UNIVERSITÉ LAVAL

Impact du stress de la culture in vitro sur la survie et le transcriptome embryonnaire chez le bovin

« Entre adaptation et viabilité »

Thèse

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Résumé

Malgré l'amélioration des techniques de procréation médicalement assistée (PMA), les données recensées depuis 40 ans montrent un faible taux de gestation après transfert embryonnaire et une incidence élevée de certains syndromes périnataux. Parmi les causes de l'insuccès de la PMA, les conditions de culture de l'embryon sont sous-optimales pour le développement normal précoce, occasionnant différents stress qui affectent la qualité de l'embryon et sa compétence à produire une gestation. Afin de mieux comprendre l'impact de la PMA sur la qualité embryonnaire, des analyses de micro-puce ont montré des changements dans l'expression de plusieurs centaines de gènes chez les embryons produits par culture in vitro en comparaison à ceux produits in vivo. Cependant, les changements transcriptomiques spécifiquement associés à la baisse de qualité embryonnaire restent encore indéterminés. En hypothèse, nous supposons que l'étude des différences transcriptomiques résultant spécifiquement du stress de la culture permette de déterminer les profiles d'expression génique directement associés à la mauvaise qualité des embryons en culture. Dans ce contexte, nos objectifs consistaient à moduler le niveau de stress en culture afin d'affecter la survie embryonnaire, puis de comparer les gènes différentiellement exprimés entre embryons contrôles et embryons stressés (analyse par miro-puce et RT-qPCR). Pour ce faire, l'exposition à un stress énergétique, oxydatif ou lipidique a été utilisée séparément pour départager les différents effets de la culture sur le développement de l'embryon bovin. Les résultats de ce projet ont mis en évidence l'impact progressif du stress énergétique en culture sur le métabolisme de l'effet Warburg, un processus développemental permettant une adaptation pathologique aux dysfonctions mitochondriales. Par la suite, l'impact du stress oxydatif a révélé des réactions inflammatoires et fibrotiques en association à la baisse de qualité embryonnaire. Enfin, l'impact du sérum et des lipides s'est traduit par un profil indiquant des perturbations inflammatoires et métaboliques, complétant notre étude des mécanismes impliqués dans la réponse au stress de la culture. En conclusion, ce projet a permis de caractériser des bio-marqueurs récurrents du stress embryonnaire chez le bovin, ouvrant à des perspectives du diagnostique de la viabilité embryonnaire et du développement d'alternatives pour mieux cultiver les embryons précoces.

Abstract

For 40 years, assisted reproductive technologies have given life to millions of offspring (human and others mammals), however numerous studies have reported lower gestational survival after embryo transfer and higher risk of perinatal syndromes. One reason for ART disappointment is the lower quality of produced embryos as a result of suboptimal condition of in vitro culture (IVC). In vitro environment induces stresses that affect viability and then gestational competence. To better understand the impact of ART on embryo quality in the bovine, transcriptomic analyses have detected differential expression in hundreds of genes in IVC embryos compared to theirs in vivo counterparts. However, how the differentially expressed genes translate into developmentally compromised embryos is unresolved. Here, we hypothesized that analyzing the gene expression specifically associated to increased stress conditions of in vitro culture could identify the transcriptomic signature associated with the compromised quality of ART-derived embryos. Therefore, our strategy used microarray technology to characterize transcriptomic markers expressed by bovine blastocysts cultured in conditions which are known to impair embryo development. Separate exposure to high glucose stress, oxidative stress and high lipid stress conditions were used to exaggerate the IVC impact on embryo viability in the bovine model. Results highlighted the progressive impact of energetic stress on the Warburg metabolism, a developmental process that allows pathological adaptation to mitochondrial dysfunction. In addition, the analysis of embryonic response to oxidative stress showed the implication of inflammatory and fibrosis-like reaction to pro-oxidant exposure, and the association with embryonic quality. Finally, our last study showed the impact of serum and lipids on both metabolic and inflammatory response, complementing the identification of the developmental mechanisms underlying the stress response to sub-optimal IVC conditions. To conclude, we have characterized biomarkers of embryonic stress in the bovine, offering perspectives in the diagnostic of embryonic viability and the development of alternatives to ameliorate the culture conditions for early embryos.

Avant-propos

Il est important de définir l'ensemble des auteurs qui ont participé aux recherches présentées dans ce manuscrit. Isabelle Dufort et Isabelle Laflamme ont joué un rôle prépondérant dans la réalisation technique des différentes expériences. Christian Vigneault nous a fourni les bases des milieux de culture ainsi qu'un appui dans l'écriture de l'article présenté en chapitre 2. Jeremy Thompson et Mélanie McDowall ont été impliqués dans les expériences menées sur l'embryon au stade morula et présentées dans le chapitre 3. Rachel Gervais et Michelline Gingras ont été décisives dans la validation des concentrations lipidiques présentée dans le chapitre 5. Mon directeur de thèse Marc-André Sirard a supervisé la direction des travaux et façonné l'écriture des différentes publications. Moi-même, Gael Cagnone, a effectué la majeure partie des expériences et l'analyse des résultats, et également rédiger le présent manuscrit. Les chapitres 2 et 4 ont été publiés, le chapitre 5 est soumis pour publication tandis que le chapitre 3 est en cours de soumission pour publication.

Remerciements

Je remercie mon directeur Marc-André Sirard pour son soutien et son dynamisme. Il m'a fait confiance et je lui en suis reconnaissant. Je remercie également Claude Robert pour son aide dans l'analyse scientifique et l'interprétation du vivant. Un grand merci à Isabelle Laflamme et Isabelle Dufort pour leurs efforts et leur aide avec les techniques de « FIV » et de « biomol », mais aussi pour leur compréhension envers mes défauts de « gars ». En ce qui concerne les collaborations, je remercie Mélanie McDowall et Jeremy Thompson de leur accueil très chaleureux à Adélaide, Christian Vigeault de chez Boviteq pour ses bonnes idées et son humour, et Sara Scantland pour sa complicité. Merci à tous les membres de l'équipe du laboratoire de l'INAF et du Comtois, et pour en nommer quelques-uns : Rémi le Beauceron, Nico Papito, la french team Anne-Laure, Audrey, Maella et Nicolas; Ernesto le mexicain et Luis le péruvien, Eric le papa Geek (V), Elise Mondou, Gab et Angus mes colocs, Isabelle Gilbert et ses 2 kids. Je tiens également à remercier les filles de nutrition (dont celle que j'aime : Gege), mon partenaire de tennis et ami Benoit Duinat, toute ma famille (du Québec et de France) dont Hugo le frérot, Marie la maman et Alain le papa.

A mes proches et à Ge

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%: pourcentage
25-OHC: 25-hydroxycholesterol
AAPH: 2,2'-azobis (2-amidinopropane) dihydrochloride
ACTA1: actin, alpha 1, skeletal muscle
ACTA2: actin, alpha 2, smooth muscle, aorta
ACTB: actin, beta
ACTC1: actin, alpha, cardiac muscle 1
ACTG2: actin, gamma 2, smooth muscle, enteric
ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif, 1
ADN: acide desoxyribo-nucléique
ADP: adenosine diphosphate
AG : acide gras
AGE: advanced glycation end-products
AHR: aryl hydrocarbon receptor
AKT: v-akt murine thymoma viral oncogene homolog
ALDOA: aldolase A, fructose-bisphosphate
AMACR : alpha-methylacyl-CoA racemase
AMP: adenosine monophosphate
AMPc: adenosine monophosphate cyclique
AMPK: kinase dépendante de l'AMPc
ANXA2: Annexin 2
APEX: APEX nuclease (multifunctional DNA repair enzyme) 1
ARN (ou RNA): acide ribo-nucléique
ARNm: ARN messager
ARRB1: arrestin, beta 1
ARRB2: arrestin, beta 2
ARRDC2: arrestin domain containing 2
ART: assisted reproductive technology
ATP: adenosine triphosphate
ATP7B: ATPase, Cu++ transporting, beta polypeptide
B2M: beta-2-microglobulin
BMP: bone morphogenic protein
BOLA: MHC class I antigen clone 2
BSA: bovine serum albumin
BSO: buthionine sulfoximine
C1QTNF3: C1q and tumor necrosis factor related protein 3
C6H4ORF31: chromosome 4 open reading frame 31 ortholog
CA2: carbonic anhydrase II
CARD10: caspase recruitment domain family, member 10
CASP3: Caspase 3

CASP4: Caspase 4
CDX2: caudal type homeobox 2
CHUK: conserved helix-loop-helix ubiquitous kinase
CLDN6: claudin 6
CLU: clusterin
CNRIP1: cannabinoid receptor interacting protein 1
CO2: dioxyde de carbone
COC: cumulus oocyte complex
CPT1: carnitine palmoyl transférase
CRAT : carnitine O-acetyltransferase
CSRP3 : cysteine and glycine-rich protein 3 (cardiac LIM protein)
CTHRC1: collagen triple helix repeat containing 1
CTL: control
CXCR2: chemokine (C-X-C motif) receptor 2
CXCR4: chemokine (C-X-C motif) receptor 4
CYR61: cysteine-rich. angiogenic inducer. 61
DCBLD2: discoidin, CUB and LCCL domain containing 2
DEG: differentially expressed gene
DNMT3: DNA (cytosine-5-)-methyltransferase 3
ECM: extracellular matrix
EDN3 endothelin 3
EDTA: acide éthylène diamine tétraacétique
EGA: embryonic genome activation
EGF: epidermal growth factor
EIF2: eukaryotic translation initiation factor 2
EPB41L3: erythrocyte membrane protein band 4,1-like 3
ERK1/2: mitogen-activated protein kinase 3/1
FAM136A: family with sequence similarity 136, member A
FBXO25: F-box protein 25
FC: fold-change
FGF: fibroblast growth factor
FIV (ou IVF): fécondation in vitro
FSH: follicle-stimulating hormone
g: gramme
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GATA6: GATA binding protein 6
GCSH: glycine cleavage system protein H (aminomethyl carrier)
GEM: GTP binding protein overexpressed in skeletal muscle
GEO: gene expression omnibus
GFP: green-fluorescent protein
GJA1: gap junction protein, alpha 1, 43kDa
Glut: glucose transporter facilitator (SLC2A)
GM-CSF: granulocyte macrophage colony growth factor
GPCR: G protein coupled receptor

GPR77 : G protein-coupled receptor 77
GPX: glutathione peroxidase
GPX8: glutathione peroxidase 8 (putative)
GSH: glutathione
GSK-3: glycogene synthase kinase 3
GSSH: oxidized glutathione
H2O2: hydrogen peroxide
HDL: high density lipoprotein
HEY2: hairy/enhancer-of-split related with YRPW motif 2
HG: high glucose
HIF: hypoxia inducible factor
HIF1A: hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HIST1H1C: histone cluster 1, H1c
HKG: house keeping gene
HLA: human leukocyte antigen
HMGB1: high-mobility group box 1
HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A
HMGCS1: 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)
HNF4A: hepatocyte nuclear factor 4 alpha
HO°: anion superoxide
HSA: human serum albumin
HTRA1: HtrA serine peptidase 1
ICM: inner cell mass
ID3: inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
IFIH1: Interferon induced with helicase C domain 1
IFITM2: interferon induced transmembrane protein 2
IFITM3: interferon induced transmembrane protein 3
IFN: interferon
IFNA2: interferon, alpha 2
IFNGR2: interferon gamma receptor 2 (interferon gamma transducer 1)
IFNT: interferon, tau
IGF: insulin growth factor
IGF2: insulin-like growth factor 2 (somatomedin A)
IGFBP: insulin growth factor binding protein
IGFBP7: insulin-like growth factor binding protein 7
IL5: interleukin 5 (colony-stimulating factor, eosinophil)
ING5: inhibitor of growth family, member 5
INSR: insulin receptor
IPA: ingenuity pathway analysis
IRX5: iroquois homeobox 5
IVC: in vitro culture
IVF: in vitro fertilization
IVM: in vitro maturation

IVP: in vitro production
JAM2: junctional adhesion molecule 2
JNK: c-Jun N-terminal kinases
KCNIP4: Kv channel interacting protein 4
KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
L: litre
LDHA : lactate dehydrogenase A
LDL: low-density lipoprotein
LDLR: low-density lipoprotein receptor
LH: hormone lutéinisante
LOC100125763 : neuronal protein 3.1
LOC100295130: similar to lysosomal-associated protein transmembrane 4B
LOC100295797: hypothetical protein LOC100295797
LOC100298356: similar to bone marrow stromal cell antigen 2
LOC100298789: similar to protein phosphatase 2, regulatory subunit B, alpha
LOC100336625: hypothetical protein LOC100336625
LOC100336840: hypothetical protein LOC100336840
LOC100336997: hypothetical protein LOC100336997
LOC100337018: hypothetical protein LOC100337018
LOC100337420: hypothetical LOC100337420
LOC100337434: hypothetical LOC100337434
LOC513329: similar to Equ c1
LOC518785: similar to ATPase family AAA domain-containing protein 2B
LOC782971: similar to histone cluster 1, H2bd
LOC782977: similar to pol protein
LOS: large offspring syndrom
LPS: lipopolysaccharide
LUM: lumican
M: mole
MANSC1: MANSC domain containing 1
MAPK: mitogen-activated protein kinase
MET: maternal-embryonic transition
MKL1: megakaryoblastic leukemia (translocation) 1
MLLT11: myeloid/lymphoid or mixed-lineage leukemia; translocated to, 11
MMD: monocyte to macrophage differentiation-associated
MPF: mitotic promoting factor
MRO: maestro
MSMO1: methylsterol monooxygenase 1
MSRB3: methionine sulfoxide reductase B3
MTOR: mammalian target of rapamycin
MYL6: myosin, light chain 6, alkali, smooth muscle and non-muscle
MYL7: myosin, light chain 7, regulatory
NADH : nicotinamide adénie dinucléotide
NADPH: Nicotinamide adenine dinucleotide phosphate

NDP: Norrie disease (pseudoglioma)
NDUFS2: NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase)
NFkB: nuclear factor of kappa light polypeptide gene enhancer in B-cells
NO: oxyde nitrique
NRF2: nuclear factor (erythroid-derived 2)-like 2
NRIP1: Nuclear receptor-interacting protein 1
NULL: Novel Transcribed Region; evidence: embryonic ESTs
O2: dioxygène
O2°-: radical superoxide
OAS1: 2'.5'-oligoadenylate synthetase 1. 40/46kDa
OLR1: oxidized low density lipoprotein (lectin-like) receptor 1
Osm: osmole
OX-PHOS: oxidative phosphorylation
P38MAPK: p38 mitogen-activated protein kinases
P4: progesterone
p53: protein 53
p66shc: p66 Src homology 2 domain containing) transforming protein 1
PAF: Platelet Activating Factor
PBS: phosphate buffered saline
PDGFC: platelet derived growth factor C
PFK: phospho-fructokinase
PGC1A: peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A)
PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC: protein kinase C
PKR: protein kinase R
PLAGL1: pleiomorphic adenoma gene-like 1
PLAT: plasminogen activator, tissue
PLOD2: procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
PMA: procréation médicalement assistée
POU5F1: POU class 5 homeobox 1
PPAR: Peroxisome proliferator-activated receptor
PPIA: peptidylprolyl isomerase A (cyclophilin A)
PPP: pentose phosphate pathway
PRDX: peroxyredoxine
PRM1: protamine 1
PTPLAD1: protein tyrosine phosphatase-like A domain containing 1
PTPRU: protein tyrosine phosphatase. receptor type. U
PTX3: pentraxin 3, long
PUFA: poly unsaturated fatty acid
RARRES1: retinoic acid receptor responder (tazarotene induced) 1
RDX: radixin
RHOC: ras homolog family member C
RNF20: ring finger protein 20, E3 ubiquitin protein ligase

RO-: alkoxyl radical
ROO-: peroxyl radical
ROS: reactive oxygen specie
rRNA: ribosomal RNA
RT-qPCR: reverse transcripase-quantitative polymerase chain reaction
RXR: retinoid X receptor
S100A11: S100 calcium binding protein A11
SCG2: secretogranine 2
SDF1: CXCL12 chemokine (C-X-C motif) ligand 12
SDHC: succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa
SELF: serum-extracted lipid fraction
SER: serum
SER-D: serum delipidisé
SERPINA5: serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5
SERPINE1: serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
SER-T: serum total
SET: single embryo transfer
SF3B1: splicing factor 3b, subunit 1, 155kDa
SGLT: Na+-coupled glucose transporters
SH3BP5: SH3-domain binding protein 5 (BTK-associated)
SHMT1: serine hydroxymethyltransferase 1 (soluble)
SLC16A7: solute carrier family 16, member 7 (monocarboxylic acid transporter 2)
SNCA: synuclein, alpha (non A4 component of amyloid precursor)
SNX16: sorting nexin 16
SOD: superoxide dismutase, soluble
SOF: synthetic oviductal fluid
SOX2: SRY (sex determining region Y)-box 2
SP1: Sp1 transcription factor
SPP1: secreted phosphoprotein 1
SREBP: Sterol reponse element binding protein
STAR: the steroidogenic acute regulatory protein
TCA: tricarboxylic acid
TE: trophectoderme
TFPI: tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)
TFPI: tissue factor protein inhibitor
TG: triglyceride
TGF: transforming growth factor
TGFB: transforming growth factor, beta
TGFB3: transforming growth factor, beta 3
THBS1: thrombospondin 1
TKDP1: Trophoblast Kunitz domain protein 1
TL STOCK: Tyrode’s Lactate solution
TLH: HEPES-buffered Tyrode's Lactate solution
TNF: tumor necrosis factor

TNFAIP6: tumor necrosis factor, alpha-induced protein 6
TNFAIP8L3: tumor necrosis factor, alpha-induced protein 8-like 3
TNFRSF1A: tumor necrosis factor receptor superfamily, member 1A
TNFSF9: tumor necrosis factor (ligand) superfamily, member 9
TNN: tenascin N
TOPBP1: topoisomerase (DNA) II binding protein 1
TP53: tumor protein p53
TP53BP2: tumor protein p53 binding protein, 2
TPI1: triosephosphate isomerase 1
TSC22D1: TSC22 domain family. member 1
TSP1: THBS1
UPK1B: uroplakin 1B
UTR: untranslated region
VEGF: vascular endothelium growth factor
VIVO: group of in vivo produced bovine blastocysts
XCL2: chemokine (C motif) ligand 2
XIRP1: xin actin-binding repeat containing 1
YWHAB: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide
ZFAND5: zinc finger, AN1-type domain 5
ZNF385B: zinc finger protein 385B

Chapitre 1: Introduction

Ce chapitre d'introduction traite en premier lieu des mécanismes impliqués dans la formation de l'embryon précoce, décrivant les aspects cellulaires (fécondation, prolifération et différenciation) et moléculaires (expression du génome embryonnaire et métabolisme) du développement de l'embryon précédent l'ancrage à l'utérus. En second lieu est introduit l'émergence des technologies de procréation médicalement assistée et les étapes qui ont permis la culture d'embryon in vitro. Par la suite, plusieurs évidences sont rapportées quand à l'impact de la culture sur le développement et la qualité de l'embryon. Cela se termine par l'énoncé des hypothèses et objectifs de mon projet de recherche visant à identifier des marqueurs transcriptomiques associés au stress embryonnaire pendant la culture « in vitro ».

1.1 L'embryon précoce

1.1.1 Production des gamètes et fécondation

Chez les mammifères, l'embryon a pour origine l'union du gamète male avec le gamète femelle. Dans les gonades, les spermatogonies et ovogonies sont les cellules souches de la spermatogénèse et de l'ovogénèse respectivement. Dans l'ovaire fœtal, les ovogonies se multiplient et entrent en méiose afin de générer les ovocytes. La maturation méiotique de l'ovocyte a lieu dans le follicule qui comprend une couronne radiaire de cellules somatiques aplaties, elles-mêmes entourées d'une membrane basale, et formant un microenvironnement isolé du stroma ovarien. Les cellules folliculaires vont supporter l'ovocyte dans sa maturation méiotique, se traduisant par des changements cytoplasmiques et moléculaires préparant à la fécondation et au développement embryonnaire précoce (Brunet and Verlhac; Pepling 2006; Sirard et al. 2006). De façon cyclique, l'ovaire pubère expulsera le ou les ovules provenant des follicules dominants. La décharge ovulante induit l'ouverture du follicule et l'expulsion de l'ovule dans l'oviducte afin d'y rencontrer les spermatozoïdes (Evans 2002).

Sous contrôle des hormones sexuelles, les tubes séminifères du testicule sont le lieu de la spermatogénèse. Tout au cours de la vie, les cellules de Sertoli vont aider les spermatogonies à proliférer puis à se différencier en spermatozoïdes qui seront ensuite éjaculés après leur

maturation dans l'épididyme (Zaneveld et al. 1991). Une fois dans le tractus génital femelle, les spermatozoïdes subiront la capacitation et la réaction acrosomiale nécessaire à la fusion avec l'ovule (Kupker et al. 1998). De façon très régulée, l'activation de l'ovule par l'entrée d'un seul spermatozoïde va induire des mouvements de calcium et la complétion méiotique (Homa et al. 1993; Williams 2002). L'arrangement du pro-noyau male (1N fourni par le spermatozoïde) et femelle (1N fourni par l'ovule) conduit à la formation d'une cellule diploïde (2N) : le zygote (Evans and Florman 2002; Kaji and Kudo 2004; Kupker et al. 1998; Stitzel and Seydoux 2007).

1.1.2 La formation de l'embryon précoce

La cellule zygotique entame tout d'abord des divisions mitotiques appelées clivages ou segmentation (Zernicka-Goetz 2005) et les cellules formées sont appelées blastomères. Chez la souris, l'orientation du clivage zygotique serait dépendante d'une polarité définie par la localisation du globule polaire (pole animal) mais également de la position de l'entrée du spermatozoïde (Zernicka-Goetz 2005). Les divisions successives des blastomères n'augmentent pas la taille de l'embryon car chaque cellule fille occupe la moitié du volume de la cellule mère. Les blastomères clivent de façon plus ou moins synchrone grâce au maintien de communications cellulaires (ponts) et à l'échange des facteurs mitotiques. Au cours des premiers clivages, le cycle cellulaire est rapide car les cellules enchainent des phases S et M sans passer par de réelles phases G1. En conséquence, les stades 2 cellules et 4 cellules, consécutifs aux premier et deuxième clivages respectivement, sont brefs chez le bovin (Lequarre et al. 2003). On observe un ralentissement du cycle cellulaire au niveau du 3ème clivage (formation de l'embryon 8 cellules), composé des phases S, G2 et M. Enfin, le cycle cellulaire du 4ème clivage entre le stade 8 cellules et 16 cellules redevient similaire au cycle cellulaire de cellules somatiques, comportant les 4 phases G1, S, G2 et M. Le 4ème cycle montre une phase de latence de longue durée correspondant à l'activation du génome embryonnaire (voir paragraphe sur l'expression du génome) et qui dure environ 48h chez le bovin.

Après le stade 8-16 cellules, l'embryon change de morphologie. L'embryon se compacte et forme alors une sphère appelée morula contenant entre 32 et 64 cellules. La compaction est caractérisée par l'augmentation des contacts entre cellules et l'établissement de jonctions

adhérentes en complexes cadhérine/ caténine. Au centre de la morula se forme une cavité appelée blastocœle correspondant à la formation d'un jeune blastocyste et à la séparation de deux lignées cellulaires : les cellules du trophectoderme (TE) et les cellules de la masse interne (ICM). La séparation des lignages de l'ICM et du TE est déterminée par l'établissement d'une asymétrie pendant le développement embryonnaire (Dard et al. 2008; Zernicka-Goetz 2005). Chez la souris, le modèle de polarisation prédit que la destinée du lignage TE ou ICM serait définie au stade 8 cellules, engendrant des divisions asymétriques et des différences dans l'adhésion entre cellules (Duranthon et al. 2008). Cependant, il existe plusieurs théories et sans doute aussi des différences interspécifiques quand au stade de ségrégation des lignages embryonnaires. Notamment, l'embryon de souris se caractérise par l'absence de cellules internes au moment de la compaction, contrairement au bovin.

Les cellules du trophoblaste, situées en périphérie de l'embryon (trophectoderme) délimitent le microenvironnement du blastocœle tandis que les cellules à l'intérieur constituent le bouton embryonnaire. Le jeune blastocyste passe par une étape d'expansion puis se libère de son enveloppe protectrice qu'est la zone pellucide. Ce stade correspond au stade de blastocyste éclos. Les cellules du TE sont reliées entre elle par des complexes de jonctions apicales, desmosomiales et de micro-filaments intracellulaires rigidifiant la barrière trophoblastique (Mohr and Trounson 1981). L'expansion et l'éclosion du blastocyste sont dues à l'entrée progressive d'eau dans le blastocœle via l'activité des pompes Na+ K+ dépendantes de l'ATP. L'entrée d'osmolytes crée un appel d'eau par réaction osmotique dans la cavité et les protéines membranaires aquaporines permettent l'élargissement du blastocœle (Duranthon et al. 2008).

Les blastomères sont considérés totipotents car ils peuvent, à eux seul et de façon isolée des autres blastomères, conduire à la formation d'un organisme entier. Avec la différenciation des lignages après compaction, les cellules de l'ICM vont maintenir une pluripotence pour former l'ensemble des cellules du futur fœtus tandis que les cellules du TE vont se différencier et seront à l'origine des tissus extra-embryonnaires permettant la communication avec l'utérus (Dard et al. 2008).

1.1.3 L'implantation/ attachement

Le développement précoce est suivi par l'implantation/attachement de l'embryon à la paroi utérine. Ce processus est contrôlé par la réceptivité de l'utérus selon une fenêtre temporelle variable en fonction des espèces (Aplin 1997). Chez la femme, cela correspond à une période de 4-5 jours, qui commence environ 7 jours après le pic de LH déclencheur de l'ovulation. Une cascade hormonale est impliquée dans l'installation de la fenêtre de réceptivité utérine. L'embryon pré-implantatoire émet différents signaux informant l'utérus qu'une fécondation a eu lieu. Au contact embryon/utérus, une communication importante via des récepteurs, des molécules adhésives ainsi que des cytokines se met en place et permet de maintenir la gestation (Aplin 1997). Chez le bovin, le blastocyste s'attache progressivement et tardivement à l'épithélium tandis que chez l'homme il y a une invasion rapide du stroma utérin par les trophoblastes. Chez la femme, l'implantation est un processus destructeur pour l'utérus car les cellules trophoblastiques sécrètent des enzymes à activité protéolytique agissant sur la matrice extracellulaire et permettant l'invasion du stroma (Zhang et al. 1994). La destruction de l'endomètre utérin induit une réaction immunitaire du système maternel et plusieurs types cellulaires, dont les cellules « Natural Killer » interviennent dans la réponse inflammatoire et la régulation de l'invasion trophoblastique (Dekel et al. 2011; Granot et al. 2012). Un équilibre entre adhésion et réponse immunitaire est alors nécessairement établie entre l'embryon et la mère afin de créer une interface viable pour les deux partis (van Mourik et al. 2009). L'implantation et le remodelage de la paroi utérine permettront ensuite la placentation et la vascularisation du fœtus en lien avec la circulation maternelle.

1.2 Le programme développemental

Les différentes étapes du développement précoce sont programmées par l'expression du génome et l'interaction de signaux de communication intracellulaires ou extracellulaires entre les différentes composantes embryonnaires.

1.2.1 L'expression du génome

Le génome est le dépositaire de l'information génétique retenue par la sélection naturelle pour sa capacité à produire un organisme le mieux adapté à son environnement (survie et

reproduction). Chez les mammifères, la fécondation permet d'unir deux génomes en un nouveau génome totalement unique et ainsi créer la diversité nécessaire à la survie de l'espèce. Pour permettre l'union des génomes parentaux, les gamètes montrent une augmentation de la transcription et la traduction qui s'arrêtent ensuite progressivement avec la maturation méiotique, le noyau est alors en quiescence. Dans l'ovaire, l'ovocyte mature contient ainsi un stock d'ARNm, de protéines, d'organelles (mitochondries par exemple) et de ressources énergétiques (ATP, lipides) qui permettront de soutenir la fécondation et les premiers clivages embryonnaires (Hamatani et al. 2006; Smith and Alcivar 1993).

Après fécondation, la traduction et/ou la dégradation des ARNs maternels est requise pour le développement précoce et, bien que plusieurs protéines soient présentes pour soutenir le développement, la néosynthèse des cyclines est par exemple essentielle pour le passage des cycles cellulaires (Seidel 1983). Une plus forte proportion d'ARNm est retrouvée dans les blastomères comparée aux cellules somatiques (20% vs 5%) mais les mécanismes de traduction ne sont pas nécessairement dépendant de la quantité de l'ARN (Seydoux 1996). Principalement, la traduction est contrôlée par des ribonucléoprotéines et le statut de poly-adénylation de l'extrémité 3' (UTR) des ARNm (Curtis et al. 1995; Gilbert et al. 2009; Oh et al. 2000). En fonction de motifs présents dans la séquence en 3'UTR, la longueur de la queue poly A est différentiellement allongée et les ARNm sont éventuellement recrutés pour être traduits en protéine (Brevini et al. 2002; Scantland et al. 2011). Pendant les premiers clivages, des vagues de poly-adénylation et de déadénylation des ARNm sont ainsi observées et contrôlent la synchronie de la synthèse protéique en fonction du cycle cellulaire (Oh et al. 2000; Vasudevan et al. 2006).

Avec la mise en place des phases G1 et G2 au stade 8-16 cellules, l'embryon bovin active la transcription de son propre génome (Lequarre et al. 2003). A ce stade, la dégradation des ARN d'origine maternelle, dont la traduction a contribué à l'activation du génome embryonnaire (Vigneault et al. 2004), est complétée et les ARN néo transcrits prennent alors le relais (voir Figure 1). Ce changement prononcé de l'état transcriptionnel de l'embryon correspond à la transition materno-embryonnaire (MET) et permet la poursuite du développement. En effet, l'ajout d'inhibiteur de transcription (alpha amanitine) bloque le développement embryonnaire au stade 8 cellules (Barnes and First 1991; Mondou et al.

2012; Vigneault et al. 2009). La MET est un processus commun à beaucoup d'espèces animales mais le stade où elle a lieu est spécifique à chacune d'entre elles. Par exemple, la MET est observée au stade 2 cellules chez l'embryon de souris, au stade 4 cellules chez l'embryon humain et porcin, et au stade 4000 cellules (12ème cycle cellulaire) chez l'embryon de Xénope (Tadros and Lipshitz 2009). Bien que l'activation majeure de la transcription du génome embryonnaire ait lieu au moment de la MET, des études ont cependant détecté une faible activité transcriptionnelle dans les stades précédant le stade 8-16 cellules chez le bovin (Memili and First 1999; Memili and First 2000; Mondou et al. 2012), délimitant une période d'activation mineure et majeure du génome embryonnaire.

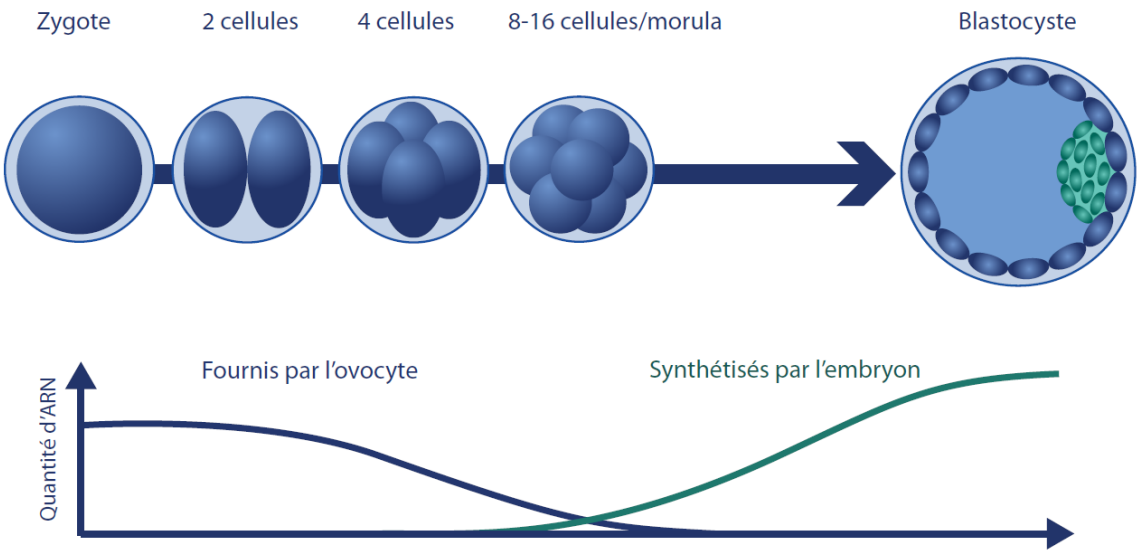

Figure 1 Programme développemental chez l'embryon bovin.

Le graphique exprime la quantité d'ARNm présent à plusieurs étapes précoces du développement embryonnaire bovin (zygote à blastocyste), caractérisant la transition materno-embryonnaire (MET) entre les transcrits du programme maternel (fournis par l'ovocyte) et ceux du programme embryonnaire (synthétisés par le génome embryonnaire).

Après la MET, l'activation du génome embryonnaire conduit à l'augmentation de la quantité d'ARNm soutenant la prolifération et la compaction des cellules de la morula. Le contrôle nucléaire de la transcription est similaire aux cellules somatiques et les fonctions biologiques sont multiples (facteurs de croissances, enzymes métaboliques, jonctions

adhérentes, etc.) (Galan et al. 2010; Hamatani et al. 2006). Avec la formation du blastocyste, différents profils transcriptomiques caractérisent les cellules embryonnaires qui se différencient en cellules du trophectoderme ou maintiennent leur pluripotence au niveau de la masse interne. La transcription de certains gènes spécifiques devient exclusive aux lignages cellulaires du TE (Cdx2) ou de l'ICM (POU5F1, Nanog, SOX2) et influence la destinée des cellules filles (Duranthon et al. 2008). Cette restriction des profils transcriptomiques conduit à la divergence des fonctions cellulaires (Adjaye et al. 2005). Les cellules du TE vont exprimer un profil de gènes impliqués dans l'implantation (Aghajanova et al. 2012) et la signalisation fœto-maternelle (Chakrabarty and Roberts 2007). Les cellules de la masse cellulaire interne vont exprimer un profil de signalisation dépendant du FGF conduisant à la ségrégation de deux types de cellules: l'épiblaste pluripotent (Nanog) et l'endoderme primitif (Gata6) (Kuijk et al. 2011). Le blastocyste prend de l'expansion et éclos afin de permettre les contacts cellules-cellules avec l'utérus. La croissance du blastocyste coïncide avec l'expression de facteurs impliqués dans l'adhésion cellulaire, la digestion de la matrice extracellulaire et l'attachement (Rekik et al. 2011). Dans ce contexte, les différents signaux embryonnaires d'implantation/attachement à l'endomètre utérin montrent des différences interspécifiques en fonction du type de placentation (Aplin 1997; Robertson et al. 2011).

Le contrôle de l'expression du génome est associé à d'importants changements épigénétiques pendant le développement embryonnaire. Cela concerne le remodelage de la chromatine ainsi que l'inscription de marques (groupements methyl entre autres) sur les histones ou les bases ADN (Albert and Peters 2009; Zaitseva et al. 2007). Tout d'abord, le cytoplasme de l'ovocyte contrôle la reprogrammation des génomes parentaux après la fécondation et les marques épigénétiques sont effacées au cours des premiers clivages du zygote. Ensuite, l'épigénome est réinscrit afin de contrôler l'état de pluripotence transitoire de l'embryon. Cependant, certains gènes gardent une empreinte parentale (paternelle ou maternelle) qui est maintenue au moment du remodelage épigénétique et reprogrammée dans la lignée germinale en fonction du sexe de l'individu (Duranthon et al. 2008). L'établissement de divergence dans le statuts épigénétique au moment de la différentiation des lignages cellulaires pendant le développement permettra de générer à partir d'un génome commun, l'ensemble des différents types cellulaires de l'organisme (Hemberger et al. 2009).

1.2.2 La signalisation intra et extracellulaire

Les fonctions biologiques exprimées par le génome sont contrôlées par différentes voies de signalisation intra et extracellulaires qui permettent une régulation fine et rapide du développement de l'embryon. En ce qui concerne la signalisation extracellulaire, plusieurs facteurs de croissance provenant de l'environnement maternel (paracrine) ou des cellules embryonnaires (autocrine) régulent l'activité et l'expression du génome afin d'influencer le développement ou la mort de l'embryon (Hardy and Spanos 2002). Par exemple, la famille des Insuline-like Growth Factors (IGF) est présente dans le milieu maternel ainsi que les IGF binding proteins (IGFBP), permettant de contrôler l'activité métabolique de l'embryon. De même, l'oviducte sécrète plusieurs facteurs de croissance tels qu'EGF, GM-CSF, TGF, VEGF, PDGF etc. Les concentrations en facteurs de croissance augmentent au moment de la phase lutéale et l'embryon exprime une batterie de récepteurs pendant son développement préimplantatoire (Artus et al. 2010; Richter 2008). D'autre part, certaines cytokines, ayant généralement un rôle immunitaire telles que TNF et IFN, ont un effet néfaste sur la croissance de l'embryon, suggérant une régulation entre maintien et perte de la survie en fonction du signal inflammatoire (Robertson et al. 2011).

En réponse aux facteurs de croissance, plusieurs cascades interviennent pour permettre la transduction du signal extracellulaire. Parmi elles, la famille des MAPK comprend différentes voies de signalisation (ERK, p38, JNK) exprimées pendant le développement embryonnaire (Zhang et al. 2007) et qui contrôlent de nombreux processus tels que la prolifération, la différenciation ou bien la mort cellulaire (Watson et al. 2004). La voie des MAPK est généralement utilisée pour signaler des changements physiologiques et préparer une réponse appropriée à la poursuite du développement. Par exemple, la voie P38 MAPK (4 isoformes) régule la formation des filaments d'actine (Paliga et al. 2005) et l'inhibition de P38 MAPK aurait un impact crucial au stade 8-16 cellules dans la redistribution de l'alpha-caténine et la perte des filaments du cytosquelette embryonnaire. A ce stade, la perte des jonctions adhérentes dépendantes du réseau d'actine serait alors dommageable pour la compaction de l'embryon. Chez la souris, la voie ERK ne serait pas impliquée dans le contrôle du réseau d'actine alors que chez le bovin, le blocage du développement par inactivation de p38 requiert également l'inactivation de la kinase ERK (Madan et al. 2005).

La glycogène synthase kinase 3 (GSK-3) est également un facteur de signalisation important pour le développement embryonnaire car elle participe à la transduction du signal Wnt/beta-caténine impliqué dans l'implantation du blastocyste (Aparicio et al. 2010; Xie et al. 2008). GSK-3 intervient également dans plusieurs voies telles que PI3K/AKT, MAPK, PKC ou AMPK (kinase dépendante de l'AMPc) afin de réguler l'effet de l'insuline et d'autres facteurs de croissance. Exprimée dès le stade zygotique, la voie de signalisation PI3K/Akt est vitale pour l'embryon car elle régule plusieurs processus tel que la prolifération cellulaire ainsi que le métabolisme du glucose (Riley and Moley 2006; Zhang et al. 2007). De même, la voie de signalisation Jak/stat serait fonctionnelle pendant le développement précoce pour contrôler le cycle cellulaire en réponse au signal IFN (Truchet et al. 2004).

La voie des BMP, famille des TGF-β, serait impliquée dans la polarisation de l'embryon via l'expression différentielle du récepteur (Zhang et al. 2007). Également, la voie de signalisation Notch contrôle la destinée cellulaire et plusieurs facteurs de la voie Notch sont présents pendant le développement précoce, notamment dans les cellules de l'ICM et du trophectoderme (Cormier et al. 2004). Bien que plusieurs voies de signalisation soient mises en place par l'embryon au cours de son développement, il existe de nombreuses interactions entre chaque voie afin de coordonner l'ensemble des signaux vers une activité cellulaire appropriée.

1.2.3 Les fonctions métaboliques

L'activité appropriée des cellules embryonnaires repose sur l'établissement d'un métabolisme dynamique pendant le développement. Lors des premiers clivages, les voies métaboliques actives seraient celles utilisées par l'ovocyte (Biggers et al. 1967). Par la suite, un remaniement du métabolisme au moment de la MET permettrai de soutenir la prolifération cellulaire et la compaction de la morula. Avec la différenciation cellulaire au stade blastocyste, des différences métaboliques apparaissent entre les cellules de l'ICM et celles du TE. Les cellules de l'ICM seraient alors dans un état quiescent (Houghton 2006) tandis que les cellules du TE auraient une activité métabolique plus importante, montrant des extensions cytoplasmiques (micro-vili) abondantes ainsi que des vésicules pycnotiques afin d'absorber les substrats du fluide tubaire (Mohr and Trounson 1981).

Le taux d'activité métabolique reflète la demande biosynthétique et énergétique de l'embryon. Pendant le développement précoce, le taux de biosynthèse augmente notamment en protéines et nucléotides au moment de l'activation du génome embryonnaire (Gilbert et al. 2009). Pendant les clivages de l'embryon bovin, le taux de dégradation protéique dépasse celui de synthèse, tandis que le développement post-compaction coïncide avec une augmentation de la quantité de protéines par embryon notamment via l'incorporation de l'albumine, principale protéine du fluide tubaire (Thompson et al. 1998). La production de nucléotides est importante pendant les premiers clivages et l'activation de la voie des pentoses phosphates permet la dégradation du glucose en pentose, base ribose des purines et pyrimidines (Alexiou and Leese 1992). Également, le glucose et la voie des hexosamines participeraient à la synthèse des glycoprotéines pendant le développement précoce de l'embryon de souris (Wales and Hunter 1990).

Afin de générer l'ensemble des constituants cellulaires, le métabolisme embryonnaire utilise de l'ATP. L'analyse du taux d'ATP chez la souris montre une diminution graduelle du stade 1 cellule jusqu'au stade blastocyste, probablement due à son utilisation par les processus biosynthétiques (Quinn and Wales 1971). Chez le bovin, une plus forte activité luciférase ATP dépendante est ainsi observée dans le blastocyste par rapport à l'ovocyte (Rieger 1997). Chez l'humain, le contenu en ATP est constant de la fécondation jusqu'au stade 4 cellules, augmente au stade 6-8 cellules puis diminue avec la compaction et la blastulation (Slotte et al. 1990). Ces variations découlent de la faible demande énergétique pendant les premiers clivages, nécessitant peu de production d'ATP (fort ratio ATP/ADP). Avec le développement des stades post-compaction, la consommation d'ATP est importante pour la blastulation (diminution du ratio ATP/ADP) et la production d'ATP augmente (Thompson et al. 1996). Par rapport à l'ATP, la quantité d'ADP diminue jusqu'au stade 8 cellules puis augmente au stade morula puis blastocyste. Également, la quantité d'AMP augmente pendant le développement embryonnaire précoce (Spielmann et al. 1984). Au stade blastocyste, les cellules du TE produisent et utilisent la majorité de l'ATP afin de former le blastocœle et générer certains substrats nécessaires à l'ICM (Houghton 2006).

1.3 Les substrats métaboliques

Fournis par l'environnement (exogène) ou stockés dans le cytoplasme (endogène), l'embryon utilise différents substrats métaboliques tels que l'oxygène, les glucides, les lipides et les acides aminés qui constituent la base du support nutritionnel requis pour le développement. Généralement, l'étude de l'utilisation des substrats par le métabolisme embryonnaire repose sur plusieurs méthodes (Barnett and Bavister 1996). Une des ces méthodes consiste à étudier la capacité de l'embryon à se développer en modifiant la disponibilité en substrats tandis que d'autres méthodes ont analysé au niveau biochimique le métabolisme embryonnaire, soit en utilisant des substrats marqués et mesurant leur dégradation par l'embryon, soit en observant les modifications des concentrations des substrats induites par le métabolisme de l'embryon. Également, des études de génomique fonctionnelle ont permis de mieux comprendre comment le métabolisme est régulé au niveau de l'expression des enzymes et facteurs de transcription.

1.3.1 L'oxygène

L'oxygène est la base du métabolisme aérobie, principalement comme accepteur final de la chaine respiratoire. Il est essentiel pour générer de l'ATP ou du pouvoir réducteur lors des réactions de phosphorylation oxydative, de glycolyse et de β-oxydation des lipides. Des stades 1 à 8 cellules, l'embryon bovin utilise relativement peu d'oxygène comparé aux stades 16 cellules, morula et particulièrement blastocyste (Thompson et al. 1996). Un patron d'incorporation d'oxygène similaire est également retrouvé chez la souris (Houghton et al. 1996b; Trimarchi et al. 2000). L'incorporation d'oxygène est corrélée avec l'expansion du blastocyste (Donnay and Leese 1999) pour soutenir la production d'ATP nécessaire aux pompes Na+ K+ ATPase ainsi qu'à la synthèse protéique. Chez le blastocyste de souris, les cellules du trophectoderme montrent une incorporation d'oxygène plus élevée que celles de l'ICM ainsi qu'une plus grande quantité de mitochondries dont la morphologie diffère de celles retrouvées dans l'ICM (Houghton 2006).

Étant directement utilisable et ne nécessitant aucun transport pour faciliter son incorporation, la quantité d'oxygène métabolisée par l'embryon est en partie dépendante de sa disponibilité dans le milieu environnant. Généralement, la tension en oxygène dans le

tractus génital des mammifères est 2 à 4 fois inférieure à celle de l'atmosphère et diminue de l'oviducte à l'utérus (Fischer and Bavister 1993). Cependant, cette dernière étude a montré une augmentation de la tension en O_2 avec l'ovulation dans l'oviducte du macaque, ainsi qu'au jour 3 après copulation chez le hamster (stade morula). Tandis que l'augmentation du taux d'O_2 est maintenue dans l'oviducte du primate pendant la phase lutéale, elle retombe brusquement au jour 4 chez le rongeur. La réduction de l'O_2 dans l'environnement de l'embryon serait contrôlée par le système maternel afin de limiter l'activité du métabolisme oxydatif de l'embryon. Ce dernier est également munit de protéines HIF (Hypoxia inducible factors) dont l'activité est contrôlée par le taux d'oxygène afin d'induire l'expression de nombreux gènes du métabolisme énergétique.

L'utilisation de l'oxygène est également régulée par le taux d'activité de la chaine proto-énergétique dans les mitochondries. Exclusivement d'origine maternelle, les mitochondries sont dans un état immature/non-développé au stade ovocyte et pendant les premiers clivages. Particulièrement, leur petite taille et la faible présence de crêtes mitochondriales (repliements de la membrane interne) suggèrent une quiescence de l'activité proto-énergétique (Van Blerkom 2004). Au cours du développement embryonnaire, le pool de mitochondries se différencierait et deviendrait complètement fonctionnel. En effet, avec le passage de la MET chez le bovin, les mitochondries embryonnaires retrouvent une structure similaire à celle retrouvée dans les cellules somatiques, soit de grande taille et riches en crêtes (Crocco et al. 2011), suggérant une activité respiratoire fonctionnelle.

En rapport avec la structure dynamique des mitochondries, l'équilibre entre la demande énergétique et l'apport de nutriments est un régulateur crucial de l'activité de la chaine respiratoire (Van Blerkom et al. 2006). La dépense énergétique est faible pendant les premiers clivages, coïncidant avec la forme des mitochondries et le faible taux d'oxygène utilisé. En revanche, la dépense énergétique s'accroit avec la prolifération cellulaire et l'expansion de l'embryon ce qui accroit la respiration mitochondriale et le taux d'oxygène utilisé. De plus, les mitochondries peuvent s'associer au réticulum endoplasmique lisse ainsi qu'aux gouttelettes lipidiques (Mohr and Trounson 1981; Sturmey et al. 2009), probablement pour réguler l'échange d'intermédiaires métaboliques nécessaire au cycle de Krebs.

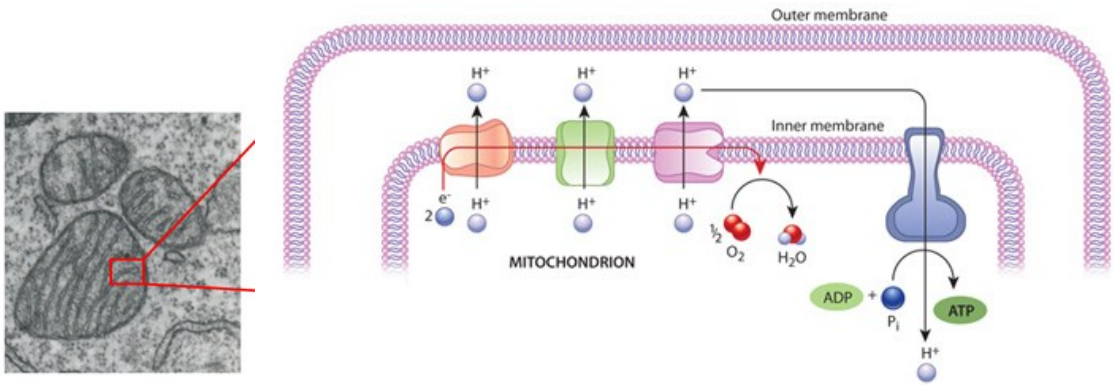

Figure 2 La chaine respiratoire mitochondriale.

Gauche : photographie de 3 mitochondries humaines (section) par microscopie électronique. Notons la présence plus ou moins abondante de crêtes (rayures sombres). Droite : représentation schématique du système de transport d'électrons et d'expulsion de proton H+ formant la chaine proto-énergétique. Source Nature Education © 2010.

1.3.2 Le pyruvate/lactate

Bien que peu consommé par l'embryon bovin en clivage, l'oxydation dans le TCA (cycle de Krebs) du pyruvate exogène ou provenant de la conversion du lactate représente la source majeure d'énergie (90% de l'ATP produit) (Thompson et al. 1996). Chez la souris, lactate et pyruvate sont également les substrats majoritairement utilisés pour les premiers clivages embryonnaires. Dans l'étude de Leese et Barton en 1984, la quantité de pyruvate utilisée pas l'embryon bovin est supérieure à celle du glucose et reste constante de l'ovocyte jusqu'au stade 16 cellules / morula. A partir du stade de 16 cellules chez le bovin, la consommation en pyruvate augmente fortement (Khurana and Niemann 2000) et son utilisation par phosphorylation oxydative génère environ 80% de l'ATP (Thompson et al. 2000; Thompson et al. 1996). Chez l'embryon humain, l'utilisation du pyruvate est croissante pendant le développement préimplantatoire, et ne chute pas au stade blastocyste (Hardy et al. 1989b; Leese et al. 1993). Ce patron métabolique du pyruvate ressemble à celui de l'embryon bovin mais diffère de celui observé chez la souris et le rat, espèces ou le développement du stade blastocyste montre une fortement augmentation de l'utilisation du glucose (Leese and Barton 1984). Ces différences illustrent la demande importante en pyruvate (et potentiellement d'autres intermédiaire du cycle de Krebs) pour soutenir l'expansion au stade blastocyste chez l'homme et le bovin (Waugh and Wales 1993), alors que la souris et le rat

montrent une dépendance métabolique accentuée pour l'utilisation de glucose (Leese and Barton 1984; Leese et al. 1993).

La disponibilité en pyruvate et lactate dans l'environnement de l'embryon contrôle en partie leur utilisation. Dans l'oviducte de bovin, la concentration en lactate et pyruvate est respectivement de 6 mM et de 0,1 mM et tombe à 1 mM pour le lactate au niveau de l'utérus. De façon intéressante, le lactate dans l'oviducte bovin est 8 fois plus concentrée que dans le plasma ou dans l'utérus, indiquant un taux élevé du métabolisme lactique de la part des cellules épithéliales, ce qui favoriserait l'utilisation du lactate par l'embryon en clivages (Hugentobler et al. 2008; Lane and Gardner 2000). D'autre part, l'entrée du pyruvate dans l'embryon est contrôlée par différents transporteurs. Dans l'ovocyte de souris, le pyruvate diffuse dans l'embryon via des transporteurs passifs et actifs (Gardner and Leese 1988; Leese and Barton 1984). L'expression de ces transporteurs pendant le développement va réguler l'utilisation du pyruvate, notamment le transporteur SLC16A7, qui est surexprimé en absence de glucose dans le milieu, afin de compenser le manque énergétique par une entrée plus importante de pyruvate (Jansen et al. 2008).

1.3.3 Les glucides

Les glucides les plus couramment utilisés par la cellule sont les hexoses. Les hexoses sont des monosaccharides formés par une chaine de 6 carbones comprenant soit une fonction cétone (ex : fructose) ou aldéhyde (ex : glucose). L'embryon bovin utilise différentiellement le glucose au cours de son développement. Pendant les premiers clivages chez le bovin, le glucose est faiblement utilisé et sa dégradation se fait majoritairement via la voie des pentoses phosphate PPP (Khurana and Niemann 2000; Thompson 2000). Chez la souris, la PPP a une activité maximale au stade 2 cellules (O'Fallon and Wright 1986) bien que l'utilisation du glucose soit faible au cours du développement jusqu'au stade morula. Le reste du glucose consommé est converti en lactate (Thompson et al. 1996). Avec l'activation du génome, les voies métaboliques sont modifiées et le glucose est fortement utilisé (Rieger et al. 1992; Thompson 2000). La fermentation lactique constitue une voie importante de dégradation du glucose (Javed and Wright 1991) mais le métabolisme du glucose augmente également au niveau de la voie des PPP (Rieger et al. 1992). Post-compaction, la glycolyse génère environ 15 % de l'ATP produit par l'embryon, fournissant un surplus énergétique

nécessaire à la blastulation et à l'éclosion du blastocyste. Au stade blastocyste chez la souris, le glucose devient la source principale d'énergie et sa consommation est élevée (Leese and Barton 1984) : 44 % du glucose consommé est alors converti en lactate (Gardner and Leese 1990).

Chez le porc, l'embryon utilise le glucose de façon similaire au bovin. Le glucose pourrait également servir de précurseur pour le cycle des acides carboxyliques et l'activation de la β-oxydation lipidique (Sturmey and Leese 2003). Chez l'homme, l'activité de déshydrogénation du glucose 6-phosphate vers la PPP est importante au stade embryonnaire 4 cellules et diminue progressivement jusqu'au stade 8-16 cellules puis augmente à nouveau jusqu'au stade blastocyste (Martin et al. 1993). Des différences apparaissent entre les cellules du trophectoderme qui consomment moins de glucose que celles de l'ICM, et ces dernières montrent une consommation de pyruvate et une production de lactate moins élevée (Gopichandran and Leese 2003). En conclusion, l'utilisation du glucose augmente avec le développement embryonnaire et la majeure partie du glucose métabolisé est destinée à l'oxydation via la PPP (Javed and Wright 1991; Rieger and Guay 1988) ou à la fermentation lactique (Khurana and Niemann 2000; Thompson 2000).

Plusieurs mécanismes interviennent afin de réguler l'apport et l'utilisation du glucose par l'embryon : la disponibilité en glucose dans l'oviducte, le transport du glucose dans l'embryon et l'expression/régulation des enzymes glycolytiques en fonction de la demande énergétique. La concentration en glucose dans les fluides de l'oviducte et de l'utérus est de 2,5 et 4 mM respectivement. Cette concentration en glucose est bien inférieure à celle retrouvée dans le sang (environ 5,5 mM) (Hugentobler et al. 2008), ce qui suggère que la diffusion du glucose plasmatique vers les fluides génitaux à travers la barrière épithéliale est limitée. De plus, l'augmentation de la concentration en glucose le long du tractus génital proviendrait de différences dans les propriétés de diffusion entre les cellules épithéliales tubaires et utérines. Cette régulation du taux de glucose par l'oviducte protégerait l'embryon d'une dose élevée de glucose avant la MET. Ces variations suggèrent que la régulation de la disponibilité en glucose est un facteur important dans la régulation du métabolisme de l'embryon (Rieger et al. 1995).

Au niveau des cellules embryonnaires, les transporteurs du glucose sont différentiellement exprimés pendant le développement (Augustin et al. 2001) pour réguler l'entrée de glucose (Gardner and Leese 1988). Les familles des transporteurs du glucose sont classées selon leur mode de fonctionnement : les transporteurs passifs (GLUT) et actifs (SGLT). Les transcrits de *GLUT3*, principal transporteur du glucose chez l'embryon bovin et murin, sont présents au niveau du zygote jusqu'au stade blastocyste, montrant une augmentation de leur expression au stade morula. De façon intéressante, l'augmentation de la transcription du transporteur GLUT3 chez l'embryon de souris serait induite par la présence de glucose et l'activité de la voie de biosynthèse des hexosamines (Pantaleon et al. 2008). L'embryon aurait donc la capacité de ressentir des signaux exogènes et d'initier une différenciation métabolique adaptée aux substrats présents dans son environnement. Pendant l'expansion du blastocyste bovin, les transporteurs GLUT 2 et 4 sont exprimés (Augustin et al. 2001), ce qui corrèle avec l'augmentation des besoins énergétiques. L'expression du transporteur SGLT1 est constante de l'ovocyte jusqu'au blastocyste. Il semblerait que son rôle ne soit pas dédié uniquement au transport du glucose, mais aussi à la balance ionique. SGLT1 étant un co-transporteur du glucose et du Na+, son activité induirait une entrée de sodium, ce qui par osmose contrôlerait le volume d'eau intracellulaire. L'expression forte de SGLT1 au stade ovocyte et blastocyste permettrait de réguler la pression osmotique nécessaire au maintien d'un grand volume intracellulaire (ovocyte) ou extracellulaire (blastocœle).

Au cours des étapes développementales, les changements d'utilisation du glucose par l'embryon sont dus également à la régulation de l'expression des différentes enzymes de la PPP ou de la glycolyse. Les transcrits de la glucose 6 phospho-déshydrogénase, enzyme limitante de la PPP, sont fortement présents pendant les premiers clivages embryonnaires bovins et diminuent après le stade 4 cellules, probablement à cause de la dégradation des ARNs maternels (Lequarre et al. 1997). Chez l'humain, les ARNs codant pour l'hypoxanthine phosphoribosyl transférase, enzyme de la PPP, sont fortement présents dans l'ovocyte et pendant le premier clivage, diminuant rapidement au stade 4 cellules et ce jusqu'au stade blastocyste (Taylor et al. 2001). Chez le singe rhésus, les ARNm codant pour la phosphogluconate déshydrogénase et la transaldolase 1, deux enzymes de la PPP, sont prédominants en tant que facteurs maternels, ce qui suggère une activité non reliée à la

production d'ATP mais à la biosynthèse de nucléotides pendant les premiers clivages de l'embryon (Zheng et al. 2007).

A partir des stades 8-16 cellules, l'activation majeure de la glycolyse serait dépendante d'une néosynthèse des enzymes glycolytiques à partir des ARNs embryonnaires. Le pool de transcrits de l'hexokinase, première enzyme de la glycolyse, diminue avec les premiers clivages embryonnaires bovins (dégradation des ARNs maternels) et augmente fortement au moment de la MET (Lequarre et al. 1997). Chez la souris en revanche, l'abondance des transcrits codant pour l'hexokinase (isoforme 1) reste plus ou moins constante tout au long du développement embryonnaire (Houghton et al. 1996a; Johnson et al. 1997). Bien que le contrôle de la transcription du gène de l'hexokinase puisse réguler l'abondance de cette enzyme au stade blastocyste chez la souris, le taux d'activité de l'hexokinase semble aussi contrôlé de façon allostérique pendant le développement préimplantatoire (Barbehenn et al. 1974). En effet, l'activité héxokinase fluctue, étant basse de l'ovocyte jusqu'au stade morula puis augmentant fortement avec la blastulation (Houghton et al. 1996a). Chez l'homme et de façon similaire au bovin, l'activité héxokinase diminue entre les stades 2 et 8 cellules puis augmente au stade morula, correspondant à l'augmentation de la consommation en glucose (Leese et al. 1993). L'activité hexokinase étant inhibée par son produit, le glucose-6-phosphate, la faible utilisation de glucose-6-phosphate durant les premiers clivages limiterait l'entrée de glucose dans la glycolyse. Avec l'augmentation des besoins métaboliques après la MET, l'utilisation de glucose-6-phosphate lèverait l'inhibition de l'hexokinase, ce qui entrainerait une activation de la glycolyse (Houghton et al. 1996a).

Une autre enzyme glycolytique, la phospho-fructokinase (PFK), est présente tout au long du développement embryonnaire et régule également l'utilisation du glucose (Barbehenn et al. 1974). Chez l'embryon humain, l'activité phosphofructokinase diminue entre les stades embryonnaires de 4 à 8 cellules et augmente avec le stade morula (Martin et al. 1993). La PFK est activée par l'ADP ainsi que le fructose-1,6 -diphosphate et inhibée par l'ATP et le citrate. Pendant les clivages embryonnaire, le fort ratio ATP/ADP et le citrate produit par le cycle de Krebs inhiberaientt l'activité de la PFK et donc l'activité de la glycolyse. Avec l'augmentation de l'utilisation de l'ATP au stade post-compaction, l'inhibition de la PFK serait levée et la glycolyse pleinement fonctionnelle. D'autre part, l'augmentation de

fructose-1,6 -diphosphate due à la forte activité de l'hexokinase et de la PFK2 au stade morula, stimulerait l'activité de la PFK1 (Barbehenn et al. 1974).

Comme le glucose, le fructose est un substrat de la glycolyse. Le fructose entre dans la chaine glycolytique via la fructokinase qui produit le fructose-6-phosphate avec utilisation d'une molécule d'ATP. Le fructose est présent dans le tractus génital et utilisé au stade morula et blastocyste (Guyader-Joly et al. 1996). L'expression de GLUT 5, un transporteur du fructose, a lieu au cours de la MET (Augustin et al. 2001) ce qui suggère un changement métabolique dans l'utilisation du fructose, probablement pour soutenir la forte demande énergétique des stades morula et blastocyste.

Bien qu'une large partie du glucose soit utilisée pour répondre aux demandes biosynthétiques et énergétiques de l'embryon, ce dernier pourrait également stocker le glucose sous forme de glycogène via l'activité de la glycogène synthase pour le réutiliser au moment de forte activité métabolique. Cependant, l'accumulation de glycogène est quasiment nulle chez l'embryon souris (Houghton et al. 1996b) et sa quantité reste stable jusqu'au stade blastocyste, stade où une déplétion en glycogène est observée (Ozias and Stern 1973). De plus, une étude a montré que la capacité d'utiliser les réserves glycogéniques n'apparaîtrait qu'à partir du stade morula chez la souris, via l'activation de la glycogène phosphorylase (Hsieh et al. 1979). Chez le bovin, l'utilisation du glycogène par les cellules embryonnaires serait contrôlé par la famille des glycogene synthase kinase, et notamment les isoformes 3A et 3B, qui deviennent phosphorylées avec le développement du stade blastocyste (Aparicio et al. 2010; Garcia-Herreros et al. 2012). La phosphorylation de ces kinases étant associée à une inactivation enzymatique, il est suggéré que l'activité de synthèse du glycogène soit réduite au moment de la blastulation, en parallèle à l'augmentation de l'utilisation du glucose par la glycolyse.

Comme vu précédemment, le glucose utilisé par la glycolyse génère du pyruvate qui est majoritairement converti en lactate, alors que le pyruvate exogène est oxydé dans la mitochondrie. La destinée du pyruvate produit par la glycolyse serait dirigée en lactate par sa compartimentation ou canalisation subcellulaire l'empêchant d'être conduit dans la mitochondrie. Dans ce scénario, la réaction transformant le phosphoenolpyruvate en

pyruvate (enzyme pyruvate kinase) serait couplée avec la lactate déshydrogénase. De façon plus radicale, la pyruvate kinase pourrait être inhibée pendant le développement de l'embryon pré-attachement, empêchant la métabolisation du glucose vers le TCA (Dworkin and Dworkin-Rastl 1991; Rieger and Guay 1988). La réduction de la tension en O_2 dans l'utérus et l'inhibition/incapacité du transport de NADH cytoplasmique dans la mitochondrie seraient également des facteurs favorisant la dégradation du glucose en lactate afin de générer le NAD+ nécessaire à la glycolyse (Lane and Gardner 2000; Thompson 2000). De plus, cet effet est associé au métabolisme des cellules cancéreuses (effet Warburg) qui favoriserait l'utilisation du glucose de façon anabolique (PPP), permettant de soutenir la demande biosynthétique nécessaire à une prolifération cellulaire et une croissance rapide (Flynn and Hillman 1978; Krisher and Prather 2012; Redel et al. 2011). Le lactate converti à partir du pyruvate glycolytique est alors soit exporté de la cellule soit transporté dans la mitochondrie via l'échangeur Lactate/pyruvate afin de maintenir l'équilibre redox de la chaine proto-énergétique. D'autre part, les métabolites intermédiaires du TCA sont également exportés vers le cytoplasme et contribue à la glycolyse lactique ou la synthèse d'acides gras (figure 3).

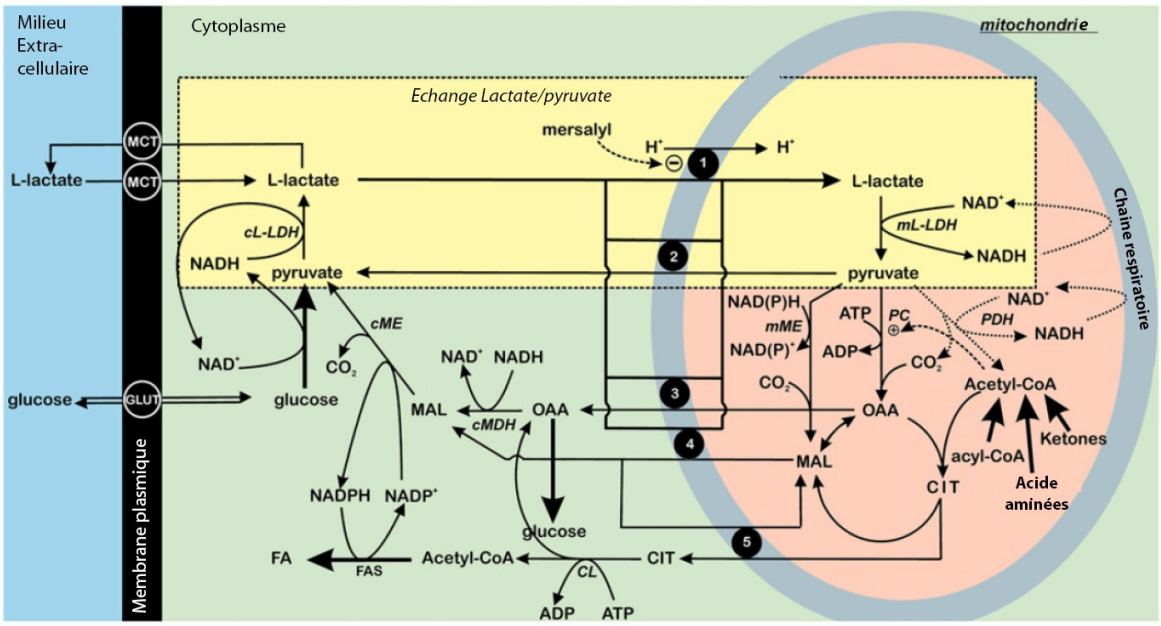

Figure 3 Entrée du glucose et échange lactate/pyruvate dans le métabolisme cellulaire de l'effet Warburg.

Ce schéma représente la destiné des substrats énergétiques et les différentes voies métaboliques cytoplasmiques et mitochondriales. Le pyruvate généré par glycolyse est réduit en lactate qui est exporté de la cellule ou transporté via la navette Lactate/pyruvate dans la mitochondrie (1&2). Cet échange contribue à la réduction du NAD+ en NADH nécessaire à la chaine respiratoire, et au renouvellement du NAD+ cytoplasmique nécessaire à la glycolyse. D'autre part, les intermédiaires du TCA (oxaloacetate OAA, malate MAL, citrate CIT) sont exportés de la mitochondrie (3,4&5) afin de contribuer à la glycolyse lactique ou à la synthèse d'acides gras (FA). Adapté de **(Pizzuto et al. 2012)** *avec permission de Elsevier © 2012.*

1.3.4 Les lipides

Les lipides représentent une source d'acides gras (AG) utilisés comme des composants des membranes cellulaires, des facteurs de signalisation (O'Neill 2008; Wang and Dey 2005) et également comme source énergétique (Kane 1979). Dans l'ovocyte bovin, la répartition de lipides comprend 50% de triglycérides, 20 % de phospholipides, 20 % de cholestérol et 10 % d'acides gras libres (McEvoy et al. 2000). Les acides gras majoritaires sont par ordre décroissant l'acide palmitique (16, n=0), oléique (18, n=1), stéarique (18, n=0), linoléique (18, n=2), vaccénique (18, n=1), myristique (14, n=0), lignocérique (24, n=0), palmitoléique (16, n=1) ; et la majorité des acides gras contenus dans les phospholipides et les triglycérides sont saturés. Les triglycérides (TG) sont une classe de lipides stockés par la cellule, encapsulés dans des vacuoles (gouttelettes lipidiques) dont la fonction majeure est de libérer des AG. Chez le bovin et le porc, la quantité de TG est maximale au stade ovocyte (bovin : 58 ng ; porc : 156 ng), diminue après maturation et reste stable jusqu'au stade blastocyste (Ferguson and Leese 1999; Kikuchi et al. 2002; Sturmey et al. 2009). L'ovocyte de porc contient une quantité plus importante de triglycérides et de phospholipides que le bovin ou l'humain tandis que la quantité de triglycéride par volume embryonnaire est extrêmement basse chez la souris (McEvoy et al. 2000). Les différences dans la proportion de triglycéride entre différentes espèces seraient dues aux périodes de temps plus ou moins longue avant l'implantation/attachement de l'embryon et donc la nécessité d'avoir des réserves d'énergie endogènes pour soutenir le développement (Sturmey et al. 2009).

Le métabolisme des lipides est encore peu caractérisé dans l'embryon précoce en comparaison aux autres substrats tel que le glucose ou le pyruvate. Sturmey et Leese proposent que la β-oxydation des acides gras provenant des TG serait une source

énergétique pendant la maturation (Sturmey and Leese 2003). Les mitochondries sont fortement associées aux vacuoles lipidiques au moment de la maturation de l'ovocyte et pendant le développement embryonnaire (Sturmey et al. 2006). D'autre part, l'inhibition de l'entrée des AG dans la mitochondrie diminue la consommation d'oxygène et le développement chez le bovin, suggérant que les AG contribuent au métabolisme oxydatif de l'embryon (Ferguson and Leese 2006). En ce qui concerne l'oxydation des acides gras intracellulaires, le transcrit de la carnitine palmoyl transférase (CPT1), enzyme qui permet l'entrée des acides gras dans la mitochondrie, n'est pas exprimé avant le stade blastocyste (Dunning et al. 2011b). Cependant, l'ajout de L-carnitine, cofacteur du transport des acides gras via CPT1, améliore le développement de l'embryon murin (Abdelrazik et al. 2009; Dunning et al. 2011a), suggérant que la fonction protéique de CPT1 soit maintenue pendant le développement précoce. De plus, l'inhibition de l'oxydation des acides gras chez l'ovocyte/zygote de souris affecte le développement de l'embryon précoce ainsi que le métabolisme des cellules de l'ICM qui compensent par davantage d'oxydation du glucose (Dunning et al. 2011b; Hewitson et al. 1996). En effet, l'utilisation des acides gras est sensible à la demande énergétique et l'activité de β-oxydation pourrait être contrôlée par l'AMP-Kinase, un régulateur majeur du statut énergétique (Downs et al. 2009; McKeegan and Sturmey 2012).

L'oviducte de mammifère contient peu de lipides mais toutes les classes sont représentées : phospholipides, triglycérides, cholestérol, acides gras libres, etc (Menezo and Guerin 1997). Dans le fluide oviductal, les lipides sont majoritairement associés à l'albumine ou présent dans des lipoprotéines (Fujimoto et al. 2010). L'incorporation des acides gras dans l'embryon pourrait s'effectuer via la diffusion passive ou par des molécules de transport tel que les Fatty Acid Binding Proteins (Duttaroy 2009) mais peu d'information est disponible sur l'embryon précoce. L'embryon de souris exprime des récepteur aux LDL ce qui pourrait permettre l'incorporation de cholestérol (Sato et al. 2003). Également, la liaison des lipides à la BSA représenterait un moyen de transport majeur pour l'incorporation des acides gras dans l'embryon (Hughes et al. 2011; Mackenzie et al. 1970). L'incorporation d'acide oléique et arachidonique a été observée pendant le développement embryonnaire chez le lapin, et notamment dans la synthèse de triglycérides (Waterman and Wall 1988). Certains acides gras exogènes sont également oxydés, tel que l'acide palmitique chez la souris dont le

taux d'oxydation augmente avec le développement du blastocyste (Hillman and Flynn 1980). Chez l'humain, l'embryon incorpore activement les acides gras du milieu avec une préférence pour l'acide linoléique au stade blastocyste tandis que l'utilisation d'acide palmitique devient négligeable (Haggarty et al. 2006). Dans cette étude, l'accumulation d'acides gras insaturés (linoléique et oléique) se trouve d'ailleurs corrélée à une diminution du taux d'acide gras saturés au cours du développement post-compaction.

Un enrichissement en lipide dans la nutrition animale, l'état de balance énergétique négative ainsi que l'obésité maternelle sont des facteurs qui se répercutent sur le profil lipidique des fluides reproducteurs et donc sur le niveau d'activité métabolique de l'embryon (Leroy et al. 2010; McKeegan and Sturmey 2012). L'embryon précoce exprime des récepteurs nucléaires aux acides gras : les PPARs (Peroxisome proliferator-activated receptors) (Kang et al. 2011) ainsi que leur partenaire de régulation : les RXR (retinoid X receptor) (Mohan et al. 2002). En réponse à des stimuli nutritionnels, l'activation des PPARs par dimérisation avec le RXR entraine la régulation transcriptionnelle de nombreux gènes impliqués dans le métabolisme énergétique (Desvergne and Wahli 1999) ainsi que la synthèse des prostaglandines (Pakrasi and Jain 2007). Également, les SREBP (Sterol Response Element Binding Protein) permettent le contrôle transcriptionnel des gènes impliqués dans le métabolisme des stérols et autres lipides dans l'embryon bovin (Al Darwich et al. 2010). Cependant, sachant l'importance majeure de l'utilisation endogène des lipides par l'embryon, les mécanismes moléculaires régulant l'entrée des acides gras et la dépense énergétique pendant le développement précoce sont encore mal caractérisés.

1.3.5 Les acides aminés

Dans le métabolisme de l'embryon, les acides aminés jouent un rôle dans plusieurs voies telles que la biosynthèse protéique, la production énergétique, la régulation du pH/osmolarité et comme chélateur de métaux lourds (Baltz and Tartia 2010; Leese 2012; Van Winkle 2001). Pendant son développement, l'embryon bovin consomme et libère différents acides aminés et ce de façon dynamique (Booth et al. 2005). Pendant les premiers clivages, l'utilisation et la production d'acides aminés est faible puis augmente rapidement pendant le développement jusqu'au stade blastocyste, stade où les cellules de l'ICM semblent consommer plus d'acides aminés que celles du trophectoderme (Gopichandran and

Leese 2003). Également, les classes d'acides aminés sont différentiellement utilisées par le métabolisme embryonnaire. Le métabolisme de la glutamine est très actif au stade 2-cellules et diminue progressivement jusqu'à la MET, probablement à cause de la perte des enzymes maternelles (Rieger et al. 1992). Avec l'activation du génome embryonnaire, la glutamine est à nouveau métabolisée par l'embryon mais son taux d'utilisation reste faible, potentiellement en raison de l'activité encore quiescente des mitochondries (Mates et al. 2009). En revanche, la glutamine est fortement consommée au stade blastocyste, soutenant la demande accrue d'énergie et d'osmolytes pendant l'expansion de l'embryon (Guyader-Joly et al. 1996). La glycine est également incorporée pendant le développement embryonnaire afin de maintenir l'osmolarité intracellulaire de l'embryon et servir aussi de combustible énergétique (Hammer et al. 2000).

Les acides aminés sont présents en grande quantité dans le fluide tubaire des mammifères (Groebner et al. 2011). L'alanine et la glycine sont les acides aminés prédominants, suivis de la taurine et l'hypotaurine. Dans les cellules de mammifère, l'entrée des acides aminés se fait via des transporteurs acceptant généralement plusieurs types d'acides aminés en fonction de leur similarité au niveau du site de liaison. Par marquage de certains acides aminés et l'utilisation de compétiteurs inhibiteurs, Pelland et collaborateurs (2009) ont étudié les différents mécanismes de transport des acides aminés et leur cinétique d'activation pendant la croissance de l'ovocyte (Pelland et al. 2009). Les études de Van Winkle ont également caractérisé l'expression et la fonction des transporteurs des acides aminés pendant le développement embryonnaire précoce (Van Winkle 2001).

1.4 L'homéostasie oxydative

Pendant le développement embryonnaire, l'embryon doit maintenir un équilibre ou homéostasie de son activité métabolique dans un intervalle physiologique. Particulièrement, les variations du métabolisme oxydatif ainsi que la régulation du taux d'acidité et d'osmolarité sont des processus finement régulés pendant le développement de l'embryon car potentiellement dangereux pour l'intégrité physique de ses cellules. Plusieurs mécanismes sont donc mis en place pour réguler les variations homéostatiques et l'environnement in vivo fournit un support idéal pour contrôler le taux d'activité

métabolique à chaque stade du développement embryonnaire. Particulièrement, l'embryon est très sensible au stress oxydative, déséquilibre entre production et élimination des radicaux libres. L'état Redox (réduit vs oxydé) est balancé par l'activité des fonctions oxydantes et anti-oxydantes provenant de l'embryon et de l'environnement (Cheong et al. 2009). Dans cette partie, seuls les mécanismes impliqués dans l'homéostasie oxydative sont décrits, et le lecteur peut se référer à l'étude de Baltz et Tartia en ce qui concerne l'homéostasie acido-basique et osmotique (Baltz and Tartia 2010).

1.4.1 Les facteurs oxydants

L'utilisation de l'oxygène produit des dérivés oxygénés appelés radicaux libres ou ROS (Reactive Oxygen Species), qui représentent une source de produits oxydants tel que l'anion superoxyde ($O_2^{\circ -}$), le peroxyde d'hydrogène (H_2O_2) et l'ion hydroxyle (HO°) (Guerin et al. 2001; Summers and Biggers 2003). Il existe plusieurs fonctions cellulaires produisant des ROS. De façon endogène, la production de ROS provient majoritairement des réactions de phosphorylation oxydative mitochondriales et dans une certaine mesure de l'activité cytoplasmique de la NADPH oxydase et du système xanthine oxydase (Filler and Lew 1981). Dans la mitochondrie, les électrons de la chaine de phosphorylation oxydative réagissant avec l'O_2 génèrent le radical superoxyde $O_2^{\circ -}$. Par l'activité de la PPP, l'enzyme NADPH oxydase convertit le NADPH en NADP+ en libérant également l'anion radical superoxyde et un proton, conduisant à la formation du peroxyde d'hydrogène H_2O_2. Par dégradation de l'hypoxanthine, la xanthine oxydase conduit à la production d' H_2O_2 et d'acide uréique.

Après la fécondation et précédant le premier clivage, un pic de ROS coïncide avec l'augmentation de la consommation d'oxygène (Lopes et al. 2010), suggérant la production de superoxyde par la chaine respiratoire. La libération d' H_2O_2 par l'activité de NADPH oxydase serait acquise 4 à 5 jours après la fécondation chez le blastocyste de lapin (Manes 1992; Manes and Lai 1995). Chez la souris, le niveau d' H_2O_2 produit par l'embryon in vitro augmente avec le passage successif des clivages et se stabilise au stade blastocyste (Nasr-Esfahani et al. 1990). L'H_2O_2 en conjonction avec l'anion superoxyde produit le radical hydroxyle extrêmement réactif et dommageable pour la cellule. Cette réaction, appelée réaction d'Haber-Weiss, est accélérée par la présence exogène d'ions ferriques (Khan and

Kasha 1994). La production physiologique de radical hydroxyle n'est pas caractérisée pendant le développement précoce des mammifères. En revanche, des études ont montré l'impact de concentrations élevées en ROS sur l'intégrité des constituants cellulaires tels que l'ADN et les protéines, et la présence de radical hydroxyle contribuerait à la peroxydation des lipides embryonnaire (Guerin et al. 2001; Nasr-Esfahani et al. 1990).

Bien que délétères à haute dose, les ROS sont impliqués dans diverses fonctions cellulaires et développementales (Covarrubias et al. 2008). En effet, les ROS peuvent servir de messagers dans diverses voies de signalisation. Particulièrement, le monoxyde d'azote (oxyde nitrique NO) est produit par l'embryon précoce via la NO synthétase à partir de l'arginine (Tranguch et al. 2003), et serait impliqué dans la régulation de la respiration mitochondriale et l'apoptose (Manser and Houghton 2006). Dès lors, la synthèse de NOs s'avère une fonction cruciale dans le développement et l'implantation de l'embryon (Chwalisz et al. 1999; Gouge et al. 1998). Il est important de rappeler que la tension en oxygène est fortement réduite dans le tractus génital femelle des mammifères. Ce paramètre exogène est donc également un facteur majeur dans la régulation de la production de ROS par les oxydases embryonnaires.

1.4.2 Les facteurs antioxydants

Pour maintenir le taux de ROS à un équilibre physiologique, des mécanismes stabilisateurs interviennent pour réduire les radicaux libres présents dans la cellule. Deux voies d'action principales existent: la voie non-enzymatique et la voie enzymatique. Parmi les voies non-enzymatiques, l'hypotaurine a un pouvoir antioxydant en recyclant les radicaux hydroxyles. Le produit de la réaction génère de la taurine (Aruoma et al. 1988), un dérivé d'acide aminé ayant également un rôle antioxydant en neutralisant les produits de peroxydation lipidiques tel que l'aldéhyde (Guerin et al. 2001). L'enzyme cystéine sulphinate décarboxylase est essentielle dans la voie de biosynthèse de l'hypotaurine et de la taurine, dont le substrat initial est la cystéine (Guerin et al. 2001). Hypotaurine et taurine sont produites par les cellules tubaires de mammifères (Guerin et al. 1995) et importées dans l'embryon via des transporteurs Na^+ dépendant chez la souris (Van Winkle 2001). Elles représentent 59% de la quantité d'acides aminés présents dans le fluide tubaire chez la souris (Dumoulin et al. 1992). L'incorporation de taurine augmente entre le stade deux cellules et

le stade blastocyste in vivo chez la souris (Van Winkle and Dickinson 1995) et la perte de fonction du transporteur de la taurine (TauT) réduit la fertilité chez la souris (Heller-Stilb et al. 2002).

Les dérivés thiol (Beta-mercaptol, cysteamine, cystéine, cystine) sont également capables d'éliminer les ROS et participent au cycle du glutathione impliqué dans la détoxification cellulaire. Formé à partir de glycine, cystéine et glutamate, le glutathion est un substrat qui, sous sa forme réduite (GSH), participe à l'élimination des ROS produits par le métabolisme oxydatif ou provenant de l'environnement extracellulaire. L'enzyme qui catalyse cette réaction est la glutathion peroxydase (GPX) qui oxyde la GSH en GSSH. Le GSSH est ensuite réduit en GSH par la glutathion réductase via la consommation de NADPH. Importante pour la décondensation du noyau du spermatozoïde, la concentration de GSH embryonnaire diminue avec le développement du blastocyste chez la souris (Gardiner and Reed 1994; Nasr-Esfahani and Johnson 1992). Cette diminution implique d'une part l'utilisation du potentiel réducteur du GSH dans l'élimination des ROS mais également l'incapacité de l'embryon à synthétiser de novo le GSH pendant le développement du blastocyste (Harvey et al. 1995; Salmen et al. 2005). Le stock de GSH présent dans l'ovocyte est donc un facteur majeur du développement embryonnaire bien que GSH et GPX soient présents dans le fluide tubaire pour limiter le stress oxydatif (Salmen et al. 2005).

L'acide ascorbique et d'autres vitamines présentes dans le fluide de l'oviducte auraient également un pouvoir antioxydant. Outre son rôle énergétique, le pyruvate permet aussi la réduction du peroxyde d'hydrogène. De même, certaines protéines, telles que la transferrine et l'albumine présentes en forte concentration dans l'environnement de l'embryon, préviennent de la peroxydation lipidique en neutralisant les ions métalliques et les radicaux libres (Guerin et al. 2001). Enfin, la réduction des ROS est également dépendante du métabolisme énergétique et de l'équilibre dynamique du couple NAD(P)H/ NAD(P)+ (Dumollard et al. 2009). NADPH est le donneur d'électrons dans les réactions d'oxydation cytoplasmiques tandis que NADH est principalement produit dans la mitochondrie pour soutenir la chaine électronique proto-énergétique. Le métabolisme du NADH et du NADPH est donc vital pour l'homéostasie embryonnaire et plusieurs mécanismes sont impliqués dans

le recyclage du NAD(P)+, notamment les réactions de déshydrogénation du lactate ou du citrate (Dumollard et al. 2007).

En ce qui concerne les antioxydants enzymatiques, l'enzyme superoxyde dismutase (SOD) participe aux premières étapes de désintoxication cellulaire en radicaux libres. Elle convertit l'anion superoxyde $O_2^{\circ -}$ en H_2O_2 qui est ensuite éliminé par la glutathion peroxydase GPX ou la catalase. La SOD nécessite l'apport d'ions métalliques pour son activité enzymatique (Cuivre/Zinc ou Manganèse selon la localisation cytoplasmique ou mitochondriale). Chez la souris et l'humain, les transcrits codant pour la SOD (Mn et Cu/Zn), la catalase et la GPX sont présents dans l'ovocyte et pendant le développement embryonnaire (El Mouatassim et al. 1999; Harvey et al. 1995). Chez l'embryon bovin, le profile d'expression de la catalase, GPX et Cu/Zn-SOD ressemble à celui de la souris alors que les transcrits de la Mn-SOD montrent une expression variable entre les embryons (Lequarre et al. 2001). De plus, les transcrits de GPX sont fortement retrouvés au stade blastocyste, suggérant une relation avec l'augmentation du métabolisme oxydatif et la production de ROS après compaction, la GPX étant la seule enzyme capable de protéger contre la peroxydation lipidique. Dans le fluide tubaire de souris sont retrouvés les transcrits codant la SOD, la catalase et le GPX alors que ceux de la Mn-SOD sont absents dans l'oviducte humain (El Mouatassim et al. 2000). L'activité de la catalase a été démontrée dans les fluides du tractus génital humain, bovin et porcin (Lapointe et al. 1998), indiquant un rôle dans la protection des gamètes et de l'embryon par rapport aux ROS extracellulaires.

En plus des enzymes antioxydantes SOD, GPX et Catalase, la famille des peroxirédoxines (PRDX) est impliquée dans l'élimination du peroxyde d'hydrogène et les transcrits de différents membres de PRDX ont été détectés dans l'embryon bovin, suivant des profils d'expression variables selon l'isoforme considéré (Leyens et al. 2004). L'expression des régulateurs de l'homéostasie oxydative sont sous le contrôle de facteurs de transcription sensibles à l'équilibre Redox intracellulaire (Harvey et al. 2002). Dans cette revue, Harvey et al 2002 mentionnent l'implication des facteurs HIF et NFκB dans la réponse aux changements de l'homéostasie oxydative pendant le développement embryonnaire (Parrott and Gay 1998). Le facteur NFκB joue un rôle clé dans la régulation de la prolifération/mort cellulaire ainsi que dans les processus inflammatoires via le controle

des gènes du cycle cellulaire et de l'adhésion. Également, d'autres régulateurs contribueraient au maintien de l'état redox de l'embryon (Velez-Pardo et al. 2007), tel que p66shc ou p53 qui ont étaient impliqués dans la réponse au stress oxydatif (Favetta et al. 2007a; Favetta et al. 2004; Favetta et al. 2007b; Takahashi 2012).

1.5 La production d'embryon in vitro

La possibilité de produire des embryons in vitro a été expérimentée dès les années 1878 par Shenk chez le lapin, suivi par Onanoff, Pincus, Meakin et Rock chez l'homme, sans succès cependant. Pendant les années 1950-60, les premières preuves de fécondation in vitro ont été fournies (revue de Chang, 1968) et après de nombreuses expériences sur l'animal, le premier bébé humain issu d'une fécondation in vitro a vu le jour en 1978 (Steptoe and Edwards 1978). A l'heure actuelle, il est possible de contrôler in vitro la fécondation et le développement embryonnaire jusqu'au stade blastocyste. Cette biotechnologie est largement utilisée en clinique et recherche fondamentale. En effet, les enjeux sont multiples. En médecine humaine, la production d'embryons in vitro est utilisée comme technique de reproduction assistée (ART), également appelé procréation médicalement assistée (PMA). l'ART a pour vocation d'apporter un traitement de l'infertilité. Les embryons produits in vitro sont transférés dans l'utérus maternel afin d'induire une gestation et une naissance. L'ART est également un outil dans la sélection et l'amélioration des races d'animaux d'élevage (traits phénotypiques ou sexe). Pour la recherche fondamentale, la production d'embryon in vitro permet d'étudier les mécanismes cellulaires et moléculaires impliqués dans la maturation ovocytaire, la fécondation et le développement précoce du zygote. Cette connaissance a ouvert la voie aux expériences sur le clonage et la pluripotence des cellules souches embryonnaires, et leurs potentielles applications en médecine régénérative. Historiquement, l'ensemble de ces technologies a reposé sur l'élaboration de milieux de culture capables de soutenir les premiers clivages de l'ovocyte fécondé ainsi que le développement de l'embryon avant son implantation.

1.5.1 Les types de culture

Partant de la notion que l'environnement immédiat entourant les tissus vivants est responsable de leur activité et leur survie, les premiers biologistes cellulaires ont élaboré des

milieux de culture appropriés à l'étude ex vivo des organes et tissus. En ce qui concerne la culture des embryons, les milieux appropriés aux cellules somatiques ne convenaient pas au développement précoce et le passage de la MET était synonyme d'un blocage développemental (Tervit et al. 1972). L'embryon précoce se développe au sein de l'oviducte, dans lequel des interactions complexes ont lieu avec les cellules maternelles et le système endocrinien. Ce concept a ouvert la voie aux expériences de co-culture de l'embryon sur un tapis ou avec une suspension cellulaire qui se rapprochait des conditions in vivo, ce qui a permis les premiers clivages embryonnaires in vitro (Ellington et al. 1990; Gandolfi and Moor 1987). La formulation des milieux de co-culture s'élabore sur la base de milieux de culture tissulaire et plusieurs lignées de cellules somatiques sont utilisables (Marquant-Leguienne and Humblot 1998). Bien que formant un support de contact, il est généralement admis que le rôle bénéfique des cellules somatiques repose sur leur capacité à modifier la composition du milieu de culture, et notamment l'élimination ou la réduction des composants embryotoxiques (Edwards et al. 1997). De plus, le pré-conditionnement par des cellules somatiques et les changements sur la composition du milieu en carbohydrates (dégradation du glucose en pyruvate et lactate) et la tension en oxygène sont bénéfiques pour le développement de l'embryon (Edwards et al. 1997; Rieger et al. 1995). Enfin, les cellules somatiques sécrètent des facteurs paracrines aux fonctions embryotrophiques, dont certains facteurs de croissance (Rief et al. 2002).

Bien que la co-culture ait permis les premiers pas pour la production d'embryon in vitro et l'étude des relations chimiques avec le tissu maternel, la présence d'un métabolisme somatique empêche la compréhension des demandes nutritives de l'embryon et soulève des considérations sanitaires quant aux risques d'infections potentielles véhiculées par les cellules d'origines diverses. De ce fait, la culture in vitro évolua vers l'utilisation de milieux chimiquement définis. Montrant tout d'abord un déficit à soutenir le passage du bloc développemental lors de la MET, l'élaboration de milieux définis s'est principalement basé sur des expériences empiriques d'essais-erreurs en évaluant l'impact de différents facteurs à différentes concentrations sur le succès développemental du zygote (Summers and Biggers 2003). Lawitts et Biggers ont ainsi élaboré le milieu KSOM, une solution saline permettant de franchir le blocage développemental chez la souris (Lawitts and Biggers 1993) et qui soutenait la production de blastocystes (Erbach et al. 1994). Parallèlement, l'étude de la

composition des fluides retrouvés in vivo dans l'oviducte a permis de formuler des solutions synthétiques appelées SOF : Synthetic Oviduct Fluid (Tervit et al. 1972). La composition des milieux SOF se base sur les concentrations des composants in vivo pour soutenir le développement in vitro. En parallèle, la formulation de solutions avec une composition complexe contenant des vitamines et autres facteurs tels que présents dans les milieux de culture tissulaire a également permis la culture embryonnaire (Pinyopummintr and Bavister 1991).

L'augmentation des connaissances sur la dynamique de la demande métabolique embryonnaire et des changements de concentration des substrats composant les fluides de l'oviducte et de l'utérus a mis en évidence le besoin d'utiliser des milieux de culture séquentiels dont la formulation diffère en fonction des stades développementaux (Gardner and Lane 1998). L'embryon est d'abord cultivé dans des conditions soutenant le développement pre-MET puis transféré dans des milieux plus appropriés au développement des stades post-compactions et à l'éclosion du blastocyste. De même, le renouvellement du milieu de culture a l'avantage d'éliminer les substances embryotoxiques telles que l'ammonium produit par dégradation des acides aminés. Cependant, la culture embryonnaire en continu (pas de changement de milieux) est encore pratiquée, argumentant que manipuler l'embryon au début de son développement et le transférer dans de nouvelles conditions nécessite un ajustement brusque de son activité métabolique (Gandhi et al. 2000) et induit une perte des facteurs embryotrophiques sécrétés auparavant par les cellules embryonnaires. Ainsi donc, de nouveaux systèmes de culture sont en cours d'élaboration, permettant d'apporter/d'éliminer les nutriments/déchets du métabolisme de façon dynamique sans manipuler l'embryon (Smith et al. 2012), et ceci grâce à des flux constants de milieu nutritif (micro-fluidique).

En plus d'investiguer les bénéfices de nouveaux système de culture, plusieurs études ont montré que le taux de développement est amélioré lorsque les embryons sont cultivés en groupe et inter distants d'environ 150 µm (Gopichandran and Leese 2006). L'hypothèse soulevée est que les embryons sécrètent des facteurs embryotrophiques qui se concentrent dans le milieu de culture et bénéficient au développement du groupe (Gardner et al. 1994). Ainsi, l'approche « well-of-the-well » permettant la culture embryonnaire dans des volumes

réduits viserait à augmenter la concentration en facteurs de croissance et le taux de développement en blastocystes (Vajta et al. 2000). Parmi les facteurs soupçonnés, l'EGF (Epidermal Growth Factor) aurait un effet positif sur le développement. Enfin, le PAF (Platelet Activating Factor) semble contrer l'effet négatif de l'éloignement entre embryons (Gopichandran and Leese 2006).

1.5.2 La composition des milieux

La culture d'embryon in vitro nécessite des milieux enrichis en macromolécules, nutriments métaboliques et régulateurs homéostatiques. Initialement, l'ajout de sérum a été utilisé en co-culture comme source essentielle de nutriments, de facteurs de croissance, de chélateurs de métaux lourds et également comme apport protéique via l'albumine. Dénommé « indéfini » en raison de sa composition variable et d'éléments non-identifiés, le sérum a permis la transition de la co-culture à la culture embryonnaire sans cellules somatiques (Vanroose et al. 2001). Cependant, l'effet protecteur du sérum est hautement variable selon le lot et le type de sérum, et son effet serait plutôt négatif au début du développement. Une alternative à l'utilisation de sérum était l'ajout d'albumine extraite du sérum ou recombinante (BSA pour le bovin et HSA pour l'homme). L'albumine représente une source protéique essentielle pour le maintien du pool d'acides aminés (Thompson et al. 1998) et joue également un rôle de chélateur ionique et de protéine chaperonne pour un grand nombre d'éléments embryotrophiques (vitamines, stéroïdes, acides gras et cholestérol). Afin d'éliminer tout effet potentiellement inconnu de produits vivants, l'albumine peut être remplacée par un apport en macromolécules synthétiques telles que le polyvinyl alcool ou le polyvinyl pyrrolidone, bien que l'avantage soit généralement moins élevé pour soutenir le développement préimplantatoire (Gardner et al. 1994; Holm et al. 1999; Lim et al. 2007; Mingoti et al. 2009; Takahashi and First 1992). A l'heure actuelle, la majorité des protocoles de culture in vitro utilisent de l'albumine comme source de macromolécules (Meintjes et al. 2009), et supplémente les milieux de différents substrats métaboliques.

Dans les milieux de culture, les substrats principaux apportant l'énergie nécessaire au développement du zygote bovin sont le glucose, le lactate et le pyruvate (Pinyopummintr and Bavister 1996). Cependant, leur supplémentation serait bénéfique en fonctions de la

dose utilisées et du stade développemental concerné. Notamment, l'ajout de glucose comme seule source énergétique pendant la culture des premiers clivages embryonnaires ne permet pas de soutenir le développement chez le bovin et la souris (Kim et al. 1993; Matsuyama et al. 1993) (Brinster and Thomson 1966) (Brinster 1965). Cependant, l'exposition de l'embryon murin à une faible dose de glucose pendant la phase de clivage améliore son développement subséquent en blastocyste (Brown and Whittingham 1991), suggérant un bénéfice précoce à l'apport de faible dose de glucose notamment au niveau des fonctions anaboliques (Pantaleon et al. 2008). Contrairement au glucose, le pyruvate et le lactate sont les substrats requis pour le métabolisme de l'embryon pendant les premiers clivages. Ajouté comme seule source d'énergie, le pyruvate ou le lactate soutiennent le développement de l'embryon bovin jusqu'au stade 8 cellules (Pinyopummintr and Bavister 1996; Rieger and Guay 1988; Rosenkrans et al. 1993). Bien que la présence de lactate semble plus appropriée que le pyruvate avant la MET (Pinyopummintr and Bavister 1996; Takahashi and First 1992), la présence simultanée de ces deux substrats selon un ratio pyruvate/lactate égale à ± 10 est des plus favorable au développement post-compaction (Rosenkrans et al. 1993; Yoshioka et al. 1993).

L'augmentation de l'apport en substrat énergétique est essentielle pour le développement et l'éclosion du blastocyste, bien que l'utilisation de stocks endogènes intervienne également. Après la MET, l'ajout de glucose a permit de soutenir le développement du blastocyste bovin (Donnay et al. 2002; Kim et al. 1993) et coïncide avec l'augmentation de la glycolyse (De La Torre-Sanchez et al. 2006; Thompson et al. 2000). L'ajout d'EDTA, un chélateur des métaux lourds induisant l'inhibition des enzymes de la glycolyse (dépendance au zinc), est bénéfique au passage du bloc développemental au stade 2 cellules chez la souris et au stade 8-16 cellules chez le bovin (Gardner and Lane 1996; Gardner et al. 2000b). En revanche, l'EDTA doit être retiré des milieux de culture après la MET pour permettre l'activation de la glycolyse nécessaire à la blastulation (Nasr-Esfahani et al. 1992). Chez l'humain, le développement du blastocyste est possible sans apport de glucose, compensé par la consommation plus importante de pyruvate (Conaghan et al. 1993). Également, certaines études in vitro montrent que le fructose peut soutenir le développement embryonnaire chez le bovin (Barcelo-Fimbres and Seidel 2007; Bhuiyan et al. 2007). De même, l'augmentation de la concentration de glutamine permettrait d'augmenter l'apport

énergétique (Chatot et al. 1990) via son oxydation par le cycle de Krebs (Nasr-Esfahani et al. 1992). Ainsi, des milieux sans glucose ont montré la capacité de soutenir le développement précoce de l'embryon bovin (REF Holm). En effet, la surcharge énergétique exogène est inutile car les embryons, particulièrement chez le bovin et le porcin, sont capables d'utiliser leur réserve en glycogène et en lipide comme combustible oxydatif (Barcelo-Fimbres and Seidel 2007). Ainsi, une diminution des apports en lactate/pyruvate pourrait également faciliter le métabolisme mitochondrial et la β-oxydation des acides gras. De plus, l'apport d'intermédiaires du cycle de Krebs tel que l'acide acétique, produit dans le rumen et constituant une source énergétique importante pour les ruminants, pourrait servir de substrat du métabolisme de l'embryon bovin.

Mis à part les carbohydrates, le passage du blocage développemental nécessite l'apport d'acides aminés pendant la culture in vitro (Gardner and Lane 1996; Pinyopummintr and Bavister 1996). Particulièrement, les acides aminés dits non-essentiels selon Eagle (Eagle 1959) sont bénéfiques tandis que ceux essentiels sont inhibiteurs des premiers clivages (Lane et al. 2001; Steeves and Gardner 1999; Van Thuan et al. 2002). L'ajout de glutamine se révèle également bénéfique au développement précoce chez l'embryon de souris (Chatot et al. 1989). Cependant, une étude montre que la glutamine (1 mM) n'améliore pas le taux de développement embryonnaire, mais l'inhiberait (Devreker and Hardy 1997). En effet la glutamine peut spontanément générer de l'ammonium quand elle est dissoute en solution (Summers and Biggers 2003), et ainsi augmenter le taux d'ammoniac, substance toxique pour l'embryon. L'utilisation de la glutamine associée à un autre acide aminé et formant un dipeptide (avec l'alanine ou la glycine : GlyGlu) permettrait d'éviter sa dégradation spontanée tout en conservant sa fonction (Summers and Biggers 2003). D'autre part, le métabolisme des acides aminés pendant le développement rejette aussi de l'ammonium par désamination (Lane and Gardner 2003). Pour contrer l'effet négatif d'une accumulation d'ammonium (Kenny et al. 2002; Lane and Gardner 2003), le renouvellement du milieu aurait un effet positif sur le développement embryonnaire (Summers and Biggers 2003). D'autre part, l'utilisation d'enzymes dégradant l'ammonium pourrait être une solution pour supprimer les effets négatifs du métabolisme des acides aminés (Lane and Gardner 1995).

L'ajout d'acides gras dans le milieu de développement est peu courant. Généralement, la source en lipide provient du sérum et les effets sur l'embryon sont alors masqués par les autres constituants sériques. Une étude chez le lapin montre que quelques acides gras longs (acides myristique, palmitique, stearique, oléique) ont la capacité de soutenir le développement embryonnaire en présence de BSA (Kane 1979). En effet, la BSA a la capacité de lier les lipides et ainsi moduler leur disponibilité pour l'embryon, et le ratio de lipides lié à la BSA par rapport à la concentration de lipide total serait un facteur déterminant de l'impact des acides gras sur le métabolisme embryonnaire. Cependant, l'ajout de lipides dans les milieux de culture est potentiellement dangereux pour l'homéostasie oxydative car les lipides peuvent être oxydés par les radicaux libres (peroxydation lipidique) et conduire à une augmentation du stress oxydatif.

1.5.3 Les facteurs homéostatiques

In vitro, la concentration d'O_2 doit être suffisante pour subvenir aux besoins du métabolisme oxydatif de l'embryon (Thompson et al. 1990) sans pour autant générer d'accumulation de ROS. Ainsi, une réduction du taux d'oxygène à une valeur de 5 % semble bénéfique au maintien de l'équilibre redox embryonnaire chez la souris (Orsi and Leese 2001), le porcin (Kitagawa, 2004) et le bovin (Rodina, 2009). Également, l'ajout d'antioxydants aide l'embryon à se défendre contre les ROS et améliore son développement. L'ajout d'hypotaurine est bénéfique pour le développement du blastocyste chez le bovin, de même pour la taurine mais en présence d'une forte concentration d'oxygène (20%) (Guyader-Joly et al. 1998; Liu and Foote 1995). In vitro, l'ajout de taurine n'améliore pas le développement embryonnaire murin (Dumoulin et al. 1992) contrairement à l'hypotaurine qui est bénéfique chez la souris (Devreker and Hardy 1997) et le hamster (Barnett and Bavister 1992). Enfin, la taurine (chez l'homme) ou l'hypotaurine (chez le porc) améliorent le développement embryonnaire (Devreker et al. 1999; Suzuki et al. 2007). D'autres antioxydants ont été utilisés (catalase, SOD) mais les effets sont relativement faibles (Ali et al. 2003), notamment à cause de l'inaccessibilité de ces molécules exogènes aux lieus de production des ROS souvent endogènes (mitochondrie, peroxysome). De plus, l'ajout des substrats de la biosynthèse du glutathion tel que la cystéine pourrait moduler les capacités antioxydantes de l'embryon (Ali et al. 2003) et soutenir le développement de blastocystes

ayant un métabolisme oxydatif compromis (Edwards et al. 2001). Cependant, l'oxydation de la cystéine en cystine dans le milieu de culture réduirait l'impact de sa supplémentation sur la synthèse de glutathion (Gardiner and Reed 1994; Salmen et al. 2005).

L'établissement de conditions précises d'osmolarité et de pH est très important pour permettre le développement embryonnaire. L'osmolarité du fluide tubaire chez la souris est d'environ 300 mOsm (Collins and Baltz 1999) bien qu'in vitro l'osmolarité la plus appropriée au développement soit d'environ 270 mOsm. De par leur fonction osmotique, la glycine et l'alanine ajoutées au milieu de culture ont ainsi montré des effets bénéfiques pour le développement précoce (Baltz and Tartia 2010; Richards et al. 2010). Chez le hamster, le développement de l'embryon tolère des osmolarité de 250 à 325 mOsm et nécessite un pH entre 7 et 7,5 (McKiernan and Bavister 1990). Chez l'homme, la culture embryonnaire a lieu dans des intervalles de pH de 7,2 et 7,4 (Swain 2012). L'utilisation de tampons (HEPES) ainsi qu'un taux de CO_2 à 5% en présence de bicarbonate permet de prévenir l'acidification du milieu environnant l'ovocyte et l'embryon. La température est également un élément crucial pour maintenir l'homéostasie cellulaire, étant un facteur modulateur des activités physico-chimiques des différents constituants biologiques. La température optimale pour le développement embryonnaire in vitro est la température corporelle, soit environ 37 à 37,5°C chez le hamster et la souris contre 38,5 à 39 °C chez le bovin (McKiernan and Bavister 1990). Pour la majorité des espèces, la température utilisée in vitro est similaire à celle retrouvée in vivo dans le tractus génital. Cependant, il est suggéré qu'une légère diminution de la température pourrait favoriser le développement et la qualité embryonnaire, notamment via le ralentissement de l'activité métabolique (Leese et al. 2008), mais peu de données sont présentement disponibles (Hunter 2012).

1.6 La qualité embryonnaire

1.6.1 La compétence développementale

La compétence développementale reflète les capacités intrinsèques de l'ovocyte/embryon qui permettent le passage des étapes du développement précoce, fœtal et ultimement la génération d'un individu sain. La notion de qualité est attribuée à l'embryon pour mesurer la compétence développementale à un moment donné de sa croissance. Ainsi, un blastocyste de

bonne ou mauvaise qualité aura plus ou moins de ressources pour induire une gestation et générer un individu viable.

Les conditions in vivo de l'oviducte (stades précoces) et de l'utérus (stades morula/blastocyste) sont considérées comme optimales pour produire des embryons avec la meilleure qualité/compétence. Le transfert des zygotes non-humains dans l'oviducte d'espèces hétérologues (brebis, lapin…) a également été utilisé pour mimer les conditions de développement in vivo (Enright et al. 2000; Rizos et al. 2002c). Cependant, cette technique induit un grand nombre de pertes au moment de la collecte des embryons et cela soulève des questions éthiques, économiques et sanitaires quant à l'utilisation d'oviductes en tant qu'incubateur. Ainsi, l'utilisation de milieu de culture in vitro demeure l'unique possibilité de produire des embryons dans les cas de procréation médicalement assistée. Depuis Louise Brown, plus de 4 millions de bébés humains et d'autant plus chez les animaux d'élevage (bovin laitier notamment) ont ainsi été conçus par fécondation et culture in vitro. Cependant, les résultats chez l'homme et d'autres mammifères ont montré une réduction du taux de gestation mais aussi une plus forte incidence de syndromes développementaux (Holm et al. 1996; Lazzari et al. 2002) tels que le syndrome du gros veau chez le bovin et le syndrome de Beckwith-Wiedemann chez l'homme (Nair 2008). Également, les conditions « in vitro » auraient un impact différent sur le développement entre embryons mâles et femelles ce qui pourrait affecter le ratio des sexes (Gutierrez-Adan et al. 2001; Larson et al. 2001). Enfin, plusieurs expériences ont montré une diminution de la tolérance des embryons à la congélation par rapport aux embryons produits in vivo.

La baisse de qualité embryonnaire est en partie due au fait que les conditions de culture sont suboptimales pour l'expression normale du développement précoce. De ce fait, le transfert embryonnaire au jour 3 (morula) limiterait le temps d'exposition aux conditions de culture. Cependant, cette pratique ne permet pas d'assurer la compétence développementale et en conséquence chez l'humain par exemple, plusieurs embryons sont alors transférés dans l'utérus, augmentant le risque de grossesse multiple dangereuse pour la santé de la mère et des enfants. Par la suite, l'extension de la culture jusqu'au stade blastocyste a permis de sélectionner uniquement les embryons les plus compétents (Gardner 1998). Plusieurs reformulations des milieux de culture ont ainsi été faites afin d'améliorer la qualité des

embryons produits. A l'heure actuelle, différents milieux de culture sont disponibles sur le marché de la FIV humaine, et proposent des recettes plus ou moins variables selon les compagnies (Biggers and Summers 2008), démontrant ainsi la plasticité du développement embryonnaire et la capacité d'adaptation de l'embryon a différentes conditions de culture (De La Torre-Sanchez et al. 2006). Cependant et malgré de nombreux efforts, les progrès sur la composition des milieux de culture sont encore vain à recréer un environnement optimal pour le développement précoce et la production d'embryons de qualité semblable aux embryons *in vivo* (Lonergan et al. 2003b; Nair 2008; Rizos et al. 2002c). En conséquence, il reste encore difficile d'obtenir une gestation à partir d'embryons « in vitro » et la pratique du transfert d'un seul embryon (SET) demeure encore infructueuse et dépréciée par les cliniques de fertilité (Bromer and Seli 2008). Des lors, beaucoup d'études ont étaient menées dans la recherche et le développement d'outils capables de déterminer l'impact du stress de la culture et les raisons de la baisse de qualité embryonnaire.

1.6.2 L'impact de la culture

Afin de déterminer l'impact de la culture sur le développement embryonnaire, les premières études ont analysé de façon macroscopique la dynamique de l'embryon et tenté de définir des critères physico-chimiques pouvant refléter la compétence développementale. Dans un premier temps, il a été démontré que l'impact des conditions de maturation sur la qualité de l'ovocyte était primordial pour le développement post-fécondation et que l'utilisation de milieux de culture permissifs au passage du bloc développemental avait peu d'impact sur le taux de blastocystes (Rizos et al. 2002c). Cependant, l'observation de la cinétique développementale a permis de montrer des différences dans la vitesse de clivage induites par la culture in vitro et l'apparition précoce de blastocystes au jour 7 par rapport au jour 8 in vivo chez le bovin (Enright et al. 2000), bien que l'accélération du développement soit majoritairement attribuable à l'ajout de sérum en culture (Van Langendonckt et al. 1997). Malgré l'accélération développementale, les blastocystes cultivés in vitro montrent une difficulté à éclore par rapport aux blastocystes obtenus in vivo (Rizos et al. 2002c), bien que la corrélation entre éclosion et implantation ne soit pas significative (Lane and Gardner 1997). En revanche, les embryons ayant une blastulation retardée sont moins viables que les blastocystes précoces (Leese et al. 1998) et la vitesse de ré-expansion du blastocyste après

décongélation serait corrélée avec la compétence développementale après transfert (Shu et al. 2009). A l'heure actuelle, l'utilisation de critères cinétiques est valable afin de déterminer l'impact de la culture in vitro (Heo et al. 2010) et potentiellement la viabilité embryonnaire selon des études chez le hamster (McKiernan and Bavister 1994). Cependant, l'utilisation de facteurs externes accélérant les étapes développementales (tel que l'éclosion assistée) est pleinement débattue (Das and Holzer 2012; Harper et al. 2012).

En lien avec la cinétique développementale, plusieurs études morphologiques ont observé des différences dans le nombre de cellules embryonnaires et le ratio entre ICM et trophectoderme entre embryons cultivés in vitro par rapport à l'in vivo. La culture in vitro affecterait le chronométrage développemental pendant la compaction au stade morula (Crosier et al. 2000) ce qui interfèrerait avec la prolifération et l'allocation des lignages cellulaires au stade blastocyste (Leese et al. 1998; van Soom et al. 1997). Il apparaît que le nombre total de cellules du blastocyste et le nombre de cellules dans l'ICM sont positivement corrélés avec le taux de gestation après transfert (Ebner et al. 2003; Lane and Gardner 1997). Cependant, une prolifération cellulaire excessive in vitro est associée avec l'induction de syndrome tel que le gros veau (Lazzari et al. 2002) et l'ajout de sérum serait un facteur exacerbant l'effet de la culture in vitro sur la déviation du développement précoce.

Avec l'amélioration des techniques de microscopie, il a été montré que la culture in vitro affecte l'ultrastructure cellulaire des embryons (Fair et al. 2001). In vitro, l'espace périvitellin du zygote est réduit, les blastomères au stade 8 cellules sont morphologiquement non-uniformes et les blastocystes contiennent plus de fragmentation par rapport aux embryons obtenus in vivo (Ebner et al. 2003; Rizos et al. 2002a; Scott 2003). Ainsi, plusieurs critères morphologiques ont été établis afin d'attribuer des scores de compétence développementale (Gardner et al. 2000a). A l'heure actuelle, l'analyse qualitative se fait principalement sur des critères morpho-dynamiques tels qu'identifiés ci-dessus, mais cette méthodologie se trouve inefficace quant à la sélection du meilleur embryon à travers une population globalement similaire. Face à cette situation, des études ont analysé l'aspect moléculaire du développement et ont pu mettre en évidence des changements dans le métabolisme des embryons cultivés in vitro.

L'incorporation et consommation d'acides aminés sont affectées durant la culture et le profil d'utilisation de certains acides aminé serait associé à la compétence développementale chez l'humain (Houghton et al. 2002). D'autre part, l'augmentation de la production de lactate est observée dans les embryons en réponse à la culture in vitro (Khurana and Niemann 2000; Thompson et al. 1991). Chez l'embryon humain, l'augmentation de la consommation de glucose et de pyruvate est positivement corrélée avec la compétence à se développer en blastocyste in vitro et in vivo (Gardner et al. 2001; Renard et al. 1980). En revanche, l'activité glycolytique (production de lactate) au stade blastocyste (Johnson et al. 1991; Lane and Gardner 1996) et des taux extrêmes (trop haut ou trop bas) de consommation d'O_2 (Abe and Hoshi 2003; Barnett and Bavister 1996; Lopes et al. 2007; Thompson et al. 1996) sont négativement corrélés avec la viabilité de l'embryon après transfert. Ces informations soulèvent l'hypothèse qu'un métabolisme quiescent, the « quiet hypothesis », serait associé à la viabilité embryonnaire (Leese 2012; Leese et al. 2007), et le développement de tests sur le métabolome pourrait prédire le succès gestationnel (Bromer and Seli 2008).

Les embryons de souris produits in vitro montrent un taux plus élevé de ROS (H_2O_2) par rapport aux embryons obtenus in vivo (Goto et al. 1993; Nasr-Esfahani et al. 1990). L'augmentation du taux d' H_2O_2 dans les embryons in vitro est corrélée avec l'augmentation de l'apoptose et de la fragmentation cellulaire (Yang et al. 1998), et l'élévation du taux de ROS au jour 1 de développement coïncide avec une réduction du taux de gestation chez l'humain (Bedaiwy et al. 2004). La culture induirait également un taux d'apoptose supérieur parmi les cellules embryonnaires (Fouladi-Nashta et al. 2005). Cependant, la mort programmée joue un rôle régulateur dans le nombre et la qualité des cellules destinées à produire le futur fœtus (Leidenfrost et al. 2011), rendant alors désuète l'utilisation de marqueurs apoptotiques comme indicateur de qualité embryonnaire. En revanche, l'impact de la culture in vitro sur l'expression de marqueurs sécrétés par l'embryon (IFN-t, HLA, Plasminogene) pourrait être utilisé comme indicateur de la capacité d'induire une gestation, bien que les données soient encore peu concluantes (Aflalo et al. 2004; Katz-Jaffe et al. 2009; Lonergan and Fair 2008). D'autre part, de nombreuses études ont montré que l'intégrité du génome était affectée dans sa structure chromosomique par la culture in vitro, mettant en évidence un nombre plus important d'anomalies (polyploïdie) par rapport aux

embryons in vivo (Hyttel et al. 2000; Lonergan et al. 2004; Viuff et al. 1999). Par la suite, l'élaboration de diagnostiques génétiques préimplantatoires a permis de détecter des défauts chromosomiques par biopsie de l'embryon avant transfert (Matzuk and Lamb 2008).

1.6.3 L'outil transcriptomique

La mise en évidence de marqueurs embryonnaires a permis de mieux définir les déviations développementales induites par la culture in vitro. Cependant, la plupart de ces marqueurs apportent une représentation partielle de la qualité embryonnaire et sont parfois très subjectifs, tel que l'utilisation de critères morphologiques pour déterminer la viabilité des embryons destinés au transfert (Enders et al. 1989; Hardy et al. 1989a). De plus, nous ne comprenons toujours pas les raisons des différences observées dans les embryons in vitro et dans quelle mesure les conditions de culture sous-optimales affectent la viabilité après transfert. Des lors, de nombreux efforts ont été réalisés afin d'analyser l'expression des gènes exprimés par l'embryon, apportant une nouvelle approche pour étudier l'impact de la culture in vitro sur les fonctions biologiques et les implications potentielles sur la qualité embryonnaire (Rizos et al. 2002b). Cette nouvelle aire de recherche s'est développée avec l'amélioration des technologies d'analyse transcriptomique et la capacité de travailler avec des quantités réduites de matériel biologique. Principalement, l'utilisation de technique d'amplification de l'ARN ont permis d'observer quantitativement et qualitativement l'expression du génome et définir les profils d'expression de gènes impliqués dans le développement embryonnaire (Seli et al. 2010a).

Dans un premier temps, des analyses de gènes candidats spécifiques par transcription inverse et qPCR (RT-qPCR) ont mesuré les différences d'expression entre embryons produits dans plusieurs conditions de culture (Harvey et al. 1995; Ho et al. 1995). Par la suite, l'étude pionnière de Wrenzycki en 1996 a montré que l'expression du gène codant pour la connexin 43, dont la fonction est impliqué dans la compaction de la morula, diffère entre embryons in vivo et in vitro (Wrenzycki et al. 1996). Par la suite, plusieurs équipes ont identifié de nombreux gènes comme différentiellement exprimés en fonction de l'environnement (Lonergan et al. 2003a; Wrenzycki et al. 1998; Zheng et al. 2007), apportant des preuves supplémentaires quant à l'impact de la culture in vitro sur le développement embryonnaire. Cependant, comme plusieurs milliers de gènes sont exprimés

par l'embryon précoce, l'estimation de l'impact de l'environnement sur le transcriptome nécessitait l'utilisation d'outils d'analyse de l'expression du génome à grande échelle. L'élaboration de nouvelles technologies utilisant des micropuces a ainsi permis de mesurer la quantité d'ARN pour de multiples gènes en fonction de l'espèce ciblée (murin, humain, bovin…), avancées qui ont ouvert l'étude de la génomique fonctionnelle à l'embryon précoce (Dobson et al. 2004; Summers and Biggers 2003; Wang et al. 2004). En 2004, l'étude de Rinaudo a été la première à utiliser la technologie des micro-puces Affymetrix contenant plus de 22 000 gènes afin de montrer l'impact de la culture in vitro sur le transcriptome embryonnaire murin (Rinaudo and Schultz 2004). Par la suite, plusieurs études ont analysé les voies de signalisation et fonctions biologiques des gènes différentiellement exprimés et leur validation par RT-qPCR (Corcoran et al. 2006; Fernandez-Gonzalez et al. 2009), déterminant l'ampleur des modifications induites par l'environnement (Smith et al. 2009).

A partir des données génomiques de l'impact de la culture in vitro, il devenait nécessaire de comprendre le lien entre l'expression différentielle des gènes et la qualité embryonnaire. Des études chez le bovin et l'humain ont pratiqué des biopsies de l'embryon puis son transfert dans l'utérus afin de corréler directement la capacité d'induire une gestation avec un profil transcriptomique spécifique (El-Sayed et al. 2006; Ghanem et al. 2011; Jones et al. 2008; Salilew-Wondim et al. 2010). Les résultats de ces recherches ont mis en évidence plusieurs marqueurs transcriptomiques dont l'expression serait associée au succès développemental du blastocyste ou bien à l'arrêt de la gestation. Cependant, l'identification de marqueurs de compétence ne permettait pas de comprendre comment la culture in vitro induit une déviation du développement normal de l'embryon. Dans cette perspective, plusieurs études ont analysé le profil transcriptomique d'embryons produits dans des conditions de culture ayant plus ou moins d'impact sur la compétence développementale (Cote et al. 2011; Gad et al. 2012; Lazzari et al. 2002; Rizos et al. 2002b; Sagirkaya et al. 2006), associant des listes de gènes avec le développement/la dégénérescence du blastocyste (Boonkusol et al. 2006; Gutierrez-Adan et al. 2004; Huang et al. 2010) ou l'induction de syndrome tel que celui du gros veau (Fernandez-Gonzalez et al. 2009; Lazzari et al. 2002; McHughes et al. 2009). De meme, l'impact de la tension en oxygène (Arias et al. 2011; Rinaudo et al. 2006) ainsi que l'augmentation de la progestérone (super-ovulation) (Gad et

al. 2011) ou encore l'exposition aux facteurs de croissance (Chin et al. 2009) ont montré des effets sur l'expression génique et mis en évidence des fonctions potentiellement associées à la qualité embryonnaire. Cependant, à l'heure actuelle, les données permettant de déterminer l'impact de l'ART sur la qualité embryonnaire et les profils d'expression génique spécifiquement associés au stress de la culture sont encore peu concluantes, nécessitant une approche à grande échelle afin de définir les signaux exprimés par l'embryon malade.

1.7 Hypothèse et objectifs

Plusieurs évidences démontrent l'impact des conditions de culture sur le développement de l'embryon, mettant en avant sa capacité d'adaptation aux différents substrats de son environnement. Cependant, la réponse adaptative de l'embryon aux conditions sous-optimales de culture induirait des modifications précoces ayant des répercussions sur la qualité du blastocyste et la santé du fœtus. Parmi les technologies capables de mesurer la réponse à l'environnement de développement, l'utilisation de micropuces montre une capacité d'analyser à grande échelle les transcrits exprimés par l'embryon en culture et d'identifier des différences transcriptomiques par rapport aux embryons produits in vivo. Des lors, les modifications de l'expression génique seraient impliquées dans la baisse de qualité embryonnaire mais les profils d'expression génique en lien direct avec le stress de la culture restaient cependant indéterminés. Dans ce contexte, nous avons émis l'hypothèse que l'analyse transcriptomique d'embryons exposés à des conditions de culture connues pour affecter la survie embryonnaire permette de caractériser les patrons d'expression génique en lien avec le stress de la culture et traduisant des voies de signalisation associés à la baisse de qualité embryonnaire.

Dans ce contexte, nous avons analysé l'impact de différents stress occasionnés par la culture (énergétique, oxydatif et lipidique) sur le profil transcriptionnel des embryons bovins. Pour ce faire, 3 objectifs ont été définis :

Définir des conditions de culture afin d'augmenter le stress embryonnaire in vitro

- stress énergétique via l'augmentation de la concentration en glucose
- stress oxydatif via l'ajout de facteurs pro-oxydants

- stress lipidique via l'exposition aux lipides sériques

Comparer par micropuce et RT-qPCR le profile transcriptomique des embryons stressés par rapport aux embryons contrôles.

Identifier les voies de signalisation spécifiquement associées au profile transcriptomique du stress embryonnaire via l'utilisation d'outils d'analyse génomique fonctionelle.

Au cours de cette thèse, l'étude de l'impact du stress énergétique, oxydatif et lipidique au stade blastocyste représente les 3 articles principaux et sont introduits aux chapitres 2, 4 et 5 respectivement. Le chapitre 4 est un article supplémentaire concernant l'impact du stress énergétique au stade morula.

Chapitre 2: Impact du stress énergétique

Title: Differential Gene Expression Profile in Bovine Blastocysts Resulting from Hyperglycemia Exposure during Early Cleavage Stages.

Published in Biology of Reproduction, 86(2):50. Feb 2012.

Key words: Assisted reproductive technology, Blastocyst, Embryo culture, Gene expression, Ruminants

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Grant support: NSERC Strategic Network EmbryoGENE NETPG 340825-06

2.1 Résumé

Afin de comprendre le défaut de survie des embryons produits par procréation médicalement assistée, l'analyse transcriptomique du développement embryonnaire précoce a mis en évidence l'impact de l'environnement de la culture in vitro sur l'expression génique chez le bovin ainsi que chez d'autres espèces. Cependant, la caractérisation des gènes différentiellement exprimés en culture et traduisant des perturbations développementales de l'embryon reste irrésolue. Dans cette perspective, notre objectif est de définir des marqueurs transcriptomiques exprimés par l'embryon bovin cultivé dans des conditions de stress connues pour affecter le développement précoce. Comme une forte dose de glucose s'avère stressante pendant les premiers clivages chez les embryons de mammifères et diminue la survie subséquente du blastocyste, des zygotes provenant de maturation et fécondation in vitro ont été cultivés dans des conditions contrôles (0,2 mM) ou augmentées en glucose (5 mM) jusqu'au stade 8/16–cellules, puis transférés dans des milieux contrôles afin de continuer leur développement jusqu'au stade blastocyste. La concentration de 5 mM était choisie comme traitement de stress puisqu'elle affectait significativement le taux de blastocyste sans être létale telle qu'observée avec 10 mM. Les analyses de microarray révélèrent des différences d'expression génique non-relatives au sexe ou à l'éclosion de l'embryon. Les processus les plus représentés à travers les gènes différentiellement exprimés concernaient la matrice extracellulaire, le signal calcique et le métabolisme énergétique. Au niveau patho-physiologique, l'augmentation du glucose montrait un impact sur les voies associées au diabète et à la tumorogenèse via des gènes contrôlant l'effet Warburg, c.a.d. l'accentuation de l'utilisation de la glycolyse anaérobie plutôt que la phosphorylation oxydative. Ces résultats permettent de conclure que le stress énergétique du développement pré-attachement en culture est concomitant à des modifications dans l'expression de gènes impliqués dans le contrôle métabolique.

2.2 Abstract

To understand the compromised survival of embryos derived from assisted reproductive techniques, transcriptome survey of early embryonic development has shown the impact of *in vitro* culture environment on gene expression in bovine or others living species. However, how the differentially expressed genes translate into developmentally compromised embryos is unresolved. We therefore aimed to characterize transcriptomic markers expressed by bovine blastocysts cultured in conditions which are known to impair embryo development. As increasing glucose concentrations has been shown to be stressful for early cleavage stages of mammalian embryos and to decrease subsequent blastocyst survival, *in vitro* matured/fertilized bovine zygotes were cultured in control (0.2 mM) or high glucose (5 mM) conditions until 8/16–cells stage, and then transferred to control media until they reached the blastocyst stage. The concentration of 5 mM glucose was chosen as a stress treatment since there was a significant effect on blastocyst rate without being lethal as with 10mM. Microarray analysis revealed gene expression differences un-related to embryo sex or hatching. Over-represented processes among differentially expressed genes in treated blastocysts were extra-cellular matrix signalling, calcium signalling and energy metabolism. On a pathophysiological level, higher glucose treatment impacts pathways associated with diabetes and tumorigenesis through genes controlling the Warburg effect, *i.e*., emphasis to use anaerobic glycolysis rather than oxidative phosphorylation. These results allowed us to conclude that disruption of *in vitro* pre-attachment development is concomitant with gene expression modifications involved in metabolic control.

2.3 Introduction

For more than thirty years, assisted reproductive techniques (ART) have been established in different mammalian species and widely used around the world. However, concerns regarding the impact of embryo manipulation on the health of the offspring have been reported, notably in livestock (Thompson and Peterson 2000). In cattle, the negative impact of *in vitro* production (IVP) results in an estimated 30% of offspring suffering from abnormally high birth weight (large offspring syndrome), which creates the need for frequent caesarean sections. Furthermore, IVP is also associated with increased gestation length, skewing of the male-to-female ratio (higher proportion of males) (Camargo et al. 2010), and a higher frequency of congenital malformations and perinatal mortality. It is estimated that one-third of *in vitro*-derived conceptuses are lost during the first month of gestation (Thompson and Peterson 2000).

In investigating the cause of the short- and long-term impacts of ART, some teams have hypothesized that the *in vitro* environment is partly responsible. *In vivo*, the embryos develop in an undefined, complex environment and exhibit a dynamic metabolism, *i.e.,* slow metabolism during early cleavage stages followed by activation to support proliferation and differentiation during blastulation. Therefore, subjecting the early embryo to an *in vitro* culture (IVC) condition, inappropriate to its metabolism, may be partly responsible for embryonic stress and deviations from the normal phenotype (Khurana and Niemann 2000; Leese et al. 2007; Zheng et al. 2007).

Although some changes in IVC protocols have been made to improve embryo development in ART, no valuable tools are available to assess the stress-related impact resulting from IVC. In this context, the current challenge is to define markers indicative of embryonic stress during development of IVC embryos (Vajta et al. 2010) in order to formulate appropriate culture conditions to improve viability and the success of ART.

A promising solution for developing stress markers is to study the gene expression profile of cultured embryos (Hamatani et al. 2006). During early cleavage, transcription is inactivated and development is supported by continued synthesis of proteins from mRNA stored during oocyte maturation (Vigneault et al. 2004). Once this RNA support is degraded

after successive cleavages of the zygote, mechanisms regulating nucleus state will allow embryonic cells to activate gene transcription, a phenomenon called embryonic genome activation (EGA) (Badr et al. 2007). This critical event (8/16-cells stage in cow) allows the maternal-embryo transition (MET) and transcriptional control of embryonic development (Vigneault et al. 2009). Recent studies have shown that the environmental milieu *per se* has an important influence on gene expression in produced bovine blastocysts (Lonergan et al. 2003a). Meanwhile, transcriptomic variations in IVC embryos compared to their *in vivo* counterparts have been proposed to reflect embryonic quality (Rizos et al. 2002b) but direct mechanistic links between differential gene expression and IVC-induced embryonic stress are still lacking.

Therefore, the aim of this study is to analyze the development and gene expression of pre-attachment bovine embryos following IVC in stress condition of hyperglycemia. Indeed, numerous studies have shown that excessive glucose exposure results in a decrease viability phenotype in early embryo development. High glucose during early cleavage stages has been correlated with developmental block at the time of EGA and low quality of blastocysts (Barnett et al. 1997; Furnus et al. 1997; Gardner et al. 2000b). Moreover, hyperglycemia induces developmental delay of pre-implantation development (Moley et al. 1996). During early cleavage stages, the embryo utilizes low amounts of glucose and pyruvate/lactate/glutamine are the preferred substrates for its metabolism (Thompson 2000). In bovine oviductal fluid, glucose concentration is lower (2 mM) than blood serum (4 mM), indicating a differential regulation in the secretion of this energy substrate by the oviduct epithelium in order to match embryo metabolism (Hugentobler et al. 2008).

Experimentally, *in vitro* produced bovine zygotes were cultured with increasing glucose concentration during early cleavages (8/16-cells stage) and transferred to control media until reaching the blastocyst stage. Embryo survival was determined by assessing the development rate of 8/16-cells embryos (at day 3) then blastocyst and hatched blastocyst embryos (at day 7). Large-scale analysis of gene expression was performed in blastocyst stage by microarray slides that are specifically enriched in genes expressed during bovine pre-attachment development (Robert et al. 2011a). Functional analysis tools were used to

highlight the expressed genes and molecular pathways significantly perturbed in hyperglycemia-treated blastocysts.

2.4 Materials and methods

All chemicals were obtained from Sigma–Aldrich, unless otherwise stated.

In vitro production of bovine blastocysts

Oocyte collection and *in vitro* maturation (IVM) : Cumulus–oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of slaughtered heifers. After four washes in TLH (HEPES-buffered Tyrode's Lactate solution), groups of up to 10 selected COCs with at least five layers of cumulus were placed in 50-µl drops of medium under mineral oil in dishes (Nunc, Roskilde) and maturated for 24 h at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium was TCM-199 supplemented with 10% fetal calf serum (HyClone, ThermoScientific, USA), 0.1 µg/ml of FSH (Folltropin V, Bioniche, Canada), 0.33 mM of pyruvic acid and 50 µg/ml of gentamycin.

In vitro fertilization (IVF): Following maturation, five matured COCs were added to 48-µl droplets of IVF medium under mineral oil. The IVF medium was composed of TL STOCK (Tyrode's Lactate solution) supplemented with 0.6% fatty acid free bovine serum albumine (BSA), 0.2 mM pyruvic acid, 10 µg/ml heparin, and 50 µg/ml gentamycin. Prior to transfer, the COCs were washed twice in TLH medium. Once transferred, 2 µl of PHE (1 mM hypotaurine, 2 mM penicillamine, 250 mM epinephrine) were added to each droplet 10 min before spermatozoa was added. The spermatozoa used consisted of a cryopreserved mixture of ejaculates from five bulls (Centre d'Insémination Artificielle du Québec, St.-Hyacinthe, QC, Canada). The spermatozoa were thawed in 37 °C water for 1 min, put on a discontinuous Percoll gradient (2 ml of 45% Percoll over 2 ml of 90% Percoll), and centrifuged at 700g for 30 min at 26 °C. After discarding the supernatant, the pellet of live spermatozoa was resuspended in IVF medium after being counted on a haemocytometer to obtain a concentration of 10^6cells/ml. Finally, 2 µl of the sperm suspension (final concentration = 4.10^4 cells/ml) were added to each IVF droplet containing the matured COC and the incubation took place in a humidified atmosphere at 38.5 °C in 5% CO_2 for 16-18 h.

In vitro culture (IVC): For embryo culture, a three step modified synthetic oviduct fluid (mSOF) culture system containing MEM essential and non-essential amino acids, 0.5 mM of glycyl-glutamine and 0.4% fatty acid-free BSA under embryo tested mineral oil (#8410, Sigma) was used. The embryo culture dishes were incubated at 38.5°C with 6.5% CO_2, 5% O_2 and 88.5% N_2 in 100% humidity. Briefly, after fertilization, presumptive zygotes were mechanically denuded and washed three times in TLH supplemented with fatty acid-free BSA and were placed in groups of 10 in 10 µl droplets of SOF 1 with non-essential amino acids (1X) and 3µM EDTA. Embryos were transferred in new 10 µl droplets of SOF 2 containing non-essential (1X) and essential (0.5X) amino acids 72 h post-fertilization and once again 120 h post-fertilization in 20 µl droplets of SOF 3 containing non-essential (1X) and essential (1X) amino acids. Media was replaced three times to prevent toxicity due to ammonium accumulation and nutrients depletion caused respectively by amino acid degradation and embryo metabolism. The glucose concentration used in SOF1, 2 and 3 was respectively 0.2, 0.5 and 1.0 mM. Blastocyst development was assessed at days 7 post-fertilization. Pooled blastocysts (hatched and non-hatched) were transferred, washed 3 times in PBS, collected in groups of 10 in small volumes of PBS into 0.5 mL microtubes and stored at -80°C until RNA extraction. Cleavage rate (number of embryos with at least 2 cells out of total embryos) and 8/16-cells embryo rate (number of embryos with at least 8 cells out of total embryos) were determined during embryo transfer from SOF1 to SOF2 (Day 3). Blastocyst rate (number of early, expanded and hatched blastocysts out of total embryos) and hatching rate (number of hatched blastocysts out of total blastocysts) were calculated at the end of the culture.

Determination of hyperglycemic treatment

Glucose dose response experiment was performed by adding increasing amounts of glucose (0.2 mM = control concentration, 1 mM, 2 mM, 5 mM or 10 mM) in SOF1 during the first 3 days of embryo development after fertilization. Total embryos were then transferred to normal culture conditions until reaching the blastocyst stage. At least 3 replicates (20 to 30 presumptive zygotes per replicate) were done for each glucose concentration excepted for the lethal concentration of 10 mM (1 replicate). Increasing the glucose concentration up to 10 mM (10 mOsm) did not significantly affect the osmolarity of

culture medium (300 mOsm). As preliminary, results from glucose dose response experiment were not subjected to statistical analysis.

Production of control and high glucose treated blastocysts: As 5 mM of glucose in SOF1 appeared to be the limit dose for blastocyst development, we selected this criterion as hyperglycemic treatment to produce 7 replicates of control and high glucose treated blastocysts from different *in vitro* production runs. Each replicate contained around 10 embryos including not expanded (early), expanded and hatched blastocysts. Although increased glucose treatment decreased the hatching rate, equivalent proportions of hatched blastocysts were distributed between control and treatment replicates. Four out of 7 replicates were used for microarray experiment and 3 out of 7 replicates were used to validate the microarray results by qRT-PCR. Un-paired t test were used for statistical comparison of developmental rate between control and 5 mM glucose treatment.

Determination of differential gene expression in treated blastocysts

Total RNA from each replicate was extracted and purified using PicoPure™ RNA Isolation Kit, (Life Science). After DNase digestion (Qiagen), quality and concentration of extracted RNA was analyzed by bioanalyzer (Agilent). All extracted samples showed good quality with an RNA Integrity Number >7.5.

For microarray purposes, purified RNA was amplified by in vitro transcription by T7 RNA amplification using the RiboAmp® HSPlus RNA Amplification Kit, (Life Science), labelled with Cy3 and Cy5 using the ULS™ Fluorescent Labeling Kit, (Kreatech) and aRNA (825 ng per replicate) was hybridized on the Agilent-manufactured EmbryoGENE slides (Robert et al. 2011a) in a 2-colors dye swap design. After 17 h of hybridization at 65 °C, microarray slides were washed 1 minute in gene Expression Wash Buffer 1 (room temperature), 3 minutes in gene Expression Wash Buffer 2 (42 °C), 10 seconds in 100% acetonitrile (room temperature) and 30 seconds in Stabilization and Drying Solution (Agilent). Slides were scanned with PowerScanner (Tecan) and features extraction was done with Array-pro6.3 (MediaCybernetics). Intensity files were analyzed with FlexArray 1.6.1 (Michal Blazejczyk, Mathieu Miron, Robert Nadon (2007), FlexArray: A statistical data

analysis software for gene expression microarrays. Genome Quebec, Montreal, Canada, URL : http://genomequebec.mcgill.ca/FlexArray).

Specifically, raw data were corrected by background subtraction then normalized within and between each array (Loess and quantile, respectively). Stastistical comparison between treatments (hyperglycemia vs. control) was done with the Limma algorithm. Significant differences between treatments were determined with a P-value less than 0.05.

The data discussed in this publication have been deposited in NCBI`s Gene Expression Omnibus and are accessible through GEO Series accession number GSE33008 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33008).

Validation of microarray results was done by RT-qPCR. Total extracted RNA from independent samples (3 replicates for each condition) were reverse-transcribed using oligo-dT primer and qScript™ Flex cDNA Synthesis Kit (Quanta Biosciences). Specific primers for each selected gene were designed using PrimerQuestSM (Integrated DNA Technologies) and qPCR were performed using LightCycler 480® SYBR Green I Master and the LightCycler® 480 System (Roche). A standard curve constituted of five points of the PCR product for each primers pairs diluted from 1 pg to 0.1 fg was used for real-time quantification of PCR output. Data normalization used GeNORM normalization factor (Vandesompele et al. 2002) from expression values of 3 reference genes (ACTB, CHUK, B2M). Moreover, technical variations were assessed and corrected through quantification of exogenous GFP spike which was introduced at the time of RNA extraction (Vigneault et al. 2004). Primer sequences, product size, annealing temperature and accession numbers are provided in Supplemental Table 4.

Functional analysis of differential gene expression profile in treated blastocysts

DAVID software was used to group overrepresented functions of differentially expressed genes into clusters (Huang da et al. 2009a; Huang da et al. 2009b). Moreover, data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). IPA served to compile canonical pathways as well as gene product

interactions (networks) that are differentially expressed between treatments. We used IPA to build schematic representations of important pathways deregulated in treated blastocysts.

Network Generation: A data set containing gene identifiers and corresponding expression values was uploaded into the application. Each identifier was mapped to its corresponding object in Ingenuity's Knowledge Base. A fold-change cut-off of 1.5 with a p-value <0.05 was set to identify molecules whose expression was significantly differentially regulated. These molecules, called Network Eligible Molecules, were overlaid onto a global molecular network developed from information contained in Ingenuity's Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Human, mouse, and rat orthologs of a gene are stored as separate objects in the Ingenuity Pathways Knowledge Base, but are represented as a single node in the network. Nodes are displayed using various shapes that represent the functional class of the gene product.

Canonical Pathway Analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed; 2) Fisher's exact test was used to calculate a p-value to determine the probability that the association between the genes in the dataset and the canonical pathway would occur by chance alone. Green and red symbols represent genes respectively down- and up-regulated in treated embryos compared to controls. Grey symbols represent genes with significant expression in blastocysts but no difference between conditions, whereas white symbols represent genes not present on microarray or with below-background intensity.

2.5 Results

5 mM glucose is a critical dose for the pre-attachment development of in vitro produced bovine embryos.

In order to clearly characterize the impact of hyperglycemia on embryo development, IVP zygotes were exposed to increasing glucose doses until reaching the limit of embryonic plasticity. Different glucose concentrations were used (control: 0.2 vs 1, 2, 5, 10 mM) during the early cleavage stages of development (until the 8/16-cells stage), then all embryos were cultured in control media until reaching the blastocyst stage. Our preliminary results showed that adding 1 to 5 mM of glucose did not affect the developmental rate of IVC 8/16-cells (please see supplemental table 2). However, a 10 mM concentration of glucose decreased the rate of embryos reaching the 8/16-cells stage and prevented blastocyst development. Therefore, the increased glucose dose of 5 mM was used to produce more replicates of treated and control blastocysts. In regard to preliminary results, statistical comparison between control and 5 mM glucose treated embryos showed a significant decrease in the blastocyst rate and hatching rate after high glucose treatment (Table 1). The small discrepancy with the preliminary results could be explained by the larger sample size of cultured embryos and the overall higher rate of blastocyst development. Nonetheless, these results indicated that early exposure to 5 mM glucose was detrimental for subsequent survival of bovine embryos.

5 mM glucose exposure during early cleavages stage impacts on subsequent gene expression at blastocyst stage.

Microarray experiments

Large-scale transcriptomic comparison between control and 5 mM glucose-treated embryos at the blastocyst stage was done through microarray analysis. The microarray design covers the majority of the bovine pre-attachment transcriptome that allows analyzing most forms of gene expression in bovine blastocysts (Robert et al. 2011a). Out of 37,238 targeted gene transcripts that are represented on the microarray slide, 22,681 and 22,886 had a signal higher than the summation of background intensity plus 2 times the standard

deviation of background, indicating the presence of the mRNA for these genes in control and high glucose treated blastocysts respectively (Figure 4). Statistical analysis of microarray comparisons revealed a significantly different expression ($p<0.05$) for 490 transcript sequences and 63 had more than 1.5 fold-change differences between control and treated blastocysts (please see Supplemental Table 3). Moreover, among these 63 sequences, 55 were up-regulated in treated blastocysts.

Functional analysis

Sex-related genes: In order to determine if the differentially expressed genes were associated with skewing of the sex ratio in 5 mM glucose treated blastocysts, the proportion of genes that could be related to embryonic sex was analyzed (Bermejo-Alvarez et al. 2010). Among hyperglycemia-related genes found in bovine blastocysts, only 6.2 % showed a moderate (fold-change>1.5, p-value<0.05) overlap with sex-related genes (clusterin, myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog. drosophila) translocated to 11, SERPINE1, thrombospondin 1).

Stage-related genes: Even if similar proportion of hatched blastocyst from each pool of control and treatment were used in the microarray comparison, the proportion of genes that could be related to blastocyst stage (young, expanded, hatched) were analyzed in order to discard potential bias linked to embryonic developmental speed (Rekik et al. 2011). Among hyperglycemia-related genes found in bovine blastocysts, only 1 gene (collagen triple helix repeat containing 1) showed a moderate overlap with stage-related genes (fold-change>1.5, p-value<0.05). If several of the stages specific targeted genes would have associated with the treatment, it could have indicated a stage effect in addition to a treatment effect.

Cellular and molecular functions: DAVID software significantly clustered molecular functions of extra-cellular matrix remodelling as well as cell adhesion. These functions were associated with cell motility, defense/response to external wounding/stimulus and calcium management. Using Ingenuity Pathway analysis (IPA), the canonical pathways with significant value were high-mobility group box 1 (HMGB1) signalling, integrin-linked kinase signalling, hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix) transcription factor signalling, transforming growth factor, beta signalling, oxidative stress

response, tumor necrosis factor receptor superfamily, member 1A signalling, tight junction signalling, interferon signalling, glutathione metabolism and peroxisome proliferator-activated receptor alpha signalling. Ingenuity-generated networks reflected processes of tissue development, metabolic disease and tumor morphology. Figure 5A shows the canonical pathway of HMGB1 signalling. Figure 5B shows the regulation network of HIF1A signalling pathway.

Reverse transcription–qPCR

A total of 17 selected genes from microarray and functional analysis (*ADAMTS1, ASCL2, C1QTNF3, EDN3, GPX8, HIF1A, HTRA1, IGFBP7, JAM2, LDHA, PPARG, SERPINE1, TGM2, TKTL1, TNFRSF1A, TP53BP2, VIM*) were quantified in 3 independent biological replicates from control and treated groups by RT-qPCR (Figure 6, for gene names please see Supplemental Table 4). Results showed positive validation of differential expression for 82% of selected genes (59% [10/17] with a $p<0.05$, and 24% [4/17] with 90 % confidence interval). Among selected candidates, relative expression differences (fold change) from microarray and RT-qPCR experiments were significantly correlated ($R=0.69$, $p<0.0001$). When looking only at the selected genes that exhibited a positive 1.5-fold change in the microarray comparison, differential expression was validated by RT-qPCR for 100% of them (6/10 with a $p<0.05$ and 4/10 with a $p<0.1$). Overall, these validations allow confident interpretation of results obtained by array hybridization and statistical/functional analysis.

2.6 Discussion

Transcriptomic analysis is useful to understand the impact of *in vitro* culture on embryo development, but gene expression profile associated with metabolic stress need characterization. Therefore, this study investigated the impact of exposure to metabolic stress during IVC on gene expression modifications in IVP bovine blastocysts. Hyperglycemia during early cleavage stages was selected as the stress condition because it has been shown to be detrimental for early zygote divisions and subsequent blastocyst survival.

In our preliminary dose-response experiment, bovine zygote exposure to 10 mM glucose prevented blastocyst development due to cleavage arrest during progression to 8/16-cells stage. This observation is in agreement with previous publications which have shown that increased glucose concentration in the medium blocks early cleavage development (Lane and Gardner 2001; Moley et al. 1996). This developmental block reflects a phenomenon known as the Crabtree effect, whereby high intracellular glucose stimulates glycolysis that perturbs oxidative phosphorylation by altering mitochondrial metabolism (Barnett and Bavister 1996a; Gardner et al. 2000b; Lane and Gardner 2001; Seshagiri and Bavister 1991) (Barcelo-Fimbres and Seidel 2007). When 5 mM glucose was used, early cleavage bovine zygotes exhibit developmental plasticity as seen by the ability to reach 8-16 cells. However, after transferring the embryos into normal culture conditions, subsequent development to the blastocyst stage and hatching was decreased. These findings are consistent with other studies in hamster and cow which have shown that post-compaction development is compromised when cleavage-stage embryos are exposed to mild hyperglycemia (1.5-5 mM)(Barnett and Bavister 1996a; Furnus et al. 1997).

The molecular mechanisms underlying developmental impairment of blastocysts after early hyperglycemic treatment (5m M) are not clearly understood but are likely to result from non-blocking perturbations induced by the Crabtree effect during cleavage stages (Barnett and Bavister 1996a). Transcriptomic analysis revealed that high glucose treated blastocysts showed, in association with lower survival rate, a dysregulation of gene expression. These results are in agreement with other findings showing that environmental

conditions influence embryonic gene activation (Fernandez-Gonzalez et al. 2009; Smith et al. 2009) and may reflect perturbed development (Rizos et al. 2002b). When looking at the ratio of up- and down-regulated genes in treated blastocysts, almost all are over-expressed. Gene over-expression is characteristic of *in vitro* produced embryos compared to their *in vivo* counterparts and could be associated with active, "unquiet" metabolism (Cote et al. 2011a). Thus, major up-regulation of gene expression in treated blastocysts would reflect a disruption of metabolic "quietness".

The study of differential mRNA level needs cautious interpretation as the correspondence with the protein level has not been demonstrated. Here, functional analysis of differentially expressed genes showed strong similarity with gene expression in hyperglycemia-associated diabetes. In diabetes, hyperglycemia increases intracellular glucose and results in its conversion to sorbitol by aldose-reductase and concomitant decrease in NADPH (polyol pathway). Hyperglycemic induction of polyol pathway has deleterious effects due to NADPH depletion that is required to reduce glutathione and protect cells from reactive oxygen species (H_2O_2) (Lee and Chung 1999). 5 mM glucose treated blastocysts showed increased expression of *GPX8* (glutathione peroxidase 8), a member of the anti-oxidant enzyme family involved in the reduction of H_2O_2 into water by oxidation of glutathione. *GPX* expression is highly correlated with increased aldose reductase activity in the pathology of diabetes (Hodgkinson et al. 2003). Moreover, embryos cultured with high glucose show increased sorbitol production and a delay in blastocyst stage development (Moley et al. 1996). As GPX and glutathione play a major anti-oxidant role during pre-implantation embryonic development (El Mouatassim et al. 1999; Takahashi et al. 1993), *GPX8* expression suggests the activation of oxidative stress response likely due to NADPH depletion through polyol pathway activation in treated blastocysts.

Diabetic hyperglycemia leads to production of advanced glycation end-products (AGE) that modify the expression of extra-cellular matrix (ECM) proteins (Brownlee 2001). Our data show an increased expression of lumican and decorin, ECM proteoglycan that are targeted by AGE modification and up-regulated in diabetic kidney (Sanchez and Sharma 2009; Schaefer et al. 2001). High glucose treated blastocysts up-regulate *JAM2*, a junction adhesion molecule which may be associated with angiogenesis in metabolic alterations of

diabetes mellitus (Javerzat et al. 2009). Finally, AGE modifications disrupt receptor binding and result in the expression of genes coding for a cytokine (*TGF-B*) and a pro-inflammatory molecule (*PLAT*) (Brownlee 2001; Doi et al. 1992) that are both up-regulated in treated blastocysts and suggest a critical role for AGE production in the impact of hyperglycaemia on early embryo development. These matrix-related responses like in somatic tissues either indicate that the blastocyst is reacting such as a somatic cell or hypothetically indicate a dysregulation by expressing genes that are normally quite silent in blastocysts. As AGE production is negatively correlated with reproductive health (Hao et al. 2008; Jinno et al. 2011; Konishi et al. 2004; Oliver et al. 2011); AGE-associated gene expression may be valuable markers of lower survival rate observed in treated blastocyst.

Activation of the hexosamine pathway is a hallmark of hyperglycemia in diabetes. Due to fructose-6-P diversion from the glycolytic pathway, concomitant accumulation of glucosamine-6-P induces protein glycosylation of transcription factor Sp1 which activates *SERPINE 1* and *THBS1* expression and contributes to the pathogenesis of diabetic complications (Brownlee 2001) (Du et al. 2000; Kolm-Litty et al. 1998; Raman et al. 2007) (Wang et al. 2002; Wang et al. 2003). During murine preimplantation development, hexosamine pathway activation and o-linked glycosylation mediates the embryotoxic effect of hyperglycemia (Pantaleon et al. 2010). Up-regulation of *THBS1* and *SERPINE1* in treated blastocysts suggests the increased activity of hexosamine pathway and could be markers of developmentally compromised embryos. In addition, *THBS1* expression is regulated through activation of aryl hydrocarbon receptor (AHR) in endothelial cells exposed to high glucose (Dabir et al. 2008).

Diabetes-associated hyperglycemia also affects protein kinase C (PKC) signalling. We observed an up-regulation of *S100A11*, a gene coding for a Ca^{2+}-binding protein, in high glucose treated blastocysts. S100 members are secreted proteins that are dependent on parallel activation of PKC as well as elevation of intracellular calcium concentrations (Foell et al. 2007) and their expression is up-regulated by hyperglycemia (Yao and Brownlee 2010). Moreover, phosphorylation of S100A11 by PKC likely occurs through interaction with AGE receptor signalling (Leclerc et al. 2009). Treated blastocysts showed up-regulation of *SCG2* (secretogranine II), a gene coding for a secreted protein whose

expression is controlled by calcium influx and PKC activation in bovine medulla cells (Turquier et al. 2002). Also, calcium and calmodulin activate *ADAMTS1* expression (Keightley et al. 2010), a gene coding for a thrombospondin motif-containing metalloproteinase, up-regulated in treated blastocysts. Up-regulation of vimentin was also observed and appears to occur in diabetic nephropathy (Sanai et al. 2000). Vimentin is an important structural protein of intermediate filaments that exhibits organizational changes following various stimuli and transient association with PKC (Goldman et al. 1996). Hyperglycemia would disrupt cytoskeletal organization and affects mitochondrial distribution (Barnett et al. 1997) as well as calcium signalling (Van Blerkom et al. 2006).

The unified cause of all diabetic complications originates from hyperglycemia-induced uncoupling of oxidative phosphorylation (OX-PHOS) which induces mitochondrial reactive oxygen species production (ROS). Thereafter, ROS inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity and diverts glycolytic metabolites which stimulate the polyol, AGE, PKC and hexosamine pathways (Brownlee 2001; Korshunov et al. 1997; Nishikawa et al. 2000). Collectively, these changes suggest that gene expression associated with diabetes in treated blastocysts may result from GAPDH inhibition by mitochondrial ROS produced during the period of hyperglycaemia. It is consistent with experiments showing that porcine embryos cultured with elevated glucose exhibit a rise in ROS generation at the 1-cell stage (Karja et al. 2006). Moreover, decreased GAPDH activity was observed in embryos from diabetic rats or embryos cultured in hyperglycemia conditions (Wentzel et al. 2003).

Associated with increased ROS production, uncoupling of OX-PHOS alters tri-carboxylic acid cycle (TCA) activity in diabetes-associated hyperglycemia (Kim et al. 2008). High glucose treated blastocysts showed a lower expression of citrate synthase, an enzyme involved in the entry of pyruvate-derived acetyl-CoA towards TCA and production of NADH for OX-PHOS. Decreased activity of citrate synthase is correlated with the pathology of diabetes and the potential dysregulation of mitochondrial oxidative capacity (Simoneau and Kelley 1997). Decreased TCA activity in treated blastocysts may also be reflected by up-regulation of *PPARG*, a key regulator of lipid metabolism (He et al. 2003). Preimplantation embryos undergo lipid oxidation (Dunning et al. 2010) which depends on

TCA activity in mitochondria (Kelley et al. 2002). *In vitro* cultured bovine embryos show altered lipid metabolism as seen by the lipid accumulation in embryonic cells (Rizos et al. 2002a) and over-expression of *PPARG* (unpublished data). Dysregulation of mitochondrial TCA may induce lipid accumulation and *PPARG* expression in treated blastocysts (El-Assaad et al. 2003). In addition, lipid accumulation and oxidative stress due to mitochondrial dysfunction may be the rational of higher expression of *OLR1* in treated blastocysts (Sakurai and Sawamura 2003) (Mabile et al. 1997) (Bakker et al. 2000). OLR1 (oxidized low-density lipoprotein receptor 1) is a receptor involved in the scavenging of oxidized lipids and is up-regulated in diabetes (Chen et al. 2001; Ethier-Chiasson et al. 2008), preeclampsia (Lee et al. 2005) and during cell culture under high-glucose conditions (Taye et al. 2010). Also, oxidized lipid accumulation may be responsible for the up-regulation of *TNFRSF1A* (de Mello et al. 2008; Lim et al. 2008) and *C1QTNF3* (Kopp et al. 2010) which are associated with adipose tissues inflammation in diabetes.

Post-compaction development and hatching are energy-demanding and correlate with increased mitochondrial OX-PHOS (Dumollard et al. 2009), activation of glycolysis and increased glucose up-take which contribute to ATP synthesis (Thompson 2000). Treated blastocysts showed increased expression of *LDHA*, a gene coding for the main enzyme of anaerobic conversion of pyruvate into lactate which produces NAD+ and stimulates glycolysis. Moreover, *PDGFC* and *HIF1A* are also up-regulated. These two gene products contribute to the expression of *LDHA* (Dafni et al. 2010) and promotes anaerobic glycolysis in tumor cells (Semenza 2003; Zhao et al. 2009), a phenomenon identified by Otto Warburg in 1924 (Vander Heiden et al. 2009). The "Warburg effect" describes the metabolic shift observed during tumorigenesis when malignant cells increase glucose metabolism toward production of lactate rather than through mitochondrial OX-PHOS (Cairns et al. 2011). Transketolase 1, a gene coding an enzyme that catalyzes the non-oxidative part of the pentose phosphate pathway, was up-regulated in treated blastocysts. This enzyme is expressed in tumor cells in order to enhance glucose usage and lactate production (Langbein et al. 2006), and is known to be associated with the Warburg effect (Kayser et al. 2010; Krockenberger et al. 2010; Xu et al. 2009).

The observed *HIF1A* up-regulation and the associated Warburg effect (Archer et al. 2008; Ashrafian et al. 2010; Harvey et al. 2007; Selak et al. 2005) may result from mitochondria failing to perform OX-PHOS in treated embryos. Stimulation of *HIF1A* transcription may be mediated by overproduction of ROS and subsequent activation of PIK3-AKT/PKC/histone deacetylase pathways (Koshikawa et al. 2009). Moreover, hyperglycaemia is correlated with defects in mitochondria biogenesis in diabetes (Mootha et al. 2003) (Kim et al. 2008; Ren et al. 2010) and mitochondria maturation is influenced by in vitro culture during the pre-implantation development growth (Crocco et al. 2011a). Perturbed mitochondria maturation would participate in Warburg effect induction in treated blastocysts.

Diabetic hyperglycemia is often associated with cancer predisposition (Kellenberger et al. 2010; Yeung et al. 2008) and may be a driving force for the metabolic shift observed in treated blastocysts (Moley et al. 1994). However, even if embryonic cells could adapt their metabolism through a common mechanism with cancer cells, it is likely that, at some point, early development would avoid a tumor fate. Treated blastocysts show up-regulation of *IGFPB7* and *TP53BP2*, genes which are known to act as tumor suppressor (Ruan et al. 2007). TP53BP2 and p53 have synthetic action in regulating energy metabolism in cancer cells (Feng and Levine 2010). TP53BP2 mediates apoptosis associated with mitochondria death pathway and depression of the mitochondrial trans-membrane potential (Kobayashi et al. 2005). Moreover, TP53 regulates the IGF1/MTOR pathway and controls *IGFBP7* expression (Suzuki et al. 2010). As hyperglycemia induces apoptosis through TRP53 signalling in mouse blastocysts (Keim et al. 2001), the establishment of a tumor-like metabolism associated with Warburg effect may activate *TP53BP2* and *IGFBP7* in order to drive embryonic cell death and compromise the development of treated blastocysts. This hypothesis is reinforced by the correlation between early embryonic lactate production and gestational demise in mouse (Gardner and Lane 1996) and human (Gardner et al. 2011).

In conclusion, we show here the detrimental impact of early high glucose stress on subsequent pre-attachment embryo survival and its association with differential gene expression at blastocyst stage. Functional analysis suggests that high-glucose exposure during early cleavage stages increases ROS production (Figure 7-IIb), a hypothesis that would imperatively require molecular analysis at earlier stages in the embryo development.

However, gene expression associated with diabetes reveals altered glucose utilization during post-compaction development of treated embryos (Figure 7-II-c,d). Accordingly, the embryonic response would be an increase of anaerobic glycolysis (Warburg effect) to compensate impairment of energy metabolism as well as limit ROS generation (Le et al. 2010), although this metabolic alternative would be inefficient to support proper blastocyst development and subsequent gestation (Betts and Madan 2008; Gardner and Lane 1996; Wilding et al. 2009).

Taken together, these results shed new light on how the environment affects early embryo development. In order to complement these findings, overlapping the present results with other stress conditions would allow estimating the potential contribution of mitochondrial dysfunction in the developmental impairment of hyperglycemia-treated embryos. Nevertheless, these functional genomic analyses are now providing original insights for the development of stress marker examination in IVC embryos and eventually the design of optimized culture conditions in animal and human ART.

2.7 Acknowledgments

We thank Isabelle Laflamme and Julie Nieminen (Université Laval, Québec, CANADA) for technical assistance in the procedure of *in vitro* embryo production and manuscript writing respectively; and Christine Power (École des Arts Visuels, Université Laval, Québec, CANADA) for assistance in figure designing.

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2.9 Tables and figures

Table 1 Effect of high glucose concentration (5 mM) during *in vitro* culture on cleavage rate, blastocysts rate and hatching rate of *in vitro*-produced bovine embryos.

Culture medium Days 0 to 3	Number of presumptive zygotes	Number of cleaved embryos day 3	Cleavage rate day 3	Number of 8-16 cells embryos day 3	8-16 cells rate day 3	Culture medium days 3 to 7	Number of blastocysts day 7	Blastocyst rate day 7	Hatching rate day 7
Control	253	224	88.5%[a]	125	49.4%[a]	Control	96	37.9%[a]	43.8%[a]
5 mM Glucose	311	257	82.6%[a]	127	40.8%[a]	Control	71	22.8%[b]	28.8%[b]

After in vitro maturation and in vitro fertilization, presumptive zygotes were cultured in SOF with 0.2 mM (Control) or 5 mM glucose during cleavage stages (days 0 to 3), and total embryos were then transferred to control media to complete their development until the blastocyst stage. Un-paired t test was used for statistical analysis. a-b Values in the same column with different superscripts are significantly different (P<0.05). Standard Error of Mean in blastocyst rate and hatching rate were respectively: control group= 4.9% and 7.3%; 5 mM glucose group = 2.1% and 7.0%.

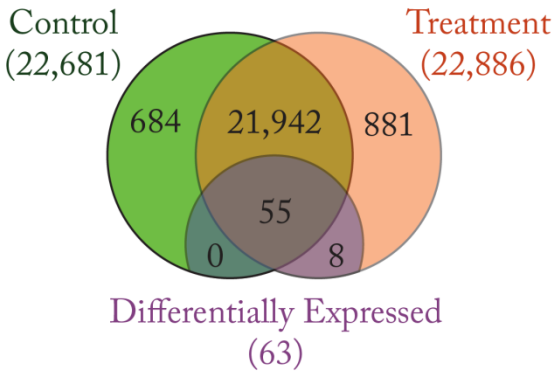
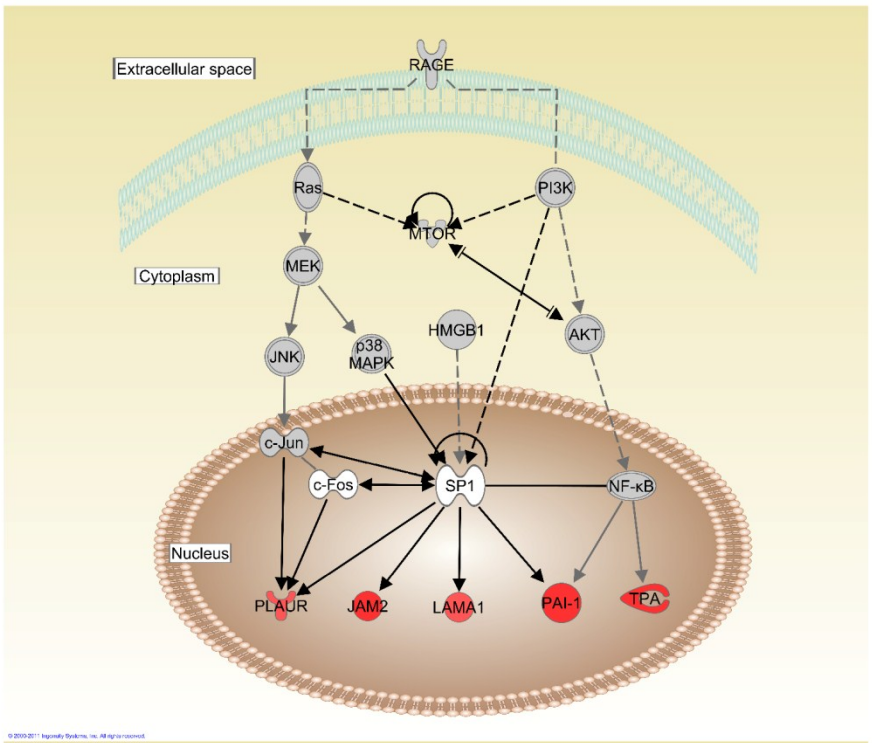


Figure 4 Number of expressed gene in control or treated blastocysts as well as differentially expressed between these two conditions.

In control and treatment conditions, embryonic gene expression was assessed through microarray technology. Results indicate the number of probes with an intensity signal higher than the summation of background intensity and 2 time its standard deviation for each condition (green and brown). Moreover, overlaping with significantly differential expressed genes between conditions (violet) is represented (superior to 1.5 fold-change, $p<0.05$)

A


Extracellular space
RAGE
Ras
PI3K
MTOR
Cytoplasm
MEK
HMGB1
AKT
JNK
p38 MAPK
c-Jun
c-Fos
SP1
NF-κB
Nucleus
PLAUR
JAM2
LAMA1
PAI-1
TPA

B

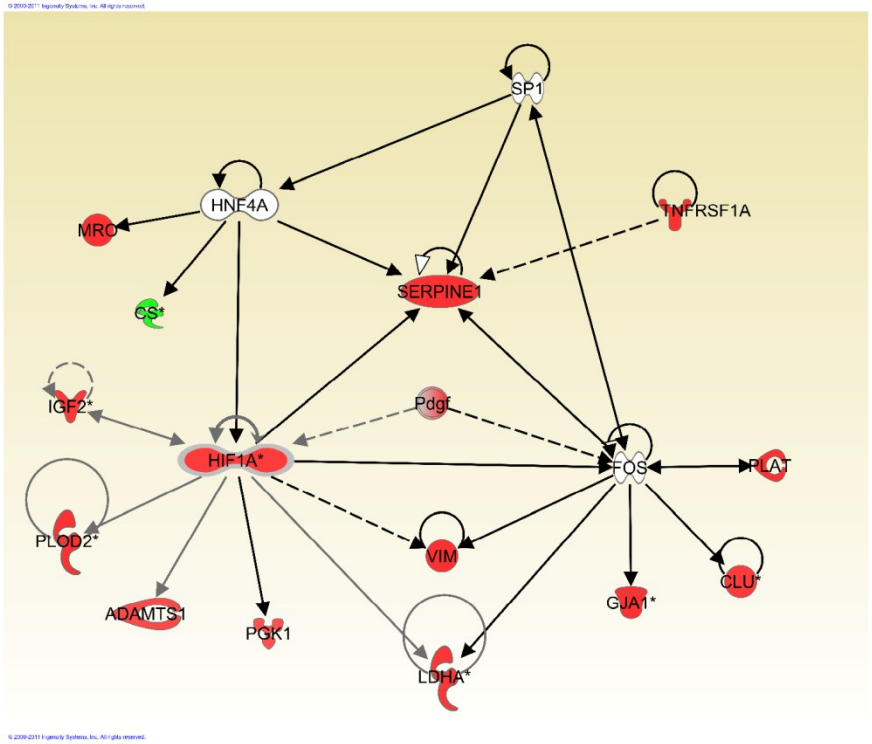

SP1
HNF4A
MRC
TNFRSF1A
SERPINE1
CS*
IGF2*
Pdgf
HIF1A*
FOS
PLAT
PLOD2*
VIM
CLU*
GJA1*
ADAMTS1
PGK1
LDHA*

Figure 5 Significantly represented pathways within differentially expressed genes in treated blastocysts compared to control.

A) IPA library of canonical pathways determined HMGB1 (high-mobility group box 1) signaling pathway as the most affected at the mRNA level in treated blastocysts. B) First network generated by IPA showing only relationships based on expression regulation between gene products. Red and green symbols show up- and down-regulated genes respectively. For gene names, please see Supplemental Table 3.

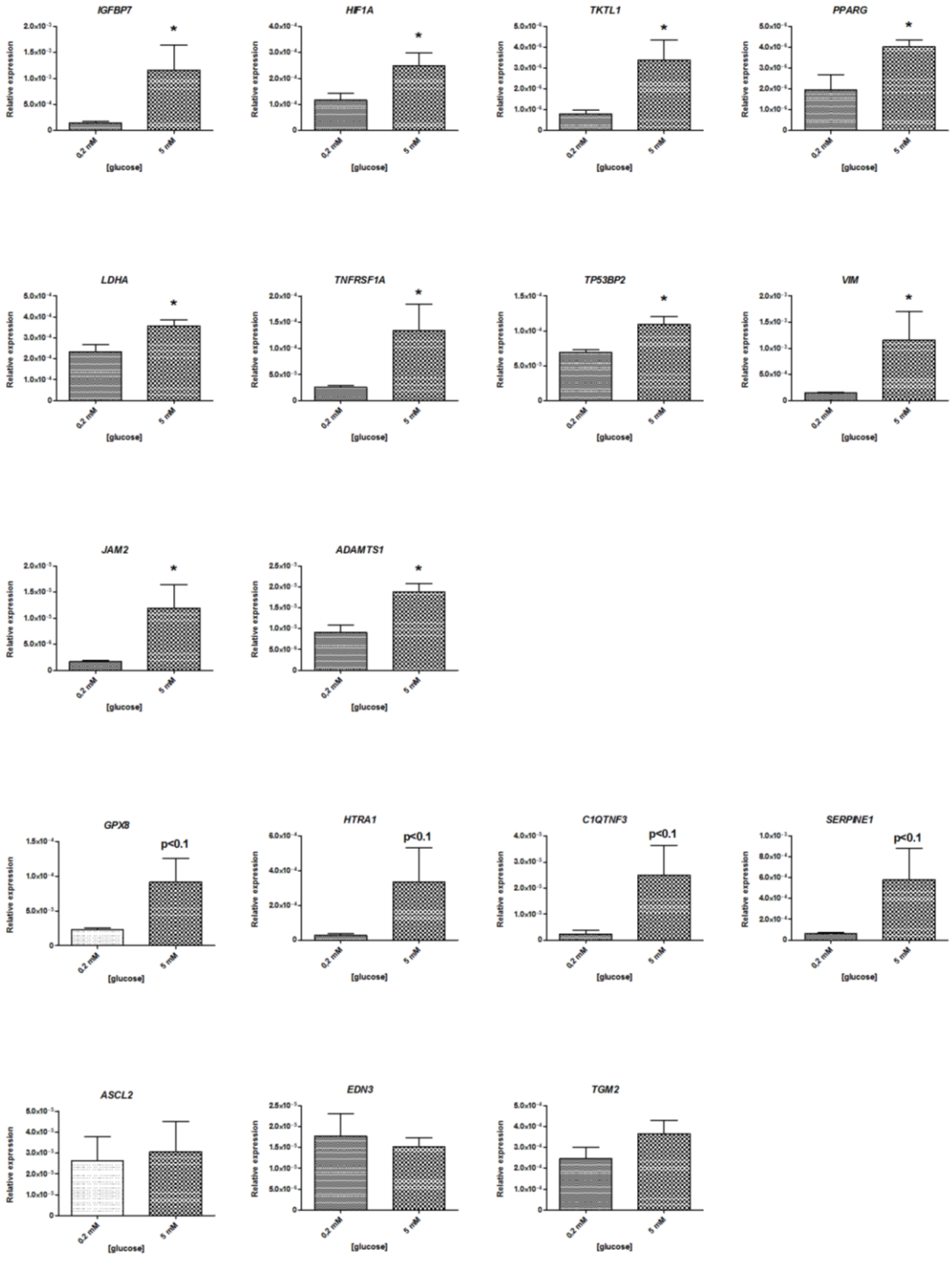

IGFBP7
HIF1A
TKTL1
PPARG
LDHA
TNFRSF1A
TP53BP2
VIM
JAM2
ADAMTS1
GPX8
HTRA1
C1QTNF3
SERPINE1
ASCL2
EDN3
TGM2
Relative expression
[glucose]
0.2 mM
5 mM
*
p<0.1

Figure 6 Quantification by reverse transcription–qPCR of the mRNA profile in bovine blastocysts cultured with 0.2 mM (control) or 5 mM glucose during early cleavage stages.

*Analysis was done in triplicate (pools of 10 blastocysts each) and the amount of mRNA represents the mean±SEM of each transcript corrected with GFP and 3 housekeeping genes. *Significantly different from control with P<0.05. p<0.1Significantly different from control with P<0.1.*

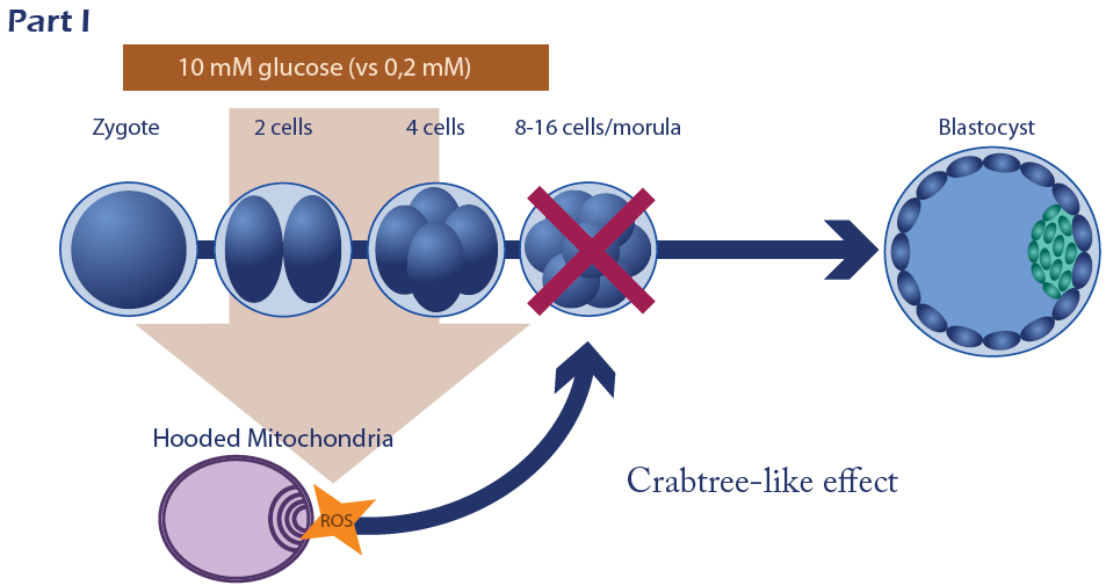
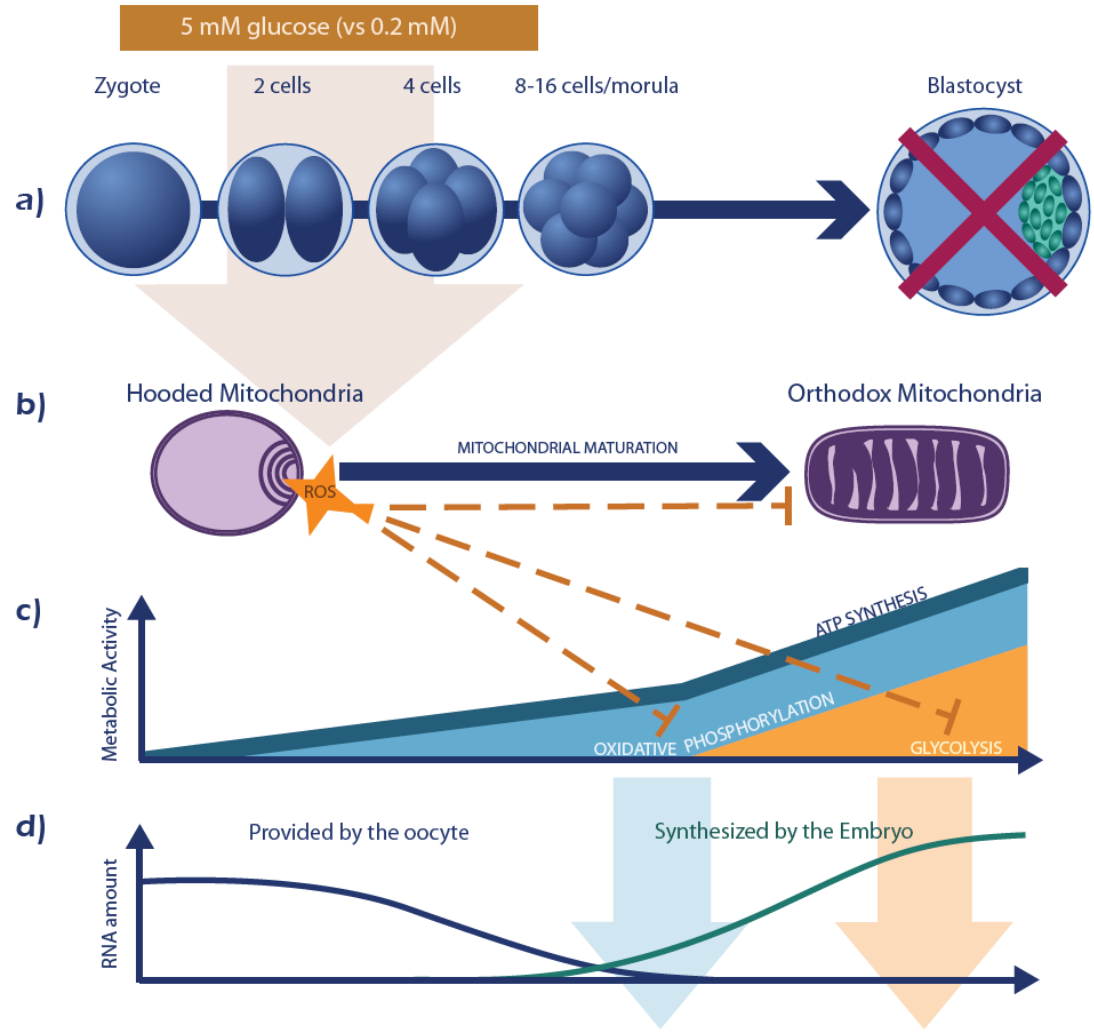


Figure 7 Summary of experimental design and results.

Part I shows the developmental block at 8/16-cells after a 10 mM glucose exposure during in vitro culture of bovine embryos. Mitochondrial perturbation is thought to result from a Crabtree effect that would alter embryo metabolism and progression beyond embryonic genome activation.

Part II (on the right) shows how a non-blocking dose of glucose (5 mM) during cleavage stage affects subsequent survival (a) and gene expression at the blastocyst stage (d). Functional analysis of differentially expressed genes showed a massive up-regulation of signalling pathways associated with diabetic complications. These results reinforce the hypothesis that early hyperglycemia would induce mitochondrial ROS production (b) that perturbs energetic balance (c) which would drive the embryo into a response similar to the Warburg effect.

Part II



Abbreviations :
ADAMTS1 : ADAM metallopeptidase with thrombospondin type 1 motif, 1
AGE : Advanced Glycation End-product
C1QTNF3 : C1q and tumor necrosis factor related protein 3
DCN : decorin
GPX8 : glutathione peroxidase 8
HIF1A : hypoxia-inducible factor alpha
IGFPB7 : insulin-like growth factor binding protein 7
JAM2 : junctional adhesion molecule 2 (or B)
LDHA : lactate dehydrogenase A
LUM : lumican
OLR1 : oxidized lipid receptor 1
PDGFC : platelet-derived growth factor c
PLAT : plasminogene activator, tissue
PPARG : peroxisome proliferator-activated receptor gamma
ROS : Reactive Oxygen Species
SCG2 : secretogranin II
THBS1 : thrombospondin 1
TKTL1 : transketolase 1
TGFB : transforming growth factor beta
TNFRSF1A : tumor necrosis factor receptor superfamily member 1A
TP53BP2 : tumor protein p53 binding protein, 2

Direct impact (similar to diabetic complications)

- Lipid accumulation (*PPARG*)
- Lipid oxidation (*OLR1*)
-Inflammation (*C1QTNF3, TNFRSF1A*)

- Hexosamine pathway (*SERPINE1,THBS1*)
- PKC signalling pathway (*S100A11, SCG2, ADAMTS1*)
- Polyol pathway (*GPX8*)
- AGE production (*LUM, DCN, JAM2, PLAT, TGFB*)

Metabolic response (Warburg-like effect)

- Increase of anaerobic glycolysis (HIF1A, PDGFC, LDHA, TKTL1)
- Tumor supressor induction (TP53BP2, IGFBP7)

2.10 Supplemental data

Supplemental table 2 Effect of different increasing glucose concentrations in culture medium on cleavage rate, blastocysts rate and hatching rate of *in vitro*-produced bovine embryos.

Culture medium Days 0 to 3	Number of presumptive zygotes	Number of cleaved embryos day 3	Cleavage rate day 3	Number of 8-16 cells embryos day 3	8-16 cells rate day 3	Culture medium days 3 to 7	Number of blastocysts day 7	Blastocyst rate day 7	Hatching rate day 7
Control	225	168	74.7%	97	43.1%	Control	43	19.1%	40.8%
1 mM Glucose	85	68	80.0%	39	45.9%	Control	15	17.6%	60,0%
2 mM Glucose	64	46	71.9%	25	39.1%	Control	10	15.6%	31,0%
5 mM Glucose	104	87	83.7%	38	36.5%	Control	19	18.3%	32.1%
10 mM Glucose	31	19	61.3%	4	12.9%	Control	0	0.0%	-

After in vitro maturation and in vitro fertilization, presumptive zygotes were cultured in SOF with 0.2 mM (Control), 1 mM, 2 mM, 5 mM or 10 mM glucose during cleavage stages (days 0 to 3), and total embryos were then transferred to control media to complete their development until the blastocyst stage.

Supplemental table 3 List of differentially expressed transcripts between treated and control embryos.

Gene Symbol	Description	Target Location	Chromosome	Symmetrical raw fold change	P-value
LUM	lumican	Constitutive	5	3.711165	0.04275859
GEM	GTP binding protein overexpressed in skeletal muscle	Constitutive	14	2.851785	0.0230952
NDP	Norrie disease (pseudoglioma)	UTR3_Alt	X	2.669314	0.04892486
THBS1	thrombospondin 1	Constitutive	10	2.649551	0.03386412
MRO	maestro	Constitutive	24	2.63449	0.04355748
PLOD2	procollagen-lysine. 2-oxoglutarate 5-dioxygenase 2	UTR3_Alt	1	2.413614	0.01659458
JAM2	junctional adhesion molecule 2	Constitutive	1	2.365624	0.00222938
PLAT	plasminogen activator. tissue	Constitutive	27	2.295987	0.02249675
LOC782977	similar to pol protein	Constitutive	6	2.209126	0.02872206
HTRA1	HtrA serine peptidase 1	Constitutive	26	2.177118	0.0346412
SERPINE1	serpin peptidase inhibitor. clade E (nexin. plasminogen activator inhibitor type 1). member 1	Constitutive	25	2.150497	0.00573239
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	Constitutive	5	2.094754	0.03207349
ZNF385B	zinc finger protein 385B	Constitutive	2	2.073843	0.03593344
MMD	monocyte to macrophage differentiation-associated	Constitutive	19	2.028929	0.02974268
TNN	tenascin N	Constitutive	16	2.005405	0.03044687
CSRP3	cysteine and glycine-rich protein 3 (cardiac LIM protein)	Constitutive	29	1.998442	0.04565942
AMACR	alpha-methylacyl-CoA racemase	Constitutive	20	1.960288	0.01070676
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-	2	1.90746	0.00806099
PDGFC	platelet derived growth factor C	UTR3_Alt	17	1.89552	0.04226591
TNFAIP8L3	tumor necrosis factor. alpha-induced protein 8-like 3	Constitutive	10	1.875146	0.03869829
OAS1	2'.5'-oligoadenylate synthetase 1. 40/46kDa	Constitutive	17	1.834778	0.04658248
MLLT11	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog. Drosophila); translocated to. 11	UTR3_Alt	3	1.830352	0.02156465
LOC100295797	hypothetical protein LOC100295797	Constitutive	7	1.77893	0.02583769
RARRES1	retinoic acid receptor responder (tazarotene induced) 1	Constitutive	1	1.778098	0.03003668

XIRP1	xin actin-binding repeat containing 1	Constitutive	22	1.761856	0.0419245
TNFRSF1A	tumor necrosis factor receptor superfamily. member 1A	Constitutive	5	1.68621	0.00460907
MSRB3	methionine sulfoxide reductase B3	UTR3_Alt	5	1.682021	0.04718952
LOC100125763	neuronal protein 3.1	Constitutive	10	1.672302	0.02858995
IFIH1	interferon induced with helicase C domain 1	Constitutive	2	1.672151	0.02942048
GJA1	gap junction protein. alpha 1. 43kDa	UTR3_Alt	9	1.662923	0.04702752
CA2	carbonic anhydrase II	Constitutive	14	1.662889	0.03058051
CNRIP1	cannabinoid receptor interacting protein 1	Constitutive	11	1.651665	0.01881978
CTHRC1	collagen triple helix repeat containing 1	Constitutive	14	1.640481	0.03835929
KCNIP4	Kv channel interacting protein 4	Constitutive	6	1.639528	0.03850156
GPX8	glutathione peroxidase 8 (putative)	Constitutive	20	1.634375	0.0242371
NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	15	1.63068	0.03042861
C6H4ORF31	chromosome 4 open reading frame 31 ortholog	Constitutive	6	1.628935	0.00399406
GPR77	G protein-coupled receptor 77	Constitutive	18	1.628203	0.04817612
TSC22D1	TSC22 domain family. member 1	UTR3_Alt	12	1.597125	0.00865304
LDHA	lactate dehydrogenase A	Constitutive	29	1.592336	0.0334947
LDHA	lactate dehydrogenase A	Constitutive	X	1.585353	0.03453575
IFNGR2	interferon gamma receptor 2 (interferon gamma transducer 1)	Constitutive	1	1.575809	0.01217431
LOC100295130	similar to lysosomal-associated protein transmembrane 4B	Constitutive	19	1.565683	0.00649668
UPK1B	uroplakin 1B	Constitutive	1	1.553055	0.03434387
IRX5	iroquois homeobox 5	Constitutive	18	1.55045	0.02749228
NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	20	1.546891	0.00848578
HIF1A	hypoxia inducible factor 1. alpha subunit (basic helix-loop-helix transcription factor)	UTR3_Alt	10	1.544799	0.01886654
CLU	clusterin	Constitutive	8	1.54085	0.00243847

NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	9	1.539884	0.01096445
NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	9	1.536731	0.00805193
CSRP3	cysteine and glycine-rich protein 3 (cardiac LIM protein)	170^338	29	1.530509	0.04387436
PLOD2	procollagen-lysine. 2-oxoglutarate 5-dioxygenase 2	UTR3_Alt	1	1.52919	0.00700624
NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	11	1.525917	0.04934857
PTPRU	protein tyrosine phosphatase. receptor type. U	Constitutive	2	1.523917	0.04280317
NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	8	1.502874	0.01158911
NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	6	-1.504402	0.03176114
NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	17	-1.534463	0.04217633
BOLA	MHC class I antigen clone 2	Constitutive	23	-1.566756	0.0162385
NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	19	-1.571037	0.03764016
NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	28	-1.57179	0.01646698
NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	13	-1.579429	0.0129824
EDN3	endothelin 3	Constitutive	13	-1.603645	0.04498944
NULL	Novel Transcribed Region; evidence: embryonic ESTs	+	X	-1.64523	0.01175036

After hybridization, background correction, normalization and finally Limma statistical test were performed to determine the significant fluorescent intensity differences between treatments for each spot out of 44k present on the slide. Spots (named EMBV3_XXXXX) which were up- or down-regulated in treated blastocysts compared to control with a 1.5 fold change and a p-value<0.05 and their associated annotations are presented.

Supplemental table 4 Sequences of reverse transcription qPCR-specific primers of candidate genes expressed in bovine blastocysts.

Gene Symbol	Name	Accession number	Forward sequence	Reverse sequence	Product size (bp)	Annealing temperature (°C)
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	NM_001101080	ACAATCCCTGCTTC CTGATGCT	TGCTGGGCAGTCC TGAATTCTT	228	58
ASCL2	achaete-scute complex homolog 2 (Drosophila)	NM_001040607	TGACCCAAGGCTA GTGTGCAA	AGTGTCCCTGAGC AGTTCAAGT	209	58
C1QTNF3	C1q and tumor necrosis factor related protein 3	NM_001101138	TGTGTTCCACATTC ATGTCAGTC	CCTATTGGCAATGC ACTTCAAGG	248	55
EDN3	endothelin 3	NM_001101979	GCCAAGGTGCTAA TGCAATGGT	TGCGTTTCCGAGAT ACCATCCT	344	58
GPX8	glutathione peroxidase 8 (putative)	NM_001046088	ACTGCGAAGTGCTT GGAGAAGA	ACGATGCCTTCAAA CAGCTCCT	393	58
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	NM_174339	AGCAGTACATGGTA GCCGCAAT	AGGGTGGGCAGAA CATTTAGGT	411	58
HTRA1	HtrA serine peptidase 1	XM_002698550	AATTGACCCATAGG CAGAGGCA	AGCATAAACACGC CCAGTACCA	405	58
IGFBP7	insulin-like growth factor binding protein 7	NM_001102300	ACTGGTGCCCAGG TGTATTTGA	AAGCCTGTCCTTG GGAATTGGA	255	58
JAM2	junctional adhesion molecule 2	NM_001083736	ATCAGCTACATGCA CCCTCTGT	ATTCTGCCACCGTT CTGTGACT	173	58
LDHA	lactate dehydrogenase A	NM_174099	TGCTGGATGGCAA GGAATGGTT	TGGACTAGGCACC TTGGCTAAA	216	58
PPARG	peroxisome proliferator-activated receptor gamma	NM_181024	TGCTGAACGTGAA GCCCATTGA	TTCTGGAGCAGCTT GGCAAAGA	114	59

SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	NM_174137	ACCATCCAACTTCG GCTCACTT	TACTGAGTGTGGCT GTCACTGT	494	57
TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	NM_177507	AGTTGCCTATGCTG ATCACCCA	TCAGTGCTCGCTGT TTGTAGCT	429	58
TKTL1	transketolase-like 1	NM_001045972	TGGTGTTTGCCTTC ATCCCTCT	ATCTGACCAGAGA GCAGAGTGT	145	57
TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A	NM_174674	TGCAGTGCGTGTG TTTGTGTCT	ACGACTGAGCGAC TGAACTGAA	378	58
TP53BP2	tumor protein p53 binding protein, 2	XM_002693966	TGCCCTGTGTTGTA CTCCGAAA	ACGGTGCTACGAG GAATCATACCA	313	58
VIM	vimentin	NM_173969	TCTGAAGCTGCTAA CCGCAACA	TCGTGATGCTGGG AAGTTTCGT	493	58
ACTB	actin-beta	NM_173979	ATCGTCCACCGCA AATGCTTCT	GCCATGCCAATCTC ATCTCGTT	101	59
B2M	beta-2-microglobulin	NM_173893	AGACACCCACCAG AAGATGG	GGGGTTGTTCCAA AGTAACG	234	54
CHUK	conserved helix-loop-helix ubiquitous kinase	NM_174021	TGATGGAATCTCTG GAACAGCG	TGCTTACAGCCCAA CAACTTGC	180	57
GFP	green-fluorescent protein		GCAGAAGAACGGC ATCAAGGTGAA	TGGGTGCTCAGGT AGTGGTTGT	143	59

Chapitre 3 : Impact du stress énergétique au stade morula

Title: Transcriptomic profiling of early energetic stress response in bovine morulae

Cet article est en attente de soumission pour fin de publication.

Key words: Embryo, Genomics, Metabolism, Bovine, In vitro culture, Assisted Reproductive Technology

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Grant support: NSERC Strategic Network EmbryoGENE NETPG 340825-06

3.1 Résumé:

L'exposition précoce de l'embryon de mammifère à des conditions de glucose élevé (HG) réduit le taux de blastocyste et affecte l'expression des gènes associés au métabolisme énergétique. Pour déterminer les évènements à l'origine du phenotype relié au stress HG, cette étude a analysé les stades développementaux précédent la formation du blastocyste. Par rapport au taux de développement, les résultats ne montrent aucun effet phénotypique jusqu'au stade en morula, suggérant alors un effet du stress HG à la transition morula-blastocyste. Cependant, l'analyse microarray des morulas traitées au HG montre des différences par rapport aux morulas contrôle, et un profil global associé avec le stress oxydatif et la dysfonction mitochondriale. Il est important de noter que ce profile documente un groupe de morula dont la compétence à donner un blastocyste est variable. De ce fait, la validation par RT-qPCR des résultats a été performée sur des embryons individuels et indépendants de ceux utilisés pour l'analyse microarray. De façon attendue, de larges variations inter-individuelles sont observées dans l'expression des gènes à l'intérieur de la population de morulas. Cependant, certains patrons transcriptomiques spécifiques sont significativement différents entre morulas ayant subi le traitement contrôle ou HG. Notament, la population traitée à l'HG contenait très peu de morulas avec une expression positive pour HNF4A tandis que PLAGL1 était significativement plus élevé. Ces résultats indiquent des modifications précoces dans l'embryon et ceux avant tout impact sur le taux de developpement. De plus, une analyse génomique suggère que l'expression précoce du facteur de transcription HNF4A au stade morula serait potentiellement connectée aux modifications transcriptomiques associées à l'effet Warburg dans les blastocystes ayant survecu au stress. Dans son ensemble, cette étude illustre comment le stress énergétique peut conduire l'embryon précoce en culture à des modifications progressives et parfois létales de son profil transcriptomique.

3.2 Abstract:

Early exposure of the mammalian embryos to high glucose (HG) condition is known to decrease the blastocyst rate and to affect metabolic gene expression translating energetic stress. To determine the early events causing the phenotype of HG stress, this study analyses the early developmental stage prior to blastocyst formation. Developmental rate showed no phenotypical effect until morula stage, suggesting of a HG effect at the morula-to-blastocyst transition. However, microarray analysis of HG-treated morulae showed transcriptomic differences in comparison to control embryos, difning a global profile associated with oxidative stress and mitochondrial dysfunction. It is important to note that this profile translate pooled morulae with variable competence to develop to the blastocyst stage. Thereofer, microarray validation were assessed by RT-qPCR analysis on single morulae. As expected, results showed large inter-individual variations in gene expression within the morulae's population. However, specific transcriptomic patterns were significantly different between morulae from control or HG treatment. Notably, HG-treated population contained very few morulae with positive HNF4A expression while PLAGL1 expression was significantly higher. This result indicates early modifications of embryonic development before any impact on blastocyst rate. Moreover, further genomic analysis suggested that the expression of HNF4A transcription factor at the morula stage was connected to the transcriptomic changes related to the Warburg effect in stress surviving blastocysts. Taken together, this study illustrates how energetic stress may drive early IVC embryos to progressive and sometimes lethal modifications of their transcriptomic profile.

3.3 Introduction

Despite the fact that millions of human babies and farm animals have been born through assisted reproductive technology (ART), a large body of literature indicates that in vitro handling of gametes and embryos might affect the normal physiology of pregnancy (Watt et al. 2011) and post-natal growth (Camargo et al. 2010; Dumoulin et al. 2009). In particular, the potential impact of in vitro culture (IVC) conditions used during early embryo development has been pointed out (Thompson 2000; Thompson et al. 2007; Thompson and Peterson 2000) and numerous reports have correlated early IVC stress with health impairments (Farin and Farin 1995; Wakefield et al. 2011), a concept consistent with the developmental origin of health and disease hypothesis (Lazzari et al. 2002). In order to understand how IVC affects embryo development, investigation is required in order to characterize the mechanisms that underlie embryonic stress during IVC and how they are associated with lower viability. Ultimately, it could permit the identification of biomarkers of embryonic stress that will help to optimize the culture medium to improve embryonic quality (Nelissen et al. 2012).

Among different strategy to assess the stress of IVC, transcriptomic analysis has emerged as a relevant tool to understand embryonic physiology and identify developmentally competent embryos (Jones et al. 2008). After early cleavage stage, transcription of the embryonic genome is activated (EGA) and this characterizes the maternal embryo transition (8-16 cells stage in cow) (Vigneault et al. 2004). EGA is crucial to support subsequent post-compaction development (Vigneault et al. 2009) and enables embryos to respond to environmental changes (Watkins et al. 2008). Analysis of embryonic transcriptome has shown that gene expression is modified in IVC embryos compared to their in vivo counterpart (Lonergan et al. 2003a; Rizos et al. 2002b), indicating the plasticity of embryonic gene expression (Fernandez-Gonzalez et al. 2009). Moreover, impact of IVC on gene expression has been hypothesized to reflect the lower quality of resulting embryos, but specific modifications associated with embryonic stress are still poorly understood (Gardner and Lane 1997; Moley et al. 1996; Rizos et al. 2002b).

Appropriate metabolism is important to support embryonic development and artificial in vitro culture conditions have been shown to affect the activity of energetic pathway resulting in suboptimal embryo development (Barnett and Bavister 1996b; Chason et al. 2011; Leese et al. 2007; Moley et al. 1996). Recently, analysis of early cleavage stage exposure to high glucose (HG) has been used to characterize the energetic stress associated with IVC (Cagnone et al. 2012a). In this study, an increased expression of metabolic genes was observed in blastocysts surviving to HG stress. This transcriptomic profile was associated to the Warburg effect, a metabolic shift used to complement the limited oxidative phosphorylation during post-compaction. Warburg metabolism allows the cell to proliferate in low-oxygen tension by using the glycolytic pathway to generate building-blocks and maintain oxidative and energetic homeostasis through lactate production (Krisher and Prather 2012; Thompson et al. 1996). This metabolism contrasts with the quiescent activity during the early cleavage (Thompson 2000) and high glucose before the EGA would induce mitochondrial dysfunction, leading to subsequent enhancement of the Warburg effect at blastocyst stage. However, deregulated oxygen consumption and enhanced lactate production, hallmarks of the Warburg effect, are associated with lower post-transfer viability in mouse (Gardner and Lane 1996) and human (Gardner et al. 2011).

Analysing end-point perturbations relative to IVC stress is informative of embryonic quality preceding the blastocyst transfer; however the early events causing development perturbation are still unresolved. Based on the results obtained with blastocyst exposed to high glucose, this study aims to characterize the transcriptomic impact of energetic stress at early stages of embryo development. Since embryonic genome activation is established at morula stage (day 5), microarray and RT-qPCR analysis were performed with morula samples, using pooled and single embryos. This is the first attempt to describe the transcriptomic profile in bovine morula.

3.4 Materials and methods

All chemicals were obtained from Sigma–Aldrich, unless otherwise stated.

In vitro production of bovine embryos

All media were pre-equilibrated before use and maturation, fertilization and culture took place under embryo-tested mineral oil. Procedure is described in (Cagnone et al. 2011).

Oocyte collection and in vitro maturation (IVM): cumulus–oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of slaughtered heifers (Holstein), washed and then matured for 24h in groups of 10 COC/drop in a humidified atmosphere at 38.5 °C in 5% CO2. The maturation medium was TCM-199 supplemented with 10% fetal calf serum (HyClone, ThermoScientific, USA), FSH (0.1 μg/ml Folltropin V, Bioniche, Canada), pyruvic acid (0.33 mM) and 50 μg/ml of gentamycin.

In vitro fertilization (IVF): After 2 washes in TLH medium (HEPES-buffered Tyrode's Lactate solution), groups of 5 matured COCs were added to 50 μL droplets of IVF medium (Tyrode's Lactate solution supplemented with 4 mg/ml BSA and 10μg/ml heparin) and frozen-thawed spermatozoa suspension (final concentration = 4.104 cells/ml) were added to each IVF droplet. The incubation took place in a humidified atmosphere at 38.5 °C in 5% CO_2 for 16-18 h.

In vitro culture (IVC): embryo culture used a two-step modified synthetic oviduct fluid culture system (mSOF1 during day 0 to 3 and mSOF2 during day 3 to 5) containing 0.4% fatty acid-free BSA in a humidified atmosphere at 38.5 °C in 5% CO_2 and 5 % O_2. Media was replaced to prevent toxicity due to ammonium accumulation and nutrients depletion caused respectively by amino acid degradation and embryo metabolism. Cleavage rate (number of embryos with at least 2 cells out of total embryos) and 8/16-cells embryo rate (number of embryos with at least 8 cells out of total embryos) were determined during embryo transfer from SOF1 to SOF2 on day 3. Morula rate (number of embryos with at least 32 cells out of total embryos) was calculated at the end of the culture (day 5). Morulae were

washed 3 times in PBS, collected in groups of 10 (pool) or individually in small volumes of PBS and transferred into 0.5 mL microtubes and stored at -80°C until RNA extraction.

In vitro model of high glucose (HG) exposure

As an increase from 0.2 to 5 mM of glucose before compaction (days 0 to 3) appeared to be the critical dose to allow in vitro pre-hatching development (Cagnone et al. 2011), this criterion was selected to produce replicates of control and high glucose (HG) treated embryos from different in vitro production runs. Four replicates of control and HG treated morulae were produced (10 embryos/ replicate) to be used for microarray experiment. Subsequently, 12 morulae from control and 12 morulae from HG-treatment were produced and processed individually by RT-qPCR without prior amplification.

Determination of differential gene expression in HG-treated morulae

As already described in (Cagnone et al. 2011), total RNA from each replicate was extracted, purified and assessed for quality. For microarray purposes, purified RNA was amplified by in vitro transcription, labelled with Cy3 and Cy5 and hybridized on the Agilent-manufactured EmbryoGENE slides (Robert et al. 2011a) in a 2-colors design with biological swap (RNA from the first and second biological replicates were stained in green for control and red for HG treatment, RNA from the 3rd and 4th biological replicates were stained in red for control and green for HG treatment). After 17 h of hybridization, microarray slides were washed and scanned. Intensity files were corrected by background subtraction then normalized within and between each array (Loess and quantile, respectively). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE40521.

For RT-qPCR, total extracted RNA from independent samples was reverse-transcribed and qPCR were performed using specific primers for each selected gene. Data normalization used GeNORM normalization factor (Vandesompele et al. 2002) from expression values of 3 reference genes (ACTB, CHUK, B2M). Moreover, technical variations were assessed and corrected through quantification of exogenous GFP spike which was introduced at the time of RNA extraction (Vigneault et al. 2004). RT-RNA quantification was performed in the

range of detection given by the standard curve and were assigned as non-detectable when no PCR product were obtained from sample but HKGs were expressed. Primer sequences, product size, annealing temperature and accession numbers are provided in Supplemental Table 6.

Analysis of differentially expressed genes functions

As already described in (Cagnone et al. 2011), data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com) to build schematic representations of important pathways deregulated in HG-treated morulae. IPA served to compile canonical pathways as well as gene product interactions (networks) that are differentially expressed between treatments or according to the developmental stage.

Statistical analysis

For microarray results, statistical comparison between high glucose and control treatments was done with the Limma algorithm. For RT-qPCR results, statistical comparison between treatments used an un-paired t test in case of homogeneous variance and a non-parametric test with Welch-correction in case of heterogeneous variance. When expression was positive for house-keeping gene expression but negative for any other gene (case of HNF4A and SNCA), the non-parametric Fisher-exact test was used to compare frequency of absolute expression within the samples of each treatment. RT-qPCR results from single embryo analysis were processed into Cluster and TreeView to hierarchically cluster (Pearson correlation) the more similar profiles among control and HG-treated morulae. Fisher-exact test was also used to compare the frequency of HG and control treated morulae which were clustered according to individual gene expression similarities.

3.5 Results

High glucose (HG) exposure does not impact the development rate until morula stage

After in vitro maturation and fertilization, early cleavage stages of bovine zygotes were exposed to high glucose (HG) or control IVC conditions until day 3, and then all embryos were transferred to identical IVC conditions until day 5. Table 5 shows that HG exposure does not significantly affect the development rate to 8/16 cell stage at day 3 (29.6% vs. 38.9%, p=0.09) or morula stage at day 5 (28.7% vs. 35.4%, p=0.14) when compared to control IVC respectively. After early cleavage stage exposure, the percentage of 8-16 cells (observed at day 3) that reached the morula stage (observed at day 5) was not different either, with 96% (93/97) and 89 % (95/107) in HG and control treated groups respectively.

HG-treated morulae express different transcriptomic profiles

Our experimental design compared pools of HG and control-treated morulae and resulted in the detection of 13,199 and 11,625 expressed sequences, respectively (Fig 8A). 10,640 sequences were present in embryos from both treatments. Statistical analysis revealed a significant different expression ($p<0.05$) for 1,732 transcript sequences. Among them, 613 transcripts had more than 1.5-fold differences between the two conditions with 366 and 247 being up- and down-regulated in HG-treated morulae, respectively. Only a small proportion of DEG (44/613) showed exclusive expression (18 in control and 26 in HG). Ninety-nine transcripts showed a differential expression with a symmetrical fold-change superior to 2; and 4 transcripts had a fold-change superior to 3. HNF4a was the only transcript showing an absolute symmetrical fold-change > 4. Fig 8B shows the principal affected canonical pathways associated with HG-related DEG in bovine morulae.

Single morula analysis shows individual variability in gene expression profile

Morulae were produced in HG and control treatment and then individually assessed (n= 12) by RT-qPCR (Fig 9). Individual House-keeping genes expression was similar, except for 3 morulae that were found to have low expression of ACTB (HG2), B2M (HG3) or CHUK (CTL2) and were not considered for gene expression quantification. Results showed no

significant difference between HG and control-treated morulae in their individual expression of CRAT, SDHC, TOPBP1 or TP53, albeit TP53 and CRAT showed a large distribution. SNCA was undetectable in the majority of individual morulae and this was independent of treatment. In contrast, a significant proportion of individual embryos from the control treatment (10/12) showed detectable expression of HNF4a, whereas a high proportion of HG-treated morulae (8/12) showed no detectable transcript. Expression levels of two genes (NDUFS2 and PLAGL1) showed different variances between treatments and utilization of a non-parametric test revealed an almost significant ($p=0.07$) increased number of HG-treated morulae with a lower expression of NDUFS2. Comparatively, PLAGL1 expression rate was significantly higher in individual embryos from the HG-treatment compared to control ($p=0.03$).

RT-qPCR results for TP53, SNCA, TOPBP1, SDHC, CRAT, PLAGL1, and HNF4A expression on single embryo allowed to drow a personal transcriptomic profile for each single morula and group particular individuals according to similarities in their gene expression profiles. To do so, we submitted to hierarchical clustering all morulae from HG and control treatments (Fig 10), and order each individual using Pearson correlation of their average gene expression pattern. This hierarchical clustering resulted in 2 branches (entitled clusters A and B) containing 10 and 11 individuals respectively. When examining the distribution of control and treated morula among each cluster, significantly higher frequencies of control morula among clusters A and HG-treated morulae among cluster B were observed (Fisher exact test, $p=0.005$). As expected, PLAGL1 and HNF4A had the most influence in the differential distribution of individuals between each cluster.

Pathway analysis of differentially expressed gene functions after HG exposure

IPA connecting tools was used to generate networks between DEG in HG-treated morula and DEG previously published from HG-treated blastocysts (Cagnone et al. 2011). Based only on IPA knowledge of gene expression connectivity, HNF4a was linked to HIF1A network as well as PPARG (Fig 11), recapitulating the metabolic changes and relationships of key transcription factors during the morula to blastocyst transition. Red and green colors reflect the direction of gene expression in embryos incubated under HG-conditions.

3.6 Discussion

Survival rate

During in vitro culutre, sub-optimal condition affects the embryonic development and blastocyst quality (Crosier et al. 2000; Rizos et al. 2002c). Using the bovine model, this study illustrates how gene expression would be affected before early developmental compromised. Moreover, we propose a genomics hypothesis to describe how surviving embryos could adapt to energetic stress.

Our first result showed that high glucose (HG) exposure did not impact significantly the rate of embryo development at morula stage. This is in contrast with another study which reported an early reduction on morula developmental rate (Furnus et al. 1997). This is likely to be due to differences in culture medium composition and the capacity of early embryos to resist to high glucose-induced Crabtree effect. The Crabtree effect, as consistent with other studies in hamster (Barnett et al. 1997; Seshagiri and Bavister 1991), ovine (Thompson et al. 1992), bovine (Kim et al. 1993b) and human (Bavister 1999; Conaghan et al. 1993), described how high glucose exposure impairs mitochondrial oxidative activity in early embryos and induces reactive oxygen species (Betts and Madan 2008; Diaz-Ruiz et al. 2008). As an anti-oxidant support is provided in SOF culture conditions and early morula still have enough glutathione defense (Gardiner and Reed 1995a), it would allow adaptation to HG-induced oxidative stress before compaction.

In the litterature, 5.56 mM glucose concentration is shown to block the bovine development to 8-16 cells stage, resulting in no blastocyst development (Kim et al. 1993b). In our culture condition, morula rate was not affected by 5 mM of glucose, but we have shown that exposure to 5 mM HG conditions during early cleavage reduce significantly the rate of blastocyst stage development (Cagnone et al. 2012a). Therefore, this indicates impairment of morula-to-blastocyst transition under HG-stress, which is consistent with the study of Pinyopummintr et al. showing similar rate of morula but reduced blastocyst development after culture in Glucose + Pi (Pinyopummintr and Bavister 1991). The morula to blastocyst transition is highly energy demanding and both oxidative and glycolytic

activities are increased (Thompson et al. 1996). The decreased morula-to blastocyst transition may coincide with incapacity to manage perturbations in oxidative or energy homeostasis after compaction (Dalvit et al. 2005; Scott and Whittingham 2002). This brings consideration in regard to the loss of embryos at the morula-to-blastocyst transition in extended culture condition (Devreker et al. 2001; Gardner and Lane 1996).

Transcriptomic outcome

Proper embryonic transcription is crucial for developmental competence through the expression of different sets of genes whose functions allow the proliferation and differentiation of embryonic cells (Misirlioglu et al. 2006). Therefore, assessing gene expression may characterize causal pathways leading to developmental block at morula stage after HG-exposure. Microarray technology has been extensively used to discover large-scale transcriptomic variations associated with physiologic changes (Lonergan et al. 2003a; Plourde et al. 2012b; Smith et al. 2009). Here, pools of morulae from control and HG treatment were compared in order to minimize individual variations within tissue samples (Cagnone et al. 2011). This strategy gives a global perspective of HG impact on the morula population and showed pathway associated with oxidative stress and mitochondrial dysfunction. However, the interpretation of differentially expressed genes according to a mixed population with different individual competences is awkward. Moreover, failure in RT-qPCR validation suggested distortion in the proportion of potentially blocking morula in harvested replicates (data not shown). Nevertheless, these results suggested an important variability between individual morulae that could be associated with different capacity to manage energetic stress.

To explore the difference between morula subjected to HG, a PCR experiment was designed to assess gene expression in single morulae. In the HG-treated group, individual showed a large distribution in their expression for 5 out of 8 selected genes, confirming the important difference among a population subjected to identical stress condition. To determine if a significant profile of DEG was able to indicate the response to HG treatment, application of Pearson correlation algorithm was able to cluster individual morulae according to control and HG-treated, with only a few individual morulae having an

intermediary profile. In this context, HNF4a and PLAGL1 expression profiles had the most power in discriminating treated and non-treated morulae, indicating an important association with the HG impact. Therefore, their differential profiles within HG-treated individuals could be associated with the higher rate of morula block as observed in stress embryo population. Further investigation would be necessary to understand the implication of HNF4A and PLAGL1 in the embryonic stress response and the competence to reach the blastocyst stage.

HNF4A and energetic programming

HNF4A expression was absent in a significantly higher number of HG-treated morulae compared to control embryos. These HNF4A-negative morulae were nonetheless expressing housekeeping genes as well as other genes. The exact role of HNF4A is not yet characterized in bovine embryos, but this gene is a conserved transcription factor involved in energy metabolism (Odom et al. 2004; Wang et al. 2000). In the mouse, HNF4 mRNA is observed in the primary endoderm at embryonic day 4.5 (Duncan et al. 1994) and HNF4 deletion showed first morphological impact at E6.5 (Duncan et al. 1997). At the molecular level, HNF4-/- impact is associated with apoptosis and protein secretion in visceral endoderm. Moreover, the presence of a dominant-negative variant of HNF4a in beta-cells is associated with reduced ATP production, most likely due to defective mitochondrial enzyme activity or partial uncoupling of the respiratory chain (Wang et al. 2000). Energy expenditure is closely controlled by the level of ATP (Wallace and Fan 2010) and Ingenuity pathway analysis linked HNF4A with PGC1A and AMPK, 2 factors that are involved in mitochondrial biogenesis and ATP sensing in cells (Mootha et al. 2003). In response to energetic demand, appropriate activation of PGC1A or AMPK controls the expression of HNF4A and down-stream metabolic factors (Zhang et al. 2004). This data suggest that HG-induced disturbance in mitochondrial metabolism (Crabtree effect) may have interfered with energy sensor factors which insure proper HNF4A expression in morula.

Integrative analysis of connectivity between DEG in HG-treated morula and HG-treated blastocysts (Cagnone et al. 2011) linked HNF4A with HIF1A and PPARG, two important regulators of glucose and lipid metabolism. HIF1A controls HNF4A gene expression but

also form heterodimer with HNF4A in order to drive glycolytic gene expression in response to hypoxia (Tsuchiya et al. 2002). PPARG is under HNF4A regulation and control cholesterol as well as lipid accumulation in concert with SREBP action (Malerod et al. 2003). As HIF1A and PPARG are key factors of Warburg effect (Cairns et al. 2011), the metabolic shift associated with glycolysis (Cagnone et al. 2012a) as well as lipolyse (Sturmey et al. 2009) could be associated with HNF4A during the morula to blastocyst transition and loss of HNF4A expression may be critical for adapting to mitochondrial stress in regard to the Crabtree-effect induced by high glucose exposure. Thereby, individuals that can't activate HNF4A expression may fail to enhance the Warburg metabolism and overcome the morula block. However, enhancement of Warburg metabolism would lead to increased glycolytic end-product (lactate) as well as lipid accumulation, hallmarks of low quality IVC embryos.

PLAGL1 and long term impact

Early embryonic development coincides with a remarkable epigenetic changes and the morula stage is concomitant with major re-methylation of DNA (Albert and Peters 2009; Duranthon et al. 2008). In our study, a significant number of HG-treated morulae showed high expression of PLAGL1, a gene under imprinting control (Arima et al. 2001). PLAGL1 is a transcriptional regulator involved in tumorogensis and indirect control of insulin release and PLAGL1 deletion reduces fertility performance in mice (Hensen et al. 2004). In bovine, PLAGL1 is progressively methylated during oocyte growth and correlate with expression of the demethylase enzyme DNMT3 (O'Doherty et al. 2012). Since mitochondrial activity has an epigenetic effect on methyl group synthesis (Wallace and Fan 2010) and inhibiting DNA methylation affect gene expression and morula-to-blastocyst transition (Lee et al. 2011b), it is possible that the impact of energetic stress on embryonic mitochondria would affect the methylation process during early development, leading to gene expression aberrations that potentially induce morula block. Moreover, PLAGL1 expression is associated with imprinting disorder in Type 2 diabetes (Mackay and Temple 2010) and Beckwith-Wiedemann syndrome (Bliek et al. 2009). These syndromes might be at higher risk in ART-derived embryos and numerous reports have correlated early environmental stress with long term effect on the post-natal physiology (Moley et al. 1994; Strawn et al. 2010). These data

bring forward the potential impact of IVC on proper establishment/maintain of epigenetics during early development which may have future implication for health and disease (Fernandez-Gonzalez et al. 2009; Fernandez-Gonzalez et al. 2007).

Conclusion

ATP requirement is lower in early embryonic development (Quinn and Wales 1973) but clearly increases during blastulation and can be related to the shift to a high-energy production period required for embryonic growth (Dumollard et al. 2009). Numerous studies in several species have shown that metabolic stress compromises the early development by impairing the mitochondrial activity (Barnett and Bavister 1996a; Barnett et al. 1997; Cagnone et al. 2012a; Moley et al. 1996). Our study shows that early exposure to high glucose compromises gene expression at morula stage, suggesting that different gene expression in response to energetic stress would correlate with differential competence to develop to blastocyst stage. Particularly, absolute expression of HNF4A might be required in order to enhance the Warburg metabolic program and counteract the mitochondrial failure caused by high glucose exposure (Cagnone et al. 2011; Krisher and Prather 2012). Taking together, these phenotypes confirm the usefulness of transcriptome analysis at temporal periods of environmental sensitivity. Moreover, it brings relevant information about the metabolic control after EGA as well as the transcriptomic modifications associated with energetic stress adaptation.

3.7 Aknowlegment

This work was supported by NSERC of Canada and the EmbryoGENE network.

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3.9 Figures and tables

Table 5 Effect of HG during early cleavage stage on developmental rate until morula stage.

	Day 0	Day 3	Day 5
IVC treatment (replicate number)	Number of Presumptive zygotes	Number of 8-16 cells embryos (% Survival rate ± SD)	number of morulae (% Survival rate ± SD)
Control (n=9)	271	107 (38.9^a ± 2.8)	95 (35.4^a ± 3.4)
High glucose (n=9)	328	97 (29.6^a ± 4.1)	93 (28.7^a ± 2.6)

In vitro produced bovine zygotes were exposed to control (0.2 mM) or High Glucose (5 mM) culture condition from day 0 to day 3, analyzed for 8-16 cells rate then total embryos were transferred to control culture condition until development to day 5 morula stage. Different superscripts represent significant differences between treatments (Unpaired t-test, $p<0.05$)

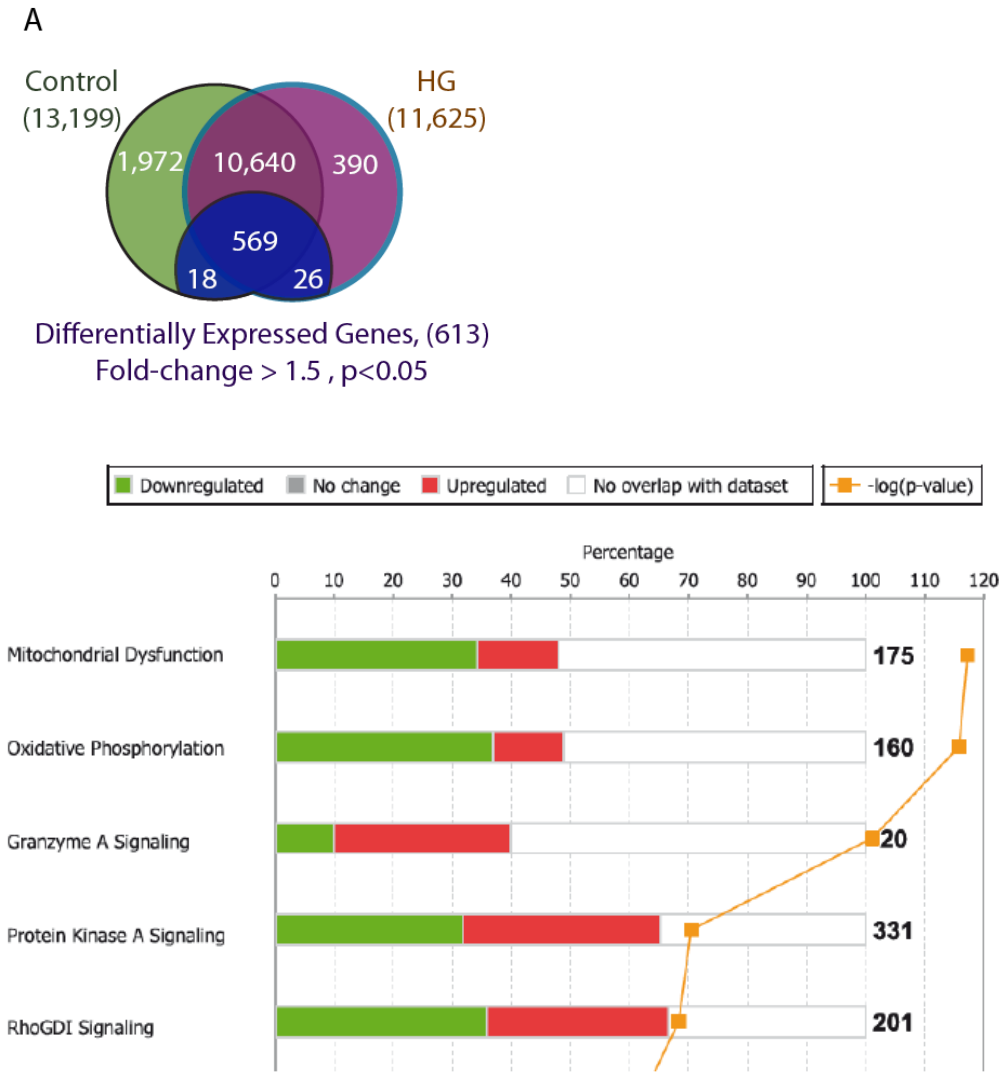
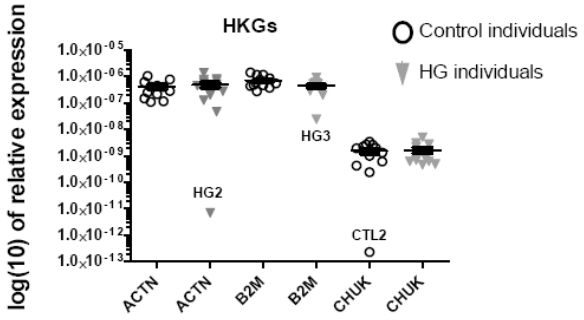
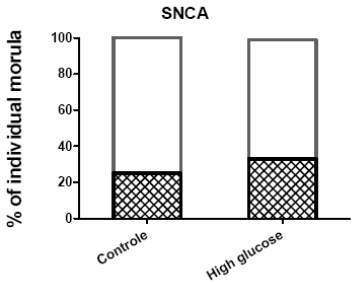
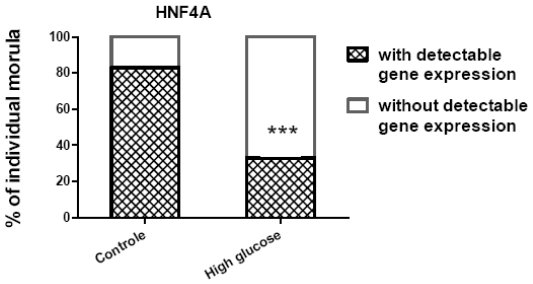


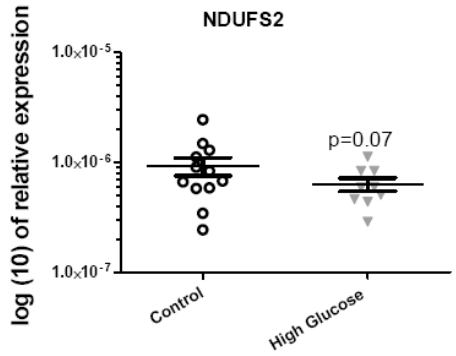
Figure 8 Impact of HG on gene expression in pooled bovine morulae.

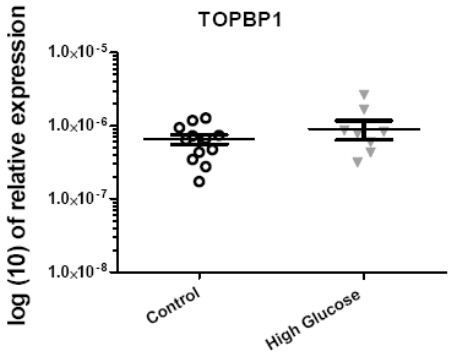
Independent pools of morulae were analyzed by microarray. A: Venn diagram indicates the number of microarray probes that were detected (green and violet) as well as the number of differentially expressed genes (blue) between control and HG-treated bovine morulae. B: Using Ingenuity Pathway Analysis, result show the most significant canonical pathways associated with the differentially expressed genes from microarray comparison between HG and control treated morulae.

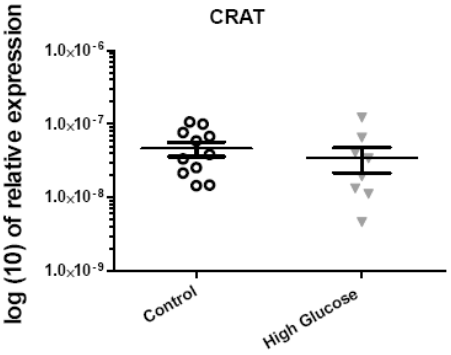

HKGs
Control individuals
HG individuals
log(10) of relative expression
HG2
HG3
CTL2
ACTN
ACTN
B2M
B2M
CHUK
CHUK

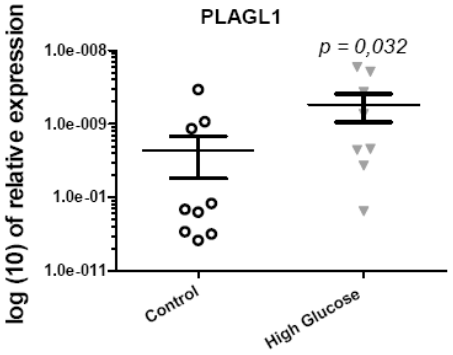

SNCA
% of individual morula
Controle
High glucose


HNF4A
% of individual morula
***
with detectable gene expression
without detectable gene expression
Controle
High glucose


NDUFS2
log (10) of relative expression
p=0.07
Control
High Glucose


TOPBP1
log (10) of relative expression
Control
High Glucose


CRAT
log (10) of relative expression
Control
High Glucose


PLAGL1
p = 0,032
log (10) of relative expression
Control
High Glucose

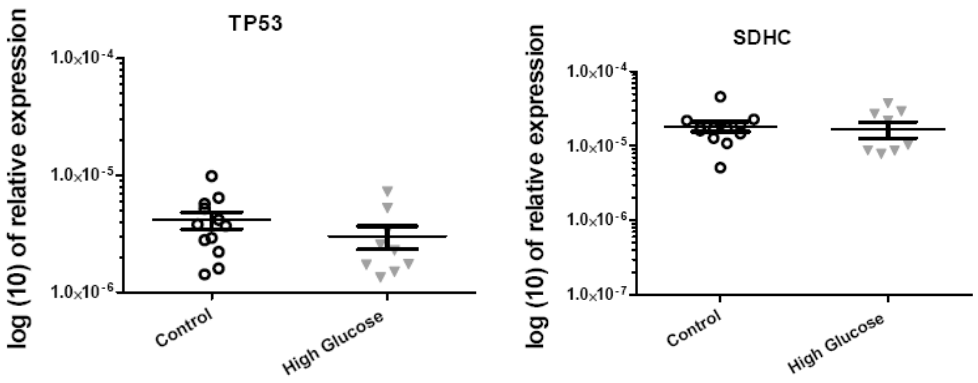
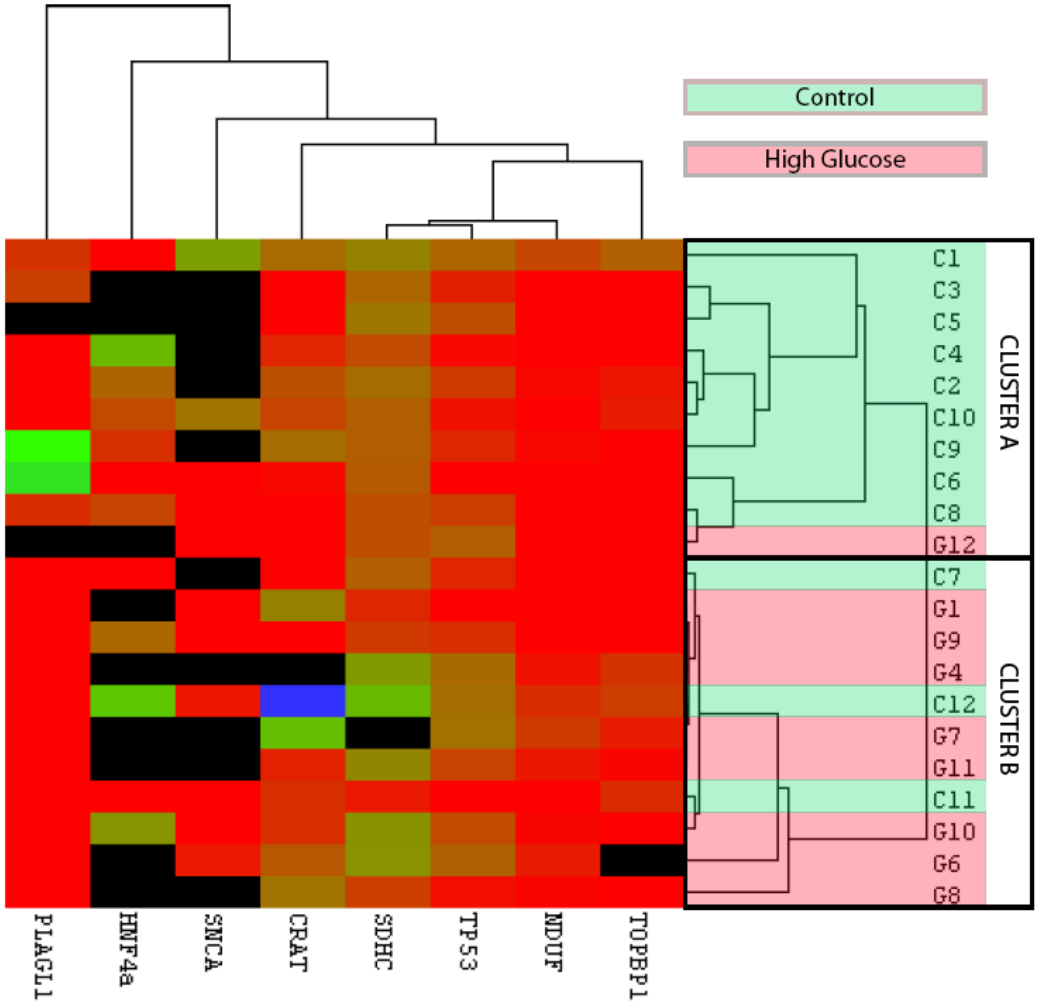


Figure 9 Gene transcript quantification in single bovine morulae after HG or control treatment.

RT-qPCR was done in 12 individual morulae from control or HG-treatment. Each point represents the amount of mRNA of the selected transcript after correction with GFP and 3 housekeeping genes. In case of detectable or not detectable gene expression, data indicate the proportion of individuals with positive or negative output.


Control
High Glucose
C1
C3
C5
C4
C2
C10
C9
C6
C8
G12
C7
G1
G9
G4
C12
G7
G11
C11
G10
G6
G8
CLUSTER A
CLUSTER B
PLAGL1
HNF4a
SNCA
CRAT
SDHC
TP53
MDUF
TOPBP1

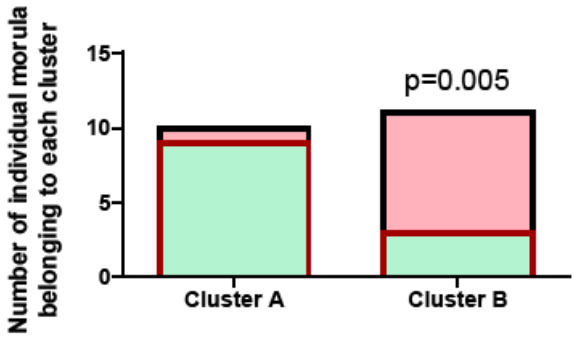
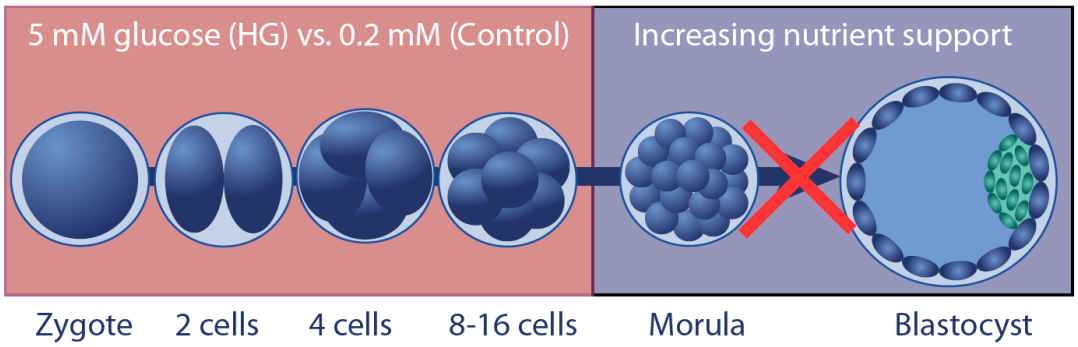

Number of individual morula belonging to each cluster
15
10
5
0
p=0.005
Cluster A
Cluster B

Figure 10 Correlation in gene expression profile among individual morulae.

Data represent the hierarchical classification of individual morulae based on similarity of RT-qPCR gene expression via an average Pearson Correlation algorithm. Red and green colors mean high and low expression respectively. Black color means no expression of the gene but expression of the 3 house-keeping genes by the individual. Blue color means that gene expression was not determined (out of material). C = control, G = high Glucose, numbers correspond to individual morulae ID. Bar chart represents the proportion of C and G individuals within cluster A or B. The probability of difference in the distribution was assessed by a Fisher exact test of frequency.



Gene Network of Energetic Program
(Warburg-like Effect)

Morula :

HNF4alpha : hepatic nuclear factor 4, alpa

Blastocyst :

LDHA : Lactate deshydrogenase A

HIF1A: Hypoxia inducible factor 1, alpha

SERPINE1 = PAI1, plasminogene activaror inhibitor 1

PPARG : Peroxisome proliferator activated receptor gamma

Up-stream Factors :

PPARGC1A = PGC1A, Peroxisome proliferator activated receptor gamma co-activator 1, alpha

HNF1A : Hepatic nuclear factor 1, alpha

SP1 : Sp1 transcription factor

PRKAA2 = AMPK : protein kinase, AMP-activated, alpha 2 catalytic subunit

HDAC1 : Histone deacetylase 1

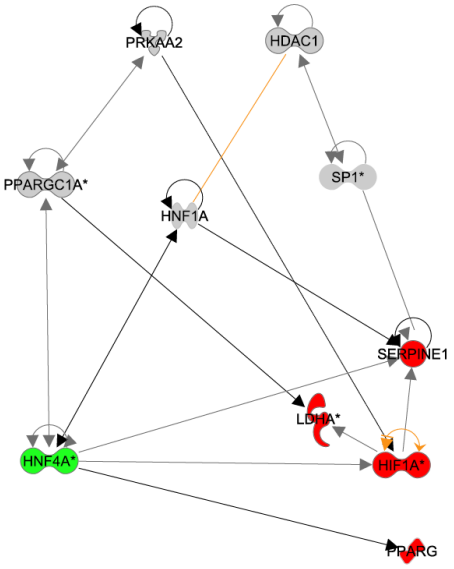


Figure 11 Ingenuity Pathway Analysis during post-compaction after HG treatment.

This figure represents the transcriptomic changes after HG-treatment and their potential connections during morula to blastocyst transition. Red and green colors indicate respectively up- and down-regulated genes in HG-treated embryos compared to control.

3.10 Supplemental table

Supplemental table 6 Sequences of reverse transcription qPCR-specific primers of candidate genes expressed in bovine morulae.

Gene Symbol	Name	Accession number	Forward sequence 5'-3'	Reverse sequence 5'-3'	Product size (bp)	Annealing temperature (°C)
CRAT	carnitine O-acetyltransferase	NM_001075587	TCAGCTCTGCCCAGTTTCTGTT	ACCAGGACATGGGTTCAGGAAT	267	57
HNF4A	hepatocyte nuclear factor 4, alpha	NM_001015557	GACAAACGGGAGCAGAGCCTTG	GTTGGGTGGCAGTGACAGGG	232	57
NDUFS2	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase)	NM_001075137	TTTGGCGTGTACCTGGTGTCTGAT	TGCACAGAAAGCCTGACAGCTA	299	58
PLAGL1	pleiomorphic adenoma gene-like 1	NM_001103289	TGTGCCAATCTGTCCTGAGTGTTCAT	ACTCACATTGTAGCATCCTGTGGAAA	469	57
SDHC	succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa	NM_175814	GCCATGTGAAGAGCTGAAGTTCCC	CTAAAGACCCTGGGCTCTGACCAA	475	59
SNCA	synuclein, alpha (non A4 component of amyloid precursor)	NM_001034041	ACAACAGTGGCTGAGAAGACCA	ACAGCACACAAAGACCCTGCTA	492	57
TOPBP1	topoisomerase (DNA) II binding protein 1	XM_002685092	GCTGCCCAGAATGTATACTGCTTGAA	TGGTTTAACGACAAGCTAGCTGAGGA	435	59
TP53	tumor protein p53	NM_174201	TCGGGAGAGGTCAGAATGTGTTCC	CTTTGGCACTGAGGTTCACCAAGG	408	59
ACTB	actin-beta	NM_173979	ATCGTCCACCGCAAATGCTTCT	GCCATGCCAATCTCATCTCGTT	101	59
B2M	beta-2-microglobulin	NM_173893	AGACACCCACCAGAAGATGG	GGGGTTGTTCCAAAGTAACG	234	54
CHUK	conserved helix-loop-helix ubiquitous kinase	NM_174021	TGATGGAATCTCTGGAACAGCG	TGCTTACAGCCCAACAACTTGC	180	57
GFP	green-fluorescent protein		GCAGAAGAACGGCATCAAGGTGAA	TGGGTGCTCAGGTAGTGGTTGT	143	59

Chapitre 4: Impact du stress oxydatif

Title: Transcriptomic signature to oxidative stress exposure at the time of embryonic genome activation in bovine blastocysts

This article has been accepted for publication in the journal Molecular Reproduction and Development.

Key words: reactive oxygen species, glutathione, inflammation, embryo quality, gene expression, degeneration, quiet metabolism

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Grant information: NSERC Strategic Network EmbryoGENE NETPG 340825-06.

4.1 Résumé

Afin de comprendre comment la culture in vitro affecte la qualité embryonnaire, nous avons analysé la survie et l'expression génique globale des blastocystes bovins après exposition à des conditions de stress oxydatif accru. Deux agents pro oxydants, l'un agissant de façon extracellulaire en promouvant la production de radicaux libres (0.01 mM 2,2'-azobis (2-amidinopropane) dihydrochloride [AAPH]), et l'autre en agissant de façon intracellulaire pour inhiber la synthèse de glutathionne (0.4 mM buthionine sulfoximine [BSO]) ont été ajoutés séparément au milieu de culture à partir du jour 3 (stade 8-16 cellule). Au jour 7, les blastocystes ayant survécu ont par la suite été soumis à une analyse transcriptomique. Dans la littérature, les deux pro-oxydants ont démontré une influence sur la dégénérescence retardée d'une proportion de blastocystes au jour 8. Dans nos expériences, aucune différence morphologique n'était visible bien qu'AAPH tende à réduire le taux de blastocyste et que BSO induise une diminution significative de celui-ci, indiquant un impact différent sur la survie de la population. Au niveau transcriptomique, les blastocystes survivant aux facteurs pro-oxydants montraient une réponse au stress oxydatif et à l'inflammation (ARRB2), bien qu'AAPH induise de plus fortes perturbations dans l'homéostasie cellulaire (SERPINE1). En revanche, la génomique fonctionnelle du profil BSO identifia l'expression différentielle de gènes reliés au métabolisme de la glycine ainsi qu'au métabolisme énergétique. Ces différentes caractéristiques pourraient être indicatives de blastocystes pré-dégénératifs (IGFBP7) dans la population AAPH tandis que l'exposition au BSO sélectionnerait les individus les plus viables (TKDP1). Ensemble, ces résultats illustrent comment la perturbation oxydative du développement pré-attachement est associée à une sur-régulation de plusieurs marqueurs métaboliques. Par ailleurs, une meilleure capacité à survivre à l'appauvrissement en antioxydants permettrait la survie de blastocystes avec un métabolisme plus stable après compaction.

4.2 Abstract

In order to understand how *in vitro* culture affects embryonic quality, we analyzed survival and global gene expression in bovine blastocysts after exposure to increased oxidative stress conditions. Two pro-oxidant agents, one that acts extra-cellularly by promoting ROS production, (0.01 mM 2,2'-azobis (2-amidinopropane) dihydrochloride [AAPH]) or another that acts intra-cellularly by inhibiting glutathione synthesis, (0.4 mM buthionine sulfoximine [BSO]) were added separately to IVC media from day 3 (8-16 cells stage) onward. Transcriptomic analysis was then performed in resulting day 7 blastocysts. In the literature, these two pro-oxidant conditions were shown to induce delayed degeneration in a proportion of day 8 blastocysts. In our experiment, no morphological difference was visible but AAPH tended to decrease the blastocyst rate while BSO significantly reduced it, indicating a differential impact on the surviving population. At the transcriptomic level, blastocysts that survived either pro-oxidant exposure showed oxidative stress and inflammatory response (*ARRB2*), although AAPH induced higher disturbances in cellular homeostasis (*SERPINE1*). However, functional genomics of the BSO profile identified differential expression of genes related to glycine metabolism and energetic metabolism (*TPI1*). These differential features might be indicative of pre-degenerative blastocysts (*IGFBP7*) in the AAPH population while BSO exposure would select the most viable individuals (*TKDP1*). Together, these results illustrate how oxidative disruption of pre-attachment development is associated with systematic up-regulation of several metabolic markers. Moreover, it indicates that a better capacity to survive anti-oxidant depletion may allow the survival of blastocysts with quieter metabolism after compaction.

4.3 Introduction

In assisted reproductive technology (ART), the suboptimal environment encountered by early embryos during *in vitro* culture (IVC) is one cause of poor blastocyst quality. Moreover, IVC is associated with long-term effects on health demonstrated by the higher incidence of developmental syndrome in the ART-offspring population, (Alukal and Lipshultz 2008) notably in animal studies. In cattle, ART-derived pregnancies are sometimes associated with high birth-weight calves (large offspring syndrome) and peri-natal mortality (Farin et al. 2006; Young et al. 1998). Early developmental deviation during IVC would correlate with the developmental origins of health and disease hypothesis (Lazzari et al. 2002). Although numerous changes have been made to IVC protocols, the complex interactions between medium compositions, physical equilibrium and incubation conditions are still sub-optimal for proper embryo development. Therefore, investigation is required to measure the impact of IVC on embryonic plasticity in regard to the reduced blastocyst quality. Defining markers of embryonic stress response may allow the development of alternative strategies to empirical reformulation of culture conditions.

In the last decade, the advancement of functional genomics and the capacity to work with minute samples, have allowed the study of gene expression in the early embryo. During the first cleavages, transcription is largely silenced, and early metabolism is supported by the transcripts and proteins provided by the oocyte. This phase of transcriptomic quiescence is followed by activation of the embryonic genome that corresponds to the maternal-embryonic transition (8- to 16-cell stage in bovine). After the MET, the transcription of the embryonic genome is extremely dynamic to control morula compaction and the pursuit of blastocyst development (Hamatani et al. 2006; Rodriguez-Zas et al. 2008). Numerous studies have shown the range of gene expression modulation under different *in vitro* culture conditions (Duranthon et al. 2008; Smith et al. 2009). While transcriptional changes may be required for the embryo to adapt its homeostasis to the environmental conditions, it is believed that perturbations of developmentally important genes may translate into a stress response affecting embryonic quality (Rizos et al. 2002b). However, the genes related to the embryonic stress response need further characterisation. In this perspective, we hypothesized

that analyzing the transcriptomic profile from embryos cultured under enhanced stress conditions would define specific gene patterns associated with compromised quality.

The maintenance of oxidative homeostasis, *i.e*. the equilibrium between reactive oxygen species production and anti-oxidant defence, is critical for normal cell division and differentiation. *In vivo*, the oviduct provides the optimal environment for the embryo to maintain its oxidative homeostasis. This is supported by the limited free radical content in the environment, the normal stimulation of oxidative metabolism and the availability of anti-oxidant molecules (Guerin et al. 2001). Associated with lower embryo quality, IVC affects oxidative homeostasis (Agarwal et al. 2006; Gardiner et al. 1998) by increasing ROS exposure or mitigating anti-oxidant protection. Variations in oxygen concentration (Lequarre et al. 2003; Rodina et al. 2009) and temperature (Sakatani et al. 2008) have been shown to modulate the rate of ROS production while supplementation with antioxidant molecules could facilitate blastocyst development (Liu et al. 1999; Takahashi et al. 1993). Accordingly, red-ox unbalance has deleterious impacts on cell constituents, organelles and developmental kinetics, that may in turn affect implantation success (Agarwal et al. 2006).

Considering the important impact of oxidative stress on IVC, previous reports analyzed the effect of exposure to pro-oxidant agents (AAPH or BSO) during post-compaction development of bovine embryos (Feugang et al. 2005; Feugang et al. 2003; Takahashi et al. 1993). With a half-life of 175 h in hydrophilic environment, AAPH (2,2'-azobis (2-amidinopropane) dihydrochloride) is known to induce continued free radical formation that initiates cell membrane lipid peroxidation and generates ROS. BSO (buthionine sulfoximine), on the other hand, inhibits the key enzyme of glutathione synthesis, gamma-glutamylcysteine synthase, and results in depletion of cellular glutathione (GSH) content in bovine oocytes and embryos (de Matos et al. 1996; Takahashi et al. 1993). BSO activity could be maintained for 5 days in culture medium. Here, exposure to AAPH or BSO was conducted at the time of embryonic genome activation (from day 3 to day 7), a period when embryonic homeostasis is challenged by increasing oxidative metabolism (Donnay and Leese 1999; Fischer and Bavister 1993; Thompson 2000) and low glutathione content (Gardiner and Reed 1994; Gardiner and Reed 1995b). The objective of this study was to

gain insight as to how the embryonic genome responds to subletal, extracellular or intracellular oxidative stress. This is the first report using this type of analysis.

4.4 Results

Differential impact of AAPH and BSO exposure on blastocyst survival

In order to investigate the impact of oxidative stress on early bovine development, in vitro produced bovine zygotes were cultured in control conditions until day 3. Afterwards, zygotes were transferred to either control or separate treatment conditions containing a pro-oxidant agent, either AAPH or BSO, and cultured until day 7. Phenotypically visible results were not present at the morula stage (day 5). The blastocyst rate was not significantly affected by 0.01 mM AAPH treatment but was significantly reduced after exposure to 0.4 mM of BSO ($p=0.04$) (Fig 12). Higher standard deviation in the blastocyst rate was noted in AAPH replicates (9.8%) compared to BSO (6.4%) or control replicates (4.4%). The hatching rate of day 7 blastocysts was not significantly affected by either pro-oxidant agent when compared to control. Moreover, no visible impact on embryo morphology was observed in blastocysts produced in each treatment.

Gene expression profile in blastocysts after AAPH or BSO exposure

Microarray technology was used for large-scale assessment of bovine embryo gene expression in response to mild oxidative stress. Of the 38,732 gene-targeted probes that cover the microarray slides, more than 17,000 probes exhibited an intensity signal higher than the summation of background intensity plus 2 times the standard deviation, regardless of the treatment. Only these probes were considered for genomics analysis.

Statistical analysis of gene expression differences between treatments and controls was performed using a Limma test which determined a p-value and fold-change for all the genes expressed in AAPH and BSO blastocysts compared to CTL. Flexarray tools generated a scatter plot of p-values which depicts the number of DEGs (probe count) in intervals of p-value (interval of 0.0125), irrespective of the fold-change. Results showed a higher number of DEGs in low-p-value ranges (<0.05) for BSO compared to AAPH (Fig 13A). A scatter plot of fold change was also generated with the fold-change of all the DEGs from AAPH and BSO, irrespective of p-value. In this plot, the identity line would correspond to 100% correlation in DEG fold-changes in both treatments. Results demonstrated a correlation of

DEG fold-change of R2=0.36 and a preferential abundance of DEGs under the identity line, i.e. in the AAPH treatment (Fig 13B).

Significant differential expression was considered for probes showing a symmetrical fold-change superior to ±1.5 with significance at $p<0.05$. With these criteria, 226 and 476 probes were shown to be differentially expressed in AAPH- and BSO-treated blastocysts, respectively (Fig. 13C). In AAPH-treated blastocysts, 70.7% (160/226) of DEGs were up-regulated, while 60.2% (287/476) of DEGs were down-regulated in BSO-treated blastocysts. Sixty-four DEGs overlapped both AAPH and BSO conditions. When symmetrical fold-change cut-off was set at ± 2 ($p<0.05$), AAPH-treated blastocysts had 59 DEGs (55 up- and 4 down-regulated) while BSO-treated blastocysts had 40 DEGs (8 up- and 32 down-regulated genes). With these criteria, only 5 DEGs were common to AAPH and BSO treatments. AAPH-related DEGs showed the higher interval of fold-change (from -2.24 to 7.15) compared to BSO-related ones (-3.2 to 5.19). Lumican was the most up-regulated gene in both treatments.

To validate the microarray results, a total of 11 genes, including 4 housekeeping genes (ACTB, MYL6, PPIA, YWHAB), plus 7 predicted DEGs (ARRB2, GCSH, IFNT, SERPINE1, TKDP1, TPI1, IGFPB7) were analyzed by RT-qPCR on 3 independent samples (Fig. 14). GeNorm results calculated consistent expression of ACTB, MYL6 and PPIA throughout conditions. AAPH-predicted DEGs were ARRB2, SERPINE1 and IGFBP7, while BSO-predicted DEGs were ARRB2, TKDP1, TPI1, GCSH and IFNT. The use of an ANOVA statistical test validated the difference ($p=0.05$) in relative expression for 2 out of 3 selected DEGs from the AAPH treatment (ARRB2, IGFBP7) and 3 out of 5 selected DEGs from the BSO treatment (ARRB2, TKDP1, TPI1). The AAPH treatment had a non-significant impact in gene expression of SERPINE1 ($p=0.44$) but the AAPH-related variance was significantly different compared to control or BSO groups.

Overlapping DEGs associated with different physiological status/developmental conditions

As the impact of oxidative stress has been potentially associated with the skewing of sex ratio in early embryos (Feugang et al. 2005), the proportion of AAPH- and BSO-related

DEGs that could be associated with embryonic sex were determined (Bermejo-Alvarez et al. 2010). Among annotated AAPH- and BSO-related DEGs, only 7.0% (8/113) 7.3% (17/230) moderately overlapped with sex-related genes, respectively. If several of the sex-specific targeted genes had been associated with the treatments, it could have indicated a sex effect in addition to the treatment effect.

Increased oxidative stress has been associated with mitochondrial stress in hyperglycemia-treated embryos (Hashimoto et al. 2000; Karja et al. 2006; Leunda-Casi et al. 2002). The proportion of AAPH- and BSO-related DEGs overlapped with the list of 63 DEGs previously described in high-glucose treated blastocysts (Cagnone et al. 2012b). Among AAPH-related DEGs, 34 showed a consistent overlap with energetic stress-related genes (Fig. 15A) and resulted in a significant correlation of fold-change ($R=0.83$, $p<0.0001$, results not shown). Among BSO-related DEGs, only 3 showed a consistent overlap with energetic stress-related genes with similar fold-changes.

Since increased oxidative stress has been associated with lower embryo quality, the proportion of AAPH- and BSO-related DEGs that correspond to the DEGs from comparison of in vivo (VIVO) and in vitro produced (IVP) bovine blastocysts was determined. This correspondence was possible since the reference IVP blastocysts for both pro-oxidant and VIVO transcriptomic comparisons were derived using an identical IVP protocol from a consistent batch of abattoir ovary's oocytes. Among the genes detected with significant intensity, VIVO blastocysts showed 3,002 DEGs (fold-change $> \pm 1.5$, $p<0.05$) when compared to IVP blastocysts, with 1,587 down-regulated DEGs and 1,415 up-regulated DEGs. When subjected to overlapping analysis, the AAPH-related profile had 96 DEGs in common with the VIVO profile (Fig. 15B), most of which were up-regulated in AAPH and down-regulated in VIVO when compared to control (Fig. 15C). In contrast, BSO showed 160 DEGs in common with VIVO, (Fig. 15B) most of which were both up- or down-regulated when compared to control (Fig. 15D). Only 7 DEGs showed common association with both pro-oxidant and VIVO. These DEGs showed inconsistent fold-changes except for the up-regulated gene lumican (pro-oxidant and high glucose treatment) which was highly down-regulated in VIVO (-13.4, $p=0.015$).

Pathway analysis of AAPH and BSO gene expression profiles

DAVID analysis software significantly clustered DEGs into functionally related groups. AAPH-related DEGs clustered with extracellular matrix organization and cell adhesion, and BSO-related DEGs clustered with structural constituents of ribosomes and glycolysis. Ingenuity pathway analysis revealed a significant enrichment of AAPH-related DEGs throughout 14 canonical pathways. The first 5 pathways were: coagulation system, TGF-beta signaling, hepatic fibrosis, pattern recognition of bacteria/viruses, and atherosclerosis signaling. BSO-related DEGs were significantly associated with 38 canonical pathways, and the first 5 pathways were: EIF2 signaling, inositol metabolism, NRF2-mediated oxidative stress response, clathrin-mediated endocytosis signaling, and regulation of actin-based motility by rho. The 64 DEGs overlapping AAPH and BSO treatment were not significantly associated with any canonical pathways. DEGs that were specific to each pro-oxidant treatment did not reveal any association with new canonical pathways. However, up-regulated genes in BSO-treated blastocysts showed a significant association with function in glycine catabolism and estrogen receptor signalling.

From the list of DEGs associated with AAPH treatment, the first gene network generated by IPA represented the impact of ROS on inflammatory response and was annotated with cancer and infectious and respiratory disease (Fig. 16, network 1). The second AAPH-related DEG network represented the impact of ROS on energetic metabolism and was annotated with organism injury and abnormalities, cardiovascular disease and cancer (Fig. 16, network 2). Twelve genes in this network overlapped DEGs associated with high glucose stress in bovine blastocysts (GJA1, HIF1A, IGF2, JAM2, LUM, MMD, OLR1, PDGFC, PLAT, PLOD2, SERPINE1 and THBS1). Networks 1 and 2 were both associated with ROS induction of NFKB signaling. The first 5 potential upstream regulators of total AAPH-related DEGs were endogenous signaling of retinoic acid (activated), KRAS enzyme activity (inhibited), IFNA2 signaling (activated), LPS effect (activated) and TNF signaling (activated).

From the list of DEGs associated with BSO treatment, the first networks were annotated to cell cycle, cellular compromise and death, and protein synthesis (Fig. 17). The second

network was associated with developmental functions of cellular assembly and organization, and tissue development. Interestingly, this network overlapped with 10 DEGs associated with in vivo produced blastocysts (VIVO) compared to in vitro (TNFSF9, RNF20, SF3B1, ZFAND5, RHOC, ACTA2, ACTA1, ACTC1, ACTC2 and MYL7), as well as NRF2 mediated oxidative stress response (ACTA1, ACTA2, ACTC1, ACTG2, and PTPLAD1). The first potential up-stream regulators of total BSO-related DEGs were rapamycine (activated), synthetic retinoid CD437, MKL1 (inhibited), IL5 and HEY2 (activated).

4.5 Discussion

The data presented in this work illustrates the genomic response of mammalian embryos to subletal oxidative stress and reveals how they become compromised, especially those that survive the stress. This is the first study exploring this concept with global gene analysis in cattle.

AAPH and BSO agents were selected as stress conditions because they were shown to affect blastocysts in a dose-dependent manner (Feugang et al. 2005; Feugang et al. 2003). AAPH generates 2 potent ROS (RO- and ROO-) that are similar to those physiologically active during IVC and which initiate extra- and intracellular responses to oxidative stress (Guerin et al. 2001). Our first result showed that adding 0.01 mM AAPH during post-compaction did not affect the day 7 blastocyst rate or hatching rate. After the MET, several cellular mechanisms regulated the adaptability to ROS and allowed the early embryo to resist to an increasing dose of oxidative stress caused by AAPH exposure (Stover et al. 2000). The effect of AAPH was, however, apparent at day 8 when a proportion of produced blastocysts started to degenerate (Feugang et al. 2003), suggesting a delayed impact on oxidative metabolism and differences in the individual capacity to react to oxidative stress. In our study, we did not observe morphological differences in the day 7 blastocyst population, consistent with the result of Feugang et al showing the appearance of a degenerating phenotype at day 7.5. However, AAPH treatment induced considerable variability on the blastocyst rate; indicative of increasing impact according to differences in weekly IVC run results. Embryo survival rate is sensitive to inherent stress during IVC and culture conditions are rapidly modulated by oxygen exposure (Agarwal et al. 2006; Martin-Romero et al. 2008). These independent variables could have exacerbated the fluctuating impact of ROS on embryo survival after AAPH treatment.

As an inhibitor of glutathione synthase, BSO was chosen to stress the limited anti-oxidant protection coming from the oocyte that might serve to adapt to IVC conditions. Our results show that post-compaction exposure to 0.4 mM BSO significantly decreased the day 7 blastocyst rate, which is consistent with another study showing impairment of blastocyst development after 24-h exposure to 1 mM BSO at 6-8–cell stage (Takahashi et al. 1993).

During post-compaction development, oxidative metabolism is increased to sustain energy production but embryonic anti-oxidant defence would normally allow maintenance of homeostasis. However, glutathione metabolism during early development would have limited response to BSO effect (Gardiner and Reed 1995b). Thus, individuals with higher oxidative metabolism or lower anti-oxidant competence may suffer from oxidative damage after GSH depletion and exhibit developmental arrest at the morula stage (Gardiner and Reed 1995a). Interestingly, Feugang et al. 2005 showed that, similar to AAPH, 0.4 mM BSO induced dose-dependent blastocyst degeneration apparent at day 8. In our study, the earlier impact may be due to the absence of serum in the medium and to the potential embryotrophic effect of growth factors that can compensate for homeostatic unbalance (Kurzawa et al. 2004; Lott et al. 2011). Moreover, BSO would have a more stringent effect than AAPH on the red/ox equilibrium maintenance of less competent blastocysts that would normally degenerate on day 8.

To study the mechanisms underlying the impact of oxidative stress on embryo survival, separate transcriptomic analyses were used to compare AAPH- and BSO-treated blastocysts against controls. Results showed high fold-changes in the transcriptomic profile of AAPH-treated blastocysts but also more variability. In contrast, BSO-treated blastocysts showed moderate modifications of gene expression and higher consistency. This indicates highly disturbed physiology in AAPH-surviving blastocysts compared to the BSO population. Also, it highlights differences in profile uniformity among AAPH- or BSO-treated blastocysts. Emerging evidence demonstrates that transcriptomic profiles are widely susceptible to intrinsic variability among the same pool of embryos with close resemblance (Smith et al. 2007). The different survival rates after both pro-oxidant treatments may be associated with a broader range of responses among individuals from the AAPH-treated population, and a reduction in individual transcriptomic diversity after BSO selection. Consistent with our microarray results, AAPH replicates showed a greater standard deviation than BSO replicates in gene expression assessed by RT-qPCR on selected candidates such as SERPINE1.

Transcriptomic analysis requires cautious interpretation as the correspondence between mRNA and protein level has not been demonstrated. Nevertheless, identification of

particular pathways significantly enriched with differentially expressed genes would indicate the global response to pro-oxidant treatment. Here, AAPH and BSO showed moderate overlapping impact on gene expression in surviving blastocysts. Among common DEGs, lower expression was validated for ARRB2, a gene coding for arrestin B2 that is involved in modulation of G protein coupled receptor (GPCR) internalization. Recruitment of ARRB2 to beta2-adrenergic receptor induces ROS generation through ERK1/2 signaling (Singh and Moniri 2012). Interestingly, loss of function of ARRB2 is associated with higher activation of MAPK after CXCR2 activation (Zhao et al. 2004). Here, CXCR4 was up-regulated after AAPH-treatment. CXCR4 is the GPCR of SDF-1, a system present in trophoblast cells and acting through ERK1/2 signalling pathway to promote survival. Alterations in SDF-1 and/or CXCR4 expression may be associated with pregnancy disorders (Jaleel et al. 2004). In relation to SDF-1 signalling, carma3 (CARD10) was down-regulated in both AAPH and BSO treatment. Carma3 is part of the molecular complex which plays a critical role in SDF-1/CXCR4-dependent induction of NF-κB and the survival of carcinoma (Brzoska et al. 2011; Rehman and Wang 2009). The differential expression of ARRB2, CXCR4 and CARD10 would indicate the implication of ERK1/2 in promoting survival in response to oxidative stress.

Carma3 functions with ARRB2 to mediate the pro-inflammatory signal of NF-κB in endothelial cells, (Delekta et al. 2010) and oxidative stress is known to trigger inflammation in somatic tissue (Cui et al. 2004). Here, both AAPH- and BSO-related transcriptomes indicate an inflammatory response in surviving blastocysts. Particularly, TNF signalling, which is central in inflammation, was well represented in AAPH-related DEGs. TNFAIP8L3 has been shown to be up-regulated in energetically stressed blastocysts (Cagnone et al. 2012b), as were the TNF receptor (TNFRSF1A), the adipokine-coding gene C1QTNF3 and the receptor of oxidized lipoprotein 1. These genes are linked with oxidative stress and inflammation of adipose tissue. As AAPH exposure results in increased cell membrane permeabilization in bovine blastocysts, (Feugang et al. 2005; Yoshida et al. 2004) up-regulation of inflammation-responsive genes may be correlated with ROS-induced lipid peroxidation and production of oxidized lipids (Kim et al. 2010; Kopp et al. 2010).

Compared to BSO, AAPH showed a greater impact on inflammatory associated genes. Coding for the prototypic, evolutionarily conserved, long pentraxin 3, PTX3 is unequivocally involved in innate immunity and inflammation (Deban et al. 2011). PTX3 is also up-regulated in cumulus cells just prior to ovulation (in mouse, human and cow) and, as the cumulus cells are rapidly destroyed by the oviduct afterward, could act as a suicide signal. In addition, several interferon-responsive genes were up-regulated after AAPH, such as IFITM2 and 3. These genes code for IFN-induced transmembrane proteins, and are involved in cell adhesion during embryogenesis (Siegrist et al. 2011). Interestingly, IFITM3 expression is restricted to the inner cell mass and is up-regulated in cloned bovine blastocysts (Smith et al. 2007). As IFITM3 over-expression reduces proliferation in human cell lines, it may affect ICM growth in AAPH-treated blastocysts. Moreover, IFITM3 physically interacts with secreted phosphoprotein 1 (SPP1 or osteopontin) (El-Tanani et al. 2010), a cytokine and adhesion molecule up-regulated at the RNA level in both AAPH- and BSO-treated blastocysts. In response to oxidative stress in vitro and in vivo (Urtasun et al. 2012), SSP1 down-regulation is associated with enhanced trophoblastic growth and migration during human implantation (Hannan and Salamonsen 2008). Therefore, analysis of cytokine secretion could be a useful marker of pro-inflammatory signal before implantation (Johnson et al. 2003).

AAPH- and BSO-treated blastocysts showed common DEGs associated with extracellular matrix signalling and adhesion molecules. Coding for secreted proteoglycans, lumican was up-regulated in both conditions while decorin expression was only increased after AAPH treatment. ROS stimulate the production of advanced glycosylation end-products (AGE) that up-regulate the expression of lumican and decorin (Brownlee 2001; Pantaleon et al. 2010; Schaefer et al. 2001). Similarly, AGE induces protein glycosylation of the transcription factor SP1 (Brownlee 2001), and leads to the up-regulation of collagen 3A1 (Luna et al. 2009; Santra et al. 2008) as observed in AAPH-treated blastocysts. Betaglycan and ECM molecules play an important role in extracellular signalling and regulation of TGF-B bio-action (Massague and Chen 2000). Here, expression of TGFB3 was increased in AAPH treated blastocysts. Up-regulation of TGFB3 may predispose the pregnancy to preeclampsia while reduced expression is linked to trophoblastic invasiveness (Caniggia et al. 1999). Similarly, NDP, encoding the putative extracellular factor Norrin with homology to the

TGF-B super-family, was up-regulated after AAPH exposure and would have an important role in reproductive tissues around implantation and possibly in the embryo (Luhmann et al. 2005). Along with TGF-B and other ECM factors (Edwards 2012), analysing the external signal produced by the embryos could indicate a potential inflammatory response (Lohr et al. 2012) and the subsequent attraction of neutrophils which may be detrimental for implantation (Hayashi et al. 2010).

As for inflammation, AAPH induced a higher impact than BSO on ECM remodelling. Periostin, a matrix protein involved in cell adhesion as well as tissue remodelling, was up-regulated after AAPH exposure. Highly expressed during fibrosis, increased periostin expression could result from inflammatory signal through several cytokines such as TGF-β (Marotta et al. 2009; Yang et al. 2012). Moreover, AAPH treated blastocysts up-regulated thrombospondin, a gene coding for a glycoprotein in the ECM found at the porcine maternal fetus interface (Edwards et al. 2011) that binds several ligands including glycosaminoglycans as well as plasminogen. Interestingly, genes involved in plasminogen function were up-regulated after AAPH exposure. Plasminogen activator inhibitor (SERPINE1) and tissue-type plasminogen activator (PLAT) controls the proteolytic degradation of plasminogen and modulation of thrombosis. Under oxidative stress conditions, secretion of SERPINE1 and PLAT is associated with ERK1/2 activation (Banfi et al. 2003). Furthermore, TGFB3 induces expression of collagen (Verrecchia and Mauviel 2002) and SERPINE1 (Liu 2008) during fibrosis. Plasminogen activity was suggested to participate in implantation (Aflalo et al. 2004; Kubo et al. 1981) and differential expression of SERPINE1 during placental oxidative stress is associated with preeclampsia (Meade et al. 2007; Wikstrom et al. 2009). Changes in gene expression relative to ECM proteases may have important consequences at the time of attachment and could be associated with miscarriage after IVC.

With implications to fibrosis-like remodelling, the AAPH-related profile is associated with growth factor signalling. Up-regulated in AAPH-treated blastocysts, platelet-derived growth factor-C (PDGFC) regulates numerous genes involved in tissue remodelling and organogenesis (Jinnin et al. 2005). PDGFC is up-regulated in growing uterine fibroids (Suo et al. 2009) and is inversely correlated with lymphocyte infiltration in carcinomas (Bruland

et al. 2009). PDGFC receptor is expressed by early embryos, suggesting at least a paracrine effect on the proliferation of the ICM and derived tissues (Osterlund et al. 1996). PDGF supplementation would partially rescue TNF-alpha/IFN-gamma induction of trophoblast apoptosis (Smith et al. 2002). Similarly, IGF2, up-regulated in AAPH-treated blastocysts, has been shown to rescue embryo development after exposure to oxidative stress (Artus et al. 2010; Kawamura et al. 2007; Kurzawa et al. 2004; Smith et al. 2002). IGF signaling plays a fundamental role in embryo-maternal crosstalk and in early embryo development (Thieme et al. 2012). IGF2 is up-regulated in ovine parthenotes (Bebbere et al. 2010) as well as in hyperglycemia-treated blastocysts in rabbit (Thieme et al. 2012) and cattle (Cagnone et al. 2012b). Considering that growth factors for the ICM play a key role in activating the ERK pathway, PDGF and IGF2 up regulation may counteract inflammatory signal and maintain survival after oxidative stress.

Both AAPH- and BSO-related profiles indicate the impact of oxidative stress but surviving blastocysts exhibited different anti-oxidant responses. On the one hand, AAPH-treated blastocysts show up-regulation of anti-oxidant enzymes. Methionine sulfoxide reductase beta 3 (Weissbach et al. 2002) responds to peroxide-induced cellular oxidation of methionine (Chao et al. 1997; Zhang et al. 2011) while glutathione peroxidase 8 (Guerin et al. 2001) eliminates the methionine sulfoxidation caused by AAPH (Cui et al. 2011; Nguyen et al. 2011). On the other hand, BSO-related DEGs indicated a significant representation of nrf2 signalling. Nrf2 transcription factor is involved in mediating the response to oxidative stress after BSO treatment (Lee et al. 2008). However, the BSO-related DEGs concordant with Nrf2 targets were mostly down-regulated in blastocysts. For example, the gene coding for the stress-induced phosphoprotein that coordinates the folding activity of chaperone was down-regulated, as were two HSP genes. This suggests that while AAPH-treated blastocysts exhibit an anti-oxidant response, embryos that survived the BSO would have better anti-oxidant capacity and therefore lower Nrf2 activity (Lee et al. 2008).

Instead of antioxidant response, BSO-treated blastocysts showed an up-regulation of genes coding for two glycine cleavage system proteins involved in the catabolism of amino acids. Glycine is the enzymatic substrate of the γ-glutamyl-cystein synthase that produces the reduced glutathione and which is specifically inhibited by BSO. It has been

demonstrated that preimplantation embryos can up-regulate de novo GSH synthesis in response to oxidative stress but not to GSH depletion (Stover et al. 2000). Therefore, inhibition of GSH turn-over would increase glycine content (Zhang et al. 2009) which, even if required to maintain embryonic cell osmolarity in culture (Baltz and Tartia 2010), may be damaging for cellular homeostasis (Leipnitz et al. 2009). Thus, the up-regulation of the glycine cleavage system could improve the clearance of glycine accumulation (Oda et al. 2007) as a side effect of inhibition of GSH synthesis. Accordingly, SHMT1 expression was down-regulated in both AAPH- and BSO-treated blastocysts. This gene codes for the serine dehydrogenase that catalyzes the reversible conversion of serine and tetrahydrofolate to glycine and 5,10-methylene tetrahydrofolate. Moreover, folate receptor 1, a membrane-bound receptor responsible for uptake of folate in hESC and bovine embryos (Kwong et al. 2010; Steele et al. 2005), was down-regulated in BSO-treated blastocysts. Folate is a methyl donor and depletion of folate results in elevated intracellular cystein and GSH. Although further studies are required to validate changes in amino acid content in BSO-treated embryos, differential expression of genes related to glycine and folate turn-over may have potential implication in methyl group metabolism in surviving blastocysts.

The notion elaborated in the quiet hypothesis proposed that embryos with internal damage exhibit a more active metabolic state than embryos able to maintain normal homeostasis (Leese et al. 2007). AAPH- and BSO-treated blastocysts showed different responses associated with metabolic stress when compared to high-glucose treated blastocysts (Cagnone et al. 2012b). High glucose is thought to compromise embryonic metabolism by targeting the mitochondrial function (Chi et al. 2002) and increasing the expression of glycolytic enzymes, a metabolic enhancement of the Warburg effect during post-compaction development (Cagnone et al. 2012b; Krisher and Prather 2012; Marin-Hernandez et al. 2009). The Warburg effect corresponds to the use of aerobic glycolysis as a complementary source of building blocks and energy to the mitochondrial respiration. Here, AAPH increased the expression of IGFBP7, a gene coding for the secreted IGF-binding protein 7 which reflects metabolic perturbation of mitochondrial oxidative phosphorylation in mammalian somatic and cancer cells (Cervera et al. 2009). Moreover, mitochondrial failure is concomitant with ROS production which induces NFκB signalling pathways as well as activation of the AhR/HIF1A complex (De Palma et al. 2007; Harvey et al. 2002), an

important site of cross-talk at the level of transcription in AAPH-related gene networks (Dimova et al. 2004; Meade et al. 2007). In contrast to AAPH, BSO-surviving blastocysts showed up-regulation of the TCA-related citrate synthase gene but down-regulation of ALDOA expression which is involved in glucose metabolism and positively associated with a higher glycolytic rate (Paczkowski and Krisher 2010; Sugiura et al. 2005). We also observed down-regulation of TPI1 coding for a triose phosphate isomerase, an important glycolytic enzyme under the regulation of HIF1A in conditions of metabolic stress (Hamaguchi et al. 2008). Lower glycolytic activity in the BSO group could also be related to the lower expression of the insulin receptor (INSR1), a positive marker of diabetes and obesity (Cai et al. 2012). Taken together, these differential patterns of metabolic genes suggest that AAPH impacts mitochondrial oxidation and therefore energy production. In contrast, lower expression of glycolytic enzymes may translate to efficient oxidative activity in BSO-surviving blastocysts.

Feugang et al. has shown that surviving blastocysts to AAPH or BSO treatments exhibit comparable hatching rates, mean cell numbers and apoptotic cell rates, while degenerating embryos have higher cellular and molecular damage (Feugang et al. 2005). Here, AAPH had more impact on inflammatory response than BSO. IFN-gamma/TNF-alpha induced apoptosis under PI3K signalling in trophoblast and early embryos (Kawamura et al. 2007; Loureiro et al. 2007) and up-regulation of responsive genes may be correlated with increased apoptotic rate in degenerating blastocysts (Feugang et al. 2003). In relation to energetic stress and mitochondrial failure, IGFPB7 up-regulation is associated with lower proliferation and invasiveness of trophoblast cells under TGF-B signalling (Liu et al. 2012). Finally, ID3, which codes for the inhibitor of differentiation/DNA binding 3, was up-regulated in AAPH-treated blastocysts. ID3 acts under p38MAPK as a redox sensor of cell proliferation in response to increased ROS (Mueller et al. 2002; Nickenig et al. 2002). In sheep, ID3 is up-regulated in degenerated blastocysts as part of the transcriptomic regulation of TGFB-signaling (Li et al. 2012). The different gene expression patterns between AAPH- and BSO-surviving blastocysts lead us to hypothesize the existence of a differential contribution of pre-degenerating blastocysts in each pro-oxidant comparison.

Overall gene up-regulation is concomitant with the environmental stress response to IVC conditions when compared to the in vivo environment (Cote et al. 2011b; Lazzari et al. 2002; Robert et al. 2011b). Associated with major up-regulation of gene expression (>70%), AAPH treatment enhanced the impact of the in vitro production system (IVP) when compared to the in vivo production system (VIVO), and the majority of these genes were associated with inflammatory response. In contrast, the majority (>60%) of BSO-related DEGs were down-regulated and showed similar changes as observed in VIVO-derived blastocysts. Cautious interpretation is required as common DEGs from pro-oxidant–surviving blastocysts represent a low percentage of those found between IVP and VIVO blastocysts. However, this transcriptomic similarity coincides with a major down-regulation of ribosomal genes in BSO group. Under the control of the nucleolus, and representing a high proportion of total RNA content, ribosomal RNA abundance has been associated with embryo quality (Zheng et al. 2012). Higher total RNA content is found in compromised blastocyst stage embryos after heat-induced metabolic stress (Edwards et al. 2009; Payton et al. 2011). Moreover, rRNA synthesis during blastocyst development would be kept low in quiet and more viable embryos (El-Sayed et al. 2006; Leese et al. 2007).

With regard to viability, two genes inversely related to developmental competence (TKDP1 and ANXA2) were down-regulated in either BSO or VIVO blastocysts when compared to IVP controls. Annexin 2 (ANXA2), a calcium-dependent phospholipid binding protein involved in membrane fusion and signal transduction, is correlated to be up-regulated in pregnancy loss and abortion (El-Sayed et al. 2006). Moreover, aberrant expression of ANXA2 in trophoblastic cells during decidualization leads to pro-inflammatory signalling and preeclampsia (Menkhorst et al. 2012). TKDP1 is hypothesized to function as a maternal-recognition factor, and both TKDP 3 and 4 are up-regulated in blastocysts derived from in vitro culture as compared to artificial insemination (Smith et al. 2009). Little is known about TKDP function but their temporal expression profile and localisation at the fetal-maternal interface strongly suggests a role during pregnancy (Chakrabarty and Roberts 2007). Prior to any embryo transfer experiment, the differential expression of competence markers by BSO-treated blastocysts may indicate higher embryonic quality in the surviving population. However, testing the metabolic state of

blastocysts that survive BSO treatment is required to validate any potential selection of the best embryos after GSH depletion.

In conclusion, our analysis showed that both extra- and intra-cellular oxidative stress disrupted pre-attachment development, and that surviving blastocysts showed transcriptomic modifications associated with inflammation and ECM remodelling, featuring a fibrosis-like reaction similar to that seen in somatic tissues (Bhattacharyya et al. 2012). Although required for adaptability, this stress response may profoundly alter cellular signalling by either inducing embryo degeneration, or affecting subsequent maternal recognition. In addition, genomic analysis of the BSO-related profile suggests a response to high glycine in blastocysts after glutathione depletion. Moreover, these surviving blastocysts showed lower expression of metabolic genes like their in vivo counterparts ("quiet" hypothesis), suggesting a selection of better quality embryos based on their capacity to maintain homeostasis after antioxidant depletion. However, more investigations are required to ensure that stress response does not affect the potentially higher viability of BSO-selected individuals.

4.6 Materials & methods

All chemicals were obtained from Sigma-Aldrich, unless otherwise stated.

In vitro production (IVP) of bovine blastocysts

Oocyte collection and in vitro maturation (IVM)

Cumulus–oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of slaughtered heifers. After four washes in TLH (HEPES-buffered Tyrode's Lactate solution), groups of up to 10 selected COCs with at least five layers of cumulus were placed in 50-µl drops of medium under mineral oil in dishes (Nunc, Roskilde, Denmark) and matured for 24 h at 39°C under an atmosphere of 5% CO2 in air with maximum humidity. The maturation medium was TCM-199 supplemented with 10% fetal calf serum (HyClone, ThermoScientific, USA), 0.1 µg/ml of FSH (Folltropin V, Bioniche, Canada), 0.33 mM of pyruvic acid and 50 µg/ml of gentamycin.

In vitro fertilization (IVF)

Following maturation, five matured COCs were added to 48-µl droplets of IVF medium under mineral oil. The IVF medium was composed of TL STOCK (Tyrode's Lactate solution) supplemented with 0.6% fatty acid free bovine serum albumin (BSA), 0.2 mM pyruvic acid, 10 µg/ml heparin, and 50 µg/ml gentamycin. Prior to transfer, the COCs were washed twice in TLH medium. Once transferred, 2 µl of PHE (1 mM hypotaurine, 2 mM penicillamine, 250 mM epinephrine) were added to each droplet 10 min before spermatozoa was added. The spermatozoa used consisted of a cryopreserved mixture of ejaculates from five bulls (Centre d'Insémination Artificielle du Québec, St.-Hyacinthe, QC, Canada). The spermatozoa were thawed in 37°C water for 1 min, put on a discontinuous Percoll gradient (2 ml of 45% Percoll over 2 ml of 90% Percoll), and centrifuged at 700g for 30 min at 26°C. After discarding the supernatant, the pellet of live spermatozoa was counted on a haemocytometer to obtain a concentration of 106 cells/ml and was resuspended in IVF medium. Finally, 2 µl of the sperm suspension (final concentration = 4x104 cells/ml) was

added to each IVF droplet containing the matured COCs and was incubated for 16-18 h in a humidified atmosphere at 38.5°C in 5% CO2.

In vitro culture (IVC)

For embryo culture, a three-step modified synthetic oviduct fluid (SOF) culture system containing MEM, essential and non-essential amino acids, 0.5 mM of glycyl-glutamine, and 0.4% fatty acid-free BSA under embryo-tested mineral oil (#8410, Sigma) was used. The embryo culture dishes were incubated at 38.5°C with 6.5% CO2, 5% O2 and 88.5% N2 in 100% humidity. Briefly, after fertilization, presumptive zygotes were mechanically denuded and washed three times in TLH supplemented with fatty acid-free BSA and were placed in groups of 10 in 10-µl droplets of SOF1 with non-essential amino acids (1X) and 3 µM EDTA. Embryos were transferred to new 10-µl droplets of SOF2 containing non-essential (1X) and essential (0.5X) amino acids 72 h post-fertilization, and again 120 h post-fertilization to 20-µl droplets of SOF3 containing non-essential (1X) and essential (1X) amino acids. The medium was replaced 3 times to prevent toxicity due to ammonium accumulation and nutrient depletion caused by amino acid degradation and embryo metabolism, respectively. The glucose concentration used in SOF1, 2 and 3 was 0.2, 0.5 and 1.0 mM, respectively. Cleavage rate, (number of embryos with at least 2 cells out of total embryos) and 8/16-cell embryo rate, (number of embryos with at least 8 cells out of total embryos) were determined during embryo transfer from SOF1 to SOF2 (Day 3). Blastocyst rate, (number of early, expanded and hatched blastocysts out of total embryos) and hatching rate, (number of hatched blastocysts out of total blastocysts) were calculated at the end of the culture period.

Exposure to pro-oxidant treatments

Concentrations of 0.01 mM for AAPH and 0.4 mM for BSO were selected as they appeared to be detrimental for blastocyst development (Feugang et al. 2005; Feugang et al. 2003), and were used from day 3 to produce 7 replicates of control, AAPH- or BSO-treated blastocysts from different in vitro production runs. Blastocyst development was assessed at day 7 post-fertilization. Pooled blastocysts (hatched and non-hatched) were washed 3 times in PBS, collected in groups of 10 in small volumes of PBS into 0.5 mL microtubes, and

stored at -80°C until RNA extraction. Each replicate contained about 10 embryos including non-expanded (early), expanded and hatched blastocysts. Equivalent proportions of hatched blastocysts were kept between control and treatment replicates. Four out of 7 replicates were used for microarray experiment and 3 out of 7 replicates were used to validate the microarray results by RT-qPCR.

Determination of differential gene expression profile

Total RNA from each replicate was extracted and purified using a PicoPure™ RNA Isolation Kit (Life Science). After DNase digestion (Qiagen), the quality and concentration of extracted RNA was analyzed with a Bioanalyzer (Agilent). All extracted samples showed good quality with an RNA Integrity Number >7.5.

For microarray purposes, purified RNA was amplified by in vitro transcription with T7 RNA amplification using the RiboAmp® HSPlus RNA Amplification Kit (Life Science) and labelled with Cy3 and Cy5 using the ULS™ Fluorescent Labeling Kit (Kreatech). aRNA (825 ng per replicate) was hybridized on the Agilent-manufactured EmbryoGENE slides in a 2-color dye swap design. After 17 h of hybridization at 65°C, microarray slides were washed 1 minute in Expression Wash Buffer 1 (room temperature), 3 minutes in gene Expression Wash Buffer 2 (42 °C), 10 seconds in 100% acetonitrile (room temperature) and 30 seconds in Stabilization and Drying Solution (Agilent). Slides were scanned with PowerScanner (Tecan) and features extraction was done with Array-pro6.3 (MediaCybernetics). Intensity files were analyzed with FlexArray (Michal Blazejczyk, Mathieu Miron, Robert Nadon (2007), FlexArray: A statistical data analysis software for gene expression microarrays. Genome Quebec, Montreal, Canada, URL: http://genomequebec.mcgill.ca/FlexArray).

Specifically, intensity raw data were corrected by background subtraction then normalized within (green/red) and between each array (Loess and quantile, respectively). Statistical comparison between treatments (AAPH vs. Control or BSO vs. Control) was done with the Limma algorithm which attributed to each probe a probability to fold-change difference between treatment and control. The data discussed in this publication has been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series

accession number GSE42281: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42281.

Validation of microarray results was done by RT-qPCR. Total extracted RNA from independent samples (3 replicates for each condition) were reverse-transcribed using oligo-dT primer and a qScript™ Flex cDNA Synthesis Kit (Quanta Biosciences). Specific primers for each selected gene were designed using PrimerQuestSM (Integrated DNA Technologies) and qPCR was performed using the LightCycler 480® SYBR Green I Master and the LightCycler® 480 System (Roche). A standard curve composed of five points of the PCR product for each primer pair diluted from 1 pg to 0.1 fg was used for real-time quantification of PCR output. A GeNORM normalization factor (Vandesompele et al. 2002) from expression values of 3 reference genes (ACTB, MYL6, PPIA) was used for data normalization. Moreover, technical variations were assessed and corrected through quantification of an exogenous GFP spike which was introduced at the time of RNA extraction (Vigneault et al. 2004). ANOVA was used for statistical comparison of developmental rate and RT-qPCR results between control, AAPH and BSO treatments. Primer sequences, product size, annealing temperature and accession numbers are provided in Supplemental Table 7.

Functional analysis of differentially expressed genes

Based on Gene ontology databases reporting transcriptomic analysis in mammalian embryos, Microsoft Access was used to compare the differentially expressed genes (DEGs) that are associated with blastocyst sex status (Bermejo-Alvarez et al. 2010) or detected in day 7 IVP bovine blastocysts exposed to high glucose stress conditions (Cagnone et al. 2012b). Moreover, a microarray profile generated with in vivo produced blastocysts (VIVO) was also compared to AAPH and BSO profiles. VIVO blastocysts resulted from artificial insemination of superovulated eggs and embryo flushing on day 7 after AI (Plourde et al. 2012a). Gene expression in VIVO blastocysts was compared to control blastocysts (CTL) which were produced using the IVP protocol described earlier. Microarray analysis between VIVO and CTL was performed using the experimental procedure described earlier.

DAVID software was used to analyze the functions of differentially expressed genes into clusters (Huang da et al. 2009a; Huang da et al. 2009b). Moreover, data were analyzed with Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com). IPA was used to compile canonical pathways as well as gene product interactions (networks) that were differentially expressed between treatments. We used IPA to build schematic representations of important pathways that were dysregulated in treated blastocysts.

Network Generation

A data set containing gene identifiers and corresponding expression values was uploaded into the application. Each identifier was mapped to its corresponding object in Ingenuity's Knowledge Base. A fold-change cut-off of 1.5 with a p-value <0.05 was set to identify molecules whose expression was significantly differentially regulated. These molecules, called Network Eligible Molecules, were overlaid onto a global molecular network developed from information contained in Ingenuity's Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Human, mouse, and rat orthologs of a gene are stored as separate objects in the Ingenuity Pathways Knowledge Base, but are represented as a single node in the network. Nodes are displayed using various shapes that represent the functional class of the gene product.

Canonical Pathway Analysis

IPA identified the pathways that were most significant to the dataset from the Ingenuity Pathways Analysis library of canonical pathways. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed; 2) Fisher's exact test was used to calculate a p-value to determine the probability that the association between the genes in the dataset and the canonical pathway would occur by chance alone. Green and red symbols

represent genes respectively down- and up-regulated in treated embryos compared to controls. Grey symbols represent genes with significant expression in blastocysts but no difference between conditions, whereas white symbols represent genes not present on microarray or with below-background intensity.

4.7 Acknowledgements

We would like to thank Isabelle Laflamme and Isabelle Dufort for technical support. The in vivo embryos were provided by L'Alliance Boviteq, Canada. This study was supported by NSERC and The EmbryoGENE network of Canada. Authors declare no conflict of interest.

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4.9 Figures

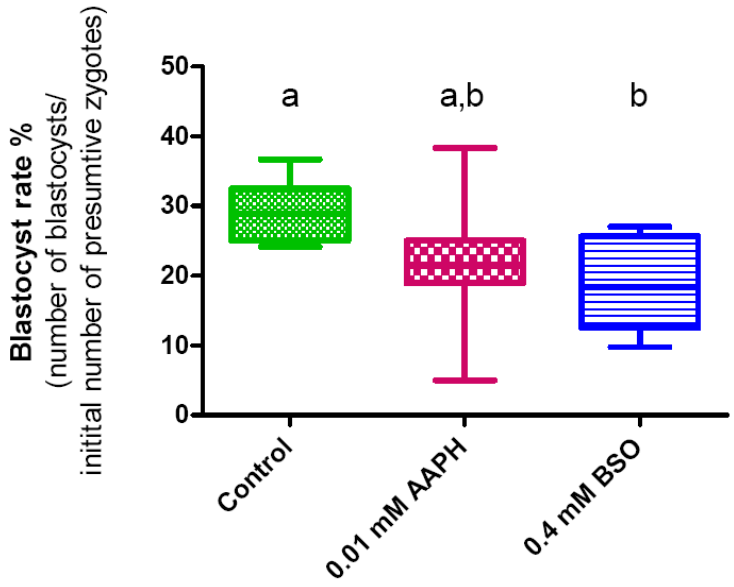
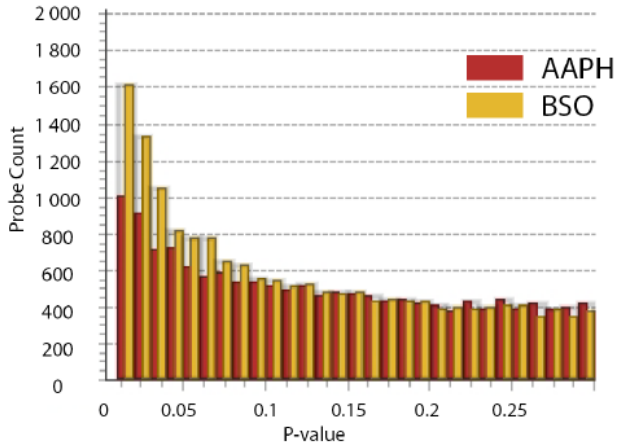


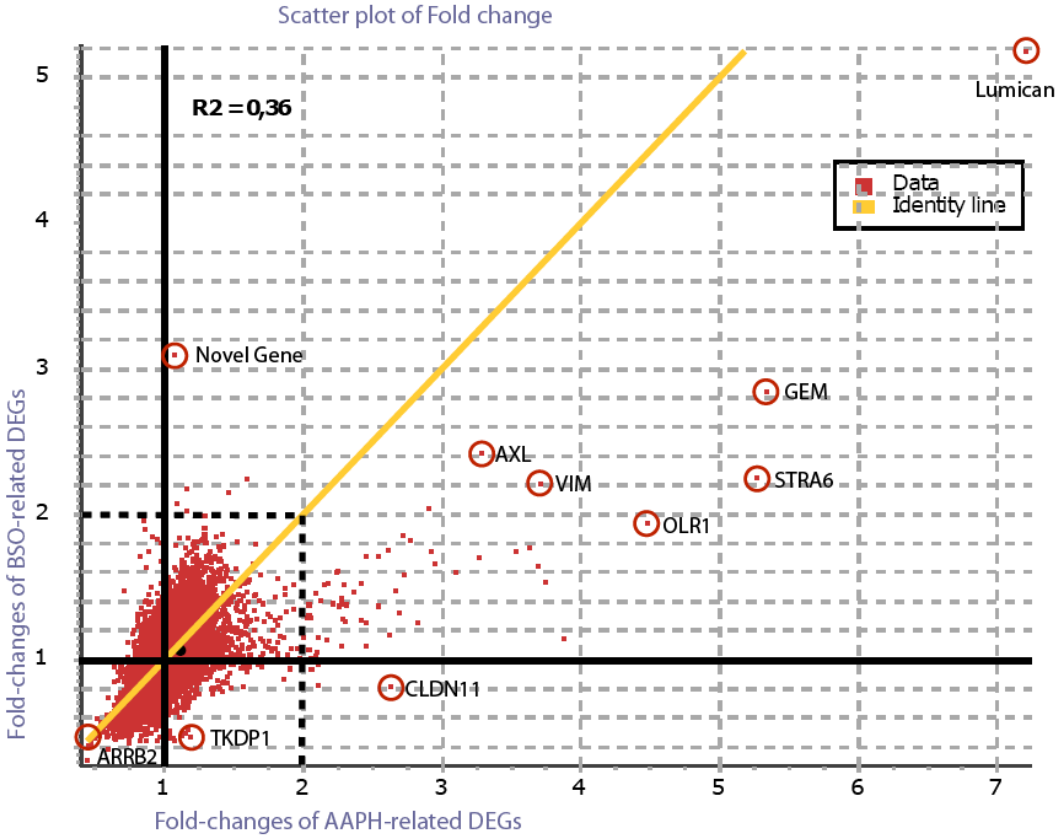
Figure 12 Survival rate to pro-oxidant conditions.

Data represent the mean blastocyst rate (± SD) after in vitro culture exposure to either control, 0.01 mM AAPH or 0.4 mM BSO supplemented condition during post-compaction development (day 3 to 7). Different superscripts represent significant difference between groups (ANOVA $p<0.05$).

A


AAPH
BSO
Probe Count
2 000
1 800
1 600
1 400
1 200
1 000
800
600
400
200
0
0
0.05
0.1
0.15
0.2
0.25
P-value

B


Scatter plot of Fold change
R2 = 0,36
Data
Identity line
Lumican
Novel Gene
GEM
AXL
VIM
STRA6
OLR1
CLDN11
TKDP1
ARRB2
Fold-changes of BSO-related DEGs
Fold-changes of AAPH-related DEGs
1
2
3
4
5
6
7

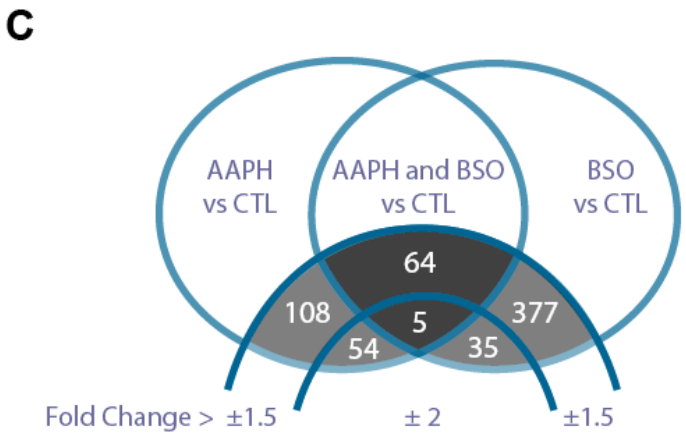
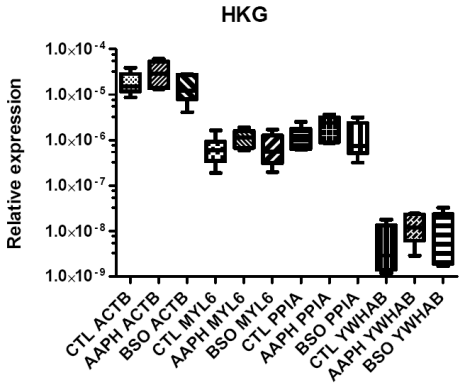
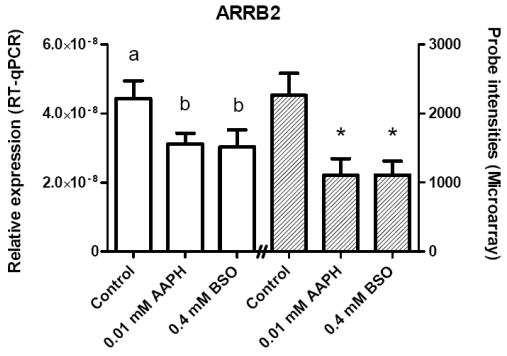
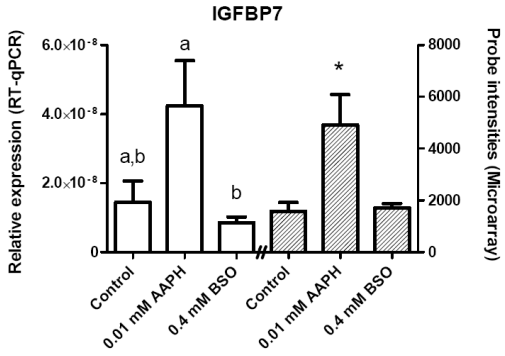


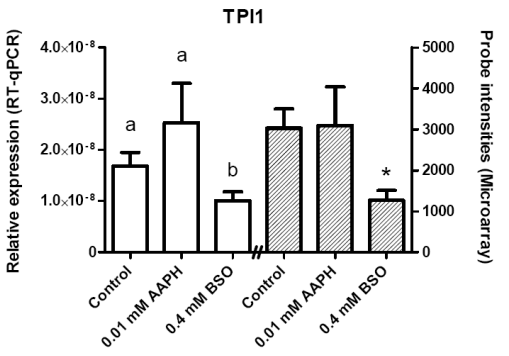
Figure 13 Microarray analysis of differential gene expression profile in blastocysts from AAPH and BSO conditions compared to control.

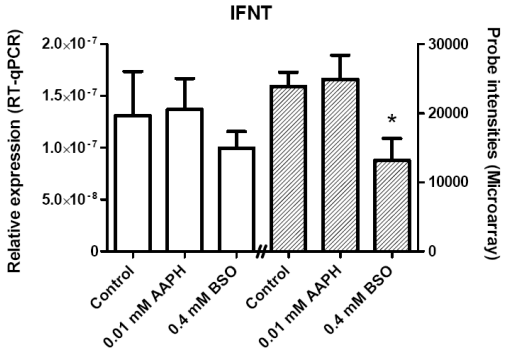
A) Data represent the number of differentially expressed genes (probe) in AAPH or BSO groups when compared to control as a function of the corresponding p-value. B) Data represent the scatter plot of all DEG fold-changes induced by AAPH and BSO. C) Venn diagram represents the number of differentially expressed genes in AAPH- and/or BSO-treated blastocysts with $p<0.05$ and fold-change $> \pm 1.5$.

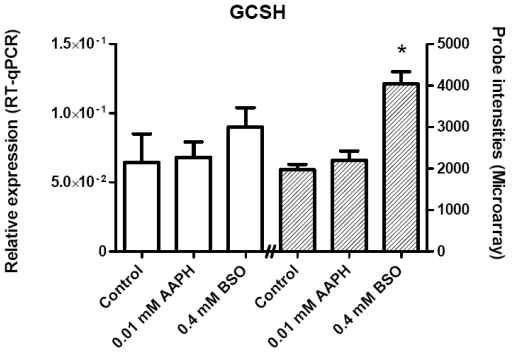

HKG
Relative expression
1.0×10-4
1.0×10-5
1.0×10-6
1.0×10-7
1.0×10-8
1.0×10-9
CTL ACTB
AAPH ACTB
BSO ACTB
CTL MYL6
AAPH MYL6
BSO MYL6
CTL PPIA
AAPH PPIA
BSO PPIA
CTL YWHAB
AAPH YWHAB
BSO YWHAB


ARRB2
Relative expression (RT-qPCR)
Probe intensities (Microarray)
6.0×10-8
4.0×10-8
2.0×10-8
0
3000
2000
1000
0
a
b
b
*
*
Control
0.01 mM AAPH
0.4 mM BSO
Control
0.01 mM AAPH
0.4 mM BSO


IGFBP7
Relative expression (RT-qPCR)
Probe intensities (Microarray)
6.0×10-8
4.0×10-8
2.0×10-8
0
8000
6000
4000
2000
0
a,b
a
b
*
Control
0.01 mM AAPH
0.4 mM BSO
Control
0.01 mM AAPH
0.4 mM BSO


TPI1
Relative expression (RT-qPCR)
Probe intensities (Microarray)
4.0×10-8
3.0×10-8
2.0×10-8
1.0×10-8
0
5000
4000
3000
2000
1000
0
a
a
b
*
Control
0.01 mM AAPH
0.4 mM BSO
Control
0.01 mM AAPH
0.4 mM BSO


IFNT
Relative expression (RT-qPCR)
Probe intensities (Microarray)
2.0×10-7
1.5×10-7
1.0×10-7
5.0×10-8
0
30000
20000
10000
0
*
Control
0.01 mM AAPH
0.4 mM BSO
Control
0.01 mM AAPH
0.4 mM BSO


GCSH
Relative expression (RT-qPCR)
Probe intensities (Microarray)
1.5×10-1
1.0×10-1
5.0×10-2
0
5000
4000
3000
2000
1000
0
*
Control
0.01 mM AAPH
0.4 mM BSO
Control
0.01 mM AAPH
0.4 mM BSO

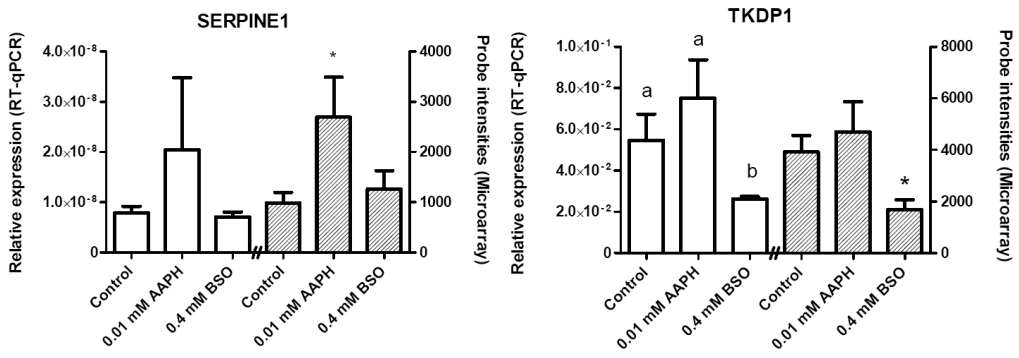


Figure 14 RT-qPCR results on selected candidates from microarray analysis.

*Graphs represent the gene expression analysis performed in independent samples using RT-qPCR (white bars) as well as the corresponding probe intensities from microarray results (gray bars). Different superscripts represent significant difference between groups (ANOVA $p<0.05$). *$p<0.05$, ANOVA was done on normalized data (see Materials and Methods).*

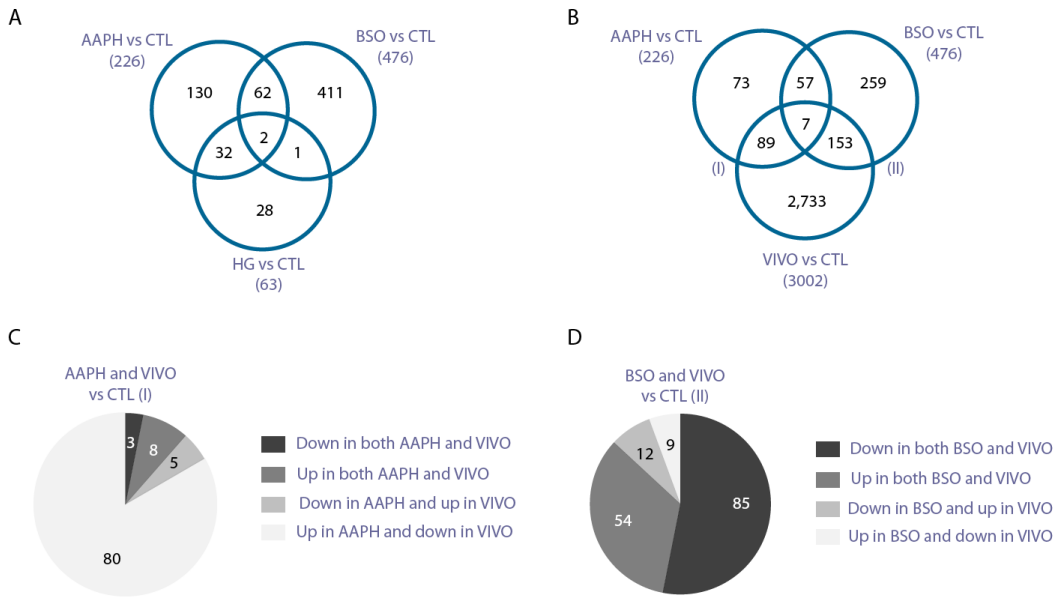


Figure 15 Overlapping the DEGs in bovine blastocysts resulting from different conditions of culture.

Using control IVP embryos as the same reference for each microarray analysis, Venn diagrams represent the DEGs in AAPH- and BSO-treated blastocysts that overlap the DEGs in high glucose (HG)-treated blastocysts (A) or in vivo (VIVO) produced blastocysts (B). Based on common DEGs only, pie charts represent the proportion of DEGs related to AAPH (C) or BSO (D) treatment that exhibit similar or different expression fold-changes to DEGs related to VIVO treatment.

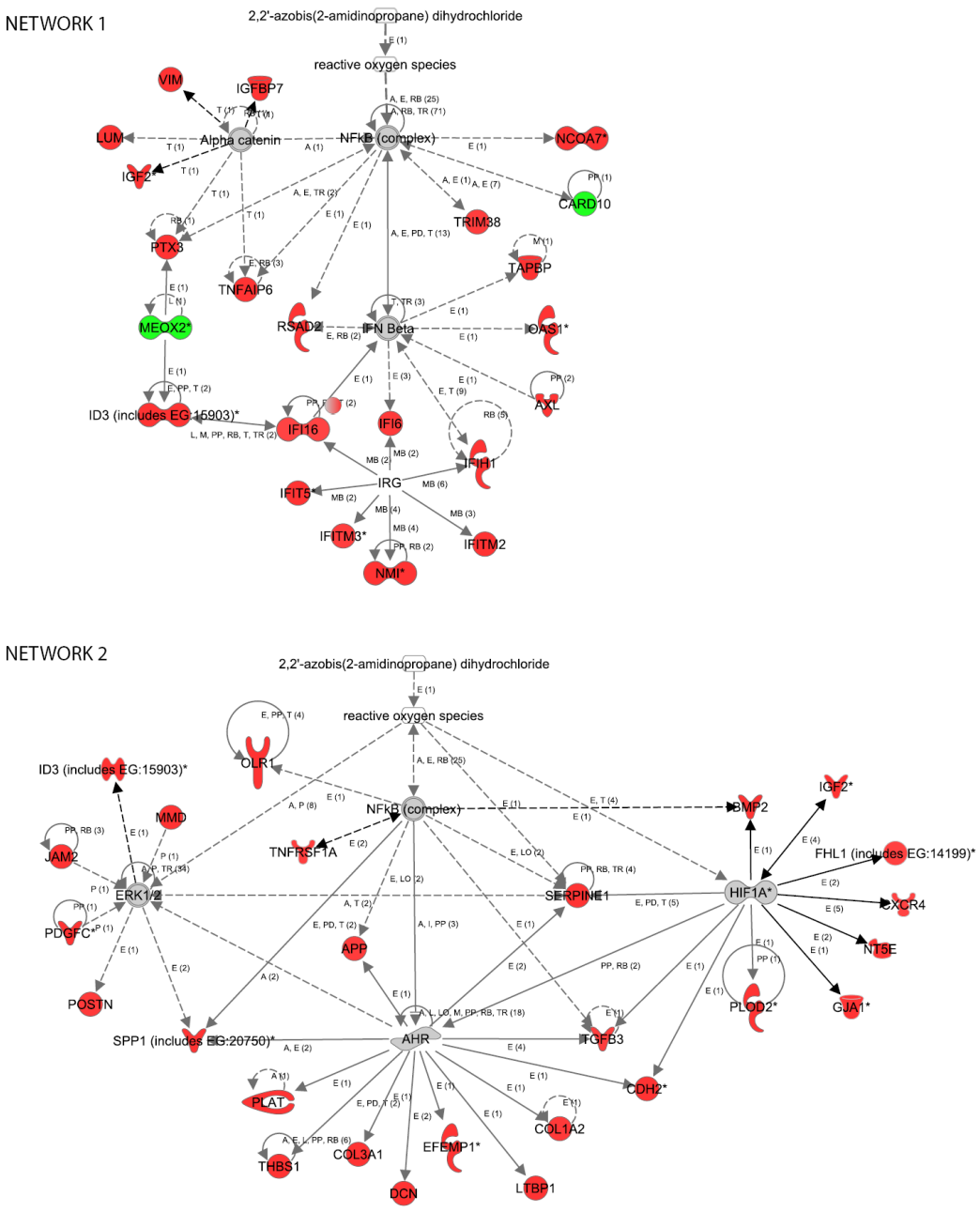


Figure 16 Ingenuity pathway analysis of AAPH impact.

Based on gene expression connectivity, Networks 1 and 2 show the significant associations of DEGs from AAPH-treated blastocysts with the inflammatory response to oxidative stress (network 1) and the impact on mitochondrial metabolism and extracellular matrix fibrosis (network 2). Red and green colors respectively represent up- and down-regulated DEGs in AAPH-treated blastocysts.

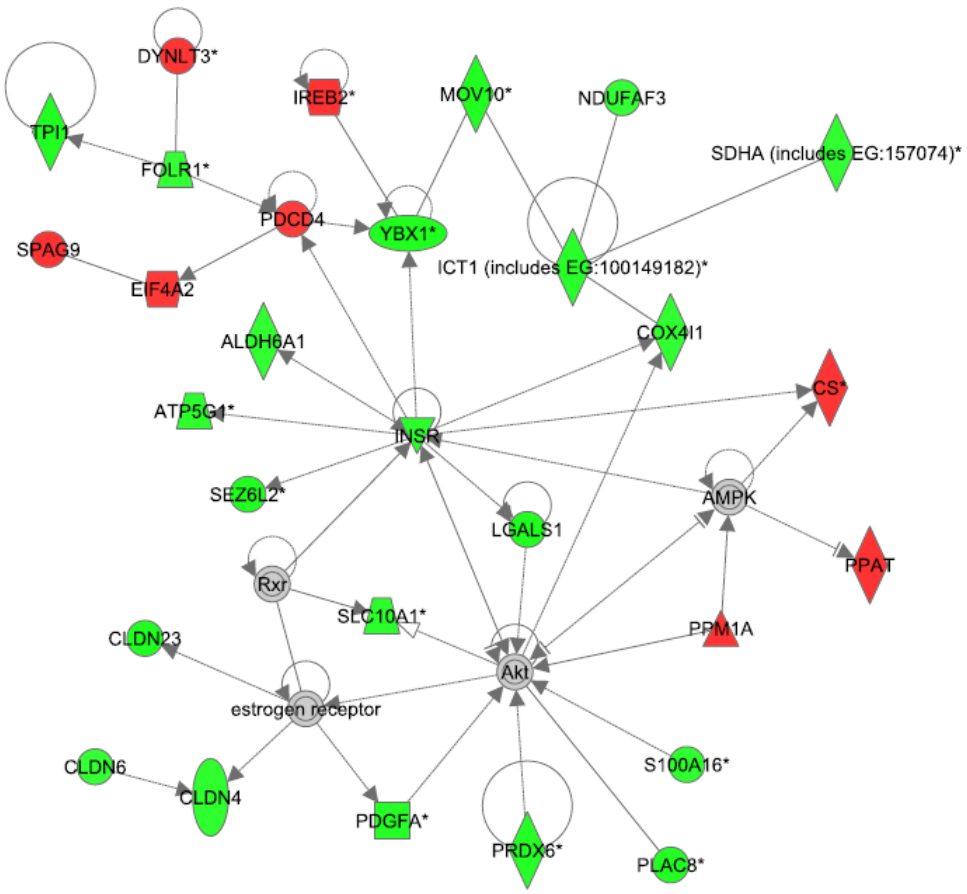


Figure 17 Ingenuity pathway analysis of BSO impact.

First network representing the associations between BSO-related DEGs based on the literature knowledge provided by IPA. Links between molecules are based on gene expression connectivity as well as experimental proofs of co-functionality. Red and green colors respectively represent up- and down-regulated DEGs in BSO-treated blastocysts.

4.10 Supplemental table

Supplemental table 7 Sequences of reverse transcription qPCR-specific primers of candidate genes expressed in bovine blastocysts.

Gene Symbol	Name	Accession number	Forward sequence	Reverse sequence	Product size (bp)	Annealing temperature (°C)
IGFBP7	insulin-like growth factor binding protein 7	NM_001102300	ACTGGTGCCCAGGTGT ATTTGA	AAGCCTGTCCTTGGGA ATTGGA	255	58
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	NM_174137	ACCATCCAACTTCGGC TCACTT	TACTGAGTGTGGCTGT CACTGT	494	57
ARRB2	arrestin, beta 2	XM_002695776	TTGTGAAGGAGGGTGC CAACAA	TTCCTAGCAGAACTGG TCGTCA	350	57
GCSH	glycine cleavage system protein H (aminomethyl carrier)	XM_002688105	AATGGTGTCGGAACAG TGGGAA	TCAGCCAACCGTCTTC GTAACA	244	58
TKDP1	trophoblast Kunitz domain protein 1	NM_205776	TCCTCCATTGGTCAAC GTGTCT	ATTCCACCTTAGCCACC CACAA	294	58
IFNT	interferon-tau 3g	NM_001168279	TGCCACATCACCTTCGT ACACT	AACATCAGGGCAGGCA TCACTT	178	58
TPI1	triosephosphate isomerase 1	NM_001013589	AGCAAACCAAGGTCAT CGCAGA	ACTGCATCAGAGACGT TGGACT	166	58
ACTB	actin-beta	NM_173979	ATCGTCCACCGCAAAT GCTTCT	GCCATGCCAATCTCAT CTCGTT	101	59
MYL6	myosin, light chain 6, alkali, smooth muscle and non-muscle	NM_175780	TTCGGGTGTTTGACAA GGAAGGGA	ATCCTCAGCCATTCAG CACCAT	228	58
PPIA	peptidylprolyl isomerase A (cyclophilin A)	NM_178320	TTTATGTGCCAGGGTG GTGACT	TCTTGCTGGTCTTGCCA TTCCT	287	58
GFP	green-fluorescent protein		GCAGAAGAACGGCATC AAGGTGAA	TGGGTGCTCAGGTAGT GGTTGT	143	59

Chapitre 5: Impact du stress lipidique

Title: The impact to serum's lipid exposure during in vitro culture on the transcriptome of bovine blastocyst

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5.1 Résumé

L'exposition au sérum est connue pour influencer le développement du jeune blastocyste en interférant avec le métabolisme lipidique, interférence associée avec une qualité embryonnaire compromise. Pour définir les mécanismes moléculaires sous-jacents aux stress lipidique induit par le sérum, l'impact d'une exposition à la fraction lipidique du sérum (SELF) ajoutée à la BSA à partir du stade 8-16 cellules pendant la culture a été étudié au niveau transcriptomique chez le blastocyste bovin. Quatre conditions lipidiques ont été comparées: BSA seule, BSA + lipides sériques (SELF) concentrés, sérum total et sérum délipidé. Aucune différence n'a été observée dans le taux de développement entre chaque condition de culture, bien que le traitment SELF tende à diminuer le taux de blastocystes éclos. Utilisant la technique de microarray, le profil d'expression génique des blastocystes du traitement BSA+SELF a était comparé au BSA-contrôle. Les résultats montrèrent des différences dans l'expression de gènes associés au stress oxydatif et l'inflammation induit par la céramide. De plus, les blastocystes traités au SELF ont subi un impact significatif au niveau des gènes du métabolisme du cholestérol (LDLR, HMGCS1), possiblement via la régulation des facteurs de transcription SREBP et PPAR. Également, l'expression des gènes de pluripotence (APEX, CLDN6) était régulée à la baisse dans les blastocystes soumis au traitement SELF mais aussi avec le sérum total, suggérant un lien avec l'effet du sérum sur le développement anomal des veaux issue de procréation médicalement assistée. Dans leur ensemble, ces résultats illustrent la réponse transcriptomique à l'exposition à un taux élevé de lipides via une signature inflammatoire et métabolique, offrant une approche génomique pour mieux comprendre la baisse de viabilité embryonnaire après l'exposition à des conditions sous-optimales de culture.

5.2 Abstract

Serum supplementation to in vitro culture medium is known to influence the early embryo development by interfering with lipid metabolism, a feature associated with compromised blastocyst quality. To define the molecular mechanisms underlying the serum induced-lipidic stress, bovine embryos were culture from 8-16 cells stage in different protein-lipid supplemented culture media, i.e BSA alone, BSA + serum lipid fraction (SELF), delipidated serum and total serum. Then, embryonic transcriptome was profiled in resulting blastocysts. SELF supplementation significantly increased the fatty acid exposure during culture, and showed tendency to decrease the hatching rate of developing blastocysts compared to other treatments. Moreover, result of microarray comparison between blastocysts cultured in BSA ± SELF revealed differential gene expression associated with ceramide-induced oxidative stress and inflammation. Interestingly, SELF-treated blastocyst showed significant impact on genes involved in cholesterol metabolism (LDLR, HMGCS1), with the potential up-stream control of the transcription factors SREBP and PPAR, two major regulators of lipid metabolism. In addition, the expression of pluripotence-related genes (APEX, CLDN6) was down-regulated in blastocysts subjected to either SELF or total serum, suggesting a link with the impact of serum supplementation on the abnormal fetal development in ART-derived offspring. Taken together, these results illustrate the transcriptomic response of the early embryo to increased lipid exposure through inflammatory and metabolic signature, offering a genomic understanding to the embryonic stress under suboptimal culture conditions.

KEY WORDS: serum, lipid, mitochondria, energy, blastocyst, transcriptome, embryo quality, assisted reproductive technology.

5.3 Introduction

A successful pregnancy requires an embryo of high developmental competence and assisted reproductive technology, especially prolonged culture may have a negative effect on embryo quality. In argument, numerous studies have shown the detrimental impact of in vitro culture (IVC) condition on blastocyst development as well as fetal, placental and post-natal growth (19,95,100), and notably in cattle with the incidence of large offspring syndrome (LOS). Compared to the natural condition of the oviduct, suboptimal IVC environment would generate a stress to the early embryo and compromise its viability. Although numerous changes in IVC protocols have been made to decrease the embryonic stress, culture conditions are still suboptimal and their impact on embryo development is still poorly understood. Therefore, characterizing the embryonic stress during IVC is important to ultimately optimize the medium composition in a way that produces blastocysts of higher quality (64).

With the development of microarray technology and the ability to use minute amount of mRNA, transcriptomic analysis has emerged as a relevant tool to study the embryonic response to culture conditions (42) (99). After the early cleavage stages, transcription of the embryonic genome is activated (EGA) and this characterizes the maternal to embryonic transition (8-16 cells stage in cow) (94). EGA is crucial to support subsequent development (93) and several analysis of global RNA content have shown significant modifications in gene expression in IVC embryos compared to their in vivo counterpart (51,72), indicating the plasticity of the embryonic development (21). Although these early modifications are likely to impact embryonic quality (26,62,72), direct links between differentially expressed genes and the resulting developmental compromise are still missing. Therefore, this study analyses the transcriptomic changes specifically associated with serum's lipids-related IVC stress in order to identify specific biomarkers of embryonic demise.

Serum is usually added during in vitro culture, providing beneficial elements as energy substrates, growth factors, amino acids and anti-oxidants for the embryonic metabolism. However, the non-defined nature of serum has demonstrated detrimental effect on embryonic quality, notably by enhancing the rate of abnormal foetal growth (LOS) (88).

Hallmark of serum exposure is increased embryonic lipid droplets in association with lower cryopreservation success (3,80). Lipid droplets are found in nearly all living cells for energy storage and contain primarily triglycerides and cholesterol-ester. It has been reported that triglycerides content of in vivo cattle embryos remained stable from the two cells to the blastocyst stage, whereas in vitro-derived embryos exposed to serum (10%) double their triglycerides reserves from the four-cell to the blastocyst stage (20). This effect of serum on lipid accumulation in the embryo is thought to be multifactorial, causing the inhibition of endogenous lipid degradation by mitochondrial oxidation as well as the increase of lipid neosynthesis (1,2,71). Oocyte and embryo can take up fatty acids in their environment from BSA or LDL which can transport lipids (36,50), and fatty acid profiles of the embryos is modified in a similar proportion to those detected in the serum (77). Thus, it is hypothesized that the lipid fraction present in the serum could be incorporated into the developing embryo and results in the adverse phenotype seen after serum exposure (3,20,35).

Since the lipid content in the serum represents a stress factor for embryonic quality (43,49), we aim to define the specific impact of high lipid conditions on cellular pathways expressed during early development. Methodologically, embryos from 8-16 cells to blastocyst stage were subjected to four lipids conditions, BSA alone, BSA + serum lipid fraction (SELF), delipidated serum (SER-D) and total serum (SER-T), and then embryonic transcriptome was profiled in resulting blastocysts. This is the first study that specifically targets the lipid impact from the serum on large-scale gene expression in bovine blastocysts.

5.4 Materials & methods

All chemicals were obtained from Sigma–Aldrich, unless otherwise stated.

Oocyte collection and in vitro maturation (IVM)

Cumulus–oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of slaughtered heifers. After four washes in TLH (HEPES-buffered Tyrode's Lactate solution), groups of up to 10 selected COCs with at least five layers of cumulus were placed in 50-µl drops of medium under mineral oil in dishes (Nunc, Roskilde, Denmark) and maturated for 24 h at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium was TCM-199 supplemented with 10% fetal calf serum (HyClone, ThermoScientific, UT), 0.1 µg/ml of FSH (Folltropin V, Bioniche, Canada), 0.33 mM of pyruvic acid and 50 µg/ml of gentamycin.

In vitro fertilization (IVF)

Following maturation, five matured COCs were added to 48-µl droplets of IVF medium under mineral oil. The IVF medium was composed of TL STOCK (Tyrode's Lactate solution) supplemented with 0.6% fatty acid free bovine serum albumine (BSA), 0.2 mM pyruvic acid, 10 µg/ml heparin, and 50 µg/ml gentamycin. Prior to transfer, the COCs were washed twice in TLH medium. Once transferred, 2 µl of PHE (1 mM hypotaurine, 2 mM penicillamine, 250 mM epinephrine) were added to each droplet 10 min before spermatozoa was added. The spermatozoa used consisted of a cryopreserved mixture of ejaculates from five bulls (Centre d'Insémination Artificielle du Québec, St.-Hyacinthe, QC, Canada). The spermatozoa were thawed in 37 °C water for 1 min, put on a discontinuous Percoll gradient (2 ml of 45% Percoll over 2 ml of 90% Percoll), and centrifuged at 700g for 30 min at 26 °C. After discarding the supernatant, the pellet of live spermatozoa was resuspended in IVF medium after being counted on a haemocytometer to obtain a concentration of 10^6 cells/ml. Finally, 2 µl of the sperm suspension (final concentration = 4.10^4 cells/ml) were added to each IVF droplet containing the matured COC and the incubation took place in a humidified atmosphere at 38.5 °C in 5% CO_2 for 16-18 h.

In vitro culture (IVC)

Embryo culture used a three step modified synthetic oviduct fluid (mSOF) culture system containing MEM essential and non-essential amino acids, 0.5 mM of glycyl-glutamine and 0.4% fatty acid-free BSA under embryo tested mineral oil (#8410, Sigma). The embryo culture dishes were incubated at 38.5°C with 6.5% CO_2, 5% O_2 and 88.5% N_2 in 100% humidity. Briefly, after fertilization, presumptive zygotes were mechanically denuded and washed three times in TLH supplemented with fatty acid-free BSA and were placed in groups of 10 in 10 µl droplets of SOF 1 with non-essential amino acids (1X) and 3µM EDTA. Embryos were transferred in new 10 µl droplets of SOF 2 containing non-essential (1X) and essential (0.5X) amino acids 72 h post-fertilization and once again 120 h post-fertilization in 20 µl droplets of SOF 3 containing non-essential (1X) and essential (1X) amino acids. The glucose concentration used in SOF1, 2 and 3 was respectively 0.2, 0.5 and 1.0 mM. Media replacement allows to prevent toxicity due to ammonium accumulation and nutrients depletion caused respectively by amino acid degradation and embryo metabolism. Cleavage rate (number of embryos with at least 2 cells out of total embryos) and 8/16-cells embryo rate (number of embryos with at least 8 cells out of total embryos) were determined during embryo transfer from SOF1 to SOF2 (Day 3). Blastocyst rate (number of early, expanded and hatched blastocysts out of total embryos) and hatching rate (number of hatched blastocysts out of total blastocysts) were calculated at the end of the culture (168 h post fertilization).

Production of bovine blastocysts in BSA-control, BSA+SELF or serum (SER) supplemented culture

After the first 3 days of IVC (i.e. SOF1), embryos were randomly assigned to four separate conditions of culture containing 0.1% of ethanol, i.e. BSA-control, BSA+SELF, SER-D and SER-T. BSA-control condition is described above in the IVC section. BSA+SELF condition is the culture in BSA control supplemented with serum-extracted lipid fraction. Specifically, 10 mL of FBS (Hyclone, Thermoscientific, U.S origin) was processed into a lyophilizator (Virtis Virtual 50L EL8) then reduced into powder. By regular 10 mL passage, 150 mL of petroleum ether were passed into a Soxlet-containing the FBS powder

and the passed ether was evaporated under nitrogen flux. The resulting lipid fraction was solubilised in ethanol (10µL) and supplemented to 10 ml of control BSA culture media. Condition of IVC with total FBS (SER-T) or delipidated FBS resulting from SELF exclusion (SER-D) corresponded to the addition of 0.4% of lyophilisated serum (before or after ether passage) to control medium without external BSA. Renewal of medium was done on day 5 according to the assigned treatment and blastocysts were harvested on day 7. Four replicates of blastocysts exposed to BSA and SELF-supplemented BSA were produced for microarray analysis. Three independent replicates of blastocysts exposed to BSA, SELF-supplemented BSA and delipidised and total serum were produced for RT-qPCR analysis.

Measurement of fatty acid concentration in culture media

Fatty acid content and composition in medium supplemented with BSA, BSA+SELF or serum was analyzed by gas chromatography. Triplicate of culture medium from embryo-free droplets (200 µL) were harvested before and after 12 hours of incubation under oil-overlay and submitted to basic and acid methylation. For full description of the methodology, please refer to Gervais et al, 2009 (27).

Determination of differential gene expression profile

Total RNA from each replicate was extracted and purified using PicoPure™ RNA Isolation Kit, (Life Science, New-York, NY). After DNase digestion (Qiagen, Toronto, Ontario, Canada), quality and concentration of extracted RNA was analyzed by bioanalyzer (Agilent, Mississauga, Ontario, Canada). All extracted samples showed good quality with an RNA Integrity Number >7.5.

For microarray purposes, purified RNA was amplified by in vitro transcription using the RiboAmp® HSPlus RNA Amplification Kit, (Life Science, New-York, NY). Amplified RNA was labelled with Cy3 and Cy5 using the ULS Flurorescent Labeling Kit (Kreatech, NC) and aRNA (825 ng per replicate) was hybridized on the Agilent-manufactured EmbryoGENE slides in a 2-colors dye swap design. After 17 h of hybridization at 65 °C, microarray slides were washed 1 minute in Gene Expression Wash Buffer 1 (room temperature), 3 minutes in gene Expression Wash Buffer 2 (42 °C), 10 seconds in 100%

acetonitrile (room temperature) and 30 seconds in Stabilization and Drying Solution (Agilent). Slides were scanned with PowerScanner (Tecan, Mannedorf, Switzerland) and features extraction was done with Array-pro6.3 (MediaCybernetics, MD). Intensity files were analyzed with FlexArray (Michal Blazejczyk, Mathieu Miron, Robert Nadon (2007), FlexArray: A statistical data analysis software for gene expression microarrays. Genome Quebec, Montreal, Canada, URL : http://genomequebec.mcgill.ca/FlexArray). Specifically, intensity raw data were corrected by background subtraction then normalized within (green/red) and between each array (Loess and quantile, respectively). Statistical comparison between treatments was done with the Limma algorithm which attributed to each probe a probability of fold-change difference between treatment and control.

Validation of microarray results was done by RT-qPCR. Total extracted RNA from independent samples was reverse-transcribed using oligo-dT primer and qScript Flex cDNA synthesis kit (Quanta Bioscience, MD). Specific primers for each selected gene were designed using PrimerQuestSM (Integrated DNA Technologies, Inc., IA). Primer sequences, product size, annealing temperature and accession numbers are provided in Supplemental Table 9. qPCR were performed using LightCycler 480® SYBR Green I Master and the LightCycler® 480 System (Roche, Mannhein, Germany). A standard curve constituted of five points of the PCR product for each primers pairs diluted from 1 pg to 0.1 fg was used for real-time quantification of PCR output. Data normalization used GeNORM normalization factor (91) from expression values of 3 reference genes (ACTB, MYL6, PPIA). Moreover, technical variations were assessed and corrected through quantification of exogenous GFP spike which was introduced at the time of RNA extraction (94).

Functional analysis of differentially expressed genes

DAVID software was used to analyse functions of differentially expressed genes into clusters (38,39). Moreover, data were analyzed with Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com). IPA generated canonical pathways as well as gene product interactions (networks) that are differentially expressed between treatments. We used IPA to build schematic representations of important pathways dysregulated in treated blastocysts.

Network Generation:

A data set containing gene identifiers and corresponding expression values was uploaded into the application. Each identifier was mapped to its corresponding object in Ingenuity's Knowledge Base. A fold-change cut-off of 1.5 with a p-value <0.05 was set to identify molecules whose expression was significantly differentially regulated. These molecules, called Network Eligible Molecules, were overlaid onto a global molecular network developed from information contained in Ingenuity's Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Human, mouse, and rat orthologs of a gene are stored as separate objects in the Ingenuity Pathways Knowledge Base, but are represented as a single node in the network.

Canonical Pathway Analysis:

IPA identified the pathways that were most significant to the data set from the Ingenuity Pathways Analysis library of canonical pathways. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed; 2) Fisher's exact test was used to calculate a p-value to determine the probability that the association between the genes in the dataset and the canonical pathway would occur by chance alone. Green and red symbols represent genes respectively down- and up-regulated in treated embryos compared to controls. Grey symbols represent genes with significant expression in blastocysts but no difference between conditions, whereas white symbols represent genes not present on microarray or with below-background intensity.

Statistical analysis

ANOVA and Tukey's Multiple Comparison Test were used for statistical comparison of blastocyst and hatching rate between treatments. Equally, relative difference in fatty acid concentration between treatments was assessed by ANOVA and Tukey's Multiple Comparison Test. Relative expression of candidate genes in BSA+SELF or serum conditions (SER-D and SER-T) was compared to BSA-control and the ratio of gene expression was subjected to statistical analysis using a One-sample t test to determine if mean ratio of gene expression was significantly different than 1.

5.5 Results

Lipid profile of culture medium containing BSA, BSA+SELF or serum

In order to measure the fatty acid concentration in the culture medium, gas chromatography analysis was performed on embryo-free medium before and after 12h of incubation under oil overlay in a humidified atmosphere at 38.5 °C in 5% CO_2. Oil overlay allows protecting embryos from external injuries (Oxygen, pH, plastic hardware) and medium evaporation. Miller et al reported a loss of around 50% of lipophilic compound from culture medium into oil overlay after 24 hours of incubation (61). Here, no changes in lipid concentration were observed before and after 12 h of incubation in embryo-free condition, suggesting a stable binding of fatty acid amount to the BSA in the SELF supplemented medium and a low rate of free-fatty acid. Moreover, statistical comparison showed significant higher lipid concentration in SELF-containing medium compared to BSA or delipidated serum (Figure 18), but not total serum. No significant difference in lipid concentration was observed between BSA and serum conditions.

In order to determine the fatty acid composition in the culture media, the fatty acid profile was analyzed in the four different treatment conditions at 12 h of incubation (Supplemental figure 22). Results show that lipid fraction in BSA, SELF or serum condition was mainly composed of palmitic acid (C16) and stearic acid (C18). However, differences between SELF and the 3 other treatments were visible in the proportion of numerous fatty acids and notably palmitic acid (C16:0), stearic (C18:0) and oleic acid (C18:1c9).

Developmental impact of SELF and serum during in vitro culture

Result of ANOVA showed that exposure to BSA, BSA+SELF, SER-T or SER-D did not change the rate of blastocyst development although serum supplemented media tend to support higher blastocyst rate (figure 19A). Equally, hatching rate was not significantly different for any culture condition (fig 19B) although SELF-treated blastocysts tended to have a lower hatching rate.

Comparison of transcriptomic profile between BSA-control and BSA+SELF-treated blastocysts

Large-scale transcriptomic comparison between BSA-control and BSA+SELF-treated blastocysts was performed using the EmbryoGENE microarray platform (73). 9068 probes showed an intensity signal higher from the background intensity signal + 2 x STD. Within these positive probes, 509 genes were found differentially expressed ($p<0.05$) between BSA+SELF and BSA-control. Moreover, 50 DEG had more than ±1.5 fold-change difference (14 up-regulated and 36 down regulated) and 6 had more than ±2 fold-change difference (Figure 20A). Among these later, 5 genes were up-regulated (SERPINEA5, XCL2, HIST1H1C, SERPINE1 and NDP) and 1 gene was down-regulated, coding for a hypothetical protein. IDs, symmetrical fold-changes and p-values of DEGs with ±1.5 fold-change difference are provided in supplemental table 8.

RT-qPCR validation of differential gene expression of selected candidates from SELF-related transcriptomic profile

A total of 7 genes (APEX1, CLDN6, LDLR, HMGCS1, ARRB2, ARRB1, and CYR61) that were predicted as differentially expressed from microarray analysis were processed for qPCR validation in 4 independent biological replicates from BSA-control or BSA+SELF-treated blastocysts. 3 House Keeping Genes (ACTB, MYL6, and PPIA) were used to normalize the data and showed consistent expression among treatment. Gene expression in treated blastocysts (BSA+SELF, SER-D or SER-T) was compared to the BSA-control blastocysts (base-line) and figure 20B depicts the log 2 of the gene expression ratio of treatment/BSA-control. Results showed significant lower expression ratio for 3 genes at $p<0.05$ (APEX1, CLDN6 and HMGCS1) and 1 genes at $p<0.01$ (LDLR) in BSA+SELF-treated blastocysts when compared to BSA-control. Similarly, blastocysts cultured in total serum (SER-T) showed lower ratio of CLDN6 ($p<0.05$) as well as APEX1, LDLR, and HMGCS1 ($p<0.1$). Finally, blastocysts cultured in delipidated serum (SER-D) demonstrated a lower expression ratio of LDLR ($p<0.05$). Differential expression in ARRB1, ARRB2 and CYR61 were not validated in BSA+SELF-treated blastocysts and showed high standard deviations in their expression ratio independently of the treatment.

Functional links between DEG in SELF-exposed blastocysts

DAVID software significantly clustered SELF-related DEG in molecular function of peptidase inhibitor activity, chromatin organization and DNA binding. Ingenuity Pathway Analysis of SELF-related DEG showed a significant association with molecular and cellular function of protein synthesis, cell morphology, cell-to-cell signaling and interaction, lipid metabolism and molecular transport. Top impact on physiological system correlated with embryonic and organ development and morphology. Top canonical pathways affected by SELF-exposure were associated with the role of tissue factor in cancer, the coagulation system, the Granzyme A signaling, the inhibition of angiogenesis by TSP1 and the role of PKR in Interferon Induction and Antiviral Response. The first network (figure 21) generated by IPA knowledge database reflected cardiovascular and hematological diseases as well as cell death and cell survival.

5.6 Discussion

This study illustrates how embryos are reacting to high serum's lipids in culture. Results show that higher serum's lipids environment in culture medium does not prevent blastocyst's development but significantly affect their transcriptomic profile.

Our result showed that serum-extracted lipid fraction (SELF) supplementation to BSA increases fatty acid content in the medium compared to BSA alone (figure 18). SELF were extracted using ether, a non-polar solvent capable of extracting all class of lipids (14), from cholesterol ester and triglycerids (high affinity) to phospholipids and non-esterified fatty acids (low affinity). In SELF supplemented culture conditions, the only acceptor for non-polar lipid is the BSA since the synthetic oviduct fluid does not contain any lipoprotein. SELF treatment contained high proportion of palmitic and stearic acid while other types of fatty acids are under represented compared to total serum. This lipid composition is consistent with other studies (77) but the amount of fatty acids is, as planned, under the threshold of embryo toxicity. Lipid extraction was not total since delipidated serum still contained a measurable lipid fraction. The extraction rate of serum lipid may be limited due to high lipid affinity of the BSA. Moreover, we observed a residual content of lipid in the BSA-control medium, suggesting that production of free-fatty acid BSA is far from 100% efficient and could explain observed variations from different lots of BSA.

Although the fatty acid concentration was higher in the SELF-supplemented medium, result showed similar rate of blastocyst development than with control BSA. This is consistent with other studies showing no impact on blastocyst rate after increasing doses of fatty acid supplementation (43,69). Moreover, we observed a slight decrease in hatching rate in SELF-treated blastocyst, consistent with another study showing significantly lower hatching rate in embryos cultured in fat serum-supplemented media compared with those cultured in control serum (49). In this study, they supplemented IVC medium with bovine serum that was nutritionally enriched in fat and carbohydrate. In the serum, presence of lipoprotein has been shown to adversely affect blastocyst quality (74) but this vector of lipid supplementation was not associated with fatty acid uptake (101). In contrary, BSA was validated as the major/sole serum fraction that leads to the net uptake of FA during embryo

culture (40). Investigating the changes in lipid composition of SELF-treated blastocysts would be useful to better characterise the developmental impact of BSA-associated lipid stress. Nevertheless, our culture conditions reflect the conditions reported in the literature where embryos are moderately affected by serum lipids and the transcriptomic response should reveal how surviving blastocysts are coping with this lipid stress.

Using the EmbryoGENE microarray platform, SELF-treated blastocysts were compared to blastocysts cultured in BSA-control media and transcriptomic profile showed 50 differentially expressed genes with more than 1.5 fold-changes. While other studies have analysed specific candidates involved in specific pathways, our results provide a global analysis of significant enriched pathways in SELF-related DEGs using both DAVID and Ingenuity Pathway Analysis softwares. Among significant pathways, SELF affected the signaling of tissue factors and coagulation. In particular, SERPINA5 and SERPINE1 appeared to be up-regulated in blastocysts produced under SELF exposure. Coding for plasminogen inhibitors, SERPIN genes play a pivotal role in regulating plasmin synthesis, the latter being increased during early development (44,59). SERPINE1 is up-regulated by PPARgamma after oxidized linoleic acid supplementation in endothelial cells (56). In mice fed a high fat diet, higher SERPINE1 expression is associated with an increase in ceramide production while SERPINE1 knock-out protects against ceramide accumulation (81). Ceramide accumulation is part of cellular stress response as inflammation (30,31) and ceramide addition in culture medium induces apoptosis in murine and bovine blastocyst (16,29). As SERPINE1 and other SELF-related DEG were also up-regulated in blastocysts cultured in condition that favour lipid peroxidation (12,22) and potentially ceramide production (52,76), this would involve the ceramide-associated pathway to the detrimental effect of excessive lipid exposure on blastocyst quality (6,66).

Transcriptomic profiling also indicates a response to inflammation in SELF-treated blastocysts. The cytokine tumor necrosis factor-alpha (TNF-a) and IFN are major mediators of inflammation and an increased level is associated with miscarriage of ART-pregnancy (13). Here, both IFIH1 (Interferon induced with helicase C domain 1) and TNFRSF1A (Tumor necrosis factor receptor superfamily member 1A) were up-regulated after SELF exposure. With increasing expression during early embryonic development (45),

TNFRSF1A is one of the major receptors of TNF-a in the regulation of inflammation while IFIH1 is important for viral recognition and is up-regulated in bovine uterus during severe negative energy balance (98). In addition, CASP4 (Caspase 4) was up-regulated in SELF-treated blastocysts. Initially supposed to be involved in endoplasmic reticulum stress-induced apoptosis, CASP4 is classified as one of the inflammatory caspases (82) and acts as an essential protein in the NFkB activation of cytokine production as well as a factor required for TNF-a signalling (65). A study showed that CASP4 is up-regulated after 6 month of rich n3 fatty acid diet and its expression rose with enhanced EPA plasma level (92). XCL2 (chemokine (C motif) ligand 2) was also up-regulated after SELF. Natural killer cells expressed XCL2 after cytokine activation and up-regulation of XCL2 likely acts to increase the immune response and inflammation (33). Recently, XCL2 expression has been found in the bovine conceptus at day 16 (55), a time when maternal immune system and recognition is crucial for uterine attachment of the embryo. All together, SELF transcriptomic signature of excessive inflammatory signal may not be a good signal for maternal recognition and implantation (8).

The expression of the LDL receptor is down-regulated in SELF treated blastocyst. This receptor is sensitive to the cholesterol load in the blood. A down-regulation of LDLR was shown in mouse blastocysts that developed in genital tract of obese mothers induced by high fat diet (9), and this was also observed in ovine blastocyst after PUFA exposure during culture (40). Oocytes and early embryos are able to accumulate and metabolize cholesterol from the environment (20,77) but the role of lipoprotein is still unclear (78). Cells can accumulate cholesterol via BSA carrier or specific receptor of lipoproteins as HDL or LDL (67). Interestingly, we observed that LDLR down-regulation was enhanced by adding serum without SELF, indicating that the lipoprotein components of serum may impact LDLR expression although the non-polar lipids fraction (SELF) could have additive effect (figure 20B). Among these non-polar lipids, one analog of cholesterol, the 25-hydroxycholesterol (25-OHC), is present in fetal calf serum and can diffuse throughout cell membrane (79). 25-OHC belongs to oxysterol derivatives that are generated by auto-oxidation of cholesterol and exert pro-inflammatory effect in placental trophoblast cells (7). When dissolve in ethanol, oxygenated sterol such as 25-OHC is more potent than LDL cholesterol in reducing the activity and the expression of LDLR receptor (54). In relation to oxidative stress and

inflammation, this data indicates the potential impact of cholesterol and oxido-sterols in culture supplemented with serum.

Cholesterol and 25-OHC influx and degradation influence the sterol regulatory element-binding proteins (SREBP) witch modulate LDLR expression as well as other genes involved in sterol metabolism (4,86). PUFA supplementation has shown to decrease the level of SREBP in bovine embryo and to down-regulate the expression of genes involved in lipid metabolism (6). Amongst the validated candidates (figure 20B), we show that HMGCS1 (3-hydroxy-3-methylglutaryl-CoA synthase 1) is down-regulated by SELF or serum supplementation in bovine blastocysts. Involved in the synthesis of the cholesterol precursor HMG-CoA, HMGCS1 is down-regulated under hypoxia-dependant decrease of SREBP expression (18). In addition, expression of MSMO1, a gene coding for the enzyme methylsterol monooxygenase 1, is also down-regulated in SELF-treated blastocysts. Culture of bovine blastocysts during early cleavage induced major down-regulation of MSMO1 as well as other lipid metabolism genes (24). PPAR-alpha is a major transcription regulator of lipid metabolism and show cross-talk with SREBP in regulation of HMGCS1 and MSMO1 (90). Altogether, the lower expression of genes involved in cholesterol uptake/ synthesis may probably represent a SREBP/PPAR response to excessive cholesterol accumulation due to SELF exposure (58). However, further investigation would be important to validate the impact of serum on the cholesterol profile of bovine blastocysts (85).

Based on the microarray probes, STAR (the steroidogenic acute regulatory protein STAR) as well as STARD3NL and STARD7 showed high intensity signal compared to other STAR family genes (D3, D4, D8, D9, D10, D13). Moreover, STAR expression was higher in BSA+SELF treated blastocysts. Literature regarding STAR expression during preimplantation in mammal is surprisingly inconsistent, except for the porcine model where RNA trace has been revealed in day 6 blastocysts and day 25 conceptuses (10). STAR functions to increase the cholesterol metabolism into the mitochondria and its transformation into steroids. The synthesis of steroid hormones like progesterone is critical for preimplantation development and blastocyst implantation of mammalian embryos (17). Progesterone (P4) is considered as Nature's immunosuppressant because it promotes survival of the foetus against uterine inflammatory signal (Peltier et al. 2008) and TNF-a

induction of apoptosis in fetal membranes (53). Embryonic steroid metabolism indicates production of progesterone and the presence of the progesterone receptor suggests an autocrine/paracrine effect on blastocyst development and implantation (5,37). In our case, it could be interesting to measure P4 in the media of SELF treated blastocysts to differentiate between a physiological induction by SELF and a biosynthetic response through increased cholesterol oxidation.

Here, NRIP1 (Nuclear receptor-interacting protein 1) was up-regulated after SELF exposure. NRIP1 is a ligand-dependent transcriptional repressor for several nuclear receptors (PPAR family and others) that controls the balance between energy storage and energy expenditure. NRIP1 null mice show resistance to high fat diet induced obesity and have increased oxygen consumption (48). Oocyte and embryo use their own stock of lipids (triglycerides) to produce energy via oxidative pathway (co-localisation of lipid droplets with mitochondria) and support membrane synthesis. Lipids are needed to sustain TCA activity and block of fatty acid transport between lipid droplets and mitochondria impairs normal embryo development (83). Moreover, bovine embryos cultured in the presence of the lipid derivatives acetoacetate and/or beta hydroxybutyrate are able to reach the blastocyst stage (28), indicating the ability in this species to use ketone bodies as a primary energy source. As serum in culture affects mitochondrial maturation at morula and blastocyst stage (3), expression of energy regulators might translate an impact to mitochondrial metabolism in SELF treated blastocyst.

SELF-treated blastocysts increased gene expression of triose phosphate isomerase (TPI1), an enzyme which catalyzes the conversion of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. Associated with body weight increase, TPI1 is up-regulated in adipose tissue after rosiglitazone-induced PPAR activation and increase TG storage or decrease TG utilisation by adipose tissue (103). In the liver of transgenic mice resistant to high fat diet, TPI1 up-regulation allow to adapt to energy demand through glycolysis and fatty acid degradation (41), a metabolic adaptation seen in cancer cells (60). This adaptation used the Warburg metabolism for rapid cell proliferation in response to limited mitochondrial oxidation and ATP generation. Early embryos are also programmed to use the Warburg effect in order to sustain rapid proliferation in complementation to mitochondrial

ATP production (46,89). Moreover, up-regulation of aerobic glycolysis and the Warburg effect-related genes has been shown in surviving blastocyst after mitochondrial stress (11). Here, 3 DEGs (SERPINE1, TNFRSF1A, MLLT11) related to SELF impact were common to the impact on mitochondrial metabolism in bovine blastocysts (11). Moreover, these DEGs are also related to the impact of TNF (47) on mitochondrial depolarisation in embryos (45) (15). This transcriptomic features associated with SELF exposure suggest a metabolic adaptation to mitochondrial demise in treated blastocysts, perturbations that has showed potential consequences for subsequent fetal and placental development in the mouse (95,96).

Expression of APEX1 was down-regulated in blastocysts exposed to SELF and total serum. APEX1 codes for an apurine/apyrimidine endonuclease base-excision repair gene and its function is central in the redox control of several transcription factor such NF-kB, HIF1A and p53 (23). Higher APEX1 level increases cell growth and resistance to programmed cell death while lower expression of APEX1 is observed in senescent stem cells (34). APEX1 is ubiquitously expressed in murine morula and immuno-localized in nucleus (57) and its role in early development has been shown to be critical following blastocyst formation, shortly after implantation (102). Equally essential for blastocyst formation (63), tight junction-associated CLDN6 was down-regulated after SELF and total serum supplementation. CLDN6 is specifically expressed in undifferentiated stem cell and RNA level decreases with concomitant down regulation of pluripotent genes (97). Genes involved in pluripotency are down-regulated in embryonic stem cell from rhesus embryo after in vitro culture when compared to in vivo (32), and BSA-bound lipids have been shown to affect hESC self-renewal (25). In regard to the transcriptomic impact of SELF on pluripotence associated genes, this may correlate with the observed higher incidence of large offspring syndrome from embryos cultured in serum condition (88).

In summary, our study depicts the transcriptomic signature to high lipid exposure during early development, unravelling associations with inflammatory response and lipid metabolism. Induction of inflammation related cytokines may promotes hypercoagulability and interfere with normal pregnancy (68,87) while the impact on lipid utilization could result in hyperlipidemic embryos with lower cryotolerance (75,80,84). Moreover, significant pathways of high lipid exposure appear to be associated with mitochondrial function and

HIF gene family. In the liver, HIF2 up-regulation is associated with impaired fatty acid oxidation as well as increase lipid storage capacity that results in the development of severe steatosis (70). Although metabolic validations are required, the similarities between HIF2-related profile in liver cells and SELF-related profile in bovine blastocysts suggest a common dysregulated mechanism in mitochondrial metabolism. Finally, we show the impact of SELF on pluripotence-related genes, transcriptomic signature that could explain how hyperlipidemic stress contributes to the detrimental effect of serum on embryonic quality and long term health.

5.7 References

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5.8 Figures

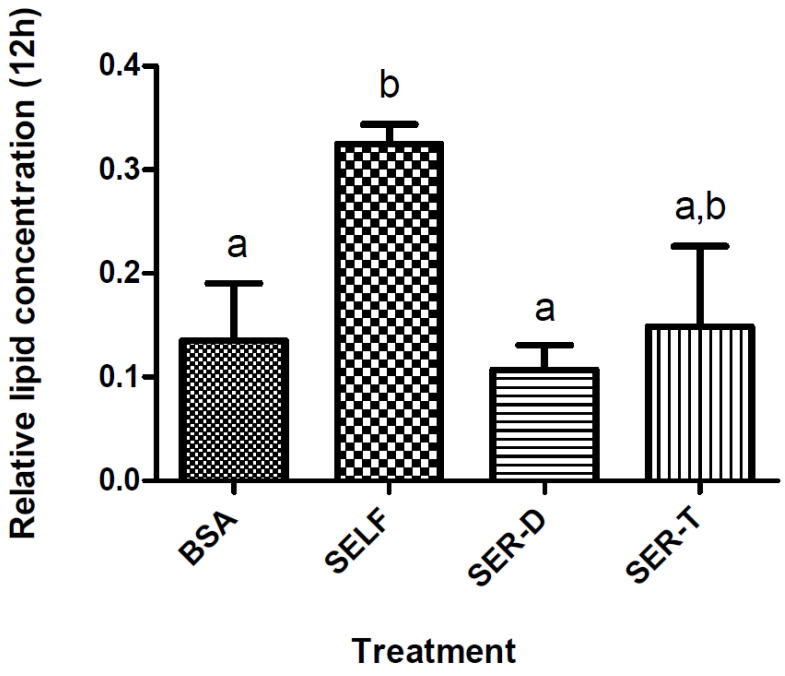


Figure 18 Fatty acid concentrations in the four different treatments of embryo culture.

Embryo-free medium from culture droplets (n=3) containing BSA alone (BSA), BSA+SELF (SELF), delipidised serum (SER-D) or total serum (SER-T) were harvested after 12 h of incubation under proper culture condition and analyzed by gas chromatography. ANOVA and Tukey's Multiple Comparison test was used for statistical analysis of difference in relative concentration between each treatment. Bar errors represent SEM. Different superscripts represent significant differences with $p<0.05$.

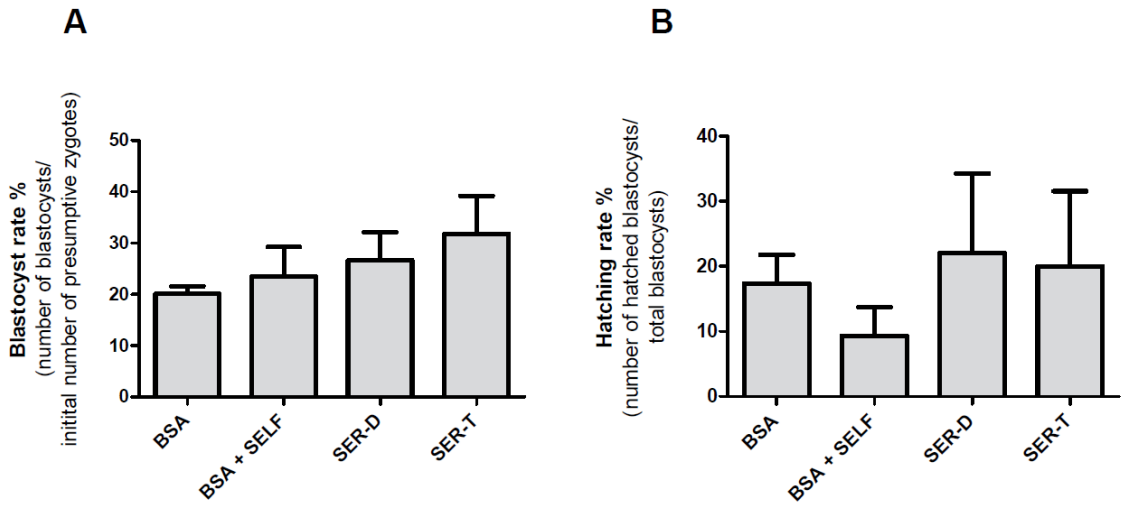
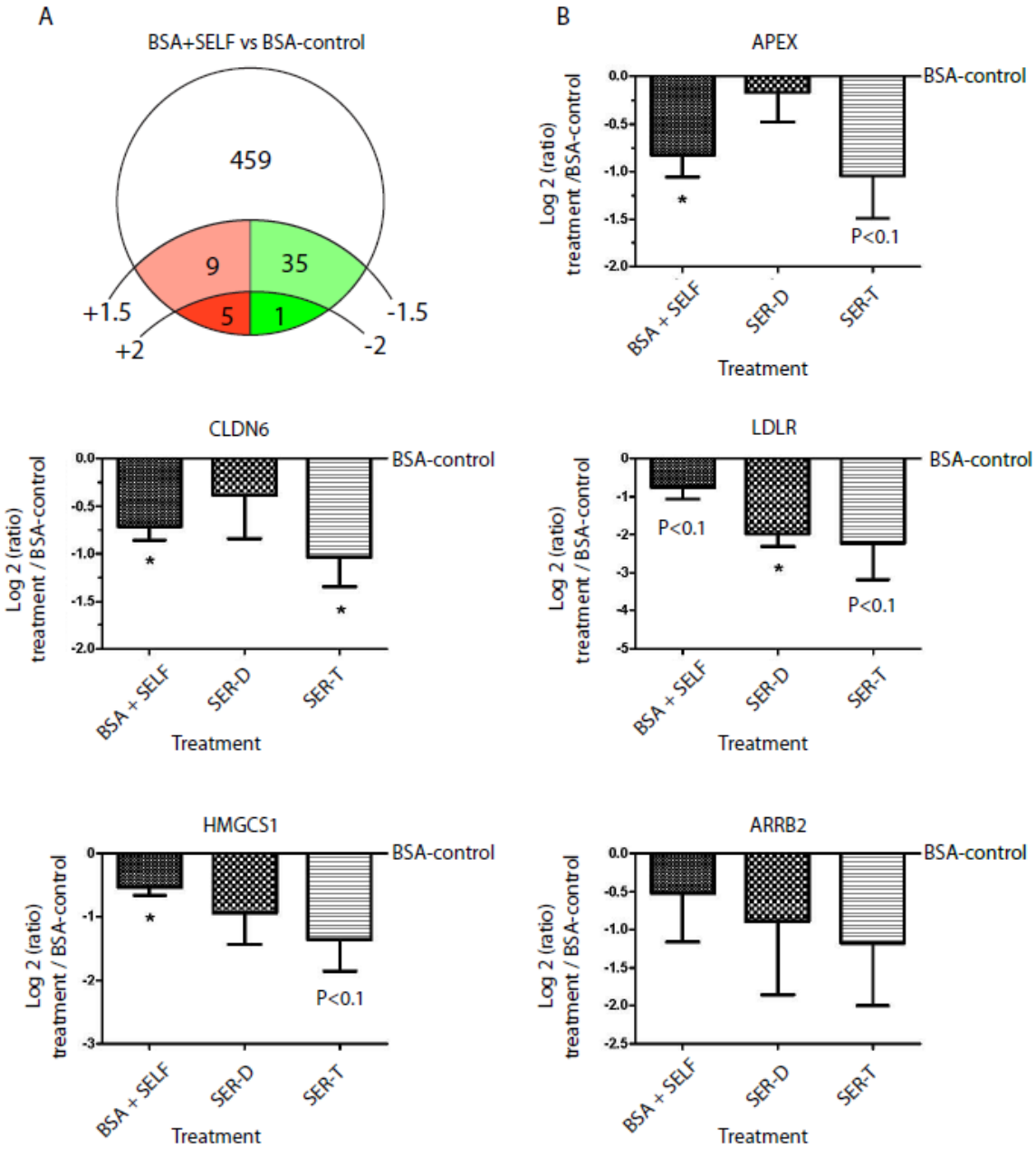


Figure 19 Effect of SELF and serum during in vitro culture at the time of embryonic genome activation on development rate to the blastocyst stage (A) and the hatching rate (B) of in vitro produced bovine embryos.

At the 3rd day of IVC, total embryos were randomly transferred into culture condition containing BSA, BSA+ serum-lipid fraction (SELF), delipidated serum (SER-D) or total serum (SER-T), and then blastocyst development was analyzed at day 7. ANOVA and Tukey's Multiple Comparison test was used for statistical comparison between each treatment. Error bars represent SEM.


A
BSA+SELF vs BSA-control
459
9
35
5
1
+1.5
-1.5
+2
-2
B
APEX
Log 2 (ratio) treatment /BSA-control
BSA-control
P<0.1
BSA + SELF
SER-D
SER-T
Treatment
CLDN6
Log 2 (ratio) treatment / BSA-control
BSA-control
Treatment
LDLR
Log 2 (ratio) treatment / BSA-control
BSA-control
P<0.1
Treatment
HMGCS1
Log 2 (ratio) treatment / BSA-control
BSA-control
P<0.1
Treatment
ARRB2
Log 2 (ratio) treatment / BSA-control
BSA-control
Treatment

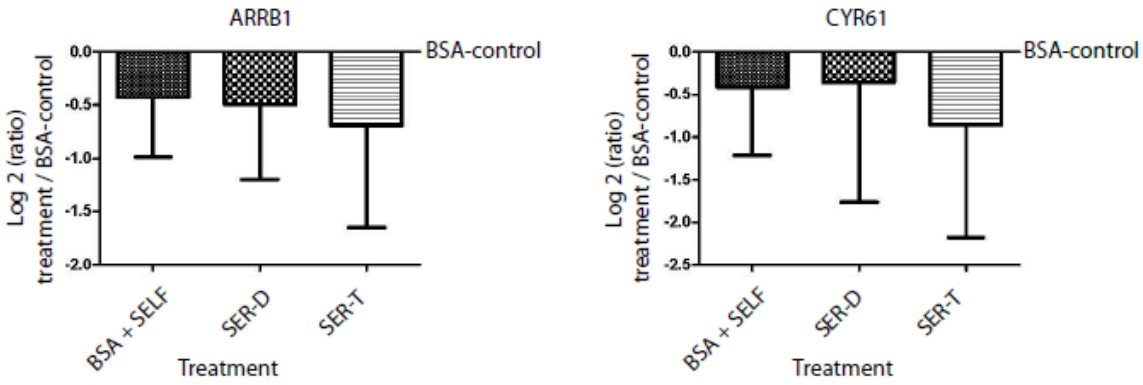


Figure 20 Analysis of gene expression in bovine blastocysts according to culture conditions.

*(A) Number of differentially expressed genes (DEG) in BSA+SELF-treated blastocyst compared to BSA-control treated blastocyst by microarray analysis. Total DEG and DEG with ± 1.5 or ± 2 fold-change P<0.05, are represented. (B) Quantification of mRNA expression of selected candidates by RT-qPCR in SELF-treated blastocysts as well as in delipidated (SER-D) or total serum (SER-T) treated-blastocysts. Analysis was done in triplicate (pools of 10 blastocysts each) and the data represents the mean±SEM of the expression ratio between BSA+SELF or serum (SER-T and SER-D) treatments compared to BSA-control. One-sample t test determinates if mean ratio of gene expression was significantly different than 1 (mean log2 ratio different from 0). *Significantly different from BSA-control with p<0.05.*

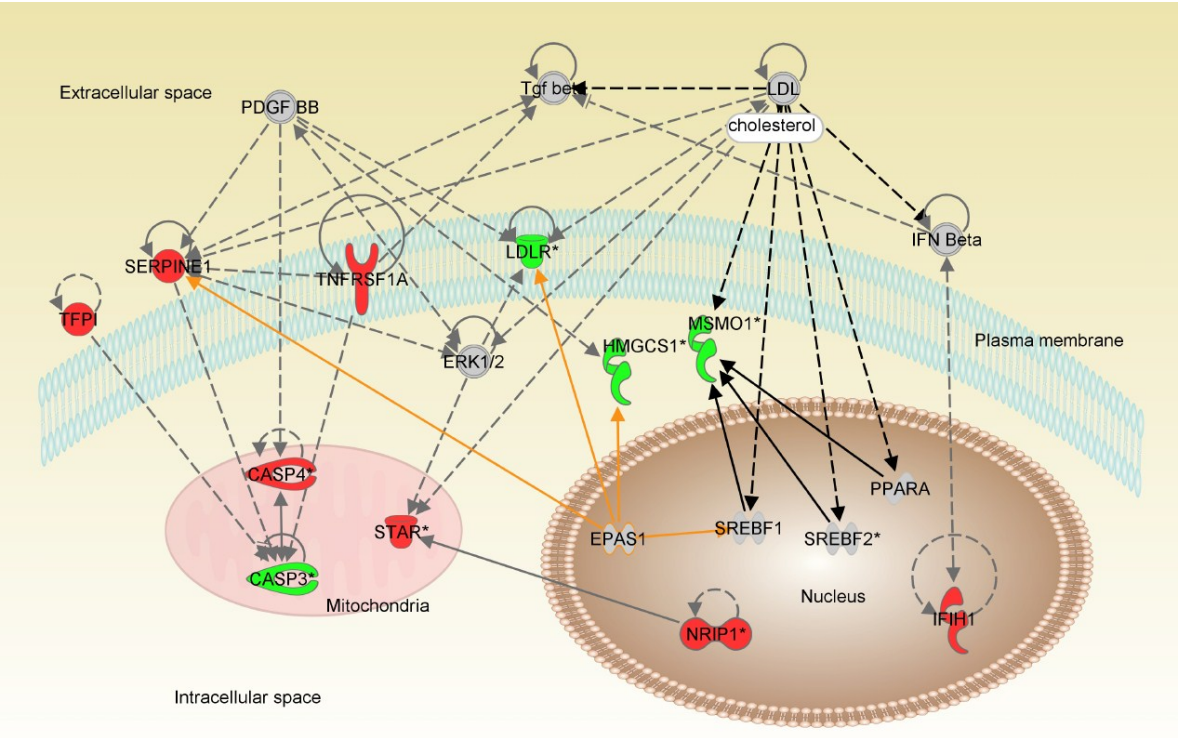
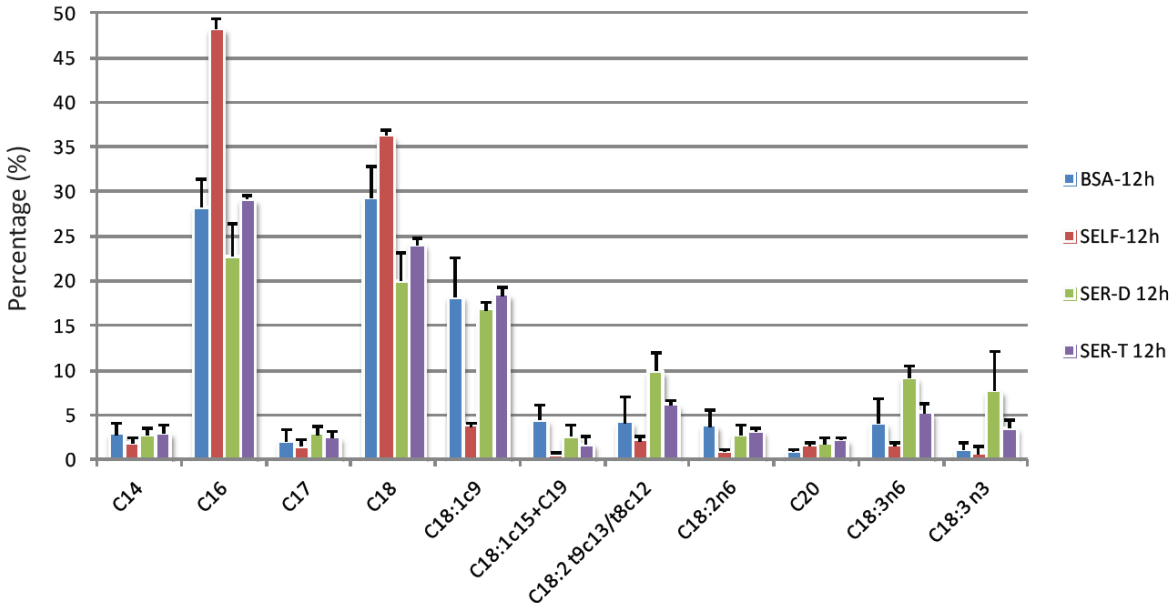


Figure 21 Ingenuity pathway analysis of differentially expressed genes in SELF-treated blastocyst compared to BSA-control.

The diagram represents the first generated network by IPA knowledge database showing functional relationships between DEG and potential up-stream factors. Red and green symbols show significantly up- and down-regulated genes in SELF-treated blastocyst compared to BSA, respectively (±1.4 fold-change, $p<0.05$).

5.9 Supplemental data



Supplemental figure 22 Fatty acid profile found in the four different culture media after 12 h of incubation.

Supplemental table 8 List of DEG in SELF-treated blastocyst based on microarray data and limma statistical test.

Gene_Symbol	Description	Symmetrical raw fold change	P-value
SERPINA5	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5	3,123253	0,012161266
XCL2	chemokine (C motif) ligand 2	2,693487	0,004333404
HIST1H1C	histone cluster 1, H1c	2,209251	0,027381418
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	2,183203	0,036250075
NDP	Norrie disease (pseudoglioma)	2,026026	0,028787049
LOC513329	similar to Equ c1	1,872102	0,046954341
MLLT11	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 11	1,841719	0,028130431
TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A	1,689999	0,032138898
LOC100298356	similar to bone marrow stromal cell antigen 2	1,584901	0,044610118
TPI1	triosephosphate isomerase 1	1,575779	0,016121929
TFPI	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	1,553935	0,026007926
LOC782971	similar to histone cluster 1, H2bd	1,542397	0,007931335
NULL	Novel Transcribed Region; evidence: embryonic ESTs	1,536373	0,021440531
MANSC1	MANSC domain containing 1	1,515097	0,049061611
RDX	radixin	-1,501109	0,019644782
FAM136A	family with sequence similarity 136, member A	-1,506509	0,040269366
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,507328	0,015680902
LOC518785	similar to ATPase family AAA domain-containing protein 2B	-1,513681	0,018441646
LOC100336625	hypothetical protein LOC100336625	-1,515716	0,017050969
SNX16	sorting nexin 16	-1,516078	0,034998011

LOC100298789	similar to protein phosphatase 2, regulatory subunit B, alpha	-1,521534	0,040571315
ARRDC2	arrestin domain containing 2	-1,529777	0,025034205
EPB41L3	erythrocyte membrane protein band 4,1-like 3	-1,539947	0,019291469
HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	-1,542409	0,048582632
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,549036	0,030095444
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,55252	0,031250838
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,554	0,004992159
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,560753	0,026043072
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,564522	0,023755223
SNX16	sorting nexin 16	-1,569982	0,035251356
FBXO25	F-box protein 25	-1,574765	0,040611188
LOC100337018	hypothetical protein LOC100337018	-1,576329	0,008602807
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,579595	0,001753681
SH3BP5	SH3-domain binding protein 5 (BTK-associated)	-1,591623	0,030665301
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,625769	0,022520637
PRM1	protamine 1	-1,641736	0,002130152
LOC100337018	hypothetical protein LOC100337018	-1,659381	0,035433432
LDLR	low density lipoprotein receptor	-1,659424	0,021898158
DCBLD2	discoidin, CUB and LCCL domain containing 2	-1,664058	0,008749797
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,690368	0,005427572
LOC100337420	hypothetical LOC100337420	-1,698997	0,029058475
ING5	inhibitor of growth family, member 5	-1,706567	0,033911936
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,716865	0,015845299
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,741323	0,009122755
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,801893	0,048902498

LOC100336997	hypothetical protein LOC100336997	-1,803843	0,006083548
LOC100337434	hypothetical LOC100337434	-1,868968	0,015677055
NULL	Novel Transcribed Region; evidence: embryonic ESTs	-1,892017	0,040608594
LOC100336997	hypothetical protein LOC100336997	-1,916181	0,012492401
LOC100336840	hypothetical protein LOC100336840	-2,14975	0,006406115

After hybridization, background correction, normalization and finally Limma statistical test were performed to determine the significant fluorescent intensity differences between treatments for each spot out of 44k present on the slide. Spots which were up- or down-regulated in treated blastocysts compared to control with a 1.5 fold change and a p-value<0.05 and their associated annotations are presented.

Supplemental Table 9 Primer sequences, product sizes, annealing temperature and accession numbers.

Gene_Symbol	Name	Accession number	Forward sequence	Reverse sequence	Product size (bp)	Annealing temperature (°C)
ARRB2	arrestin. beta 2	XM_002695776	TTGTGAAGGAGGGTGCCAACAA	TTCCTAGCAGAACTGGTCGTCA	350	57
CLDN6	claudin 6	XM_002697936	ACAAGCCTTTCCTTGCTGGTCA	AGTAACAACTGGTGAGTGTGGGCA	227	59
LDLR	low density lipoprotein receptor	NM_001166530	TGGCTGGTGTCCATCTGAAACA	TCAACCCACTCAGACGTTTCCA	188	58
HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	XM_001789611	TTGTGGCTGTGATCCTTCCCTT	AACATGGGTTGAGGCTGTCAGT	176	58
APEX	APEX nuclease (multifunctional DNA repair enzyme) 1	NM_176609	ATGCTGGCTTCACTCCACAAGA	TGTCACACAATGCAGGCAACAG	207	58
ARRB1	arrestin. beta 1	NM_174243	ACTTTGCCCGCCAGAGACTAAA	AAGTGATGCAGTGAGAGGGTGA	318	57
CYR61	cysteine-rich. angiogenic inducer. 61	NM_001034340	ATGGTAGAAGGGAGGCATTGCT	ACGTCAACACCACAAGCTCCAA	168	58
ACTB	actin-beta	NM_173979	ATCGTCCACCGCAAATGCTTCT	GCCATGCCAATCTCATCTCGTT	101	59
MYL6	myosin, light chain 6, alkali, smooth muscle and non-muscle	NM_175780	TTCGGGTGTTTGACAAGGAAGGGA	ATCCTCAGCCATTCAGCACCAT	228	58
PPIA	peptidylprolyl isomerase A (cyclophilin A)	NM_178320	TTTATGTGCCAGGGTGGTGACT	TCTTGCTGGTCTTGCCATTCCT	287	58
GFP	green-fluorescent protein		GCAGAAGAACGGCATCAAGGTGAA	TGGGTGCTCAGGTAGTGGTTGT	143	59

Conclusion générale

Afin de comprendre l'impact des technologies de procréation assistée sur la viabilité des embryons produits, nos recherches visaient à définir le profil transcriptomique de la réponse au stress et d'identifier des biomarqueurs de l'impact de la culture sur la qualité embryonnaire. Via l'exposition à différentes conditions de stress (énergie, oxydation, acides gras), le transcriptome des blastocystes survivants a été analysé par micro-puce, mettant en évidence des profils de gènes différentiellement exprimés qui ont ensuite été validés par RT-qPCR. Grâce aux outils bioinformatiques d'analyse génomique fonctionnelle des profils transcriptomiques, les voies de signalisation sous-jacentes à l'impact du stress ont pu être révélées, apportant de nouvelles informations sur les mécanismes embryonnaires affectés en condition de culture sous-optimale. En parallèle, la réponse au stress énergétique au niveau morula a montré des modifications précoces dans le métabolisme et le transcriptome, suggérant un impact progressif de la culture pendant le développement post-compaction. Au vu de ces résultats, la conclusion est alors faite que l'adaptation de l'embryon aux conditions sous-optimales de culture entraine des changements transcriptomiques en réponse au stress qui seraient préjudiciables pour la viabilité du blastocyste. De plus, le recoupement des différents profils de réponse au stress procure un panel de gènes associés de façon récurrente aux symptômes d'un embryon « malade ». Bien que nos objectifs soient atteints (déterminer l'impact du stress sur le transcriptome embryonnaire), certains points demeurent limitatifs dans l'implication biologique de nos résultats, et ouvrent alors des perspectives sur des recherches futures.

L'outil micropuce est un puissant générateur d'hypothèses biologiques basées sur la quantité d'ARNm de plusieurs milliers de gènes. L'expression « hypothèse biologique » réfère à la nature des résultats dont le but n'est pas de valider l'impact d'un traitement sur un aspect biologique attendu, mais de proposer l'implication d'une fonction biologique à partir d'une liste de gènes dont l'expression est affectée par le traitement. Les résultats informent de la quantité de gènes différentiellement exprimés (DEG) et leur direction (« up-régulé » ou « down-régulé »), mais également de la nature des processus cellulaires représentés par un enrichissement significatif en DEGs. Cet outil est très performant pour comprendre les mécanismes globaux affectés par le traitement en rapport aux modifications du profile

transcriptionnel de l'embryon. Cependant, beaucoup de précautions doivent être prises quant à l'interprétation des conséquences sur l'activité cellulaire puisque le lien entre différence de quantité d'ARN et différence fonctionnelle n'est pas toujours direct. Dans l'ovocyte et avant l'activation du génome embryonnaire par exemple, la présence de l'ARN n'est pas forcément associée à la présence de la protéine, et l'analyse d'ARNs poly-ribosomaux pourrait être utile pour déterminer quels gènes sont effectivement traduits en protéine. Aux stades post-EGA, la transcription serait liée à la traduction, bien que le taux de corrélation reste à déterminer. L'utilisation de gel 2D et les analyses de spectrométrie de masse pourraient alors permettre de connaitre l'ampleur des répercussions du transcriptome sur le protéome, et ainsi valider des biomarqueurs consistants avec le stress embryonnaire.

Pour valider le lien entre ARNm et protéine, les techniques d'immunohistochimie s'avèrent également utiles pour étudier une protéine cible. Cependant, le modèle bovin entraine des problèmes de spécificité inter-espèce de l'anticorps souvent destiné à l'humain ou aux rongeurs. Ce fut le cas dans notre analyse de lumican, un gène ayant une forte différence d'expression embryonnaire en réponse aux différents stress appliqués. Avec un anticorps fonctionnel, nous aurions pu estimer l'accumulation de la protéine, prédite comme facteur sécrété dans la matrice extracellulaire. Évidemment, il serait nécessaire de tester d'autres anticorps présents sur le marché ou développés spécifiquement pour le bovin. Le développement d'anticorps spécifiques pourrait aussi être utile en kit ELISA afin d'analyser la teneur des protéines sécrétées dans le milieu, telles que TNF, IFN ou plasmine en relation avec les profils inflammatoire (TNFRSF1A) et fibrotique (SERPINE1) observés dans les embryons stressés. Le niveau d'expression du sécrétome embryonnaire pourrait alors servir de diagnostique pré-implantatoire pour le choix du « meilleur » embryon destiné au transfert. En effet, les mécanismes immunitaires et remodelant la matrice extracellulaire sont finement régulés au moment de l'implantation/attachement. Il serait donc intéressant de valider comment les protéines de gènes candidats sont impliquées dans le processus d'implantation. L'ajout d'inhibiteurs/anticorps spécifiques de la protéase SERPINE1 pendant le développement post-compaction serait un moyen d'étudier le rôle du plasminogène dans l'élongation et la communication embryo-maternelle. Également, l'analyse de la réponse immunitaire (quantité de macrophages ou taux de TNF) dans l'utérus

en fonction de l'expression embryonnaire du récepteur au TNF permettrait de mieux comprendre comment le niveau de stress embryonnaire est reconnu par le système maternel.

Dans nos travaux, la réponse transcriptomique au stress énergétique a été corrélée à une réduction du taux d'ATP dans les embryons stressés. Cette analyse a permis de renforcer l'impact fonctionnel prédit par les profils des DEG et la résultante pour le métabolisme de l'embryon. Cependant, il serait intéressant d'étudier l'implication spécifique des gènes candidats dans la réponse au stress de l'embryon. Dans ce contexte, le modèle souris permet de réaliser des expériences de délétion de gène et d'analyser le phénotype résultant de la perte de fonction. Chez le bovin, la création d'animaux transgéniques est beaucoup plus complexe et couteuse. Une alternative à la délétion transgénique serait l'utilisation des techniques d'interférence à ARN. Par micro injection d'une séquence complémentaire, il est possible de diminuer la quantité de messagers spécifiques d'un gène, affectant le nombre de protéines synthétisées et donc la fonctionnalité de la voie de signalisation. L'injection de séquences complémentaires est faisable au stade ovocyte (une seule cellule) mais difficile au stade post-compaction (plusieurs dizaines de cellules). L'utilisation de morpholino, séquence d'ARN complémentaire pouvant être transférée par électroporation dans les cellules, serait une alternative à la micro injection. De plus, l'hybridation d'ARN in situ permettrait de localiser les ARNm et définir les types cellulaires à l'origine de la réponse au stress. Enfin, il serait également possible d'utiliser des inhibiteurs chimiques des voies de signalisation de réponse au stress (PI3K par exemple) et d'observer l'impact sur l'adaptation de l'embryon aux conditions sous-optimales de culture.

Par rapport aux conditions in vivo, la culture induit une augmentation de la production de lactate à partir du glucose, des variations du taux de respiration et d'oxydation des acides aminés, et l'accumulation d'acides gras. Ces changements proviendraient d'une adaptation homéostatique de l'embryon aux dommages induits par les conditions sous-optimales de son environnement. Ces différences métaboliques sont corrélées avec la viabilité embryonnaire, l'hypothèse étant qu'un embryon au métabolisme quiescent (selon « the quiet embryo hypothesis » d'Henry J. Leese) aurait une plus grande viabilité. En accord avec cette hypothèse, nos résultats montrent que la réponse au stress induit une dérégulation de l'expression des gènes du métabolisme énergétique dont notamment les facteurs de l'effet

Warburg. L'effet Warburg a d'abord été étudié dans les cellules cancéreuses dont les conditions de prolifération ainsi que les demandes métaboliques sont similaires aux besoins de l'embryon (oxydation des acides gras et des acides aminés dans la mitochondrie, production de lactate à partir du glucose pour maintenir le statut redox, utilisation du pyruvate pour l'échange des métabolites du cycle de Krebs). En réponse au stress de la culture, l'embryon aurait la capacité de détourner l'effet Warburg comme adaptation métabolique aux conditions sous-optimales, notamment en s'appuyant sur la glycolyse pour contrebalancer un déséquilibre énergétique et oxydatif. L'implication de l'effet Warburg dans l'analyse du métabolome pourrait servir de diagnostique qualité, perspective déjà utilisable (et améliorable) en ce qui concerne le profil en acides aminés (Marhuenda-Egea et al. 2010; Seli et al. 2010b; Vergouw et al. 2012). De plus, cela ouvre de nouvelles perspectives dans notre vision du métabolisme embryonnaire et la comparaison avec celui des cellules cancéreuses (Krisher and Prather 2012).

Afin de mieux comprendre l'effet progressif du stress sur le développement précoce, l'analyse de la réponse au stress énergétique a montré un impact au moment clé de la transition entre le stade morula et blastocyste. Cette transition est critique pendant le développement post-compaction et les conditions de culture permettant de dépasser le bloc MET montrent tout de même un nombre important d'embryons bloqués au stade morula (Devreker et al. 2001; Gardner and Lane 1996; Roth et al. 1994). En analysant les populations de morula contrôles et traitées, nos résultats montrent que l'impact du stress induit une réponse différente entre les individus, notamment dans l'expression du facteur de transcription HNF4A impliqué dans le contrôle énergétique hépatique et l'effet Warburg. L'absence d'expression d'HNF4A serait d'ailleurs corrélée au blocage développemental morula puisque le nombre d'individus HNF4A négatifs (absence du transcrit) est plus élevé dans les conditions de stress énergétique. Cette information apporte de nouvelles perspectives quant à l'interprétation des données transcriptomiques utilisant des groupes d'embryons et les différences de compétence intra-population. L'analyse individuelle serait alors une approche expérimentale utile pour définir l'ensemble des différentes réponses à un même traitement. D'autre part, il serait intéressant de déterminer le profil transcriptomique des embryons HFN4 positifs et HNF4 négatifs en réponse au stress afin de mieux définir les gènes associés à l'adaptation ou au bloc développemental. L'utilisation des outils d'analyse

fonctionnelle évoqués auparavant permettrait alors de valider l'implication de ce facteur de transcription dans la survie de l'embryon.

De plus en plus d'études de l'impact de la culture sur l'embryon précoce montrent l'implication des mitochondries dans le développement et la qualité embryonnaire. Cette organelle proto-énergétique héritée des bactéries est très sensible aux quantités de substrats disponibles ainsi qu'au taux d'oxygène de l'environnement. Bien que le métabolisme mitochondrial puisse utiliser différentes sources de carbone en réponse à la demande énergétique, la surcharge de substrats ou le déséquilibre redox peuvent affecter l'activité de la chaine respiratoire et le bon fonctionnement de l'organelle. Afin de mieux contrôler l'activité mitochondriale des embryons, l'élaboration de conditions de culture capables de faciliter les fonctions respiratoires ainsi que l'utilisation endogène des substrats serait potentiellement favorable à l'amélioration de la viabilité. De plus, les fonctions de la mitochondrie contribuent à la gestion du calcium intracellulaire et également à l'induction de la mort cellulaire programmée. Bien que ces propriétés soient encore mal comprises, l'analyse de la santé mitochondriale (imagerie par auto fluorescence du NADH par exemple) pourrait s'avérer un déterminant crucial dans la sélection du meilleur embryon. D'autre part, le métabolisme mitochondrial est un producteur de groupements methyl, molécules impliquées dans le control épigénétique de l'expression du génome (Wallace and Fan 2010). L'impact in utero de la demande énergétique est bien connu pour affecter la physiologie de l'individu, et des modifications épigénétiques très tôt dans le développement embryonnaire pourraient être responsables du phénotype à long terme. De même, l'impact de la culture est associé à des changements épigénétiques, notamment au niveau de gènes impliqués dans le control énergétique tel qu'IGF2. Dans nos expériences, ce gène est fréquemment up-régulé en réponse au stress. Il serait alors intéressant de voir l'interaction des profils épigénétiques et transcriptomiques en relation avec l'impact du stress mitochondrial sur la qualité embryonnaire.

Pour conclure, ce projet permet de développer un regard critique quant à l'impact potentiel des manipulations embryonnaires précoces et les enjeux pour le futur individu. En guise d'avertissement, la régulation des conditions de culture semble encore mal contrôlée par les cliniques pratiquant la PMA, et les changements de formulation des milieux sont

généralement associés au «lobby» du secteur pharmaceutique plutôt qu'à de vraies preuves scientifiques (Harper et al. 2012). Par exemple, le GM-CSF « Granulocyte-Macrophage Colony-Stimulating Factor » (ou CSF2), un facteur retrouvé dans l'oviducte, est depuis peu ajouté au milieu de culture dans différentes cliniques afin d'améliorer le taux de gestation lors de fausse-couche à répétition (Kawamura et al. 2012; Scarpellini and Sbracia 2009). Cependant, l'ajout de facteurs de croissance reste encore amplement débattu (Richter 2008), principalement à cause de potentiels effets épigénétiques pendant le développement embryonnaire ayant des répercussion a long terme sur l'individu. Bien que notre société engendre le cycle du profit, il serait dangereux de confronter le destin d'une partie de l'humanité aux décisions des comités d'administration (Leese et al. 1998). Dans ce contexte, la mesure éthique restera une arme capitale dans la bataille du progrès contre l'infertilité.

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