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Transcriptome profiling of bovine blastocysts developed under alternative culture conditions during specific stages of development

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Dedicated to my parents, my wife and my lovely sons Zeyad & Hazem

Transcriptome profiling of bovine blastocysts developed under alternative culture conditions during specific stages of development

The aim of this study was to determine the influences of different environmental conditions during specific stages of early bovine embryos on the transcriptome profile of produced blastocysts and subsequent effects on molecular mechanisms and pathways controlling embryo development. Using the advent of transvaginal endoscopy-mediated technology, different bovine blastocyst groups were produced under alternative culture conditions. In the first experiment, transcriptome analysis was performed between day 7 blastocysts which were developed either in superovulated heifers (High P4) or in unstimulated recipients (Normal P4) from day 2 onward using Affymetrix GeneChip Bovine Genome Array. In the second experiment, four different blastocyst groups were produced under alternative in vivo and in vitro culture conditions before or after major embryonic genome activation (EGA) stage. Completely in vitro and in vivo produced blastocysts were used as contrasts. Transcriptome profile of each blastocyst group has been compared to in vivo control group using EmbryoGENE's bovine microarray. Abnormal environmental conditions either in vivo or in vitro showed a dramatic effect on the transcriptome profiles of produced blastocysts. A total of 454 genes were differentially regulated between blastocysts derived from superovulated animals and those which cultured in unstimulated recipients from day 2. Blastocysts which developed under high P4 conditions due to superovulation treatment showed higher cellular and metabolic activities, as genes involved in the oxidative phosphorylation pathway and different metabolic processes, in addition to genes expressed in response to stress, were highly expressed compared to embryos which developed in the oviduct of unstimulated animals. In vitro culture conditions during EGA stage have critically influenced gene expression patterns, irrespective to embryo origin. Compared to complete in vivo group, blastocyst groups which spent EGA stage under in vitro conditions showed higher number of differentially regulated genes than those which spent EGA stage under in vivo conditions. Ontological classification showed a clear contrast in expression patterns for lipid metabolism and oxidative stress between blastocysts generated in vivo *vs.* in vitro, with opposite trends. These results will help for future efforts to modify culture conditions at the critical stages of development which will allow more efficient production of developmentally competent blastocysts.

Transkriptionsprofile boviner Blastozysten unter alternativen Kulturbedingungen während den spezifischen Entwicklungsstufen

Ziel dieser Studie war es, den Einfluss verschiedener Umweltbedingungen auf die frühe Embryogenese zu untersuchen. Transkriptionsprofile wurden von den erzeugten Blastozysten erstellt, um die Effekte verschiedener Umweltbedingungen auf molekularer Mechanismen und Pathways im Bezug auf die embryonale Entwicklung zu erfassen. Durch die Anwendung transvaginaler Endoskopietechniken, konnten unterschiedliche bovine Blastozysten Gruppen unter verschiedenen Kulturbedingungen erzeugt werden. Im ersten Experiment wurden Tag 7 Blastozysten zum einen in superovulierenden Färse (High-P4) oder zum anderen ab Tag 2 in unstimmulierten Rezipienten (Normal-P4) entwickelt. Das Transkriptionsprofil der Blastozysten wurde mittels des Affymetrix GeneChip Bovine Genome Array untersucht. In einem zweiten Experiment wurden vier alternativen Blastozystengruppen, sowohl vor als auch nach der zentralen embryonalen Genom Aktivierung (EGA), unter verschiedenen *in vivo* und *in vitro* Kulturbedingungen erzeugt. Als Kontrolle wurden gänzlich *in vivo* oder *in vitro* erzeugte Blastozysten verwendet. Transkriptionsprofile jeder Blastozystengruppe wurden mittels des bovinen EmbryoGENE Mikroarrays erstellt und mit der *in vivo* Kontrollgruppe verglichen. Abnormale Umweltbedingen zeigten einen dramatischen Einfluss auf das Transkriptionsprofil der erzeugten Blastozysten. Ingesamt waren 454 Gene unterschiedlich reguliert bei dem Vergleich von Blastozysten aus superovolierten Tieren und Blastozysten kultiviert ab Tag 2 in unstimmulierten Rezipienten. Blastozysten, die unter High-P4 Bedingungen auf Grund der Superovulation entwickelten, zeigten höhere zelluläre und metabolische Aktivitäten von Gene, die am Pathway der oxidativer Phosphorylierung und verschiedener metabolischer Prozesse beteiligt waren. In dieser Gruppe waren im Vergleich zur Gruppe der unstimmulierten Rezipienten, Gene der Stressantwort sehr hoch exprimiert. *In vitro* Bedingungen während der EGA haben einen kritischen Einfluss auf die Genexpressionsmuster, ungeachtet der Embryoherkunft. Im Vergleich zur gänzlich *in vivo* Gruppe, zeigten Blastozystengruppen, die während dem EGA Stadium unter *in vitro* Bedingungen verbrachten, deutlich mehr unterschiedliche regulierte Gene als auch die Gruppen, die das EGA Stadium unter *in vivo* Bedingungen waren. Ontologische Klassifizierungen zeigten deutliche Unterschiede in den Genexpressionsmustern des Lipidmetabolismus und des oxidativen Stresses zwischen *in vivo* und *in vitro* erzeugten Blastozysten. Diese Ergebnisse werden zukünftige Bestrebungen von modifizierten Kulturbedingungen während den kritischen Stadien der embryonalen Entwicklung fördern, welches zu einer effizienteren Erzeugung entwicklungskompetenter Blastozysten beiträgt.

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List of abbreviations

$A_{260/280}$	:	Absorbance at 260/280 mm wavelength ratios
ANOVA	:	Analysis of variance
ARE	:	Antioxidant response element
aRNA	:	Amplified ribonucleic acid
ART	:	Assisted reproduction technology
ATP	:	Adenosine-5'-triphosphate
bp	:	Base pairs
cAMP	:	Cyclic adenosine monophosphate
cDNA	:	Complementary Deoxyribonucleic acid
CL	:	Corpus luteum
COH	:	Controlled ovarian hyperstimulation
cRNA	:	Complementary ribonucleic acid
Ct	:	Cycle threshold
Cy-3	:	Cyanine 3
Cy-5	:	Cyanine 5
DEGs	:	Differentially expressed genes
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleoside triphosphate
EGA	:	Embryonic genome activation
FDR	:	False discovery rate
Fig	:	Figure
FSH	:	Follicle stimulating hormone
GAPDH	:	Glyceraldehyde 3- phosphate dehydrogenase
GEO	:	Gene Expression Omnibus
GnRH	:	Gonadotropin releasing hormone
h	:	Hours
H_2O	:	Water
IPA	:	Ingenuity pathway analysis software
IVC	:	In vitro culture
IVF	:	In vitro fertilization
IVM	:	In vitro maturation

IVP	:	In vitro production
IVT	:	In vitro transcription
Kg	:	Kilogram
LH	:	Luteinizing hormone
LIMMA	:	Linear models for microarray data analysis
µg	:	Microgram
min	:	Minute
ml	:	Milliliters
µl	:	Microliters
mM	:	Milimole
mm	:	Millimetr
MOET	:	Multiple ovulation and embryo transfer
mRNA	:	Messenger ribonucleic acid
ng	:	Nanograms
nM	:	Nanomole
NTC	:	No template control
^{o}C	:	Degree celsius
P4	:	Progesterone
PCA	:	Principal component analysis
PCR	:	Polymerase chain reaction
PG	:	Prostaglandins
PGF2α	:	Prostaglandin F2α
pH	:	pH value
RT-PCR	:	Reverse transcriptase polymerase chain reaction
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
s	:	Seconds
SAS	:	Statistical analysis system
S.D.	:	Standard deviation
vs.	:	Versus
W	:	Watt
XCI	:	X chromosome inactivation

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2.1 Chapter 1

1.1 Introduction

Early embryonic development, the period from maturation until blastocyst formation, is one of the most critical periods of mammalian development involves various morphological, cellular, and biochemical changes related to genomic activity. This period including three major steps: oocyte maturation, oocyte fertilization and embryo culture. Under normal in vivo conditions, mammalian oocytes and embryos develop in a complex and dynamic environment. Bovine oocyte grows and matures in the ovarian follicle, subsequently undergoes fertilization in the midpoint of the oviduct (Hunter 1988). First cleavage occurs approximately 1 to 2 days after fertilization. Between days 3 and 4 after fertilization, at the 8- to 16-cell stage, the embryo moves from the oviduct to the uterus (Hackett et al. 1993). The embryo reaches the 16- to 32-cell stage between days 5 and 6 and the cells begin to form intimate junctions (Boni et al. 1999), forming a compact ball of cells termed the morula. Compaction is a prerequisite to trophectoderm differentiation and is essential for blastocyst formation (van Soom et al. 1997). At Day 7 to 8, a blastocoelic cavity develops and the cells of the early blastocyst differentiate into inner cell mass cells, mainly destined to form the fetus, and trophectoderm cells, destined to form the placental tissues. At this stage, the blastocyst comprises about 120 cells with the inner cell mass constituting about 25% and the trophectoderm about 75% of the total cell number (Rizos et al. 2010). This embryonic development is initially supported by maternal RNAs and proteins synthesized during oogenesis. As development proceeds these proteins degrade, and embryogenesis becomes solely dependent on embryonic derived genes. Transcriptional activation of the embryonic genes is the result of a gradual degradation of the maternal RNAs and proteins due to the reprogramming of the cell nucleus (Memili and First 2000). Thus a well-orchestrated expression of genes derived from both the maternal and embryonic genomes is required to allow normal development. Clearly any modification to the culture environment can have profound effect on gene expression pattern and subsequently on the quality and developmental competence of the resulting embryos (Lonergan et al. 2003b).

Over the past three decades, applications of assisted reproductive technology (ART) include multiple ovulation and embryo transfer (MOET) and in vitro production (IVP) of embryos have been widely increased. These technologies offers several advantages

over natural breeding through their great potential for speeding up genetic improvement in farm animals, as well as greatly advanced our basic understanding of embryo development. Despite ongoing advances in ARTs, pregnancy rates remain low and embryos produced in this way still differ than 'golden standard' embryos (i.e. embryos derived from natural cycles). The differences involve morphological and molecular aspects that impair embryo quality and developmental efficiency.

MOET is one of the most widely used technology in order to increase the number of offspring during the lifetime of individual animals. Females of high genetic merit are typically stimulated with gonadotrophins to induce the ovulation of a variable but a large number of follicles. These superovulated animals are then artificially inseminated and the resulting embryos are non-surgically flushed from the uterus on Day 7 for transfer to surrogate recipients or for cryopreservation and transfer at a later date. It is clear that oocytes and embryos in superovulated animals develop under abnormal endocrine conditions, which differ substantially from those in unstimulated (i.e. single-ovulating) animals. This modification of oviductal conditions and/or composition of oviduct fluid due to ovarian steroids subsequently affect the embryonic developmental competence (Greve and Callesen 2001, Murray et al. 1995). However, the exact influences of this abnormal conditions on the transcriptome profile of produced embryos are still unknown. Although these embryos are differ than golden standard embryos, it is well recognised that bovine embryos derived in vivo, typically following superovulation, artificial insemination and non-surgical recovery, are of higher quality than those derived from in vitro maturation (IVM) fertilization (IVF) and culture (IVC). The superiority of in vivo derived embryos is reflected in the most recent published data from commercial cattle embryo transfer practice (Stroud 2011) with 990,993 bovine embryos were transferred worldwide in 2010 of which 63% were in vivo derived and the remaining 37% being produced in vitro. Moreover, approximately 55% of in vivo derived embryos were transferred frozen compared with only 7% of those produced in vitro, reflecting the poorer quality and cryotolerance of such embryos.

In vitro production of embryos has emerged as a reliable alternative method to conventional ovarian superstimulation methods and as an important research tool for animal and human embryology (Bavister 1995). However, embryos developed under in vitro conditions have been associated with several deviations from the normal in vivo development that resulted in development rate limited to 30–40% (Gutierrez-Adan et al.

2001, Niemann and Wrenzycki 2000), higher incidence of chromosomal abnormalities (Lonergan et al. 2004, Viuff et al. 2002), gross morphological abnormalities (Abd El Razek et al. 2000, Pollard and Leibo 1994) and a dramatic effect on gene expression pattern in embryos, which in turn has serious implications for the quality of blastocyst, lowering cryo-tolerance and decreasing pregnancy rates (Lonergan et al. 2003a, Rizos et al. 2003, Tesfaye et al. 2004). Previous studies have clearly demonstrated that, while the intrinsic quality of the oocyte determines the proportion of oocytes developing to blastocysts (i.e., oocyte developmental competence), the post-fertilization culture environment has the biggest influence on blastocyst quality, irrespective of the origin of the zygote (Lonergan et al. 2003a, Rizos et al. 2002a, Russell et al. 2006). The period of post-fertilization culture involved several major developmental events, including: (1) the first cleavage division, (2) embryonic genome activation (EGA), when the embryo transfers from a reliance on maternal RNA derived from the oocyte to expression of its own genome, (3) compaction of the morula, which involves the establishment of the first intimate cell to-cell contacts in the embryo and (4) formation of the blastocyst, involving the differentiation of two cell types, the trophectoderm and the inner cell mass. However, the exact influence of culture conditions during each of these critical events is still unknown. Major EGA in bovine embryos occurs around the 8- to 16-cell-stage (Memili and First 2000). This event found to be one of the most critical events during preimplantation period and also associated with the first differentiative events, successful embryo implantation, and fetal development (Misirlioglu et al. 2006).

Gene expression has a fundamental role in the coordination of homeostatic and metabolic mechanisms during preimplantation period of development. This period involves various morphological and biochemical changes related to genomic activity and comprise a complex set of physiological processes, many of which are still unknown. These processes are controlled by a harmonized expression of about 10,000 sequential and temporal genes and strongly influenced by culture conditions (Niemann and Wrenzycki 2000). Transcriptomics have been employed successfully to contrast gene expression in mammalian oocytes and early embryos. Recent advances in bioinformatics and high-throughput technologies such as Next-generation sequencing and microarray analysis have revolutionized the way we can analyze the entire transcriptome within a population of cells which improve our understanding of the molecular mechanisms underlying normal and dysfunctional biological processes

(Marguerat and Bahler 2010, Marioni et al. 2008, Wang et al. 2009). Comparative analysis of mRNA expression patterns between embryos produced under different in vitro culture systems and in vivo allows the isolation of genes associated with embryo quality and investigation of the influence of suboptimal culture conditions on embryonic gene expression (Cote et al. 2011, Lee et al. 2001, Natale et al. 2001, Smith et al. 2009). These studies confirmed that there are alterations in gene expression of embryos developed under different culture conditions, which consequently resulted in reduced qualities and subsequently associated with fetal and neonatal abnormalities. Moreover, alterations in gene expression profile of oocytes and embryos which have different developmental potential have been reported (Mamo et al. 2006). A common finding between different studies in different mammalian species is that most genes in embryos cultured in vitro were down-regulated compared to their in vivo counterparts (Bauer et al. 2010, Miles et al. 2008) and that might be a result of decreased expression of genes associated with transcription and translation processes (Corcoran et al. 2006). In addition, It has been shown that stress response genes are up-regulated during in vitro development while metabolism related genes are down-regulated compared to in vivo derived embryos (Gutierrez-Adan et al. 2004). As a result of studying gene expression profiles to compare in vivo and in vitro derived blastocysts, some genes emerged and suggested to be as candidate genes for developmental competency and embryonic quality which can be used to modify the current culture systems. An example of these genes is the genes controlling the process of pluripotency during embryo culture: *Oct4, Nanog,* and *Sox2* (Botquin et al. 1998, Chambers et al. 2003, Masui et al. 2007, Nichols et al. 1998). In different studies, the expression patterns of these candidate genes showed higher relative amount in in vivo produced embryos when compared to in vitro derived ones (Kumar et al. 2007, Magnani and Cabot 2008) which support the idea that inadequate culture conditions alter gene expression and contribute to impaired development.

In accordance with many studies done in this field as well as future perspectives, there is an inevitable need for combining different culture systems during critical stages of embryonic development to facilitate a comprehensive understanding of early embryo development regulation and to improve success of embryo culture. For that, two different experiments were conducted in this thesis to achieve the following aims:

1- Examine the effect of an abnormal endocrine environment due to superovulation treatment on embryo development and its influence on the blastocyst transcriptome profile.
2- Examine the effect of alternative in vivo and in vitro culture conditions during the time of major EGA on embryo development and the transcriptome profile of bovine blastocyst.
3- Determine molecular mechanisms and pathways that control embryo development and influenced by alternative culture conditions during specific stages of preimplantation bovine embryos.

1.2 Materials and methods

To achieve the objectives of this research several materials and methods were used. The materials and methods are described in details in the different chapters of this thesis. The importance of some methods and their descriptions are briefly summarized here.

1.2.1 Transvaginal endoscopy-mediated method for embryo recovery and transfer

Transvaginal endoscopy promises to be an approach for embryo transfer and collection by introduce tubal-stage embryos into the physiological environment of the oviducts as well as recover specific developmental stage embryos (Besenfelder et al. 2008, Besenfelder et al. 2001). As a result of progress in optimization of this nonsurgical endoscopic access to the oviduct, there has been a steady increase in pregnancy rate after transfer of early cleavage-stage embryos to the oviduct (Besenfelder et al. 2010). Therefore, it is suggested that this could be a good system to produce embryos under conditions appropriate for addressing limits for physiological mechanisms during in vitro production as well as for production of embryos for commercial purposes. For embryo transfer, a universal tube including a traumatic trocar was introduced through the mid-dorsal area of the fornix vagina. After removal of the trocar, a bi-tubular inlay was inserted bearing the endoscopic as well as the transfer system. The transfer system consisted of a 1-mL syringe connected to a perfusor tube and the curved glass capillary was filled with embryos (n=25-50) and the contents were transferred into the oviduct ipsilateral to the corpus luteum with as little as 100 µl medium. For embryo collection, procedure was composed of the deposition of a uterine embryo flushing catheter, as is routinely done on day 7 in superovulated cows, and the endoscopic technique similar to the described transfer. However, we used a metal flushing catheter for insertion into the oviduct. The oviducts were flushed using 40 to 60 mL of medium leading to visible medium flow in the embryo filter, which was attached to the uterine flushing catheter. Additionally, each uterine horn was flushed with about 300 mL medium.

With the advent of this technology two different experiments have been done to produce bovine blastocysts cultured under alternative environmental conditions at specific time points during embryonic preimplantation period. For the first experiment, two different bovine blastocyst groups (3 replicates; each with 5 blastocysts) were produced. In the first group blastocysts were developed in superovulated heifers until day 7. In the

second group blastocysts were flushed by endoscopy at day 2 from superovulated heifers and then transferred (in groups of 25-50) to the ipsilateral oviduct of 4 unstimulated (i.e. single-ovulating) synchronized recipients by endoscopic tubal transfer. At day 7 all animals were non-surgically flushed to collect bovine embryos. For the second experiment, four different blastocyst groups (3 replicates; each with 10 blastocysts) were produced under alternative in vivo and in vitro culture conditions. The first two groups (Vitro_4-cell and Vitro_16-cell) were transferred from in vitro conditions to synchronized recipients at 4- and 16-cell stage, respectively and cultured in vivo until day 7 blastocyst stage. The second two groups (Vivo_4-cell and Vivo_16-cell) were flushed out from donor heifers at 4- and 16-cell stage, respectively and cultured in vitro until day 7 blastocyst stage. In addition complete in vitro (IVP) and in vivo produced blastocysts were produced and used as contrasts.

1.2.2 RNA isolation, amplification and microarray hybridization

Total RNA isolation was performed using the PicoPure™ RNA Isolation Kit (Arcturs, Munich, Germany). Genomic DNA contamination was removed by performing on-column DNA digestion using RNase-free DNase (Qiagen GmbH, Hilden, Germany). Quantity and quality of the extracted total RNA was verified on a 2100 Bioanalyzer (Agilent Technologies Inc, CA, USA). In the first experiment (chapter 1), two rounds of RNA amplification were performed as described in the GeneChip® Expression Analysis Technical Manual using 100 ng of total RNA as a starting material. The resulting double-stranded cDNA in the second round was then amplified and labelled using a biotinylated nucleotide analog/ribonucleotide mix in the second in vitro transcription (IVT) reaction using GeneChip IVT Labeling Kit (Affymetrix, Inc., Santa Clara, CA, USA). The biotin labelled cRNA was fragmented and analyzed in the Bioanalyzer. The samples were then hybridized to the Affymetrix GeneChip Bovine Genome Array consisting of 24128 probesets. For each group, three biotin-labelled cRNA hybridizations were performed. The arrays were washed and stained using the Fluidics Station 450/250 and scanned using the GeneChip® scanner 3000 integrated with Affymetrix® Microarray Suitesoftware. In the second experiment (chapter 2), two rounds of RNA amplification were performed using RiboAmp HS RNA amplification kit (Applied Biosystems). Two micrograms of aRNA from each sample were conjugated with either Cy-3 or Cy-5 dyes using ULS Fluorescent labelling kit for

Agilent arrays (Kreatech Diagnostics, Amsterdam, Netherlands). Samples from the three pools (biological replicates) of each blastocyst group and in vivo control blastocyst were hybridized on EmbryoGENE's bovine microarray (Made by Agilent 4 x 44k) using a dye-swap design (technical replicates) for a total of six arrays. Slides were scanned using Agilent's High-Resolution C Scanner (Agilent Technologies, CA, USA) and features were extracted with the Agilent's Feature Extraction software (Agilent Technologies, CA, USA).

1.2.3 Quantitative real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) has emerged as a robust and widely used methodology for biological investigation. It can detect and quantify very small amounts of specific nucleic acid sequences in rapid and accurate assessment. The cDNA synthesized from blastocyst samples was subjected to real-time PCR using *GAPDH* to test for any variation in the expression of this internal control gene. After confirming that there were no differences in the relative abundance of *GAPDH* between samples, all transcripts were quantified using independent real-time PCR runs. In the first experiment (chapter 1), a serial dilution of 10^1-10^9 copy number of molecules for each quantified gene was prepared from the plasmid DNA. Quantitative real-time PCR was performed using cDNA samples from three independent biological replicates of each blastocyst group in addition to the samples used for array analysis. In each run, beside each cDNA sample, plasmid standards for the standard curves and no-template control (NTC) were used. The PCR was performed in a 20 µl reaction volume containing iTaq SYBR Green Supermix with ROX (Bio-Rad) in ABI PRISM® 7000 instrument (Applied Biosystems). Melting curve analysis was constructed to verify the presence of gene-specific peak and the absence of primer dimer. Final quantitative analysis was carried out using the relative standard curve method and results were reported as the relative expression after normalization of the transcript amount relative to the endogenous control. In the second experiment (chapter 2), each pair of primers was tested to achieve efficiencies close to 1. The PCR was performed in a 20 µl reaction volume containing iTaq SYBR Green Supermix with ROX (Bio-Rad) in StepOnePlus™ real time PCR system (Applied Biosystem) and the comparative cycle threshold (Ct) method was used to quantify expression levels.

1.2.4 Data analysis

Data were analysed using the Statistical Analysis System (SAS) version 8.0 (SAS Institute Inc., Cary, NC, USA) software package. Mean developmental rates of embryos were analysed by ANOVA followed by a multiple pair wise comparison (Tukey Test). The relative expression data were analysed using General Linear Model (GLM) of SAS, and differences in mean values were tested using ANOVA followed by a multiple pair wise comparison using t-test. Differences of p-value < 0.05 were considered to be significant. Array data analyses were performed using R version 2.12.1 and Linear Models for Microarray Data Analysis (LIMMA) package version 3.6.9. For Affymetrix array data analysis (chapter 1), data normalization and background correction were performed using Guanine Cytosine Robust Multi-Array Analysis method. However, for Agilent array data analysis (chapter 2), data were submitted to a simple background correction, a Loess within array normalization and Quantile between array normalization. Differentially expressed genes (DEGs) were obtained using LIMAA (Smyth 2005). Genes were considered differentially expressed at a fold-change >2 with adjusted P-value of < 0.05 using the Benjamini and Hochberg false discovery rate (FDR) method. A list of the DEGs from each comparison was uploaded into the Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, www.ingenuity.com) to identify relationships between the genes of interest and to uncover common processes and pathways.

1.3 Results

Some of the important results are briefly described here. The detailed results can be found in the respective chapters in this thesis.

1.3.1 Effect of maternal environment on developmental rates and blastocyst transcriptome profile

In the first experiment (Chapter 1), embryos which were developed either in the reproductive tract of superovulated heifers (High P4) or in unstimulated recipients (Normal P4) from day 2 onward showed differences in developmental rate and blastocyst:morula ratio. The percentage of embryos which developed to the morula or blastocyst stage was higher ($P < 0.05$) when cultured in superovulated heifers compared to unstimulated heifers (68.3% versus 52.1%). However, significantly more embryos developed to the blastocyst stage in the unstimulated heifers compared to those developing in superovulated heifers (33.6% versus 22.1%). The blastocyst:morula ratio was significantly higher ($P < 0.05$) in unstimulated heifers compared to superovulated heifers (1.81 vs. 0.48). A total of 454 DEGs with $p < 0.05$, fold change ≥ 2 and FDR ≤ 0.3 were differentially regulated between blastocysts derived from superovulated animals and those which cultured in unstimulated recipients from day 2 onward. The majority of the DEGs (429 gene) were up-regulated in blastocysts which developed under superovulation conditions. A high proportion (40%) of these DEGs fell into functional categories related to metabolic processes including carbohydrate, lipid, nucleic acid, amino acid, vitamin and mineral metabolism. Other functional categories, including protein synthesis, RNA post-transcriptional modification, gene expression, cell-to-cell signalling, energy production and molecular transport were also overrepresented in the same blastocyst group. Pathway analysis using IPA revealed that the oxidative phosphorylation pathway was the dominant pathway among 5 different canonical pathways and all DEGs (26 genes) involved in this pathway were highly abundant in the blastocysts derived from superovulated heifers compared to those derived from unstimulated heifers. These genes can be classified into 4 main categories: NADH dehydrogenase, cytochrome c reductase, cytochrome c oxidase and ATP synthase, representing 4 out of 5 protein complexes involved in the electron transport and oxidative phosphorylation pathway. The results of microarray have been validated

using quantitative real-time PCR that confirmed the expression profile of 11/14 (79%) of the selected genes for validation.

1.3.2 Effect of alternative culture conditions during major EGA on developmental rates and blastocyst transcriptome profile

In the second experiment (Chapter 2), there was no effect of culture conditions (in vivo or in vitro) either before or after the time of major EGA on embryo developmental rates. However, the origin of the oocyte was the main factor determines the developmental rates. In vitro originated blastocyst groups (Vitro_4-cell, Vitro_16-cell and IVP) showed lower developmental rates (24.1, 27.5 and 33.6%, respectively) than in vivo originated blastocyst groups (Vivo_4-cell and Vivo_16-cell; 64.4 and 68.2%, respectively). Vitro_16-cell group showed the lowest blastocyst:morula ratio (0.2). In contrast, Vivo_4-cell group showed the highest blastocyst:morula ration (9.9) comparing to all other groups. Principal component analysis (PCA) of microarray data from different blastocyst groups revealed that the source of greatest variation in the transcriptional profile was the in vitro culture conditions during the time of major EGA. Blastocyst groups which spent the time of major EGA under in vitro conditions either originated in vitro (Vitro_16-cell) or in vivo (Vivo_4-cell) showed higher number of differentially expressed genes (882 and 1918 DEGs, respectively) than those which spent the time of major EGA under in vivo culture conditions either originated in vitro (Virtro_4-cell) or in vivo (Vivo_16-cell) (633 and 311 DEGs, respectively), or than IVP group (841 DEGs) compared to the complete in vivo control group. Chromosome distribution comparison between all groups for DEGs showed a higher percentage of genes to be located on X-chromosome and most of these genes were up-regulated in all blastocyst groups compared to their complete in vivo blastocyst counterparts. Ontological classification of DEGs indicated that metabolic processes including: lipid, carbohydrate, nucleic acid and amino acid, cell related functions including: cell death, cellular growth and proliferation, cell cycle and cell-to-cell signalling, as well as embryonic development and gene expression processes were the most significant common functions affected in all blastocyst groups exposed to some in vitro conditions compared to the in vivo control group. Interestingly, lipid metabolism was the most significant biological function affected in Vitro_16-cell group with down-regulation for most of the genes involved in this function comparing to in vivo control group. The

same pattern of lipid metabolism related genes has been found in Vitro_4-cell and IVP blastocyst groups but with lower number of genes involved. In contrast, Vivo_4-cell group showed opposite pattern in which most of DEGs involved in lipid metabolism were up-regulated compared to in vivo group. Pathway analysis using IPA revealed that NRF2-mediated oxidative stress response pathway was the dominant pathway in Vivo_4-cell group and showed up-regulation for most of the involved genes, especially genes encoding for antioxidant enzymes (*PRDX1, SOD, TXNRD1, GPX2* and *CAT*). Antioxidant related genes in this pathway showed an opposite pattern in the groups which spent major EGA time under in vitro culture conditions with up-regulation in Vivo_4-cell group and down-regulation in Vitro_16-cell group which showed also down-regulation of *NRF2* gene, central gene acting as a transcriptional factor and regulating the expression of sets of genes including antioxidant, detoxifying, stress response and proteasomal degradation related genes. Quantitative real-time PCR confirmed the differences in the expression of 14 out of 15 (93%) genes selected to validate the results of microarray.

1.4 Conclusions

In this study we identified the effect of culture environment at specific stages of preimplantation bovine embryos on the transcriptome profiles of the resulting blastocysts. The results showed that abnormal culture conditions either in vivo due to superovulation treatment or in vitro due to suboptimal culture conditions can influence the development and gene expression patterns of embryos. Culture of embryos under hormonal stimulation conditions until day 7 lead to a delay in development, as greater proportion of morula than blastocysts flushed from the superovulated heifers at day 7 compared to unstimulated heifers has been observed. This delay in embryo developmental rate under superovulation conditions has been reported in mice (Ertzeid and Storeng 2001, Van der Auwera and D'Hooghe 2001). Likewise, embryos from superovulated hamsters had significantly reduced mean cell numbers and lower viability than controls (McKiernan and Bavister 1998). However, the reasons for the developmental retardation, loss of developmental competence and low quality of embryos from superovulated donors are still unknown. We also showed that, although changing culture conditions from in vivo to in vitro or vice versa either before or after the time of major EGA have no effect on the embryo developmental rates, oocyte origin seem to have a critical effect on the developmental rates. In which, in vivo originated embryos had higher overall transferable embryo rates compared to in vitro originated ones. These results were in agreement with previous studies which proved that while the in vivo or in vitro origin of oocyte (the oocyte quality) is the main factor determining embryo developmental rate (Hendriksen et al. 2000, Rizos et al. 2002b), the post fertilization culture environment is known to be the most important factor determining the quality of the resulting embryos (Rizos et al. 2003, Rizos et al. 2002a).

Expression of genes during early embryonic developmental stages is one of the good indicators for embryo quality and viability. In this study, microarray analysis revealed that embryos developing to the blastocyst stage in superovulated heifers showed significant up-regulation for most of the DEGs which are involved in metabolic processes, protein synthesis and energy production via the oxidative phosphorylation pathway compared to embryos developing to the blastocyst stage in unstimulated heifers. This indicated that embryos under superovulation conditions are metabolically active and they are in high demand for ATP to reach the blastocyst stage. In addition,

higher expression of heat shock protein related genes (*HSPA14* and *HSPE1)* has been observed in blastocysts derived from superovulated heifers which may indicate that the oviductal environment under superovulation conditions provided some stress factors to the preimplantation embryos. These findings are supported by the 'quiet embryo hypothesis' put forward by Leese (2002) which proposed that viable embryos have a 'quieter' metabolism than those with less viability and this quietness can be lost in response to environmental stress (Leese et al. 2008). In accordance, metabolic processes including lipid, carbohydrate, nucleic acid and amino acid were the most significant functions affected in all blastocyst groups exposed to in vitro conditions compared to the in vivo control group. Blastocysts which spent the time of major EGA under in vitro conditions showed higher number of DEGs than those spent major EGA time under in vivo conditions compared to in vivo control blastocyst. This disturbance in gene expression highlight the sensitivity of embryos towards in vitro culture conditions during major EGA time. Interestingly, lipid metabolism and oxidative stress response related genes were the most affected genes in the two blastocyst groups which were cultured in vitro during major EGA time either originated in vivo or in vitro. However, they showed opposite patterns of expression. A clear pattern of down-regulation for lipid metabolism and oxidative stress response related genes has been found in the in vitro originated group compared to in vivo originated one. This was also reflected on the morphology of in vitro originated embryos which showed a darker cytoplasm at different developmental stages as indicator for lipid accumulation in the cytoplasm. This finding could highlight the critical influence of culture conditions during maturation and early developmental stages on the metabolic activity and the ability of embryos to adapt with changing culture conditions.

This is the first comprehensive study provided more information about molecular mechanisms and pathways that influenced by altered culture conditions during specific embryonic developmental time points. This will enable to launch strategies to modify culture conditions at the critical stages of development to enhance the development of competent blastocyst and will aid in determining new candidate genes to be used as markers of embryo quality.

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2.1 Chapter 1 (Published in: *Human Reproduction*, 2011. 26(7):1693-707)

Effect of reproductive tract environment following controlled ovarian hyperstimulation treatment on embryo development and global transcriptome profile of blastocysts: implications for animal breeding and human assisted reproduction

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Abstract

In mammals, the reproductive tract plays a crucial role in the success of early reproductive events and provides an optimal microenvironment for early embryonic development. However, changes in the reproductive tract environment associated with controlled ovarian hyperstimulation and the influence on the embryo transcriptome profile have not been investigated. Therefore, we investigated differences in development rate and transcriptome profile of bovine blastocysts developing in the reproductive tract of unstimulated or superovulated heifers. Nineteen Simmental heifers were synchronized, superovulated and artificially inseminated; nine heifers were flushed on day 2 after insemination and 2- to 4-cell stage embryos were recovered and endoscopicaly transferred to the ipsilateral oviduct of unstimulated (i.e. single-ovulating) synchronized recipients (n=4 recipients; 25-50 embryos per recipient). The remaining ten superovulated heifers and the unstimulated recipients were then non-surgically flushed on day 7 to collect embryos. The blastocyst transcriptome profile was examined using the Affymetrix GeneChip Bovine Genome Array. The proportion of embryos which developed to the blastocyst stage was lower in superovulated heifers than unstimulated heifers ($P<0.05$). Blastocysts which developed under the abnormal endocrine conditions associated with superovulation showed higher cellular and metabolic activities, as genes involved in the oxidative phosphorylation pathway, different metabolic processes and translation and transcription processes, in addition to genes expressed in response to stress, were highly expressed compared to embryos which developed in the oviduct of unstimulated animals. The environment in which the embryo develops in the oviduct/uterus significantly alters gene expression patterns, especially those genes which regulate metabolic activity in the embryo.

Introduction

Controlled ovarian hyperstimulation (COH) is a routine procedure utilized in assisted reproduction to stimulate the growth of multiple follicles in naturally single-ovulating species including cattle and humans. In cattle, in order to increase the number of offspring during the lifetime of individual animals, females of high genetic merit are typically stimulated with gonadotrophins to induce the ovulation of a variable but large number of follicles (hence the routine use of the term 'superovulation' in domestic

species, as ovulation actually takes place). These embryo ‘donors’ are then artificially inseminated with semen from a high genetic merit sire and the resulting embryos are non-surgically flushed from the uterus on day 7 for transfer to surrogate recipients, or for cryopreservation and transfer at a later date (see Lonergan and Boland 2011 for review). Similarly, women undergoing IVF treatment undergo COH to induce the development of multiple dominant follicles. In contrast to cattle, multiple mature oocytes are recovered by transvaginal follicle aspiration just prior to ovulation, inseminated in vitro with partner/donor sperm and then, in contrast to cattle, because of the very different objectives, embryos are transferred back to the same women, often in the same cycle. Thus, while the objective of producing multiple mature oocytes is the same, the term 'superovulation' is inappropriate in routine human IVF, as ovulation does not take place. Therefore, while in cattle embryos are transferred into a synchronised recipient animal with a normal endocrine profile, in humans, the embryo(s) is routinely transferred into an endocrinologically abnormal uterine environment associated with controlled ovarian stimulation.

Despite significant advances in assisted reproduction technology (ART) protocols in humans, pregnancy rates are still relatively low and have not increased significantly in the last decade (Andersen et al. 2007). Similarly, in cattle, despite constant refinement of superovulation protocols, mainly driven by more efficient management demands, embryo yield per superovulated donor has not changed significantly (Bo et al. 2008). The yield and quality of embryos produced following superovulation of cattle is highly variable and may be affected either through effects on the oocyte during follicular growth (Sirard et al. 2006) or directly during embryo development in the oviduct and/or uterus (Killian 2004, Looney and Pryor 2010). It is clear that oocytes and embryos in superovulated animals develop under abnormal endocrine conditions which differ substantially from those in unstimulated (i.e, single-ovulating) animals and that they may exhibit a reduced developmental potential (Greve and Callesen 2001). Previous studies in mice have shown that COH procedures can result in delayed embryo development, decreased implantation and increased postimplantation loss (Ertzeid and Storeng 1992, 2001). Similarly, Van der Auwera and Hooghe (2001) reported delayed blastocyst formation, increased incidence of zona lysis and blastocyst collapse of embryos derived from stimulated mice compared with naturally cycling controls. In rabbits, embryos removed from superovulated donors at the one-cell stage and

immediately transferred to unstimulated recipients tended to have higher cell numbers than embryos residing continuously in superovulated animals (Carney and Foote 1990).

The oviduct and the fluid contained in it provide a beneficial environment for gamete maturation, gamete transportation, fertilization and early embryonic development. These events are key processes in mammalian reproduction and are under the control of steroids (Murray et al. 1995) and prostaglandins (PG) (Lim et al. 1997). Studies in mice and humans have shown that COH can lead to incorrect genomic imprinting and decreased embryo quality (Fauque et al. 2007, Rossignol et al. 2006, Sato et al. 2007), suggesting that ovarian hormonal stimulation may affect embryo development and gene expression in many different ways. In addition, an increase in chromosomal abnormalities has been reported in human embryos after conventional stimulation, mainly resulting from an increased incidence of chromosome segregation errors during the first embryonic divisions, and abnormalities were lowest in embryos which divided within the expected time frame (Baart et al. 2009).

Development of transvaginal endoscopy in cattle (Besenfelder et al. 2008, Besenfelder et al. 2001) has allowed unrivalled access to the bovine oviduct for the recovery and/or transfer of embryos at different developmental stages, and offers an excellent tool for studying the early kinetics of embryo development as well as the effects of hormonal stimulation on embryo physiology. As a result of progress in optimization of this nonsurgical endoscopic access to the oviduct, there has been a steady increase in pregnancy rate after transfer of early cleavage-stage embryos to the oviduct (Besenfelder et al. 2010).

To our knowledge, the changes in the oviduct and uterine environment related to hormonal treatments and subsequent influence on transcriptome profile of embryos have not been investigated. It is well known that COH leads to deviant oocyte maturation in the follicle (Hyttel et al. 1989) as well as an abnormal endocrine environment in the reproductive tract. Therefore, this study aimed to separate these two processes and examine the effect of an abnormal endocrine environment on embryo development to day 7 and its influence on the blastocyst transcriptome. To do this, a state-of-the-art nonsurgical endoscopy method was used to recover 2- to 4-cell stage embryos from superovulated donors and transfer them to non-stimulated single-ovulating recipients. The proportion of embryos which developed to the blastocyst stage and the transcriptome of the resulting blastocysts was compared between groups. These

data have relevance not only for cattle embryo production but also for assisted human reproduction where oocytes derived from women undergoing COH are routinely replaced in the uterus of the same women during the same cycle, in contrast to the situation in cattle, where transfer to unstimulated surrogate recipients is the norm. This relevance is highlighted by the fact that high-quality embryos transferred into women who are acting as surrogate mothers have a higher likelihood of implanting than if they are transferred back into the donors (Check et al. 1992, Stafford-Bell and Copeland 2001).

Materials and Methods

Animals for embryo collection

All experimental animals were handled according to the animal protection law of Germany. Simmental heifers (n= 23) aged between 15 and 20 months and weighing between 380 and 500 kg were used in this study. All animals were kept under identical farm conditions within the same herd. A total of 19 heifers were superovulated using FSH (see below) and artificially inseminated three times with the same frozen-thawed commercial bull semen. Nine animals were flushed by endoscopy at day 2 to recover 2- to 4-cell stage embryos which were then transferred (in groups of 25-50) to the ipsilateral oviduct of 4 unstimulated (i.e. single-ovulating) synchronized recipients by endoscopic tubal transfer. The remaining 10 superovulated heifers were not flushed until day 7. At day 7 all animals were non-surgically flushed to collect bovine embryos which have thus developed either in a superovulated or an unstimulated environment. The overall experimental design is indicated in Figure 1.

Hormonal treatment

Pre-synchronization was performed by i.m. administration of 500 µg cloprostenol (PGF2α, Estrumate®; Essex Tierarznei, Munich, Germany) twice within 11 days. Two days after each of the PGF2α treatments animals received 10 µg GnRH (Receptal®; Intervet, Boxmeer, The Netherlands). Twelve days after the last GnRH injection heifers received the first of eight consecutive FSH-injections over 4 days in decreasing doses (in total 300–400 mg FSH equivalent according to body weight; Stimufol, University of Liege, Belgium). Two PGF2α treatments were performed 60 and 72 h after the initial FSH injection. Finally, 48 h after the first PGF2α application, ovulation was induced by

simultaneous administration of 10 μg GnRH with the first of a total of three artificial inseminations within a 12 h interval. The time of the second insemination (60 h after the first PGF2α application) was defined as day 0 (D0). The oestrous cycles of recipient animals were synchronized as mentioned above but were not inseminated.

Blood sampling and progesterone assay

Daily blood samples were collected from each animal from day 1 until the day of embryo recovery (D7) by jugular venipuncture. Following collection, blood samples were refrigerated at 4°C for 12-24 h before being centrifuged at 1500 g at 4°C for 20 min. Serum was separated and stored at -20°C until assayed to determine progesterone concentration by time-resolved fluoroimmunoassay using an AutoDELFIA™ Progesterone kit (Perkin Elmer, Wallac Oy, Turku, Finland). The sensitivity of the assay was 0.01 ng/ml.

Collection of embryos at 2- to 4-cell stage

Embryos at the 2- to 4-cell stage were collected 48-54 h after the expected onset of ovulation (D0). Flushing was accomplished as described previously (Besenfelder et al., 2001). Briefly, after restraining the recipients, administering 5 mL of a 2% lidocaine-solution (Xylanest purum1, Richter Pharma,Wels, Austria) for epidural anesthesia and disinfecting the vulva (Octenisept1, Schülke/Mayer, Vienna, Austria), a trocar set consisting of an universal metal tube (12.5 mm _ 52 cm, Storz, Vienna, Austria) and an atraumatic mandrin was placed caudodorsal of the fornix vagina. The mandrin was replaced by a sharp trocar and the trocar set was inserted through the vaginal wall into the peritoneal cavity. The trocar was replaced by a shaft bearing the endoscope (5.5 mm 08 forward Hopkins endoscope, Storz, Vienna, Austria) and the transfer system. The site was illuminated by a fiberoptic cold light (250 W, Storz, Austria) and visualized with a camera (Telecam PAL-Endovision, Storz, Vienna, Austria) connected to a monitor.

The flushing system consisted of a 20 ml syringe connected to a perfusor tube (No. 08272514; Braun, Melsungen, Germany) and a metal tube (14 cm x 2.5 mm) with numerous lateral holes covered by a silicon tube. Thus, after the metal tube was inserted via the infundibulum into the ampulla, the careful management of the flushing pressure allowed the balanced adjustment of tubal sealing to avoid medium reflux. Oviducts

were flushed with 50 ml flushing medium (phosphate-buffered saline supplemented with 1% fetal calf serum). Flushing medium (approx. 50 ml) was forced through the uterotubal junction into the uterine horn and from there was collected via the uterus flushing catheter (CH15, Wörrlein, Ansbach, Germany) into an embryo filter (Emcon filter®, No. 04135; Immuno Systems Inc., Spring Valley, WI, USA).

Tubal transfer of 2- to 4-cell stage embryos

Tubal transfer was performed as described previously (Besenfelder and Brem, 1998) by transvaginal endoscopy. Briefly, after getting access into the peritoneal cavity (described above), the glass capillary of the transfer system, consisting of a 1 mL syringe attached to a perfusor tube (no. 08272514, Braun, Meslungen, Germany) and a glass capillary (Transferpettor Caps1, 100–200 mL, no. 701910, Brandt, Wertheim, Germany) which was crosier shaped at the tip, was filled with embryos (n=25-50) and the contents were transferred into the oviduct ipsilateral to the corpus luteum with as little as 100 µl medium.

Embryo recovery at day 7

The embryos were collected on day 7 after insemination. Access into the peritoneal cavity and endoscopic tubal flushing was performed in the same way as described above for flushing of 2- to 4-cell stage embryos. Following flushing of the oviduct towards the uterus, the uterus was flushed nonsurgically as described above.

RNA isolation and biotin labeled cRNA synthesis

Total RNA extraction from blastocysts was performed at two different time points during the whole experiment: (1) a total of six pools (three from superovulated and three from unstimulated heifers; each with 5 blastocysts) was used for array hybridization after amplification, and (2) a total of six pools (three from superovulated and three from unstimulated heifers; each with 5 blastocysts) was used for quantitative real-time PCR validation of array results. In all cases, total RNA isolation was performed using the PicoPure™ RNA Isolation Kit (Arcturs, Munich, Germany) according to the manufacturer's instruction. Genomic DNA contamination was removed by performing on-column DNA digestion using RNase-free DNase (Qiagen GmbH, Hilden, Germany). The concentration (ng/µl) and the quality of the extracted total RNA

were assessed using the Nanodrop 8000 spectrophotometer (Biotechnology GMBH, Erlangen, Germany). RNA integrity was evaluated using the Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip® Kit (Agilent Technologies Inc, CA, USA). Two rounds of RNA amplification were performed as described in the GeneChip® Expression Analysis Technical Manual using 100 ng of total RNA as a starting material. After cDNA synthesis in the first cycle, an unlabeled ribonucleotide mix was used in the first cycle of IVT amplification using MEGAscript® T7 Kit (Ambion, Inc., Austin, TX, USA). The unlabeled cRNA was then cleaned using GeneChip® IVT cRNA Cleanup Kit (Affymetrix, Inc., Santa Clara, CA, USA) and reverse transcribed in the first-strand cDNA synthesis step of the second cycle using random primers. Subsequently, the T7-Oligo (dT) Promoter Primer was used in the second-strand cDNA synthesis to generate double-stranded cDNA template containing T7 promoter sequences. The resulting double-stranded cDNA was then amplified and labelled using a biotinylated nucleotide analog/ribonucleotide mix in the second IVT reaction using GeneChip IVT Labeling Kit (Affymetrix, Inc., Santa Clara, CA, USA). The biotin labelled cRNA was fragmented and analyzed in the Bioanalyzer. The success of fragmentation was evaluated by the RNA peaks in the Bioanalyzer.

Affymetrix array hybridization and scanning

A Bovine Genome GeneChip 100 Format Array was used for hybridization. For this, a hybridization cocktail consisting of fragmented and labelled cRNA (15 µg), control oligonucleotide B2 (3 nM), 20X eukaryotic hybridization controls (bioB, bioC, bioD, cre), 2X hybridization mix, dimethylsulphoxide and RNAse-free water was mixed to a final volume of 200 µl and heated to 99°C for 5 min followed by warming at 45°C for 5 min. The samples were then hybridized to the Affymetrix GeneChip Bovine Genome Array consisting of 24128 probesets. Hybridization was performed for 16 h. For each group, three biotin-labelled cRNA hybridizations were performed. The arrays were washed and stained using the Fluidics Station 450/250 and scanned using the GeneChip® scanner 3000 integrated with Affymetrix® Microarray Suitesoftware.

Affymetrix data analysis

The microarray data normalization and background correction were performed using Guanine Cytosine Robust Multi-Array Analysis as described previously (Vardhanabhuti

et al. 2006). The quality of the arrays after hybridization was assessed by the absent and present calls of the control probesets. Differentially expressed genes were obtained using Linear Models for Microarray Data Analysis (Smyth 2005). The differentially regulated genes were selected based on $p < 0.05$, fold changes > 2 and false discovery rate (FDR) ≤ 0.3. The raw data have been submitted to Gene Expression Omnibus under series GSE21030.

Pathways and networks analysis

A list of the differentially expressed genes (DEGs) was uploaded into the Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, www.ingenuity.com) to identify relationships between the genes of interest and to uncover common processes and pathways. Networks of the genes were then algorithmically generated based on their connectivity. The ‘‘Functional Analysis’’ tool of the IPA software was used to identify the biological functions that were most significant to the data set. Canonical pathway analysis was also utilized to identify the pathways from the IPA library of canonical pathways that were most significant to the data set. Fishers exact test was used to calculate a *p*-value determining the probability that each biological function or canonical pathway assigned to the data set was because of chance alone. In addition, the significance of the association between the data set and the canonical pathway was calculated as the ratio of the number of genes from the data set that mapped to the pathway divided by the total number of genes that mapped to the canonical pathway

Validation of results using quantitative real-time PCR

Sequence specific primers (Table 1) were designed using Primer Express v 2.0 (Applied Biosystems, Foster City, CA, USA). For each primer a serial dilution of 10^1-10^9 copy number of molecules was prepared from the plasmid DNA. Quantitative real-time PCR was performed using cDNA samples from three independent biological replicates of each blastocyst group in addition to the samples used for array analysis. The cDNA synthesized from blastocyst samples was subjected to real-time PCR using GAPDH to test for any variation in the expression of this internal control gene. After confirming that there were no differences in the relative abundance of GAPDH between samples, all transcripts were quantified using independent real-time PCR runs. The PCR was performed in a 20 µl reaction volume containing iTaq SYBR Green Supermix

with ROX (Bio-Rad laboratories, Munich, Germany), the cDNA samples, the specific forward and reverse primer in ABI PRISM® 7000 sequence detection system instrument (Applied Biosystems). The thermal cycling parameter was set as 95 °C for 3 min, 40 cycles of 15 sec at 95 °C and 45 sec at 60 °C. Final quantitative analysis was carried out using the relative standard curve method and results were reported as the relative expression after normalization of the transcript amount relative to the endogenous control.

Statistical analysis

Data were analysed using the Statistical Analysis System (SAS) version 8.0 (SAS Institute Inc., Cary, NC, USA) software package. Developmental rates of embryos were analysed by χ^2 -test. The relative expression data were analysed using General Linear Model of SAS. Differences in mean values were tested using analysis of variance followed by a multiple pairwise comparison using *t*-test. Differences of $P \leq 0.05$ were considered to be significant.

Results

Embryo recovery and development

In superovulated heifers (n=10), the overall embryo recovery rate following non-surgical flushing on day 7 was 78% [calculated based on number of corpora lutea (CL)] with a mean of 14.5 structures recovered per donor. Of these flushed embryos, 68.3% had developed to the morula or blastocyst stage (Table 2). Heifers (n=9) flushed at day 2 delivered a total of 177 embryos; 164 of these embryos were transferred by endoscopy to the oviducts of 4 synchronized unstimulated recipients, while 13 embryos were discarded owing to retarded development. Following nonsurgical flushing on day 7, 146 embryos (89%) were recovered of which 76 (52.1%) were of transferable quality. Based on the number of heifers which had been superovulated initially, the mean embryo number per heifer was 16.2. Based on the estimated total number of CL (174) of the superovulated heifers (n=9) the recovery rate was 84%. The percentage of embryos which developed to the morula or blastocyst stage was higher ($p<0.05$) when cultured in superovulated heifers compared to unstimulated heifers (68.3% versus 52.1%). However, significantly more embryos developed to the blastocyst stage in the unstimulated heifers compared to those developing in superovulated heifers (33.6%

versus 22.1%). The developmental rate of the embryos (defined as Blastocyst : Morula ratio) was significantly higher ($P < 0.05$) in unstimulated heifers compared to superovulated heifers (1.81 versus 0.48) (Table 2).

Circulating progesterone profile

The progesterone profiles of the superovulated and unstimulated heifers are shown in Figure 2. The mean progesterone concentration from day 1 to day 6 was 11.37 ± 1.3 and 0.62 ± 0.11 ng/ml in the superovulated and unstimulated heifers, respectively. The progesterone concentrations diverged significantly from day 1 onwards between the two groups ($P < 0.01$).

Blastocyst transcriptome profile

The microarray data analysis revealed a total of 454 DEGs with $p < 0.05$, fold change ≥ 2 and FDR ≤ 0.3 between the two groups of blastocysts (Supplementary Table S1). From these, 429 genes were highly abundant in blastocysts derived from superovulated heifers and 25 were more abundant in blastocysts derived from unstimulated recipients. The overall expression pattern of the differentially regulated genes in all hybridizations and the hierarchical clustering are indicated in Figure 3a. The most highly abundant genes (with fold change ≥ 8) in the blastocysts derived from superovulated heifers included *HSPA14, ZNF238, PTGES3, HSPE1, IFT122, RPS17, CPSF3, POLR2F, SLC30A5* and *TMEM184B*. Blastocysts derived from unstimulated heifers were enriched with genes such as *CLGN, FUT1, MGC* and *CHMP1B* (Fig. 3b).

Biological functions, canonical pathways and gene networks identified

Of the 454 DEGs, 333 could be assigned to a specific functional group based on the information in the IPA. A high proportion of the DEGs (40%) which were enriched in the blastocysts derived from superovulated heifers fell into functional categories related to metabolic processes including carbohydrate, lipid, nucleic acid, amino acid, vitamin and mineral metabolism. Other functional categories, including protein synthesis, RNA post-transcriptional modification, gene expression, cell-to-cell signalling, energy production and molecular transport were also overrepresented in the same blastocyst group. A graphical representation of this functional classification of the genes overexpressed in blastocysts derived from superovulated heifers is shown in Figure 4, in

which the 13 functional groups with the lowest *p*-values ($P \leq 0.05$) are highlighted. The genes classified into each of these groups are listed in Table 3. In addition, the IPA analysis assigned 57 of the DEGs to 5 different canonical pathways. The five selected pathways had ratios (proportion of DEGs involved in the pathway out of the total genes of this pathway) ranging from 5% to 16% and *p*-values lower than 0.05 (Figure 5). The genes assigned to these pathways are presented in Table 4. Interestingly, the oxidative phosphorylation pathway was the dominant pathway and all DEGs (26 genes) involved in this pathway were highly abundant in the blastocysts derived from superovulated heifers compared to those derived from unstimulated heifers (Table 5). These genes can be classified into 4 main categories: NADH dehydrogenase, cytochrome c reductase, cytochrome c oxidase and ATP synthase, representing 4 out of 5 protein complexes involved in the electron transport and oxidative phosphorylation pathway (Figure 6).
Finally, the DEGs in our data set were mapped onto 17 networks, each containing 8–29 genes that shared direct or indirect relationships. The transcripts involved in each network and the top functions for each network are presented in Supplementary Table S2. An example of networks created from our data is shown in Figure 7. In this network the relationships between molecules overexpressed in the blastocysts derived from superovulated heifers and involved in cytochrome c reductase (Complex III in oxidative phosphorylation pathway) are represented by arrows. DEGs involved in the four protein complexes of oxidative phosphorylation pathway were mapped in four different networks (Networks 2, 7, 12 and 14; Supplementary Table S2).

Validation of microarray results

Quantitative real-time PCR validation on an independent group of blastocysts in addition to that used for the microarray analysis confirmed the expression profile of 79% (11/14) of the selected genes (Figure 8). The expression of 9 up-regulated genes (*MDH2*, *HSPE1*, *COX7B*, *ALDH7A1*, *POMP*, *ATPIF1*, *HSPA14*, *COX5A* and *CDC2*) and 2 down-regulated genes (*PNRC2* and *CLGN*) was found to be significantly different between blastocysts derived from superovulated heifers and those derived from unstimulated heifers. The expression of *TEMTM* was not different between the two groups and the expression of *KRT18* and *CPSF3* showed a different pattern than the microarray results with no significant differences.

Discussion

The majority of spontaneous human conceptions do not result in a live birth; the maximal chance of achieving successful implantation is ~40% per cycle, and this rate declines with age (Ferrara et al. 2002, Achard et al. 2005). Similarly, in cattle despite high fertilization rates following artificial insemination (>90%), calving rates to a single insemination are approximately 55-60% indicating embryonic mortality of about 30-40%. The majority of this loss is believed to occur prior to maternal recognition of pregnancy at Day 16 (Diskin et al. 2006, Diskin and Morris 2008) and is likely to the result of a combination of poor embryo quality and suboptimal reproductive tract environment (Lonergan 2009).

We have previously demonstrated that the postfertilization culture environment of the bovine embryos can significantly alter the quality of the resulting blastocysts, assessed in terms of morphology, cryotolerance and the expression of key candidate genes (Lonergan et al. 2003, Rizos et al. 2002). In particular, culture of in vitro-derived bovine zygotes in vivo in the sheep (Enright et al. 2000, Rizos et al. 2002) or bovine (Tesfaye et al. 2007) oviduct significantly improves the quality of the resulting blastocysts.

Under normal conditions, the oviduct provides the ideal environment for sperm transportation and final capacitation, mature oocyte transportation and fertilization, and supports the development of early mammalian embryos. The modification of oviductal conditions and/or composition of oviduct fluid caused by ovarian steroids subsequently affects the embryonic developmental competence during these critical processes (Murray et al. 1995). Thus, understanding the molecular changes that occur in preimplantation embryos because of changes in the female reproductive tract environment is fundamental to improving pregnancy rates using ART.

In humans, the luteal phase of all stimulated IVF cycles is abnormal (see review by Fatemi 2009). The supra-physiological concentrations of steroids associated with the development of multiple dominant follicles during the early luteal phase seems to be the main cause of the so-called luteal phase defect in stimulated cycles in women undergoing IVF, and this in turn directly inhibits LH release via negative feedback actions at the hypothalamic–pituitary axis level (Fauser and Devroey 2003). In addition, recent reports have suggested that the neonatal outcome of singletons born after embryo cryopreservation was better than those born after fresh embryo transfer (Pinborg et al.

2010, Veleva et al. 2009, Shih et al. 2008). Although the reason for the better outcome after cryopreservation is not known, adverse effects of hormone stimulation in fresh cycles is one of the mechanisms which has been put forward (reviewed by Wennerholm et al. 2009).

Elevated concentrations of circulating progesterone in the immediate post-conception period have been associated with an advancement of conceptus elongation (Carter et al. 2008, Garrett et al. 1988, Satterfield et al. 2006), an associated increase in interferon-tau production and higher pregnancy rates in cattle (Inskeep 2004, McNeill et al. 2006, Stronge et al. 2005) and sheep (Ashworth et al. 1989, Satterfield et al. 2006). This effect is likely a result of downstream effects of well described progesterone-induced changes in gene expression in the tissues of the uterus (Bauersachs et al. 2006, Forde et al. 2009, Satterfield et al. 2006) resulting in changes in composition of the histotroph to which the developing embryo is exposed. Our previous data would suggest that elevated progesterone does not influence the proportion of oocytes reaching the blastocyst stage (Carter et al. 2008, Clemente et al. 2009). Furthermore, we have more recently shown that elevating progesterone concentrations in vivo, within physiological ranges, by inserting an intravaginal progesterone-releasing device on day 3 does not alter blastocyst developmental rates but does lead to subtle changes in blastocyst gene expression (Carter et al. 2010). Here we build on these observations and describe significant changes in the global transcriptome of bovine blastocysts, induced by the supra-physiological progesterone concentrations associated with superovulation.

The potential implications of circulating progesterone on the composition of oviduct and uterine fluid are interesting but data on the composition of these fluids are sparse. Hugentobler et al. (2007, 2008) described the concentration of ions (Na^+, K^+, Ca^+, Mg^+, Cl^-, PO_4^{--}, SO_4), eighteen amino acids and energy substrates (glucose, lactate, pyruvate) in oviduct, uterine fluid and blood throughout the oestrous cycle in beef heifers. More recently, the same group (Hugentobler et al. 2010) characterized the effects of changes in systemic progesterone on amino acid, ion and energy substrate composition of oviduct and uterine fluids on day 3 and day 6, respectively, of the oestrous cycle. Infusion of progesterone had no effect on oviduct fluid secretion rate, however, uterine fluid secretion rate was lowered. Progesterone increased uterine glucose, decreased oviduct sulphate and, to a lesser extent, oviduct sodium but had no effect on any of the ions measured in the uterus. The most marked effect of progesterone was on oviductal

amino acid concentrations with a 2-fold increase in glycine, while in the uterus only valine was increased. These data provide evidence of progesterone regulation of oviduct amino acid concentrations in cattle; while the effect of superovulation on oviduct fluid composition has not, to our knowledge, been reported, such changes may partly explain the differences in embryo development and gene expression observed in the current study.

The number of embryos in the oviducts of unstimulated and stimulated animals in this study was different. This is an unavoidable consequence of the inherent individual variability in response associated with superstimulation treatments. While, in theory, this may have affected development, our previous studies in sheep (Enright et al. 2000, Rizos et al. 2002) and cattle (Tesfaye et al. 2007, Rizos et al. 2010) strongly suggest that there is a wide range in the number of embryos that can be successfully developed in the oviduct (up to 100 embryos) without a deleterious effect on blastocyst development. The greater proportion of morula than blastocysts flushed from the superovulated heifers at day 7 compared to unstimulated heifers, most likely results from a negative effect of hormonal stimulation on embryo developmental rate. In support of this fact several studies have shown a delay in embryo developmental rate under superovulation conditions in mice (Ertzeid and Storeng 2001, Van der Auwera and D'Hooghe 2001, Van der Auwera et al. 1999). Likewise, embryos from superovulated hamsters had significantly reduced mean cell numbers and lower viability than controls (McKiernan and Bavister 1998). The reasons for the developmental retardation, loss of developmental competence and low quality of embryos from superovulated donors are still unknown.

Understanding changes in the molecular profile of embryos developing in different oviductal/uterine environments could help to clarify the reasons for the developmental retardation and loss of developmental competence of embryos under superovulation conditions. To our knowledge, this is the first study to investigate differences in gene expression between bovine blastocysts produced in vivo in superovulated versus unstimulated conditions. The ‘quiet embryo hypothesis’ put forward by Leese (2002) proposed that viable embryos have a ‘quieter’ metabolism than those with less viability. This quiet metabolism represents the basal turnover required for normal cellular and developmental processes. This quietness can be lost in response to environmental stress (Leese et al. 2007, 2008) in which less viable embryos have more molecular/cellular

damage or are less well equipped at the transcriptome and proteome levels to cope with damage present. Damaged cells may carry out repair and/or resort to rescue strategies or undergo apoptosis with the consumption of a greater quantity of nutrients, reflected as a more 'active' metabolism and consequently cells will expend energy to repair damage as part of normal 'housekeeping' processes. In agreement with this hypothesis we found that the majority of the DEGs were upregulated in blastocysts which developed under superovulation conditions. Functional grouping of the DEGs using IPA indicated a number of distinct differences between the two groups of blastocysts (Figure 4). A higher proportion of genes which were over-expressed in the blastocysts derived from superovulated heifers were involved in metabolic processes, including cellular metabolic process, macromolecule metabolic process, protein metabolic process and RNA metabolic process. Our findings also reveal progressive up-regulation of different genes involved in protein synthesis and cellular growth and proliferation processes.

The increase in metabolic processes as the preimplantation embryo develops to more advanced stages has been associated with the increase in energy demand for the initiation of the processes of compaction and blastocyst formation (Harvey et al. 2004, Thompson et al. 1996). Like most mammalian cells, preimplantation embryos derive ATP predominantly by oxidative phosphorylation, initially from pyruvate, lactate and amino acids (Leese 1995). Based on our data, oxidative phosphorylation was the main pathway and all the DEGs which mapped to this pathway were over-expressed in the blastocysts derived from superovulated heifers (Table 5). These genes can be classified into 4 main categories representing 4 out of 5 protein complexes involved in the pathway: NADH dehydrogenase, cytochrome c reductase and cytochrome c oxidase, which are members of electron transport chain complexes, and ATP synthase which is the enzyme complex that carries out the oxidative phosphorylation reaction (Fig. 6). Bovine pre-elongation embryos are highly dependent on oxidative phosphorylation as the primary energy production pathway (ATP generating pathway): this is particularly true during pre-compaction development, during which it is estimated that approximately 90% of all ATP is derived from oxidation (Thompson et al. 1996). During compaction and blastulation, the demand for ATP increases during an increase in protein synthesis (Thompson et al. 1998). The increased demand for ATP causes increases in consumption of the major substrates, including oxygen, pyruvate and glucose (Thompson et al. 1996). There is evidence that the O_2 tension of the

reproductive tract decreases as the embryo passes from the oviduct to the uterine cavity (Fischer and Bavister 1993) and subsequently there is a shift in the metabolic pathway preference for embryonic ATP production from oxidative phosphorylation to glycolysis, to correspond with development within the uterus, an environment with limited O_2 availability. This shift in metabolic pathway has been reported in human (Gott et al. 1990) and rat (Brison and Leese 1991) embryos. Partial down-regulation of mitochondrial ATP production via oxidative phosphorylation during the compaction and blastulation stages improves in vitro development of bovine embryos (Thompson et al. 2000). This may indicate that bovine embryos produced in vivo tend to shift the metabolic pathway, as in human and rat embryos, during compaction and blastulation stages in response to the low O_2 tension in the reproductive tract. However, under the abnormal endocrine environment associated with superovulation, as reported in this study, the oxidative phosphorylation pathway was the main metabolic pathway in blastocyst stage embryos. This supports the notion that embryos developing in a superovulated environment need more ATP to reach the blastocyst stage and any shift in metabolic pathway will not provide the required energy.

Protein synthesis processes, including transcription and translation, appear to be one of the main biological processes which are over-expressed in the blastocysts derived from superovulated heifers. One of the main regulatory molecules which is derived from ATP and plays an important role in intracellular signal transduction and the induction of transcription of many eukaryotic genes is cAMP (Roesler et al. 1988). The downstream actions of cAMP are dependent upon its binding to cAMP-dependent protein kinase A (*PKA*) which mediates the intracellular actions of cAMP via phosphorylation of specific target proteins (Büchler et al. 1988). In humans and mice, there are four regulatory subunit genes: *PRKAR1A, PRKAR1B, PRKAR2A*, and *PRKAR2B*. Of the four regulatory subunits, *PRKAR1A* was found to be the most highly and ubiquitously expressed (Amieux et al. 2002). In our study, we found that *PRKAR1A* was highly abundant in the blastocysts derived from superovulated heifers. Moreover, one of the networks generated by IPA showed the involvement of FSH and LH in the expression of *PRKAR1A* (Fig. 7). In the same network the protein-protein interaction showed a direct relationship between *PRKAR1A* and *UQCRB*. *UQCRB,* which is member of the ubiquinol-cytochrome-c reductase complex in the oxidative phosphorylation pathway. This may explain the indirect relationship between superovulation hormone treatment

and the higher abundance of genes involved in oxidative phosphorylation pathway. A significant elevation of two heat shock protein transcripts (*HSPA14* and *HSPE1)* was noted in blastocysts produced under superovulation conditions. Heat shock proteins play a protective role within a cell, also exerting antiapoptotic actions (Betts and King 2001), and they also act as molecular chaperones and prevent from protein degradation (Levy 2001). As expression of the heat shock proteins is induced by stress factors, the transcript level reflects both cell response and stress intensity (Neuer et al. 2000). The higher expression of *HSPA14* and *HSPE1* in blastocysts derived from superovulated heifers may indicate that the oviductal environment under superovulation conditions provided some stress factors to the preimplantation embryos. This stress is may be related to an increase in expression of genes related to metabolic processes, protein synthesis and ATP production to face the developmental delay which is abnormal when compared to embryos produced under normal conditions. In addition, as byproducts of oxidative phosphorylation, reactive oxygen species resulting from in situ leakage from the mitochondrial electron transport chain are stress factors which can damage cellular components including nucleic acids, proteins and membranes (Burton et al. 2003).

In conclusion, the present study demonstrated the differences in transcriptome profile of the blastocysts cultured under different oviductal/uterine conditions, either in superovulated heifers until day 7 or in unstimulated heifers from the 2- to 4-cell stage onwards. Embryo development under the supra-physiological progesterone concentrations which are associated with superovulation was slower compared to that in the unstimulated reproductive tract. Microarray analysis revealed that embryos developing to the blastocyst stage in superovulated heifers showed significant up-regulation for most of the DEGs which are involved in metabolic processes, protein synthesis and energy production via the oxidative phosphorylation pathway.

Author's roles

AG: responsible for implementation of the experiment and write up of the draft paper; MH, UB and VH: participated in the design and implementation of the experiment by performing nonsurgical endoscopic transfer of embryos in the oviduct; FR: was responsible for handling of embryos after flushing, NG, DSW and AB contributed in array hybridization, scanning and data analysis; UC: participated in molecular analysis

part; DT, PL and KS: contributed to the design and supervision of the experiment and critical reading of the manuscript.

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Table 1 Details of primers used for real-time PCR quantitative analysis.

Gene	Accession number	Primer sequences	Annealing temperature (°C)	Product size (bp)
HSPA14	NM_001046388	F: 5´- GGGAGTTGATGAATCAGGAG -3´ R: 5´- AATCTTGGAGCACAACCTGT -3´	52	191
KRT18	XM_582930	F: 5´- CCTGGACTCCATGAGAAATC -3´ R: 5´- CTTGACCTTGACATTCAGCA -3´	52	190
CPSF3	NM_174284	F: 5´- AATGCACGTTTACAGCAAGA -3´ R: 5´- TCTTCACTGCCTTCTTCACA -3´	50	165
HSPE1	NM_174346	F: 5´- GTGAGAGCAGGGTACGAACT -3´ R: 5´- GAATCTCTCCACCCTTTCCT -3´	54	216
TMEM184B	NM_001081522	F: 5´- CCTTCCTAGGATGTGTAGCC -3´ R: 5´- ACTCATGGCAGTCATACACG -3´	54	164
COX7B	NM_175795	F: 5´- TCGTCTGAGAGTTCAAAGCA -3´ R: 5´- ATGGGTTCCACTCTATTCCA -3´	52	179
COX5A	AY528254	F: 5´- AGACGAGGAGTTTGATGCTC -3´ R: 5´- GAGGTCCTGCTTTGTCCTTA -3´	52	212
ATPIF1	NM_175816	F: 5´- AAGAAATTGAGCGGCATAAG -3´ R: 5´- GGAAGCACACAGGTTGATTT -3´	50	154
ALDH7A1	NM_001045969	F: 5´- AAGGAGGTTCTCCATCTTCC -3´ R: 5´- TCAGAGAAAACTTCTGGAGTCA -3´	52	158
POMP	NM_001034376	F: 5´- AAATTTCCAGCTCAACCAAG -3´ R: 5´- TCATCGTTACCCCTCAAAAT -3´	50	171
CDC2	NM_174016	F: 5´- AGCATCCCATGTCAAAAACT -3´ R: 5´- TTGACACAACACAGGGAAAC -3´	50	178
MDH2	NM_001013587	F: 5´- CCCTTTCGAAGAGAAGATGA -3´ R: 5´- AGGGGTGGTTCGTTAATACA -3´	52	218
PNRC2	NM_001076957	F: 5´- TTTCGACAGCTTGTGTAACC -3´ R: 5´- ACTGGGATGGCTGTTGTAAT -3´	52	176
CLGN	NM_001034205	F: 5´- AGATGGGCCAATAAAGTCAG -3´ R: 5´- AGTAGAAAAGGGGTGTGCAG -3´	52	151
GAPDH	BC102589	F: 5´-ACCCAGAAGACTGTGGATGG-3´ R: 5´-ACGCCTGCTTCACCACCTTC-3´	60	247

Table 2 Overall and structures recovery rate between superovulated and unstimulated heifers.

	Estimated no. CLs	No. embryo transferred	Structures recovered	Recovery rate	Total transferable N (%)	Morula N (%)	Blastocyst N (%)	Blastocyst/Morula Ratio
Superovulated, flushed Day 7(n=10)	187	-	145	0.78*	99 (68.3)[a]	67 (46.2)[a]	32 (22.1)[a]	0.48[a]
Superovulated, flushed Day 2 (n=9)	177	-	177	1.00*	-	-	-	
Unstimulated, flushed Day 7 (n=4)	-	164	146	0.89**	76 (52.1)[b]	27 (18.5)[b]	49 (33.6)[b]	1.81[b]

- Not applicable

* Recovery rate = number of structure(s) recovered/number of CL x 100

** Recovery rate = number of structure(s) recovered/ transferred embryos x 100

[a,b] values with different superscripts within columns differ significantly ($p < 0.05$)

Table 3 The functional categories and the corresponding genes that were over-expressed in blastocysts derived from superovulated heifers

Functional category	*p*-value[a]	Transcripts
Protein Synthesis	2.75E-10 - 3.66E-02	*RPL24, FN1, RPL27A, PDPK1, RPL31, RPL14, EIF4E, HNRNPK, IGF2BP1, NCL, RPL6, PEPD, RPS3A, EIF5, RPL19, ERRFI1, RPL13, RPL3, RPS17, EIF3F, RPL34, RPS19, THOP1, NDUFA13, RPS12, LAMP2, RPS10, RPS5, RPL39, RPL28, SNRNP70, AARS, RPL38, UBE2E1, UBE2C, HSPB1, SSB*
RNA Post-Transcriptional Modification	2.46E-08 - 4.94E-02	*LUC7L3, RPS17, SYNCRIP, RPS28, NOP56, RPS19, RPL14, WDR43, HNRNPD, IMP4, CDC2, ARL6IP4, RPS7, CPSF6, WDR75, RPS16, SNRNP70, HNRNPUL1, CPSF3, AARS, LSM4, HNRNPAB, SNRNP40, SSB*
Gene Expression	1.33E-03 - 3.66E-02	*AKAP12, FN1, LGALS3, RAC1, PDPK1, KLF13, KLF4, EIF4E, HNRNPK, CDC2, NBN, YY1, SSBP1, SREBF1, EIF5, DAB2, PUF60, CTNNB1, HNRNPAB, LGALS1, SSB*
Carbohydrate Metabolism	1.74E-03 - 3.66E-02	*AP2M1, FN1, KIT, PSAP, ANXA2, NANS, CTNNB1, LGALS1*
Lipid Metabolism	1.74E-03 - 3.77E-02	*GSTP1, AP2M1, ACSL3, RAB4A, FN1, ANXA2, ADH5, EBP, INSIG2, PTGES3, SREBF1, KIT, PSAP, PTGS2, SFN, PDHB, LGALS1, PRDX2*
Cell-To-Cell Signaling and Interaction	2.36E-03 - 4.46E-02	*AP2M1, FN1, C3, LGALS3, RAC1, LGALS4, KLF4, LRPAP1, NCL, KRT8, KRT18, EPCAM, DAB2, DSG2*
DNA Replication, Recombination, and Repair	3.57E-03 - 3.66E-02	*MCM6, GSTP1, LMNA, EIF4E, CDC2, MLH1, FUS, NBN, NCL, ATPIF1, YY1, TOP2A, HNRNPAB*
Cellular Growth and Proliferation	6.42E-03 - 4.05E-02	*AKAP12, LGALS3, C3, FN1, PA2G4, RPS19, LGALS4, GATA2, KLF4, CTSL2, NCL, EPCAM, KIT, PTGS2, SFN, CTNNB1, SIRT1, UBE2C*
Energy Production	7.61E-03 - 1.39E-02	*ATPIF1, ATP5O, ATP5B, TOP2A, ATP5L, MLH1, CDC2, ATP5I*
Nucleic Acid Metabolism	7.61E-03 - 1.39E-02	*ATPIF1, ATP5O, ATP5B, TOP2A, ATP5L, MLH1, CDC2, ATP5I*
Vitamin and Mineral Metabolism	1.24E-02 - 3.66E-02	*ADH5, EBP, INSIG2*
Molecular Transport	2.25E-02 - 3.66E-02	*UROD, FN1, RAB4A, PRDX1, PRDX5, PSAP, PTGS2, LGALS1, PRDX2*
Amino Acid Metabolism	3.66E-02 - 3.66E-02	*ADH5, ANXA2*

[a]Numbers in the *p*-value column showed a range of *p*-values for the genes from each category

Table 4 The canonical pathways from the Ingenuity Pathway Analysis Knowledge Base that involve transcripts over-expressed in the blastocyst derived from superovulated heifers

Pathway	**-Log (P-value)**	**Ratio**	**Transcripts**
Oxidative Phosphorylation	15.17	0.157	*ATP5B, ATP5G3, ATP5H, ATP5I, ATP5L, ATP5O, ATP6VOD2, COX4I1, COX5A, COX7B, CYC1, NDUFA1, NDUFA5, NDUFA6, NDUFA12, NDUFA13, NDUFA9, NDUFB2, NDUFB8, NDUFB10, NDUFC2, NDUFS2, NDUFV1, UCRC, UQCR, UQCRB*
Ubiquinone Biosynthesis	7.23	0.109	*FTSJ2, NDUFA1, NDUFA5, NDUFA6, NDUFA12, NDUFA13, NDUFA9, NDUFB2, NDUFB8, NDUFB10, NDUFC2, NDUFS2, NDUFV1*
Methan Metabolism	2.07	0.045	*PRDX1, PRDX2, PRDX5*
Integrin-linked kinase Signalling	2.03	0.059	*ACTB, ACTN1, CTNNB1, FN1, GSK3A, KRT18, MYL6, PDPK1, PPP2R5E, PTGS2, RAC1*
Pentose Phosphate Pathway	1.85	0.045	*PDHB, PRPSAP2, RBKS, TKT*

Table 5 Gene symbol, Affymetrix probe ID, gene description and fold change of transcripts involved in oxidative phosphorylation pathway and up-regulated with ≥2 fold change in blastocysts derived from superovulated heifers.

Gene Symbol	Affy Probe ID	Gene description	Fold change
NDUFA6	Bt.63.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	7.5
COX7B	Bt.400.1.S1_at	cytochrome c oxidase subunit VIIb	7.3
COX5A	Bt.9826.1.S1_at	COX5A protein	7.2
ATP5B	Bt.4431.1.S1_a_at	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	6.2
UCRC	Bt.1819.1.S1_at	ubiquinol-cytochrome c reductase complex 7.2 kDa protein	5.6
UQCR	Bt.5040.1.S1_at	ubiquinol-cytochrome c reductase (6.4kD) subunit	5.7
ATP5G3	Bt.8047.1.S1_at	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3 (subunit 9)	5.3
NDUFA12	Bt.298.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12	4.4
COX4	Bt.16025.1.S1_at	cytochrome c oxydase subunit 4	4.2
NDUFA1	Bt.69.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa	4.0
NDUFA5	Bt.4704.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa	3.5
ATP5I	Bt.378.1.S1_at	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit e	3.4
NDUFB2	Bt.60.1.S1_at	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8kDa	3.2
NDUFC2	Bt.21.1.S1_at	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5kDa	3.1
ATP5O	Bt.442.1.S1_at	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	3.0
ATP5L	Bt.2816.1.S1_at	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G	3.0
NDUFB8	Bt.5378.1.S1_at	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa	2.9
UQCRB	Bt.25083.1.A1_at	Ubiquinol-cytochrome c reductase binding protein	2.8
NDUFA13	Bt.8950.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	2.4
NDUFS2	Bt.4475.1.S1_at	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa	2.3
CYC1	Bt.1808.1.S1_at	cytochrome c-1	2.2
ATP6V0D2	Bt.11010.1.S1_at	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	2.2
NDUFV1	Bt.4072.1.S1_at	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	2.2
NDUFB10	Bt.70.1.S1_at	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	2.1
NDUFA9	Bt.5541.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9, 39kDa	2.1
ATP5H	Bt.5029.1.S1_at	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	2.1

Table S1 Gene symbol, Affymetrix probe ID, gene description and fold change of DEGs with ≥2 fold difference in blastocysts derived from superovulated heifers.

Gene Symbol	Affy Probe ID	Gene description	Fold change
IFT122	Bt.8829.1.S1_at	intraflagellar transport 122 homolog (Chlamydomonas)	28.1
ZNF238	Bt.15860.1.S1_at	zinc finger protein 238	20.8
HSPA14	Bt.19575.1.S1_at	heat shock 70kDa protein 14	16.6
KRT18	Bt.1745.1.S1_at	keratin 18	14.8
LOC517284	Bt.16595.1.A1_at	Similar to Protein piccolo (Aczonin)	13.6
CPSF3	Bt.5045.1.S1_at	cleavage and polyadenylation specific factor 3, 73kDa	12.2
PTGES3	Bt.7247.1.S1_at	prostaglandin E synthase 3 (cytosolic)	12.1
NAT11	Bt.15937.1.A1_at	N-acetyltransferase 11	11.1
AGPAT5	Bt.22170.2.A1_at	1-acylglycerol-3-phosphate O-acyltransferase 5 (lysophosphatidic acid acyltransferase, epsilon)	10.5
MKRN1	Bt.11395.1.A1_at	makorin ring finger protein 1	10.2
LOC533514	Bt.7150.2.S1_at	Similar to KIAA1289 protein	10.1
HSPE1	Bt.504.1.S1_at	heat shock 10kDa protein 1 (chaperonin 10)	9.7
HNRNPUL1	Bt.21035.2.S1_a_at	heterogeneous nuclear ribonucleoprotein U-like 1	9.5
JARID2	Bt.7008.1.S1_at	jumonji, AT rich interactive domain 2	9.5
CISD1	Bt.10154.1.S1_at	CDGSH iron sulfur domain 1	9.1
RPS17	Bt.3839.1.S1_at	ribosomal protein S17	9.0
MAPT	Bt.2289.1.S1_at	microtubule-associated protein tau	8.7
TMEM184B	Bt.10312.1.A1_at	transmembrane protein 184B	8.7
MYO1D	Bt.22679.1.S1_at	myosin ID	8.6
LOC540136	Bt.5542.1.A1_at	similar to Nucleosome assembly protein 1-like 1 (NAP-1-related protein) (hNRP)	8.6
GOLGA4	Bt.26662.1.S1_at	golgi autoantigen, golgin subfamily a, 4	8.4
POLR2F	Bt.11138.1.S1_at	polymerase (RNA) II (DNA directed) polypeptide F	8.3
SLC30A5	Bt.26811.1.S1_at	Solute carrier family 30 (zinc transporter), member 5	8.2
KIT	Bt.26445.1.A1_at	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	8.2
HSPA8	Bt.12309.1.S1_at	heat shock 70kDa protein 8	8.0
LOC100138102	Bt.9459.1.S1_at	similar to 3-phosphoinositide-dependent protein kinase 1 (hPDK1)	7.9
PAN3	Bt.1861.1.S1_at	PAN3 polyA specific ribonuclease subunit homolog (S. cerevisiae)	7.8
CCDC47	Bt.15698.2.A1_at	Coiled-coil domain containing 47	7.8
LOC784987	Bt.16464.1.A1_at	Hypothetical protein LOC784987	7.7
PSAP	Bt.5467.1.S2_at	prosaposin	7.5

Gene Symbol	Affy Probe ID	Gene description	Fold change
NDUFA6	Bt.63.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	7.5
COX7B	Bt.400.1.S1_at	cytochrome c oxidase subunit VIIb	7.3
CALM3	Bt.12896.1.S1_at	calmodulin 3 (phosphorylase kinase, delta)	7.3
LOC533514	Bt.7150.1.S1_at	Similar to KIAA1289 protein	7.2
HNRNPF	Bt.23314.1.A1_s_at	heterogeneous nuclear ribonucleoprotein F	7.2
COX5A	Bt.9826.1.S1_at	COX5A protein	7.2
RPL6	Bt.5348.1.S2_at	ribosomal protein L6	7.0
RAB11A	Bt.19522.1.S1_at	RAB11A, member RAS oncogene family	7.0
C18H16ORF61 /// LOC100138086	Bt.29655.1.S1_a_at	chromosome 16 open reading frame 61 ortholog /// similar to MGC137594 protein	6.9
RANBP1	Bt.8189.2.A1_at	RAN binding protein 1	6.8
ATPIF1	Bt.380.1.S1_at	ATPase inhibitory factor 1	6.7
ANKS1A	Bt.19167.1.A1_at	Ankyrin repeat and sterile alpha motif domain containing 1A	6.7
CLTA	Bt.1617.2.S1_a_at	clathrin, light chain (Lca)	6.6
HSD17B11	Bt.11993.1.S1_at	hydroxysteroid (17-beta) dehydrogenase 11	6.5
H11CXORF26	Bt.24704.1.S1_at	Chromosome X open reading frame 26 ortholog	6.5
RPL13A	Bt.1432.1.S1_a_at	ribosomal protein L13a	6.4
LMNA	Bt.28432.1.S1_at	lamin A/C	6.4
RPL38	Bt.7658.1.S1_at	ribosomal protein L38	6.4
RPS24	Bt.8015.1.S1_a_at	ribosomal protein S24	6.4
LOC518469	Bt.20788.1.A1_at	Similar to RP5-1022P6.2	6.3
VPS53	Bt.13360.1.S1_at	vacuolar protein sorting 53 homolog (S. cerevisiae)	6.3
USMG5	Bt.5571.1.S1_at	up-regulated during skeletal muscle growth 5 homolog (mouse)	6.3
PA2G4	Bt.4896.2.S1_at	proliferation-associated 2G4, 38kDa	6.3
CLIC1	Bt.23174.2.S1_at	chloride intracellular channel 1	6.3
ATP5B	Bt.4431.1.S1_a_at	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	6.2
TOPORS	Bt.15703.1.S1_at	topoisomerase I binding, arginine/serine-rich	6.2
SUZ12	Bt.24502.1.S1_at	suppressor of zeste 12 homolog (Drosophila)	6.1
MGC128036	Bt.9457.1.S1_at	similar to COX17 homolog, cytochrome c oxidase assembly protein	6.1
CROP	Bt.1595.1.S1_at	cisplatin resistance-associated overexpressed protein	6.1
UBA6	Bt.2174.2.A1_at	Ubiquitin-like modifier activating enzyme 6	6.1
RPL36A	Bt.9814.1.S1_at	ribosomal protein L36a /// similar to mCG7611	6.1
TOP2A	Bt.20277.1.S1_at	topoisomerase (DNA) II alpha 170kDa	6.0

Gene Symbol	Affy Probe ID	Gene description	Fold change
PRDX1	Bt.1823.1.S1_at	peroxiredoxin 1	6.0
CPT2	Bt.1065.1.S1_at	carnitine palmitoyltransferase II	5.9
C3	Bt.4209.2.S1_at	complement component 3	5.9
PPP2R5E	Bt.1549.2.S1_at	protein phosphatase 2, regulatory subunit B', epsilon isoform	5.7
CCDC72 /// MGC148581	Bt.6032.1.S1_s_at	coiled-coil domain containing 72 /// hypothetical LOC615251	5.7
RPL34	Bt.23548.1.S1_at	ribosomal protein L34	5.7
UQCR	Bt.5040.1.S1_at	ubiquinol-cytochrome c reductase (6.4kD) subunit	5.7
TKT	Bt.4750.1.S1_at	transketolase	5.7
UCRC	Bt.1819.1.S1_at	ubiquinol-cytochrome c reductase complex 7.2 kDa protein	5.6
RPL27	Bt.4721.1.S1_at	ribosomal protein L27	5.5
SAFB2	Bt.8079.1.S1_a_at	scaffold attachment factor B2	5.4
RPL27A	Bt.23343.2.S1_a_at	ribosomal protein L27a	5.3
ATP5G3	Bt.8047.1.S1_at	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3 (subunit 9)	5.3
HSPA4	Bt.22168.1.A1_at	heat shock 70kDa protein 4	5.3
RPS13	Bt.2623.1.S1_at	ribosomal protein S13	5.3
BSG	Bt.3791.1.S1_at	basigin	5.3
GSK3A	Bt.1147.1.S1_at	glycogen synthase kinase 3 alpha	5.3
MRPS31	Bt.13610.1.A1_at	mitochondrial ribosomal protein S31	5.2
ALDH7A1	Bt.22086.2.A1_at	aldehyde dehydrogenase 7 family, member A1	5.2
THOP1	Bt.21827.1.A1_s_at	thimet oligopeptidase 1	5.2
TACSTD1	Bt.9569.1.S1_at	tumor-associated calcium signal transducer 1	5.2
ACTN1	Bt.7700.1.S1_at	actinin, alpha 1	5.2
TMSB10	Bt.2798.1.S1_a_at	thymosin beta 10	5.1
POMP	Bt.6111.1.S1_at	proteasome maturation protein	5.1
RPS20	Bt.23001.1.S1_a_at	ribosomal protein S20	5.0
EBP	Bt.3786.1.S1_a_at	emopamil binding protein (sterol isomerase)	5.0
LOC505221	Bt.28640.1.A1_at	similar to BTB/POZ domain-containing protein KCTD3 (Renal carcinoma antigen NY-REN-45)	5.0
RPLP2	Bt.5346.1.S1_at	ribosomal protein, large, P2	4.9
KRT8	Bt.23608.1.S1_s_at	keratin 8	4.8
TNRC6C	Bt.19479.2.A1_a_at	trinucleotide repeat containing 6C	4.8
ERCC6L	Bt.7620.1.S1_at	excision repair cross-complementing rodent repair deficiency, complementation group 6-like	4.7
RPLP1	Bt.8125.1.S1_a_at	ribosomal protein, large, P1	4.7

Gene Symbol	Affy Probe ID	Gene description	Fold change
GATA2	Bt.6408.2.A1_at	GATA binding protein 2	4.7
FN1	Bt.23418.1.S1_at	fibronectin 1	4.7
BZW1	Bt.20692.1.A1_at	Basic leucine zipper and W2 domains 1	4.7
WDR43	Bt.6782.1.A1_at	WD repeat domain 43	4.7
SLC25A5	Bt.9559.1.S1_at	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	4.6
CKB	Bt.5084.1.S1_at	creatine kinase, brain	4.6
KIAA1715	Bt.26009.1.A1_at	KIAA1715	4.6
SSB	Bt.15513.1.S1_at	Sjogren syndrome antigen B (autoantigen La)	4.6
DAB2	Bt.3814.1.S1_at	Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	4.6
LASP1	Bt.7980.1.S1_at	LIM and SH3 protein 1 /// similar to LIM and SH3 protein 1	4.6
EZR	Bt.3583.1.S1_at	ezrin	4.5
CD99	Bt.13829.1.S1_at	CD99 molecule	4.5
SAPS1	Bt.21710.1.S1_at	SAPS domain family, member 1	4.5
NDUFA12	Bt.298.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12	4.4
DCP1B	Bt.24459.1.A1_at	DCP1 decapping enzyme homolog B (S. cerevisiae)	4.4
MRPL22	Bt.17490.1.S1_a_at	mitochondrial ribosomal protein L22	4.4
ZNF207	Bt.13679.1.S1_at	zinc finger protein 207	4.4
RPS10	Bt.3921.1.S1_at	ribosomal protein S10	4.4
LOC100141158	Bt.11588.1.A1_at	similar to ubiquitin specific protease 48	4.4
PPHLN1	Bt.27337.1.S1_at	Periphilin 1	4.4
RPS28	Bt.22772.1.S1_at	ribosomal protein S28	4.3
LOC654400	Bt.24023.1.A1_at	GATA-6	4.3
TAF9	Bt.1121.1.S1_at	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kDa	4.3
THOC4	Bt.1768.1.A1_at	THO complex 4	4.3
LOC613525	Bt.7358.1.S1_at	similar to Selenoprotein H	4.3
LOC616065	Bt.4283.1.S1_at	similar to 60S ribosomal protein L22 (Heparin binding protein HBp15)	4.2
RPS3A	Bt.4967.1.S1_at	ribosomal protein S3A	4.2
COX4	Bt.16025.1.S1_at	cytochrome c oxydase subunit 4	4.2
RPS21	Bt.12469.1.S1_at	ribosomal protein S21	4.2
HSPB1	Bt.4415.1.S1_at	heat shock 27kDa protein 1	4.2
NARS	Bt.6688.1.S1_at	asparaginyl-tRNA synthetase	4.2
SYNCRIP	Bt.23038.1.A1_at	Synaptotagmin binding, cytoplasmic RNA interacting protein	4.2
RPS12	Bt.1269.1.S1_a_at	ribosomal protein S12	4.1

Gene Symbol	Affy Probe ID	Gene description	Fold change
RPS5	Bt.1874.1.S1_at	ribosomal protein S5	4.1
FBXL11	Bt.9278.1.S1_at	F-box and leucine-rich repeat protein 11	4.1
EFHD2	Bt.15561.1.S1_at	EF-hand domain family, member D2	4.1
UQCRB	Bt.23361.1.S1_at	Ubiquinol-cytochrome c reductase binding protein	4.1
CDC2	Bt.2.1.S1_at	cell division cycle 2, G1 to S and G2 to M	4.1
NME2	Bt.1582.1.S1_at	non-metastatic cells 2, protein (NM23B) expressed in	4.1
ADH5	Bt.2561.1.S1_at	alcohol dehydrogenase 5 (class III), chi polypeptide	4.1
AHCYL2	Bt.15900.1.A1_at	S-adenosylhomocysteine hydrolase-like 2	4.1
TFRC	Bt.13834.1.S1_at	transferrin receptor (p90, CD71)	4.0
SLC11A2	Bt.8364.1.S1_at	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	4.0
DSTN	Bt.15705.1.S1_at	destrin (actin depolymerizing factor)	4.0
MDH2	Bt.7915.1.S1_at	malate dehydrogenase 2, NAD (mitochondrial)	4.0
FAM60A	Bt.15602.1.S1_at	family with sequence similarity 60, member A	4.0
NDUFA1	Bt.69.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa	4.0
ACTB	Bt.14186.1.S1_at	actin, beta	3.9
RPL14	Bt.15931.1.S1_at	ribosomal protein L14	3.9
DSG2	Bt.16441.1.A1_at	desmoglein 2	3.9
RBM26	Bt.14007.3.A1_at	RNA binding motif protein 26	3.8
UBE2E1	Bt.13684.1.S1_at	ubiquitin-conjugating enzyme E2E 1 (UBC4/5 homolog, yeast)	3.8
PTPN2	Bt.24652.1.A1_at	protein tyrosine phosphatase, non-receptor type 2	3.8
LGALS4	Bt.9082.1.S1_at	lectin, galactoside-binding, soluble, 4	3.8
LRPAP1	Bt.2982.1.S1_at	low density lipoprotein receptor-related protein associated protein 1	3.8
LOC504861	Bt.6372.1.A1_at	similar to cationic amino acid transporter 5	3.8
LOC532409	Bt.11711.1.A1_at	similar to high-mobility group box 3	3.8
RPL28	Bt.1226.1.S1_a_at	ribosomal protein L28	3.8
ANXA2	Bt.4314.1.S1_at	annexin A2	3.7
FCF1	Bt.20900.1.S1_at	FCF1 small subunit (SSU) processome component homolog (S. cerevisiae)	3.7
HNRNPD	Bt.3809.2.S1_a_at	heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)	3.7
TAF13	Bt.26935.1.A1_at	TAF13 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 18kDa	3.7
WDR57	Bt.20560.1.S1_at	WD repeat domain 57 (U5 snRNP specific)	3.7
PSMB4	Bt.4966.1.S1_at	proteasome (prosome, macropain) subunit, beta type, 4	3.7
C15H11orf46	Bt.24242.1.S1_at	chromosome 11 open reading frame 46 ortholog	3.7
LSM4	Bt.20499.1.S1_at	LSM4 homolog, U6 small nuclear RNA associated (S. cerevisiae)	3.7

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NOL5A	Bt.10277.2.S1_a_at	nucleolar protein 5A (56kDa with KKE/D repeat)	3.7
RPL36	Bt.14228.1.S1_at	ribosomal protein L36	3.7
RPL21	Bt.5211.1.S1_at	ribosomal protein L21	3.7
PRDX2	Bt.2689.1.S1_at	peroxiredoxin 2	3.7
OXA1L	Bt.1719.1.S1_at	oxidase (cytochrome c) assembly 1-like	3.6
RBM47	Bt.22438.1.S1_at	RNA binding motif protein 47	3.6
EIF4E	Bt.1148.1.S1_at	eukaryotic translation initiation factor 4E	3.6
ARS2	Bt.20029.1.S1_at	arsenate resistance protein 2	3.6
LGALS3	Bt.1416.1.S1_at	lectin, galactoside-binding, soluble, 3	3.6
RPL36A /// RPL36AL	Bt.3404.1.S1_at	ribosomal protein L36a /// similar to mCG7611 /// ribosomal protein L36a-like	3.6
PICALM	Bt.9107.1.S1_a_at	phosphatidylinositol binding clathrin assembly protein	3.6
MTBP	Bt.26326.1.A1_at	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) binding protein, 104kDa	3.6
HINT2	Bt.11196.1.S1_at	histidine triad nucleotide binding protein 2	3.6
NANS	Bt.3388.1.S1_at	N-acetylneuraminic acid synthase	3.6
NDUFA5	Bt.4704.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa	3.5
FUS	Bt.2474.1.S1_at	fusion (involved in t(12;16) in malignant liposarcoma)	3.5
H3F3A /// H3F3B	Bt.5388.1.S1_at	H3 histone, family 3A /// similar to Histone H3.3B CG8989-PA	3.5
UBE2O	Bt.24878.1.A1_at	ubiquitin-conjugating enzyme E2O	3.5
SR140	Bt.6832.1.S1_at	U2-associated SR140 protein	3.5
NCL	Bt.7403.1.S1_at	Nucleolin	3.5
HERC4	Bt.15687.1.S1_at	Hect domain and RLD 4	3.5
RPS7	Bt.7187.1.S1_at	ribosomal protein S7	3.5
RPS8	Bt.1034.1.S1_at	ribosomal protein S8	3.5
PTRH2	Bt.15787.1.S1_at	peptidyl-tRNA hydrolase 2	3.5
DC2	Bt.3114.1.A1_at	DC2 protein	3.5
MGC165939	Bt.17889.1.A1_at	Hypothetical LOC517231	3.4
ATP5I	Bt.378.1.S1_at	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit e	3.4
SFRS9	Bt.23552.1.S1_at	splicing factor, arginine/serine-rich 9	3.4
TMED7	Bt.12099.1.A1_at	Transmembrane emp24 protein transport domain containing 7	3.4
SCPEP1	Bt.6357.1.S1_at	serine carboxypeptidase 1	3.4
EIF3C	Bt.2516.1.S1_at	eukaryotic translation initiation factor 3, subunit C	3.4
C10H14orf179	Bt.18569.1.A1_at	chromosome 14 open reading frame 179 ortholog	3.4
ABCB6	Bt.14310.1.S1_a_at	ATP-binding cassette, sub-family B (MDR/TAP), member 6	3.3

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WDR82	Bt.24362.1.A1_at	WD repeat domain 82	3.3
RAB11FIP4	Bt.5300.1.S1_at	RAB11 family interacting protein 4 (class II)	3.3
GLTSCR2	Bt.1429.3.A1_s_at	glioma tumor suppressor candidate region gene 2	3.3
Rpl3	Bt.3616.1.S1_at	ribosomal protein L3	3.3
CASK	Bt.6686.1.S1_at	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	3.3
LOC535156	Bt.9425.3.A1_at	Similar to presenilin-like protein 4	3.2
WDR75	Bt.28680.1.S1_at	WD repeat domain 75	3.2
SREBF1	Bt.15667.1.S1_at	Sterol regulatory element binding transcription factor 1	3.2
ANLN	Bt.4995.1.A1_at	anillin, actin binding protein	3.2
ATP5E	Bt.5510.1.S1_at	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	3.2
RPL13	Bt.23317.1.S1_at	ribosomal protein L13	3.2
OSTF1	Bt.2888.1.S1_at	osteoclast stimulating factor 1	3.2
HNRNPAB	Bt.3928.1.S1_at	heterogeneous nuclear ribonucleoprotein A/B	3.2
RBM39	Bt.12348.1.A1_at	RNA binding motif protein 39	3.2
RPS16	Bt.2686.1.S1_at	ribosomal protein S16	3.2
DNMT3A	Bt.22981.1.S1_at	DNA (cytosine-5-)-methyltransferase 3 alpha	3.2
NDUFB2	Bt.60.1.S1_at	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8kDa	3.2
CTSL2	Bt.3987.1.S1_at	cathepsin L2	3.2
NBN	Bt.24703.1.S1_at	Nibrin	3.2
C22H3orf60	Bt.8279.2.S1_at	Chromosome 3 open reading frame 60 ortholog	3.1
NDUFC2	Bt.21.1.S1_at	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5kDa	3.1
ARL6IP4	Bt.20547.1.S1_a_at	ADP-ribosylation-like factor 6 interacting protein 4	3.1
CTBP2	Bt.6440.1.S1_at	C-terminal binding protein 2	3.1
C7H5orf32	Bt.10278.1.S1_at	chromosome 5 open reading frame 32 ortholog	3.1
GPRC5C	Bt.21454.1.S1_at	G protein-coupled receptor, family C, group 5, member C	3.1
BZW2	Bt.5495.1.S1_at	basic leucine zipper and W2 domains 2	3.1
DYRK1A	Bt.27934.1.S1_at	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	3.1
PRKAR1A	Bt.10903.2.S1_at	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	3.1
ALAD	Bt.9585.1.S1_at	aminolevulinate, delta-, dehydratase	3.1
INSIG2	Bt.20716.2.A1_at	insulin induced gene 2	3.1
NBEAL1	Bt.16647.1.S1_at	neurobeachin-like 1	3.1
UBE2C	Bt.2725.1.S1_at	ubiquitin-conjugating enzyme E2C	3.1
CCDC59	Bt.13559.1.S1_at	coiled-coil domain containing 59	3.1

Gene Symbol	Affy Probe ID	Gene description	Fold change
PDPN	Bt.12295.1.S1_at	podoplanin	3.0
ATP5L	Bt.2816.1.S1_at	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G	3.0
IDH2	Bt.5520.1.S1_at	isocitrate dehydrogenase 2 (NADP+), mitochondrial	3.0
MYL6	Bt.2847.1.S1_a_at	myosin, light chain 6, alkali, smooth muscle and non-muscle	3.0
ATP5O	Bt.442.1.S1_at	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	3.0
COL11A2	Bt.4741.1.S1_at	collagen, type XI, alpha 2	3.0
RPL39	Bt.9548.1.S1_at	ribosomal protein L39	3.0
SIRT1	Bt.20465.1.S1_at	sirtuin (silent mating type information regulation 2 homolog) 1 (S. cerevisiae)	2.9
ZNRF1	Bt.15887.2.S1_a_at	zinc and ring finger 1	2.9
LGALS1	Bt.5472.1.S1_at	lectin, galactoside-binding, soluble, 1	2.9
RPL23	Bt.3091.1.S1_at	ribosomal protein L23	2.9
NDUFB8	Bt.5378.1.S1_at	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa	2.9
ELMO2	Bt.26400.1.A1_at	engulfment and cell motility 2	2.9
RBM8A	Bt.6706.1.S1_at	RNA binding motif protein 8A	2.9
PEPD	Bt.3082.1.S1_at	peptidase D	2.9
CTNNB1	Bt.1125.1.S1_at	catenin (cadherin-associated protein), beta 1, 88kDa	2.9
LOC535277	Bt.9589.1.S1_at	hypothetical LOC535277	2.9
LOC530342	Bt.6001.1.S1_s_at	similar to Chromosome 16 open reading frame 24	2.9
PAK1IP1	Bt.27921.1.S1_at	PAK1 interacting protein 1	2.9
ARPC2	Bt.6846.1.S1_at	actin related protein 2/3 complex, subunit 2, 34kDa /// similar to actin related protein 2/3 complex subunit 2	2.9
SLC15A4	Bt.20185.1.A1_at	solute carrier family 15, member 4	2.8
LOC784459	Bt.6063.1.S1_at	Similar to Heat shock 70kDa protein 9B (mortalin-2)	2.8
LOC533763	Bt.6589.1.A1_at	Similar to glycogen branching enzyme	2.8
UQCRB	Bt.25083.1.A1_at	Ubiquinol-cytochrome c reductase binding protein	2.8
MGC152585	Bt.13422.2.A1_at	Hypothetical LOC507035	2.8
LZTFL1	Bt.22150.2.S1_at	leucine zipper transcription factor-like 1	2.8
LOC614731	Bt.13966.1.S1_at	similar to BolA-like protein 2	2.8
ABCA9	Bt.8673.1.S1_a_at	ATP-binding cassette, sub-family A (ABC1), member 9	2.8
EIF3F	Bt.21463.1.S1_at	eukaryotic translation initiation factor 3, subunit F	2.8
YY1	Bt.1718.3.S1_at	YY1 transcription factor	2.8
GSTP1	Bt.13949.1.S1_at	glutathione S-transferase pi 1	2.8
CPSF6	Bt.27146.1.A1_at	Cleavage and polyadenylation specific factor 6, 68kDa	2.7
RER1	Bt.7896.1.S1_at	RER1 retention in endoplasmic reticulum 1 homolog (S. cerevisiae)	2.7

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MLH1	Bt.5658.1.S1_s_at	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	2.7
EIF5	Bt.9042.1.A1_at	eukaryotic translation initiation factor 5	2.7
NUPL1	Bt.17812.1.S1_at	nucleoporin like 1	2.7
PUF60	Bt.5143.1.A1_at	poly-U binding splicing factor 60KDa	2.7
C8H9orf80	Bt.14380.1.S1_at	Chromosome 9 open reading frame 80 ortholog	2.6
LOC511121	Bt.6419.1.S1_at	Similar to Ubiquitin carboxyl-terminal hydrolase 47 (Ubiquitin thioesterase 47) (Ubiquitin-specific-processing protease 47) (Deubiquitinating enzyme 47)	2.6
CCT3	Bt.5096.1.S1_at	chaperonin containing TCP1, subunit 3 (gamma)	2.6
ATP6V1D	Bt.3998.1.S1_at	ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D	2.6
SS18L2	Bt.20445.1.S1_at	synovial sarcoma translocation gene on chromosome 18-like 2	2.6
SFN	Bt.9957.1.S1_at	stratifin	2.6
LUC7L	Bt.13181.9.S1_at	LUC7-like (S. cerevisiae)	2.6
RPL31	Bt.1601.1.S1_at	ribosomal protein L31	2.6
HCCS	Bt.26718.1.S1_a_at	holocytochrome c synthase	2.6
RAB18	Bt.13979.1.S1_at	RAB18, member RAS oncogene family	2.6
CCDC127	Bt.3386.1.S1_at	coiled-coil domain containing 127	2.6
GHDC	Bt.9402.1.S1_at	GH3 domain containing	2.6
RPL19	Bt.23191.1.S1_at	ribosomal protein L19	2.6
AARS	Bt.12364.1.S1_at	alanyl-tRNA synthetase	2.6
SLC6A20	Bt.28747.1.S1_at	Solute carrier family 6 (proline IMINO transporter), member 20	2.6
LOC782444	Bt.27075.1.A1_at	similar to tripartite motif-containing 8	2.5
VPS37C	Bt.9228.1.A1_at	vacuolar protein sorting 37 homolog C (S. cerevisiae)	2.5
COX6B	Bt.75.1.S1_at	cytochrome c oxidase subunit VIb	2.5
RNASEH2C	Bt.21833.1.S1_at	ribonuclease H2, subunit C	2.5
TGIF1	Bt.20778.3.A1_at	TGFB-induced factor homeobox 1	2.5
ERRFI1	Bt.23905.2.S1_at	ERBB receptor feedback inhibitor 1	2.5
IMP4	Bt.5241.1.S1_at	IMP4, U3 small nucleolar ribonucleoprotein, homolog (yeast)	2.5
FTSJ2	Bt.20021.1.S1_at	FtsJ homolog 2 (E. coli)	2.5
KLF4	Bt.1424.1.S1_at	Kruppel-like factor 4 (gut)	2.5
LOC767909	Bt.23496.1.S1_at	6.8 kDa mitochondrial proteolipid	2.5
HNRPK	Bt.3183.3.A1_at	Heterogeneous nuclear ribonucleoprotein K	2.5
RPL10A	Bt.23381.1.S1_at	ribosomal protein L10a	2.5
TP53I13	Bt.11370.1.A1_at	tumor protein p53 inducible protein 13	2.5

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CCNC	Bt.16826.1.A1_s_at	cyclin C	2.4
PTGS2	Bt.15758.1.S1_at	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	2.4
NOL7	Bt.4096.1.S1_s_at	nucleolar protein 7, 27kDa	2.4
IFT122	Bt.8829.1.S1_a_at	intraflagellar transport 122 homolog (Chlamydomonas)	2.4
PSMA6	Bt.15837.1.S1_at	proteasome (prosome, macropain) subunit, alpha type, 6	2.4
AKAP12	Bt.10458.2.S1_at	A kinase (PRKA) anchor protein (gravin) 12	2.4
RBKS	Bt.13335.1.S1_at	ribokinase	2.4
EEF1G	Bt.3631.1.S1_at	eukaryotic translation elongation factor 1 gamma	2.4
RPS19	Bt.7648.1.S1_at	ribosomal protein S19	2.4
NDUFA13	Bt.8950.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	2.4
ERAS	Bt.21077.1.S1_at	ES cell expressed Ras	2.4
PHF3	Bt.15306.1.A1_at	PHD finger protein 3	2.4
NT5DC1	Bt.18484.1.A1_at	5'-nucleotidase domain containing 1	2.4
NDUFS2	Bt.4475.1.S1_at	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase)	2.3
LOC614048	Bt.9905.1.S1_at	Similar to bromodomain-containing 4	2.3
DDX27	Bt.2644.1.S1_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27	2.3
SSBP1	Bt.21217.1.S1_at	single-stranded DNA binding protein 1	2.3
EFHA1	Bt.28154.1.S1_at	EF-hand domain family, member A1	2.3
DRG1	Bt.26551.1.S1_at	developmentally regulated GTP binding protein 1	2.3
AHCTF1	Bt.13722.2.A1_at	AT hook containing transcription factor 1	2.3
RPL24	Bt.5054.1.S1_a_at	ribosomal protein L24	2.3
TRIB2	Bt.18822.1.A1_at	tribbles homolog 2 (Drosophila)	2.3
APOO	Bt.27908.1.S1_at	apolipoprotein O	2.3
PWWP2A	Bt.9719.2.A1_at	PWWP domain containing 2A	2.3
UCHL5	Bt.4689.1.S2_at	ubiquitin carboxyl-terminal hydrolase L5	2.3
AHNAK	Bt.23331.1.S1_at	AHNAK nucleoprotein	2.3
LAMP2	Bt.2606.1.S1_at	lysosomal-associated membrane protein 2	2.2
TCEB2	Bt.6661.1.S1_at	transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B)	2.2
LOC782444	Bt.9660.1.A1_s_at	similar to tripartite motif-containing 8	2.2
UROD	Bt.26622.1.S1_a_at	uroporphyrinogen decarboxylase	2.2
AEBP2	Bt.9715.1.S1_at	AE binding protein 2	2.2
MCM6	Bt.21433.1.S1_at	Minichromosome maintenance complex component 6	2.2

Gene Symbol	Affy Probe ID	Gene description	Fold change
PSMB1	Bt.7040.1.S1_at	proteasome (prosome, macropain) subunit, beta type, 1	2.2
NDUFV1	Bt.4072.1.S1_at	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	2.2
ATP6V0D2	Bt.11010.1.S1_at	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	2.2
KIF5B	Bt.14058.1.S1_at	Kinesin family member 5B	2.2
RAC1	Bt.22865.1.S1_at	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	2.2
IGF2BP1	Bt.27949.1.A1_at	insulin-like growth factor 2 mRNA binding protein 1	2.2
PSMD4	Bt.22831.1.A1_s_at	proteasome (prosome, macropain) 26S subunit, non-ATPase, 4	2.2
CYC1	Bt.1808.1.S1_at	cytochrome c-1	2.2
PRDX5	Bt.4355.1.S1_at	peroxiredoxin 5	2.2
LOC505941	Bt.4937.1.S1_at	similar to KIAA1398 protein	2.2
PLDN	Bt.7131.1.S1_at	pallidin homolog (mouse)	2.2
LOC508529	Bt.23131.1.S1_s_at	hypothetical LOC508529	2.2
TNP1	Bt.333.1.S1_at	transition protein 1 (during histone to protamine replacement)	2.2
SLC2A5	Bt.19805.1.A1_at	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	2.2
YWHAZ	Bt.2128.1.S1_at	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	2.1
RAB4A	Bt.21708.1.S1_at	RAB4A, member RAS oncogene family	2.1
AP2M1	Bt.7552.1.S1_at	adaptor-related protein complex 2, mu 1 subunit /// similar to adaptor-related protein complex 2, mu1 subunit	2.1
KLF13	Bt.20394.1.A1_at	Kruppel-like factor 13	2.1
FAU	Bt.4618.1.S1_at	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	2.1
NDUFB10	Bt.70.1.S1_at	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	2.1
NDUFA9	Bt.5541.1.S1_at	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9, 39kDa	2.1
NFU1	Bt.25072.1.S1_at	NFU1 iron-sulfur cluster scaffold homolog (S. cerevisiae)	2.1
CBX3	Bt.10198.1.S1_at	chromobox homolog 3 (HP1 gamma homolog, Drosophila)	2.1
NBEAL1	Bt.16647.2.A1_at	neurobeachin-like 1	2.1
SNRP70	Bt.12748.1.S1_at	small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen)	2.1
PDHB	Bt.2973.2.S1_a_at	pyruvate dehydrogenase (lipoamide) beta	2.1
LHFPL2	Bt.3015.1.A1_at	lipoma HMGIC fusion partner-like 2	2.1
MAPK1IP1L	Bt.1400.1.S1_at	mitogen-activated protein kinase 1 interacting protein 1-like	2.1
ATP5H	Bt.5029.1.S1_at	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	2.1
MTDH	Bt.13983.2.S1_at	metadherin	2.0
MPV17	Bt.11135.1.S1_at	MpV17 mitochondrial inner membrane protein	2.0
COMMD8	Bt.2125.1.A1_at	COMM domain containing 8	2.0
MGC148714	Bt.1334.1.S1_at	similar to Cytochrome c oxidase polypeptide VIc-2	2.0

Gene Symbol	Affy Probe ID	Gene description	Fold change
SPC24	Bt.9517.1.S1_a_at	SPC24, NDC80 kinetochore complex component, homolog (S. cerevisiae)	2.0
CLGN	Bt.17314.2.A1_at	calmegin	-116.2
LOC510240	Bt.6982.1.S1_at	similar to RRP1-like protein B	-33.2
CHMP1B	Bt.803.1.A1_at	chromatin modifying protein 1B	-11.7
LOC100174924	Bt.16614.1.A1_s_at	hypothetical LOC100174924	-10.9
MGC152033	Bt.22756.1.A1_at	Hypothetical LOC515055	-10.4
CLGN	Bt.17314.1.S1_at	calmegin	-10.1
FUT1	Bt.16029.1.S2_at	fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase, H blood group)	-10.1
NHLRC2	Bt.20833.3.A1_at	NHL repeat containing 2	-8.5
PNRC2	Bt.16143.2.A1_at	proline-rich nuclear receptor coactivator 2	-6.9
ZNF45	Bt.5423.2.A1_at	Zinc finger protein 45	-4.1
PRPSAP2	Bt.21104.1.S1_at	phosphoribosyl pyrophosphate synthetase-associated protein 2	-4.0
SIRT2	Bt.19604.1.A1_at	Sirtuin (silent mating type information regulation 2 homolog) 2 (S. cerevisiae)	-3.8
NAT9	Bt.2329.1.A1_at	N-acetyltransferase 9	-3.7
LOC505941	Bt.24973.1.A1_at	similar to KIAA1398 protein	-3.1
PPP3CC	Bt.2191.1.A1_at	Protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform	-3.0
LRRFIP2	Bt.13852.3.S1_a_at	Leucine rich repeat (in FLII) interacting protein 2	-2.8
CNPY2	Bt.27267.1.S1_at	canopy 2 homolog (zebrafish)	-2.8
ZC3H18	Bt.20159.1.S1_at	zinc finger CCCH-type containing 18	-2.3
LOC616839	Bt.24278.1.A1_at	hypothetical LOC616839	-2.3
SOSTDC1	Bt.27202.1.A1_at	sclerostin domain containing 1	-2.2

Table S2 The networks from the IPA Knowledge Base that involve molecules differentially expressed (bold) in the blastocyst derived from superovulated heifers compared to those derived from synchronized heifers.

ID	Molecules in Network	Top Functions
1	↑***ACTB***, ↑***ACTN1***, *Alpha Actinin, Alpha catenin,* ↑***ANLN***, ↑***ANXA2***, *Cadherin, Calpain,* ↑***CD2AP***, ↑***CTBP2***, *ERK1/2, GNRH,* ↑***HNRNPK***, ↑***KLF13***, ↑***MRPS31***, ↑***RAB4A***, ↑***RPL13***, ↑***RPL14***, ↑***RPL19***, ↑***RPL28***, ↑***RPL31***, ↑***RPL34***, ↑***RPL27A***, ↑***RPS5***, ↑***RPS7***, ↑***RPS8***, ↑***RPS10***, ↑***RPS13***, ↑***RPS19***, ↑***RPS20***, ↑***RPS12***, ↑***RPS3A***, ↑***SNRNP70***, ↑***TGIF1***, ↑***UBE2O***	Protein Synthesis, RNA Post-Transcriptional Modification, Cell Cycle
2	↑***ABLIM1***, *adenosine-tetraphosphatase,* ↑***ALAD***, ↑***ALDH7A1***, *Alpha tubulin,* ↑***ANKS1A***, ↑ ***ATP5B***, ↑***ATP5G3***, ↑***ATP5H***, ↑***ATP5I***, ↑***ATP5L***, ↑***ATP5O***, ↑***ATP6V0D2***, ↑***EPCAM***, ↑***ERRFI1***, *H+-exporting ATPase, H+-transporting two-sector ATPase,* ↑***HSPE1***, *MAP2K1/2, NFkB (complex), Pak,* ↑***PAK1IP1***, ↑***PDPK1***, *peroxidase (miscellaneous),* ↑***PRDX1***, ↑***PRDX2***, ↑***PRDX5***, ↑***PTPN2***, *Raf,* ↑***SFN***, ↓***SIRT2***, ↑***SLC2A5***, *TCR,* ↑***TOP2A***	Energy Production, Nucleic Acid Metabolism, Small Molecule Biochemistry
3	*Akt, Caspase, Cdc2, CDC2, CTSL2, Cyclin A, Cyclin E, Cytochrome c, E2f,* ↑***EIF4E***, *Eif4g,* ↑***ERCC6L***, ↑***GSK3A***, ↑***HNRNPD***, *Hsp27,* ↑***HSPA4***, ↑***HSPB1***, ↑***JARID2***, ↑***KRT8***, ↑***KRT18***, ↑***LGALS3***, ↑***MCM6***, ↑***MLH1***, ↑***MPV17***, ↑***NBN***, *p70 S6k,* ↑***PA2G4***, ↓***PPP3CC***, *Rb,* ↑***RPL24***, ↑***RPL27***, ↑***RPLP1***, ↑***RPLP2***, ↑***RPS16***, ↑***UBE2C***	Cellular Assembly and Organization, Hepatic System Development and Function
4	*Adaptor protein 2, Ap1,* ↑***AP2M1***, *Calcineurin protein(s), Ck2, Clathrin,* ↑***CLTA***, ↑***DAB2***, ↑***FUS***, ↑***GATA2***, ↑***GSTP1***, ↑***HELZ***, *Histone h3, Hsp70, Hsp90,* ↑***HSPA8***, ↑***HSPA9***, ↑***HSPA14***, ↑***IGF2BP1***, *Jnk,* ↑***KIT***, ↑***NCL***, ↑***PICALM***, ↑***POLR2F***, ↑***PTGES3***, ↑***PUF60***, ↑***RBM8A***, *RNA polymerase II, Rsk,* ↑***SIRT1***, ↑***SSB***, ↑***SYNCRIP***, ↑***TAOK3***, ↑***TCEB2***, ↑***ZNF238***	RNA Damage and Repair, Cell Cycle, Cellular Development
5	*Calmodulin,* ↑***CASK***, ↑***CBX3***, ↑***CTNNB1***, ↑***DNMT3A***, ↑***EIF3F***, *Histone h4,* ↑***HNRNPF***, *IKK (complex), Insulin, Interferon alpha,* ↓***LRRFIP2***, ↑***MKRN1***, *NADH2 dehydrogenase,* ↑***NDUFA1***, ↑***NDUFA9***, ↑***NDUFC2***, ↑***NDUFV1***, ↑***PDHB***, ↑***PEPD***, *peptidase, Pka, Pkc(s),* ↑***RBM39***, ↑***RPL6***, ↑***RPL10A***, ↑***SSBP1***, ↑***SUZ12***, *Tgf beta,* ↑***THOP1***, ↑***TKT***, ↑***TRIB2***, ↑***YWHAZ***, ↑***YY1***, ↓***ZNF45***	Genetic Disorder, Metabolic Disease, Organ Development
6	*AGT, ALPP,* ↑***ARL6IP4***, *ARRB2, BMP2,* ↑***BOLA2***, *CDKN1A, CER1,* ↓***CLGN***, *CUL4B,* ↑***CXORF26***, ↑***DDX27***, ↑***EBP***, *GRWD1, HIRA, KIF2C,* ↑***LUC7L3***, *MIR194-2, MIR298,* ↑***NAT11***, ↑***NFU1***, *PPP1R16A,* ↑***PSAP***, ↑***RPL14***, *RPL15,* ↑***RPL28***, ↑***RPS8***, ↑***RPS13***, ↑***RPS17***, ↑***SLC30A5***, ↑***SNRNP40***, ↓***SOSTDC1***, ↑***SPIN1***, *WDR5,* ↑***WDR82***	Cell Death, Renal Necrosis/Cell Death, Cell Morphology
7	*ADI1,* ↑***COMMD8***, *EIF2C2,* ↑***FAM173A***, *HSP90AB1, HTT,* ↑***LUC7L***, *ME2,* ↑***MYO1D***, *NADH2 dehydrogenase (ubiquinone),* ↑***NANS***, *NDUFA2,* ↑***NDUFA5***, ↑***NDUFA6***, ↑***NDUFA12***, ↑***NDUFA13***, *NDUFA10, NDUFA4L2,* ↑***NDUFA9***, *NDUFAF1, NDUFB1,* ↑***NDUFB2***, *NDUFB4,* ↑***NDUFB8***, ↑***NDUFB10***, ↑***NDUFC2***, *NFKBIE,* ↑***NUPL1***, ↑***OSTC***, ↑***OXA1L***, *oxidoreductase,* ↑***RPL38***, ↑***SAPS1***, *TM7SF2, TSC22D1*	Carbohydrate Metabolism, Small Molecule Biochemistry, Amino Acid Metabolism

ID	Molecules in Network	Top Functions
8	*20s proteasome, 26s Proteasome, AKAP12, COL11A2, Collagen type I, Collagen(s), FN1, Ikb, Immunoproteasome Pa28/20s, Integrin, LAMP2, LMNA, Mapk, NADH dehydrogenase, NAP1L1, NDUFA5, NDUFB2, NDUFB8, NDUFS2, Pdgf, PDGF BB, PI3K, POMP, PP2A, PPP2R5E, Proteasome PA700/20s, PSMA6,* ↑***PSMB1,*** ↑***PSMB4,*** ↑***PSMD4,*** ↑***TAF9****,* ↑***TAF13****, Tcf/lef, Ubiquitin,* ↑***UCHL5***	Connective Tissue Disorders, Genetic Disorder, Ophthalmic Disease
9	*Actin,* ↑***ARPC2,*** ↑***CCNC,*** ↑***CCT3****, CD3,* ↑***CKB****, Cofilin, Creatine Kinase,* ↑***DSTN,*** ↑***EZR,*** *F Actin, Fascin,* ↑***KLF4,*** *Laminin,* ↑***LASP1,*** ↑***LGALS4,*** ↑***LRPAP1,*** ↑***LZTFL1****,* ↑***MAPK1IP1L****, MIR1, MIR124, Nfat (family), P38 MAPK,* ↑***PLDN,*** ↑***RAC1,*** ↑***RANBP1****, Ras homolog,* ↑***RBM47****, Rock,* ↑***SFRS9,*** ↑***SLC15A4,*** ↑***TPM4,*** ↑***USP48****, VitaminD3-VDR-RXR,* ↑***VPS37C***	Cellular Assembly and Organization, Cellular Function and Maintenance, Cell Morphology
10	↓***CNPY2****,* ↑***EEF1G,*** ↑***EIF5,*** ↑***ELMO2****, ERN1, GBP2,* ↑***HCCS****, IFNB1, IKBKE,* ↑***IMP4****, NDUFB9, NR3C1, PFDN5,* ↑***PTGES3,*** ↑***PTRH2,*** *Rab11,* ↑***RAB11A****, RAB11FIP2, RAB11FIP3,* ↑***RAB11FIP4****, RAB11FIP5,* ↑***RPL3****, RPL18,* ↑***RPL36AL****,* ↑***RPS19,*** ↑***RPS28****, RSP5, SPI1,* ↑***SSB****, ST13, TMEM189-UBE2V1,* ↑***TOPORS,*** ↑***UBE2E1****, XIAP,* ↑***ZNRF1***	Protein Synthesis, Genetic Disorder, Hematological Disease
11	↑***ACSL3,*** *BCCIP,* ↓***CCDC93****,* ↑***CPSF3****, DCAF7, DLST,* ↑***DYRK1A,*** ↑***HNRNPAB,*** ↑***HNRNPF****, IL2, KITLG, MDM2,* ↑***MTBP****,* ↓***NAT9****, NCBP1, PARD3B, PRKX, PRPF19, PRPS1,* ↓***PRPSAP2****,* ↑***PSMB4,*** ↑***PTRH2,*** ↑***RPL23,*** ↑***RPL13A,*** ↑***RPS7,*** ↑***RPS21,*** ↑***SCPEP1****, SFRS4, SLC25A3, SLC2A4, SMAD1, YWHAG, YWHAQ,* ↓***ZC3H18****,* ↑***ZNF207***	Cell Cycle, Cell Death, Cellular Growth and Proliferation
12	↑***AARS,*** ↑***ATPIF1,*** ↑***C3,*** *Cbp/p300,* ↑***CPSF6****, Creb,* ↑***CYC1****, ERK,* ↑***FAU****, Fgf, FSH, Gsk3, hCG, IgG, IL1,* ↑***INSIG2****, LDL,* ↑***LGALS1****, Lh, Mek, N-cor, NADPH oxidase,* ↑***OSTF1****, PLC,* ↑***PRKAR1A****,* ↑***PTGS2****, Ras,* ↑***RPL39****,* ↑***SREBF1,*** ↑***THOC4****, ubiquinol-cytochrome-c reductase,* ↑***UCRC,*** ↑***UQCR,*** ↑***UQCRB****, Vegf*	Cancer, Reproductive System Development and Function, Digestive System Development and Function
13	*ARL6IP1, BRF1,* ↑***BZW1,*** ↑***BZW2,*** ↑***C9ORF80,*** ↑***CISD1****, DNAJA2,* ↑***EPCAM****, ERH,* ↑***HNRNPUL1****, HUWE1,* ↑***IGF2BP1****, IPO9, MARS, MIRLET7B,* ↑***MTDH****, MYC,* ↑***NAP1L1,*** ↑***NARS****, NDC80, NUF2, PERP, PPAT, PRKAB1,* ↑***RPS7,*** ↑***SPC24****, SPC25,* ↑***SR140****, STK11,* ↑***TMED7,*** ↑***TNRC6C****, Top2,* ↑***TOP2A****, TP53,* ↑***UBA6***	Cell Cycle, Cellular Development, Cellular Assembly and Organization
14	↑***ABCB6,*** ↑***AHCYL2,*** ↑***CCDC59****, COX1, COX2, COX3, COX17,* ↑***COX4I1,*** ↑***COX5A****, COX5B,* ↑***COX7B****, Cytochrome c oxidase,* ↑***DCP1B****, DDR2, DNAJC13,* ↑***DRG1****,* ↑***DSG2,*** *GEM,* ↑***HDGFRP3****, HSPA1L, IFNG, IRAK2, iron, LOC100289404, MIR297-2,* ↑***NDUFAF3****, NRF1,* ↑***RAB18****, SMURF1, ST13, TGFB1,* ↑***TMEM184B****, TRAF6,* ↑***UNQ1887,*** ↑***UROD***	Molecular Transport, Small Molecule Biochemistry, Cellular Growth and Proliferation

ID	Molecules in Network	Top Functions
15	*ARL1, ↑**CCDC47,** CEACAM1, CHMP1A, ↓**CHMP1B**, ↑**CPT2**, DNAJB9, ↑**GBE1,** ↑**GLTSCR2,** ↑**GOLGA4,** ↑**GPRC5C**, GSTK1, HNF4A, HTRA2, ↑**KIAA1715**, LSM3, ↑**LSM4**, LSM5, ↑**MRPL22,** ↑**MYL6**, ↑**NOL7**, NR1H4, NR2F1, ↑**RBKS**, SART3, ↑**SLC25A5**, SPAST, STAMBP, USP8, VDAC1, VDAC2, VPS4A, VPS4B, WASF2, ZNF277*	Gene Expression, Lipid Metabolism, Molecular Transport
16	*beta-estradiol, CHAT, CLIC1, CRADD, CYB5A, cyclic AMP, DIO1, DIO3, E2F1, ↑**ERAS**, ERH, ↑**FAM60A**, ↓**FUT1**, GRB10, H19, ↑**HSD17B11,** ↑**IDH2,** ↑**IFT122,** ↑**LHFPL2**, MIRN330, MMP16, ↑**NOP56**, NRP2, ↑**PA2G4**, PDE4B, ↑**PHF3**, PTGER3, RAF1, ↑**RER1**, SELE, SELPLG, SFRS5, ↑**SLC6A20**, SP4, ↑**TOP2A***	Neurological Disease, Skeletal and Muscular Disorders, Reproductive System Development and Function
17	*↑**ADH5**, APOA1, APOA2, APOB, APOC3, CHD3, dehydroisoandrosterone, HDL, ↑**KDM2A**, KPNB1, linoleic acid, ↑**LMNA,** MEN1, mevalonic acid, MSTN, NFATC2, Nos, NOS2, NOS3, NR2F1, ↑**PDPK1,** PLA2G2A, PLA2G4A, ↑**PPHLN1**, PPL, RIPK2, Rock, ↓**RRP1B**, ↑**SAFB2**, SMAD3, ↑**SRRT**, SUMO2, TOMM20, TXN, VIM*	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry

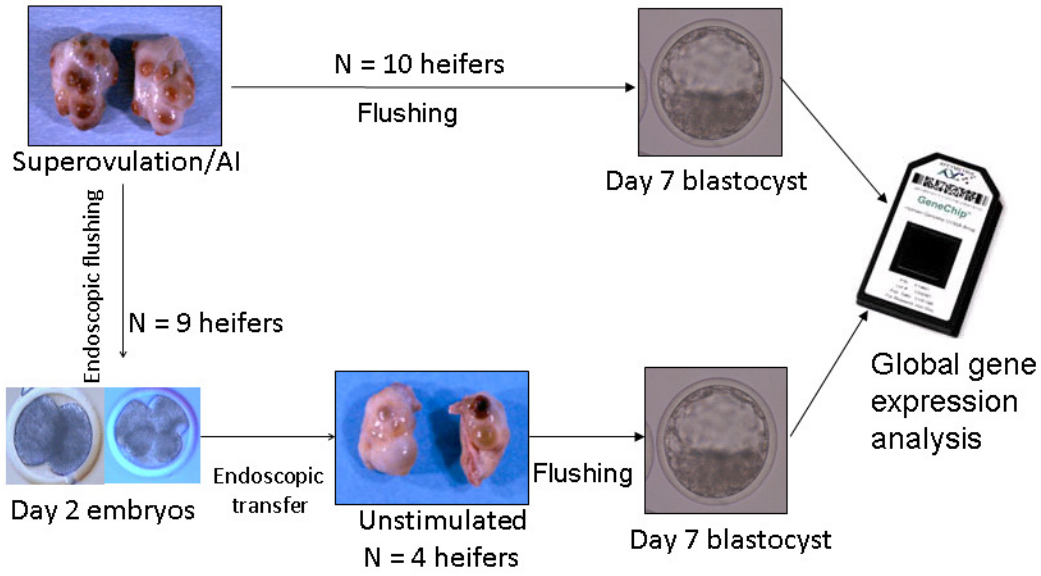

Figure 1 An overview of the overall experiment to investigate the effect of controlled ovarian hyperstimulation treatment on embryo development and the global transcriptome profile of blastocysts.

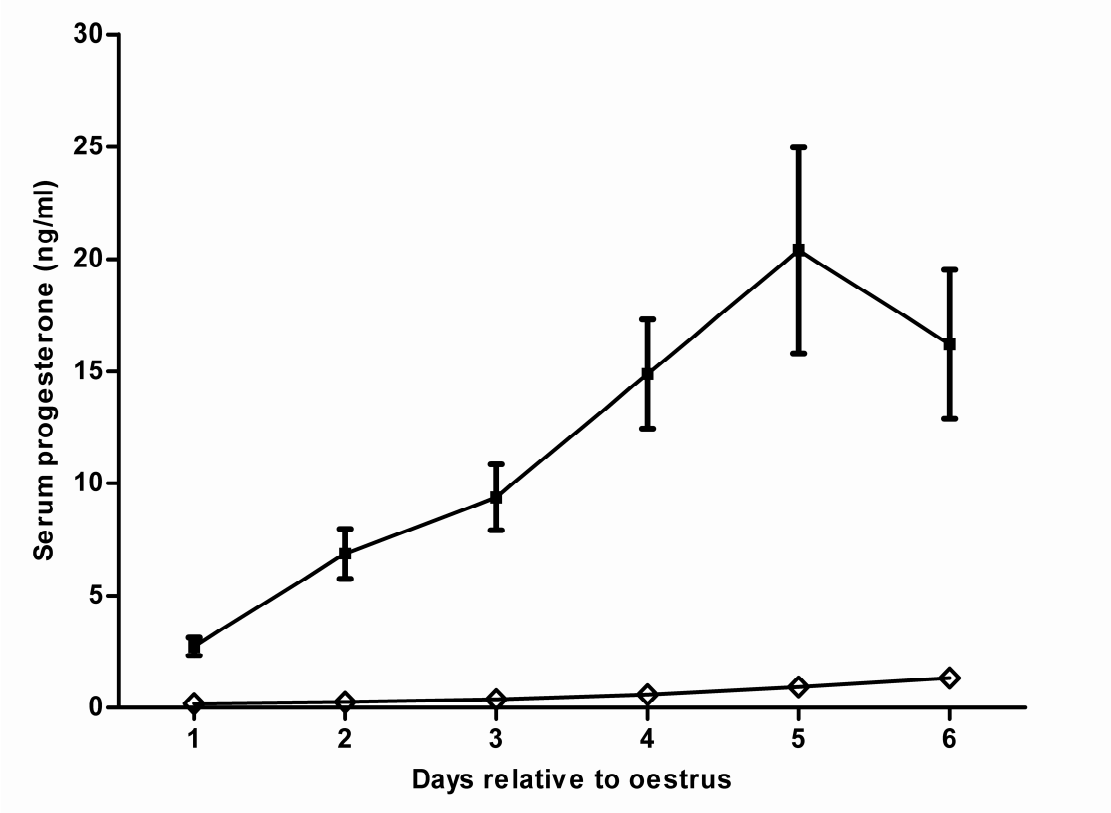

Figure 2 Progesterone concentrations (mean ± s.e.m.) from Day 1 to Day 6 in unstimulated recipients (n=4; ◊) and superovulated heifers (n=9; ■).

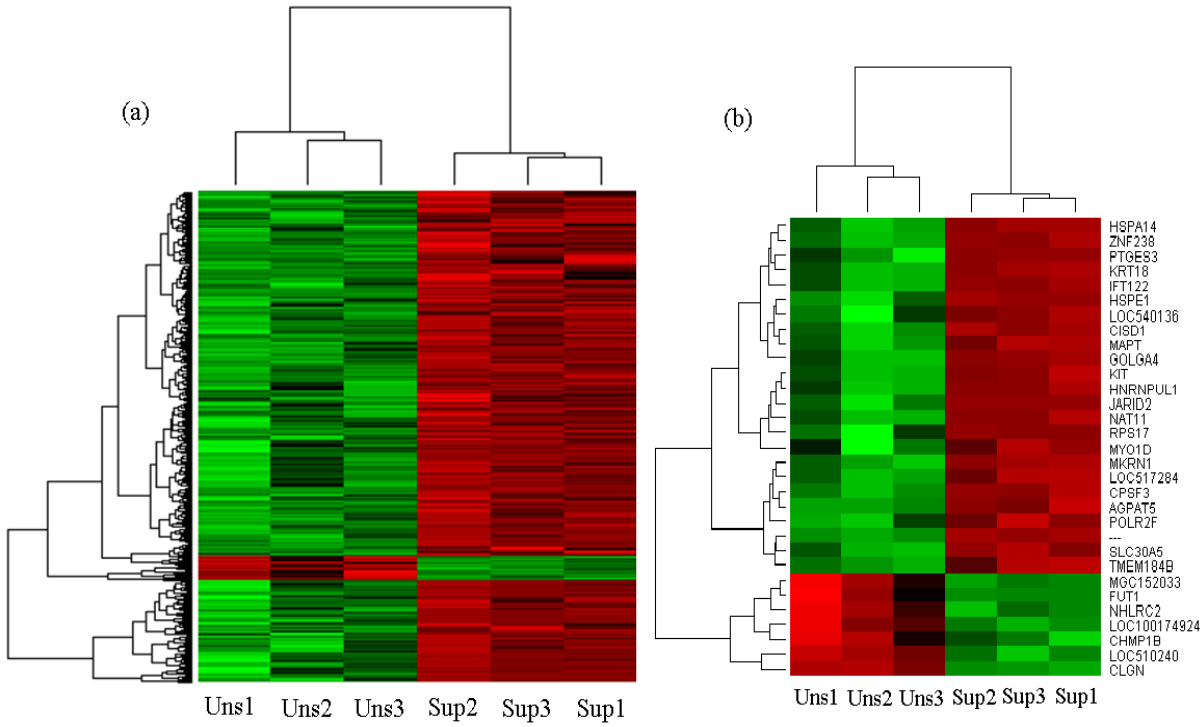

Figure 3 Hierarchical clustering and heatmap for all differentially expressed genes (a) and for those genes with fold change ≥ 8 (b) in blastocysts derived from superovulated (Sup) or unstimulated (Uns) heifers. The red blocks represent higher expression of genes while the green blocks represent lower expression of genes. Columns represent individual hybridizations, rows represent individual genes.

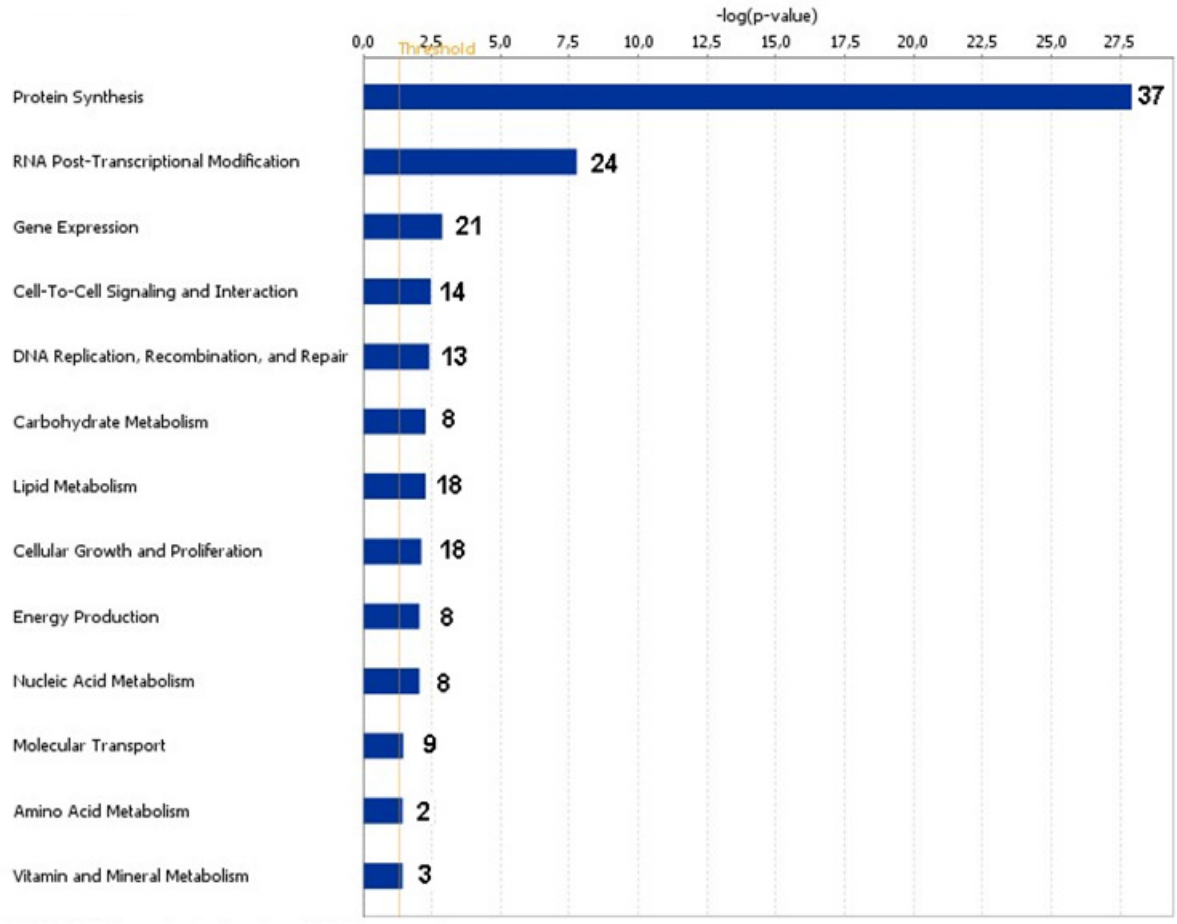

Figure 4 Functional grouping of differentially expressed genes in the two blastocysts groups (derived from superovulated or unstimulated heifers) using Ingenuity Pathways Analysis software. The most significant functional groups ($p<0.05$) are presented graphically. The bars represent the *p*-value on a logarithmic scale for each functional group. The numbers on each bar represent the number of differentially expressed genes involved in each function.

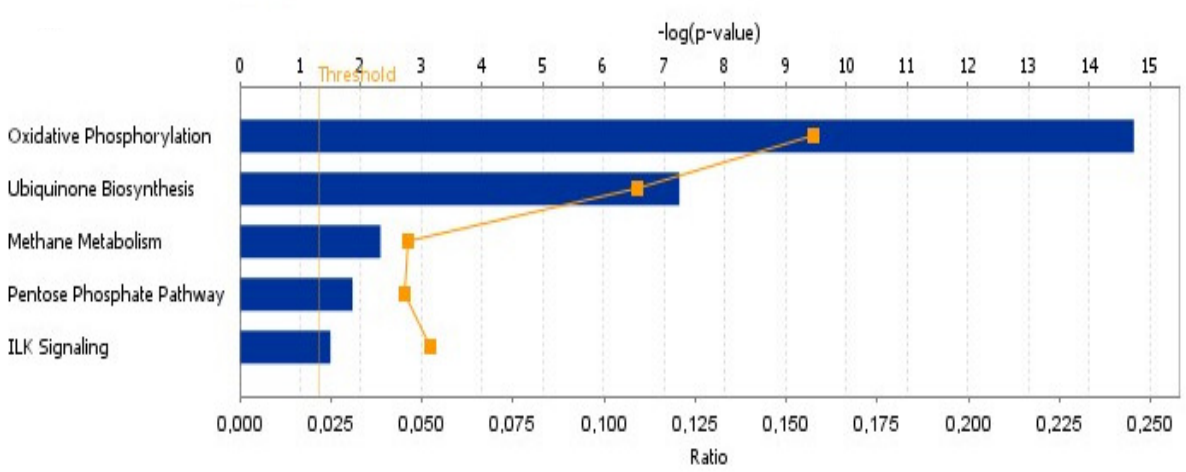

Figure 5 The most prominent canonical pathways related to the data set ($p<0.05$). The bars represent the *p*-value for each pathway. The orange irregular line is a graph of the ratio (genes from the data set/total number of genes involved in the pathway) for the different pathways.

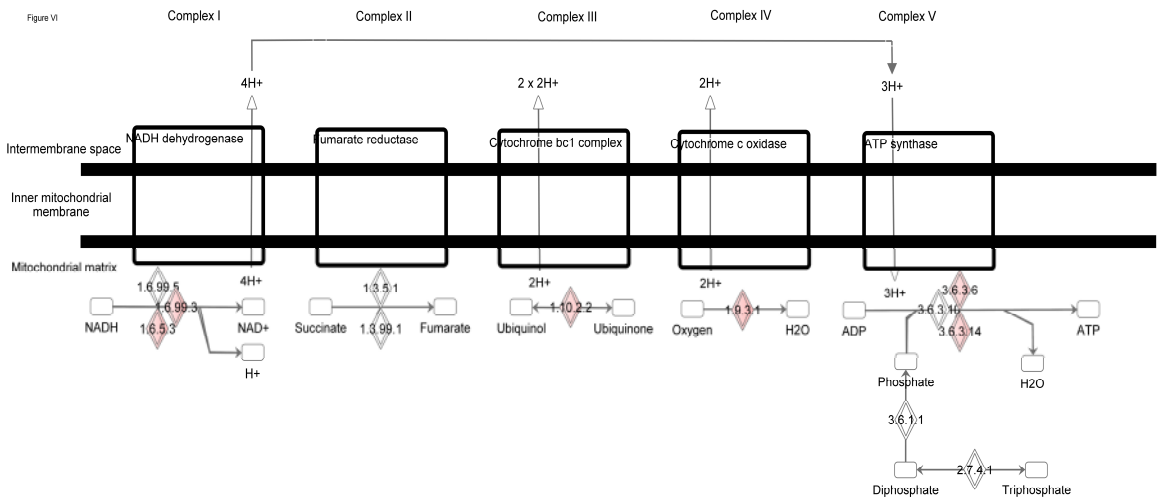

Figure 6 Diagrammatic illustration showing the five protein complexes involved in electron transport and oxidative phosphorylation pathway. As the electrons are passed down the electron transport chain (Complex I-IV), they lose much of their free energy. Some of this energy can be captured and stored in the form of a proton gradient that can be used to synthesize ATP from ADP (Complex V). Dark circles represent the gene complexes which are overexpressed in the blastocysts derived from superovulated heifers.

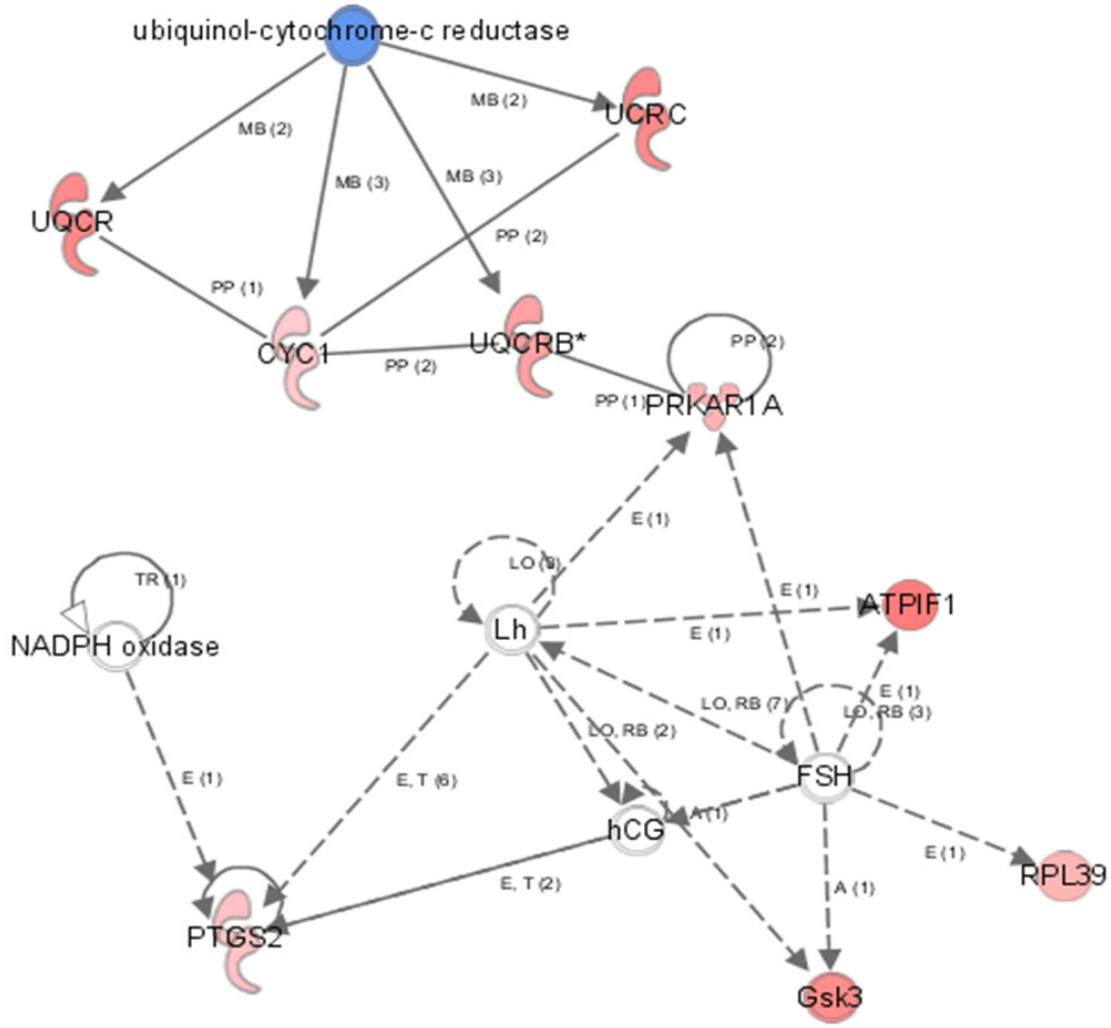

Figure 7 A network showing the relationships between members of the ubiquinol-cytochrome-c reductase complex, one of oxidative phosphorylation pathway complexes, which are overexpressed in the blastocysts derived from superovulated heifers. The type of association between two molecules is shown as a letter on the line that connects them. The number in parenthesis represents the number of bibliographic references currently available in the Ingenuity Pathways Knowledge Base that support each one of the relationships. Direct or indirect relationships between molecules are indicated by solid or dashed connecting lines, respectively. P, phosphorylation; A, gene activation; E, involved in expression; PP, protein-protein interaction; PD, protein-DNA binding; MB, membership in complex; LO, localization.

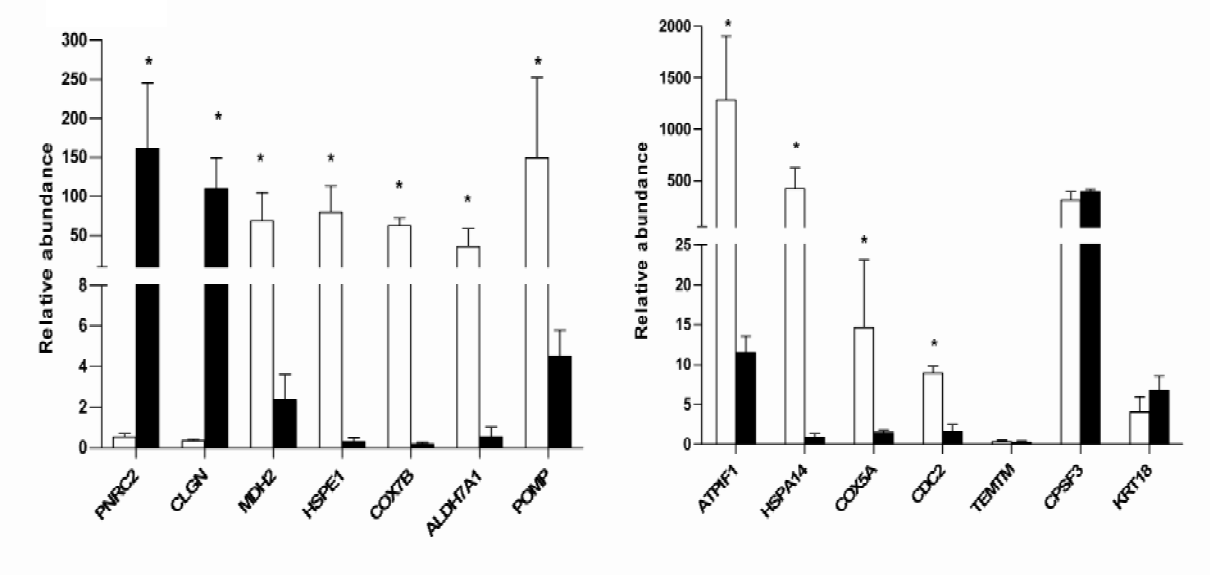

Figure 8 Quantitative real-time PCR validation of 14 differentially expressed genes between blastocysts derived from superovulated heifers (Sup) and those derived from unstimulated heifers (Uns) as identified by microarray analysis. (*) indicates significant differences (P<0.05) between Sup group (white bar) and Uns group (black bar).

2.2 Chapter 2 (Submitted to *Biology of Reproduction 2.2.2012*)

Molecular Mechanisms and Pathways Involved in Bovine Embryonic Genome Activation and Influenced by Alternative In vivo and In vitro Culture Conditions

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Short title: Transcriptome profile of bovine blastocysts.

Key words: embryonic genome activation, gene expression, culture conditions, bovine, lipid metabolism, oxidative stress.

Abstract

Understanding gene expression patterns in response to altered environmental conditions during different time points of preimplantation period would improve our knowledge on regulation of embryonic development. Here we aimed to examine the effect of alternative in vivo and in vitro culture conditions during the time of major embryonic genome activation (EGA) on development and transcriptome profile of bovine blastocysts. Four different blastocyst groups were produced under alternative in vivo and in vitro culture conditions before or after major EGA. Completely in vitro (IVP) and in vivo produced blastocysts were also produced and used as controls. We compared gene-expression pattern between each blastocyst group and in vivo blastocyst control group using EmbryoGENE's bovine microarray. The data showed that changing culture conditions from in vivo to in vitro or vice versa either before or after the time of major EGA had no effect on the developmental rates, however in vitro conditions during that time critically influenced the transcriptome of produced blastocysts. The source of oocyte critically determined the developmental rates and the ability of embryo to react with changing culture conditions. Ontological classification evidenced the presence of a clear contrast in expression patterns for lipid metabolism and oxidative stress between blastocysts generated in vivo vs. in vitro, with opposite trends. Molecular mechanisms and pathways that are influenced by altered culture conditions during EGA were defined. These results will help in development of new strategies to modify culture conditions at this critical stage to enhance development of competent blastocysts.

Introduction

Early embryonic development is one of the most critical periods of mammalian development. This early phase involves various morphological and biochemical changes related to genomic activity and comprise a complex set of physiological processes, many of which are still unknown. These processes are controlled by several molecular mechanisms and pathways that have a fundamental role in the coordination of homeostatic and metabolic processes. During the post-fertilization developmental period, several critical events occur in the embryo which should be regulated by a harmonized expression of genes under ideal culture conditions (Niemann and Wrenzycki 2000). However, the exact influence of in vitro culture conditions during

each of these critical events/steps is still unknown. Among these events is the time at which the embryo switches from using the mRNA derived from the maternal genome to those resulted from the embryonic genome activation (EGA). Initiation of EGA is a species-specific time point, which occurs at the 2-cell stage in mouse (Schultz 1993), at the 4-cell stage in human (Braude et al. 1988) and at the late 8-cell stage in bovine embryos (Memili and First 2000). EGA is considered as the most critical event for viability during early development (Meirelles et al. 2004) and it is associated with the first differentiation events, successful embryo implantation, and fetal development (Niemann and Wrenzycki 2000). The time between fertilization until the initiation of EGA is known to be a transcriptionally silent period which is required for DNA-chromatin epigenetic reprogramming (Baroux et al. 2008). Epigenetic modifications are involved in gene expression regulation in the embryo and play a crucial role in controlling reprogramming events during early embryogenesis (Li 2002) which appears to be particularly sensitive to culture conditions (Doherty et al. 2000). Accordingly, the impact of environmental factors during this window of development, from maturation until EGA, requires much more investigations.

There are evidences demonstrating that the environment to which the oocyte is exposed during maturation can influence the pattern of transcripts in the matured oocyte and in the resulting blastocyst (Lonergan et al. 2003, Russell et al. 2006). However, other evidences suggest that the pattern of mRNA abundance in the blastocyst is dictated by the post-fertilization culture conditions (Lonergan et al. 2006, Rizos et al. 2002a, Tesfaye et al. 2004). Nevertheless, oocyte quality, beside the process of fertilization, seems to be the main critical factor affecting the proportion of immature oocytes developing to the blastocyst stage (see Merton et al. 2003 for review). This raise a question, how ‘high quality’ zygotes, produced by in vivo maturation and fertilization, leads to the production of high number of poor quality blastocysts when they cultured in vitro?

To resolve this fundamental question, there is an inevitable need for an experiment combining in vitro and in vivo culture systems during specific developmental time points. This analysis would facilitate a comprehensive understanding of early embryo development yielding insights into the molecular pathways controlling early development and to improve our knowledge in regulation of embryonic development. In the current study, we explored the influences of alternative culture conditions (in vivo or

in vitro) before and during EGA event on embryonic developmental rate, gene expression pattern and subsequent influences on pathways and biological functions controlling bovine embryo development. For this purpose we have used the combination of in vivo culture in oviduct of recipients and a new bovine micro-array harbouring 40,000 different targets based on the completed bovine genome and RNAseq to identify splice variants, non translated regions and alternative UTR variant from bovine embryo libraries.

Materials and Methods

Embryo production

Six different blastocyst groups were produced under alternative in vivo and in vitro culture conditions (Figure 1). The first two groups (Vitro_4-cell and Vitro_16-cell) were matured, fertilized and cultured in vitro until 4- and 16-cell stage, respectively then transferred to synchronized recipients and cultured in vivo until day 7 blastocyst stage. The second two groups (Vivo_4-cell and Vivo_16-cell) were matured, fertilized and cultured in vivo until 4- and 16-cell stage, respectively then flushed out and cultured in vitro until day 7 blastocyst stage. Complete in vitro (IVP) and in vivo (control group) produced blastocysts were used as controls in this comparison.

In vitro embryo production, endoscopic tubal transfer and embryo recovery

Bovine oocytes were recovered from slaughterhouse ovaries and embryos were produced in vitro as described previously (Tesfaye et al. 2003). For fertilization, semen was used from the same bull that had been used for AI of the donor animals. Embryos were harvested at different time points (see above) and transferred directly to synchronized recipients by transvaginal endoscopic tubal transfer (Besenfelder and Brem 1998) to generate blastocysts of Vitro_4-cell and Vitro_16-cell groups. At day 7, blastocysts were collected by nonsurgical uterine flushing as described previously (Besenfelder et al. 2001) and pooled in groups of 10. In vitro blastocysts were produced with the same procedure and harvested at day 7 to be pooled in groups of 10.

In vivo embryo production and collection of embryos

Simmental heifers were superovulated and artificially inseminated two times with the same frozen–thawed commercial bull semen and embryos were produced in vivo as described previously (Tesfaye et al. 2004). Embryos were flushed out at different time points as described previously (Besenfelder et al. 2001) and transferred to the in vitro culture to generate blastocysts of Vivo_4-cell and Vivo_16-cell groups. Blastocysts were harvested at day 7 and pooled in groups of 10. Complete in vivo blastocysts were produced with the same procedure and flushed out at day 7 by nonsurgical uterine flushing (Besenfelder et al. 2001).

RNA isolation, amplification and microarray hybridization

Total RNA isolation was performed using the PicoPure RNA isolation kit (Arcturs, Munich, Germany) according to the manufacturer's instruction. Genomic DNA contamination was removed by performing on column DNA digestion using RNase-free DNase (Qiagen GmbH, Hilden, Germany). RNA was eluted in 11 µl of elution buffer and the quantity and quality of the extracted RNA was verified on a 2100 Bioanalyzer (Agilent Technologies Inc, CA, USA). Samples were stored at -80°C until used. Amplified RNA (aRNA) was produced with a two round amplification protocol using RiboAmp HS RNA amplification kit (Applied Biosystems). Quantity of aRNA was determined using a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Two micrograms of aRNA from each sample were conjugated with either Cy-3 or Cy-5 dyes using ULS Fluorescent labelling kit for Agilent arrays (Kreatech Diagnostics, Amsterdam, Netherlands). The labelled product was then purified using the PicoPure RNA extraction kit (Applied Biosystems) to remove uncoupled dyes. Samples from the three pools (biological replicates) of each blastocyst group and the in vivo control group were hybridized together on EmbryoGENE's bovine microarray (Made by Agilent 4 x 44k) using a dye-swap design (technical replicates) for a total of six arrays for each comparison as described previously (Robert et al. 2011). In total, 30 array slides were scanned using Agilent's High-Resolution C Scanner (Agilent Technologies, CA, USA) and features were extracted with the Agilent's Feature Extraction software (Agilent Technologies, CA, USA).

Global data analysis

Microarray data analysis was performed using the Flex Array version 1.6 (Genome Quebec, genomequebec.mcgill.ca/FlexArray). Data were submitted to a simple background correction, a Loess within array normalization, a Quantile between array normalization, and statistically analyzed using Linear Models for Microarray Data Analysis (LIMMA) (Smyth 2005). Genes were considered differentially expressed at a fold-change > 2 with adjusted *p*-value of < 0.05 using the Benjamini and Hochberg false discovery rate method. All data analyses procedures are performed using R version 2.12.1 and limma package version 3.6.9. Principal component analysis (PCA) was performed with the PAST 2.07 software (Hammer et al. 2001) (http://folk.uio.no/ohammer/past). The raw data have been submitted to Gene Expression Omnibus (GEO) under series GSE33314.

Pathways and networks analysis

A list of the DEG's (differently expressed genes) from each comparison was uploaded into IPA (Ingenuity Systems, www.ingenuity.com) to identify relationships between the genes of interest and to uncover common processes and pathways. The ''Functional Analysis'' tool of the IPA software was used to identify the biological functions that were most significant in the data set. Canonical pathway analysis was also utilized to identify the pathways from the IPA library of canonical pathways that were most significant. In addition, the significance of the association between the data set and the canonical pathway was calculated as the ratio of the number of genes from the data set that mapped to the pathway divided by the total number of genes that mapped to the canonical pathway.

Validation of results using quantitative real-time PCR

To confirm the ability of this microarray analysis to resolve the differences in expression levels, fifteen genes that showed significant differences in four experimental groups (Vitro_4-cell, Vitro_16-cell, Vivo_4-cell and IVP) compared to in vivo control group were selected and GAPDH was used as endogenous control. Sequence specific primers (Table 1) were designed using Primer Express v 2.0 (Applied Biosystems, Foster City, CA). Each pair of primers was tested to achieve efficiencies close to 1. Independent RT-PCR for the aRNA (3 replicates for each group) has been done and

quantitative real-time PCR was performed in a 20 µl reaction volume containing iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Munich, Germany), the cDNA samples and the specific forward and reverse primer in StepOnePlus™ real time PCR system (Applied Biosystem). The thermal cycling parameter was set as 95°C for 3 min, 40 cycles of 95°C for 15 s and at 60°C for 1 min. After the end of the last cycle, dissociation curve was generated by starting the fluorescence acquisition at 60°C and taking measurements every 7 s interval until the temperature reached 95°C. The comparative cycle threshold (CT) method was used to quantify expression levels as previously described (Bermejo-Alvarez et al. 2010).

Statistical analysis

Data were analysed using the Statistical Analysis System (SAS) version 8.0 (SAS Institute Inc., Cary, NC, USA) software package. Mean developmental rates of embryos were analysed by ANOVA followed by a multiple pair wise comparison (Tukey Test). The relative expression data were analysed using General Linear Model (GLM) of SAS. Differences in mean values were tested using ANOVA followed by a multiple pair wise comparison using t-test. Differences of $p< 0.05$ were considered to be significant.

Results

Embryo recovery and developmental rates

Alternative culture conditions (in vivo or in vitro) either before or after the time of EGA have no effect on the developmental rates, however the origin of the oocyte determines the developmental rates. Total developmental rates based on pre- and post-transfer development of in vitro originated embryos which transferred to in vivo conditions at 4-cell (Vitro_4-cell) or 16-cell (Vitro_16-cell) until day 7 were 24.1 and 27.5%, respectively. On the other hand, total developmental rates based on pre- and post-flushing development of in vivo originated embryos which flushed at 4-cell (Vivo_4-cell) or 16-cell (Vivo_16-cell) and cultured in vitro until day 7 were 64.4 and 68.2%, respectively. The percentage of total oocytes that developed to morula/blastocyst stage under complete in vitro conditions (IVP) was 33.6 % (Table 2). In general, development rates of in vivo originated embryos were significantly higher than in vitro originated ones ($P<0.05$). The lowest blastocyst:morula ratios have been found in Vitro_16-cell and IVP groups (0.2 and 0.6, respectively). However, the highest blastocyst:morula

ratio has been found in Vivo_4-cell group with 9.9 (Table 2). The morphology of bovine embryos produced in vivo and in vitro at different developmental stages is shown in Figure 2. In vitro-generated embryos tended to have a darker overall appearance at all stages compared to in vivo originated ones.

Global transcriptome analyses: transcription dynamics

Principal component analysis (PCA) revealed that the source of greatest variation in the transcriptional profile of the produced blastocysts was the in vitro culture conditions during the time of EGA. Blastocyst groups which spent the time of EGA under in vitro conditions, originated either from in vitro (Vitro_16-cell) or in vivo conditions (Vivo_4-cell), clustered away from the other groups (Figure 3). Compared to the complete in vivo control groups, these two groups showed higher number of differentially expressed genes (882 and 1918 DEGs, respectively) than those which spent the time of EGA under in vivo culture conditions, originated either from in vitro (Vitro_4-cell) or in vivo conditions (Vivo_16-cell) (633 and 311 DEGs, respectively), or than IVP group (841 DEGs). Numbers of common DEGs between different groups are presented in Supplemental Figure S1. Chromosome distribution of DEGs from all groups showed a higher percentage of genes to be located on X-chromosome and most of these genes were up-regulated in all blastocyst groups compared to complete in vivo blastocyst group (Figure 4).

Functional classification and pathway analyses

Ontological classification of DEGs based on the information in the IPA indicated that metabolic processes including lipid, carbohydrate, nucleic acid and amino acid, cell related functions including cell death, cellular growth and proliferation, cell cycle and cell-to-cell signalling, as well as embryonic development and gene expression processes were the most significant common functions affected in all blastocyst groups exposed to in vitro conditions compared to the in vivo group (Supplemental Table S1). Comparative analysis of DEGs in all groups (Figure 5) showed that lipid metabolism was the most significant biological function affected in Vitro_16-cell group with down-regulation for most of the genes involved in this function comparing to in vivo control group. An example of these genes is shown in one of the networks created from Vitro_16-cell group data set (Figure 6), as genes involved in metabolism of cholesterol

(*HSD17B7, CYP11A1*), metabolism of steroids (*HSD3B1, CYP11A1, APOA1*), excretion and translocation of lipid (*ABCC2*) and metabolism of lipid (*MSMO1, ANXA1, ANXA2*) were down-regulated. The same pattern of lipid metabolism genes has been found in Vitro_4-cell and IVP blastocyst groups but with lower number of genes involved. In contrast, Vivo_4-cell group showed opposite pattern in which most of DEGs involved in lipid metabolism were up-regulated compared to in vivo group. Cellular development related genes involved in cell death and apoptosis, gene expression and cellular proliferation were significantly up-regulated in the 2 blastocyst groups which spent the EGA time under in vitro culture conditions (Vitro_16-cell and Vivo_4-cell), irrespective to the origin of these blastocyst and they were highly significant in Vivo_4-cell group (Figure 5). Pathway analysis using IPA generated several pathways for each group with ratios ranging from 3 to 22% (proportion of DEGs involved in the pathway out of the total genes of this pathway) and p-value <0.05 (Table S2). NRF2-mediated oxidative stress response pathway was the dominant pathway in Vivo_4-cell group (Figure 7) and showed up-regulation for most of the involved genes, especially genes encoding for antioxidant enzymes. In this pathway, nuclear factor (erythroid-derived 2)-like 2 gene (*NFE2L2*, also known as *NRF2*) is the central gene which is acting as a transcriptional factor and regulating the expression of sets of genes including antioxidant, detoxifying, stress response and proteasomal degradation related genes. Antioxidant genes in this pathway (*PRDX1, SOD1, TXNRD1, GPX2, CAT*) showed an opposite pattern in the groups which spent EGA under in vitro culture conditions with up-regulation in Vivo_4-cell group and down-regulation in Vitro_16-cell group which showed also down-regulation of *NRF2* gene compared to in vivo control blastocyst group. Retinoic acid receptor activation pathways (LXR/RXR and FXR/RXR) and signalling pathways (ILK, and Integrin signalling) were the dominant pathways in Vitro_4-cell and Vitro_16-cell groups. Genes involved in retinoic acid receptor activation pathways were found to be down-regulated in Vitro_16-cell group. An indirect relationship between retinoic acid and different lipid metabolism genes are shown in the same network generated from Vitro_16-cell data set compared to in vivo group (Figure 6).

A group of 68 genes found to be exclusively differentially expressed in the two groups which spent the time of EGA under in vitro conditions (Vitro_16-cell and Vivo_4-cell). Most of these genes showed an opposite pattern between the two groups with 50 genes

up-regulated in Vivo_4-cell and down-regulated in Vitro_16-cell compared to the complete in vivo control group (Figure 8). Amongst these genes were a group of genes involved in lipid metabolism (*MSMO1, ANXA1, ANXA3, HMGCR, HSD17B11, LDLR, ACAT2*) and oxidative stress response (*PRDX1*).

Array data validation by quantitative real-time PCR

A total of 15 selected genes (Table 1) from microarray analysis were quantified in 3 biological replicates from control and four experimental groups by quantitative real-time PCR. Results showed the expression of the genes to be in agreement with the array results except in one case (*PGRMC1* in IVP group). All transcripts showed significant differences in expression ($P<0.05$) between each blastocyst group and in vivo control group (Figure 9).

Discussion

In this study, we analysed the transcriptome of bovine blastocysts developed under in vivo and in vitro alternative culture conditions during the embryonic genome activation (EGA). Indeed, combination of in vitro and in vivo culture systems during specific time points of early mammalian embryonic development should enhance our understanding of molecular mechanisms and pathways controlling early development and could highlight potential candidate genes which could be used as markers for competent embryos. Moreover, with this study we could be able to identify specific stage of bovine embryo development which is critically influenced by the culture environment. One of the most critical time points of preimplantation embryogenesis is the major EGA at which the embryo switches from using the mRNA and proteins derived from the maternal genome to those resulted from de novo transcription from the embryonic genome (Memili and First 2000). During that time, availability of transcription factors, which are regulated by cell cycle-dependent mechanisms, are required (Latham and Schultz 2001). These mechanisms are highly influenced by a change in environmental conditions and subsequently affected embryo quality with potentially severe effects on fetal, prenatal and postnatal viability (Niemann and Wrenzycki 2000).

Previous studies have shown that while the in vivo or in vitro origin of oocyte is the main factor determining embryo developmental rate (Hendriksen et al. 2000, Rizos et al. 2002b), the post fertilization culture environment is known to be the most important

factor determining the quality of the resulting embryos (Rizos et al. 2003, Rizos et al. 2002a). This is not only true when in vitro and in vivo culture systems are compared, but also when comparisons of different in vitro culture systems are made (Doherty et al. 2000, Fair et al. 2004, Natale et al. 2001). In agreement to these studies, our results showed that changing culture conditions from in vivo to in vitro or vice versa either before or after the time of major EGA have no effect on the developmental rates, however in vitro conditions during that time was critically influenced the gene expression pattern of the resulting blastocysts. The highest number of DEGs amongst all groups compared to in vivo produced blastocyst has been found in Vivo_4-cell group followed by Vitro_16-cell group, indicating the sensitivity of embryos towards in vitro culture conditions during EGA time. Although both groups spent the time of EGA under in vitro conditions, they showed opposite patterns for most of the differentially expressed transcripts. Ontological classification of these transcripts revealed that lipid metabolism and oxidative stress response related genes were the most affected genes and they were up-regulated in Vivo_4-cell and down-regulated in Vitro_16-cell groups.

Previous studies on mammalian oocytes and embryos reported that intracellular lipid contents may determine embryo quality, developmental potential and cryotolerance (Jeong et al. 2009, Nagano et al. 2006). During preimplantation embryo development, excess lipid may be sequestered within the cell and used by mitochondria for increased production of ATP required for compaction and blastocyst formation (Tarazona et al. 2006). However, one of the common characteristic of in vitro produced embryos is darker cytoplasm and a lower buoyant density as a consequence of lipid accumulation in these embryos compared with their in vivo counterparts (Abe et al. 2002, Rizos et al. 2002b). Lipids can accumulate in the embryo by uptake from the culture environment (McEvoy et al. 2001), or as a result of culture-induced impaired activity of mitochondria and their inability to metabolise complex lipids (Barcelo-Fimbres and Seidel 2007). In our results, a clear pattern of down-regulation for lipid metabolism related genes has been found in the in vitro originated groups with high significance level especially for the Vitro_16-cell group. This down-regulation indicated that embryos originated and cultured under in vitro conditions until the time of EGA were unable to use internal lipids for the production of ATP and this could be due to in vitro environmental factors impaired mitochondrial activity. This was also reflected on the morphology of in vitro originated embryos which showed a darker cytoplasm at

different developmental stages (Figure 2). However, in vivo originated groups showed up-regulation of lipid metabolism related genes and a clear transparent cytoplasm. This finding could highlight a critical influence of culture conditions during maturation and early developmental stages on the metabolic activity and the ability of embryos to adapt with changing culture conditions. Supporting to this argument, a recent study by Somfai et al. (2011) showed that supplementation of IVM medium with L-carnitine, ammonium compound play a primary role in the transportation of fatty acids from the cytosol into mitochondria, increased mitochondrial activity, enhanced lipid metabolism and improved cleavage rates in porcine embryos, suggesting the importance role of mitochondria and lipid metabolism in the maturation process and early development. Another study (Sturmey et al. 2006) reported that inhibition of fatty acid oxidation during IVM has been found to impair porcine embryo development, giving further evidence for the importance of lipid metabolism for developmental competence. This could explain the higher developmental rates for in vivo originated groups, as they had acquired in vivo the ability to metabolise lipids during culturing in vitro, compared to in vitro originated ones. Moreover, recent study in human embryos provided further evidence for a developmental role of lipoproteins in early embryonic development (Mains et al. 2011). In that study it is reported that apolipoprotein A-I (*APOA1*), the major lipoprotein of HDL cholesterol, is upregulated by human preimplantation embryos of higher morphologic grade. In our results, this gene was amongst the lipid metabolism related genes which were down-regulated in Vitro_16-cell blastocyst group compared to in vivo control group.

During oocyte maturation and early embryo development, various environmental stress factors such as oxidative stress can impair mitochondrial integrity and activity that results in either apoptosis or abnormal embryonic development (Kadenbach et al. 2004). Oxidative stress occurred as an imbalance between antioxidants and reactive oxygen species (ROS) production which is believed to be induced by endogenous and exogenous factors during in vitro culturing of embryos (Guerin et al. 2001). Enhanced embryo development under lower oxygen conditions is thought to be attributable to improved embryo metabolism and decreased ROS production (Du and Wales 1993).

Several defence mechanisms are present in embryos to protect against oxidative stress and to decreased level of ROS. One mechanism by which cells defend themselves against oxidative stress is through the transcriptional up-regulation of cytoprotective

genes like heat-shock proteins. Under oxidative stress conditions, the transcription factor NRF2 binds to the antioxidant response element (ARE) to induce antioxidant and phase II detoxification enzymes (Wild et al. 1999). Recent studies provided evidence that NRF2 function was not only associated with induction of genes involved in oxidative stress response, but also with lipogenic pathways that result in inhibition of lipid accumulation in mouse liver (Huang et al. 2010; Tanaka et al. 2008). However, the role of NRF2 in oxidative stress and lipid metabolism in preimplantation embryos has not yet been investigated. In the present study, NRF2-mediated oxidative stress response pathway was the dominant pathway in Vivo_4-cell blastocyst group with high ratio (13%) and up-regulation for most of the antioxidant related genes which are controlled by the transcription factor NRF2. This indicates that embryos flushed out and transferred to in vitro condition before the time of EGA were highly sensitive to oxidative stress compared to embryos which were transferred to in vitro conditions after the time of EGA (Vivo_16-cell group), which showed a much lower ratio (3%) of DEGs involved in this pathway. In contrast, Vitro_16-cell blastocyst group showed the opposite pattern of expression for the same pathway with significant down-regulation of *NRF2* and its down stream genes related to antioxidant and detoxification enzymes. These data emphasize the role of culture conditions before the EGA in determining embryonic ability to adapt with suboptimal culture conditions.

The oviduct and the uterus environment probably contain many substances that sustain embryo development or remove toxic factors. To mimic these conditions in vitro, different substances that decrease the concentrations of ROS in embryos have been added to culture. For example, addition of superoxide dismutase (SOD) or catalase to murine (Orsi and Leese 2001) and bovine (Iwata et al. 1999) embryos improved their development with up-regulation of glutathione (GSH) synthesis controlling the redox environment. Another example, the addition of retinoic acid as an antioxidant reagent to the oocyte maturation culture medium was found to be beneficial to improve subsequent development of porcine (Atikuzzaman et al. 2011) and bovine (Livingston et al. 2004) embryos. Interestingly, retinoic acid receptor activation pathways (LXR/RXR and FXR/RXR activation) were significantly affected in Vitro_4-cell and Vitro_16-cell groups with down-regulation for most of the genes involved in these pathways compared to in vivo control group. We have previously demonstrated that the transcript levels of retinoid X receptors (*RXRA, RXRB, RXRG*) were consistently lower in bad-

quality embryos in comparison with good-quality embryos (Mamo et al. 2005). In addition, the network generated from Vitro_16-cell group data set (Figure 6) showed the indirect relationship between retinoic acid and different lipid metabolism related genes. These results with the recent evidences on the function of *NRF2* gene in lipid metabolism described above, may suggest a crucial role of antioxidants during maturation and early stages of embryo development.

Epigenetic reprogramming during early embryonic development is an essential process to ensure a proper selection of the genes to be transcribed during EGA time (Baroux et al. 2008). Epigenetic based primarily on the methylation of the DNA sequence which controls imprinting, X chromosome inactivation (XCI), genome stability and tissue-specific expression (Bird and Wolffe 1999). During early preimplantation embryonic development, paternally imprinted XCI is established in all cells of XX embryos (Okamoto et al. 2004). Since in vitro production systems may have an influence on DNA methylation patterns which could interfere with the gene expression patterns and the XCI process and subsequently could compromise embryo quality and pregnancy rates (Ferreira et al. 2010, Lucifero et al. 2004, Shi and Haaf 2002). In our results, the chromosome distribution for the total differentially expressed transcripts in the five blastocyst groups showed a high number of DEGs to be located on X-chromosome and a high proportion of these genes were up-regulated in all 5 groups of blastocysts compared to in vivo control group (Figure 4). This may suggest a negative impact of in vitro culture conditions during preimplantation development on the DNA methylation pattern which lead to incomplete paternal XCI process. However, this point needs more investigation to provide more evidence of XCI disturbance under in vitro culture conditions in preimplantation bovine embryos.

In conclusion, this is the first comprehensive study to provide detail information on molecular mechanisms and pathways that are influenced by altered culture conditions during specific embryonic developmental time points. Our results evidenced the significant impact of in vitro culture conditions during the time of EGA on the transcriptome profile of bovine blastocyst which showed different patterns between distinct originated embryos. This data will improve our knowledge on regulation of embryonic development and could be used to launch new strategies to modify the in vitro culture conditions at this critical stage of development to enhance the development of competent embryos.

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Table 1 Details of primers used for real-time PCR quantitative analysis.

Gene	Accession number	Primer sequences	Annealing temperature (°C)	Product size (bp)
GAPDH	NM_001034034	F:acccagaagactgtggatgg R:acgcctgcttcaccacctt	60	247
CYP51A1	NM_001025319	F:gagctcatcgggagatcaag R:ccatccaggcactggtagtt	55	207
MSMO1	NM_001098863	F:caatcctctgcaggaaccat R:tccattggttttcccatgtt	51	209
ANXA1	NM_175784	F:aagcccctggatgaagttct R:ctgaggcgatgtctttagcc	55	244
PTGS2	NM_174445	F:cgatgagcagttgttccaga R:gaaagacgtcaggcagaagg	55	215
HSD17B11	NM_001046286	F:ggtgaaggcagaagttggag R:aagaaggggaccccagtatg	55	228
PRDX1	NM_174431	F:tggatcaacacacccaagaa R:gtctcagcgtctcatccaca	53	217
ABCC2	NC_007327	F:ctgattgggaacctgatcgt R:caacagccacaatgttggtc	54	172
HMOX1	NM_001014912	F:caaggagaaccccgtctaca R:ccagacaggtctcccaggta	56	225
SOD1	NM_174615	F:agaggcatgttggagacctg R:cagcgttgccagtctttgta	54	189
NFE2L2	NM_001011678	F:cccagtcttcactgctcctc R:tcagccagcttgtcattttg	54	165
RARRES1	NM_001075430	F:gcagtgtcaagcagtggaaa R:gaggcctcttctggtgtctg	55	163
IFNT2	NM_001015511	F:gcagtgcttcaacctcttcc R:tccttcccatgtcagagtcc	55	155
SFN	NM_001075912	F:cacccagaacctgaccactt R:gcagacatgctttccctctc	55	217
POU5F1	NM_174580	F:gttttgaggctttgcagctc R:ctccaggttgcctctcactc	55	182
PGRMC1	NM_001075133	F:gcctttgcatctttctgctc R:atgagtatacgcgggtcctg	54	163

Table 2 Overall data and transferable rates.

Group	Total	Pre trans/flush Development (PD)		Transfer	Post trans/flush Development (PP)						Total Development
		4-cell stage	16-cell stage		Subtotal	FR	Morula (Ma)	Blastocysts (Bla)	Mo/Bla	Bla : Mo	PD x PP
	N	(%)	(%)	N	N	(%)	N (%)	N (%)	N (%)	Ratio	(%)
Vitro_4-cell	1048	(61.3)	-	642	568	(88.5)	72 (12.7) [a]	151 (26.6) [b]	223 (39.3) [a]	2.1 [b]	(24.1) [A]
Vitro_16-cell	1667	-	(48.7)	811	620	(76.4)	284 (45.8) [c]	66 (10.6) [a]	350 (56.5) [b]	0.2 [a]	(27.5) [A]
IVP	681	-	-	-	681		146 (21.4) [b]	83 (12.2) [a]	229 (33.6) [a]	0.6 [a]	(33.6) [A]
Vivo_4-cell	280 *	(70.6)		-	95**		8 (8.4) [a]	79 (83.3) [c]	87 (91.6) [c]	9.9 [c]	(64.4) [B]
Vivo_16-cell	347 *	-	(72.3)	-	53**		13 (24.5) [b]	37 (69.8) [c]	50 (94.3) [c]	2.9 [b]	(68.2) [B]

*: total number of flushed embryos
**: only embryos of 4- or 16-cell stage embryos were evaluated for subsequent developmental capacity
Values with different superscript within colums differ significantly (a:b:c: $p<0.05$, A:B: $p< 0.01$, ANOVA, Tukey Test)
FR: Flushing rate

Table S1 The functional categories and the corresponding genes that were differentially expressed in blastocyst groups compared to in vivo group.

Group	Functional category	*p*-value[1]	Transcripts
Vitro_4-cell	Nucleic Acid Metabolism	2.35E-05 - 1.64E-02	*CRMP1, SLC23A1, TDG, UGP2, ERH, DPM1, UPP1, PRPSAP2, CDA, ELOVL6, ACOT8, PRNP*
	Cellular Development	4.47E-05 - 1.64E-02	*ENAH, CD55, LGALS4, IL6, APP, BARX2, CHEK1, CD47, GP1BA, HNF4A, ACADM, CITED2, PRNP, EFNA2, EPHB4, PAK4, IL4R, MAEL, CNP, TDGF1, RAC1, MAPK8, VIM, PAWR, KLF4, NDN, AVIL, EFNA1, ABCC2, IL18, NANOG, RND3, ZAP70, LFNG, IL2RA, EDN3, PLAU, AKR1B1, SFN, CXCL2, ITGA7, A2M, POU5F1, GZMB*
	Cell-To-Cell Signaling and Interaction	8.37E-05 - 1.64E-02	*CD55, LRP6, LGALS4, IL6, APP, CD47, GP1BA, CDC42EP1, DMBT1, PRNP, PHB, EFNA2, IL4R, RHOC, RAC1, MAPK8, VIM, KLF4, EFNA1, IL18, CDH2, HSPG2, LTA, SERPINA5, ZAP70, SNX1, TFRC, IL2RA, LFNG, PLAU, CXCL2, DSG2, ITGA7, A2M, POU5F1, GZMB*
	Protein Synthesis	1.41E-04 - 1.64E-02	*ENAH, IL18, CD47, RHOQ, NCKIPSD, RAC1, CDC42EP1*
	Cell Death	1.95E-04 - 1.64E-02	*CHRNA1, DNMT3A, UNC5B, LGALS4, IL6, DNAJC15, LMNB1, HNRNPA1, IP6K3, AHSA1, VAMP3, JUND, CALB1, PHB, EFNA2, IL4R, YWHAE, CNP, RAC1, TDGF1, PAWR, KLF4, NDN, RASD1, SLAMF6, ITPK1, IL18, CDH2, NANOG, RND3, TFRC, SRXN1, ITGA7, INPP1, LRP6, CD55, RAD9A, FKBP1A, SYCP3, ASNS, APP, PTPN5, CHEK1, AQP3, CD47, UBE2K, MAP1LC3B, DUSP14, HNRNPC, CITED2, PRNP, PAK4, EPHB4, MAEL, RHOC, ELL, MAPK8, SCIN, EFNA1, SERPINB4, LTA, LINGO1, TEX11, IL2RA, PLAU, CXCL2, SFN, A2M, DSG2, AMIGO2, GZMB, POU5F1, PINK1*
	Cell Cycle	2.67E-04 - 1.64E-02	*FKBP1A, RAD9A, LGALS4, SYCP3, IL6, ASNS, APP, CHEK1, CARD10, JUND, HNF4A, CITED2, PRNP, PHB, IL4R, MAEL, YWHAE, NUBP1, MAPK8, RAC1, PAWR, SCIN, KLF4, DDB1, KIF15, NANOG, TEX11, TFRC, LFNG, ASCL2, EDN3, PLAU, SFN, AKR1B1, A2M, TM4SF1*
	Embryonic Development	7.92E-04 - 1.64E-02	*EPHB4, RHOC, LRP6, TDGF1, RAC1, MAPK8, IL6, APP, CHEK1, IL18, NANOG, ASCL2, PLAU, EDN3, CCL16, CITED2, POU5F1*

Group	Functional category	*p*-value[1]	*Transcripts*
Vitro_4-cell	Gene Expression	2.58E-03 - 1.64E-02	*YWHAE, APBB1IP, EID1, ELL, LRP6, ADCY6, MAPK8, RAC1, ETV1, IL6, HNRNPA1, IL18, ERBB2IP, CARD10, ZAP70, IL2RA, JUND, CXCL2, HNF4A, A2M, CITED2, POU5F1, PRNP*
	Lipid Metabolism	2.58E-03 - 1.64E-02	*ARV1, BCO2, SC4MOL, FKBP1A, IL6, APP, ELOVL6, ABCC2, SUCLA2, RHOQ, FADS2, SLC27A3, HNF4A, FADS1, ACOT8*
	Post-Translational Modification	2.99E-03 - 1.63E-02	*EFNA2, PRMT3, DPM1, CD55, FKBP1A, IL6, DDB1, APP, EFNA1, IMPACT, CD47, TCEB2, SUV420H2, CARD10, ZAP70, NHLRC1, UBE2E1*
	Amino Acid Metabolism	3.84E-03 - 8.91E-03	*CTNS, ENPEP, SLC1A4, NAALAD2*
	Molecular Transport	3.84E-03 - 1.64E-02	*CTNS, ABCC2, HSPG2, SLC1A4, YWHAE, OSTM1, TFRC, PAWR, HNF4A, NHLRC1, PHAX, APP*
Vitro_16-cell	Lipid Metabolism	4.1E-07 - 2.24E-02	*LTA4H, GSTP1, ACAT2, IDI1, XDH, MYC, FDFT1, HRH1, ALDH1A1, GPD2, CHPT1, SERPINE1, CALB1, SLCO2B1, BDH2, SC4MOL, ANXA2, ELOVL1, ABCC2, IGF2, IL1RN, SLC22A5, FADS2, AKR1B1, EEA1, SCD, SQLE, IL1A, HSD3B1, SLC10A1, APOA1, RLBP1, UGCG, GK, HMOX1, GPC1, INSIG2, ANXA1, PI4K2B, AGPAT9, NFE2L2, CYP51A1, PLSCR1, PRNP, CYP11A1, STX12, LIPH, PDZK1, HSD17B7, ACADSB, SEPP1, FDPS, B4GALT6, LDLR, FABP5, S1PR1, PSAP, PLAU, PTGS2, HMGCR, PTGR1, LGALS1*
	Vitamin and Mineral Metabolism	4.1E-07 - 3.14E-04	*CYP11A1, SCD, SQLE, HSD3B1, ACAT2, IDI1, APOA1, RLBP1, HSD17B7, FDPS, FDFT1, LDLR, INSIG2, ALDH1A1, CALB1, HMGCR, CYP51A1*
	Cellular Development	3.55E-05 - 2.02E-02	*RAC2, LGALS3, LHX2, XDH, CCL17, NCK1, NID1, MYC, HRH1, ALDH1A1, EZR, SMARCB1, RNF128, SERPINE1, DSP, BDH2, RAB27A, TDGF1, ANXA2, TPD52, KLF13, RAP1A, KLF4, NDN, ABCC2, IGF2, NANOG, GAB1, IL1RN, GFI1, ZAP70, AKR1B1, AGRN, CYR61, CDK5RAP2, SCD, HIST1H1C, IL1A, RAB1A, EPB41L3, MCC, AKTIP, HSPB8, HIF1A, CLIC4, KLHL20, HMOX1, GPC1, WASL, TFAP2A, ANXA1, SLC11A2, STAP2, ARID3A, PLAC8, NFE2L2, PLSCR1, PEX11A, PRNP, MUC1, ENPP1, KHDRBS1, TXNIP, GABBR1, SCIN, ERBB3, KIFAP3, EFNA1, ERBB2IP, FABP5, ENO1, S1PR1, PLAU, PTGS2, SFN, A2M, CTNND1, LGALS1*

Group	Functional category	*p*-value[1]	*Transcripts*
Vitro_16-cell	Cell Death	3.85E-05 - 2.24E-02	*RAC2, GSTP1, LGALS3, XDH, NCK1, DNAJC15, GSTZ1, MYC, FDFT1, ALDH1A1, EZR, KRT18, SMARCB1, SERPINE1, CALB1, SH3KBP1, DSP, S100A10, TNFRSF21, DDIT4, TIA1, TDGF1, TPD52, KLF13, KLF4, NDN, EI24, UBD, ANKRD1, IGF2, GAB1, IL1RN, GFI1, SRXN1, GNG2, CYR61, HIST1H1C, SCD, IL1A, APOA1, PRDX1, AKTIP, HSPB8, UGCG, HIF1A, NUAK2, CLIC4, GLIPR1, PTPN5, PTPRF, LOXL2, HMOX1, GPC1, CD5L, KRT19, TFAP2A, ANXA1, ZFAND5, NFE2L2, PLAC8, CCBL1, GMFB, PRNP, PLSCR1, MUC1, MAP4K2, PDZK1, DUT, SDC1, KHDRBS1, TXNIP, GABBR1, ERBB3, SCIN, KIFAP3, EFNA1, LDLR, SWAP70, ENO1, LGALS3BP, CIDEB, S1PR1, PROCR, PSAP, TEX11, PTGS2, PLAU, CTH, SFN, A2M, TXNDC17, PINK1, LGALS1, AKAP1*
	Cell-To-Cell Signaling and Interaction	7.24E-05 - 2E-02	*IL1A, LGALS3, APOA1, ACTA2, CCL17, NCK1, HIF1A, PTPRF, MYC, HRH1, GPC1, ANXA1, SPAM1, SERPINE1, NFE2L2, PRNP, S100A10, MUC1, SDC1, FERMT2, ANXA2, ERBB3, SNAP25, RAP1A, P2RY2, IGF2, LDLR, TRIM63, IL1RN, LGALS3BP, GFI1, S1PR1, GNG2, PTGS2, PLAU, CYR61, PTPRA, A2M, CTNND1, LGALS1, EEA1*
	Carbohydrate Metabolism	3.65E-04 - 2.15E-02	*SCD, SLC10A1, APOA1, HIF1A, TPI1, GK, PTPRF, MYC, FUT8, SLC23A1, GPD2, CXCL14, SMARCB1, TKTL1, CHPT1, DHDH, SPAM1, SERPINE1, STX12, PFKFB3, NAGPA, ERBB3, CHST12, ITPKA, SEPP1, IGF2, MGAT4A, FABP5, IL1RN, PTGS2, PLAU, AKR1B1*
	Embryonic Development	5.01E-04 - 1.28E-02	*RAC2, IL1A, ENPP1, XDH, EFNA1, HMOX1, HRH1, IGF2, KRT19, GAB1, IL1RN, KRT18, PTGS2*
	Gene Expression	5.01E-04 - 2.24E-02	*MYC, LGALS3, NFE2L2, LGALS1*
	Cellular Growth and Proliferation	5.1E-04 - 2E-02	*RAC2, XDH, KLK6, NCK1, BCAT1, HRH1, URGCP, EZR, SMARCB1, RNF128, SERPINE1, THEM4, TNFRSF21, ANXA2, TPD52, NDN, ABCC2, GAB1, GFI1, FADS2, GNG2, CYR61, PTPRA, PRDX1, HSPB8, CXCL5, TFAP2A, STARD13, STAP2, PLAC8, PDZK1, IGF2BP3, ENPP1, KHDRBS1, TXNIP, EFNA1, KIF15, B4GALT6, SWAP70, FABP5, ENO1, IMPDH1, S1PR1, PSAP, PTGS2, CTH, PLAU, SFN, CTNND1, GSTP1, LGALS3, NUDC, MYC, FDFT1, ALDH1A1, TNN, CRIP1, LAMA1, IFI30, DSP, CALM1, PFKFB3, TDGF1, NUMBL, KLF4, EI24, ANKRD1, IGF2, NANOG, IL1RN, ZAP70, LIMA1, AKR1B1, IL1A, RAB1A, EPB41L3, APOA1, AKTIP, MCFD2, RARRES1, UGCG, HIF1A, PTPRF, GPC1, HMOX1, ANXA1, ARID3A, SPAM1, PLSCR1, PRNP, MUC1, UPP1, QSOX1, GABBR1, SCIN, ERBB3, KIFAP3, ZDHHC17, ERBB2IP, NFIB, YME1L1, HMGCR, A2M, LGALS1*

Group	Functional category	*p*-value[1]	*Transcripts*
Vitro_16-cell	Cell Cycle	5.29E-04 - 2.24E-02	*IL1A, LGALS3, KPNA2, HIF1A, MYC, BCAT1, HMOX1, GPC1, KRT19, SMARCB1, PLAC8, PRNP, BANP, TXNIP, ERBB3, SCIN, KLF4, UBD, IGF2, GAB1, GFI1, S1PR1, TEX11, PSAP, PTGS2, PLAU, SFN, CYR61, PTPRA, TM4SF1, LGALS1, CTNND1*
	Cellular Function and Maintenance	1.1E-03 - 2.03E-02	*HIST1H1C, RAC2, IL1A, LGALS3, LHX2, AKTIP, CCL17, HIF1A, NCK1, CKB, MYC, WASL , EZR, ANXA1, DSTN, RNF128, SLC40A1, STAP2, DSC2, NFE2L2, PRNP, MUC1, ERBB3, KLF13, KIFAP3, EFNA1, P2RY2, ABCC2, IGF2, IL1RN, GFI1, ZAP70, S1PR1, PTGS2, CYR61, PTPRA, LGALS1*
	Molecular Transport	1.27E-03 - 2.24E-02	*SCD, IL1A, SLC10A1, ACAT2, APOA1, XDH, HIF1A, PTPRF, MYC, HRH1, FDFT1, HMOX1, INSIG2, GPD2, ANXA1, SLC11A2, SLC40A1, AGPAT9, SERPINE1, NFE2L2, SLCO2B1, PRNP, STX12, CYP11A1, GCH1, PDZK1, GGCT, ERBB3, ABCC2, IGF2, LDLR, IL1RN, SLC16A1, SLC22A5, FADS2, PSAP, PTGS2, PLAU, SFN, HMGCR, AKR1B1, EEA1, LGALS1*
IVP	Lipid Metabolism	8.54E-05 - 3.34E-02	*PDPN, SCD, HSD3B1, IL1A, CLN8, C3ORF57, BCO2, FKBP1A, F2, HMOX1, SUCLA2, ALDH1A1, PRKAA1, FAR2, CALB1, MUC1, CYP11A1, SC4MOL, ELOVL6, ABCC2, SCP2, SREBF2, AKR1B10, FADS2, NR5A2, CPNE7, PLAU, SLC27A3, PTGR1, FADS1*
	Cellular Development	1.53E-04 - 3.59E-02	*UPK3A, F2, ALDH1A1, AHSA1, EZR, LAMA1, C1QBP, TMBIM1, PEG3, DMBT1, EFNA2, PRKCQ, TWIST2, EID1, OSTM1, DDX4, TDGF1, CITED1, KLF4, RASD1, OTX2, ABCC2, HSPG2, PPP1R13L, GAB1, ZAP70, FADS2, LFNG, AGRN, NOTCH1, ITGA7, PDPN, RAP1B, SCD, IL1A, EPB41L3, PA2G4, NID2, NFKB1, HNRNPK, BARX2, CCNA2, HMOX1, NFKBIA, NLK, TFAP2A, GP1BA, LAMP1, CITED2, PRNP, MUC1, PAK4, EPHB4, AGFG1, MAEL, S1PR2, SDC1, SYNE2, MAPK8, GABBR1, SCIN, AVIL, EFNA1, TRPM6, F11R, ERBB2IP, ENO1, LTA, ASCL2, PLAU, SFN, CXCL2, A2M, GZMB*
	Embryonic Development	1.53E-04 - 3.54E-02	*SBDS, EPHB4, SLC31A1, DNMT3A, LRP6, TDGF1, MAPK8, RAD9A, F2, EFNA1, PLCD3, KRT19, GINS1, TFAP2A, GAB1, TPM3, KRT18, LAMA1, NR5A2, LFNG, ASCL2, NOTCH1, CITED2, DMBT1*

Group	Functional category	*p*-value[1]	*Transcripts*
IVP	Cell Death	4.28E-04 - 3.56E-02	*SLC31A1, CLN8, DNMT3A, DNAJC15, GRIA4, F2, PLCD3, ALDH1A1, AHSA1, EZR, KRT18, PRKAA1, C1QBP, CALB1, SH3KBP1, PEG3, EFNA2, PRKCQ, YWHAE, DDX4, TDGF1, PDE4B, KLF4, RASD1, OTX2, UBD, SLAMF6, ITPK1, PPP1R13L, GAB1, SCP2, SRXN1, GNG2, AGRN, ITGA7, NOTCH1, SCD, IL1A, INPP1, PA2G4, LRP6, CD55, RAD9A, FKBP1A, SYCP3, NFKB1, PTPN5, RPLP0, PTPRF, CCNA2, HMOX1, NLK, KRT19, PSMG2, NFKBIA, TFAP2A, RNPS1, LAMP1, MAP1LC3B, CITED2, PRNP, MUC1, EPHB4, PAK4, SDC1, S1PR2, MAEL, PPP1R8, RHOC, ELL, MAPK8, CD48, GABBR1, SCIN, PLK1, LYAR, FCN2, EFNA1, SERPINB4, TPM3, SREBF2, ENO1, LTA, EEF1A1, MSH6, TEX11, PLAU, SFN, CXCL2, A2M, PTGR1, GZMB*
	Cell Cycle	1.61E-03 - 3.56E-02	*IL1A, YWHAE, MAPK8, RAD9A, PLK1, NFKB1, DDB1, KLF4, PTPRF, F2, UBD, CCNA2, HMOX1, NFKBIA, ASCL2, PLAU, SFN, NOTCH1, PRNP*
	Cellular Growth and Proliferation	2.16E-03 - 3.3E-02	*TRIM24, F2, CTSZ, PLCD3, ALDH1A1, CRIP1, LAMA1, PRKAA1, IFI30, SCPEP1, C1QBP, PRKCQ, DDX4, TDGF1, CITED1, KLF4, RASD1, OTX2, ABCC2, HSPG2, GAB1, LIMA1, ZAP70, AKR1B10, GNG2, NOTCH1, ITGA7, RAP1B, PDPN, ENPEP, IL1A, PA2G4, PRDX4, LRP6, RAD9A, FKBP1A, IL20RA, NFKB1, PTPRF, HNRNPK, CCNA2, HMOX1, NFKBIA, CITED2, PRNP, MUC1, EPHB4, S1PR2, PPP1R8, RHOC, MAPK8, CD48, QSOX1, GABBR1, SCIN, PLK1, IL20RB, EFNA1, F11R, KIF15, ERBB2IP, TACC2, TPM3, ENO1, LTA, EEF1A1, NR5A2, ASCL2, PLAU, CXCL2, SFN, A2M, CARM1, GZMB*
	Carbohydrate Metabolism	2.5E-03 - 3.28E-02	*SCD, GNPDA1, IL1A, SCP2, ENO1, LTA, MAPK8, PRKAA1, C1QBP, TPI1, F2*
	Gene Expression	6.07E-03 - 3.53E-02	*TRIM24, DNMT3A, ETV1, F2, SNRPC, PRKCQ, TWIST2, YWHAE, EID1, TDGF1, SPIC, SAP30, CITED1, DDB1, KLF4, OTX2, BRD7, DDX6, ATXN7L3, ZAP70, GNG2, AGRN, NOTCH1, IL1A, PA2G4, LRP6, RAD9A, ZNF219, FKBP1A, NFKB1, ZBTB33, HNRNPK, BARX2, CCNA2, NLK, NFKBIA, RNPS1, TFAP2A, SORBS3, CITED2, PRNP, MUC1, MAEL, S1PR2, TDG, APBB1IP, PPP1R8, TAF15, RHOC, ELL, ADCY6, MAPK8, ZNF345, ERBB2IP, SREBF2, ENO1, LTA, LRRFIP1, RCOR2, EEF1A1, NR5A2, PLAU, CXCL2, A2M, STAG2, CARM1*
	Molecular Transport	6.07E-03 - 3.54E-02	*CTNS, SCD, IL1A, AGFG1, SLC1A4, SLC31A1, YWHAE, U2AF2, F2, RAE1, ABCC2, HMOX1, HSPG2, SCP2, FADS2, NR5A2, C1QBP*

Group	Functional category	*p*-value[1]	*Transcripts*
IVP	Cell-To-Cell Signaling and Interaction	3.34E-03 - 3.55E-02	*RAP1B, IL1A, CD55, F2, NFKBIA, GP1BA, CDC42EP1, C1QBP, DMBT1, PAK4, SDC1, S1PR2, PRKCQ, RHOC, FERMT2, CD48, EFNA1, F11R, HSPG2, TPM3, LTA, ZAP70, SNX1, GNG2, PLAU, AGRN, CXCL2, ITGA7, NOTCH1, GZMB*
	Amino Acid Metabolism	6.07E-03 - 2.07E-02	*CTNS, ALDH4A1, ENPEP, SLC1A4, NAALAD2, GABBR1*
	Cellular Function and Maintenance	6.07E-03 - 2.87E-02	*AGFG1, PRKCQ, PTPRF, F2, EFNA1, CKB, ABCC2, KRT18, EZR, ZAP70, C1QBP, PLAU, NOTCH1, PRNP*
Vivo_4-cell	Cellular Growth and Proliferation	2.95E-07 - 1.4E-02	*XDH, IL6, PTPRC, LAMC1, LUM, JARID2, SERPINE1, HNF4A, AHCY, PDIA5, EGLN2, AKT2, IL4R, PRKCQ, JAG2, PRKRIR, OTX2, NDNL2, CTSL1, ARAF, FADS2, TFRC, PRDX1, PRDX4, RBBP7, HSPB8, MTCH1, CD47, GADD45A, NDUFAB1, NGFR, CD38, CASP8, TMSB4X, ABCB1, EPHB4, GPN3, MECP2, RHOC, VIM, MAD2L1, LDLR, IMPDH1, SHMT1, SLC6A4, PIN1, GZMB, LOXL4, ARL6IP5, PTTG1, CCT2, CSNK1A1, LIMK1, CTSD, ANXA11, JUND, CAPZA1, SMAD1, TSPAN31, TJP2, THBS1, EPOR, CNP, TDGF1, BAMBI, DNAJC2, DDB1, FOSB, APBB1, ANKRD1, DUSP1, AKR1B10, ZAP70, IGFBP3, EPCAM, CAPN2, ACTN4, DNAJB6, S100A11, B2M, PEBP1, TNFAIP8, CRLF2, GTF2I, NRG1, MCFD2, RAD9A, CCL24, ETS2, IL20RA, PDCD10, APP, WDR6, MLH1, HNRNPK, TOM1L1, CCNA2, BAI1, ARID3A, ENPP2, LASP1, SOD1, UPP1, SMARCE1, CD36, APAF1, ERO1L, STK3, SKP1, SHB, TACC2, PSMD2, CAT, WNK1, CXCL2, A2M, GATA4, CTGF, TCIRG1, TYRO3, POMC, EEF1D, RFFL, CCNG1, CTSL2, AHSA1, BAG1, EZR, PSMD10, MGAT4B, PDXK, MCL1, TIMP2, NDRG1, RBM3, DDX4, RPA1, MMP2, L1CAM, LDOC1, CDH2, HSPG2, BSG, ACP1, RTN4, SPINT2, HPRT1, GNG2, EDN3, ITGA7, NOTCH1, LDHA, CASC3, ENAH, EMILIN2, PPP1R12C, PTPN13, CD55, LRP6, CTNNA1, FKBP1A, NAA35, RCHY1, STARD13, BID, TBX2, FKBP5, CITED2, GSTM1, PDZK1, ENPP1, PPP1R8, ELL, MAPK8, PLK1, TSG101, CDK1, EFNA1, FIS1, F11R, KIF20B, SPRY2, CNN1, LTA, NR5A2, IHH, HOXB4, IL2RA, PTGS2, FBN2, UTP6, GAB2, CHRNA1, MAPK1, UNC5B, SHFM1, LGALS4, CDA, TOP1, RPS3A, TNN, ALDH1A1, LAMB1, PRKAA1, UBE2E3, CDC37, EID2, CLTC, RAC1, SH3BP2, PAWR, BCAR3, RASD1, EIF2AK4, IL33, PRDX3, IL18, NANOG, S100B, PTPRS, AK2, FADS1, PDPN, EPB41L3, PA2G4, SAT1, DTYMK, NFKBIA, HNRNPR, PARK7, MAP3K7, ANXA1, GMNN, MMP12, PIK3IP1, PLSCR1, PAK4, DAP, TFAP2C, S1PR2, CD48*

Group	Functional category	*p*-value[1]	*Transcripts*
Vivo_4-cell	Cell Death	4.06E-06 - 1.52E-02	*XDH, DNAJC3, IL6, PTPRC, VAMP3, SERPINE1, EGLN2, IL4R, AKT2, JAG2, PRKCQ, PRKRIR, OTX2, NDNL2, SCP2, PPP2R2B, TFRC, PRDX1, HSPB8, NUF2, AQP3, CD47, NAIP, GADD45A, NGFR, NDUFAB1, ZFAND5, UBE2K, CD38, CASP8, RAD21, TMSB4X, ABCB1, EPHB4, MECP2, RHOC, APBB3, SAP18, MAD2L1, SERPINB4, LDLR, MSH2, PIN1, AMIGO2, GZMB, PTTG1, CCT2, CSNK1A1, DNAJC15, CTSD, BLOC1S2, PRR13, JUND, SMAD1, TJP2, YWHAE, THBS1, EPOR, CNP, TDGF1, PDE4B, FOSB, C14ORF153, APBB1, ANKRD1, RND3, DUSP1, IGFBP3, DNAJB6, ACTN4, S100A11, THOC5, TNFAIP8, PEBP1, B2M, CRLF2, NRG1, MCFD2, MPG, RAD9A, ETS2, CLIC4, EIF2S1, PDCD10, APP, MLH1, CCNA2, EGLN1, KRT19, MAEL, SOD1, ATF1, PTP4A2, CD36, SMARCE1, APAF1, UGT8, STK3, CAT, TEX11, CXCL2, A2M, GATA4, CTGF, DENND4A, TYRO3, SH3BGRL3, POMC, MDH1, GRIA4, GLO1, EEF1D, CCNG1, CTSL2, NDUFS1, CD2AP, AHSA1, BAG1, EZR, PDXK, TIMP2, MCL1, EFNA2, WAPAL, TWIST2, ZC3HC1, NDRG1, DDX4, L1CAM, MMP2, RPA1, CDH2, BSG, TMX1, RTN4, SPINT2, HPRT1, GNG2, NOTCH1, ITGA7, LDHA, AZI2, INPP1, EMILIN2, PTPN13, LRP6, CD55, CTNNA1, NRF1, FKBP1A, NAA35, SYCP3, RCHY1, ASNS, PTPN5, PSMG2, LAMP1, BID, FAIM, MAP1LC3B, DUSP14, FKBP5, CITED2, GSTM1, PDZK1, PPP1R8, ELL, SPC25, MAPK8, PLK1, TSG101, CDK1, EFNA1, FIS1, SREBF2, LTA, SPRY2, IHH, IL2RA, PTGS2, GAB2, CHRNA1, MAPK1, CLN8, DNMT3A, UNC5B, SHFM1, LGALS4, PLA2G7, LMNB1, TOP1, WDR85, ALDH1A1, RPS3A, ADCY5, SUB1, PRKAA1, M6PR, CDC37, RAC1, PAWR, RASD1, IL33, EIF2AK4, ITPK1, PRDX3, IL18, S100B, UTP11L, CREBL2, PA2G4, SAT1, DTYMK, NFKBIA, PARK7, MAP3K7, ANXA1, GMNN, PEBP4, MMP12, ACTC1, PIK3IP1, PLSCR1, TFAP2C, DAP, PAK4, S1PR2, GSTA1, PERP, CD48, NDUFS3, ATP6AP2, GNAS,*
	Gene Expression	7.35E-06 - 1.52E-02	*CTGF, TLE1, POMC, IL6, PAIP2, EEF1D, THRAP3, BAG1, JARID2, STK16, SERPINE1, HNF4A, AKT2, JAG2, PRKCQ, TWIST2, BCOR, NUFIP1, REEP5, RAP1A, OTX2, BRD7, NDNL2, ATXN7L3 (includes EG:56970), GNG2, SLC44A2, NOTCH1, AZI2, LRP6, CD55, RBBP7, FKBP1A, NRF1, ABLIM1, ZXDC, NAA15, MACF1, CD47, GADD45A, GRINL1A, CARD10, TBX2, FKBP5, CASP8, RAD21, CITED2, RSF1, MECP2, TDG, RHOC, PPP1R8, ELL, MRPL12, ADCY6, MAPK8, TSG101, CDK1, SAP18, TRAPPC2, SREBF2, LTA, NR5A2, IHH, IL2RA, HOXB4, PIN1, PTGS2, GAB2, POLR2D, MAPK1, DNMT3A, PTTG1, ETV1, LGALS4, TOP1, BLOC1S2, SUB1, UCHL5, JUND, SMAD1, CDC37, EPOR, EID2, EID1, RAC1, TDGF1, PAWR, SNW1, DDB1, CCRN4L, IL33, FOSB, APBB1, IL18, ANKRD1, DDX6, NANOG, DUSP1, ZAP70, IGFBP3, ELP4, LCOR, DNAJB6, MTDH, TAGLN2, CREBL2, PEBP1, NR2C1, RBM25, TFEB, PA2G4, GTF2I, NRG1, MPG, RBBP8, MEIS2, ETS2, ZNF219, BTF3, EIF2S1, APP, HNRNPK, TOM1L1, BARX2, CCNA2, AP1G2, NFKBIA, PARK7, MAP3K7, SENP3, PSMG4, ARID3A, SORBS3, SUDS3, NFYB, ENY2, TFAP2C, S1PR2, MAEL, APBB1IP, ATF1, TAF15, SMARCE1, STRAP, GNAS, HRSP12, ZNF345, ERBB2IP, ZNF326, LRRFIP1, RCOR2, WNK1, IL10RB, NKRF, UTP15, CXCL2, A2M, CARM1, GATA4*

Group	Functional category	*p*-value[1]	*Transcripts*
Vivo_4-cell	Cell Cycle	1.41E-05 - 1.52E-02	*POMC, IL6, RFFL, PTPRC, CCNG1, CD2AP, CENPE, JARID2, HNF4A, AHCY, TIMP2, IL4R, PRKCQ, ZC3HC1, RPA1, ARAF, PPP2R2B, TFRC, EDN3, SGOL1, NOTCH1, RAB35, FKBP1A, NUP214, SYCP3, NUF2, RCHY1, ASNS, CETN2, PSMG2, GADD45A, CARD10, PROS1, NGFR, RHOU, BID, CD38, ZWILCH, RAD21, CITED2, GPN3, APBB3, SPC25, MAPK8, PLK1, TSG101, CDK1, MAD2L1, FIS1, MSH2, SPRY2, KIF20B, HOXB4, PTGS2, PIN1, TM4SF1, GAB2, MAPK1, PTTG1, TOP3A, CSNK1A1, LGALS4, LIMK1, TOP1, DSTN, SUGT1, JUND, YWHAE, THBS1, EPOR, RAC1, SH3BP2, DNAJC2, PAWR, DDB1, APBB1, NANOG, DUSP1, IGFBP3, LFNG, PEBP1, PDPN, SH3BP4, PA2G4, GTF2I, NRG1, RBBP8, MPG, RAD9A, DTYMK, WDR6, APP, MLH1, PFDN1, CCNA2, KRT19, NFKBIA, MAP3K7, CCDC99, ANXA1, MPHOSPH6, GMNN, ARID3A, SOD1, MAEL, S1PR2, SMARCE1, APAF1, GNAS, CENPI, CAT, PNPT1, TEX11, A2M*
	Amino Acid Metabolism	1.42E-04 - 1.16E-02	*MAPK1, GCSH, MAPK8, SHMT1, PCYOX1, GLDC, GATM, LIMK1*
	Post-Translational Modification	1.42E-04 - 1.16E-02	*MAPK1, UBE2L3, XDH, DPM1, IL6, LIMK1, RFFL, PTPRC, FBXW2, ALDH1A1, NHLRC1, EFNA2, PDIA5, AKT2, EPOR, THBS1, STK38L, LMAN1, RPN1, UGT1A1, DDB1, UBL4A, CAND1, ERCC8, ERP29, TPST1, BSG, ARAF, DUSP1, ZAP70, ACP1, S100B, ERO1LB, IGFBP3, CAPN2, DNAJB6, SUV420H1, UBE2E1, PEBP1, EPB41L3, PTPN13, PRDX1, PRDX4, NRG1, CD55, FKBP1A, RCHY1, APP, PTPN5, CD47, PARK7, CARD10, MAP3K7, SENP3, CD38, FKBP5, PFDN4, GATM, STT3B, PPME1, MECP2, CD36, CD48, MAPK8, ERO1L, VWF, CANX, SENP2, RELN, TSG101, CDK1, EFNA1, SHB, SUV420H2, GCSH, CAT, GLDC, CARM1*
	Cell-To-Cell Signaling and Interaction	7.54E-04 - 1.2E-02	*GAB2, CTGF, MAPK1, ITGB1BP1, TYRO3, JAM2, LGALS4, IL6, PTPRC, CTSL2, LAMC1, CD2AP, BAG1, LAMB1, SERPINE1, CCL16, DMBT1, AKT2, JAG2, PRKCQ, THBS1, RAB13, TDGF1, RAC1, MMP2, L1CAM, RAP1A, IL33, CDH2, IL18, HSPG2, SELP, BSG, RND3, TRIM63, ACP1, ZAP70, IGFBP3, EPCAM, CAPN2, GNG2, EDN3, ACTN4, C1GALT1, NOTCH1, ITGA7, PDPN, NID2, NRG1, CD55, CTNNA1, APP, ITGAE, CD47, NFKBIA, ANXA1, GP1BA, BAI1, CD38, ROBO2, CASP8, TMSB4X, PAK4, S1PR2, RHOC, PERP, CD48, CD36, TPM2, VIM, VWF, RELN, F11R, MAD2L1, TPM3, SPRY2, CDH17, SLC6A4, IL2RA, PTGS2, CXCL2, DSG2, GZMB*
	Embryonic Development	1.2E-03 - 1.29E-02	*SBDS, TFAP2C, NDRG1, MAPK1, LRP6, RAC1, TDGF1, ETS2, STK3, LAMC1, GINS1, IHH, SMAD1, DMBT1*

Group	Functional category	*p*-value[1]	*Transcripts*
Vivo_4-cell	Lipid Metabolism	1.49E-03 - 1.49E-02	*ACAT2, CLN8, ARV1, XDH, C3ORF57, POMC, IL6, PLA2G7, ALDH1A1, FAR2, PRKAA1, SULT1E1, HNF4A, M6PR, ACSL3, IMPA1, PON2, THBS1, PEX7, RAC1, SC4MOL, UGT1A1, PRDX3, PIP5K1A, NUDT7, SCP2, AKR1B10, SLC22A5, DLD, FADS2, CPNE7, ACOT4, PNLIP, FADS1, PDPN, HAO2, APP, SUCLA2, ANXA1, LAMP1, ENPP2, ABCB1, SOD1, MECP2, CD36, FAR1, UGT8, LARGE, RDH8, SEPP1, MYO5A, LDLR, SREBF2, CAT, NR5A2, PTGS2, SLC27A3, HMGCR*
	Nucleic Acid Metabolism	2.28E-03 - 1.01E-02	*DPYS, ERH, UPP1, DGUOK, REXO2, CRMP1, MSH2, SCP2, NUDT7, IMPDH1, SLC22A5, HPRT1, ACOT4, PRPSAP2, AK2, HNF4A*
	Free Radical Scavenging	2.3E-03 - 2.3E-03	*CD47, APBB3, XDH, CD36, CCL24, APP*
	Molecular Transport	2.3E-03 - 1.52E-02	*ACAT2, XDH, CCL24, POMC, IL6, DNAJC15, APP, CD47, PTS, LAMP1, SULT1E1, HNF4A, M6PR, ABCB1, PON2, SOD1, MECP2, APBB3, THBS1, CD36, PRDX3, LDLR, SREBF2, SCP2, CAT, PTPRS, SLC6A4, NR5A2*
Vivo_16-cell	Cell Cycle	1.39E-04 - 2.04E-02	*MAEL, SPP1, TNP1, KRAS, MLH1, UBD, SKA2, NANOG, PEA15, JARID2, TEX11, ASCL2, A2M, PLAC8, TM4SF1, MMP9, TIMP2, PRNP*
	Embryonic Development	1.39E-04 - 2.04E-02	*SBDS, SPP1, TDGF1, KRAS, RELN, CDH2, NANOG, CASP9, ALDH1A1, PEA15, ASCL2, CASP8, MMP9, TIMP2*
	Cell-To-Cell Signaling and Interaction	2.46E-04 - 2.04E-02	*TFF3, NID2, ACTA2, DLG2, KRAS, CKAP4, GPNMB, CASP9, PPBP, CASP8, TMSB4X, PRNP, SPP1, GRM8, CNP, CD36, TDGF1, TPM2, RELN, IL33, CDH2, S100B, ZAP70, KCNMB4, AGRN, TFPI, SLC11A1, MMP9, HS3ST5*
	Cell Death	2.76E-04 - 2.04E-02	*TFF3, SPP1, CNP, CD36, KRAS, CHMP4B, MLH1, IL33, UBD, CDH2, ALDH1A1, CASP9, PPBP, S100B, PEA15, AGRN, SETMAR, CASP8, A2M, PLAC8, MMP9, TMSB4X, TIMP2, PRNP*
	Lipid Metabolism	6.84E-04 - 2.04E-02	*CYP1A1, ALDH1A1, PPBP, BCO2, CD36, A2M*
	Vitamin and Mineral Metabolism	6.84E-04 - 2.04E-02	*CYP1A1, ALDH1A1, BCO2*
	Amino Acid Metabolism	1.62E-03 - 2.04E-02	*TPST1, SLC25A12, ZAP70, TDGF1, RELN, HS3ST5*

Group	Functional category	*p*-value[1]	*Transcripts*
Vivo_16-cell	Carbohydrate Metabolism	1.62E-03 - 1.36E-02	*SPP1, PPBP, CD36, GNS, GUSB, TFPI, PRNP*
	Cellular Growth and Proliferation	1.62E-03 - 2.04E-02	*CASC3, LOXL4, TFF3, KRAS, ATP5G2, MLH1, COL1A2, TNN, CASP9, ALDH1A1, PPBP, JARID2, CASP8, PLAC8, TMSB4X, PRNP, TIMP2, CYP1A1, SPP1, ACTB, TDGF1, TPM2, IL33, NANOG, CNN1, S100B, ZAP70, PEA15, ASCL2, S100A11, A2M, MMP9*
	Post-Translational Modification	1.62E-03 - 1.99E-02	*CASP9, TPST1, ZAP70, TDGF1, CASP8, RELN, HS3ST5, TIMP2*
	Cell Signaling	3.44E-03 - 4.13E-03	*COL1A2, CDH2, DLG2, KRAS, AGRN, RELN, TIMP2*
	Molecular Transport	5.23E-03 - 2.04E-02	*ALDH1A1, SLC25A12, PPBP, CD36, SLC28A3, A2M, SLC11A1*

[1]Numbers in the *p*-value column showed a range of p-values for the genes from each category

Table S2 The canonical pathways from the IPA knowledge base that involve transcripts differentially expressed in the blastocyst groups compared to in vivo control group.

Group	Pathway	-Log (P-value)	Ratio	Transcripts
Vitro_4-cell	Integrin Signaling	2.32	0.04	*PAK4, RHOQ, RND3, RHOC, MAPK8, RAC1, CAPN2, TSPAN6, ITGA7*
	Cell Cycle: G2/M DNA Damage Checkpoint Regulation	2.24	0.08	*YWHAE, PTPMT1, SFN, CHEK1*
	Myc Mediated Apoptosis Signaling	1.73	0.06	*YWHAE, MAPK8, RAC1, SFN*
	Propanoate Metabolism	1.72	0.03	*SUCLA2, ECHS1, ABAT, SLC27A3, ACADM*
	FXR/RXR Activation	1.63	0.04	*ABCC2, IL18, MAPK8, RAC1, HNF4A*
	RAR Activation	1.62	0.03	*MAPK8, RAC1, ADCY6, SORBS3, SNW1, RDH13, CITED2*
Vitro_16-cell	ILK Signaling	4.12	0.07	*MUC1, RAC2, FBLIM1, MYL6, ACTA2, FERMT2, HIF1A, MYL7, MYC, CFL2, KRT18, ACTG2, PTGS2, DSP*
	LXR/RXR Activation	3.34	0.09	*SCD, IL1A, LDLR, IL1RN, APOA1, IL1F5, PTGS2, HMGCR*
	FXR/RXR Activation	2.76	0.08	*RAC2, ABCC2, IL1A, SLC10A1, SDC1, IL1RN, APOA1, IL1F5*
	Biosynthesis of Steroids	2.55	0.05	*FDPS, SQLE, FDFT1, IDI1, NUDT12, HMGCR*

Group	Pathway	-Log (P-value)	Ratio	*Transcripts*
Vitro_16-cell	Synthesis and Degradation of Ketone Bodies	2.22	0.15	*BDH2, ACAT2, HMGCS1*
	Tight Junction Signaling	2.33	0.05	*CLDN10, RAC2, CLDN23, CLDN8, MYL6, ACTA2, JAM2, ACTG2, MYL7*
	NRF2-mediated Oxidative Stress Response	1.69	0.05	*HMOX1, ABCC2, GSTP1, PRDX1, ACTA2, HSPB8, DNAJC15, ACTG2, NFE2L2*
IVP	TNFR2 Signaling	2.62	0.12	*NFKBIA, LTA, MAPK8, NFKB1*
	RAR Activation	2.48	0.05	*ALDH1A1, PRKCQ, TRIM24, PNRC1, MAPK8, ADCY6, SORBS3, NFKB1, CARM1, CITED2*
	TNFR1 Signaling	1.81	0.08	*PAK4, NFKBIA, MAPK8, NFKB1*
	Inositol Phosphate Metabolism	1.79	0.04	*PLCD3, ISYNA1, INPP4B, INPP1, PRKCQ, PRKAA1, MAPK8, PLK1*
	Glycerolipid Metabolism	1.75	0.04	*ALDH4A1, AGPAT5, ALDH1A1, AKR1B10, GK, PTGR1, DHRS4*
	ATM Signaling	1.65	0.07	*NFKBIA, TLK1, MAPK8, RAD9A*
Vivo_4-cell	NRF2-mediated Oxidative Stress Response	5.13	0.13	*AKR7A2, MAPK1, PRDX1, HSPB8, DNAJC3, DNAJC15, MAP3K7, UBE2K, JUND, UBE2E3, FKBP5, ACTC1, GSTM1, SOD1, PRKCQ, GSTA1, GSTM3, MAPK8, ERP29, CAT, DNAJC14, DNAJC18, CDC34, DNAJB6, PTPLAD1*
	Protein Ubiquitination Pathway	4.25	0.11	*B2M, HSPB3, USP24, UBE2L3, HSPB8, DNAJC3, DNAJC15, USP48, USO1, PSMC6, BAG1, PSMD10, UCHL5, SUGT1, UBE2E3, UCHL3, DNAJC2, SKP1, PSMB7, USP32, HSCB, PSMD2, DNAJC14, DNAJC18, PSMA4, DNAJB6, CDC34, UBC, USP34, UBE2E1*

Group	Pathway	-Log (P-value)	Ratio	*Transcripts*
Vivo_4-cell	Integrin Signaling	3.28	0.11	*PAK4, CAPN6, AKT2, MAPK1, RHOC, ARPC5, MAPK8, RAC1, BCAR3, RAP1A, MYL7, ITGAE, RHOQ, WASL, RND3, RHOU, ARPC1A, CAPN2, ACTN4, TSPAN6, ACTC1, ITGA7*
	DNA Methylation and Transcriptional Repression Signaling	2.51	0.22	*MECP2, DNMT3A, RBBP7, SUDS3, SAP18*
	Apoptosis Signaling	2.38	0.12	*CAPN6, NAIP, PRKCQ, NFKBIA, MAPK1, MAPK8, APAF1, BID, CAPN2, CASP8, CDK1*
	Ephrin Receptor Signaling	2.33	0.09	*EFNA2, PAK4, EPHB4, AKT2, MAPK1, PTPN13, ARPC5, RAC1, RAP1A, EFNA1, LIMK1, GNG10, GNAS, WASL, CFL2, ACP1, ARPC1A, GNG2*
Vivo_16-cell	NRF2-mediated Oxidative Stress Response	2.83	0.03	*ERP29, GSTM3, ACTB, ACTA2, KRAS, ACTG2*
	ILK Signaling	2.77	0.03	*ACTB, ACTA2, ACTG2, MMP9, MYL7, TMSB4X*
	Integrin Signaling	2.66	0.03	*ACTB, ACTA2, KRAS, ACTG2, TSPAN6, MYL7*
	Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	2.44	0.07	*NANOG, SPP1, JARID2*
	FAK Signaling	2.44	0.04	*ACTB, ACTA2, KRAS, ACTG2*
	Myc Mediated Apoptosis Signaling	2.06	0.05	*CASP9, KRAS, CASP8*

Group	Maturation	Fertilization	Culture							
			Zygote	2-cell	4-cell	8-cell	16-cell	32-cell	Morula	Blast.
Vitro_4-cell										
Vitro_16-cell										
IVP										
Vivo_4-cell										
Vivo_16-cell										
In vivo										

Figure 1 Overview of the experimental groups. Six different blastocyst groups were produced under alternative in vitro (gray colour) and in vivo (white colour) culture conditions at different time points of development. In vivo produced blastocysts used as a control for all other blastocyst groups.

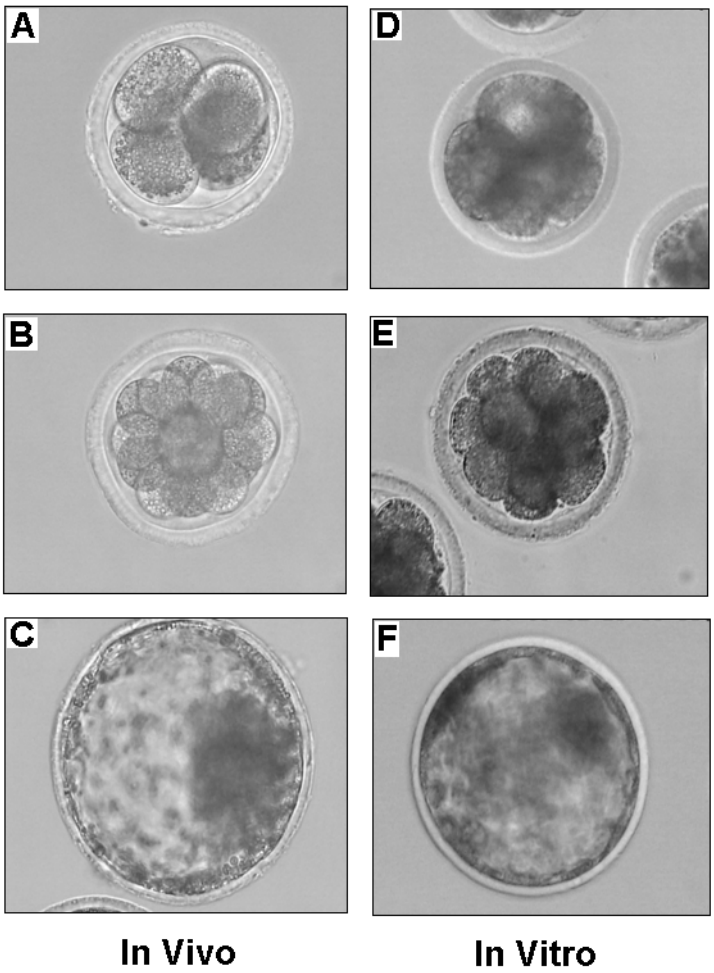

Figure 2 Morphology of bovine embryos produced in vivo (A-C) or in vitro (D-F). Images are representative of 4-cell embryos (A and D), 16-cell embryos (B and E), and blastocysts (C and F).

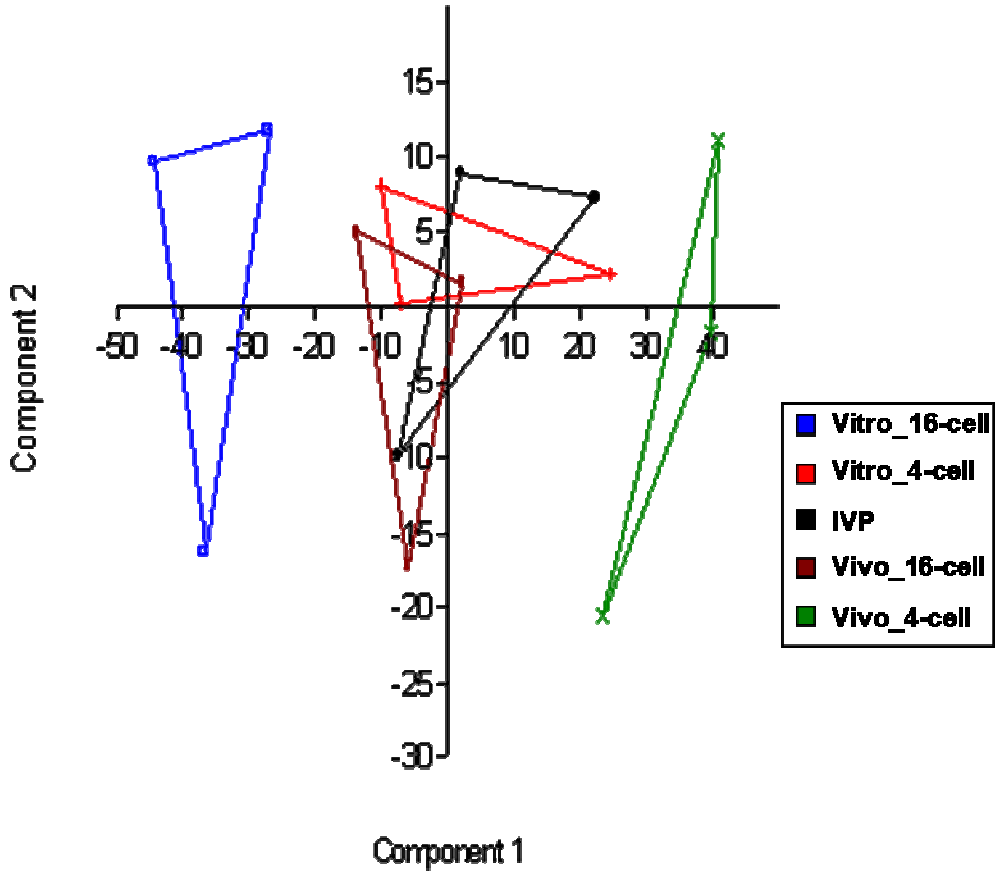

Figure 3 Principle component analysis (PCA). The PCA is a plot distribution indicating the source of greatest variation in the overall transcriptional profiles of the 5 blastocyst groups. Each dot represents one replicate. Note the clear separation of Vitro_16-cell and Vivo_4-cell blastocyst groups.

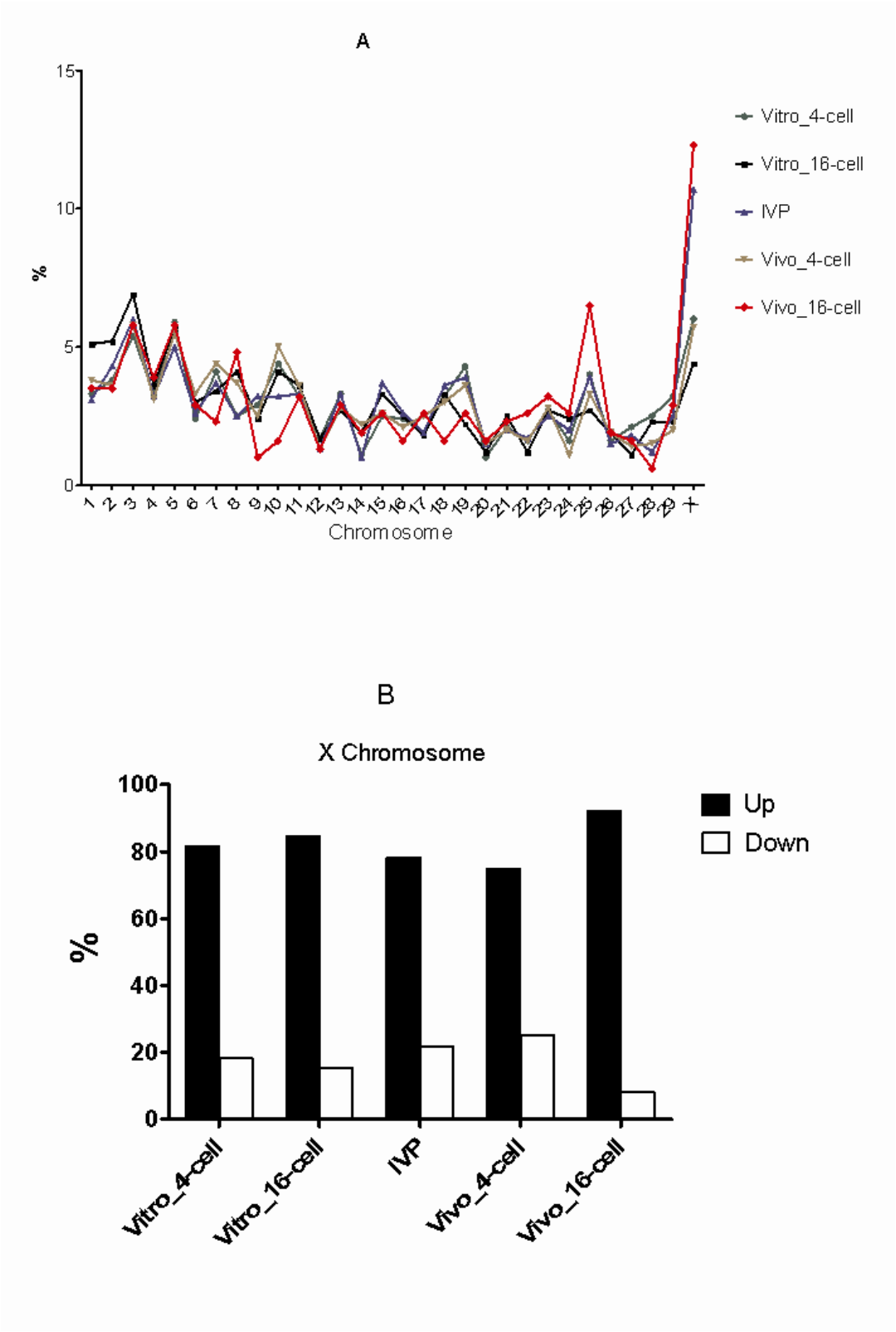

Figure 4 Chromosome distribution for the total differentially expressed transcripts in the five blastocyst groups (A). Percentages of up- and down-expressed genes located on X-chromosome for the five blastocyst groups compared to in vivo control blastocyst (B).

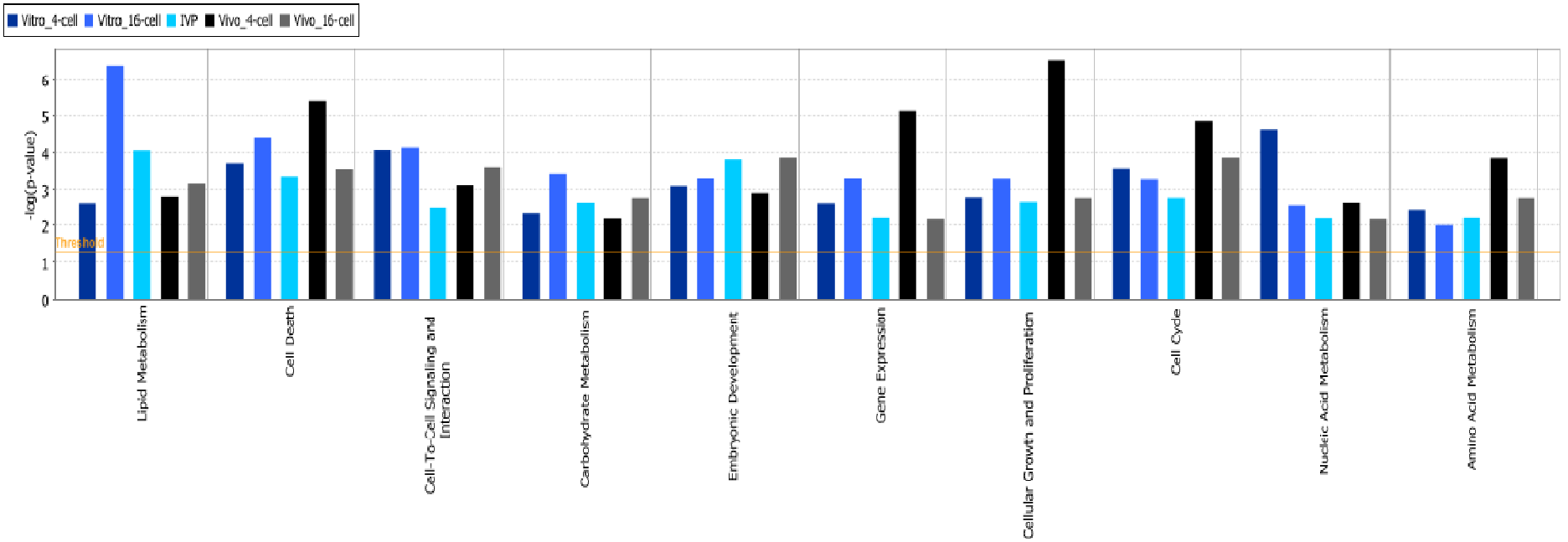

Figure 5 Functional grouping of differentially expressed genes in the five blastocyst groups using Ingenuity Pathways Analysis software. The most significant functional groups ($P < 0.05$) are presented graphically. The bars represent the *p*-value on a logarithmic scale for each functional group.

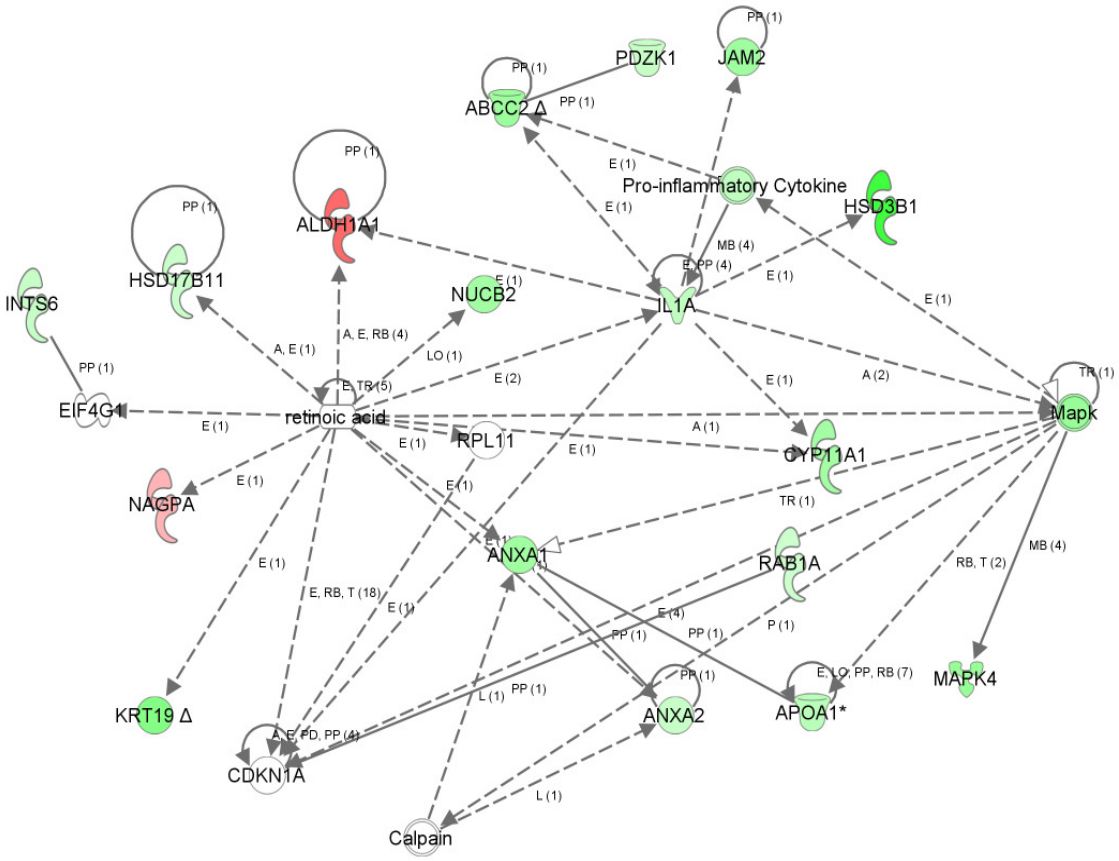

Figure 6 Ingenuity pathway interaction network analysis. Differentially expressed genes between Vitro_16-cell and In vivo control groups involved in lipid metabolism, cell-to-cell signalling and small molecule biochemistry. The network displays nodes (genes/gene products) and edges (the biological relationship between nodes). The colour intensity of the nodes indicates the fold-change increase (red) or decrease (green) associated with a particular gene on Vitro_16-cell group compared to In vivo control group. Direct or indirect relationships between molecules are indicated by solid or dashed connecting lines, respectively. The type of the association between two molecules is shown as a letter on the line that connects them. P, phosphorylation; A, gene activation; E, involved in expression; PP, protein-protein interaction; PD, protein-DNA binding; MB, membership in complex; LO, localization; RB, regulation of binding; T, transcription. The number in parenthesis next to the letter represents the nu mber of bibliographic references currently available in the Ingenuity Pathways Knowledge Base that support each one of the relationships.

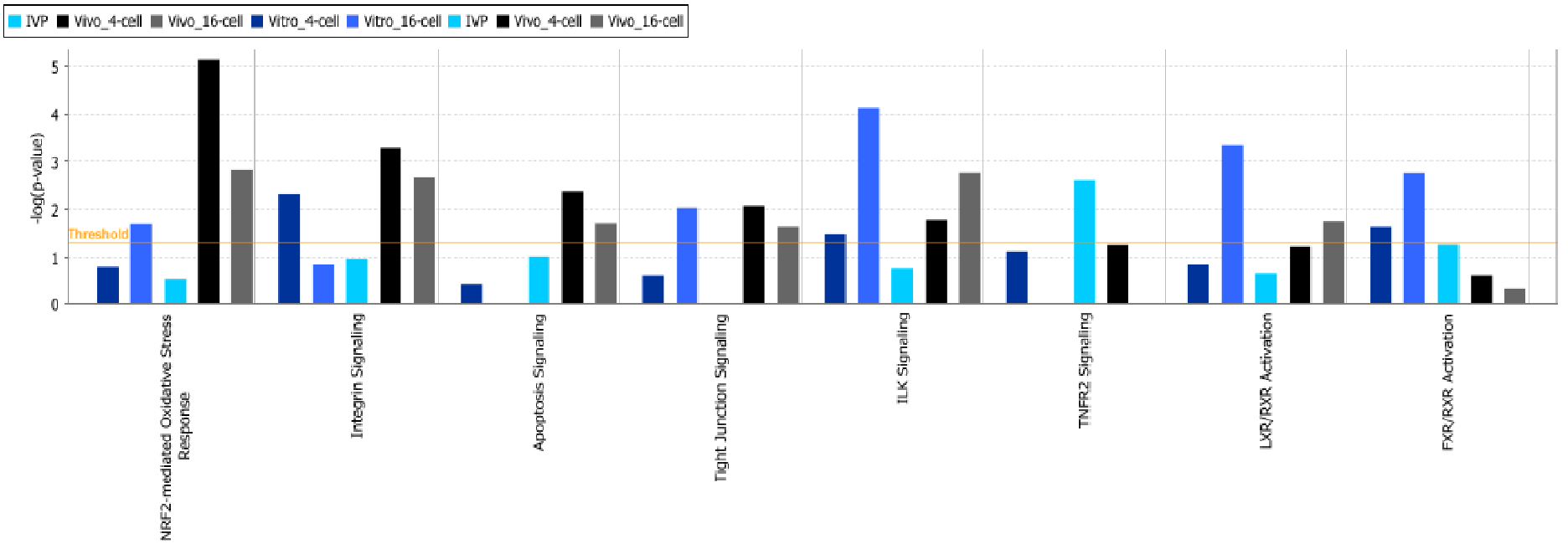

Figure 7 Comparison of the most prominent canonical pathways related to the data sets of the five blastocyst groups ($P < 0.05$). The bars represent the *p*-value for each pathway.

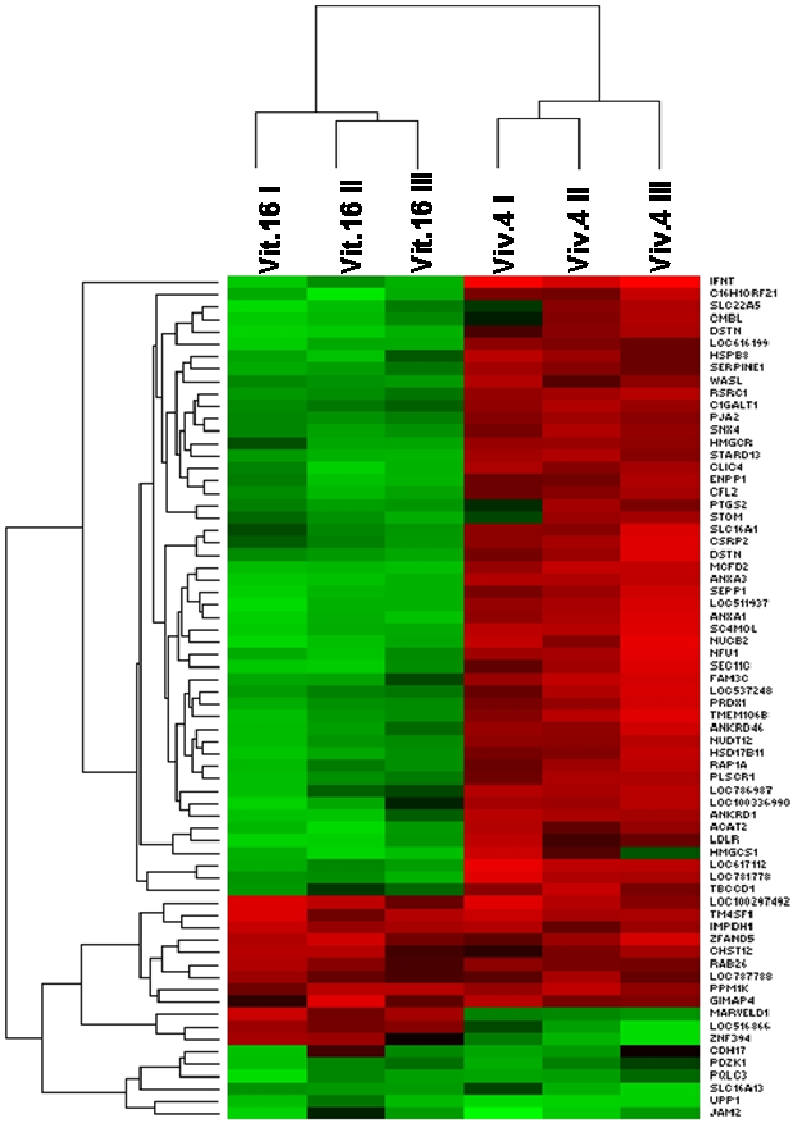

Figure 8 Heatmap of 68 transcripts found to be exclusively differentially expressed in Vitro_16-cell and Vivo_4-cell groups. The red blocks represent higher expression of genes while the green blocks represent lower expression of genes compared to In vivo control group.

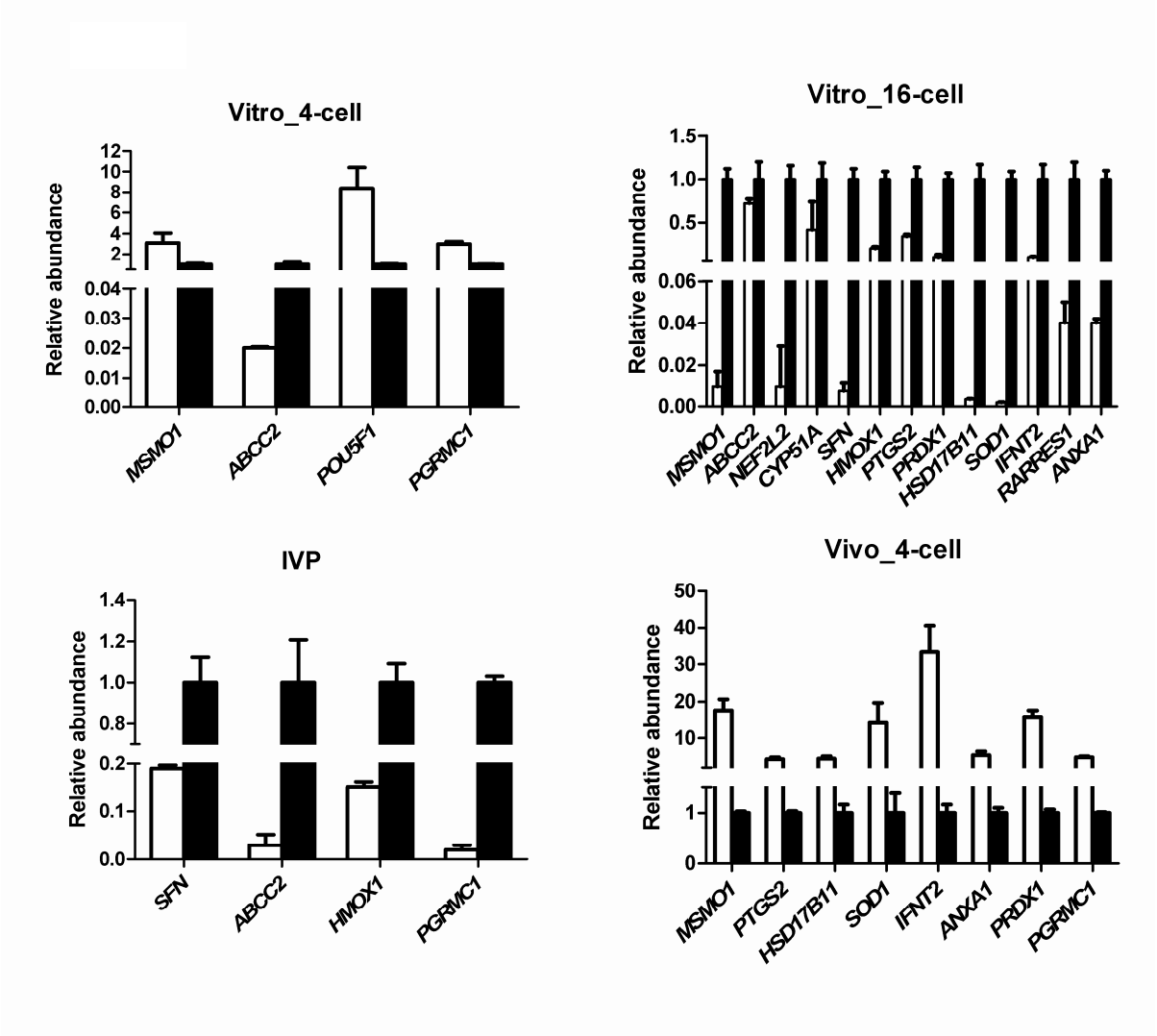

Figure 9 Quantitative real-time PCR analysis of selected genes for microarray validation. The expression pattern of 15 selected genes was consistent with the results from the microarray analysis in the 4 selected groups except in one case (*PGRMC1* in IVP group). All transcripts showed significant differences ($P < 0.05$) between each blastocyst group (white bar) and In vivo control group (black bar).

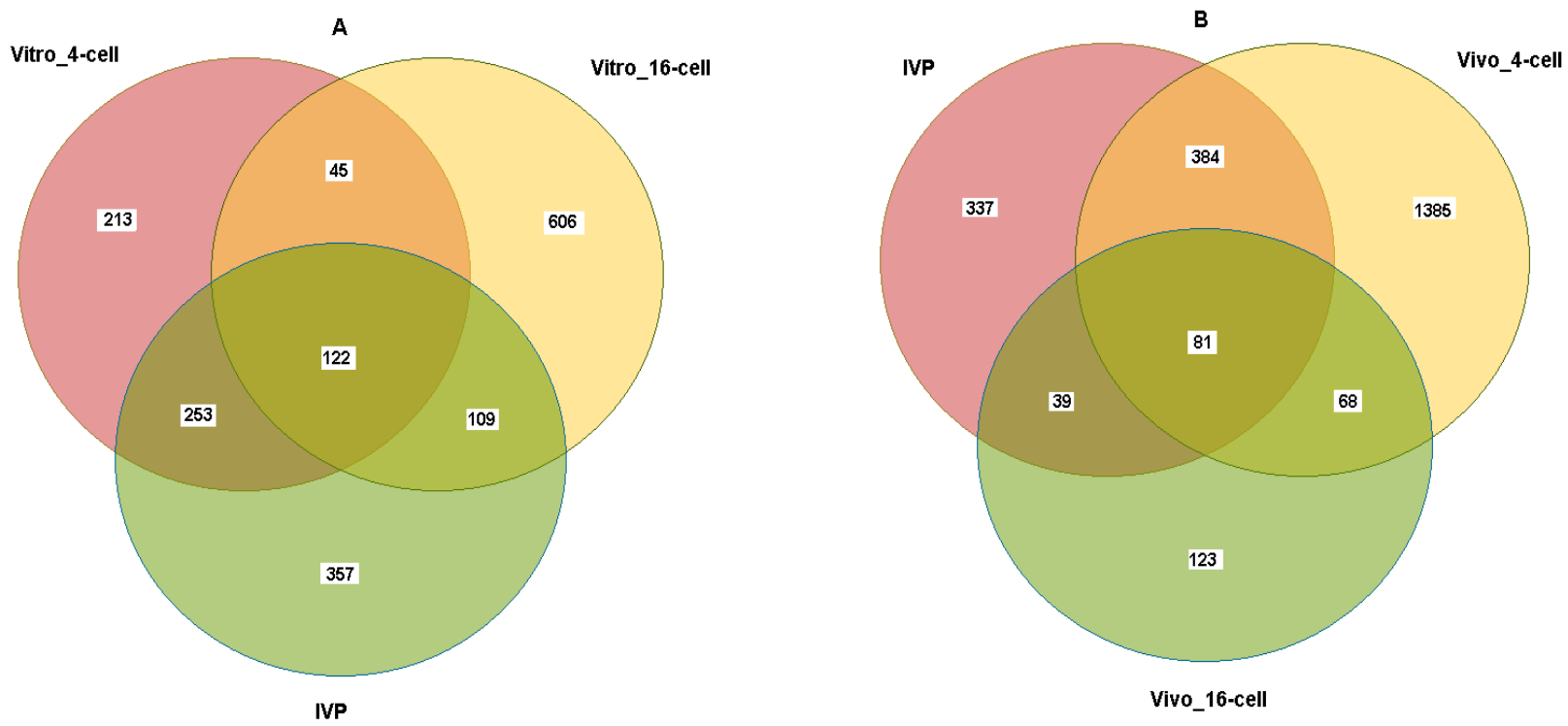

Figure S1 Venn diagrams showing the number of transcripts common or specific between Vitro_4-cell, Vitro_16-cell and IVP (A) and Vivo_4-cell, Vivo_16-cell and IVP (B). Fold-change >2, *p*-value <0.05 and false discovery rate (FDR) <0.05.

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4. Awards:

a) 1st prize of the student competition in the 25th Scientific Meeting of the European Embryo Transfer Association (AETE), Poznan, Poland. September 2009.

b) Prize of the best scientific Master Thesis in the field of Animal Production for the academic year 2006/2007, awarded by Cairo University. December 2007.

c) 1st prize of the student competition for the best scientific poster in the 13th Conference of the Egyptian Society of Animal Production (ESAP), Cairo, Egypt. December 2006.

5. Research Publications:

a) Peer reviewed published articles

1) Abdoon AS, Ghanem N, Kandil OM, **Gad A**, Schellander K, Tesfaye D (2012): cDNA microarray analysis of gene expression in parthenotes and in vitro produced buffalo embryos. Theriogenology 77, 1240-1251.

2) **Gad A**, Besenfelder U, Rings F, Ghanem N, Salilew-Wondim D, Hossain MM, Tesfaye D, Lonergan P, Becker A, Cinar U, Schellander K, Havlicek V, Hölker M (2011): Effect of reproductive tract environment following controlled ovarian hyperstimulation treatment on embryo development and global transcriptome profile of blastocysts: implications for animal breeding and human assisted reproduction. Human Reproduction 26, 1693-1707.

3) Ghanem N, Salilew Wondim D, Tesfaye D, **Gad A**, Phatsara C, Tholen E, Looft C, Schellander K, Hoelker M (2011): Bovine blastocysts with developmental competence to term share similar expression of developmentally important genes although derived from different culture environments. Reproduction 142, 551-564.

4) Abd El Naby WS, Hagos TH, Hossain MM, Salilew-Wondim D, **Gad AY**, Rings F, Cinar MU, Tholen E, Looft C, Schellander K, Hoelker M and Tesfaye D (2011): Expression analysis of regulatory microRNAs in bovine cumulus oocyte complex and preimplantation embryos. Zygote 11, 1-21.

5) El-Gendy EA, **Gad AY** and Mostageer A (2006): Sperm-mediated gene transfer in poultry 1. The relationship with cock sperm viability. Arab Journal of Biotechnology 10: 1-12.

b) Review article

1) **Gad A,** Tesfaye D, Schellander K (2012): Transcriptome profile of early mammalian embryos in response to culture environment. Anim. Reprod. Sci. *In press.*

c) Article under review

1) **Gad A,** Hölker M, Besenfelder U, Havlicek V, Cinar U, Rings F, Held E, Dufort I, Sirard MA, Schellander K, Tesfaye D (2012): Molecular Mechanisms and Pathways Involved in Bovine Embryonic Genome Activation and Their Regulation by Alternative In vivo and In vitro Culture Conditions. Biology of Reproduction (under review).

d) Thesis

1) MS thesis: **Gad A** (December 2006): A study on sperm-mediated gene transfer in poultry. Thesis submitted to Department of Animal Production, Faculty of Agriculture, Cairo University, Giza, Egypt.

e) Abstract / posters

1) **Gad A,** Besenfelder U, Havlicek V, Hoelker M, Cinar MU, Rings F, Dufort I, Sirard MA, Schellander K, Tesfaye D (2011): Transcriptome profile of bovine blastocysts derived from alternative in vivo and in vitro culture conditions at specific phases of early embryonic development. Reproduction, Fertility and Development 24: 179 (Abstr).

2) **Gad A,** Besenfelder U, Havlicek V, Hoelker M, Cinar MU, Rings F, Dufort I, Sirard MA, Schellander K, Tesfaye D (2011): Differences in transcriptome profile of bovine blastocysts derived from alternative in vivo and in vitro culture conditions. 27. Scientific Meeting of the European Embryo Transfer Association (AETE), 9./10.9.2011, Chester, England, Tagungsband, 148 (Abstr).

3) **Gad A,** Besenfelder U, Havlicek V, Hoelker M, Cinar MU, Dufort I, Sirard MA, Schellander K, Tesfaye D (2011): Molecular mechanisms and pathways involved in bovine embryonic genome activation and influenced by alternative culture conditions. 3rd Embryo Genomics Meeting, 20.-22.9.2011, Bonn, Germany, Proceedings, 56-57 (Abstr).

4) **Gad A,** Besenfelder U, Havlicek V, Hoelker M, Cinar MU, Dufort I, Sirard MA, Schellander K, Tesfaye D (2011): Transcriptome profile of early mammalian embryos in response to culture environment. 3rd Embryo Genomics Meeting, 20.-22.9.2011, Bonn, Germany, Proceedings, 9-10 (Abstr).

5) **Gad A,** Besenfelder U, Havlicek V, Hoelker M, Cinar MU, Rings F, Dufort I, Sirard M, Schellander K, Tesfaye D (2011): Alternative culture of bovine embryos (in vivo or in vitro) during embryonic genome activation and subsequent influence on transcriptome profile of blastocysts. Vortragstagung der Deutschen Gesellschaft für Züchtungskunde e.V. (DGfZ) und der Gesellschaft für Tierzuchtwissenschaften e.V. (GfT), Tagungsband, 6./7.9.2011, Freising, Deutschland, C02 (Proc.).

6) **Gad A,** Besenfelder U, Havlicek V, Cinar MU, Rings F, Dufort I, Sirard MA, Schellander K, Tesfaye D, Hölker M (2011): Molecular mechanisms and pathways involved in bovine embryonic genome activation. 38. Jahrestagung der Arbeitsgemeinschaft Embryotransfer deutschsprachiger Länder (AET-d), Tagungsband, 16./17.6.2011, Kleve, Germany, 22-23 (Abstr).

7) **Gad A**, Besenfelder U, Havlicek V, Hoelker M, Cinar MU, Rings F, Dufort I, Sirard M, Schellander K, Tesfaye D (2010): Transcriptome profile of in vitro matured and fertilized embryos cultured until blastocyst stage in bovine oviduct before and after major embryonic genome activation. Vortragstagung der Deutschen Gesellschaft für Züchtungskunde e.V. (DGfZ) und der Gesellschaft für Tierzuchtwissenschaften e.V. (GfT), Tagungsband, 15./16.9.2010, Kiel, Deutschland, C02 (Proc.).

8) Heidt H, Salilew-Wondim D, **Gad A**, Große-Brinkhaus C, Tesfaye D, Hoelker M, Looft C, Tholen E, Schellander K (2010): Expression pattern in the endometrium of genes involved in calcium signaling pathway during bovine estrous cycle. 61st Annual Meeting of the European Association for Animal Production (EAAP), Book of Abstracts, 23.-27.8.2010, Heraklion, Kreta, Greece, 211 (Abstr).

9) **Gad A**, Besenfelder U, Rings F, Ghanem N, Salilew-Wondim D, Tesfaye D, Lonergan P, Cinar MU, Schellander K, Havlicek V, Hölker M (2010): Influence of reproductive tract environment in unstimulated vs. superovulated heifers on development and global transcriptome profile of bovine blastocysts. 37. Jahrestagung der Arbeitsgemeinschaft Embryotransfer deutschsprachiger Länder (AET-d), Tagungsband, 8./9.7.2010, Bonn, Germany, 8-9 (Abstr).

10) **Gad A,** Hoelker M, Rings F, Ghanem N, Salilew-Wondim D, Tesfaye D, Phatsara C, Schellander K, Havlicek V, Besenfelder U (2010): Differences in global transcriptome profile of bovine blastocysts derived from superovulated or synchronized cyclic heifers. Symposium der Deutschen Akademie der Naturforscher Leopoldina und der österreichischen Akademie der Wissenschaften (ÖAW) - Das "gläserne" Tier: Ein- und Ausblicke in Genome und Gene von Haustieren, Tagungsband, 10.-12.3.2010, Wien, Österreich (Abstr).

11) **Gad A,** Hoelker M, Rings F, Ghanem N, Salilew-Wondim D, Tesfaye D, Schellander K, Havlicek V, Besenfelder U (2010d): Differences in global transcriptome profile of bovine blastocysts derived from superovulated or synchronized cyclic heifers. Reprod Fert Dev, Proceedings of the 36th Annual Conference of the International Embryo Transfer Society (IETS), 9.-12.1.2010, Cordoba, Argentina, 278, www.publish.csiro.au/nid/45/issue/5305.htm (Abstr).

12) **Gad A**, M. Hoelker, F. Rings , N. Ghanem, D. Salilew-Wondim, D. Tesfaye, C. Phatsara, K. Schellander, V. Havlicek and U. Besenfelder (2009). Differences in global Transcriptome profile of bovine blastocysts derived from superovulated or synchronized cyclic heifers. Reproduction, Fertility and Development. 22: 278 (Abstr).

13) Abd El Naby W, Tesfaye D, Hossain MM, **Gad A**, Salilew-Wondim D, Rings F, Phatsara C, Tholen E, Looft C, Hoelker M, Schellander K (2009): Regulatory microRNAs in the bidirectional communication of bovine oocytes and the surrounding cumulus cells, Vortragstagung der Deutschen Gesellschaft für Züchtungskunde und der Gesellschaft für Tierzuchtwisenschaften, 16./17.9.2009, Gießen, Deutschland (Proc.), pp. 44-46.

14) Tesfaye D, Abd El Naby WS, Hossain MD, **Gad A**, Salilew-Wondim D, Rings F, Phatsara C, Tholen E, Looft C, Schellander K and Hoelker M (2009). Regulatory microRNA in the bidirectional communication of bovine oocytes and the surrounding cumulus cells. Reproduction, Fertility and Development. 22: 284 (Abstr).

6. Training:

a) R program and its Applications in Statistics, July 25-29, 2011. University of Bonn, Bonn, Germany.

b) Bioinformatics for Animal and Plant Scientists, October 18-22, 2010. Humboldt Universität, Berlin, Germany.

c) miRNA Detection and Analysis, March 16-17, 2010. QIAGEN, EMBL Advanced Training Centre, Heidelberg, Germany.

d) Microarray Data Analysis: An Introductory Course to GeneChip Exon 1.0 ST arrays, December 9-11, 2009. AIRBB, Torino, Italy.

e) International Computer Driving Licence (ICDL) Version 4.0, May 2008. Ministry of Communication and Information Technology, Cairo, Egypt.

f) How to Prepare and Present Scientific Papers, August 25-26, 2007. European Association for Animal Production (EAAP), Dublin, Ireland.

g) Training Course in Microsoft Access, Microsoft PowerPoint and Statistic Programs, February 11 - March 8, 2006. Computer Center, Cairo University, Giza, Egypt.

h) Molecular techniques in nucleic acids & proteins, July 27-31, 2003. Institute of Graduate Studies & Research (IGSR), Alexandria University, Alexandria, Egypt.

i) Training Course in Statistical Analysis Software System (SAS) for Data Analysis, April 28 - May 16, 2001. Computer Center, Cairo University, Giza, Egypt.