcome. Because maternal molecules are regulated at the transcript level depending on developmental capacity, reproductive scientists have made great efforts during recent years to modify the abundance levels of the transcripts of interest through chemical supplementation. Alternatives to pursue this goal would be either culture supplementation or stimulation of oocyte/embryo donors.

In the next section, we confirmed the involvement of the developmentally important transcription factors, ATF1 and ATF2, on the regulation of preimplantation development. Molecular profiling of knock-down 8-cell embryos unveiled a potential downstream target of ATF1: The transcription factor *SP1*, which is a hub modulator of gene expression during development, underscoring the significance of ATF1 involvement in EGA programming. Further studies will confirm the relationship between ATF1 and SP1 in the embryonic context. Our survey was in agreement with the involvement of ATF1 in murine ZGA (Jin and O'Neill 2010) and confirmed the importance of maternal transcripts on EGA orchestration in cattle. Jin and O'Neill (2014) demonstrated how ATF1 is regulated at the protein level by embryotrophic factors in mice around time of ZGA. Therefore, such observations raise the question of whether this could also be the case of ATF1 in cattle embryos. Similarly, as in Jin and O'Neill's strategy, supplementation in culture seems the method of choice to address this issue in bovines.

The third experiment further explored the regulation of ATF1/2 transcription factors by studying the *cis*-motifs located in the 3'-UTR of both *ATF1* and *ATF2*. This was performed with the aim to evaluate how their translational dynamics are modulated in early embryos. Remarkably, the translation appeared regulated by the elapsed time from fertilization, but we do not discard the possibility that translation of other developmentally important mRNAs by different mechanisms, like embryonic cleavage. A particular sequence motif associated with polyadenylation signal (MAPS), previously detected by Gohin et al. (2014) to be exclusive in the long *ATF2*-UTR isoform and related with transcripts with poor adenylation status in oocytes, constitutes a candidate motif for the observed regulation. This could be investigated through specific knockdown of transcripts containing particular 3'-UTR sequences. Moreover, deletion of specific motifs (e.g. MAPS) in reporter mRNAs will help to confirm their involvement in translational synchronization. Notably, numerous molecules proposed here as candidate regulators of the synchronization of translation have been found by Labrecque et al. (2013) and Labrecque et al. (2014) at variable mRNA levels in immature oocytes of distinct quality. In this way, it is suggested the potential role of orchestrating molecular events occurring at previous stages during development, not only for transcriptional regulation but also for the control of translational recruitment. To test

such hypothesis, culture supplementation of activators or inhibitors of the molecular pathways of interest could be used to evaluate whether *ATF1/2* modify their translational recruitment behavior. In relation to the molecular switches triggering these physiological signals, CDKN1A was predicted as an upstream regulator for timely regulation of *ATF1/2* translation. This was also the case in the previous transcriptomic assessment of 2-cell embryos. This clearly positions CDKN1A as an appealing candidate in the regulation of the molecular pathways associated with developmental competence.

Future studies are required to determine the possible significance of the control of timely translation. An important question that remains would be whether the delay in the translation from the long *ATF2*-UTR is a system to ensure that the protein is not going to be translated too precociously. A translation-blocking mechanism was proposed for histone mRNAs in cow oocytes (Labrecque et al. 2015). Furthermore, it will be important to determine if a short or long *ATF1/2* isoform is preferentially used as either maternal or embryonic transcript, as well as the relative developmental importance of each mRNA isoform. In relation to this, *ATF1/2* are examples showing how indispensable it is to consider 3'-UTR tilling in developmental studies, although it will be also necessary to address whether mRNAs with crucial physiological roles are regulated by other posttranscriptional mechanisms, like alternative splicing or micro-RNA effects. This would add another level of complexity to the already intricate transcript modulation during early development. Thus, our studies corroborated that *ATF1* and *ATF2* are not only regulated at the transcript level, as observed in our first experimental section, but also at the translational level by *cis*-motifs in their 3'-UTR. Consequently, it would be interesting to ask whether an elevated proportion of molecules with pivotal roles in the embryo are also regulated both transcriptionally and translationally. This question could apply not only for the embryonic scenario, but also for other cellular contexts. Moreover, it will be necessary to research the way by which global mechanisms of gene expression control, as micro-RNAs and other non-coding RNAs, regulate the 3'-UTR of the molecules of interest.

Previous reports clearly demonstrated the tight regulation to which CREB family members, including ATF1, are subjected at the protein level around ZGA in rodents (Jin and O'Neill

2010; Jin and O'Neill 2014). In addition, Vigneault et al. (2009) observed nuclear translocation of ATF2 protein in bovine embryos just before the time of EGA, raising the question of the potential role of ATF2 and the identity of its protein partners in the embryonic cytoplasm. Concerning this matter, it would be interesting to know the mechanisms that drive ATF1/2 to the nuclear compartment, like nuclear localization signals and/or transport by specific chaperones or nuclear channels. The findings above make clear that these crucial transcription factors should also be monitored post-translationally. Therefore, it might be plausible that molecules with key roles in development must be modulated at distinct levels, before and after translation. Overall, the experimental part of the project allowed us to better understand the role of maternal transcripts during early bovine development both in a global scenario, pinpointing crucial biological functions related with competence, and with specific maternal molecules and how these can drive the embryo's outcome. Notably, the combination of *in silico* analysis together with supplementation in culture of either activating or inhibiting molecules will help to corroborate upstream regulators of the maternal transcripts with key roles for developmental competence. Such a strategy, together with the evaluation of the control of a physiologically important molecule at different levels (transcriptional, translational, and posttranslational) will provide us with a better understanding of the molecular mechanisms governing early development.

In the last chapter we addressed the challenges and potential use of biomarkers in the animal reproductive field. We consulted the available literature concerning mRNA profiling in both the germinal and somatic portions of the ovarian follicle, as well as in embryos of distinct stages up to blastocyst formation. Available data were reviewed in an attempt to depict a general panorama of the oocyte and embryo's needs through different stages, in addition to illustrate how transcriptomics is useful to monitor the physiological hurdles of the challenging *in vitro* culture environment. In a similar way, literature about mRNA assessments of distinct somatic follicular cells was discussed together with their potential physiological meaning. In this section, the usefulness of the molecular evaluation of somatic ovarian cell as a non-invasive proxy to determine the egg's intrinsic developmental competence was reviewed. Furthermore, the use of another non-invasive molecular

evaluation of physiology: Metabolites spent in culture media (Sturmey et al. 2010) or those contained in follicular fluid (Matoba et al. 2014) was discussed. Taken together, we aim to highlight the use of biomarkers as a powerful tool for the progress of basic reproductive knowledge, as well as that of animal breeding technologies.

5.1. References

Bohrer RC, Coutinho AR, Duggavathi R, Bordignon V. 2015. The Incidence of DNA Double-Strand Breaks Is Higher in Late-Cleaving and Less Developmentally Competent Porcine Embryos. *Biol Reprod* **[in press]**.

Gohin M, Fournier E, Dufort I, Sirard MA. 2014. Discovery, identification and sequence analysis of RNAs selected for very short or long poly A tail in immature bovine oocytes. *Mol Hum Reprod* **20:** 127-138.

Held E, Salilew-Wondim D, Linke M, Zechner U, Rings F, Tesfaye D, Schellander K, Hoelker M. 2012. Transcriptome fingerprint of bovine 2-cell stage blastomeres is directly correlated with the individual developmental competence of the corresponding sister blastomere. *Biol Reprod* **87:** 154.

Henrique Barreta M, Garziera Gasperin B, Braga Rissi V, de Cesaro MP, Ferreira R, de Oliveira JF, Gonçalves PB, Bordignon V. 2012. Homologous recombination and non-homologous end-joining repair pathways in bovine embryos with different developmental competence. *Exp Cell Res* **318:** 2049-2058.

Jin XL, O'Neill C. 2010. The presence and activation of two essential transcription factors (cAMP response element-binding protein and cAMP-dependent transcription factor ATF1) in the two-cell mouse embryo. *Biol Reprod* **82:** 459-468.

Jin XL, O'Neill C. 2014. The regulation of the expression and activation of the essential ATF1 transcription factor in the mouse preimplantation embryo. *Reproduction* **148:** 147-157.

Labrecque R, Lodde V, Dieci C, Tessaro I, Luciano AM, Sirard MA. 2015. Chromatin remodelling and histone m RNA accumulation in bovine germinal vesicle oocytes. *Mol Reprod Dev* **82:** 450-462.

Labrecque R, Sirard MA. 2014. The study of mammalian oocyte competence by transcriptome analysis: progress and challenges. *Mol Hum Reprod* **20:** 103-116.

Labrecque R, Vigneault C, Blondin P, Sirard MA. 2013. Gene expression analysis of bovine oocytes with high developmental competence obtained from FSH-stimulated animals. *Mol Reprod Dev* **80:** 428-440.

Matoba S, Bender K, Fahey AG, Mamo S, Brennan L, Lonergan P, Fair T. 2014. Predictive value of bovine follicular components as markers of oocyte developmental potential. *Reprod Fertil Dev* **26:** 337-345.

Sturmey RG, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ, Lonergan P. 2010. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. *Mol Reprod Dev* **77:** 285-296.

Vigneault C, McGraw S, Sirard MA. 2009b. Spatiotemporal expression of transcriptional regulators in concert with the maternal-to-embryonic transition during bovine in vitro embryogenesis. *Reproduction* **137:** 13-21.

Appendix 1

6. Molecular Markers of Fertility in Cattle Oocytes and Embryos – Progress and Challenges

Ernesto Orozco-Lucero,[1] Marc-André Sirard[1]

[1]Centre de Recherche en Biologie de la Reproduction (CRBR), Université Laval, Québec, Canada

This paper has been published in the journal "Animal Reproduction".

Reference:

Orozco-Lucero E, Sirard MA. 2014. Molecular markers of fertility in cattle oocytes and embryos: Progress and challenges. *Anim Reprod* **11:** 183-94.

6.1. Résumé

Pour améliorer les technologies de la reproduction, il est impératif de développer des méthodes plus robustes pour discriminer les ovocytes et les embryons selon leur niveau de compétence développementale afin de substituer ou de complémenter les critères morphologiques subjectifs qui sont encore très utilisés. Des marqueurs objectifs et efficaces ont été étudiés pendant les dernières décennies en tant qu'options puissantes pour sélectionner les meilleurs ovocytes et embryons lors des programmes de transfert. Ces méthodologies moléculaires comptent principalement sur les nouvelles technologies de type « OMICS ». Parmi ces dernières, la transcriptomique est la plateforme principalement appliquée aujourd'hui pour amplifier des petits échantillons. D'ailleurs, les biomarqueurs de compétence ont été un avantage non seulement pour sélectionner les meilleurs ovocytes et embryons pour les technologies reproductives, mais ils ont aussi dévoilé la physiologie moléculaire complexe qui mène à l'acquisition de la capacité développementale de l'ovocyte et la façon dont l'embryon manifeste ce potentiel pendant la culture. Cependant, une vérification moléculaire implique normalement la destruction de l'ovocyte/embryon et par conséquent empêche l'utilisation pratique des biomarqueurs dans la production in vitro des embryons et les programmes de transfert. D'un autre côté, des résultats prometteurs ont été obtenus à partir des technologies non invasives basées sur des biopsies des cellules somatiques folliculaires qui entourent l'ovocyte en développement ainsi qu'à partir de l'analyse métabolique du fluide folliculaire ou du milieu issu lors de la culture d'ovocytes/embryons. Ce travail résume les accomplissements des dernières années dans le domaine des biomarqueurs des embryons bovins compétents. Les principaux défis seront exposés, ainsi que les lignes directrices qui nous aiderons à mieux comprendre pourquoi les biomarqueurs de compétence développementale semblent si prometteurs pour le progrès dans l'amélioration des technologies reproductives.

6.2. Abstract

In order for assisted reproduction technologies to improve, better methods to discern eggs and embryos according to their level of developmental competence are urgently required to substitute or complement the subjective morphological selection criteria still broadly in use. Objective and reliable molecular markers of viability have been studied during the last decades as robust options to select the best oocytes and embryos for embryo transfer programs. These molecular methodologies rely mostly on the novel "OMICS" technologies. Among these, transcriptomics is the primary platform applied so far in animal breeding research mainly due to the possibility to amplify small samples. In addition, biomarkers of competence have not only been instrumental to select the best oocytes and embryos for reproductive technologies, but have also shed light on the intricate molecular physiology leading to the acquisition of developmental capacity within the ovary and on how the embryo manifests this potential during culture. Nevertheless, such molecular profiling usually implies the destruction of the oocyte/embryo thereby preventing the practical use of biomarkers in *in vitro* embryo production and transfer systems. Alternatively, encouraging results have been lately obtained from non-invasive technologies based on biopsies of follicular somatic cells surrounding the developing egg, as well as from metabolic analysis of follicular fluid or spent culture media. This work summarizes the achievements of recent years in the field of biomarkers of competent bovine embryos. The main challenges will be exposed, while the future guidelines will help to comprehend why biomarkers of developmental competence appear promising to take us steps forward in the amelioration of reproductive technologies.

6.3. Introduction

Over the course of the last decades assisted reproductive technologies (ARTs) have been increasingly used to enhance production in domestic animals. Among these methodologies, *in vitro* embryo production (IVP) has allowed the quick propagation of embryos from parents with genetically desired characteristics for the animal breeding industry, and the supply of biological material for reproduction research. Nonetheless, it is clear that oocytes matured *in vitro* can only produce less than half of the blastocysts than *in vivo* systems in cattle unless special care is applied to ovarian preparation prior to oocyte aspiration. Intense investigation of the possible causes of decreased embryo yields following *in vitro* culture (IVC) has highlighted the poor developmental competence, or quality, of the *in vitro*-matured oocytes in comparison to gametes matured *in vivo*, or of those oocytes collected too early or late during the antral phase (Sirard and Blondin, 1996; Merton et al. 2003). It is largely accepted that the quality of the female gamete directly depends on specific gene products: transcripts and proteins that are stored in the cytoplasm during oocyte growth and support early development during the transcriptionally inactive period from maturation up to activation of the embryonic genome (Krisher 2004; Sirard 2010). Understanding these maternal constituents has not only been used to decipher the intricate regulation of developmental capacity in oocytes and cleavage-stage embryos, but also to obtain markers of fertility during early development. Additionally, embryo culture conditions importantly impact developmental success and the molecular origin of this effect can also be assessed (Lonergan et al. 2003b; Cagnone et al. 2012; Gad et al. 2012). However, the available methods, mostly relying on transcript profiling, involve the sacrifice of the oocyte/embryo in order to perform the molecular analysis (Bols et al. 2012; Fair 2012). This has prompted the search of non-invasive techniques to evaluate competence through the assessment of the somatic compartments of the follicle (Bettegowda et al. 2008; Bunel et al. 2013; Nivet et al. 2013), follicular fluid (Matoba et al. 2014), and spent culture media (Sturmey et al. 2010; Hemmings et al. 2012), while the egg or embryo is preserved for further development. In this review, we will focus on the discovery of fertility markers in bovine through invasive methodologies, on how they have been useful to discern populations of oocytes and embryos according to their developmental capacity, and on the fact that they were crucial to comprehend the complex molecular regulation of quality in gametes and embryos.

Remarkably, this has resulted in the deciphering of some of the mechanisms responsible for developmental competence acquisition within the ovary throughout folliculogenesis and the way that this potential is then revealed after fertilization, and later on in embryogenesis. Promising non-invasive techniques to analyze developmental competence will also be discussed.

6.4. Fertility status of oocytes and embryos

"Developmental competence" or "oocyte quality" is defined as the capacity of the oocyte to successfully mature, be fertilized, and progress through development to form a viable blastocyst that can induce pregnancy and generate a healthy offspring. In cattle it is impossible to transfer all IVP blastocysts to recipient cows. Therefore, the developmental competence of oocytes is usually measured by their capacity to yield blastocysts with adequate morphology and timing for being frozen and potentially transferred (Sirard et al. 2006; Mermillod et al. 2008). For simplicity "competence" or "developmental potential" will refer here to the ability of either oocytes or early embryos to reach the blastocyst stage. Therefore, the term "markers of competence" will be used in this work as synonym of "markers of fertility".

The requirement for practical standards to identify oocytes and embryos endowed with the highest probability of becoming blastocysts able to induce healthy pregnancies successfully carried to term is one of the central challenges in the clinical application of ARTs. In human, the rate of live births obtained per embryo transferred in utero is only approximately 15%, while clinics around the world continue to try to overcome this problem by transferring multiple embryos, which represents one of the major risk issues of ARTs. Nowadays, the arbitrary morphological selection of oocytes to be subjected to IVF or intracytoplasmatic sperm injection, as well as of the embryos to be transferred is still controversial and unable to effectively discern them according to their level of developmental potential. Consequently, human ARTs urgently need far more objective and effective criteria to select, within a group of embryos, the one with the highest developmental capacity in order to transfer a single embryo; or to select the oocyte most likely able to produce such an embryo (Patrizio 2007; Hemmings et al. 2012 for review). A

similar scenario occurs in domestic animals, where the blastocyst rate obtained by routine IVP from non-stimulated animals is still limited to 35-45% of the fertilized oocytes (Bettegowda et al. 2008; Bols et al. 2012; Boni 2012 for review). It is possible that a male effect exists posterior to syngamy. However, it is widely accepted that the embryo's developmental outcome mostly depends on the intrinsic quality of the oocyte as demonstrated by the fact that existing culture systems can hardly improve blastocyst rates of IVP (Blondin et al. 2002; Krisher 2004; Sirard et al. 2006; Sirard 2010). As in human, the vast majority of cattle oocytes (Blondin and Sirard 1995) and embryos (Massip et al. 1995) selected for ARTs are still graded through subjective morphological standards that do not accurately evaluate the intrinsic developmental capacity of the oocyte/embryo increasing variability of ART procedures between laboratories. In contrast, using objective and more reliable molecular markers of competence derived from the application of "OMICS" technologies could potentially improve ARTs. These biomarkers have been uncovered in recent years through the comparison of oocytes and embryos of extreme levels of competence when applying indirect criteria of developmental potential such as follicular size and stage, morphology, metabolism, maturation media, age of donor, migration speed of oocytes/zygotes under a dielectrophoretic field, cleavage dynamics, or culture stress. Moreover, biomarkers of competence are also useful to elucidate how the mechanisms governing early embryogenesis and acquisition of quality during folliculogenesis are interrelated. Notwithstanding, amelioration in the field of developmental predictive value is still to come (Wrenzycki et al. 2007; Boni 2012; Ruvolo et al. 2013 for review) and, together with the current progress in the identification of molecular biomarkers of fertility, applications will be the central subject of this review.

6.5. Molecular Markers of Quality Prior to Fertilization

6.5.1. Immature oocytes

A robust body of evidence of competence markers is derived from the discrimination of oocytes based on their follicle size, where gametes from larger follicles are of better quality. Several teams have reported that transcripts whose levels varied between GV-oocytes of differing quality pertained to several functional categories including cyclins, histones, and other cell cycle regulators, as well as transcription factors and molecules related to mRNA

and protein processing, which are remarkably well represented on the list of potential markers of competence (Robert et al. 2000; Donnison and Pfeffer, 2004; Mourot et al. 2006; Pfeffer et al. 2007; Table 6-1). In addition, super-stimulation of cows with various protocols of FSH coasting demonstrated that oocyte quality can be modified by systemic factors, which affect ovarian follicular cell physiology first and then reach the developing gamete. Upon applying the appropriate FSH-deprivation period (44/68 h coasting) before ovum pick-up (OPU), it is possible to generate a population of follicles at the right differentiation level and the recovered oocytes resemble those of natural pre-ovulatory follicles possessing optimal developmental capacity. The decrease in circulating FSH is a physiological phenomenon that has been neglected as a tool to control the proper differentiation of follicles (Nivet et al. 2012). Notably, the transcriptome signature of GV-oocytes obtained by using this strategy demonstrated that the main biological functions that varied between oocytes of different quality were related to RNA processing and regulation of chromosome segregation (Labrecque et al. 2013). These authors confirmed 13 mRNA markers of competence (Table 6-1), including *PAIP2*, *AURKAIP1*, *CDK1*, *ENY2*, and *PMS1* (Labrecque et al. 2013). Moreover, further confirmation that hormonal dynamics closely affect the molecular regulation inside the oocyte was demonstrated through the identification of alterations of the transcriptome of immature oocytes collected after FSH-coasting and administration of a GnRH antagonist. Although Labrecque et al. (2014) did not report any significant effects of the antagonist cetrotide on blastocyst rates, transcriptomic analysis revealed that cetrotide impaired protein translation capacity, RNA processing, and chromosome segregation in oocytes. Interestingly, such findings coincide with the biological functions identified as being affected in the coasting model, demonstrating how hormonal processes influence the molecular modulation of the gamete's quality (Labrecque et al. 2013; Nivet et al. 2013).

Assessment of the activity of glucose-metabolizing enzymes has also been successfully applied to distinguish immature oocytes according to their viability. Brilliant cresyl blue (BCB) staining detects the activity of G6PDH, which catalyzes the first step of the pentose phosphate pathway (Gutierrez-Adan et al. 2004 for review). BCB+ oocytes, which remain blue due to low G6PDH cytoplasmic activity, are considered as fully-grown gametes of

higher quality than BCB- gametes (unstained) that own higher G6PDH activity (Alm et al. 2005; Bhojwani et al. 2007). Bols et al. (2012) considered BCB staining as one of the few suitable non-invasive indicators of competence. Notably, classification of immature oocytes by BCB staining followed by transcriptomic analysis has highlighted differential levels of mRNAs (Table 6-1) related to cell cycle regulation, *CCNB1*, *PTTG1*; transcription control, *SMARCA5*; and protein translation, *eIF*-, *RPL*- and *RPS*-group proteins (Ghanem et al. 2007; Torner et al. 2008), suggesting the importance of such functions for the potential to develop to the blastocyst stage.

An attractive approach for quality assessment of the developing female gamete is the analysis of proxies of fertility such as follicular fluid (FF) or biopsied follicular somatic cells (Fig. 6-1). Molecular characterization of these follicular components represents a non-invasive alternative to investigate the developmental competence of the oocyte without compromising its viability. The most external follicular compartment is theca cells and Matoba et al. (2014) observed that *ESR1* and *VCAN* mRNAs were overexpressed in thecal cells associated with competent oocytes (Table 6-2). It makes sense that increased levels of *VCAN* are correlated with higher competence as this proteoglycan may be necessary for ovulation. A larger amount of biomarker data has been derived from granulosa cells. Nivet et al. (2013) reported on four putative markers of fertility in these cells and the information was valuable not only because biomarkers of fertility were unveiled, but such findings also shed light on complete molecular pathways (prolactin, growth hormone pathways) related to the events in the granulosa compartment that lead to acquisition of competence of the gamete. In addition, the granulose transcriptomic profiling demonstrated that folliculogenesis in cattle is a highly dynamic and tightly regulated process: The pre-ovulatory differentiation of granulosa cells at the end of follicular growth, which is characterized by angiogenesis, early hypoxia and oxidative stress, contributes to the specific environment required for the oocyte to attain maximum competence (as is the case with 44/68 h FSH coasting). Then, if FSH starvation is extended (92 h), folliculogenesis enters a phase where apoptosis is increased and signs of inflammation appear (Nivet et al. 2013), while the quality of the enclosed oocyte suddenly diminishes, exemplifying what could happen if the gamete were not ovulated at the appropriate moment (Labrecque et al.

2013). In this sense, the negative influence of such a prolonged coasting period on ovulation rates and oocyte quality has been demonstrated (Dias et al. 2013).

Bunel et al. (2013) identified six markers of competence in cumulus cells by using the same coasting model as mentioned above (Table 6-2). Abundance of the *CYP11A1* and *NSDHL* transcripts increased with developmental competence. Since they are involved in progesterone biosynthesis, their highest levels at the moment of optimal quality may indicate that increased levels of this steroid and modulation of its synthesis by CYP11A1 and NSDHL favor acquisition of developmental competence. Similarly, *NRP1* and *VNN1* mRNA abundance increased in parallel with FSH-coasting and peaked in cumulus cells from over-differentiated follicles, probably reflecting the fact that angiogenesis and the need to deal with oxidative stress are required by the end of folliculogenesis, as NRP1 and VNN1 are involved in such functions, respectively. Interestingly, this expression pattern corresponds to the same profile previously observed by Nivet et al. (2013) in granulosa cells, which indicates that the level of angiogenesis and oxidative processes increases in both granulosa and cumulus cells. Additionally, increased GATM expression could reflect the elevated hypoxic condition at the end of folliculogenesis (Bunel et al. 2013). These results support the conclusions reached by Assidi et al. (2008) that the molecular regulation of cumulus cell function and differenctiation is a complex process that involves events necessary for acquisition of developmental capacity by the oocyte. As a result of an outstanding effort, O'Shea et al. (2012) identified markers of competence in cumulus cells and oocytes shared across species and concluded that some of the molecular mechanisms related to competence are conserved. Bettegowda et al. (2008) observed that transcript levels of cathepsins varied in cumulus cells according to the viability of the oocyte. In the same report, blastocyst rates were increased by inhibiting cathepsins during IVM. Nevertheless, these authors emphasized the need to understand the molecular basis of the IVP improvement observed in response to the pharmacological targeting of cathepsins.

Another appealing non-invasive strategy to identify markers of competence is the fingerprinting of the metabolome of FF. Using this method Matoba et al. (2014) observed that urea, three amino acids, two fatty acids, and total fatty acid contents varied in the FF

associated with oocytes of distinct quality levels (Table 6-2). Amino acid profiling was particularly predictive of developmental competence. Specifically, T. Fair laboratory's results (Matoba et al. 2014) of metabolic analysis of FF were in agreement with previous observations that high levels of urea (De Wit et al. 2001), total saturated fatty acids and palmitic acid (Leroy et al. 2005) have deleterious effects on oocyte competence during IVM, whereas a surplus of alanine, glycine, and glutamate appear to positively impact development (Sinclair et al. 2008). Results of FF metabolome characterization are promising and will contribute to the improvement of maturation media (Matoba et al. 2014). However, their practical use in commercial IVP programs is currently challenging because more information is still needed to establish a clear correlation of FF metabolites and oocyte fertility status (Revelli et al. 2009; Bols et al. 2012).

6.5.2. Mature oocytes

Using transcriptomic analysis, Biase et al. (2014) identified twenty-nine putative mRNA markers of quality in bovine mature oocytes (Table 6-1). It is noteworthy that the most variable biological functions between oocytes of high and low developmental competence were RNA processing and translation as observed in GV-oocytes (Labrecque et al. 2013). The metabolomic analysis, of spent IVM media identified variations in the capacity to turnover alanine, arginine, glutamine, leucine, and tryptophan between oocytes of distinct developmental competence (Hemmings et al. 2012). Overall M-II oocytes of decreased quality had higher amino acid turnover rates. This is in agreement with the quiet embryo hypothesis (Leese 2002; Baumann et al. 2007; Leese et al. 2008), which states that less competent embryos have major levels of metabolic activity. Hemmings et al. (2012) extended the notion of metabolic quietness as a sign of higher developmental potential to oocytes and suggested that the metabolism of an oocyte or cleavage stage embryo could be a reflection of its stored maternal transcripts.

6.6. Molecular Markers of Quality in Embryos

6.6.1. From fertilization to embryonic genome activation

In this section, biomarkers of competence found in zygotes, as well as in 2-cell and 8-cell embryos will be summarized. Prior to embryonic genome activation (EGA), in the absence

of *de novo* transcription, the embryo still depends on maternal stocks of mRNAs and proteins and on the metabolic machinery inherited from the oocyte for its development (Fig. 6-1; Krisher 2004; Marlow 2010; Sirard 2010). Considering that it is generally accepted that EGA occurs in cattle at the 8-16 cell stage (Barnes and First 1991; Memili and First 1998; Memili and First 1999), the first cleavages provide a relatively long time-span where biomarkers of competence are likely exclusively from maternal origin (Lechniak et al. 2008; Orozco-Lucero et al. 2014). Analysis of zygotes of distinct levels of fertility has unveiled seven potential biomarkers of competence (Table 6-3), most of them related to the functions of cell cycle regulation: *NASP*, *AURKA*, and *IQGAP1*; and transcription regulation: *DDX10*, *DNMT1*, and *SMARCA5* (Dessie et al. 2007). Amino acid (turnover of overall amino acids) profiling of spent culture medium confirmed that the most metabolically inactive zygotes were the most likely to reach the blastocyst stage (Sturmey et al. 2010). These findings are in agreement with those of Hemmings et al. (2012) described above concerning metabolically quiet oocytes.

One of the new parameters that arise upon fertilization that can be used to evaluate developmental competence is embryonic cleavage dynamics. It is generally accepted that early-cleaving embryos produce higher blastocyst rates than their slow-cleaving counterparts (Lechniak et al. 2008; Orozco-Lucero et al. 2014). Although the exact nature of this phenomenon and the way in which it impacts developmental capacity, or reflects it, is still not fully understood, the most plausible hypothesis is that the elevated competence accompanying fast embryonic division is mostly due to intrinsic characteristics of the oocyte from which the cleaving embryo originates. The fact that embryonic cleavage speed is correlated with developmental capacity has been observed across species and timing to the first zygotic division has been used as a parameter to separate embryos of variable fertility status and to try to identify the molecular mechanisms underlying early cleavage (Lechniak et al. 2008). Initial efforts to unveil markers of competence in 2-cell cattle embryos of differing cleavage speed have identified transcripts of differential abundance levels between fast- and slow-cleaving embryos. These transcripts (Table 6-3) are related to various biological functions such as structure: *CX32*, *CX43*, *PKP1* (Brevini et al. 2002; Gutierrez-Adan et al. 2004); glucose metabolism: *IDH*, *G6PDH*, *GPI*, *HK1* (Lequarre et al.

1997; Dode et al. 2006); transport: *GLUT1* (Lequarre et al. 1997); signaling: *BMP15*, *PED*, *IGF2*, *IGF1R*, *IFNT*, *FS*, *INHA*, *INHBB* (Fair et al. 2004a; Fair et al. 2004b; Gutierrez-Adan et al. 2004; Patel et al. 2007); oxidative stress: *SOD2* (Gutierrez-Adan et al. 2004); cell cycle regulation: *CCNB1* (Fair et al. 2004b; Bermejo-Alvarez et al. 2010); transcription control: *OCT4*, *YEAF1* (Brevini et al. 2002; Dode et al. 2006); DNA packaging: *H2A*, *H3A* (Fair et al. 2004b; Dode et al. 2006; Mourot et al. 2006); protein regulation: *CTSB*, *TCP1* (Dode et al. 2006); transcript processing: *PAP*, *PARN* (Brevini et al. 2002); and DNA repair: *RAD50* (Dode et al. 2006). In an ingenious study, Held et al. (2012) analyzed the transcripts from one of the blastomeres of 2-cell embryos resulting in blastocysts at either high or low rates upon individual culture of the remaining sister blastomere. The transcriptomic contrast uncovered that NRF2-mediated oxidative stress response and oxidative phosphorylation were the main biological functions varying between competent and unviable blastomeres. Ten candidate markers of fertility were validated by RT-qPCR through an independent model of time to the first zygotic cleavage (Held et al. 2012). Our laboratory compared fast- and slow-dividing 2-cell embryos by transcriptomic analysis and identified cell cycle regulation, DNA damage response, RNA processing, transcription control, and protein degradation as the main biological functions differing between 2-cell embryos of variable developmental fitness (Orozco-Lucero et al. 2014). Ten of the candidate markers of competence that were confirmed by RT-qPCR were involved in crucial functions such as DNA damage response: *ATM*, *ATR*, *MRE11A*, *MSH6*, *CTNNB1*; cell cycle: *APC*, *PCNA*, *CENPE*; and transcription control: *TAF2* (Orozco-Lucero et al. 2014). The finding that the most viable 2-cell embryos had higher levels of mRNAs related to DNA damage response could either mean that such embryos have suffered less DNA offenses and therefore had not translated these mRNAs, or that competent embryos are better equipped to deal with DNA damage prior to EGA. It is tempting to speculate that there is a possible association between reduced DNA damage in 2-cell embryos and quiet metabolism. In fact, Sturmey et al. (2009) correlated increased levels of DNA damage in pig, cow, and human embryos with elevated metabolic activity manifested as high amino acid turnover. These authors speculated that this could be due to the fact that the less viable embryos with more DNA damage attempt to avoid developmental arrest by repairing it. Consequently, the least competent embryos need to increase their metabolism (including

processes involving amino acid turnover) to perform this additional molecular "work" compared to healthier and metabolically quieter embryos. Moreover, our results with 2-cell embryos were consistent with the report of Labrecque et al. (2013) where the most viable GV-oocytes seemed to be better prepared to regulate meiosis and process mRNA. Thus, an improved maternal stock of transcripts related to cell cycle regulation in oocytes and cleavage-stage embryos might reduce the risk of aneuploidy, which is a major cause of embryonic arrest (Pers-Kamczyc et al. 2012). One of our major hypotheses concerning oocyte quality is that the most competent oocytes and their derived embryos are better supplied with maternal molecules that will help them go smoothly through EGA, when modulation of mRNA processing, transcription, cell cycle, and protein translation/degradation are key events (Sirard 2010). This notion makes sense in the light of the multiple biomarkers of competence related to these functions found in oocyte/embryo compartments even prior maturation. Ripamonte et al. (2012) reported differential abundance of *PI3KCA* and *ITM2B* mRNAs between early- and late-cleaving 8-cell embryos (which are approaching the EGA time point). Both molecules are related to apoptotic mechanisms and their presence might reflect the need for programmed cell death regulation in bovine embryos at the 8-cell stage and beyond.

6.6.2. Morulae and blastocysts

Very few biomarkers of fertility have been identified in bovine embryos at the morula stage. The dynamic distribution of the CTNNB1 protein in morulae is associated with fast embryonic cleavage and high competence, as pointed out by Modina et al. (2007). In contrast, several markers of fertility have been identified so far at the blastocyst stage. Unfortunately, the opportunities to transfer IVP-blastocysts to assess their final capacity to establish pregnancy are rare. Therefore, molecular markers at the blastocyst stage have been used mostly to characterize how blastocysts modify their quality and how they react to different *in vivo* or *in vitro* conditions, or to specific stress conditions achieved by culture medium supplementation (Fig. 6-1). By using non-invasive amino acid profiling of *in vivo*-generated and IVP-blastocysts, it was corroborated previous findings at the M-II and zygote stage, where the most metabolically quiet oocytes/embryos had higher competence. This time, it was observed that the IVP- blastocysts, likely less competent, consumed more

amino acids than their *in vivo*-generated counterparts (Sturmey et al. 2010). In relation to medium supplementation, Cagnone et al. (2012) tested hyperglycemic culture conditions and observed that the resulting blastocysts were affected in their extracellular matrix signaling, calcium signaling, as well as energetic metabolism, while such modified gene expression was also related to the Warburg effect (induction of aerobic glycolysis) as if these blastocysts were activating pathways related to cancer and diabetes. The effects of oxidative stress have been examined in culture too by supplementation with two pro-oxidant agents, AAPH and buthionine sulfoximine, which differentially impacted on blastocysts biological functions such as oxidative stress, energy metabolism, glycine metabolism, cellular homeostasis, and inflammatory response. Importantly, this work allowed us to observe that the most metabolically inactive embryos seemed to better survive to oxidative stress (Cagnone and Sirard, 2013). Subsequently, Cagnone and Sirard (2014) unveiled the changes triggered by supplementation of the culture medium with different proteins and lipids. The expression of genes related to ceramide-induced oxidative stress, inflammation, and cholesterol metabolism was altered in response to distinct supplementation and the expression of a pair of pluripotency-associated genes (*APEX*, *CLDN6*) was also modified (Table 6-3). A different perspective on how culture conditions affect early development arose from the comparison by transcriptomic analysis of blastocysts developed in the reproductive tract of super-stimulated cows with those cultured in the tracts of non-stimulated recipient cows (originally transferred to the oviduct as 2-4 cell embryos). Eleven candidate markers were validated in this study, and day 7-blastocysts flushed from the uterus of super-ovulated animals had higher expression of genes involved in transcription, translation, stress response, oxidative stress, oxidative phosphorylation, as well as cellular and metabolic activity (Gad et al. 2011). Furthermore, Gad et al. (2012) unraveled the effects of the surrounding environment on embryo development by comparing blastocysts obtained from alternation of *in vivo* and IVC (switching at either EGA or morula stage) against embryos completely cultured *in vitro* or *in vivo*. Whereas the oocyte maturation environment (*in vivo*/*in vitro*) importantly impacted developmental competence, changing culture conditions up until around the time of EGA did not affect blastocyst rates. However, changing culture conditions had a marked impact on transcript profiles demonstrating the sensitivity of embryos to their environment around the time of

EGA. In this survey, oxidative stress (including NRF2-mediated oxidative stress response) and lipid metabolism were the most altered biological functions. Outstandingly, negative environmental effects occurring as early as by the time of EGA could influence pluripotency of the analyzed blastocysts, as observed by the variable expression of OCT4 (Gad et al. 2012). In Gad et al. (2014, Faculty of Agriculture, Cairo University, Institute of Animal Science, University of Bonn, personal communication), the culture environment alternation occurred around the morula stage and transcriptional analysis revealed that cell death, lipid metabolism, NRF2-related oxidative stress, integrin signaling, and TNFR1/2 pathways were the most affected biological functions between each of the three groups of stressed embryos and the golden standard group fully cultured *in vivo*. In such study, eight putative markers of developmental competence were confirmed by RT-qPCR (Table 6-3). Noticeably, the authors suggested that embryos that develop to the blastocyst stage under harsh *in vitro* conditions try to adapt to the challenging culture environment and as a consequence their transcriptome is modified. Interestingly, a potential carry-over effect of the detrimental culture environment can affect the pluripotency status of the resulting embryos given that the mRNA level of the transcription factor *KLF4* was affected. In summary, the previous works have helped to better understand how embryos adapt to different culture conditions. Shortly after EGA a variable culture environment not only prompts remarkable metabolic changes in embryos (Gad et al. 2011; Cagnone et al. 2012; Cagnone and Sirard 2013), but also modifies the expression of pluripotency-related genes (Gad et al. 2012; Gad et al.2014; Gad, personal communication; Cagnone and Sirard 2014).

In spite of the fact that is difficult to find studies in cattle that correlated molecular biomarkers with the ultimate measure of developmental competence (calf delivery), two important surveys must be mentioned. In the first study, biopsies of IVP-blastocysts were transcriptome-profiled, while the rest of the embryo was transferred. Blastocysts that produced a calf were enriched in transcripts related to implantation and signaling. In contrast, embryos unable to generate pregnancies had increased levels of mRNAs (Table 6-3) associated with inflammation, protein binding, transcription, cell cycle control, and implantation inhibition (El-Sayed et al. 2006). Subsequently, with a similar strategy but this time using *in vivo*-derived blastocysts, Ghanem et al. (2011) reported that embryos that

produced a calf were enriched in *BMP15*, *KRT8*, *RGS2*, as well as in the marker of placental development and embryo-maternal interaction *PLAC8*; whereas blastocysts unable to establish a gestation had higher *FL405* and *HSPD1*, which are associated with mitochondrial function and stress, respectively. Interestingly, in this report, the list of markers that differed between blastocysts able and unable to produce a pregnancy was compared with the list from El-Sayed et al. (2006) in order to find shared genes. Although three markers had no correspondence, probably due to the influence of the different culture environments (*in vivo*/*in vitro*), eighteen markers were in agreement, implying that blastocysts capable of bringing pregnancy to term have similar gene expression patterns in spite of the culture environment. Therefore, both studies demonstrated the feasibility of using gene markers of implantation in cattle.

6.7. Conclusion

The ability to discern populations of oocytes/embryos of different levels of developmental capacity has been a holy grail pursued by reproductive scientists for decades. The work to achieve this now appears to be going in the right direction with the use of powerful "OMICS" technologies. Distinguishing gametes and embryos according to their fertility status is not the only major benefit of fertility markers. These molecules are helping to unravel the intricate modulation of the acquisition of competence during early development, whether in any of the follicular compartments (and their interactions) or in the developing embryo. Such molecular markers are also instrumental at comprehending the way in which the surrounding environment impacts early development and what are the possible resilience mechanisms of embryos in relation to their milieu.

The main challenges for a practical application of biomarkers of fertility in oocytes and embryos are: 1) to generate a standard and consensual list of competence markers and avoid the confusion arising from the large amount of data and the long list of candidate markers; 2) to improve the identification of non-invasive biomarkers; 3) to integrate all the information from different sources and developmental stages into a broad and comprehensive scheme of the molecular physiology leading to developmental potential acquisition; 4) to utilize this knowledge to ameliorate the protocols of super-

stimulation/oocyte recovery and IVC in order to provide through systemic (e.g. hormonal super-stimulation) or local targeting (e.g. IVC media supplementation) the developing oocyte with conditions more reflective of the natural microenvironment.

6.8. Acknowledgements

We thank Dr. Sylvie Bilodeau-Goeseels for revising the manuscript.

6.9. References

Alm H, Torner H, Lohrke B, Viergutz T, Ghoneim IM, Kanitz W. 2005. Bovine blastocyst development rate in vitro is influenced by selection of oocytes by brilliant cresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity. *Theriogenology* **63:** 2194-2205.

Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S, Sirard MA. 2008. Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate in vitro. *Biol Reprod* **79:** 209–222.

Balboula AZ, Yamanaka K, Sakatani M, Hegab AO, Zaabel SM, Takahashi M. 2010. Cathepsin B activity is related to the quality of bovine cumulus oocyte complexes and its inhibition can improve their developmental competence. *Mol Reprod Dev* **77:** 439-448.

Barnes FL, First NL. 1991. Embryonic transcription in in vitro cultured bovine embryos. *Mol Reprod Dev* **29:** 117-123.

Baumann CG, Morris DG, Sreenan JM, Leese HJ. 2007. The quiet embryo hypothesis: molecular characteristics favoring viability. Mol Reprod Dev **74:** 1345-353.

Bermejo-Alvarez P, Lonergan P, Rizos D, Gutierrez-Adan A. 2010. Low oxygen tension during IVM improves oocyte competence and enhances anaerobic glycolysis. *Reprod Biomed Online* **20:** 341-349.

Bettegowda A, Patel OV, Lee KB, Park KE, Salem M, Yao J, Ireland JJ, Smith GW. 2008. Identification of novel bovine cumulus cell molecular markers predictive of oocyte competence: functional and diagnostic implications. *Biol Reprod* **79:** 301-309.

Bhojwani S, Alm H, Torner H, Kanitz W, Poehland R. 2007. Selection of developmentally competent oocytes through brilliant cresyl blue stain enhances blastocyst development rate after bovine nuclear transfer. *Theriogenology* **67:** 341-345.

Biase FH, Everts RE, Oliveira R, Santos-Biase WK, Fonseca Merighe GK, Smith LC, Martelli L, Lewin H, Meirelles FV. 2014. Messenger RNAs in metaphase II oocytes correlate with successful embryo development to the blastocyst stage. *Zygote* **22:** 69-79.

Biase FH, Martelli L, Puga R, Giuliatti S, Santos-Biase WK, Fonseca Merighe GK, Meirelles FV. 2010. Messenger RNA expression of Pabpnl1 and Mbd3l2 genes in oocytes and cleavage embryos. *Fertil Steril* **93:** 2507-2512.

Blondin P, Bousquet D, Twagiramungu H, Barnes F, Sirard MA. 2002 Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biol Reprod* **66:** 38-43.

Blondin P, Sirard MA. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol Reprod Dev* **41:** 54-62.

Bols PEJ, Jorssen EPA, Goovaerts IGF, Langbeen A, Leroy JLMR. 2012. High throughput non-invasive oocyte quality assessment: the search continues. *Anim Reprod* **9:** 420-425.

Boni R. 2012. Origins and effects of oocyte quality in cattle. *Anim Reprod* **9:** 333-340.

Brevini TA, Lonergan P, Cillo F, Francisci C, Favetta LA, Fair T, Gandolfi F. 2002. Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. *Mol Reprod Dev* **63:** 510-517.

Bunel A, Nivet AL, Blondin P, Vigneault C, Richard FJ, Sirard MA. 2013. Cumulus cell gene expression associated with pre-ovulatory acquisition of developmental competence in bovine oocytes. *Reprod Fertil Dev* **[in press]**.

Cagnone GL, Dufort I, Vigneault C, Sirard MA. 2012. Differential gene expression profile in bovine blastocysts resulting from hyperglycemia exposure during early cleavage stages. *Biol Reprod* **86:** 50.

Cagnone GL, Sirard MA. 2013. Transcriptomic signature to oxidative stress exposure at the time of embryonic genome activation in bovine blastocysts. *Mol Reprod Dev* **80:** 297-314.

Cagnone G, Sirard MA. 2014. The impact of exposure to serum lipids during in vitro culture on the transcriptome of bovine blastocysts. *Theriogenology* **81:** 712-722.

Caixeta ES, Ripamonte P, Franco MM, Junior JB, Dode MA. 2009. Effect of follicle size on mRNA expression in cumulus cells and oocytes of Bos indicus: An approach to identify marker genes for developmental competence. *Reprod Fertil Dev* **21:** 655-664.

Camargo LS, Viana JH, Ramos AA, Serapiao RV, de Sa WF, Ferreira AM, Guimaraes MF, do Vale Filho VR. 2007. Developmental competence and expression of the Hsp 70.1 gene in oocytes obtained from Bos indicus and Bos taurus dairy cows in a tropical environment. *Theriogenology* **68:** 626-632.

Costa LFS, Machado MSN, Oliveira JFC, Zamberlan G, Goncalves PBD. 2006. Annexin II mRNA expression in bovine oocytes during follicular development. *Gen Mol Biol* **29:**396-400.

Cote I, Vigneault C, Laflamme I, Laquerre J, Fournier E, Gilbert I, Scantland S, Gagne D, Blondin P, Robert C. 2011. Comprehensive cross production system assessment of

the impact of in vitro microenvironment on the expression of messengers and long non-coding RNAs in the bovine blastocyst. *Reproduction* **142:** 99-112.

De Sousa PA, Westhusin ME, Watson AJ. 1998. Analysis of variation in relative mRNA abundance for specific gene transcripts in single bovine oocytes and early embryos. *Mol Reprod Dev* **49:** 119-130.

De Wit AA, Cesar ML, Kruip TA. 2001. Effect of urea during in vitro maturation on nuclear maturation and embryo development of bovine cumulus-oocyte-complexes. *J Dairy Sci* **84:** 1800-1804.

Dessie SW, Rings F, Holker M, Gilles M, Jennen D, Tholen E, Havlicek V, Besenfelder U, Sukhorukov VL, Zimmermann U, Endter JM, Sirard MA, Schellander K, Tesfaye D. 2007. Dielectrophoretic behavior of in vitro-derived bovine metaphase II oocytes and zygotes and its relation to in vitro embryonic developmental competence and mRNA expression pattern. *Reproduction* **133:** 931-946.

Dias FC, Khan MI, Sirard MA, Adams GP, Singh J. 2013. Differential gene expression of granulosa cells after ovarian superstimulation in beef cattle. *Reproduction* **146:** 181-191.

Dode MA, Dufort I, Massicotte L, Sirard MA. 2006. Quantitative expression of candidate genes for developmental competence in bovine two-cell embryos. *Mol Reprod Dev* **73:** 288-297.

Donnison M, Pfeffer PL. 2004. Isolation of genes associated with developmentally competent bovine oocytes and quantitation of their levels during development. *Biol Reprod* **71:** 1813-1821.

El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, Sirard MA, Schellander K, Tesfaye D. 2006. Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiol Genomics* **28:** 84-96.

Fair T. 2012. Molecular and endocrine determinants of oocyte competence. *Anim Reprod* **10:** 277-282.

Fair T, Gutierrez-Adan A, Murphy M, Rizos D, Martin F, Boland MP, Lonergan P. 2004a. Search for the bovine homolog of the murine ped gene and characterization of its messenger RNA expression during bovine preimplantation development. *Biol Reprod* **70:** 488-494.

Fair T, Murphy M, Rizos D, Moss C, Martin F, Boland MP, Lonergan P. 2004b. Analysis of differential maternal mRNA expression in developmentally competent and incompetent bovine two-cell embryos. *Mol Reprod Dev* **67:** 136-144.

Gad A, Hoelker M, Besenfelder U, Havlicek V, Cinar U, Rings F, Held E, Dufort I, Sirard MA, Schellander K, Tesfaye D. 2012. Molecular mechanisms and pathways involved

in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. *Biol Reprod* **87:** 100.

Gad A, Hoelker M, Besenfelder U, Havlicek V, El-Sayed A, Rings F, Dufort I, Sirard MA, Schellander K, Tesfaye D. 2014 (unpublished). Culture environment during the time of transition from morula to blastocyst resulted in significant alteration in the transcriptome of bovine embryos.

Gad A, Besenfelder U, Rings F, Ghanem N, Salilew-Wondim D, Hossain MM, Tesfaye D, Lonergan P, Becker A, Cinar U, Schellander K, Havlicek V, Holker M. 2011. Effect of reproductive tract environment following controlled ovarian hyperstimulation treatment on embryo development and global transcriptome profile of blastocysts: implications for animal breeding and human assisted reproduction. *Hum Reprod* **26:** 1693-1707.

Ghanem N, Holker M, Rings F, Jennen D, Tholen E, Sirard MA, Torner H, Kanitz W, Schellander K, Tesfaye D. 2007. Alterations in transcript abundance of bovine oocytes recovered at growth and dominance phases of the first follicular wave. *BMC Dev Biol* **7:** 90.

Ghanem N, Salilew-Wondim D, Gad A, Tesfaye D, Phatsara C, Tholen E, Looft C, Schellander K, Hoelker M. 2011. Bovine blastocysts with developmental competence to term share similar expression of developmentally important genes although derived from different culture environments. *Reproduction* **142:** 551-564.

Gutierrez-Adan A, Rizos D, Fair T, Moreira PN, Pintado B, de la Fuente J, Boland MP, Lonergan P. 2004. Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. *Mol Reprod Dev* **68:** 441-448.

Held E, Salilew-Wondim D, Linke M, Zechner U, Rings F, Tesfaye D, Schellander K, Hoelker M. 2012. Transcriptome fingerprint of bovine 2-cell stage blastomeres is directly correlated with the individual developmental competence of the corresponding sister blastomere. *Biol Reprod* **87:** 154.

Hemmings KE, Leese HJ, Picton HM. 2012. Amino acid turnover by bovine oocytes provides an index of oocyte developmental competence in vitro. *Biol Reprod* **86:** 165.

Katz-Jaffe MG, McCallie BR, Preis KA, Filipovits J, Gardner DK. 2009. Transcriptome analysis of in vivo and in vitro matured bovine MII oocytes. *Theriogenology* **71:** 939-946.

Krisher RL. 2004. The effect of oocyte quality on development. *J Anim Sci* **82:** 14-23.

Labrecque R, Vigneault C, Blondin P, Sirard MA. 2013. Gene expression analysis of bovine oocytes with high developmental competence obtained from FSH-stimulated animals. *Mol Reprod Dev* **80:** 428-440.

Labrecque R, Vigneault C, Blondin P, Sirard MA. 2014. Gene expression analysis of bovine oocytes at optimal coasting time combined with GnRH antagonist during the no-FSH period. *Theriogenology*, **[in press]**.

Lechniak D, Pers-Kamczyc E, Pawlak P. 2008. Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos. *Reprod Biol* **8:** 23-42.

Leese HJ. 2002. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. *Bioessays* **24:** 845-849.

Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG. 2008. Metabolism of the viable mammalian embryo: quietness revisited. *Mol Hum Reprod* **14:** 667-672.

Lequarre AS, Grisart B, Moreau B, Schuurbiers N, Massip A, Dessy F. 1997. Glucose metabolism during bovine preimplantation development: Analysis of gene expression in single oocytes and embryos. *Mol Reprod Dev* **48:** 216-226.

Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G, Van Soom A. 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction* **130:** 485-495.

Li HJ, Liu DJ, Cang M, Wang LM, Jin MZ, Ma YZ, Shorgan B. 2009. Early apoptosis is associated with improved developmental potential in bovine oocytes. *Anim Reprod Sci* **114:** 89-98.

Lingenfelter BM, Dailey RA, Inskeep EK, Vernon MW, Poole DH, Rhinehart JD, Yao J. 2007. Changes of maternal transcripts in oocytes from persistent follicles in cattle. *Mol Reprod Dev* **74:** 265-272.

Lonergan P, Gutierrez-Adan A, Rizos D, Pintado B, de la Fuente J, Boland M. 2003a. Relative messenger RNA abundance in bovine oocytes collected in vitro or in vivo before and 20 hr after the preovulatory luteinizing hormone surge. *Mol Reprod Dev* **66:** 297-305.

Lonergan P, Rizos D, Gutierrez-Adan A, Fair T, Boland MP. 2003b. Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns. *Reprod Domest Anim* **38:** 259-267.

Marlow FL. 2010. Maternal Control of Development in Vertebrates: My Mother Made Me Do It! Morgan & Claypool Life Sciences (San Rafael, CA), **186 pp**.

Massip A, Mermillod P, Dinnyes A. 1995. Morphology and biochemistry of in-vitro produced bovine embryos: implications for their cryopreservation. *Hum Reprod* **10:** 3004-3011.

Matoba S, Bender K, Fahey AG, Mamo S, Brennan L, Lonergan P, Fair T. 2014. Predictive value of bovine follicular components as markers of oocyte developmental potential. *Reprod Fertil Dev* **26:** 337-345.

Memili E, First NL. 1998. Developmental changes in RNA polymerase II in bovine oocytes, early embryos, and effect of alpha-amanitin on embryo development. *Mol Reprod Dev* **51:** 381-389.

Memili E, First NL. 1999. Control of gene expression at the onset of bovine embryonic development. *Biol Reprod* **61:** 1198-1207.

Mermillod P, Dalbies-Tran R, Uzbekova S, Thelie A, Traverso JM, Perreau C, Papillier P, Monget P. 2008. Factors affecting oocyte quality: who is driving the follicle? *Reprod Dom Anim* **43:** 393-400.

Merton JS, de Roos APW, Mullaart E, de Ruigh L, Kaal L, Vos PLAM, Dieleman SJ. 2003. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology* **59:** 651-674.

Modina S, Abbate F, Germana GP, Lauria A, Luciano AM. 2007. Beta-catenin localization and timing of early development of bovine embryos obtained from oocytes matured in the presence of follicle stimulating hormone. *Anim Reprod Sci* **100:** 264-279.

Mourot M, Dufort I, Gravel C, Algriany O, Dieleman S, Sirard MA. 2006. The influence of follicle size, FSH-enriched maturation medium, and early cleavage on bovine oocyte maternal mRNA levels. *Mol Reprod Dev* **73:**1367-1379.

Nemcova L, Machatkova M, Hanzalova K, Horakova J, Kanka J. 2006. Gene expression in bovine embryos derived from oocytes with different developmental competence collected at the defined follicular developmental stage. *Theriogenology*, **65:** 1254-1264.

Nivet AL, Bunel A, Labrecque R, Belanger J, Vigneault C, Blondin P, Sirard MA. 2012. FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction* **143:** 165-171.

Nivet AL, Vigneault C, Blondin P, Sirard MA. 2013. Changes in granulosa cells' gene expression associated with increased oocyte competence in bovine. *Reproduction* **145:** 555-565.

O'Shea LC, Mehta J, Lonergan P, Hensey C, Fair T. 2012. Developmental competence in oocytes and cumulus cells: candidate genes and networks. *Syst Biol Reprod Med* **58:** 88-101.

Opiela J, Katska-Ksiazkiewicz L, Lipinski D, Slomski R, Bzowska M, Rynska B. 2008. Interactions among activity of glucose-6-phosphate dehydrogenase in immature

oocytes, expression of apoptosis-related genes Bcl-2 and Bax, and developmental competence following IVP in cattle. *Theriogenology* **69:** 546-555.

Oropeza A, Wrenzycki C, Herrmann D, Hadeler KG, Niemann H. 2004. Improvement of the developmental capacity of oocytes from prepubertal cattle by intraovarian insulin-like growth factor-I application. *Biol Reprod* **70:** 1634-1643.

Orozco-Lucero E, Dufort I, Robert C, Sirard MA. 2014. Rapidly cleaving bovine two-cell embryos have better developmental potential and a distinctive mRNA pattern. *Mol Reprod Dev* **81:** 31-41.

Patel OV, Bettegowda A, Ireland JJ, Coussens PM, Lonergan P, Smith GW. 2007. Functional genomics studies of oocyte competence: Evidence that reduced transcript abundance for follistatin is associated with poor developmental competence of bovine oocytes. *Reproduction* **133:** 95-106.

Patrizio P, Fragouli E, Bianchi V, Borini A, Wells D. 2007. Molecular methods for selection of the ideal oocyte. *Reprod Biomed Online* **15:** 346-353.

Pers-Kamczyc E, Pawlak P, Rubes J, Lechniak D. 2012. Early cleaved bovine embryos show reduced incidence of chromosomal aberrations and higher developmental potential on day 4.5 post-insemination. *Reprod Domest Anim* **47:** 899-906.

Pfeffer PL, Sisco B, Donnison M, Somers J, Smith C. 2007 Isolation of genes associated with developmental competency of bovine oocytes. *Theriogenology* **68:** S84-90.

Plourde D, Vigneault C, Laflamme I, Blondin P, Robert C. 2012. Cellular and molecular characterization of the impact of laboratory setup on bovine in vitro embryo production. *Theriogenology* **77:** 1767-1778.

Racedo SE, Wrenzycki C, Herrmann D, Salamone D, Niemann H. 2008. Effects of follicle size and stages of maturation on mRNA expression in bovine in vitro matured oocytes. *Mol Reprod Dev* **75:** 17-25.

Revelli A, Piane LD, Casano S, Molinari E, Massobrio M, Rinaudo P. 2009. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrinol* **7:** 40-52.

Ripamonte P, Mesquita LG, Cortezzi SS, de Carvalho Balieiro JC, Fonseca Merighe GK, Watanabe YF, Caetano AR, Meirelles FV. 2012. Differential gene expression and developmental competence in in vitro produced bovine embryos. *Zygote* **20:** 281-290.

Robert C, Barnes FL, Hue I, Sirard MA. 2000. Subtractive hybridization used to identify mRNA associated with the maturation of bovine oocytes. *Mol Reprod Dev* **57:** 167-175.

Romar R, De Santis T, Papillier P, Perreau C, Thelie A, Dell'Aquila ME, Mermillod P, Dalbies-Tran R. 2011. Expression of maternal transcripts during bovine oocyte in vitro maturation is affected by donor age. *Reprod Domest Anim* **46:** e23-30.

Ruvolo G, Fattouh RR, Bosco L, Brucculeri A, Cittadini E. 2013. New molecular markers for the evaluation of gamete quality. *J Assist Reprod Genet* **30:** 207-212.

Sinclair KD, Lunn LA, Kwong WY, Wonnacott K, Linforth RS, Craigon J. 2008. Amino acid and fatty acid composition of follicular fluid as predictors of in-vitro embryo development. *Reprod Biomed Online* **16:** 859-868.

Sirard MA. 2010. Activation of the embryonic genome. *Soc Reprod Fertil Suppl* **67:** 145-158.

Sirard MA, Blondin P. 1996. Oocyte maturation and IVF in cattle. *Anim Reprod Sci* **42:** 417-426.

Sirard MA, Richard F, Blondin P, Robert C. 2006. Contribution of the oocyte to embryo quality. *Theriogenology* **65:** 126-136.

Sturmey RG, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ, Lonergan P. 2010. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. *Mol Reprod Dev* **77:** 285-296.

Sturmey RG, Hawkhead JA, Barker EA, Leese HJ. 2009. DNA damage and metabolic activity in the preimplantation embryo. *Hum Reprod* **24:** 81-91.

Torner H, Ghanem N, Ambros C, Holker M, Tomek W, Phatsara C, Alm H, Sirard MA, Kanitz W, Schellander K, Tesfaye D. 2008. Molecular and subcellular characterisation of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity *Reproduction* **135:** 197-212.

Wrenzycki C, Herrmann D, Niemann H. 2007. Messenger RNA in oocytes and embryos in relation to embryo viability. *Theriogenology* **68:** S77-83.

6.10. Tables and Figures

Table 6-1 Molecular markers of developmental competence in oocytes

Factor(s)	Type	Stage	Reference(s)
CCNB1	mRNA	GV	Robert et al. (2000); Torner et al. (2008)
CCNB2, CKS1B, CDC5L, PSMB2, SKIIP, RGS16, PRDX1	mRNA	GV	Mourot et al. (2006)
CCNA2, NDFIP1, OCT4, MSX1, ZNF198, SLBP, DNAJA1 (DJA4), GDF9, TRAPPC3	mRNA	GV	Donnison and Pfeffer (2004); Pfeffer et al. (2007)
DYNLL1, DYNC1I1	mRNA	GV	Racedo et al. (2008)
NASP, SMARCA5, RPS274A, EIF1A, ATP5A1	mRNA	GV	Torner et al. (2008)
PTTG1	mRNA	GV	Mourot et al. (2006); Ghanem et al. (2007)
H2A	mRNA	GV	Caixeta et al. (2009)
RPL24, MSX1	mRNA	GV	Ghanem et al. (2007)
MATER, YY1, MSY2, PAP, PARN, EIF4E	mRNA	GV	Lingenfelter et al. (2007)
HSP70	mRNA	GV	Camargo et al. (2007)
CTSB	Protein	GV	Balboula et al. (2010)
ATP1A1	mRNA	GV	De Sousa et al. (1998)
INHBA, INHBB	mRNA	GV	Patel et al. (2007)
ANXA2	mRNA	GV	Costa et al. (2006)
PRDX1, PRDX2	mRNA	GV	Romar et al. (2011)
G6PDH	Enzy-matic activity	GV	Alm et al. (2005); Bhojwani et al. (2007); Ghanem et al. (2007); Torner et al. (2008)
BCL2, BAX	mRNA	GV	Opiela et al. (2008); Li et al. (2009)

Table 6-1 (continued) Molecular Markers of developmental competence in oocytes

Factor(s)	Type	Stage	Reference(s)
RBM42, LSM10, HAUS8, AURKAIP1, CDK1, PAIP2, ENY2, ESCO2, PMS1, ELP4, TFDP1, SFRS7, TAF1A	mRNA	GV	Labrecque et al. (2013)
TACC3, SARNP, CTNNBL1	mRNA	GV	Labrecque et al. (2014)
CCNB1, GDF9, SOD1, SOD2	mRNA	M-II	Lonergan et al. (2003a)
CKS1B, FAM58A, NASP, NUSAP1, CDC91L, SMARCA5, RPL2, RPL8, RPL35, RPLP0, DNMT1, ANXA2	mRNA	M-II	Dessie et al. (2007)
AQP3, SEPT7, ABHD4, SIAH2	mRNA	M-II	Katz-Jaffe et al. (2009)
PABPNL1	mRNA	M-II	Biase et al. (2010)
SFRS14, DDR1, NDUFB6, UQCRH, DUSP6, NDUFS4	mRNA	M-II	Biase et al. (2014)
Alanine, arginine, glutamine, leucine, tryptophan	Amino acid	M-II	Hemmings et al. (2012)

Table 6-2 Molecular markers of developmental competence in follicles

Factor(s)	Type	Compartment	Reference(s)
VCAN, ESR1	mRNA	Theca	Matoba et al. (2014)
IGF2, NRP1, VNN1, KCNJ8	mRNA	Granulosa	Nivet et al. (2013)
LHCGR	mRNA	Granulosa	Matoba et al. (2014)
HAS, INHBA, EGFR, GREM1, BTC, CD44, TNFAIP6, PTGS2	mRNA	Cumulus	Assidi et al. (2008)
CTSB, CTSS, CTSZ	mRNA	Cumulus	Bettegowda et al. (2008)
CYP11A1, NSDHL, GATM, MAN1A1, VNN1, NRP1	mRNA	Cumulus	Bunel et al. (2013)
TNFAIP6	mRNA	Cumulus	Matoba et al. (2014)
L-alanine, glycine, glutamic acid	Amino acid	Follicular fluid	Matoba et al. (2014)
Palmitic acid, linoleic acid, total fatty acids	Fatty acid	Follicular fluid	Matoba et al. (2014)
Urea	Amino acid metabolite	Follicular fluid	Matoba et al. (2014)

Table 6-3 Molecular markers of developmental competence in embryos

Factor(s)	Type	Stage	Reference(s)
NASP, AURKA, IQGAP, SMARCA5, DDX10, DNMT1, RGS2	mRNA	Zygote	Dessie et al. (2007)
Total amino acids	Amino acid	Zygote	Sturmey et al. (2010)
CCNB1	mRNA	2-cell	Bermejo-Alvarez et al. (2010); Fair et al. (2004b)
TCP1, RAD50, YEAF1 (RYBP), CTSB, IDH	mRNA	2-cell	Dode et al. (2006)
H2A	mRNA	2-cell	Dode et al. (2006); Mourot et al. (2006)
H3A, BMP15	mRNA	2-cell	Fair et al. (2004b)
OCT4, PAP, PARN, HSP70, PKP1, CX43, CX32, PLAT	mRNA	2-cell	Brevini et al. (2002)
GLUT1	mRNA	2-cell	Lequarre et al. (1997); Brevini et al. (2002); Oropeza et al. (2004)
PED	mRNA	2-cell	Fair et al. (2004a)
CX43, IGF2, IGF1R, IFNT, GLUT5, SOD2	mRNA	2-cell	Gutierrez-Adan et al. (2004)
FS, INHA, INHBB	mRNA	2-cell	Patel et al. (2007)
G6PDH	mRNA	2-cell	Lequarre et al. (1997); Gutierrez-Adan et al. (2004)
GPI, HK1	mRNA	2-cell	Lequarre et al. (1997)
ATF1, BSG, CAT, MAPK14, NDUFS1, PRDX1, PRDX6, SFRS12, SYCP3, TEAD1	mRNA	2-cell	Held et al. (2012)
ATM, ATR, CTNNB1, MSH6, MRE11A, PCNA, APC, CENPE, GRB2, TAF2	mRNA	2-cell	Orozco-Lucero et al. (2014)
PI3KCA, ITM2B	mRNA	8-cell	Ripamonte et al. (2012)
CTNNB1	Protein	Morula	Modina et al. (2007)

Table 6-3 (continued) Molecular markers of developmental competence in embryos

PTTG1, MSX1, TNF, EEF1A1, PGK1, AKR1B1, CD9, KRT8, OCLN, COX2, CDX2, ALOX15, BMP15, PLAU, PLAC8	mRNA	Blastocyst	El-Sayed et al. (2006)
CX43	mRNA	Blastocyst	Nemcova et al. (2006)
Aspartic acid, glutamic acid asparagine, histidine, threonine, arginine, alanine, tyrosine, methionine, valine, phenylalanine, isoleucine, leucine, lysine	Amino acid	Blastocyst	Sturmey et al. (2010)
PLAC8, HMGCS1, LDHB, RPS4X, PLAU, NTR	mRNA	Blastocyst	Cote et al. (2011)
PNRC2, CLGN, MDH2, HSPE1, COX7B, ALDH7A1, POMP, ATPIF1, HSPA14, COX5A, CDC2	mRNA	Blastocyst	Gad et al. (2011)
FL405, HSPD1, S100A10, PLAC8, BMP15, KRT8, RGS2	mRNA	Blastocyst	Ghanem et al. (2011)
IGFBP7, HIF1A, TKTL1, PPARG, LDHA, TNFRSF1A, TP53BP2, VIM, JAM2, ADAMTS1	mRNA	Blastocyst	Cagnone et al. (2012)
MT1A, DNMT3A, IGFBP7	mRNA	Blastocyst	Plourde et al. (2012)

Table 6-3 (continued) Molecular markers of developmental competence in embryos

MSMO1, ABCC2, OCT4, PGRMC1, NFE2L2, CYP51A, SFN, HMOX1, PTGS2, PRDX1, HSD17B11, SOD1, IFNT, RARRES1, ANXA1	mRNA	Blastocyst	Gad et al. (2012)
ARRB2, SERPINE1, IGFBP7, TPI1, TKDP1, IFNT, GCSH	mRNA	Blastocyst	Cagnone and Sirard (2013)
APEX, CLDN6, LDLR, HMGCS1	mRNA	Blastocyst	Cagnone and Sirard (2014)
HSD3B1, SREBF2, SLC23A1, MYL7,MAPK8,FADS1, ACTA2, DNAJC15	mRNA	Blastocyst	Gad et al. (2014; personal communication)

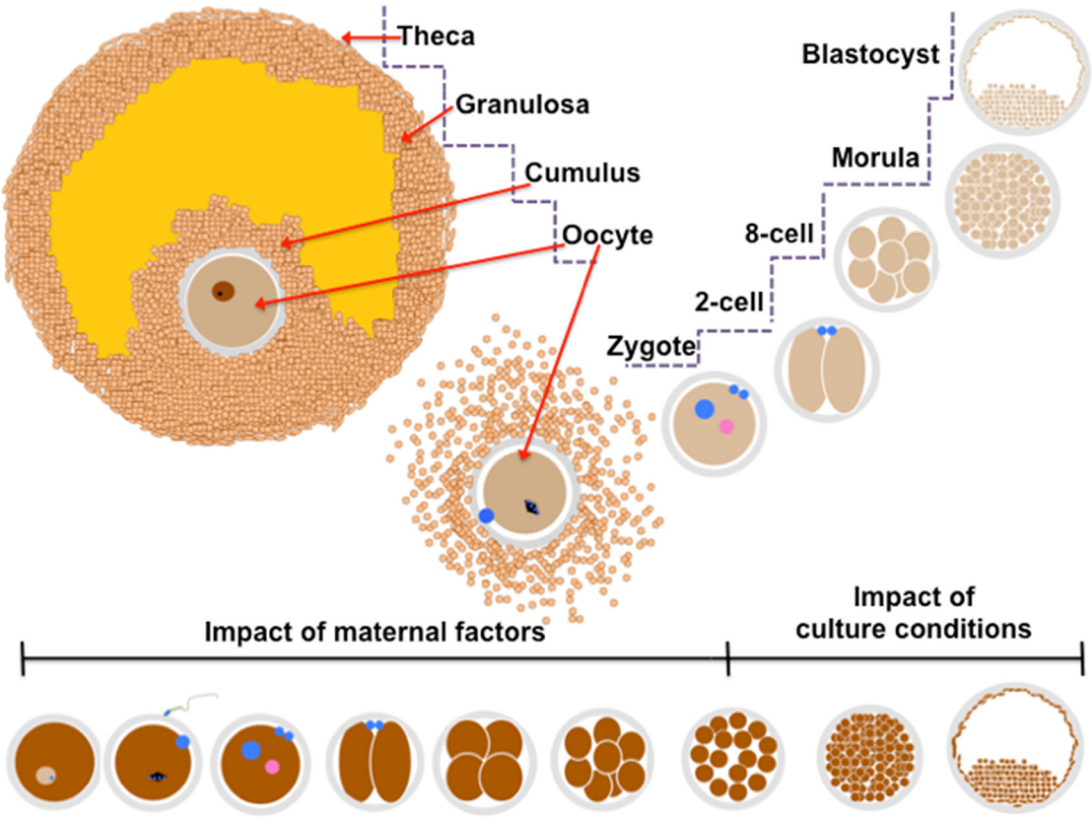

Figure 6-1 Multi-step molecular markers of competence
Distinct developmental stages and compartments where moleculars biomarkers of competence can be tested