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Transcriptional analysis of biopsies derived from in vitro produced bovine blastocysts in relation to pregnancy success after transfer to recipients

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Dedicated to my beloved mother,
Who always used to give me support, encouragement and blessings but passed away during the final phase of my study

Genexpressionsprofile von in vitro produzierten Rinderembryobiopsien in Relation zur in vivo Entwicklung nach Transfer

In dieser Arbeit wurde der Zusammenhang zwischen Genexpression in Embryonen und Trächtigkeitserfolg, basierend auf der Expressionsanalyse von vor dem Transfer an den Rezipienten gewonnenen Blastozystenbiopsien, untersucht. Hierzu wurden Biopsien (30-40% des intakten Embryos) von Tag 7 Blastozysten genommen (n=118) und der 60-70% Restteil den Rezipienten nach Re-expansion transferiert. Basierend auf dem Erfolg der Trächtigkeit wurden die Biopsien in 3 Gruppen (je 10 Biopsien) gepoolt, nämlich: jene, die in keiner Trächtigkeit resultierten (G1), resorbierte Embryonen (G2) und jene, die in Geburt eines Kalbes resultierten (G3). Die Genexpressionsanalyse dieser Gruppen wurde mit einem selbsthergestellten bovinen präimplantationsspezifischen cDNA Array (mit 219 Clonen) und mit BlueChip (mit ~2000 Clonen) durchgeführt. Die Datenanalyse mittels Significant Analysis for Microarray (SAM) Software zeigte insgesamt 52 bzw. 58 unterschiedlich regulierte Gene im Vergleich zwischen G1 versus G3 und G2 versus G3. Quantitative real-time PCR wurde zur Bestätigung der durch das Microarray-Experiment entdeckten unterschiedlich exprimierten Gene eingesetzt. G3-Biopsien exprimierten herstäarkt solche Gene, die notwendig für Implantation (COX-2 und CDX2), Kohlenhydratmetabolismus (ALOX15), Wachstum (BMP15), oxidative Stressantwort (TXN), Signalübermittlung (PLAU) und Plazentafunktion-8 (PLAC8), sind die Biopsien der resorbierten Embryonen zeigten vermehrt Transkripte, die in Protein-phosphorylation (KRT8), Plasmamembranaufbau (OCLN) und Glucosemetabolismus (PGK1, AKR1B1). Involvirt sind die Biopsien von G1-Embryonen, exprimierten vermehrt Transkripte von Zytokinen (TNF), Proteinaminoacidbindung (EEF1A1), Transkriptionfaktoren (MSX1, PTTG1), Enzymen des Glucosemetabolismus (PGK1, AKR1B1) und CD9, einem Inhibitor der Implantation. Zusammengefasst lässt sich sagen, dass wir direkte Kandidatengene identifiziert haben, die eine wichtige Rolle in der Bestimmung der Enwicklungsfähigheit des Embryos nach dem Transfer spielen könnten.

Transcriptional analysis of biopsies derived from in vitro- produced bovine blastocysts in relation to pregnancy success after transfer to recipients

This study was carried out to address the relationship between the transcriptional profile of embryos and the pregnancy success based on gene expression analysis of blastocyst biopsies taken prior to transfer to recipients. For this, biopsies (30-40% of the intact embryo) were taken from day 7. Blastocysts (n=118) and 60-70% part were transferred to recipients after re-expansion. Based on the success of pregnancy, biopsies were pooled in three groups (each 10 biopsies) namely: those resulting in no pregnancy (G1), resorption (G2) and those resulting in delivery of calf (G3). Gene expression analysis of these groups was performed using a home made bovine preimplantation specific cDNA array (with 219 clones) and BlueChip (with ~2000 clones). Data analysis using Significant Analysis for Microarray (SAM) software revealed that a total of 52 and 58 genes were differentially regulated during comparison between G1 versus G3 and G2 versus G3 respectively. Quantitative real-time PCR was used to validate the results of the microarray experiments. G3-Biopsies are enriched with genes necessary for implantation (COX-2 and CDX2), carbohydrate metabolism (ALOX15), growth factor (BMP15), response to oxidative stress (TXN), signal transduction (PLAU) and placenta-specific 8 (PLAC8). G2-Biopsies are enriched with transcripts involved protein phosphorylation (KRT8), plasma membrane (OCLN) and glucose metabolism (PGK, AKR1B1). G1-Biopsies are enriched with transcripts involved inflammatory cytokines (TNF), and factors relevant for protein amino acid binding (EEF1A1), transcription factors (MSX1, PTTG1), glucose metabolism (PGK1, AKR1B1) and CD9 which is an inhibitor of implantation. The bovine MSX1 protein detected by immunohistochemistry was localized in the cytoplasm of immature oocytes and distributed at periphery of the cytoplasm of matured oocytes. Throughout the preimplantation period the staining was apparently more concentrated around the nuclei, whereas the ICM in blastocyst showed weaker labelling for MSX1 than the trophectoderm. In conclusion, we generated direct candidates of genes which may play an important role in determining the fate of the embryo after transfer.

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List of abbreviations

A	: Adenine
AOS	: Abnormal offspring syndrome
APS	: Ammonium peroxydisulphate
ATP	: Adenosine triphosphate
BLAST	: Basic Local Alignment Search Tool
BME	: Basic Medium Eagle
bp	: base pair
BPSA	: Bovie pre-implantation specific array
BSA	: Bovine Serum Albumin
C	: Cytosine
CCD	: Charge coupled devise
cDNA	: Complementary deoxyribonucleic acid
COC	: Cumulus oocyte complex
CT	: Threshold cycle
d	: Days
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynuceotid triphosphate
DOP	: Degenerate oligonucleotid primer
DTT	: Dithiothreitol
EDTA	: Ethylenediamine tetra acetic acid
EST	: Expressed sequence tags
Fig.	: Figure
FSH	: Follicle stimulating hormone
GV	: Germinal vesicle
Hpi	: Hours post insemination

HUGO : Human genome organization
IETS : International embryo transfer society
IPTG : Isopropyl ß-D-thiogalactopyranoside
IVC : In vitro culture
IVF : In vitro fertilization
IVM : In vitro maturation
IVP : In vitro production
LOD : Log of the odds
LB : Luria-Bertani
LH : Luteinizing hormone
MEM : Minimum essential medium
mRNA : Messenger ribonucleic acid
OD : Optical density
PBS : Phosphate buffered saline
PCR : Polymerase chain reaction
PHE : Penicillamine, hypotaurine and epinephrine
RNA : Ribonucleic acid
rpm : Rotations per minute
SCNT : Somatic cell nuclear transfer
SD : Standard deviation of mean
SDS : Sodium dodecil sulphate
SSH : Suppression substractive hybridization
TAE : Tris-acetate-EDTA
TBE : Tris-Boric acid-EDTA buffer
TCM : Tissue culture medium
TIFF : Tagged image file format
tRNA : Transfer ribonucleic acid
UTR : Untranslated region

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1 Introduction

Revenue from dairy and beef farms is directly dependent upon reproductive efficiency because it affects milk production and the number of calves born. Pregnancy loss can have devastating effects on economical success in dairy and beef units. In dairy farms, it was estimated that each pregnancy lost results in an average loss of US$ 640.00 (Thurmond et al. 1990). In beef herds, pregnancy loss represents an even more important economic factor because most of the income is determined by the number of calves sold. So, pregnancy loss or in other words, embryonic mortality is a recognized cause of reproductive failure in cattle leading to the loss of a large number of potential calves, retarded genetic progress, and significant loss of money and time in rebreeding cows (Khurana and Niemann 2000, Morris et al. 2001).

Embryonic mortality results either from intrinsic defects within the embryo, or an inadequate maternal environment, or asynchrony between embryo and mother, or failure of the mother to respond appropriately to embryonic signals (Hansen 2002). With the advent of reproductive technologies this developmental failure becomes more evident. In in vitro production (IVP) of bovine embryos, most of this mortality is sustained within the first 2-3 weeks after fertilization (Diskin and Sreenan 1980, Dunne et al. 2000, Farin et al. 2001 and Sreenan et al. 2001). The explanation for this high rate of developmental failure according to the defect within the embryo (intrinsic errors) remain unclear. The extent and regulation of cell death during preimplantation development is likely to be critical for later development of the conceptus (Brison and Schultz 1997), however, the causes, roles and genetic regulation of embryo death and arrest before implantation remain to be elucidated. Implantation failure may result from failure of particular gene expression or erroneously gene expressions at a crucial point in time. In some cases, even a defect in a single but a critical gene is sufficient to cause implantation failure (Copp 1995). This is in agreement with what was shown from in vitro production of bovine embryos correlated with significant up- or down regulation, de novo induction or silencing of genes critical for undisturbed fetal and neonatal development (Blondin et al. 2000, Crosier et al. 2002, Wrenzycki et al. 2005).

The study of gene expression in preimplantation development will help to understand the development after transfer also, as it has been reported in both mice and cattle that IVP of embryos resulted in not only altered expression of metabolic and growth-related genes in preimplantation-stage embryos but also altered conceptus and fetal development following transfer (Khosla et al. 2001 and Lazzari et al. 2002). Therefore, understanding and unravelling the secrets of preimplantation embryo development has been a challenge to the investigators in the field aiming to alleviate the problems of pregnancy failure. So it is important to study the gene expression during the critical period when the embryo loss occurs, because a better knowledge of gene expression patterns during pre-implantation development would yield insights into the molecular pathways controlling early embryonic development and for understanding events that may be compromised in its mortality (Khurana and Niemann 2000).

Despite the fact that, data on transcriptional analysis of transferable blastocysts of various origins have been accumulated (Rizos et al. 2003, de A Camargo et al. 2005, Wrenzycki et al. 2005), so far no direct connection of gene expression and developmental competence has been established. To study the embryo gene expression responsible for the pregnancy failure we need to study the gene expression in blastocyst which already transferred to the animal or identical with it. What is needed is a well established biopsy technique to obtain cells from embryos prior to transfer without any lethal effect to the embryo during further development. One of the possibilities used to obtain genetically identical offspring in cattle is embryo splitting (Klein et al. 2006). This technique has been used extensively in cattle with many thousands of calves being born worldwide (Ozil 1982, Williams et al. 1984). There have been no reports of the technique producing abnormalities in the offspring (Lewis 1994). The health of calves resulted from embryo duplication is not different from normal calves. The success of the embryo bisection is greatest at the blastocyst to expanded blastocyst stage (Lewis 1994, Hygate et al. 1995).

Therefore the objectives of this study were:-

1- To compare transcriptional activity of embryo biopsies derived from blastocysts resulting in different pregnancy phenotypes after transfer to recipients.

2- To identify differentially regulated genes between three biopsy groups.

3- Further functional analysis for selected candidate gene at the protein level.

2 Literature review

2.1 Preimplantation conceptus and maternal uterine communication

After fertilization, embryo development involves differentiation, as well as development of the fetal body and extra-embryonic tissue until the moment of implantation. Implantation is a unique example of successive interaction between two tissues (embryo and endometrium) that are genetically distinct, therefore, this critical step for mammals in establishing pregnancy, requires successful completion of sequential events such as maternal uterine development, conceptus development and attachment, and placental formation (Basak et al. 2002, Robertson et al. 2003, Imakawa et al. 2004). Differing from rodents and primates, the ruminant blastocyst undergoes a longer, up to two weeks, pre-attachment period (Aplin et al. 2004, Imakawa et al. 2004). The embryo begins to form the placenta around day 20 of gestation in bovine (King et al. 1980, Yamada et al. 2002), while embryonic trophoblast and endometrial cells tightly unite to form placentomes on day 30 (Wooding and Flint 1994). A variety of molecules including adhesion, signaling, transcription, growth factors, cytokines, cell cycle, DNA replication proteins and hormones by embryonic as well as maternal tissues of both uterine and extra-uterine origins coordinate conceptus and uterine development, differentiation and structural formation during this critical phase (Carson et al. 2000, Basak et al. 2002). It is apparent that, even though embryonic development may proceed normally, there remain many opportunities for implantation failure (Carson et al. 2000). This failure in implantation or lack of sufficient placental development leads to conceptus losses. Such losses are commonly associated with in vitro fertilization procedures in human and livestock species of the agriculture industry (Imakawa et al. 2004). It is assumed that essential mechanisms for embryo implantation must be supported by redundant pathways to ensure the conception of new offspring. This predicts that a large number of genes that are important for implantation remain to be identified (Rees et al. 2001, Qin et al. 2003, Imakawa et al. 2004).

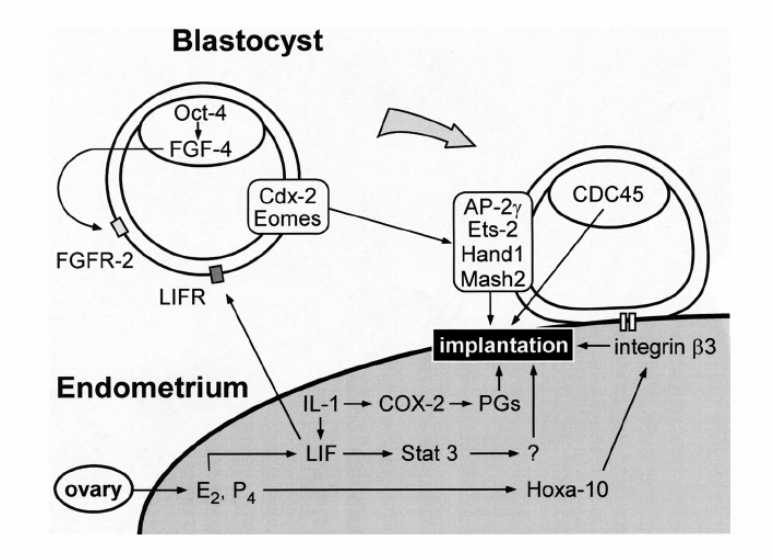


Figure 1: Gene expression model during the early implantation period in mouse (Imakawa et al. 2004)

2.2 Gene expression during preimplantation embryo development

Early embryonic development of most animals requires a specific complement or abundance of oogenetic mRNAs and proteins to confer full developmental competence following fertilization (Babinet et al. 1990, Renard et al. 1994). Formation of zygotes following successful fertilization triggers cohorts of events beginning with repeated cycles of cell division, activation of embryonic genome, compaction and differentiation into inner cell mass (ICM) and trophoblast cells resulting in the formation of blastocyst (De Sousa 1998, Lawinger et al. 1999, Ko et al. 2000). While these series of events have been the distinctive characteristics of preimplantation embryo development, temporal occurrences vary between different species (Telford et al. 1990, Lawinger et al. 1999) and reliant on the sequential and temporal expression of about 10,000 genes out of which the sequence, expression and function of only a very minor portion of these a known so far (Ko et al. 2000, Niemann and Wrenzycki 2000). Recently, cDNA microarray has been applied successfully to profile the expression pattern of a large number of transcripts involved in various developmental pathways. These include study in the analysis of preimplantation stage human embryos (Kelly and Rizzino 2000, Adjaye et al. 2005), study of gene

expression in mouse embryos using cDNA arrays (Tanaka et al. 2000, Brambrink et al. 2002), of bovine oocyte maturation using heterologous cDNA array (Dalbies-Tran and Mermillod 2003), the identification of important genes in oocyte library important for embryogenesis (Yao et al. 2004) and of novel oocyte-specific genes in three different species: bovine, mouse and *Xenopus laevis* (Vallee et al. 2005). Comparing the developmental competence between oocyte and preimplantation embryo in bovines showed that 35 genes were differentially regulated between matured oocyte and blastocyst (Mamo et al. 2005), compare the expression profiles of embryos derived from different origins (Wrenzycki et al. 2004), detect the gene expressions during bovine embryogenesis and implantation (Ushizawa et al. 2004) and recently identification of differentially expressed genes in preimplantation embryos produced by nuclear transfer to study the effect of this new technology on reprogramming the genes required for development (de A Camargo et al. 2005, Pfister-Genskow et al. 2005). Generally, the control of development in preimplantation embryos is guided by two major activation events occurring temporally at the preimplantation development stage. These are maternal and zygotic gene activation events that control development sequentially.

2.2.1 Maternal gene activation and development control

Early embryonic development in mammalian species is regulated by maternal transcripts. As a result, any activity that requires the creation and development of an embryo, whether it is in the context of infertility treatment or in the creation of a reconstructed embryo by nuclear transfer, is dependent on the intrinsic ability of that oocyte to support development (Nusser et al. 2001, Rodriguez and Farin 2004). This is, however, guided by a very stable form of RNA that accumulated in the oocyte and translated during maturation, fertilization and early embryonic development (Stutz et al. 1998). These translationally dormant mRNAs encode a variety of products which are activated in a stage and sequence specific manner in early development (De Moor and Richter 1997, Stutz et al. 1998). During this time, different factors were mentioned to be responsible for the regulation of translation in this stored mRNAs. Sub-cellular localization, cytoplasmic polyadenylation, and Y-box proteins have emerged as leading candidates to regulate the translation of maternal mRNAs

(Yu et al. 2001). Although these and a number of other mechanisms are probably responsible for the translational control of maternal mRNA, one that appears to be widespread is cytoplasmic poly (A) elongation (De Moor and Richter 1997, Charlesworth et al. 2002, Tay et al. 2003). As a result, mRNAs and proteins synthesized and stored during oogenesis initiate and support a developmental program induced by sperm penetration (Memili et al. 1998, Viuff et al. 1998, Pacheco-Trigon et al. 2002, Lonergan et al. 2003). Several gene transcripts required for oocyte competence have been identified in mouse including oocyte specific growth differentiation factor-9 (GDF-9) (McGrath et al. 1995), Bone morphogenetic protein 15 (BMP15) (McNatty et al .2004) connexin 37 (Cx37) (Simon et al. 1997), c-mos proto-oncogene (Colledge et al. 1994) and genes encoding the zona pellucida glycoprotein (Epifano et al. 1995). However, after one to four cleavage divisions, based on the species, the maternal phase gradually looses its development control (Telford et al. 1990, De Sousa et al. 1998, Watson et al. 1999). This transition from maternal to embryonic control of development is characterized by a degradation of maternal RNA and protein, arrest in embryonic development, increased sensitivity to transcriptional inhibitors such as alpha-amanitin, a burst of transcriptional activity from the embryonic genome (Natale et al. 2000) and in the replacement of transcripts previously degraded and the generation of new transcripts that were not present in the oocyte (Brunet-Simon et al. 2001).

2.2.2 Embryonic gene activation and development control

In all species, the development beyond early cleavage divisions is dependent on zygotic gene activation and subsequent maintenance of temporally and spatially appropriate zygotic transcription (Henrion et al. 2000). The trigger for the initiation of embryonic transcription remains unclear (Memili and First 1999, Ma et al. 2001). However, it involves the synthesis of proteins, which are about 40 in mouse (Latham et al. 1991). During the transition from maternal to embryonic control of development, maternal transcripts are depleted and embryo specific transcripts involved in early embryogenesis are generated (Adjaye et al. 1999). The transcription of the 18S, 5.8S, and 28S rRNA polymerase I and their subsequent processing lead to the formation of a distinct nuclear structure of the nucleus (Viuff et al.

1998). Furthermore, the transition is accompanied by modifications in chromatin structure and post-translational modifications of the transcriptional abilities in early embryos (Pacheco-Trigon et al. 2002). In addition, a dramatic reprogramming of gene expression occurs during this transition, and this is likely the molecular foundation for transforming the highly differentiated oocyte into the totipotent blastomere of the early cleavage stage preimplantation embryo (Ma et al. 2001). The timing of zygotic gene activation, or competence to sustain appreciable transcriptional activity in bovine embryos may be controlled temporally by a time dependent mechanism referred to as zygotic clock rather than by developmental stage (Nothias et al.1995, Watson et al. 1999). In bovine embryos, zygotic gene activation has definitely occurred by the 8 to 16 cells stage as evidenced by incorporation of [3H]-uridine into nuclei and nucleolei at the 8-cells stage (De Sousa et al. 1998). As in cattle embryo, a relatively constant pattern of protein synthesis is observed during the first three cell cycles (one-, two-, or four-cell embryos), but a distinctly different pattern is observed in 16-cell embryos and at later stages (Telford et al 1990). This activation is responsible for controlling subsequent development, and different transcripts are expressed in a stage specific manner. However, first transcript initiation at 2 to 4 cells stages was observed in bovine embryo development and this initiation is α- amanitin insensitive and is not required for progression of embryonic development to advanced preimplantation stage (De Sousa et al. 1998).

2.2.3 Genes expressed during the period from blastogenesis through implantation

The process of implantation involves complex cell-to-cell communications between the blastocyst trophectoderm and luminal epithelium of the receptive uterus (Wang et al. 2002). As a result, the period of pre-implantation in ruminants is of main interest for the outcome of pregnancy, since it is associated with a high rate of developmental failure. Blastocyst growth and differentiation can thus be considered as a main target period for the improvement of applications resulting from embryo technologies. In this process, the expression of different genes contributing of normal implantation and development is very important. For example, inadequate production of interferon-tau (IFN-τ) by the embryo to block uterine prostaglandin F2α production results to pregnancy failures in cattle (Hansen

2002). IFN-τ mRNA has been detected in blastocysts but not in morula, suggesting that the onset of IFN-τ production is tightly linked to blastocoel formation (Bazer et al. 1997, Lee et al. 2002, Rizos et al. 2003, Ushizawa et al. 2004). Platelet/endothelial cell adhesion molecule 1 (PECAM-1) is indeed expressed in the mouse blastocyst and it is specific for the cell-cell borders of the inner cell mass (ICM) (Robson et al. 2001). Similarly, disruption of sodium/potassium transporting ATPase (Na/K-ATPase) gene expression by antisense oligonucleotide inhibition disrupted blastocyst formation; these results have implicated the Na/K-ATPase as a key regulator of bovine blastocyst formation (Watson et al. 1999).

Furthermore, octamer-binding transcription factor 4 (Oct4) is expressed during cleavage stages and is essential for the differentiation of the blastocyst (Boiani et al. 2002). Oct4 is expressed initially in all blastomers and later restricted to the inner cell mass (ICM) and down regulated in trophectoderm (Palmieri et al. 1994, Pesce and Schöler 2000). The absence of Oct-4 leads to blastocysts of lower cell number in ICM in bovines (Nganvongpanit et al 2006) and results in periimplantation lethality from defective ICM development in the mouse conceptus (Imakawa et al. 2004). Other transcription factors involved in ICM differentiation include fibroblast growth factor-4 (FGF-4) (Rappolee et al. 1994), heparin-binding FGF like growth factor (HB-EGF) (Paria et al. 2000) and FGF receptor 2 (FGFR-2) (Rappolee et al. 1998, Haffner-Krausz et al. 1999). It was indicated that Oct-4 enhances the ICM expression of FGF-4, which affects both ICM and trophoplast development mediated through the FGFR-2 pathway. Thus, the differentiation of ICM and trophoplast cells during the process of implantation is regulated by means of factors like Oct-4, FGF-4 and FGFR-2 as summarized in figure 1 (Imakawa et al. 2004).

Immunostaining of blastocyst confirmed that HB-EGF, which is present in both the trophectoderm and the inner cell mass, promotes blastocyst growth and function in a number of species (Wang et al 2002). Similarly, ß human chorionic gonadotrophin (HCG), human leukocyte antigen (HLA)-G and pregnancy specific ß-1 glycoprotein (SP-1) are known to be the three placental markers that are expressed prior to implantation and there was a significant positive correlation between SP-1 concentrations and blastocyst cell numbers, which suggest that SP-1 may be used to select blastocysts with higher cell number, possibly resulting in higher pregnancy rates (Jurisicova et al. 1999).

Numerous transcription factors are expressed in trophoplasts including zink finger protein 42 (Rex-1) (Rogers et al. 1991), gata binding protein 3 (GATA-3) (Ng et al. 1994), T-box gene Eomesodermin (Eomes) (Ciruna and Rossant 1999), the caudal-related gene Cdx-2 (Beck et al. 1995), activating protein 2 gamma (AP-2δ) (Shi and Kellems 1998), basic helix-loop-helix (bHLH) gene Mash2 (Rossant et al. 1998), heart and neural creast derivatives expressed 1 (Hand1) (Cross et al. 1995) and erythroblastosis virus E26 oncogene homology 2 (Ets-2) (Yamamoto et al. 1998).

The T-box gene Eomes and homeobox gene Cdx-2 are required for early trophoplast development during the pre-implantation period (Chawengsaksophak et al. 1997, Russ et al. 2000). Cdx2 is one of the genes crucial for placental development, which its aberrations in embryo can result in implantation or placental defect as reported by Hall et al. (2005). AP-2 δ, Mash2, Hand1 and Ets-2 are involved in trophoplast development during the peri- and post-implantation periods (Guillemot et al. 1994, Yamamoto et al. 1998, Riley et al. 1998, Scott et al. 2000).

Also, more genes found to be expressed from blastogenesis through implantation like, matrix metalloproteinnases (MMPs) (Whiteside et al. 2001), heparanase (Kizaki et al. 2001), retinoid X receptors (Mohan et al. 2002), trophoplast-cell-specific molecules such as placental lactogenes (Pls), prolactin-related proteins (PRPs) as stated by (Ushizawa et al. 2004). Interestingly, mouse conceptuses deficient in several other genes, which expressed as DNA replication initiator such as cell division cycle 45 (CDC45) (Yoshida et al. 2001), cell cycle, checkpoint kinase 1 homolog (Chk-1) (Liu et al. 2000), SWI/SNF related, matrix associated, actin dependent regulator of chromatin subfamily (a) member 4 (Brg-1) (Bultman et al. 2000), neural precursor cell expressed, developmentally down-regulated gene 8 (NEDD-8) (Tateishi et al. 2001), and menage a trios 1 (Mat-1) (Rossi et al. 2001), are reported to die during the preimplantation period. High expression of Cox-2 during the time of the implantation suggestes an important role for the prostaglandins released by the embryo in mediating interactions with the uterus (Charpigny et al. 1997, Wang et al. 2002).

Vast numbers of genes change their expression levels during this period to support the complex mechanisms of embryogenesis and implantation. Although numerous molecules participate in trophoblast differentiation and placentation, the precise molecular and genetic pathways which lead to the formation of placenta remain difficult to clarify. Nevertheless,

recent data from animal cloning technology where a somatic or stem cell nucleus is transplanted to an enucleated unfertilized egg, strongly suggested that inappropriate expression of genes is possibly the main cause of early embryo loss (Hashizume et al. 2002, Zhang et al. 2004).

2.3 Fertility and embryo loss in cattle

Fertility is a measure of reproductive success and might be considered as two traits: inherent fertility and expressed fertility. Inherent fertility refers to the genetic potential for reproductive performance and is not directly measurable. Expressed fertility can be measured by age at puberty, quality and quantity of spermatozoa, conception rate, etc. Many factors are correlated with fertility in cattle such as nutrition (Boland and Lonergan 2003), genetics (Royal et al. 2002) and yield (Butler 2003). Fertility in males can be defined as the ability of bull to produce semen that will result in successful pregnancy whereas in females, it can be defined as the ability of a cow to cycle and conceive normally to produce a viable offspring. Such a complex feature is under the influence of numerous genes, working together to produce functional gametes; promote early embryonic and fetal development and finally the delivery of a healthy calf. The heritability, the proportion of variability in phenotypic trait that due to genetics relative to the proportion that due to the environment is relatively low for fertility, usually with value of 5% or less (Dearborn et al. 1973, Berglund and Philipson 2001). However, a number of genetically based variations is known to have direct effects on fertility and reproductive outcome in cattle. For example genetic selection programs have produced a modern dairy cow biologically efficient at producing large volumes of milk, but with an embryo loss rate close to 50% (Diskin et al. 2001). Similarly a higher increment of embryonic loss in high-yielding dairy cattle than in moderate yielding cows or in heifers was observed (Silke et al. 2002, Santos et al. 2004b). This in accordance with Lucy (2001) who reported that pregnancy rate per insemination in dairy cattle has declined in the last 30 years. Although some of this decline likely reflects failures of estrus detection, improper semen deposition, and so on, such a large decrease in pregnancy rate points to an increase in embryonic mortality. It has usually been estimated

that genetic merit has greater effects on early embryonic loss or fertilization failure than that on later stages of embryo development (Grimard et al. 2006).

2.4 Pregnancy failure and embryo loss

Embryo mortality is a major problem for the cattle breeding industry which compromises reproductive efficiency and genetic improvement, resulting in serious financial loss to farmers (Morris et al. 2001).

2.4.1 Factors associated with pregnancy loss

2.4.1.1 Oocyte quality and persistent follicles

Pregnancy begins at conception (union of egg and sperm), but factors affecting the health of gametes may ultimately determine the developmental competence of the embryo. The newly-formed zygote is composed of both genetic and nongenetic material from the oocyte and spermatozoa that produced it. Sperm mitochondria are rapidly ubiquitinated and cleared from the zygotes (Sutovsky et al. 2000). So, the cytoplasm of the zygote is largely derived from the oocyte and only maternal mitochondria survive in the zygote. Until the late four-cell to eight cell stage, when there is a major round of embryonic transcription, the early embryo undergoes only limited transcription (Memili and First 2000) and the embryo is dependent in large part on transcripts and proteins formed in the oocyte which lead to developmental competence.

The term oocyte competence has been developed to describe the potential of an oocyte to give rise to a normally developing embryo following fertilization. Mayes (2002) proposed the theory that the developmental competence of the oocyte is determined during the long period of follicular growth that precedes ovulation or in case of in vitro maturation, precede the isolation of the oocyte from its follicle. The biochemical and morphological changes in the oocyte in persistent follicles reduce fertility in cattle (Mihm et al. 1994, Inskeep 2002) due to embryo mortality before the 16-cell stage (Ahmad et al. 1995). Therefore, extending the period of follicle dominance either by exogenous progestins (Mihm et al. 1994) or when

cows have cycles with two waves of follicle growth (instead of three follicle growth waves; Townson et al. 2002) Fortunately, the presence of a persistent follicle does not alter the developmental potential of oocytes from smaller follicles; that is, if the persistent follicle regresses, normal fertility is resumed (Smith and Stevenson 1995).

It is becoming increasingly clear that environmental or nutritional stresses can adversely affect oocyte competence. Using in vitro fertilization, several factors have been reported to reduce oocyte competence, as measured by the reduction in the proportion of oocytes that successfully develop into blastocysts (Hansen 2002). These factors include diets with high amounts of degradable crude protein (Sinclair et al. 2000, Armstrong 2001), low body condition score (BCS), parity, and high genetic merit for milk yield (Snijders et al. 2000). Oocytes collected from cows with low BCS had lower rates of cleavage and blastocyst formation rates than those from cows with high BCS. Furthermore fertilization of oocytes from first lactation cows resulted in fewer blastocysts than from third lactation cows. Also oocytes of high genetic merit cows yielded fewer blastocysts and had lower rates of cleavage and blastocyst formation than oocytes from medium genetic merit cows. In addition, oocyte competence is reduced in prepubertal animals (Armstrong 2001). Using in vitro fertilization protocols, the proportion of oocytes that develop to blastocyst is greater for oocytes from larger follicles than for oocytes recovered from smaller follicles (Lonergan et al. 1994).

2.4.1.2 Heat stress

The summer decline in oocyte competence is presumably due to heat stress. In sheep, heat stress 12 d before estrus reduced fertilization and lambing rates (Dutt 1964). In addition, retrospective analysis of a large reproductive data set in lactating dairy cattle revealed a negative association between heat stress 10 d before breeding and subsequent pregnancy rate (Al-Katanani et al. 1999). Near estrus, the oocyte also appears sensitive to damage. Exposure of superovulated cows to heat stress for 10 h beginning at the onset of estrus had no effect on fertilization rate but reduced the proportion of normal embryos recovered on d 7 after estrus (Putney et al. 1989).

Exposure to high environmental temperatures compromised steroidogenesis and viability of oocytes (Zeron et al. 2001), reduced oocyte quality (Hansen 2002), and reduced fertilization rate (Sartori et al. 2002) and causes declines in oocyte competence (Al-Katanani et al. 2002). Drost et al. (1999) demonstrated that transfer of in vivo produced embryos from cows exposed to thermoneutral temperatures increased pregnancy rate in heat-stressed recipient cows compared to that in heat-stressed cows subjected to AI. Cartmill et al. (2001b) observed an extremely high pregnancy loss (42.7%), which was substantially higher than that observed for cows at similar stage of gestation not exposed to heat stress. These results demonstrate the negative effects of heat stress on oocyte quality, which compromises fertilization and early embryo development, thus further exacerbating pregnancy losses.

2.4.1.3 Insemination protocol

A wide range of hormonal programs is available to synchronize estrus or ovulation, thereby optimizing service rate. Lucy (2001) indicated that most studies evaluating late embryonic losses in cattle involved animals subjected to timed AI protocols. Based on results from Smith and Stevenson (1995), it was suggested that cows inseminated following spontaneous estrus may have lower rates of embryonic death than those bred following timed AI (Lucy 2001).

Pregnancy loss in 6 published studies (Cartmill et al. 2001a, Cartmill et al. 2001b, Cerri et al. 2003, Gümen et al. 2003, Santos et al. 2004a, Chebel et al. 2004) with cows inseminated following timed AI was 11.2%, which was similar to that of cows inseminated upon estrus detection (12.7%; Table 1). In five of the six studies, pregnancy loss was not altered by insemination protocol. In only one study (Cartmill et al., 2001b), a tendency for increased pregnancy loss was observed with timed AI and, in spite of insemination protocol, pregnancy loss was extremely high (42.7%), perhaps associated with heat stress during the study. In spite of lack of evidence that timed AI results in increased embryonic losses in cattle, it is possible that synchronization protocols that limit the length of the proestrus or result in incompetent follicles might compromise fertility by increasing pregnancy losses.

2.4.1.4 Milk yield

The rapid progress in genetics and management in the dairy industry throughout the world has created a new era in which a smaller number of dairy cows meet the growing demand for dairy products. To meet the demands of the 21st century, individual cows produce more milk and are found on farms with larger herd sizes (Nebel and McGilliard 1992). Recent genetic improvement in dairy cows has led to a dramatic increase in milk yield, which has been associated with a decrease in reproductive performance (Pryce et al. 1999, Royal et al. 1999, Washburn et al. 2002) and higher embryonic loss (Silke et al. 2002, Santos et al. 2004b). Selection for milk yield has increased blood concentration of somatotropin and prolactin, stimulators of lactation, and decreased insulin, a hormone that is antagonistic to lactation and may be important for normal follicular development. These changes in hormone concentration promote higher milk yield but maybe potentially detrimental to other physiological functions, such as reproduction (Nebel and McGilliard 1992). One challenge that will undoubtedly affect future efficiency of the dairy industry is the decline in fertility and reproductive efficiency in modern dairy cows (Lucy 2001).
In dairy cattle the loss of the embryo occurs within 40 d after the cow became pregnant (Moore et al. 2005). The same author stated that a large proportion of the loss occurs 8 to 19 d after breeding (early embryonic loss) resulting in return to heat 18 to 24 d post-breeding. Other losses occur after pregnancy is recognized by the cow at 17 to 19 d after breeding but prior to the time when pregnancy can be detected by rectal palpation at 35 to 42 d.

2.4.1.5 Nutritional effects

Intensive genetic selection for increased milk production, coupled with increased dry matter intakes has led to significant improvements in cow milk yield, however, this increase in milk output has been accompanied by decline in cow fertility through increasing the embryo loss (Silke et al. 2002, Boland et al. 2003). It has been found that embryo loss is affected by nutrition of the cow at the time of insemination where, fluctuation in energy intake can have a significant deleterious effect on embryo survival rate. A reduction in

energy intake from twice to 0.8 times maintenance requirements for a period of two weeks immediately after insemination resulted in reduction in embryo survival rate to 38% (Diskin et al. 2001).

Despite the documented relationship between energy intake and systemic progesterone in sheep and pigs, there is no evidence presented that changes in energy intake affect systemic progesterone concentration in heifers as it has found by Diskin et al. (2001), who stated that previous published reports on the effect of nutrition on progesterone concentration in cattle are equivocal.

Similarly cows fed the high rumen-degradable-protein diet during early lactation were less likely to conceive at first service and to have prolonged interval from calving to conception (Westwood et al. 2002). Diets rich in highly degradable crude protein (CP) compromises fertility through direct actions of urea on the oocyte and through diet-induced alterations in uterine pH (Ocon and Hansen 2003). Furthermore toxic plants can cause embryonic death and abortion (James et al. 1992). For instance cottonseed contains gossypol that can be toxic to mammalian cells (Santos et al. 2003, Villasenor et al. 2003) since high plasma gossypol concentrations (>5 µg/ml) reduced embryo quality and development, and conception rates. Cows fed high gossypol diets experienced more fetal losses, and reduced conception rates and fetal survival (Santos et al. 2003).

2.4.1.6 Progesterone and the uterine environment

Sub-optimal cross-talk between the conceptus and the endometrial epithelial cells leads to secretion of $PGF_{2\alpha}$ and resumption of ovulatory cycles with cessation of pregnancy (Thatcher et al. 1986, Mann and Lamming 2001, Thatcher et al. 2001). Progesterone secretion by the corpus luteum (CL) is essential for orchestrating the histotrophic environment for nourishment of the conceptus (Santos et al. 2004b). Low systemic progesterone in the first week after AI in cow has been associated with reduced embryo survival (McNeil et al. 2006).

Indeed progesterone and estradiol act as systemic regulators leading to local oviductal and endometrial timed events and they program the uterus to regress the CL if there is sub-optimal communication between conceptus and uterus via secretion of $PGF_{2\alpha}$ (Robinson et

al. 2001). Progesterone inhibits luteolysis by decreasing sensitivity to oxytocin by binding to oxytocin receptors and blocking the second messenger system (Grazzini et al. 1998). Nevertheless, the most important effect of progesterone in blocking luteolysis is by enhancing conceptus development which, in turn, stimulates secretion of interferon-tau (Mann and Lamming 2001). Because progesterone plays a major role in stimulating the production of several endometrial proteins and growth factors (Geisert et al. 1992), supplemental progesterone during the first 4 days after AI increased morphological development and biosynthetic activity of day 14 conceptus (Garret et al. 1998). Supplemental progesterone was beneficial to fertility by increasing conception rates when administered prior to day 6 after AI in lactating dairy cows (Mann and Lamming 1999). Collectively, these data indicate that progesterone availability in the early diestrus phase may benefit conception rates and embryonic survival.

2.4.1.7 Pregnancy recognition

The mononuclear cells of the trophectoderm in early stages of development are responsible for the production and secretion of interferon-tau (Thatcher et al. 2001). It is first produced by the conceptus on day 12 of pregnancy because of expression of trophoblastic interferon genes (Farin et al. 1990), but its concentrations in the uterine lumen only peak on days 15–17 of gestation. The antiluteolytic effect of interferon-tau results from the inhibition of endometrial expression of oxytocin receptors and possibly through the transduction mechanism after oxytocin-receptor binding on the endometrial cells and inhibits the episodic release of $PGF_{2\alpha}$ (Demmers et al. 2001). Compromised development of the embryo and underdevelopment of the trophectoderm are, therefore, responsible for premature luteolysis. Therefore, some of the embryonic losses in cattle are thought to be mediated by the inability of the embryo to suppress the luteolytic cascade during the period of CL maintenance (Thatcher et al. 1986).

There is compelling evidence that failure of the conceptus to produce luteotropic signals, or perhaps failure of the CL to respond to luteotropins contribute to early embryonic death in cattle (Thatcher et al. 1986, 2001). Administration of an anti-prostaglandin agent at embryo transfer increased pregnancy rates (82% versus 56%). These data indicate that suppressing

$PGF_{2\alpha}$ secretion favors establishment and maintenance of pregnancy in cattle by reducing embryonic mortality. (Elli et al. 2001)

2.4.1.8 Body condition

Changes in body condition score (BCS) have been used to monitor the energy balance of the cow (Britt 1992), who showed that pregnancy rates are less in recipients with low BCS (Mapletoft et al. 1986). Furthermore, it has been indicated that a 1 unit drop in body condition score (BCS; 1–5 scale) from calving to 30 days postpartum increased the pregnancy loss by 2.41 (Lopez-Gatius et al. 2002). Similarly, Silke et al. (2002) observed that cows losing 1 unit in BCS from days 28 to 56 of gestation had a 3.2-fold increase in pregnancy loss in the same period. Therefore, the metabolic status of the cow, as evidenced by changes in BCS, affects embryonic and fetal survival.

2.4.2 Genetic control of embryonic survival and death

Failures in physiologic and or genetic mechanisms essential for proper fetal growth and survival outside of the uterus contribute significantly to pregnancy and neonatal losses (Farin et al. 2006). A number of factors can influence the survival of embryos produced using in vitro systems including medium composition (Thompson 2000), atmosphere (Watson et al. 1994), oocyte quality (Blondin and Sirard 1995, Sinclair et al. 2000) and embryo genotype. The influence of embryo genotype can be further subdivided into the effects of sire and dam (Hasler et al.1995, Holm and Callesen 1998), embryo sex (Avery et al. 1991, Xu et al. 1992), and expression of specific alleles such as those affecting rates of development during culture (Wu et al. 1998) or metabolic capacity (Niemann and Wrenzycki 2000).

There are many possible underlying causes for embryo demise, including DNA damage, poor embryo metabolism and the effect of suboptimal culture media, all of which could result in an imbalance in gene expression and the failed execution of basic embryonic decisions (Jurisicova and Acton 2004). Also embryonic loss through death of the conceptus is observed with varying frequency among mammalian species (Diskin and Sreenan 1980,

king 1991, Dunne et al. 2000). In a variety of species, cell death is first observed during blastocyst formation, and occurs predominantly in the inner cell mass (Handyside and Hunter 1986, Jurisicova et al. 1995), which could be a means to eliminate abnormal cells and cells with inappropriate developmental potential (Hardy 1999). Programmed cell death is a precisely coordinated set of events dependent upon the actions and interactions of at least 100 genes that either repress or activate the process of cellular self-destruction (Green and Reed 1998, Vaux and Korsmeyer 1999, Adams and Cory 2001). This means that cell fate (i.e. survival/differentiation or death) is determined by the outcome of specific intracellular interactions between pro- and anti-apoptotic proteins, many of which are expressed during oocyte and preimplantation embryo development (Jurisicova and Acton 2004). The most widely studied of these genes are members of the Bcl-2 family, which can be subdivided into two groups: cell death suppressors (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1) and cell death inducers (Bax, Bak, Mtd, BH3-only proteins) (Jurisicova and Acton 2004).
Moreover, there are several mutations that cause death in the preimplantation period. For instance inactivation of the gene that encodes E-cadherin, a cell-surface adhesion molecule, yields embryos that fail to form a trophectoderm epithelium at blastocyst stage. (Copp 1995). The extent and regulation of cell death during preimplantation development is likely to be critical for later development of the conceptus (Brison and Schultz 1997). However, the causes, roles and genetic regulation of embryo death and arrest before implantation remain to be fully elucidated (Betts and King 2001).

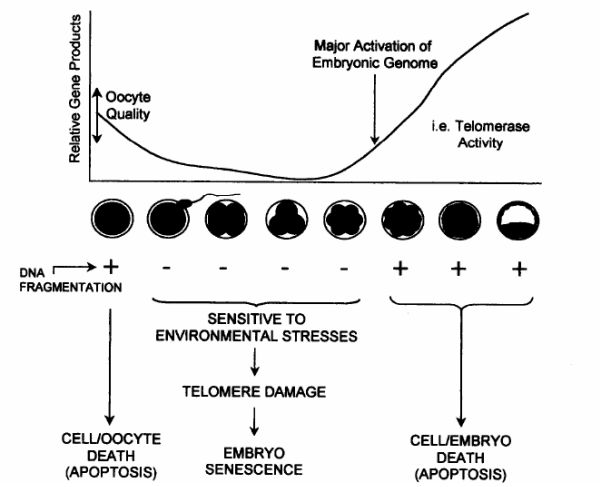


Figure 2: Proposed model for embryo death and arrest during early bovine development. (Betts and King 2001)

All these factors can adversely affect reproductive capacity. However, there is also a new element to be taken into account: epigenetics. There is no doubt that epigenetics is profoundly involved in embryo and foetal losses in cattle, not only under artificial conditions but also under normal ones. One could say that in vitro embryo production and, in particular, cloning by somatic cell nuclear transfer exaggerates the problems and thus makes it easier to deduce the cause of embryo, conceptus and neonatal mortality (Greve and Callesen 2005)

2.4.3 Embryo loss in relation to the source of the embryo

2.4.3.1 Embryo loss in inseminated cows

Reproductive failure in inseminated cattle results from poor fertilization and embryo survival. Recent studies utilizing dairy and beef cattle indicate that fertilization rates are higher for dairy and beef heifers and nonlactating beef cows than lactating beef and dairy cows and nonlactating dairy cows as reported by Santos et al. (2004b), who suggested that pregnancy losses in dairy cattle from fertilization to term might represent up to 60% as shown in figure 3.

Once fertilization has occurred, the fate of a successful pregnancy is then determined by the survival of the embryo and fetus. Losses of pregnancy are characterized by early embryonic death, which occurs prior to the period of CL maintenance in the cow at d 15–17 of the cycle, and late embryonic death, which occurs from CL maintenance to the end of the differentiation stage, at approximately 42 d of gestation. After 50 d of gestation, pregnancy losses are less frequent and characterize fetal death.

Most pregnancy losses occur prior to the period of maintenance of the CL, but in high producing lactating dairy cattle, substantial losses continue to occur up to 42–56 d after insemination (Santos et al. 2004b). This is in line with results obtained by Hubbert (1974) who reported that loss of pregnancy in cattle is greater during the embryonic period (conception to the end of organogenesis, d 1 to d 42), than during either the fetal period (completion of organ differentiation to parturition, d 42 to d 280) or the neonatal period (parturition to d 28 of extra-uterine life).

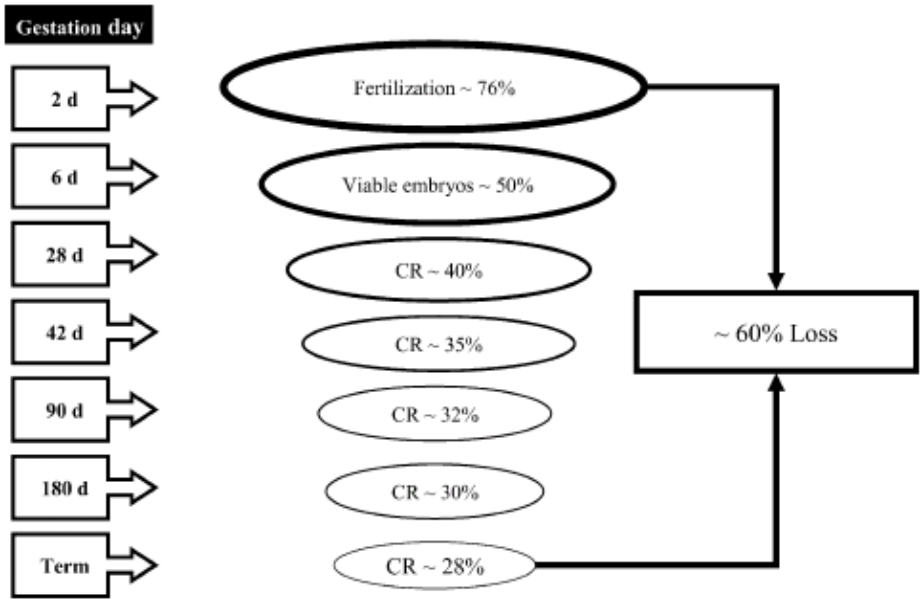


Figure 3: Timing and extent of pregnancy losses in the high producing lactating dairy cow. CR: conception rate (Santos et al. 2004b)

Specifically, early embryonic loss between Day 8 and 18 accounts for approximately 40% of all pregnancy loss in cattle (Ayalon 1978, Diskin and Sreenan 1980). This early

embryonic loss is considered as a major reproductive problem in all mammalian species (Morris et al. 2001).

Embryonic mortality results either from intrinsic defects within the embryo, an inadequate maternal environment, asynchrony between embryo and mother, or failure of the mother to respond appropriately to embryonic signals (Hansen 2002). Failures in implantation or lack of sufficient placental development or functioning lead to conceptus losses. In in vivo and in vitro embryos most of this mortality is sustained within 1-3 weeks after fertilisation (Diskin et al. 1980, Dunne et al. 2000, Farin et al. 2001, Morris et al. 2001). Estrous cycle length is normal for cows experiencing embryonic mortality prior to d 16, but extend when embryonic death occurs after d 16 of pregnancy (Northey and French 1980). Such losses are commonly associated with in vitro fertilization procedures in humans and livestock species of the agricultural industry (Imakawa et al. 2004, Farin et al. 2006). In most cases, pregnancy rates following transfer of in vitro-produced embryos have been significantly lower than those for comparison groups composed of recipient cattle bred by AI or receiving in vivo-produced embryos (Farin et al. 2001) (discussed in details below)**.**

2.4.3.2 Embryo loss after transfer of IVP embryos

Although a variety of methods has been used to produce IVP embryos in cattle, but several laboratories have demonstrated that these embryos have distinct differences in morphology, developmental competence and gene expression, compared to the embryos produced in vivo (Farin et al. 2001, Lonergan et al. 2003, Tesfaye et al. 2004, Wrenzycki et al. 2005). In addition**,** markedly higher rates of early embryonic death and abortions was shown than that seen with either artificial insemination or the transfer of in vivo-produced embryos (Bousquet et al. 1999, Hasler 2000, Farin et al. 2006). The developmental outcomes following transfer of IVP or somatic cell nuclear transfer (SCNT) embryos is shown in figure 4.

Based on numerous studies, pregnancy rates following transfer of IVP embryos have ranged from about 45% to 60% (Kruip and den Daas 1997, Bousquet et al. 1999, Lane et al. 2003, Hansen and Block 2004). Factors that have shown to influence the maintenance of pregnancy following transfer of IVP embryos include genetic abnormalities (Gianaroli et

al. 2000, Thompson et al. 2001), heterogeneity of oocyte quality (Young et al. 1998), embryo culture system, embryo quality, embryo evaluator, number of embryos transferred per recipient, synchrony of embryo development with the recipients day of estrous cycle, transfer technician, fresh versus frozen embryos, heat stress on the embryo or recipient and maternal exposure to excessively high urea diets (Young et al. 1998, Bousquet et al. 1999, Farin et al. 2001, Peterson and Lee 2003, Hansen and Block 2004, Wrenzycki et al. 2005). The reasons for this embryonic loss and abnormal development during early development may be due to one factor or a combination of these, the effect of which can be manifested by disturbances of gene expression and adverse effects on embryo survival (Niemann and Wrenzyski 2000**,** Rizos et al. 2002). Pregnancy loss following transfer of IVP of embryos occurs most frequently prior to day 21 of gestation, or within about 2 weeks of embryo transfer (Diskin and Sreenan 1980, Dunne et al. 2000, Farin et al. 2001, Morris et al. 2001). The explanation for this developmental failure remains unclear, but it is postulated that this phenomenon is accompanied by significant up- or down regulation, de novo induction or silencing of genes critical for undisturbed fetal and neonatal development (Blondin et al. 2000, Crosier et al. 2002, Wrenzycki et al. 2005).

Some genes that are transcribed exclusively in the trophectoderm, the precursor of the placenta (i.e. IFN-τ and Mash2), were affected by reproductive biotechnology (Wrenzycki et al. 2001), supporting the hypothesis that deviation from normal placentation is one major cause of pregnancy loss after transfer of embryos derived from biotechnological procedures (Hasler et al. 1995). It is important to keep in mind that differences in levels of gene expression can vary within the same medium types supplemented with different commercial brands of bovine serum albumin or other type of protein; it is not surprising that gene expression profiles from embryos at the same stage of development will differ between laboratories (Wrenzycki et al. 2001). Unfortunately, this situation increases the level of difficulty for successfully identifying key genes that critically the influence development of IVP embryos. Characterization of early deviation in gene expression would enable us to better understand the biology of early embryo development and improve in vitro culture systems. To improve the IVP systems and make these embryos as good as their in vivo counterparts it is necessary to identify gene expression patterns associated with competence

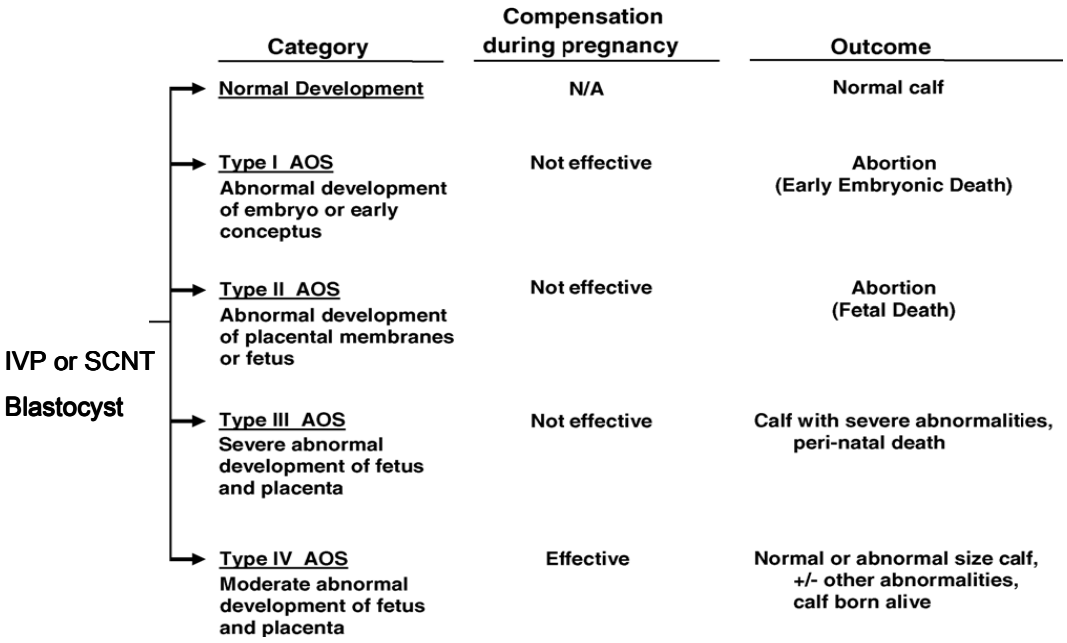


Figure 4: Classification system for conceptus development following transfer of embryos from in vitro production (IVP) or somatic cell nuclear transfer (SCNT) in cattle. AOS refers to abnormal offspring syndrome (Farin et al. 2006).

in oocytes and during early embryonic development (Rizos et al. 2003, Farin et al. 2004, Mohan et al. 2004, Wrenzycki et al. 2005). Studying the gene expression in preimplantation development will help to understand the development after transfer, as reported in both mice and cattle embryos produced in vitro using specific culture environments which resulted in altered expression of some genes in preimplantation-stage embryos and also altered conceptus and fetal development following transfer (Khosla et al. 2001, Lazzari et al. 2002). Moreover, the embryonic stage and quality at the time of embryo transfer have also been shown to be closely associated with the degree of elongation and pattern of gene expression on day 17 or 18 of development (Farin et al. 1999).

2.5 Embryo splitting and transfer

The splitting of blastocysts (multi-layered pre-embryos at the last stage before implantation is referred to as “embryo splitting” (Cohen and Tomkin 1994). In this technique, a

blastocyst is bisected into multicellular groups of non-totipotent cells, each of which is nurtured to encourage further development (Jones et al. 1994). This technique was first described by Willadsen and Polge (1981) for producing bovine monozygotic twins from the bisection or splitting of one embryo. Following this, the technology was refined, and successes were achieved with different kinds of splitting instruments and embryos of varying ages (Ozil 1982, Williams et al. 1984). Embryos could be bisected (split) using either a glass microneedle or a metal microblade (Kippax et al. 1991). Since cattle blastocysts can be easily obtained by uterine flushing, embryo splitting is the preferred means of embryo multiplication in the cattle industry. However, embryo splitting yields only 1.0 to 1.52 pregnancies per original embryo, a yield less than the ideal rate of 2.0 due to the inevitable loss of some cells by the splitting process (Fehilly and Willadsen 1986, Hasler 1992). On contrary, Nibart et al. (1989) have shown that removal of a few cells as resulted of biopsied embryo does not have a drastic effect on pregnancy initiation. Furthermore, Heyman et al. (1998) has reported that the method of splitting, stage of embryo development and parity of recipient have no effects on pregnancy rates and embryo splitting has become a relatively simple technique but is limited to twining. Also it has been found that embryo biopsy for selection of the embryos for transfer can improve the implantation rates and decrease multiple pregnancy rates (Geber and Sampaio 1999) and can be useful for sex determination (Leoni et al 2000). Regarding the source of the embryo, Nibart et al. (1989) have reported a 55% pregnancy rate following transfer of fresh, biopsied, in vivo derived embryos. On the other hand, pregnancy rates of 44% from biopsied and vitrified IVP embryos were observed (Agca et al. 1998). Regarding the genetic expression, Klein et al. (2006) have reported that this technique is found to be both a unique possibility to eliminate genetic variability and a factor potentially affecting the results of gene expression analysis. Furthermore Leoni et al. (2000) have found that this technique will enhance the use of molecular genetic methods in animal production, animal genetic resources programmes and assisted human reproduction.

3 Materials and Methods

3.1 Materials

In this section, materials used as input for the study such as embryos, all other biological materials, chemicals, kits, reagents, equipments and softwares used in different procedures are mentioned with their sources.

3.1.1 Embryos

Embryos used in this study were produced in vitro according to procedures described in section 3.2.1 below. These embryos were used to study and to compare transcript expression patterns and levels using microarray technique, and to study the expression profiles of different transcripts using real-time PCR technique.

3.1.2 Materials for laboratory analysis

3.1.2.1 Chemicals, kits, biological and other materials

Amersham Biosciences (Freiburg): CyScribe™ GFX™ purification kit, CyScribe post-labelling kit

Biomol (Hamburg): Phenol, Phenol/Chlorophorm/Isoamyl alcohol (25:24:1), Lambda DNA Eco91I (BstE II) and Lambda DNA Hind*III*

Biozym Diagnostic (Epicentre technologies): AmpliScribe™ T7 transcription kit Corning (Amsterdam): GAPS II coated slides

DYNAL biotech (Hamburg): Dynabeads. oligo (dT)25

Eppendorf (Hamburg): SYBR Green Real Master Mix

Gibco (Karlsruhe): BME (Essential amino acids), MEM (non essential amino acids), DTT,

G. Streuli & Co (Aulendorf): Streptocombin

Invitrogen life technologies (Karlsruhe): DTT, SuperScriptTM II RNase H- Reverse Transcriptase, 5 X first strand buffer, Random primers

MBI Fermentas (St. Leon-Rot): Glycogen

Mettler Instrument company (USA): Mettler AE 200 sensitive balance Nunc (Roskilde): Four well dishes, Cryotubes

Promega (Mannheim): BSA, pGEM.-T vector, RQ1 RNase-free DNase, RNasin. Ribonuclease inhibitor, 2X rapid ligation buffer, T4 DNA ligase

Qiagen (Hilden): RNeasy. Mini kit, QIAquick PCR purification kit, MiniEluteTM reaction cleanup kit

Roche Diagnostics GmbH (Mannheim): DOP PCR Master Kit

Roth (Karlsruhe): Acetic acid, Agar-Agar, Ampicillin, Ammonium peroxide sulphate (APS), Bromophenol blue, Dimethyl sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA), Ethanol, Ethidium bromide, Hydrochloric acid, Isopropyl β-D-thiogalactoside (IPTG), Kohrsolin. FF, Nitric acid, Pepton, Potassium dihydrogen phosphate, 2- Propanol, Silver nitrate, Sodium acetate, Sodium carbonate, Sodium chloride, Sodium hydroxide, Trichloromethane/chlorophorm, Tris, X-Gal (5-bromo-4-chloro-3-indolylbeta- D-galactopyranoside), Yeast extract

Sigma-Aldrich Chemie GmbH (Munich): Agarose, Ammonium acetate, Calcium chloride, Calcium chloride dihydrate, Calcium lactate, Dulbecco's phosphate buffered saline (D-PBS), Epinephrine, Formaldehyde, FSH, GenEluteTM plasmid Miniprep kit, Glutamine, Hemicalciumlacate, Heparin, Hepes, Hydroxylamin, Hypotaurin, Igepal, Isopropanol, Magnesium chloride, Magnesium chloride hexahydrate, Medium 199, 2-Mercaptoethanol, Mineral oil, Oligonucleotide primers, Penicillin, Phenol red solution, 10 X PCR reaction buffer, Potassium chloride, Sodium dodecyl sulfate (SDS), Sodium hydrogen carbonate, Sodium hydrogen phosphate, Sodium hydrogen sulfate, Sodium lactate solution (60%), Sodium pyruvate, Streptomycin sulfate, Taq DNA polymerase, yeast tRNA

STARLAB GmbH (Ahrensburg): Rigid thin wall 96 X 0.2 ml skirted microplates

Stratagene (Amsterdam): 5 α DH Escherichia coli competent cells

USB Corporation (Staufen): Exo-SAP-IT

3.1.2.2 Reagents and media

All solutions used in this investigation were prepared with deionised and demineralised (Millipore) water and where necessary the pH was adjusted with Sodium hydroxide or hydrochloric acid.

Agarose loading buffer:	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	Water added to	25 ml total
Binding buffer:	1 M Tris HCl pH 7.5	1.0 ml
	5 M LiCl	10.0 ml
	5 mM EDTA pH 8.0	20.0 ml
	Water added to	50.0 ml
Capacitation medium:	Sodium chloride	0.2900 g
	Potassium chloride	0.0115 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dihydrogen phosphate	0.0017 g
	Hepes	0.1190 g
	Magnesium chloride hexahydrate	0.0155 g
	Calcium chloride dihydrate	0.0145 g
	Sodium lactate solution	(60%) 184 µl
	Phenol red solution (5% in D-PBS)	100 µl
	Water added to	50 ml
Culture medium (CR1):	Calcium lactate	0.0273 g
	Streptomycin sulphate	0.0039 g

	Penicillin	0.0019 g
	Sodium chloride	0.3156 g
	Potassium chloride	0.0112 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium pyruvate	0.0022 g
	Glutamin	0.0073 g
	Phenol red solution (5% in D-PBS)	100µl
	Water added to	50 ml
dNTP solution:	dATP (100 mM)	10.0 µl
	dCTP (100 mM)	10.0 µl
	dGTP (100 mM)	10.0 µl
	dTTP (100 mM)	10.0 µl
	Water added to	400.0 µl
Epinephrine solution:	Sodium hydrogen sulfite	0.04 g
	Sodium lactate solution (60%)	100.00 µl
	Epinephrine	0.00183 g
Fertilization medium:	Sodium chloride	0.3300 g
	Potassium chloride	0.0117 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dihydrogen phosphate	0.0021 g
	Penicillin	0.0032 g
	Magnesium chloride hexahydrate	0.0050 g
	Calcium chloride dihydrate	0.0150 g
	Sodium lactate solution (60%)	93µl
	Phenol red solution (5% in D-PBS)	100 µl
	Water added to	50 ml

Hypotaurin solution:	Hypotaurin	0.0011 g
	Physiological salt solution (0.9%)	10.0 ml
IPTG solution:	IPTG	1.2 g
	Water added to	10.0 ml
LB-agar plate:	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Agar-Agar	12.0 g
	Sodium hydroxide (40.0 mg/ml)	480.0 µl
	Water added to	800.0 ml
LB-broth:	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Sodium hydroxide (40.0 mg/ml)	480.0 µl
	Water added to	800.0 ml
Lysis buffer:	Igepal	0.8 µl
	RNasin (40U/µl)	5.0 µl
	DTT (100mM)	5.0 µl
	Water added to	100.0 µl
Modified Parker medium:	Sodium hydrogen carbonate	0.0800 g
	Hepes	0.1400 g
	Sodium pyruvate	0.0250 g
	Glutamin	0.0100 g
	Tissue culture medium (TCM- 199)	99.0 ml

	Gentamycin (10mg/ml)	500 µl
	Calcium lactate	0.0600 g
	Water	10.0 ml
PBS:	Sodium chloride	8.766 g
	di-Sodium hydrogen phosphate	1.495 g
	Potassium dihydrogen phosphate	0.204 g
	Water added to	1000.0 ml
PHE:	0.9 % physiological solution	16.00 ml
	Hypotaurin solution	10.0 ml
	Epinephrin solution	4.0 ml
Physiological solution (0.9%):	Sodium chloride	9.0 g
	Water	1000.0ml
Prehybridization buffer:	BSA	0.5 g
	10 % SDS	0.5 ml
	20 % SSC	7.5 ml
	water added to	50.0 ml
TAE (50x) buffer, pH 8:	Tris	242.0 mg
	Acetic acid	57.1 ml
	EDTA (186.1 mg/ml)	100.0 ml
	Water added to	1000.0 ml
TE (1x) buffer:	Tris (1 M)	10.0 ml
	EDTA (186.1 mg/ml)	2.0 ml
	Water added to	1000.0 ml

SDS (10%):	Sodium dodecil sulphate	5 g
	Water	100 ml
SSC (20x):	NaCl	87.65 g
	Sodium citrate	44.1 g
	Water	500 ml
Washing buffer:	1 M Tris HCl pH 7.5	0.5 ml
	5 M LiCl	1.5 ml
	5 mM EDTA	10.0 ml
	Water added to	50.0 ml
X-gal:	X-gal	50.0 mg
	N, N'-dimethylformamide	1.0 ml

3.1.3 Equipments

ABI PRISM 7000 SDS	Applied Biosystems, Foster city, USA
CEQ 8000 genetic analysis apparatus	Beckman Coulter, Inc, USA
CO2-incubator (MCO-17AI)	Sanyo, Japan
Electrophoresis (for agarose gels)	BioRad, München, Germany
Fluorescence microscope (DM-IRB)	Leica, Bensheim, Germany
GenePix 4000A scanner	Axon Instruments, Foster City, USA
GFL 7601 hybridization chamber	GFL, Dülmen, Germany
HERA safe Bioflow safety hood	Heraeus Instruments, Meckenheim, Germany
Hot baking plate	Jenway, Essex, UK
Hybridization cassettes	TeleChem International, Inc, CA, USA
Icycler	Bio-Rad Laboratories, München, Germany
Memmert CO2 incubator	Fisher Scientific, Leicestershire, UK
Microplade (Beaver)	Minitüb GmbH, Tiefenbach, Germany
Millipore apparatus	Millipore Corporation, USA

PCR thermocycler (PTC 100)	MJ Research, USA
Power supply PAC 3000	BioRad, München, Germany
PTC-100 thermal cyclers	Waltham, MA, USA
Savant SpeedVac	GMI, Inc. Minnesota, USA
Spectrophotometer (Ultrospec™ 2100)	Amersham Bioscience, Freiburg, Germany
Thermoshake Gerhardt	John Morris scientific, Melbourne, Australia
Tuttnauer autoclave	Connections unlimited, Wettenberg, Germany
-85 °C Ultra low freezer	Labotect GmbH, Gottingen, Germany
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany

3.1.4 Used softwares

BLAST program	http://www.ncbi.nlm.nih.gov/BLAST
Entrez Gene	www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
Gene Ontology	http://www.geneontology.org
GenePix Pro Version 4.0	Axon Instruments
GPROCESSOR	http://bioinformatics.med.yale.edu/softwarelist.html
PermutMatrix	http://www.lirmm.fr/~caraux/PermutMatrix/EN/index.html
Multiple Sequence Alignment	http://searchlauncher.bcm.tmc.edu/ multialign/Options/clustalw.html
Primer Express software	Primer Express. Software v2.0
SAM	http//www-stst.stanford.edu/tips/SAM

3.2 Methods of sample preparation and molecular analysis

3.2.1 Bovine embryo production and sample preparation

3.2.1.1 Oocyte collection and in vitro maturation

Bovine ovaries were collected from slaughtered cows at a local abattoir, and transported to the laboratory within 2-4 h, in a thermosflask containing physiological NaCl solution (0.9%

NaCl supplemented with 50 µl/100 ml Streptocombin;) maintained at a temperature of 30 35°C. In the laboratory, the ovaries were washed once with 70% ethanol and twice with physiological saline to eliminate surface organisms, and were dried with sterile paper to avoid sample contamination. Subsequently, cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm-diameter follicles using a 10-ml syringe loaded with an 18-gauge needle. COCs with multiple cumulus layers and evenly granulated cytoplasm were selected under the microscope, washed three times in pre-warmed maturation medium [Modified Parker Medium (MPM) supplemented with 12 % heat-inactivated oestrous cow serum and 10 µg/ml FSH], and finally transferred to wells containing 400 µ1 maturation medium. The MPM was composed of medium 199 0.73 mg/ml of sodium bicarbonate, 50 µg/ml of gentamicin, 0.23 mg/ml of sodium pyruvate, 1.27 mg/ml HEPES and 0.55 mg/ml calcium lactate. Cumulus-oocyte complexes were maintained in groups of 50 and incubated in four-well dishes containing maturation medium and covered with mineral oil. Maturation was done at 39°C in a humidified atmosphere containing 5% CO_2 in air for 22 h.

3.2.1.2 In vitro fertilization

Two semen straws from selected bulls were thawed at a temperature of 39°C for 8 seconds in a water bath, and sperm cells were separated by means of the “swim up” technique in capacitation medium in the incubator for 50 minutes (Parrish et al. 1988). The supernatant was carefully discarded and sperms were resuspended in 3.5 ml capacitation medium, centrifuged at 1500 rpm for 10 minutes with discarding the supernatant. The sperm cells were diluted and counted in a haemocytometer. The matured oocytes in group of 50 were transferred in Fert-TALP medium (Parrish et al. 1988) and co-incubated 1 X 10^6 spermatozoa/ml and incubated for 18 h at the same conditions used for maturation.

3.2.1.3 In vitro culture

At the end of co-incubation, the presumed zygotes were treated with 0.1 % (w/v) hyaluronidase and mechanically denuded by repeated pippeting to remove the attached sperm and cumulus cells, and washed three times in the culture medium [CR-1aa] (Rosenkrans and First 1994) supplemented with 10% oestrus cow serum, 10 µl/ml of basal medium Eagle (BME) essential amino acid solution, and minimum essential medium (MEM) non-essential amino acid solution. Cumulus free zygotes were then transferred to 400 µl culture medium covered with mineral oil and incubated at the same conditions as used for maturation and fertilization till blastocyst stage.

3.2.2 Recovery of embryo biopsies

Morphologically good quality day 7 blastocysts were used for biopsing which was performed using the Beaver microblade (Minitüb GmbH, Tiefenbach, Germany) fixed to a micromanipulator under inverse microscope (Leica , Bensheim, Germany). In this procedure 30-40% of blastocyst containing both ICM and TE cells were taken as biopsy and the rest 60-70% was cultured in-vitro for two hours to allow the re-expansion before transfer to recipients.

3.2.3 Embryo transfer procedure

Healthy 2-years old Simmental heifers of our experimental herd were estrus synchronized by administration of PGF2α (2 ml Estrumate, Fa. Essex, Germany) followed by a second administration 11 days later. Re-expanded demi-blastocysts were transferred using non surgical standard procedures into the uterus at day 7 of the estrus cycle, placed to the side of the uterus where the corpus luteum was located. All recipients were monitored for coming back to estrus at day 21. Cows returning to estrus at day 21 were considered as non pregnant (Group 1). Pregnancies were checked at days 28 and 42 by ultrasonography (Pie Medical, Netherlands, 5 MHz) and by rectal palpation at day 56. Positive pregnancies at day 28 but which got lost until day 56 were categorized as resorbed (Group 2), pregnancies

which got lost after day 56 were judged as aborted. Recipients containing their pregnancies to give birth to a calf were categorized as group 3. An overview of the experimental design is shown in Figure 5.

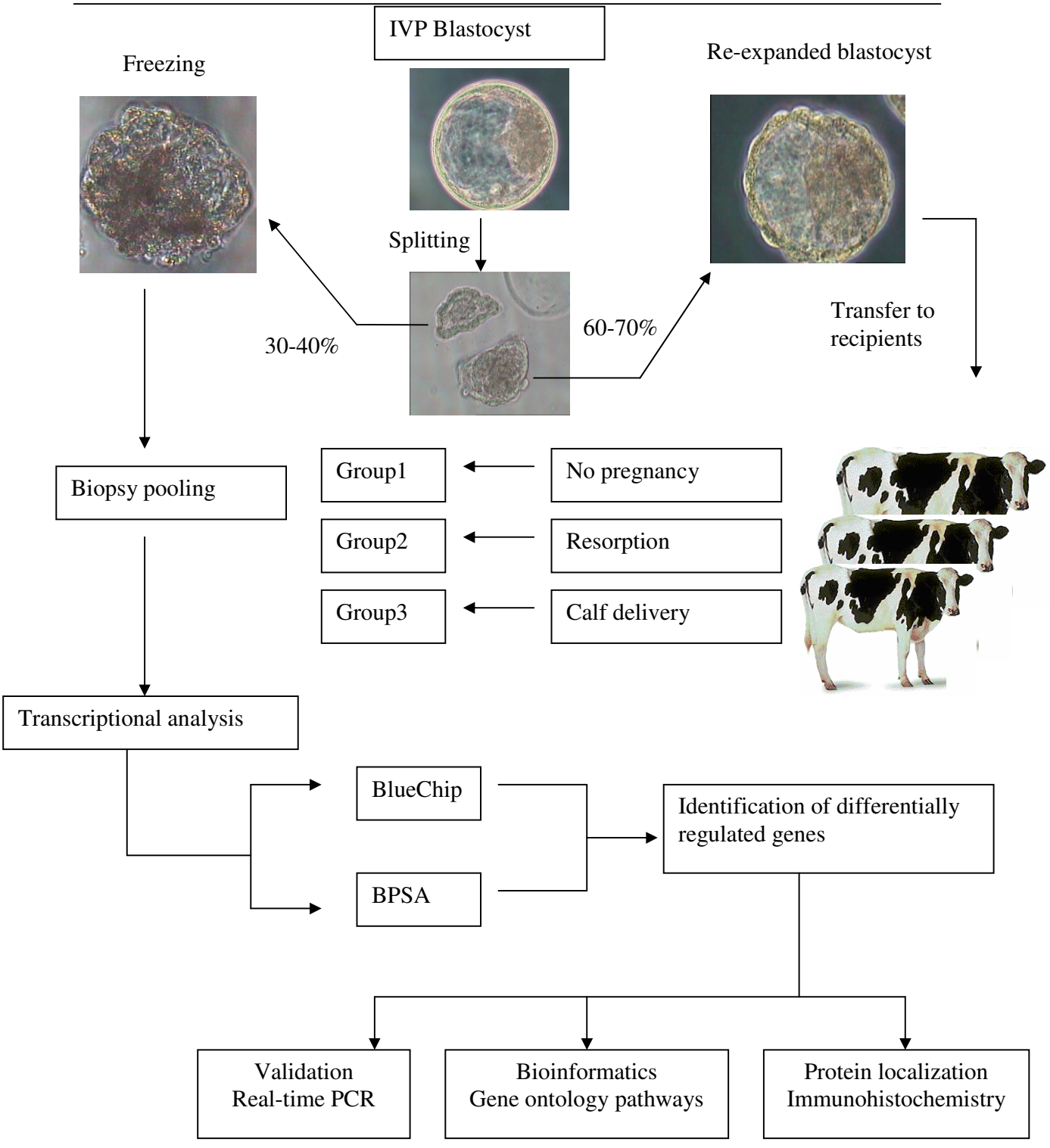


Figure 5: Overview of the experimental design for the identification of differently regulated genes between the three embryo biopsy categories and further functional characterization of selected candidate genes.

3.3 Microarray technique and hybridization

3.3.1 Preparation of probes

In our study we used two types of arrays. The first one is a custom cDNA array (Blue-chip) obtained from the working group of Dr. Sirard (Department of Animal Sciences, Laval University, Canada) which represents transcripts derived from four different suppressive subtractive hybridisation (SSH) made with bovine tissues. The first library is the result of substraction between germinal vesicle (GV) oocytes and somatic tissues (including the liver, kidney, spleen, skeletal muscles and cumulus cells). The second library comes from GV oocytes subtracted from Day-8 blastocysts. The third library is the reverse experiment of the second one and subtracted Day-8 from that of GV oocytes. The final substraction is between Day-8 blastocysts and somatic tissues. The second array is a bovine preimplantation specific array (BPSA) generated in our lab from bovine preimplantation embryo cDNA library construction (Ponsuksili et al. 2001), SSH techniques (Ponsuksili et al. 2002) and differential display analysis (Tesfaye et al. 2003). In addition to these, some specific genes that are known to be expressed during embryo preimplantation development stage have also been amplified with gene specific primers and finally cloned for probe production.

3.3.1.1 RNA isolation and synthesis of complementary DNA (cDNA)

Messenger RNA (mRNA) was isolated from embryo samples prepared using Dynabead oligo $(dT)_{25}$ following the manufacturer's instructions and finally eluted in 11 µl RNase-free water. First-strand cDNA synthesis was carried out in 20 µl reaction volumes by adding 1 µl of oligo $d(T)_{12}$ primer (100 µM) and 8 µl reverse transcription reaction mix [first-strand buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$), 0.3 mM dNTP, 0.1 mM DTT, 10 U of RNase inhibitor and 200 U of SuperScript II reverse transcriptase] to the 11µ1 mRNA sample. The reactions were carried out by using the PTC-100 thermocycler at 42°C for 90 min followed by 75°C for 15 min to terminate the reaction.

Successes of first-strand cDNA synthesis were checked with histone 2a (H2A) amplification as an internal control gene.

3.3.1.2 Primer design and PCR reaction

Gene specific primers were designed using Primer Express® sequence design software v2.0 (Applied Biosystems) using default parameters set by the software. PCR reactions have generally been carriedout in a 20 µl reaction volume containing 2 µ1 template, 2 µ1 10 X PCR buffer, 0.5 µl dNTP, 50 pmol of each (forward and reverse) primer, 0.5 units of Taq DNA polymerase and water has been added to complement the rest volume. Most PCR reactions were performed in a 'touch down'PCR protocol, for about 2-3 hours. PCR cycles were generally hot start with denaturation at 94 °C for 3-5 minutes. Annealing begins at 5 °C above the expected temperature, decreasing 0.5 °C/cycle until it reaches the expected temperature after 10 cycles. In all cases, annealing was preceded by 30 seconds of denaturation at 94 °C and followed by 30-60 seconds of extension at 72 °C. The reaction was carried out 35-40 cycles with final extension at 72 °C for 5 minute.
At the end of the PCR reactions, the products were loaded and screened on agarose gel in 1 X TAE buffer by staining with ethidium bromide.

3.3.1.3 Purification of PCR products

For purification, PCR products were loaded on 0.7%-1% (w/v) agarose gel and run in 1 X TAE buffer by staining with ethidium bromide. The bands were then visualized under UV light and specific gel slices containing the bands were cut for further processing. 600 µ1 of 1 X TE buffer were added to this gel slice and grounded with the syringe needle until it was dissolved, to which 600 µ1 of phenol chloroform was again added and centrifuged for 20 min at 14000 rpm. The supernatant was carefully transferred to another tube and 600 µ1 of chloroform was added and centrifuged for 20 min. Then the supernatant was transfered carefully in another tube, and 1:10 volume (v/v) of 3 M sodium acetate, two volumes (v/v) of 100 % ethanol were added and incubated at -20 °C overnight. The next day, the sample was centrifuged at 14000 rpm for 30 minutes, and the pellet was recovered, which was

subsequently washed with 70 % ethanol, centrifuged at 14000 rpm for 15 min, air dried and later dissolved in 8-10 µ1 of Millipore water.

3.3.1.4 Cloning of PCR products

This was done by using 2 µ1 of PCR product in a 6 µ1 total reaction volume containing 3 µ1 of 2 X rapid ligation buffer, 0.5 µ1 each of vector and ligase enzyme and incubated at 4 °C overnight.

To clone specific fragment, 3 µ1 of the ligation product and 60 µ1 of DH5α *Escherichia coli* competent cells were incubated on ice for 20 min followed by 90 seconds of heat shock at exactly 42 °C, and immediate cold shock on ice for 2 min. To this, 700 µ1 of LB-broth were added and put in a Thermoshaker kept at 37 °C and 110 rpm for 90 min. In the mean time, LB-agar plates containing ampicillin (5 µl/ml) were prepared by adding 20 µ1 of X-Gal and an equal amount of IPTG solution. At the end of the 90 min time, the cell culture was then plated on these plates and incubated at 37 °C for 12-16 h.

Four white colonies assumed to contain inserts, were selected and picked into 1% PCR buffer prepared in 30 µ1 volume. In addition to the white colonies, one blue colony assumed to have no insert, was also picked as a control in a similar volume and buffer condition. The 30 µ1 sample was boiled at 95 °C for 15 min and from this lysate, 10 µ1 product was used as a template to screen for products in a PCR reaction. The reaction conditions were similar as mentioned before. However, in this case, the differences were the use of M13 forward (5‘-TGTAAAACGACGACGGCCAGT-3‘) and M13 reverse primers (5‘-CAGGAAACAGCTATGACC -3‘) and 60 °C annealing temperature, which is specific for this reaction.

3.3.1.5 Sequencing and product confirmation

M13 products containing inserts were used as a template and sequenced from both termini. Sequencing was done by CEQ 8000 Genetic Analysis System using CEQ Dye Terminator Cycle sequencing with Quick start kit. The product was purified first using 5 µ1 M13 products then adding 1 µ1 Exo-SAP-IT. To activate the enzyme, the reaction started with

37°C for 30 min. Then the temperature was increased to 80°C to stop the reaction after 15 min. The sequencing PCR reaction was done according to the recommendation of the company with minor modification as follows: 6 µ1 purified product was added to a PCR tube then 2 µ1 of 1.6 µM M13 primer (forward or reverse), 4 µ1 DTCS Quick start master mix and 8 µ1 milli-pore water were added. The sequencing PCR reaction was done for 30 cycles at 96 °C for 20 sec, 50 °C for 20 sec and 60 °C for 4 min, followed by holding at 4°C. Precipitation and loading the samples into CEQ sample plate performed according to the instructions in the kit protocol. The sequences were then compared against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

3.3.1.6 Amplification of probes

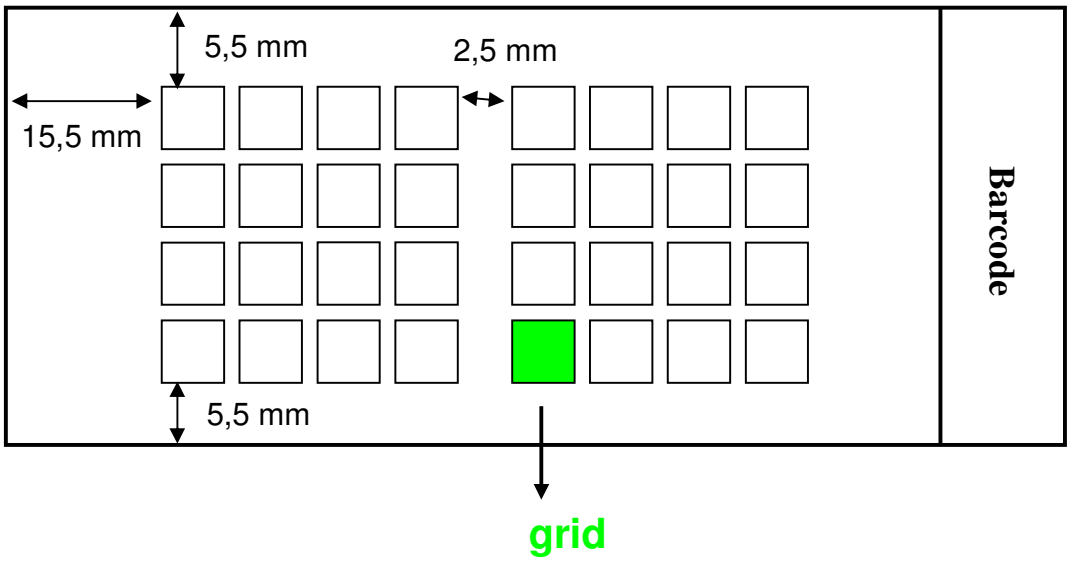
For probe amplification, 2 ul of M13 product from each clone was used as a template to amplify the probes in 40 u1 reaction volume containing 10 X PCR buffer, dNTP, each of amine modified M13 forward (5‘[AC12]TGTAAAACGACGACGGCCAGT-3‘) and M13 reverse primers (5‘-[AC12]CAGGAAACAGCTATGACC-3‘), Taq DNA polymerase (Sigma) and water add up to the volume 40 u1. Reactions were carried in PTC-100 thermal cyclers with a hot start at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72 °C for 1 minutes and final extension at 72 °C for 10 minutes. These PCR products were purified using a PCR purification kit following the manufacturer‘s instructions. Finally the product was eluted in 30 µl RNase free water from which 8 µl was taken to estimate the yield and purity of DNA by UV absorbance reading A260/280 using Ultrospec™ 2100 pro UV/Visible Spectrophotometer. The product was then stored at –20 °C until spotting.

3.3.1.7 Array spotting and description

Using the clones prepared in our lab, spotting was performed by custom service provided by the Resource centre and primary database (RZPD) Germany. Probes used to construct the bovine preimplantation specific array (BPSA) containing genes and ESTs. Each slide has two independent sub-arrays, with 16 blocks. Each block has 11 X 6 spots in which each

clone is represented by three spots in the block representing 3112 spots (or features) to avoid possible missing data and confirm reproducibility (Figure 6). ß-actin (4 spots), GAPDH (4 spots) and H2AFZ (8 spots) were printed as positive hybridization control, and 3SSC and blank as a negative hybridization control. The code of the clones, the gene names, accession numbers and the functions are shown in Table 1.

Preparation for blue-chip was done according to the Sirard Group. Briefly, the microarray slide contained 4928 spots divided into two arrays. Each array was composed of 2304 ESTs randomly selected clones obtained from four different Subtraction suppressive hybridization (SSH) made with bovine tissue (First SSH: GV oocytes subtracted from somatic tissues, second SSH: GV oocytes subtracted from Day-8 blastocyst, third SSH: Day-8 blastocyst subtracted from GV oocytes subtracted and fourth SSH: Day-8 blastocyst subtracted from somatic tissues). All the clones were spotted two times in each array for a total of four replicates. Eleven more samples namely vide (32 spots), alien1 (8 spots), alien2 (8 spots), GFP (4 spots), GFP1 (4 spots) , GFP 1/2 (4 spots), GFP 1/4 (4 spots), GFP 1/8 (4 spots) , GFP 1/16 (4 spots) and H2O/DMSO (50 spots) were spotted to be used as negative control for determination of hybridisation background during the statistical analysis. Housekeeping genes, known as tubulin (8 spots), ubiquitin (8 spots), β-actin (6 spots) and actin (8 spots) were also added as positive control.



Scheme Grid-pattern

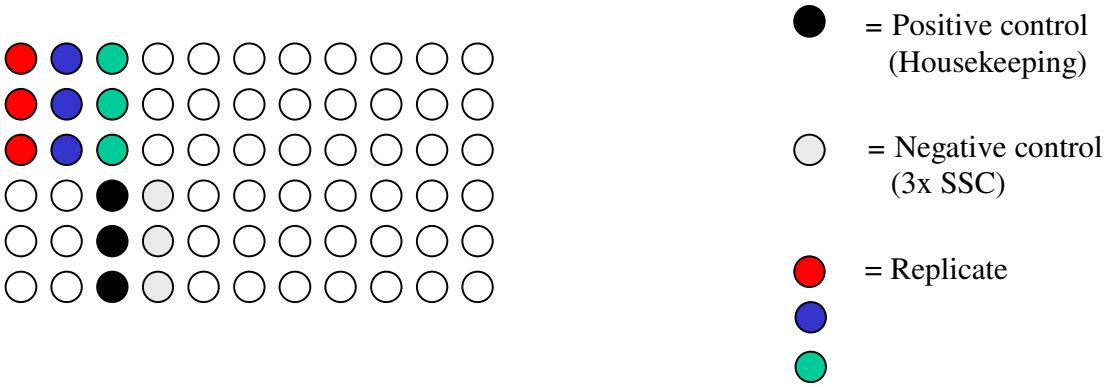


Figure 6: BPSA array lay out of the experimental slides

Table 1: The spotted clone's information for bovine pre-implantation specific array (BPSA)

Code	Gene name	Accession number	Function
MTCO1	Homo sapiens cytochrome oxidase subunit 1	LOC347799	Oxidoreductase activity
MTCO2	Homo sapiens cytochrome oxidase subunit II	LOC348871	Oxidoreductase activity
ATP5A1	Homo sapiens alpha subunit ATP synthase isoform	NM_004046	ATP binding
LGALS3	Homo sapiens lectin,galactoside-binding,soluble 3	BC001120	Sugar binding
FN1	Bos taurus fibronectin mRNA	K00800	Protein binding
KRT18	Bos taurus keratinl8	XM_582930	Structural molecule activity
C72	EST	_	Unknown
C109	Bos taurus Gamma non-muscle actin	BQ640955	Unknown
C110	EST	_	Unknown
C100	EST	_	Unknown
C3	Primary structure of bovine 1.715 satelite DNA	V00124	Unknown
C149	EST	_	Unknown
GNAS	Homo sapiens adenylate cyclase-stmulation G-protein	NM_002070	Nucleotide binding
SLC25A5	Bos taurus adenine nucleotide translocator 2	AB065433	Transporter activity
EEF1A1	Bos taurus elongation factor 1 alpha1	BTA238405	Protein amino acid binding
S100A10	Homo sapiens calpactin 1 light chain	NM_002966	Calcium ion binding
ANXA2	Bovine calpactin 1 heavy chain protein	M14056	Calcium ion binding
CCT3	Homo sapiens chaperonin containing TCP1,	AB208882	Unfolded protein binding
KRT8	Homo sapiens epithelial cytokeratin type II	NM_005556	Protein binding
ANXA3	Bos taurus annexin A3	NM_001035325	Calcium ion binding
CLIC1	Homo sapiens chloride intracellular channel 1	NM_001288	Protein binding
HNRPA1	Homo sapiens heterogeneous nuclear ribonucleoprotein Al	XM_010852	DNA binding
BRP44L	Homo sapiens brain protein 44 like	NM_016098	Unknown
DUSP16	Bos taurus MAPK phosphatase-7	XM_592862	kinase phosphatase activity
C180	EST	_	Unknown
SLC25A3	Rattus norvegicus mitochondrial phosphate carrier protein	LOC498541	Unknown
SPUVE	Homo sapiens protease serine 23	NM-007173	Protein coding
HSPA8	Bos taurus 70 Kd heat shock cognate protein	NM_174345	Unfolded protein binding
C12orf14	Homo sapiens tera protein	NM_007126	Protein binding
KIAA 764	Bos taurus KIAA1 764 protein	LOC514116	Unknown
2C16	EST	_	Unknown
7C25	EST	_	Unknown

Table 1: Continued

Code	Gene name	Accession number	Function
NAP1L1	Homo sapiens nucleosome assembly protein 1 like 1	NM_004537	Nucleosome assembly
TCF7L2	Mus musculus HMG boxtranscription factor	NM_009333	Transcription factor activity
1C19	EST	-	Unknown
N-PAC	Bos taurus cytokine-like nuclear factor n-pac	NM_001040568	Decarboxylating activity
1026A	EST	-	Unknown
1026B	EST	-	Unknown
NY-REN-58	Homo sapiens NY-REN-58 antigen	NM_016122	protein binding
1c4	EST	BTRPPST1	Unknown
PAIP1	Bos taurus polyadenylate binding protein -interacting 1	NM_001034636	Unknown
2c9	EST	-	Unknown
4c20	EST	-	Unknown
PSCD2	Homo sapiens pleckstrin Sec7 and coiled/coil domains2	NM_004227	phosphatidylinositol binding
1c14	EST	-	Unknown
CTNNDI	Bos taurus Catenin (cadherin asso. Protein)delta 1	XM_612939	Unknown
ESTBb4c2	EST	-	Unknown
ESTBb4c3	EST	-	Unknown
CAPZA1	Capping protein, muscle Z -line alpha 1	CK410523	Unknown
GA17	Rattus norvegicus dendritic cell protein	XM_215794	Unknown
HMGN2	Mus musculus high mobilitygroup nucleosomal binding P2	NM_008251	DNA binding
ESTBb8c5	EST	-	Unknown
FLJ23320	Hypothetical protein FLJ23320	BQ038041	Unknown
SMARCC1	Homo sapiens SWI/SNF related actin dependent regulator	NM_003074	DNA binding
ESTBb8c9	EST	-	Unknown
HMGB2	Homo sapiens high mobility group box 2	NM_002129	DNA binding
SC5DL	Bos taurus sterol 05 desaturase like	NM_001035356	oxidoreductase activity
MGC21654	Homo sapiens unknown MGC21654	CV876071	Unknown
NUP160	Homo sapiens nucleoporin l60 kda	D83781	Transporter activity
KLHDC2	Homo sapiens kelch domain containing 2	NM_015483	protein binding
FMO1	Homo sapiens flavin containing monoxygenase 1	BC014341	Electron transport
TBX	Homo sapiens TATA box binding protein 32 kDa	NM_016283	DNA binding
TBX3	EST	-	Unknown

Table 1: Continued

Code	Gene name	Accession number	Function
IFN	Bos taurus interEeron tau (IFN-tau-c3) gene	NM_001015511	Hormone activity
RXRA	Homo sapiens retinoid X receptor alpha	NM_002957	Transcription factor activity
RXRB	Homo sapiens retinoid X receptor beta	DQ100361	Transcription factor activity
RXRG	Homo sapiens retinoid X receptor gamma	NM_001009598	Transcription factor activity
IGFIR	Bos taurus insulin like growth factor 1 receptor	XM_606794	Receptor activity
GJA1	Bos taurus connexin 43	NM_174068	Gap junction activity
Q8	Bos taurus gene for MHC dass 1 heavy cham	XM_604652	Receptor activity
GAPD	Bos taurus glyceraldehyde-3-phosphate dehydrogenase	BC102589	NAD binding
ACTB	Bos taurus beta actin	DQ066897	Protein binding
H2AFZ	Bovine mRNA for histone H2A.Z	X52318	DNA binding
NOS3	Homo sapiens endothelial Nitic oxide synthase	NM_000603	Ion binding
NOS2A	Bos taurus inducible nitric oxide synthase mRNA	AF340236	Ion binding
CDX1	Homo sapiens caudal type homeo box transcription factor 1	NM_001804	Transcription factor activity
HSPCB	Bos taurus heat shock cognate 90 kDa protein 1, beta	NM_174345	Unfolded protein binding
IL1-ß	Bovine interleukin 1-beta	M35589	Cytokine activity
Na/K-ß2	Bos taurus Na,K-ATPase beta 2 subunit	U45944	Sodium/potassium-exchanging ATPase activity
OCT-4	Homo sapiens Oct-4 gene promoter sequence	NP_002692	Transcription factor activity
HK	Bos taurus hexokinase 1 mRNA	HSA297527	Hexokinase activity
PGK	Human mRNA encoding phosphoglycerate kinase	V00572	ATP binding
Tubulin	Homo sapiens alpha tubulin, mRNA	Bc008659	Nucleotide binding
Zo-1	Human tight junction (zonula occludens) protein ZO-1	L14837	Protein amino acid binding
Xist	Bos taurus X-inactive specific transcript	AF104906	X chromosome inactivation
Poly A	B.taurus mRNA for poly(A) polymerase	X63436	Transferase activivty
B TUBIQ	Bos taurus gene for polyubiquitin	Z18245	Protein modification
Hsp70-1	Bos taurus 70 kDA heat-shock protein	U09861	Unfolded protein binding
IGFII-R	Bovine cation-independent mannose 6-phosphate receptor	J03527	Receptor activity
Bax	Bos taurus apoptosis regulator bax-alpha mRNA	U92569	Apoptosis regulator activity
Glut-4	Bos taurus mRNA for glucose transporter type4	D63150	Transporter activity
Lamin	Homo sapiens lamin B1	XM_003777	Tight junction
Dc-II	Bos taurus type 2 desmocollin II mRNA	M81190	Protein binding
Glut-1	Bos taurus glucose transporter type I	M60448	Transporter activity

Table 1: Continued

Code	Gene name	Accession number	Function
GPI	Homo sapiens glucose phosphate isomerase	AY324386	Growth factor activity
DC-III	Bos taurus desmocollin (Dsc3) mRNA	L33774	Protein binding
PALKO	B.taurus mRNA for plakophilin	Z37975	Intermediate filament binding
Globin	Bos taurus alpha globin gene	AJ242798	Transporter activity
Glut-2	Bos taurus glucose transporter 2 mRNA	AF308828	Transporter activity
E-cad	Bos taurus e-cadherin (CDH1) mRNA	AY508164	Protein binding
Glut-3	Bos taurus glucose transporter 3 mRNA	AY033938	Transporter activity
gp130	Human membrane glycoprotein gp130 mRNA	M57230	Receptor activity
Zn-SOD	Bovine mRNA for Cu-Zn superoxide dismutase	X54799	Antioxidant activity
AP2	Bos taurus intestinal alkaline phosphataseII	AF052230	Alkaline phosphatase activity
AP3	Bos taurus intestinal alkaline phosphatase III	AF052226	Alkaline phosphatase activity
DNMT	Bos taurus cytosine-5-methyltransferase	AY173048	Transferase activivty
IGFBP-2	Bos taurus insulin-like growth factor binding protein 2	NM_174555	Insulin-like growth factor binding
CSF1	Bos taurus colony stimulating factor 1	NM_174026	Growth factor activity
GPX	Bos taurus ciliary body glutathione peroxidase	AF080228	Hydrolase activity
Catalase	Homo sapiens catalase	AY028632	Catalse activity
IGFBP-3	Bos taurus insulin-like growth factor binding protein-3	AF305199	Insulin-like growth factor binding
IL-6	Bos taurus interleukin 6 (interferon, beta2)	NM_173923	Cytokine activity
IGFBP-4	Bos taurus insulin-like growth factor-binding protein 4	AF448849	Insulin-like growth factor binding
FGF4	Bos taurus (hst) gene	U15969	Growth factor activity
Na/K-a1	Bos taurus partial mRNA for Na/K ATPase alpha-1	AJ496457	Sodium/potassium-exchanging ATPase activity
FGFR2	B.taurus mRNA for FGF-receptor	Z68150	Receptor activity
Na/K-a2	Bos taurus partial mRNA for Na/K ATPase alpha-2	NM_000702	Sodium/potassium-exchanging ATPase activity
bFGF	Bovine basic fibroblast growth factor	M13440	Growth factor activity
Pan zo-1	Homo sapiens tight junction protein 1 (zona occludens 1)	NM_003257	Protein binding
Na/K-a3	Mus musculus ATPase, Na+/K+ transporting, alpha 3	BC027114	Sodium/potassium-exchanging ATPase activity
Histon 4	Bos taurus histone H4.1 mRNA	AF001288	DNA binding
Occludin	Bos taurus occludin	AY589500	Tight junction
JAM	Bos taurus junctional adhesion molecule	AF111714	Tight junction
Na/K-ß1	Mus musculus ATPase, Na+/K+ transporting, beta 1	NM_009721	Sodium/potassium-exchanging ATPase activity
GCS	Homo sapiens glutamate-cysteine ligase, catalytic subunit	NM001498	Nucleic acid binding

Table 1: Continued

Code	Gene name	Accession number	Function
TGF-a	Homo sapiens transforming growth factor, alpha	NM_003236	Growth factor activity
5S	B.taurus 5S rRNA	X57170	Protein biosynthysis
Activin A	Bos taurus beta A inhibin/activin precursor gene	U16238	Growth factor activity
TGF-ß2	Homo sapiens transforming growth factor, beta 2	NM_003238	Growth factor activity
BTP	Bos taurus trophoblast protein-1 mRNA	M31557	Hormone activity
OSTF1	Bos taurus osteoclast stimulating factor 1	NM_174409	Protein binding
Activin B	Bos taurus beta B inhibin/activin precursor, gene	U16241	Growth factor activity
PDGFR-a	Homo sapiens platelet-derived growth factor receptor	NM_006206	Receptor activity
CREP	Bos taurus cyclic AMP responsive element binding protein	AF006042	Transcription factor activity
Activin R-I	Bos taurus activin receptor typeI	U43208	Receptor activity
Aromatse	B.taurus CYP19 mRNA for aromatase	Z32741	Ion binding
Activin R-IIa	Bos taurus activin receptor type IIB precursor	U57707	Receptor activity
CTCF	Bos taurus CCCTC-binding factor gene	AY205566	Nucleic acid binding
Cycline A	B.taurus mRNA for cyclin A	X68321	Cell cycle activity
GH	Bovine mRNA for growth hormone	V00111	Hormone activity
Activin R-IIb	Bos Taurus activin receptor type II	U58095	Receptor activity
Cycline B	Bos taurus mRNA sequence	L26548	Cell cycle activity
HIF 2a	Bos taurus mRNA for endothelial PAS domain protein	AB018399	Transcription factor activity
PRDX1	Bos taurus peroxiredoxin 1	NM_174431	Oxidoreductase activity
PGF	Bos taurus placental growth factor	NM_173950	Growth factor activity
INF α3	DNA coding of bovine interferon-alpha 3	E00135	Unknown
PRDX2	Bos taurus peroxiredoxin 2	NM_174763	Oxidoreductase activity
Trof.1 INF	B.taurus gene for trophoblast type I interferon	X65539	Hormone activity
INF α4	DNA coding of bovine interferon-alpha 4	E00136	Unknown
Inhibin A	Bos taurus Inhibin A subunit	A14416	Cytokine activity
TMSB10	Bos taurus thymosin, beta 10 (TMSB10), mRNA	NM_174623	Actin binding
INF ß2	DNA coding of bovine interferon-beta 2	E00138	Unknown
INF ß3	DNA coding of bovine interferon-beta 3	E00139	Unknown
Inhibin B	Bos taurus Inhibin B subunit	A14418	Cytokine activity
rpL 37	Bos taurus ribosomal protein L37	S79980	Protein biosynthesis
INF δ	DNA coding of bovine interferon-gamma	E01329	Unknown

Table 1: Continued

Code	Gene name	Accession number	Function
TGF ß1	Bovine transforming growth factor-beta-1	M36271	Growth factor activity
TNF α	Bos indicus tumor necrosis factor alpha	AF011927	Signal transduction
Plau	Bos taurus plasminogen activator, urokinase	NM_174147	Signal transduction
ZFX	Bos taurus Zfx mRNA	D84097	DNA binding
VEGF	Bovine heparin-binding vascular endothelial growth factor	M32976	Growth factor activity
C-mos	Bos taurus c-mos (c-mos) mRNA	AY630920	Protein kinase
HPRT	Mus musculus hypoxanthine guanine phosphoribosyl T1	BC083145	Transferase activivty
HOX B7	Homo sapiens homeobox B7	NM_004502	Transcription factor activity
GDF9	Bos taurus partial mRNA for growth and differentiation F9	AJ302697	Growth factor activity
Mater	Bos taurus maternal antigen that embryo require mRNA	AY721594	ATP binding
BMP15	Bos taurus bone morphogenetic protein 15 mRNA	AY304484	Growth factor activity
Survivin	Bos taurus apoptosis inhibitor survivin mRNA	AY606044	Ion binding activity
CDX1	Caudal type homeo box transcription factor 1	NM_001804	Transcription factor activity
ARF1	Bos taurus ADP-ribosylation factor 1	NM_176653	Protein binding
Glut-synt	Bos taurus glutamate-ammonia ligase pseudogene	NG_002443	Metabolism
U2	Human U2 small nuclear RNA gene	K02227	Unknown
Hox C9	Homo sapiens homeobox C9	NM_006897	Transcription factor activity
ApoA1	Bos taurus apolipoprotein A1	NM_174242	lipid binding
Trf	Bos taurus transferrin mRNA	U02564	Ion binding activity
Cdx 2	Homo sapiens caudal type homeobox transcription factor 2	NM_001265	Transcription factor activity
U3	Bos taurus U3 small nuclear RNA	AF176810	Unknown
Igl	Primula beesiana internal transcribed spacer 1	AF396689	Unknown
Bcl2	Bos taurus bcl-2 mRNA	U92434	Apoptosis regulator activity
Ped	Bos taurus MHC class I 4221.1 gene	AJ010865	Unknown
Hox B9	Homo sapiens homeobox B9	NM_024017	Transcription factor activity
P53	Bos taurus p53 tumor suppressor phosphoprotein	NM_174201	Transcription factor activity
ADCY1	Bos taurus adenylate cyclase 1	NM_174229	Ion binding
Bcl-XL	Bos taurus clone 1.1 anti-apoptotic regulator Bcl-xL	AF245487	Apoptosis regulator activity
FGF2	Bos taurus fibroblast growth factor 2	NM_174056	Growth factor activity
CSF1	Bos taurus colony stimulating factor 1 (macrophage	NM_174026	Growth factor activity
SLC2A3	Bos taurus solute carrier family 2	NM_174603	Transporter activity

Table 1: Continued

Code	Gene name	Accession number	Function
Glut-Dehy	Bos taurus glutamate dehydrogenase 1	NM_182652	Metabolism
ITPR1	Bos taurus inositol 1,4,5-triphosphate receptor	NM_174841	Receptor activity
MSX1	Bos taurus msh homeo box homolog 1	NM_174798	Transcription factor activity
MMP9	Bos taurus matrix metalloproteinase 9	NM_174744	Ion binding
SPARC	Bos taurus secreted protein, acidic, cysteine-rich	NM_174464	Ion binding
SMCX	Bos taurus SMCX protein	AF032367	DNA binding
AQP8	Bos taurus aquaporin 8 mRNA	AY743596	Transporter activity
P14	Bos taurus P14 (p14) mRNA	AF037349	Binding
Fibronectin	Bos taurus fibronectin mRNA	K00800	Protein binding
AchE	Bos taurus acetylcholinesterase T-subunit precursor	AF061813	hydrolase activity
Ledgf	Bos taurus lens epithelium-derived growth factor	AF474175	DNA binding
CHRNA3	Bos taurus cholinergic receptor, nicotinic, alpha polypeptide3	NM_174719	Ion transport
DBI	Bos taurus diazepam binding inhibitor	NM_181038	lipid binding
HOXD4	Homo sapiens homeobox D4	NM_014621	Transcription factor activity
PRNP	Bos taurus prion protein	NM_181015	GPI anchor binding
PENK	Bos taurus proenkephalin	NM_174141	Opioid peptide activity
PCSK1	Bos taurus proprotein convertase subtilisin/kexin type 1	NM_174412	Proteolysis
PTGER3	Bos taurus prostaglandin E receptor 3	NM_181032	Receptor activity
CPE	Bos taurus carboxypeptidase E	NM_173903	Proteolysis
TNF	Bos taurus tumor necrosis factor receptor member 1A	NM_174674	Receptor activity
NDUF	Bos taurus NADH dehydrogenase (ubiquinone)	NM_176660	oxidoreductase activity
UQC	B.taurus mRNA for ubiquinol-cytrochrome-c reductase	X59693	oxidoreductase activity
NSEP	Bos taurus nuclease sensitive element binding protein 1	NM_174815	DNA binding
tnf	Bos taurus tumor necrosis factor (ligand) member 5	NM_174624	Receptor activity
MLC	Bos taurus 155 kda myosin light chain kinase homolog	S57131	ATP binding
RAB	Bos taurus RAB3A, member RAS oncogene family	NM_174446	Nucleotide binding
PIK	Bos taurus phosphoinositide-3-kinase, alpha polypeptide	NM_174574	Transferase activivty
CSNK	Bos taurus casein kinase 2, alpha 1 polypeptide	NM_174635	ATP binding
PLAUr	Bos taurus plasminogen activator, urokinase receptor	NM_174423	Receptor activity
MAPK	Bos taurus similar to dual specificity protein phosphatase 16	XM_592862	kinase phosphatase activity
Mn-SOD	Cow manganous superoxide dismutase mRNA	L22092	Ion binding
LIF	Bovine DNA for leukemia inhibitory factor	D50337	Cytokine activity

3.3.2 Target preparation

3.3.2.1 RNA isolation and first strand synthesis

Once the respective biopsies were classified by the pregnancy phenotypes, they were categorized into three groups. Group one are those derived from blastocysts resulting in no pregnancy, group 2 those derived from blastocysts that ended with resorption and group 3 those resulting in calf delivery.
mRNAs isolation was performed using equivalents of pooled embryos (n=10 biopsies) and first strand synthesis were all carried as mentioned in section (3.3.1.1) above except the use of 20 mM of T7 Oligo d(T)21 primer [TCTAGTCGACGGCCAGTGAATTGTAATAC GACTCACTATAGGGCG(T)21] in this case. This T7 oligo d(T)21 primer has the T7 promoter sequence at its 5'-end which is recognized by RNA polymerase during in vitro transcription.

3.3.2.2 Second strand synthesis and global PCR amplification

This reaction has been carried out by using DOP PCR master kit. In addition to the 20 µl of first strand cDNA product, 40 µl of 2 X DOP PCR master mix, 1.0 µl of DOP primer (5'-CCGACTCGAG NNNNNN ATGTGG-3'), 1.0 µl of T7 oligo d(T)21 primer, and 16.0 µl water were added and properly mixed. This reaction was heated at 95 °C for 5 min to denature the sample and activate the polymerase, followed by one cycle of denaturation at 95 °C for 30 sec and annealing at 30 °C for 90 sec. Unspecific primer annealing is achieved up to this step through application of relatively low annealing temperatures of 30 °C. Subsequently the temperature was increased at 0.2 °C/sec until it reached 72 °C and incubated for 3 minutes at this temperature. To this end, second strand synthesis was completed and 1 µl each of DOP and T7 primers were added. Then, the global PCR amplification was continue for the last 10 cycles at 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 3 min. This reaction was terminated after final extension at 72 °C for 7 min and kept at 7 °C holding temperature. All reactions of amplification were carried out by using iCycler.

3.3.2.3 Purification of PCR products

DNA purification was performed by using a phenol chloroform extraction system. Equal volumes of phenol/chlorophorm/isoamyl alcohol was added to the 80 µl cDNA and mixed well before centrifuged at 14000 rpm for 15 min. The upper phase was carefully transferred to a new 2 ml tube to which 1 µl of glycogen, 0.5 volumes (v/v) of 5M NH_4OAc, and 2.5 volumes of ethanol were added and incubated at –20°C for 30 minutes. At the end of incubation time, the tubes were centrifuged at 14000 rpm for 20 min and the supernatant was discarded. The pellet was then washed with 500 µl of 70 % cold ethanol, centrifuged for 10 min at 14000 rpm and the alcohol was removed carefully. Finally the pellet was air dried and dissolved in 12 µl RNase free water for subsequent reaction.

3.3.2.4 In vitro transcription and RNA amplification

In vitro transcription was carried using AmpliScribe T7 transcription kit with some modifications of the manufacturer's protocol. Briefly, 2 µl of 10 X reaction buffer, 2 µl dNTP (100 mM each of ATP, CTP, GTP and UTP), 2 µl of DTT and 2 µl of T7 RNA polymerase were added to the 12 µl cDNA preparation, mixed well and incubated at 42 °C for 3 h. At the end of incubation, 1 µl of DNase was added and incubated at 37 °C for 30 min. Then the amplified RNA (aRNA) was purified using RNeasy Mini kit according to manufacturer's recommendations. Finally the aRNA was eluted in 30 µl RNase free water from which 8 µl were taken to estimate the yield and purity of aRNA by UV absorbance reading.

3.3.2.5 Aminoallyl labelling and dye coupling

For array hybridisation experiments, aRNA prepared as mentioned above was used for indirect labelling using the CyScribe post labelling kit. For this, 3 µg each of constructing aRNA in 10 µl volume, 1.5 µl random primer and 1.5 µl anchored oliogo(dT) were added and incubated at 70 °C for 5 min followed by incubating the reaction tube at room temperature for 10 min. Then 10 µl of master mix, containing 4 µl of 5 X first strand

buffer, 2 µl of 0.1 M DTT, 1.5 µl of dNTP mix, 1.5µl aminoallyl dUTP and 1µl CyScript reverse transcriptase was added to the reaction and incubated at 42 °C for 90 minutes. At the end, 2 µl of 2.5 M NaOH were added to the reaction to hydrolyse the mRNA and incubated at 37 °C for 15 minutes. Finally 10 µl of 2 M HEPES were added. The products were purified with the CyScribe™ GFX purification kit. The purified cDNA was then eluted in 60 µl 0.1 M sodium bicarbonate. The dye coupling reaction was performed by mixing the aminoallyl labelled cDNA with either of the dyes. The reaction was incubated at room temperature and in the dark for 1.5 h. At the end of the incubation, non reacting dyes were quenched by adding 15 µl of 4 M hydroxylamine solution and further incubated for 15 min at the same conditions. The combined reaction was then purified with CyScribe™ GFX purification kit. The sample was finally eluted in 60 µl elution buffer. Following GFX purification, Cy3-and Cy5-labelled cDNAs were combined into one tube. Then the target was speed-vacuum centrifuged in speedvac centrifuge.

3.3.3 Hybridization procedure

3.3.3.1 Prehybridization of the slides

To avoid non specific binding by deactivating the amine groups on the slide surfaces, the slides were blocked for 20 min with prehybridization buffer (3 X SSC, 1 % BSA) kept at 55 °C. The slides were then immersed in boiling water to denature the probes and wash unbound DNA from the slide surfaces, followed by immediate immersion in cold water and isopropanol consecutively. Then the slides were dried by centrifugation at ≥2000 rpm for 2 min before stored in a dry, light proof environment at room temperature.

3.3.3.2 Hybridization of the arrays

Shortly before hybridisation, array slides were treated with the prehybridization buffer as mentioned before. The dried target was then resuspended in pre-warmed (42 ^{o}C) hybridization solution (15 µl hybridization buffer, 30 µl 100% formamide, and 15 µl DEPC water) according to the recommendation of the company (Amersham Bioscience, Freiburg,

Germany). 1 µl of yeast tRNA (4 mg/ml, Sigma) and 1 µl of poly (dA) (8 mg/ml, Amersham) were also added to avoid non specific hybridisation. This mixture was incubated at 95 °C for 5 min to denature the target. After brief centrifugation, the target was put on the array slide and covered with glass cover slips. The slide was put in the hybridisation cassette (TeleChem, Inc, CA, USA) and placed in the hybridisation chamber (GFL, Dülmen, Germany) at 42 °C and incubated for 16-20 h.

3.3.3.3 Washing the slides

At the end of incubation period, the slides were washed twice with 2X SSC,0.1%SDS buffer for 5 min at 42°C followed by sequential washing by 1x SSC, 0.2x SSC and 0.1x SSC for 5 min each at room temperature. Finally slides were shortly rinsed in millipore water and then isopropanol for one min of each. Slides were then shortly centrifuged to dry and scanned immediately.

3.4 Image capture and data analysis

Slides were scanned by a GenePix 4000B scanner. Features were analyzed using GenePix Pro Version 4.0 software. The statistical analysis of the microarray's data was performed using SAM (Significant Analysis for Microarray), a free software program developed at Stanford University. First, a loess normalization of the data was performed using GPROCESSOR freeware to eliminate uninformative data. A mean of $\log_2$ ratio of the biopsies from various groups (no pregnancy /calf delivery and resorption /calf delivery) using normalized data was then calculated for the replicates to obtain one value per clone. Finally ratios were submitted to SAM analysis. The above experiments were then repeated with reverse-labeled cDNA samples. Heatmap were generated using HeatMap Builder. Heatmap reflects normalized gene expression ratios and is organized with individual hybridization for each experiment. Hierarchical clustering was carried out for up and down regulated genes using the clustering programme (Cluster & TreeView) written by Michael Eisen at the Eisen lab (http://rana.lbl.gov/index.htm). Average linkage clustering algorithm

was employed. Genes expressed equally in both samples were not included in the hierarchical clustering.

3.5 Quantitative real-time PCR analysis.

3.5.1 Plasmid isolation and preparation of serial dilution

Plasmid DNA containing the insert of each of the selected genes was used as standard for real-time PCR assay. For this the PCR products were amplified using the specific primers (Table 2) and then ligated into pGEM-T vector. After transformation in DH5α *Escherichia coli*, the positive colonies were cultured overnight at 37 ^{o}C in 5 ml LB-broth containing ampicillin. The recombinant *Escherichia coli* cultures were pelletted by centrifuging at 12000 rpm for 1 min. Plasmid DNA was isolated by using the GenElute plasmid Miniprep kit, following the manufacturers instructions. Briefly, the cells were resuspended with 200 µl of the resuspension solution, mixed thoroughly by vortexing and the cells were lysed by incubating with 200 µl of lysis solution for 4 min. The cells debris was precipitated by adding 350 µl of neutralization or binding buffer and centrifuiged at 12000 rpm for 10 min. In the mean time, columns were prepared by using column preparation solution. The cleared lysates were added to the prepared column and centrifuged at 12,000 rpm for 1 min and the flow through was discarded. The columns were again washed with 750 µl of washing solution and centrifuged at 12000 rpm for 1 min and the fllow through was discarded. Finally the plasmid DNA was eluted by adding 60 µl of distilled water. The isolated plasmid DNA was sequenced to confirm the sequence of gene of interest.

The concentration of the plasmid DNA was estimated by reading the absorbance A260/280 using Ultrospec™ 2100 pro UV/Visible Spectrophotometer. The plasmid concentration (ng/µg) was converted in number of copies (molecules) using the following program: www.molbiol.ru. Then serial dilutions were prepared for each clone from 10^1 up to 10^8 copy number in 50 µl volumes.

3.5.2 Quantitative real-time PCR

Quantitative real-time PCR was used to confirm the differentially expressed genes revealed by microarray experiments. Real-time PCR reactions were conducted in an ABI Prism ® 7000 SDS instrument and SYBER green was used as a double-strand DNA-specific fluoresecent dye. Prior to quantification, primer optimisation was performed for both forward and reverse primers. Specific primer level combinations with lower threshold cycle (C_T) value and without primer-dimer formation were selected for subsequent PCRs. Quantitative analyses of biopsies cDNA were performed in comparison with the bovine GAPDH gene (endogenous control), and were run in separate wells. Standard curves were generated for both target and internal control genes using serial dilution of plasmid DNA (10^1-10^8 molecules). Polymerase chain reactions were performed in 20 µl reaction volume containing 9 µl of 2.5 X RealMasterMix/20X SYBR Solution, optimal levels of forward and reverse primers and 2 µl cDNA. Each PCR was run for a particular biopsy group in duplicate to control the reproducibility of quantitative results. An universal thermal cycling parameter (10 s at 50 ^oC, 10 min at 95 ^oC, 45 cycles of 15 s at 95 ^oC and 60 s at 60 ^oC) was used for the quantification of each gene. After the end of the last cycle, a melting curve was generated by starting the fluorescence acquisition at 60 ^oC and taking measurements every 7 sec until the temperature reached 95 ^oC. Final quantification analysis was performed using the relative standard curve method (User bulletin # 2 ABI PRISM 7700 SDS, Http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf) and results were reported as the relative expression to the calibrator cDNA after normalization of the transcript amount to the endogenous control.

3.6 Immunofluorescence staining of oocytes and embryos

In vitro produced bovine immature and mature oocytes, zygotes; 2-cell, 4-cell, 8-cell, morula and blastocyst stage embryos were washed three times in PBS and fixed in 4 % (w/v) paraformaldehyde in PBS overnight at 4 °C. The fixed specimens were permeabilized during 2.5 h incubation in 0.5 % (v/v) Triton-X100 in PBS. In order to inhibit non-specific binding of the antibodies, samples were subsequently blocked in 3 % (w/v) bovine serum

albumin (BSA) in PBS for 1h. The oocytes and embryos were then incubated for 1 h at 39 °C with 1:100 dilution of anti-MSX1 primary polyclonal antibody. After three consecutive washes with PBS, oocytes and embryos were further incubated for 1 hour with 1:100 dilutions of secondary anti-rabbit IgG FITC conjugated antibody. Negative controls were processed in the same manner except that the primary antibody was omitted. In order to visualize the nucleus of the cells, oocytes and embryos were finally incubated with 0.5µg/ml propidium iodide. After an ultimate washing with PBS, oocytes and embryos were mounted on glass slides (Menzel GmbH & Co KG, Braunschweig, Germany) in Vectashield mounting medium, protected with coverslip, sealed with nail polish and examined under confocal laser scanning microscope.

Table 2: Details of primers used for quantitative real time PCR

Gene name	Gene Bank accession number	Forward primer	Reverse primer	Product size (bp)
GAPDH	BC102589	5´-ACCCAGAAGACTGTGGATGG-3´	3´ ACGCCTGCTTCACCACCTTC-5´	247
EEF1A1	BTA238405	5´-CCATGGCATATTAGCACTTGGTT-3´	3´-GCTTACACCCTGGGTGTGA-5´	214
PTTG1	NM_004219	5´-GAAGAGCACCAGATTGCGC-3´	3´-GTCACAGCAAACAGGTGGCA-5´	204
TNF	AF011927	5´-GTGAAGTCGCTCAGTCGTGC-3´	3´-TCTACAAGGCGGGAGACCTG-5´	170
CD9	NM_173900	5´-CACATCAGTCCAACCCAGAC-3´	3´-AATCGGAGCCATAGTCCAAC-5´	146
HNRNPA1	BC052296	5´-TGGACTCCAGGTCACAACACA-3´	3´-CTTCAGGGTGATGCCAGGTT-5´	121
AKR1B1	M31463	5´-CGTGATCCCCAAGTCAGTGA-3´	3´-AATCCCTGTGGGAGGCACA-5´	152
PLAC8	NM_016619	5´-CGGTGTTCCAGAGGTTTTTCC-3´	3´-AAGATGCCAGTCTGCCAGTCA-5´	163
COX-2	AF031698	5´-ATCTACCCGCCTCATGTTCCT-3´	3´-GGATTAGCCTGCTTGTCTGGA-5´	187
PLAU	L03546	5´CATCTACAGGAGGCATCGAGG-3´	3´GTCGGAGTTAAGCCGTGACTG-5´	162
TXN	AF104105	5´- ATGGTGAAACAGATTGAGAAG-3´	3´- CGTTGGAATACTTTTCAGAGAGAGAA-5´	154
ODC1	NM_174130	5´- CAAAGGCCAAGTTGGTTTTAC-3´	3´-CAGAGATGGCCTGCACAAAG-5´	201
ANXA2	NM_174716	5´-CGTGCTCCAGCTAACAGTTCT-3´	3´-GGAAAGCCAGGTAATGCGTA-5´	139
ATP5A1	M22465.1	5-´AAGCTTCAAATCCAGCCAAGAA-3´	3´-TTGTCTCACGTTATCAGCCAACA-5´	127
MSX1	NM_174798	5´-AAGGTATCCACAGTCCCCAGC-3´	3´-TCTGCCTCTCCTGCAAAGTTC- 5´	180
KRT8	X12877	5´-CACCAGTTCCAAGCCTGTGG-3	3´-TCAGGTCTCCTGTGCAGATGC- 5´	176

4 Results

4.1 Microarray experiments

4.1.1 Array characterization

In the present study we used two different cDNA arrays. The first is the BlueChip cDNA array which was kindly provided by the Department of Animal Science, Laval University, Canada. This array contained 4928 spots in two sub-arrays per slide. Each sub-array is composed of 2304 randomly selected clones obtained from four different suppression subtractive hybridizations (SSH). First SSH: GV oocytes subtracted from somatic tissues, second SSH: GV oocytes subtracted from Day-8 blastocyst, third SSH: Day-8 blastocyst subtracted from GV oocytes and fourth SSH: Day-8 blastocyst subtracted from somatic tissues.

The second array is bovine preimplantation specific custom cDNA array which prepared in our lab. This was constructed being enriched with bovine preimplantation stage specific clones. The majority of the clones are generated from different developmental stages of bovine preimplantation embryos using stage specific cDNA library construction (Ponsuksili et al. 2001), suppressive subtractive hybridisation (SSH) (Ponsuksili et al. 2002) and differential display (Tesfaye et al. 2003). In addition to these, some specific clones that are known to be expressed during embryo preimplantation development stage have also been amplified with gene specific primers and included in the array. A total of 219 genes and EST probes were used as a probe in this array. The genes in this array have been classified based on the available information for the bovine, human and mouse species in the gene data bank. Even some of the information has been generated from non bovine species, functional conservation of genes has been assumed in the absence of bovine specific gene information. Therefore, based on this assumption, they were classified functionally into 20 major categories (Figure7). As has been indicated in the figure, the majority of known genes were comprised of genes in the protein binding, transcription factor activity, DNA and nucleotide binding, transporters activity and ion binding categories. About 16 % of the

genes and ESTs have not yet been classified for their function either in the bovine or other species.

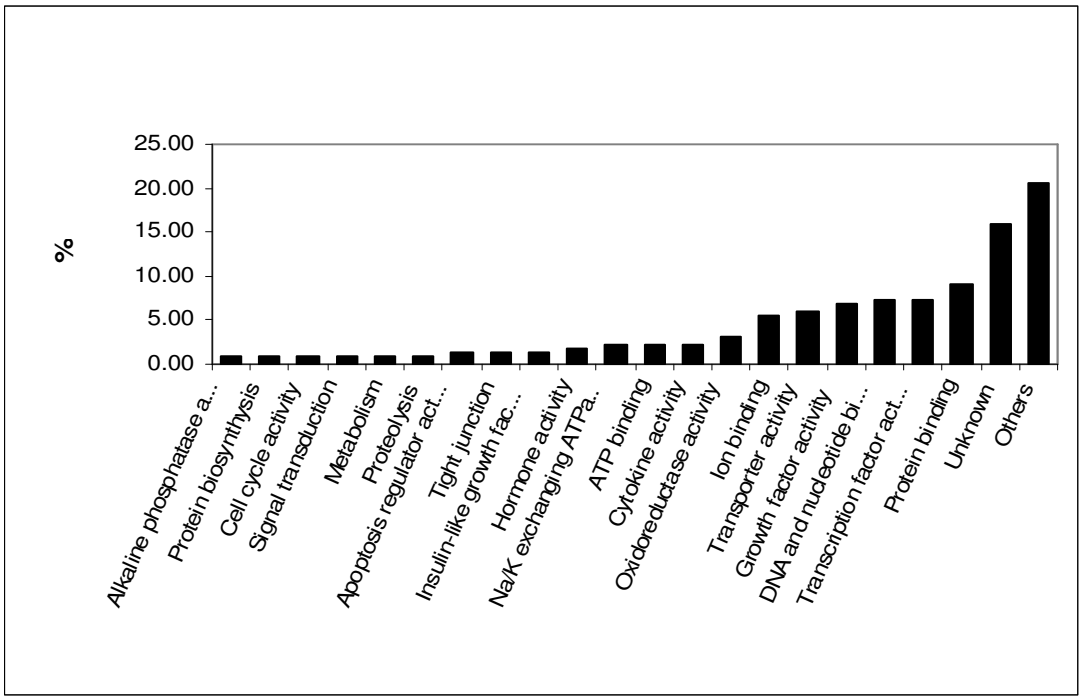
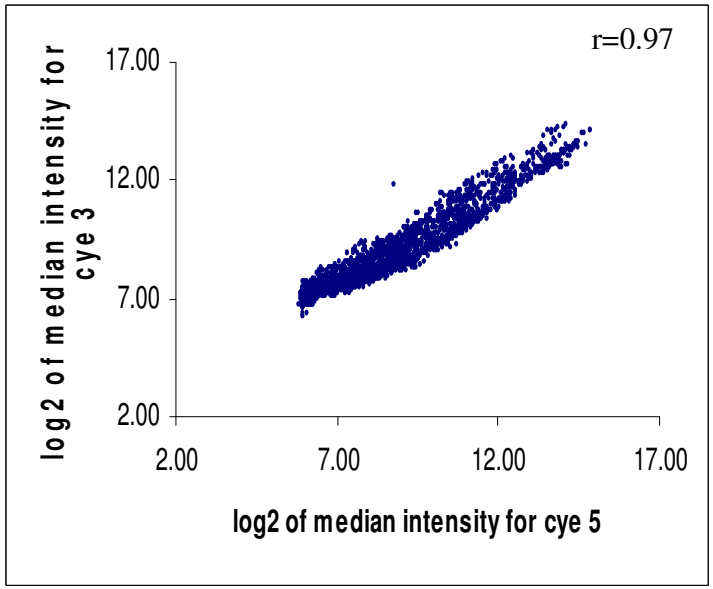


Figure 7: Distribution of genes present on in-house-produced cDNA microarray according to functional groups.

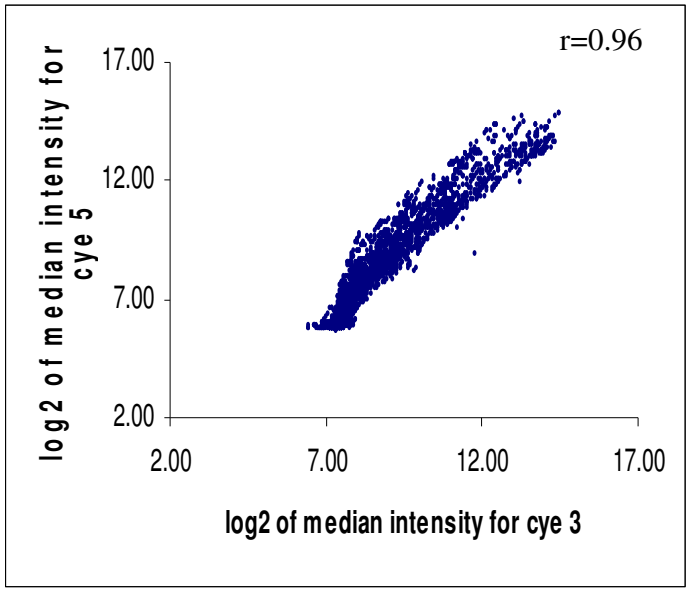
4.1.2 Differential gene expression profiles

During each experiment a series of six hybridization experiments (three biological replicates with dye swap) were conducted to minimize false positive expression changes and to identify genes truly differentially expressed ($q \leq 0.10$) between biopsies derived from blastocysts resulting in no pregnancy, resorption or calf delivery. The scatter plots of the replicates showing the reproducibility between biological or technical replicates are shown in Figure 8 A and B and 9 A and B. The heatmap (Figure 10 A and B) represents the overall view of the expression levels of genes on BlueChip and bovine preimplantation specific custom arrays during comparison between the two experiments (No pregnancy vs. calf delivery and resorption vs. calf delivery). All differentially expressed genes were

classified based on their functions according to criteria of gene ontology consortium classifications (http://www.geneontology.org). The resulting data were supplemented with additional information from Entrez Gene (http:www.ncbi.nlm.nih.gov/entrez/query.fcgi?db =gene).

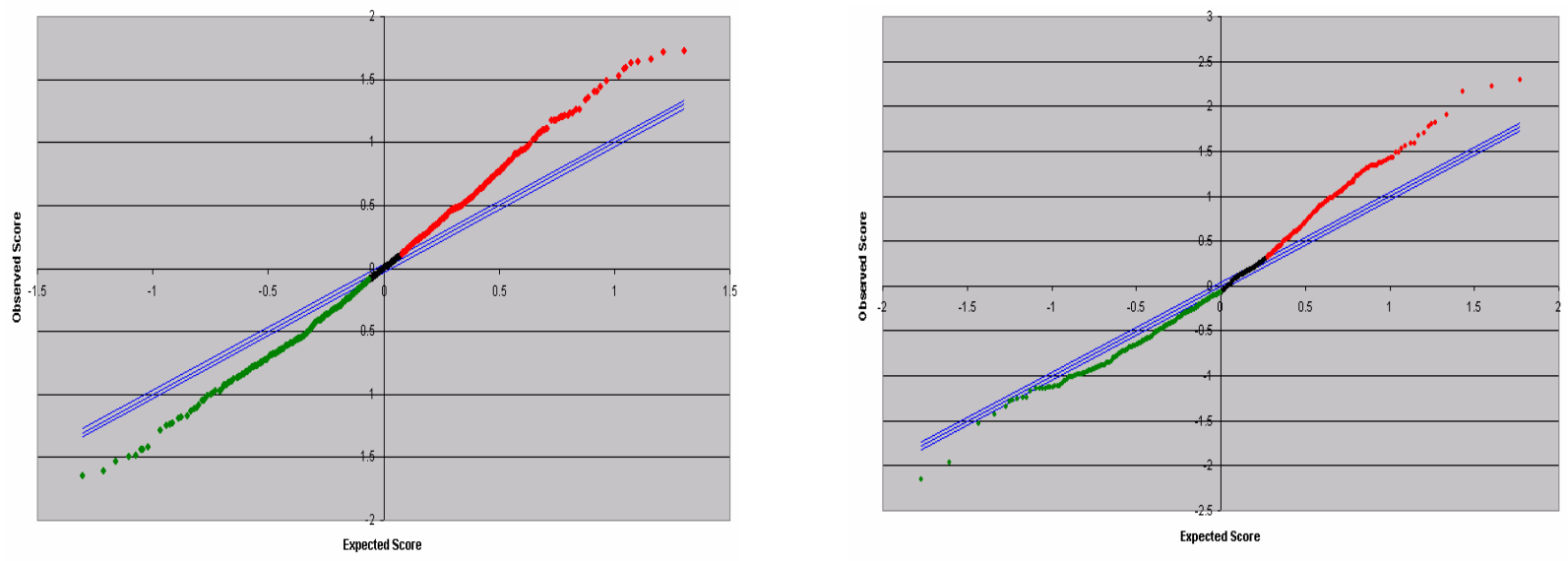


(A)



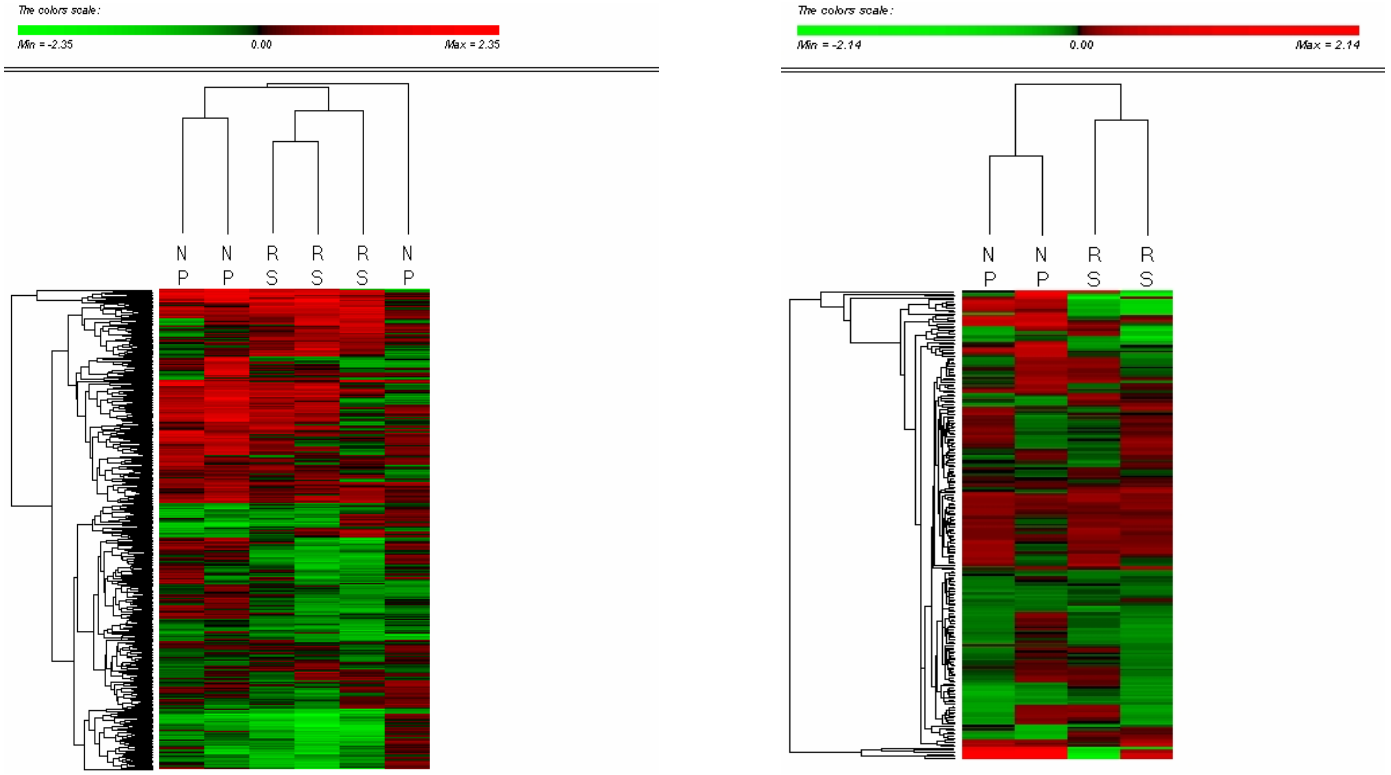
(B)

Figure 8: Scatter plots of the replicates with dyeswap showing the log2 of median signal intensity of cye3 and cye5, (A) is the three biological replicates and (B) is the dyeswap of these replicates.



(A) (B)

Figure 9: SAM plotsheet for repeated experiments using dyeswap showing the reproducibility between replicates (C) and (D). The red colur showing the up-regulated genes and the green colur showing the down-regulated.



(A) (B)

Figure 10: Heatmap showing the normalized gene expression ratios and is organized with individual hybridization for the triplicate hybridizations for each experiments using BlueChip cDNA array (A) and duplicate hybridizations using bovine preimplantation specific custom cDNA array (BPSA) (B) arranged along the x axis, with normalized expression ratios depicted by color intensity such that highest expression corresponds to the bright red, and the lowest expression corresponds to bright green.

4.1.3 Experiment 1: Transcriptional analysis of biopsies derived from blastocysts resulting in no pregnancy (G1) versus calf delivery (G3)

A cDNA array analysis between G1 and G3 biopsy groups revealed that a total of 52 genes (41 from Bluechip and 11 from BPSA array) was differentially regulated between the two groups. 46 and 6 clones were up- and down-regulated, respectively, in G1 compared with G3 (1.5≤fold change≤4.7). These differentially regulated genes represent three functional categories. The classification of the differentially regulated genes according to function is shown in figure. 11. The identity, database accession number, fold change difference and the functional category of differentially regulated genes are given in Table 3.

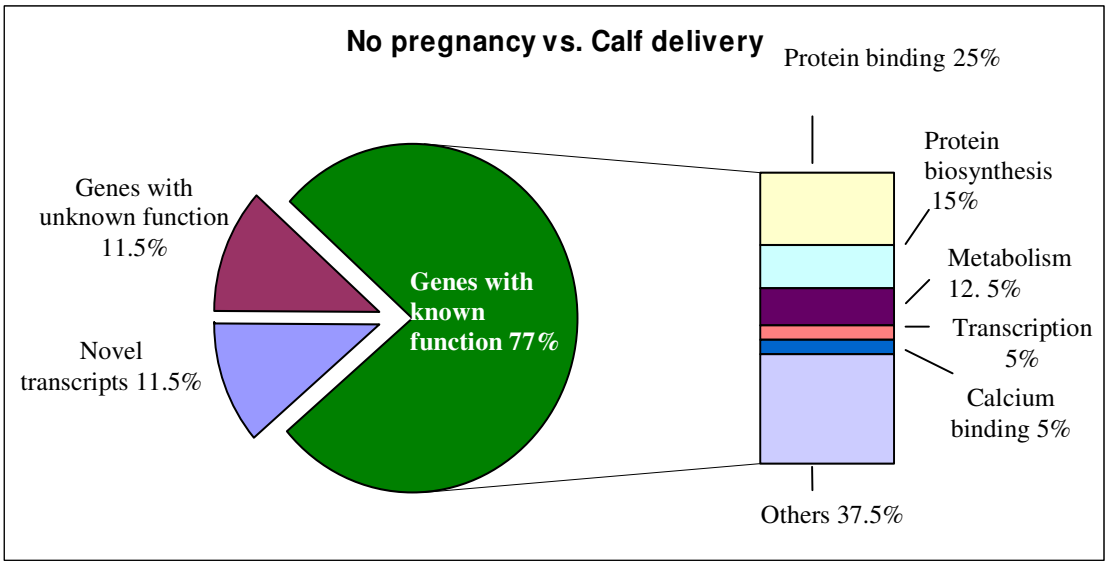


Figure 11: Ontology classification for differentially expressed transcripts between biopsies derived from blastocysts resulting in no pregnancy and calf delivery. The known genes were classified functionally based on the Gene Ontology Consortium classification (http://www.geneontology.org)

The expression of the unknown genes and novel transcripts showed profiles similar to those of the annotated genes, as determined by tree hierarchical clustering analyses (Figure 12).

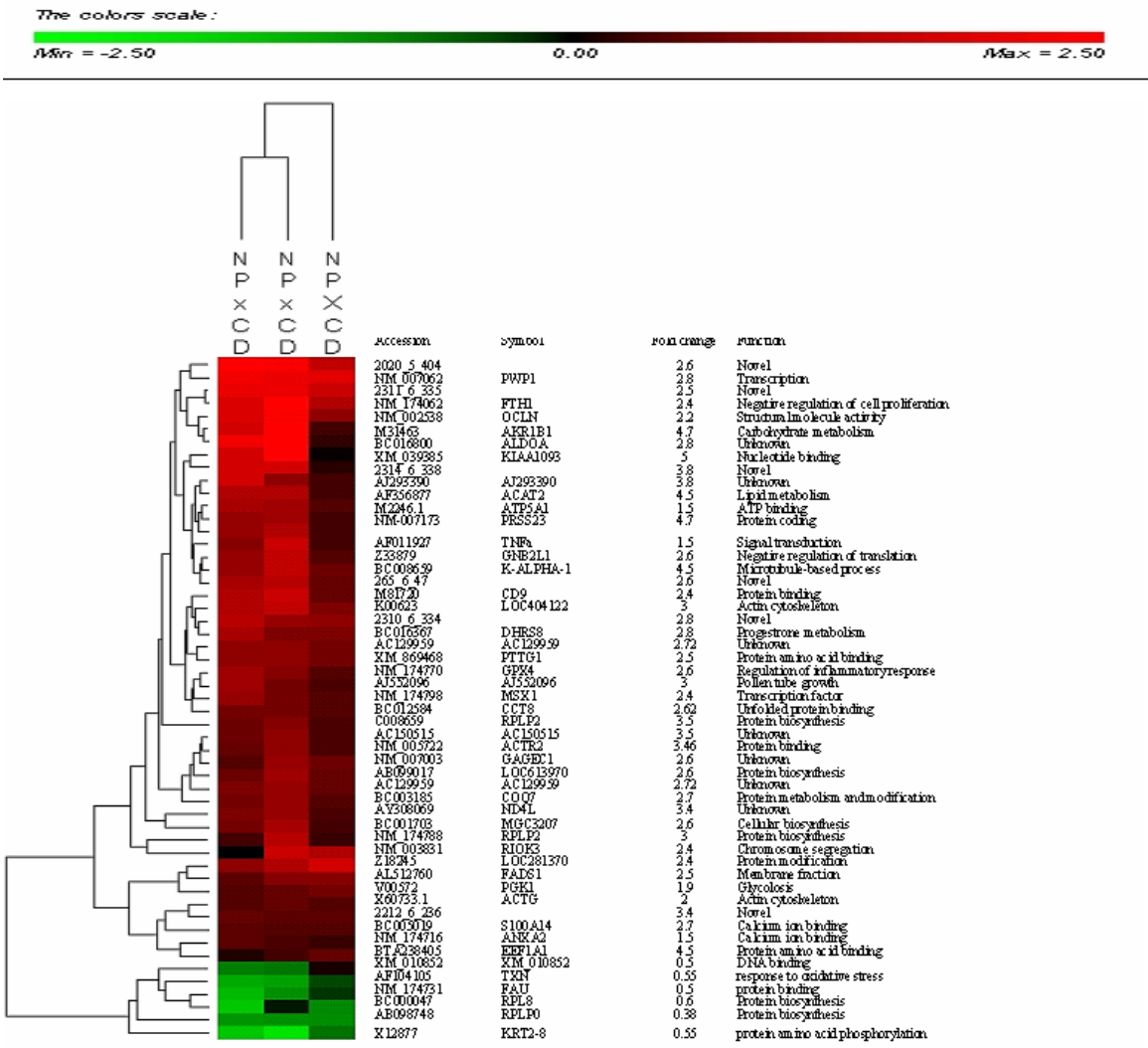


Figure 12: Hierarchical clustering for the differentially expressed genes between biopsies derived from blastocysts resulted in no pregnancy (G1) vs. calf delivery (G3). The columns represent the replicates. The rows represent 46 genes found to be up regulated (red colur) and 6 genes down regulated (green colur) in no pregnancy. The rows showing the accession number, symbol, fold change and function for each gene.

Table 3: List of differentially regulated genes in blastocyst biopsies resulting in no pregnancy (G1) compared to calf delivery (G3) at a false discovery rate (FDR) ≤ 10 %.

Gene Name	Accession No.	Fold change	Function
1-Up regulated			
Homo sapiens KIAA 1093 protein mRNA (KIAA1093)	XM_039385	5.0	Nucleotide binding
Bovine aldose reductase mRNA, 3 end (AKR1B1)	M31463	4.7	Carbohydrate metabolism
Homo sapiens Protease serine 23 (PRSS23)	NM-007173	4.7	Protein coding
Bos taurus elongation factor 1 alpha 1 (EEF1A1)	BTA238405	4.5	Protein amino acid binding
Homo sapiens tubulin alpha ubiqutous mRNA (K-ALPHA1)	BC008659	4.5	Microtubule-based process
Homo sapiens acetyl CoA transferse-like protein (ACAT2)	AF356877	4.5	Lipid metabolism
Homo sapiens mRNA, differentially expressed in malignant melanoma	AJ293390	3.8	Unknown
2314_6_338		3.8	Novel
Bos taurus BAC CH240-118E9	AC150515	3.5	Unknown
Homo sapiens ARP2 actin-related protein 2 homolog (yeast) (ACTR2)	NM_005722	3.4	Protein binding
Bos taurus acidic ribosomal protein P2 mRNA, complete cds	C008659	3.4	Protein biosynthesis
Bos taurus Isolate FL405 mitochondrion, partial genome	AY308069	3.4	Unknown
2212_6_236		3.4	Novel
Bos taurus ribosomal protein, large P2 (RPLP2)	NM_174788	3.0	Protein biosynthesis
Arabidopsis thaliana T-DNA flanking sequenze, left border,clone	AJ552096	3.0	Pollen tube growth
Bovine Actin mRNA, 3 end (LOC404122)	K00623	3.0	Actin cytoskeleton
Homo sapiens nuclear phosphoprotein similar to S.cerevisiae (PWP1)	NM_007062	2.8	Transcription
Homo sapiens dehydrogenase/reductase SDR family (DHRS8)	BC016367	2.8	Progestrone metabolism
Homo sapiens Aldose A, Fractose-biphosphate,transcript (ALDOA)	BC016800	2.8	Unknown
2310_6_334		2.8	Novel
Homo sapiens Coenzyme Q7 homolo,ubiquinone (yeast) (COQ7)	BC003185	2.7	Protein metabolism and modification
Homo sapiens S100 calcium binding protein A14 (S100A14)	BC005019	2.7	Calcium ion binding
Bos taurus, clone RP42-518P7, complete sequence	AC129959	2.7	Unknown
Bos taurus glutathione peroxidase 4 (GPX4)	NM_174770	2.6	Regulation of inflammatory response
Bos taurus mRNA for similar to ribosomal protein S3a (RPS3A)	AB099017	2.6	Protein biosynthesis
S.scrofa mRNA encoding G-beta like protein (GNB2L1)	Z33879	2.6	Negative regulation of translation
265_6_47		2.6	Novel
2020_5_404		2.6	Novel

Table 3: (Continued).

Gene Name	Accession No.	Fold change	Function
Homo sapiens hypothetical protein (MGC3207)	BC001703	2.6	Cellular biosynthesis
Homo sapiens chaperonin containing TCP1, subunit 8 theta (CCT8)	BC012584	2.6	Unfolded protein binding
Homo sapiens G antigen, family C, 1 (GAGEC1)	NM_007003	2.6	Unknown
Homo sapiens mRNA ;cDNA DKFZp762M2311 (FADS1)	AL512760	2.5	Oxidreductase activity
Homo sapiens Pitutary tumor-transforming 1 (PTTG1)	XM_869468	2.5	Protein amino acid binding
2311_6_335		2.5	Novel
Bos taurus CD9 antigen (p24) (CD9)	M81720	2.4	Protein binding
Bos taurus msh homeo box homog 1 (drosophila) (MSX1)	NM_174798	2.4	Transcription factor
Homo sapiens RIO kinase 3(yeast) (RIOK3)	NM_003831	2.4	Chromosome segregation
Bos taurus Polyubiqutin	Z18245	2.4	Protein modification
Bos taurus ferritin, heavy polypeptide 1 (FTH1)	NM_174062	2.4	Negative regulation of cell proliferation
Homo sapiens occludin (OCLN)	NM_002538	2.2	Plasma membrane
Bovine gamma non muscle actin	X60733.1	2.0	Actin cytoskeleton
Human mRNA encoding phosphoglcerate kinase	V00572	1.9	Glycolysis
Bos Taurus annexin A2(ANAX2)	NM_174716	1.5	Calcium ion binding
Bovine primary structure of 1.715 satelite DNA	V00124	1.5	Unknown
Bos taurus Tumor necrosis fator alpha gene (TNF)	AF011927	1.5	Signal transduction
Bovine alpha subunit ATP synthase isoform mRNA (ATP5A1)	M2246.1	1.5	ATP binding
2- Down regulated			
Homo sapiens ribosomal protein L8 (RPL8)	BC000047	0.6	Protein biosynthesis
Bovine mRNA fragment for cytokeratin A (no. 8) (KRT8)	X12877	0.5	Protein amino acid Phosphorylation
Bos taurus Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)	NM_174731	0.5	Protein binding
Heterogeneous nuclear ribonucleoprotein A1 (HNRPA1)	XM_010852	0.5	DNA binding
Bos taurus thioredoxin mRNA (TXN)	AF104105	0.5	Response to oxidative stress
Bos taurus similar to acidic ribosomal phosphoprotein PO (RPLPO)	AB098748	0.3	Protein biosynthesis

4.1.4 Experiment 2: Transcriptional analysis of biopsies derived from blastocysts resulted in resorption (G2) versus calf delivery (G3)

A comparison between G2 and G3 group of biopsies showed that 58 genes were differentially regulated (44 from BlueChip and 14 from BPSA array) between G2 and G3. 37 genes were up-regulated in G2 and 21 genes down-regulated compared with G3 ($1.6 \leq$ fold change ≤ 6). These differentially regulated genes represent three functional categories. The classification of the differentially regulated genes according to function is shown in figure 13. The identity, database accession number, fold change difference and the functional category of differentially regulated genes are indicated in Table 4..

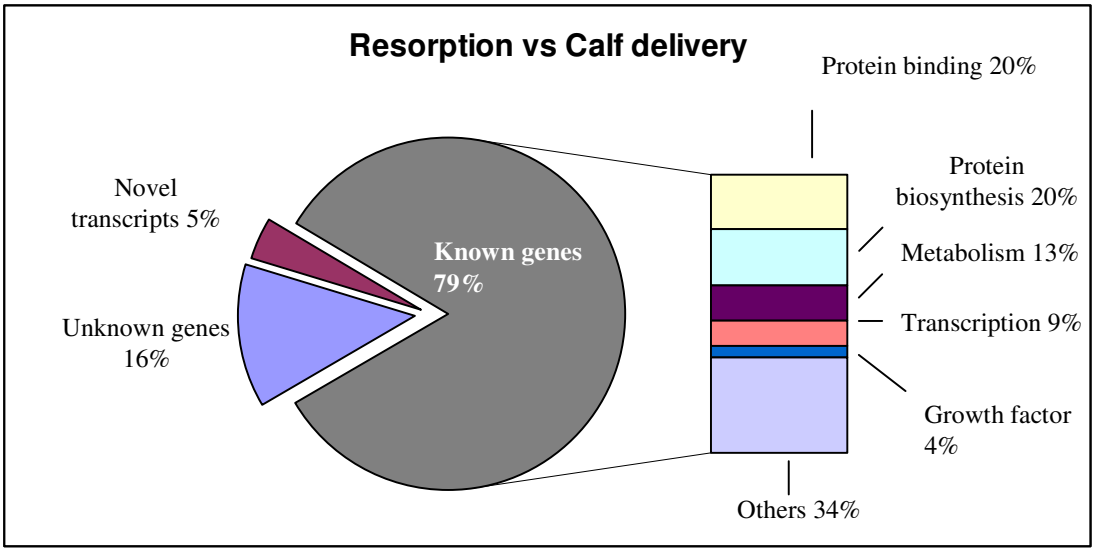


Figure 13: Ontology classification for differentially expressed transcripts between biopsies derived from blastocysts resulting in resorption and calf delivery. The known genes were classified functionally based on the Gene Ontology Consortium classification (http://www.geneontology.org)

The hierarchical clustering analysis has enabled to correlate the expression of those ESTs and genes with unknown function to those of the annotated genes (Figure 14).

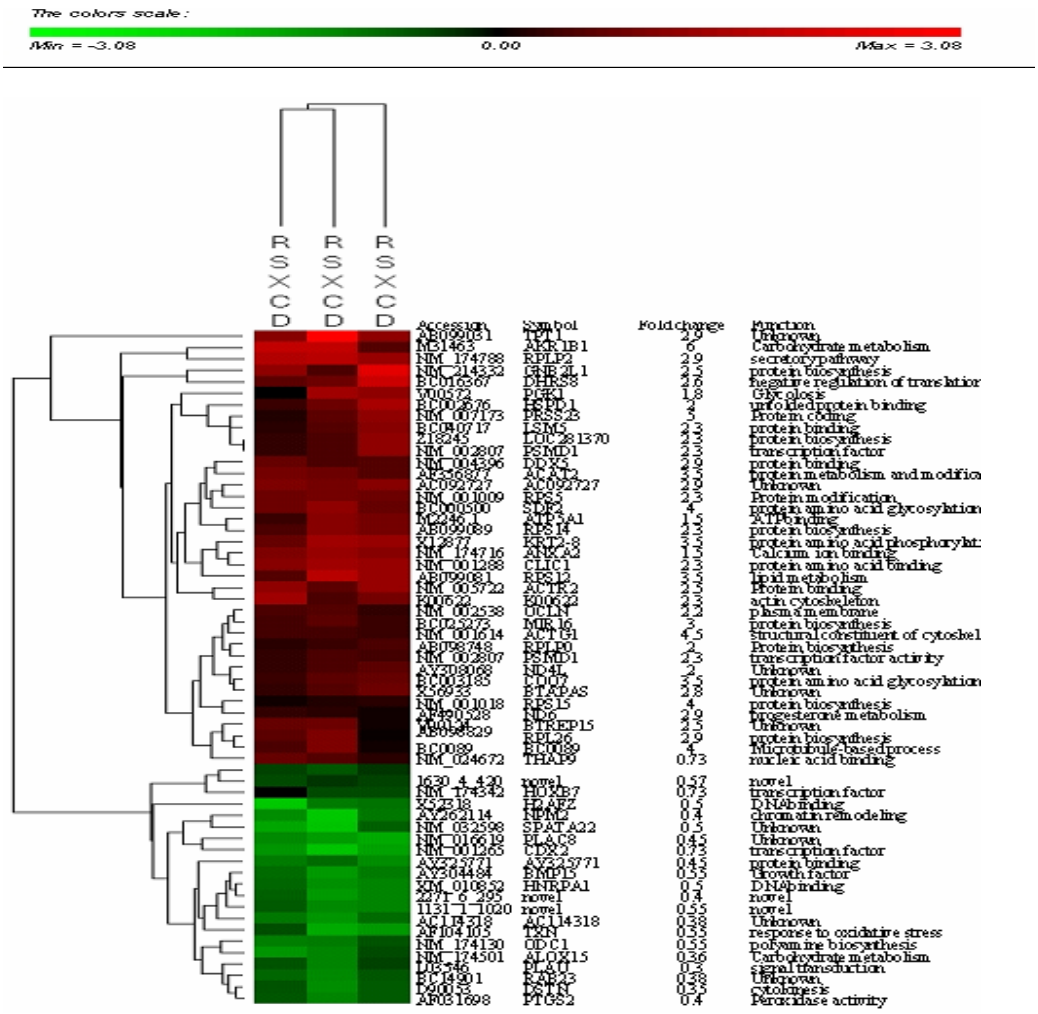


Figure 14: Hierarchical clustering for the differentially expressed genes between biopsies derived from blastocysts ended with Resorption (G2) vs. calf delivery (G3). The columns represent the replicates. The rows represent 37 genes found to be up-(red colour) and down-(green colour) regulated respectively in biopsies derived from blastocysts resulted in resorption. The rows showing the accession number, gene symbol, fold change and function category of each gene.

Table 4: List of differentially regulated genes in biopsies obtained from blastocysts resulted in resorption (G2) compared to calf delivery (G3) at a false discovery rate (FDR) of ≤ 10%.

Gene Name	Accession No.	Fold change	Function
1-Up regulated			
Bovine aldose reductase mRNA, 3' end (AKR1B1)	M31463	6.0	Carbohydrate metabolism
Protease serine 23 (PRSS23)	NM_007173	5.0	Protein coding
Homo sapiens actin, gamma 1 (ACTG1)	NM_001614	4.5	Structural constituent of Cytoskeleton
Homo sapiens ribosomal protein S15 (RPS15)	NM_001018	4.0	Protein biosynthesis
Homo sapiens stromal cell-derived factor 2 (SDF2)	BC000500	4.0	Protein amino acid glycosylation
Homo sapiens tubulin alpha ubiquitous mRNA (K-ALPHA1)	BC0089	4.0	Microtubule-based process
Homo sapiens occludin (OCLN)	NM_002538	3.6	Structural molecule activity
Bovine mRNA fragment for cytokeratin A (no. 8) (KRT8)	X12877	3.5	Protein amino acid phosphorylation
Bos taurus mRNA for similar to ribosomal protein S12 (RPS12)	AB099081	3.5	lipid metabolism
Homo sapiens acetyl CoA transferase-like protein (ACAT2)	AF356877	3.5	Protein metabolism and modification
Homo sapiens coenzyme Q7 homolog, ubiquinone (yeast) (COQ7)	BC003185	3.5	Protein amino acid glycosylation
Homo sapiens membrane interacting protein of RGS16 (MIR16)	BC025273	3.0	Protein biosynthesis
Bos taurus mRNA for similar to ribosomal protein L26 (RPL26)	AB098829	2.9	Protein biosynthesis
Bos taurus ribosomal protein, large P2 (RPLP2)	NM_174788	2.9	Secretory pathway
Bos taurus isolate 65 NADH dehydrogenase subunit 1 (ND1)	AF490528	2.9	Progesterone metabolism
Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (DDX5)	NM_004396	2.9	Protein binding
Bos taurus mRNA similar to translationally controlled tumor (TPT1)	AB099031	2.9	Unknown
Bos taurus clone RP42-351K5, complete sequence	AC092727	2.9	Unknown
B.taurus mRNA, alternative polyadenylation signals	X56933	2.8	Unknown
Homo sapiens dehydrogenase/reductase SDR family (DHRS8)	BC016367	2.6	Negative regulation of translation
Sus scrofa G-beta like protein (GNB2L1)	NM_214332	2.5	Protein biosynthesis
Homo sapiens ARP2 actin-related protein 2 homolog (yeast) (ACTR2)	NM_005722	2.5	Protein binding
Primery structure of bovine 1.715 satelte	V00124	2.5	Unknown
Homo sapiens protasome (prosome, macropain) 26S subunit (PSMD1)	NM_002807	2.3	Transcription factor activity
Bos taurus mRNA for similar to ribosomal protein S14 (RPS14)	AB099089	2.3	Protein biosynthesis
Homo sapiens ribosomal protein S5 (RPS5)	NM_001009	2.3	Protein modification
Bos taurus Polyubiqutin	Z18245	2.3	Protein biosynthesis

Table 4: (Continued)

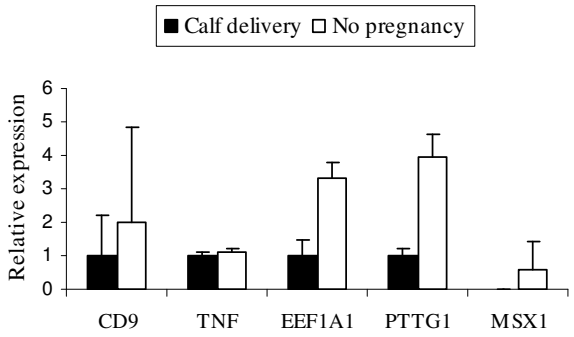
Gene Name	Accession No.	Fold change	Function
Bovine actin mRNA, 5' end	K00622	2.3	Actin cytoskeleton
Homo sapiens chloride intracellular channel 1 (CLIC1)	NM_001288	2.3	Protein amino acid binding
Proteasome (prosome, macropain) 26S subunit, non-ATPase	NM_002807	2.3	Transcription factor activity
Bos taurus acidic ribosomal phosphoprotein PO (RPLPO)	AF013214	2.0	Protein biosynthesis
Homo sapiens heat shock 60kDa protein 1 (chaperonin) (HSPD1)	BC002676	2.0	Unfolded protein binding
Bos taurus ribosomal protein L3 (Rpl3)	NM_174715	2.0	Protein biosynthysis
Bos taurus isolate FL396 mitochondrion, partial genome	AY308068	2.0	Unknown
Human mRNA encoding phosphate kinase (PGK1)	V00572	1.8	Glycolysis
Bovine alpha subunit ATP synthase isoform mRNA (ATP5A1)	M2246.1	1.5	ATP binding
Bos taurus annexin A2 (ANAX2)	NM_174716	1.5	Calcium ion binding
2-Down regulated			
Homo sapiens caudal type homeo box transcription factor 2 (CDX2)	NM_001265	0.73	Transcription factor
Homo sapiens Hypothetical protein FLJ23320 (THAP9)	NM_024672	0.73	Nucleic acid binding
Bos taurus homeo box B7 (HOXB7)	NM_174342	0.70	Transcription factor
1630_4_420		0.57	Novel
1131_1_1020		0.55	Novel
Bos taurus ornithine decarboxylase (ODC1)	NM_174130	0.55	Polyamine biosynthesis
Bos taurus thioredoxin (TXN)	AF104105	0.55	Response to oxidative stress
Bos taurus bone morphogenetic protein 15 (BMP15)	AY304484	0.55	Growth factor
Bovine mRNA for histone H2A.Z	X52318	0.50	DNA binding
Heterogeneous nuclear ribonucleoprotein A1 (HNRPA1)	XM_010852	0.50	DNA binding
Homo sapiens testes development-related NYD-SP20 (NYD-SP20	NM_032598	0.50	Unknown
Homo sapiens placenta-specific 8 (PLAC8)	NM_016619	0.45	Unknown
Bos taurus BTAB2MDS3 beta-2-microglobulin (B2M)	AY325771	0.45	Protein binding
Bos taurus prostaglandin G/H synthase-2 (PGHS-2) (COX2)	AF031698	0.40	Oxidoreductase activity
2271_6_295		0.40	Novel
Homo sapiens RAN, member RAS oncogene family (RAN)	BC14901	0.40	Unknown

Table 4: (Continued).

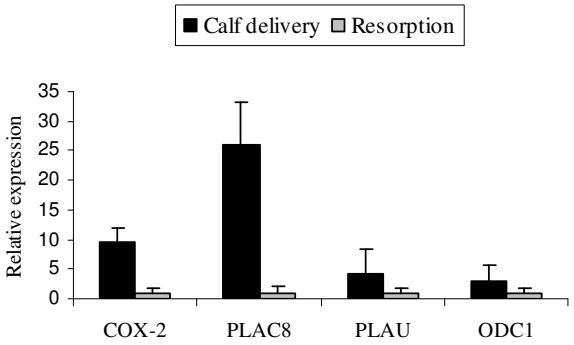
Gene Name	Accession No.	Fold change	Function
Homo sapiens chromosome 5 clone RP11-425E13	AC114318	0.38	Unknown
Homo sapiens nucleoplasmin 2 variant 2 (NPM2)	AY262114	0.38	Chromatin remodeling
Bos taurus arachidonate 15-lipoxygenase (ALOX15)	NM_174501	0.36	Carbohydrate metabolism
Sus scrofa mRNA for destrin (DSTN)	D90053	0.35	Cytokinesis
Bos taurus urokinase-type plasminogen activator (PLAU)	L03546	0.30	Signal transduction

4.2 Validation of differentially gene expression

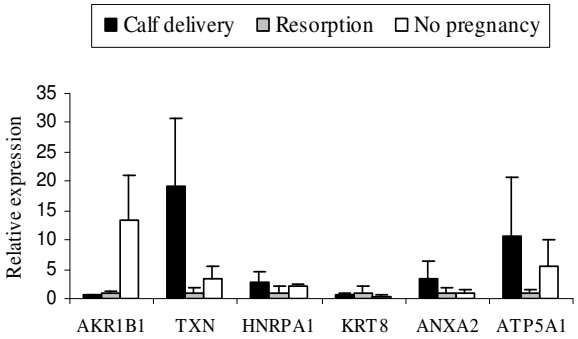
For confirming the results of microarray, two approaches were used. The first is is quantitative real-time PCR (qPCR) where a total of 15 selected genes (AKR1B1, ANXA2, ATP5A1, CD9, COX-2, EEF1A1, HNRPA1, KRT8, MSX1, ODC1, PLAC8, PLAU, PTTG1, TNF, TXN), are quantified in independent biopsy samples from the three groups (Figure 15 A, B and C). The primer sequences for these genes are mentioned in table 2. In general the quantitative real time PCR confirmed the expression of 87% (13/15) of the genes generated from the array hybridization, while the expression profiles of 3% (2/15) (ANXA2 and ATP5A1) were not in agreement with the microarray results The second is in silico analysis to check relevant expression profile data for that particular gene from literature and data bank. The results of four genes were confirmed. These includes the caudal-related homeobox protein (CDX2) which is crucial for placental development (Hall et al. 2005), phosphoglycerate kinase (PGK1) showing overexpression as a result of high glucose level (Mohan et al. 2002), polyubiquitin which is involved in programmed cell death (PCD) (Herskko and Ciechanover 1998) and Protease serine 23 (PRSS23) gene whose its specific function is unknown (Gene data Bank).



(A)



(B)



(C)

Figures 15A to C: Quantitative real time PCR confirmation of selected transcripts between biopsies derived from blastocysts resulting in no pregnancy versus calf delivery (A) and resorbed embryos versus calf delivery (B) and those resulting in no pregnancy and resorbed embryos versus calf delivery (C).

As shown in the above figures, confirmation analysis using real time PCR for 5 genes namely: PTTG, EEF1A1, TNFα and CD9 has shown that the transcripts were 3.9, 3.2, 1.2 and 2.2 fold high expressed in G1 group respectively compared to those G3 group. Interestingly, MSX1 was not detected at all in the G3 group (Figure 15A). Similar analyse for four genes COX2, PLAC8, PLAU and ODC1 have confirmed 9.6, 26.1, 4.3 and 3.1 times respectively more expression in the G3 group compared to G2 group (Figure 15B). Further confirmation of genes which were differentially regulated between both experiments showed that AKR1B1 gene was 28 and 2.1 fold more abundant in G1 and G2 group respectively. On contrary TXN gene was abundant at 19.2 and 3.3 fold in G3 and G1 group respectively compared to G2. The same trend was observed for HNRNP gene which was at 2.7 and 2.1 fold more abundant in G3 and G1 respectively compared to G2. Finally KRT8 has shown expression of 3 and 2 fold change in G2 and G3 group, respectively compared to the G1 (Figure 15C).

4.2.1 Comparison of expression levels obtained from microarray experiment and real time PCR

Overall the differential expression detected by the two methods was comparable (Figure 16 and 17). However among the differentially expressed genes confirmed by real-time PCR some genes showed notable expression difference between the two methods (AR, TRX, PLAC 8 and COX2).

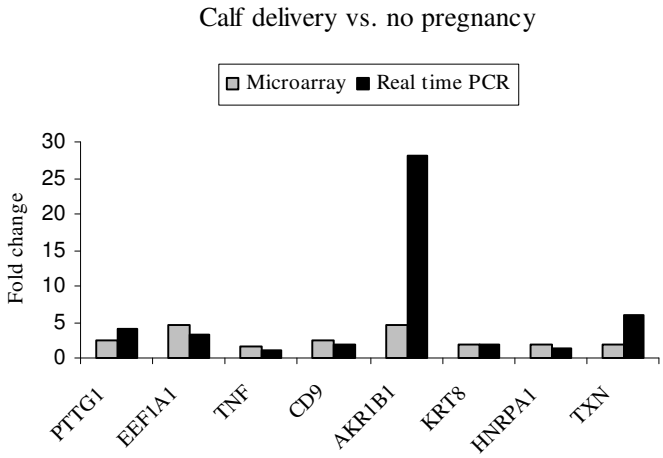


Figure 16: Comparison between results obtained by microarray and real time PCR for the first experiment (Calf delivery vs. no pregnancy).

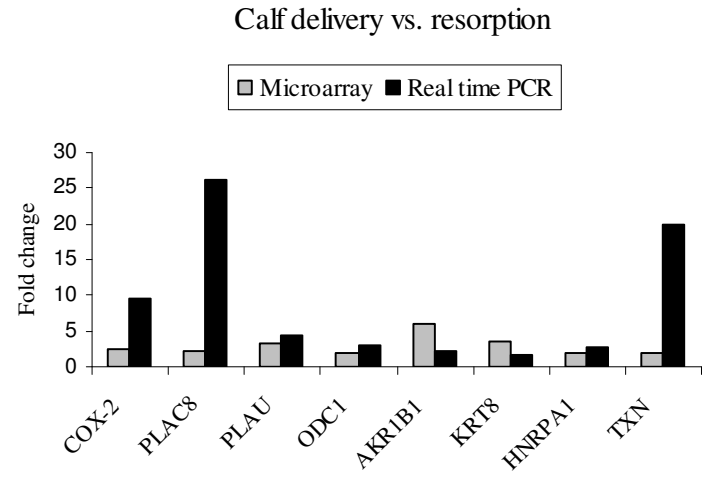


Figure 17: Comparison between results obtained by microarray and real time PCR for the second experiment (Calf delivery vs. resorption).

4.3 Immunohistochemical localization of MSX1

In order to further investigate some candidate genes at the protein level, MSX1 gene was selected. It has been shown that this gene is up regulated in the biopsies derived from blastocysts resulting in no pregnancy compared to those resulting in calf delivery (Table 3). Moreover the result obtained from real time PCR showed that this gene is not expressed in the biopsies leading to calf delivery (Figure 15 A). For this, immunohistochemistry was used localize the expression of MSX1 protein in in-vitro produced bovine embryos. MSX1 was found to be distributed in fine discrete granulae uniformly in the cytoplasm of immature oocytes (a). Staining was distributed at the periphery of the cytoplasm of matured oocytes (b). In zygote stage embryo (c), MSX1 was predominantly in the cytoplasm and the staining was most intensive towards the nucleus. Throughout the preimplantation period (2C, 4C, 8C, 16C and morula) the staining was apparently more concentrated around the nuclei, whereas the ICM in blastocyst (i) showed weaker labelling for MSX1 than the trophectoderm (Figure 18)

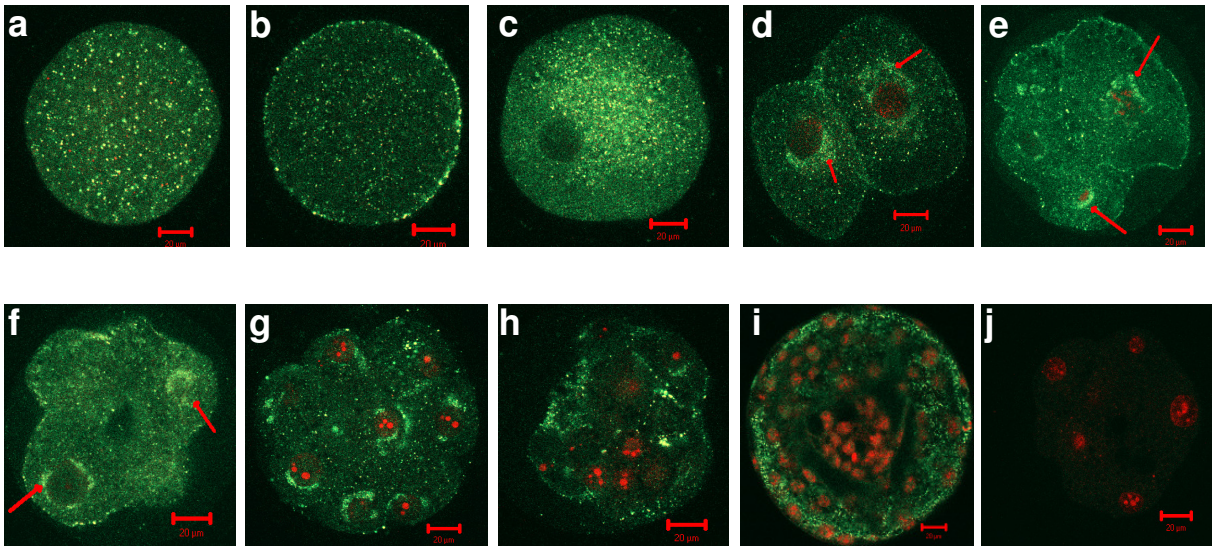


Figure 18: Subcellular localization of MSX1 protein in bovine oocytes and in preimplantation embryos [immature oocytes (a), matured oocytes (b), zygote (c), 2-cell (d), 4-cell (e), 8-cell (f), 16-cell (g), morula (h) and blastocyst (i) stages]. Control (j) was stained without primary anti-MSX1 antibody. Red arrows indicate concentrated localization of MSX1 protein. The figure is representative of 8-10 oocytes or embryos stained from each developmental stage. Nuclei are stained by propidium iodide (red). Scale bars represent 20 µm.

5 Discussion

The major reproductive waste in farm animals is early embryo loss, i.e. the anomalous development of embryos and/or an aberration of placentation (Cross et al. 1994). Precise knowledge of gene expression profile during preimplantation will enable to get insight into the molecular mechanisms controlling early embryonic survival and contribute to the effort aiming to improve the ever declining fertility in cattle (Lazzari et al. 2002, Lonergan et al. 2003, Farin et al. 2004, Wrenzycki et al. 2005). In the present study we have compared the transcriptional profile of blastocyst biopsies related to the fate of the embryo after transfer resulting in either no pregnancy, resorption or calf delivery. The study was performed using biopsies after blastocyst splitting, which is a unique possibility to eliminate genetic variability as a factor potentially affecting the results of gene expression analysis (Klein et al. 2006). The results showed that 46 genes were up-regulated in blastocysts resulted in no pregnancy, while 6 genes were down-regulated when compared with calf delivery (G1 vs. G3). In the second experiment (G2 vs. G3), 37 and 21 genes were up- and down-regulated, respectively, in blastocysts resulted in resorption when compared with blastocyst resulted in calf delivery.

We selected genes related to embryo death or survival to interpret comprehensive genes expression, because previous data had revealed their importance in early embryo development.

5.1 Genes highly abundant in blastocysts resulted in no pregnancy (G1)

Tumor necrosis factor TNF (inflammatory cytokine) which is known to be involved in fetal resorption or embryo loss (Silen et al. 1989, Chaouat et al. 1990, Gendron et al 1990, Sidhu and Bollon 1993, Chaouat 1994) is found to be up-regulated in blastocysts biopsy resulted in no pregnancy. The pathophysiology of TNF mediated fetal loss remains unclear. Elevated TNF restricts ICM proliferation in blastocyst and changes the ratio of mononucleated to multinucleated trophoblast cells (Whiteside et al. 2003). Moreover, TNF is reported to lead to cell apoptosis through cell-autonomous defects which is one of the intra-embryonic causes of death in the preimplantation period in mouse because of making

it unable to carry out specific housekeeping functions (Copp 1995, Lozano et al. 2001). These findings suggest a mechanism by which increased expression of TNF during trophoblast differentiation may be detrimental to pregnancy. It is important to recognize that low levels of TNF may be required for normal fetal and placental development, while elevated levels that occur in pathological settings, e.g., tissue damage or infection, may contribute to fetal defects. Further studies are needed to clarify the compartmentalization and role of this cytokine in both normal and abnormal gestation (Silver 1994). The ability to resist TNF depends on the de novo induction of specific gene products which can be induced by TNF (Gordon et al. 1992). In the present study some genes which are related to TNF have also been found to be differentially regulated including elongation factor 1 a1 (EF1A1)

EF1A1 is a part of elongation factor-1 complex which includes EF-1ß and EF-1γ and promotes GTP-dependent binding of aminoacyl-tRNAs to ribosomes during peptide elongation (Tatsuka et al. 1992). This gene showed a strong expression in the bovine immature oocyte (El-Halawany et al. 2004). As EF1A1 is a component of protein translation apparatus, therefore, its high expression level at immature oocyte is consistent with the fact that the oocyte shows a high rate of transcription and translation resulting in formation of RNAs and protein used for immediate oocyte growth and for storage (Fair et al. 1997). This gene has been shown to be upregulated in tumor cells (Gordon et al. 1992, Starkey and Levy 1995). Our result may provide an explanation for the selective anti-apoptotic advantage of the elevated levels of EF1a1 observed during tumor (Grant et al. 1992, Scheuner et al. 2001), where this gene is found to be up-regulated in blastocysts biopsy resulted in no pregnancy compared to calf delivery group. On contrary to our result, the analysis of the expression patterns of EF1A1 in bovine embryos demonstrated a dramatic decrease of this gene in bad quality in vitro embryos compared to good qualities in vitro and in vivo produced embryos (El-Halawany et al. 2004). Most of the studies of the role of EF1A1 in mammals have been in adult or postimplantation period. Accordingly, the EF1A1 protein level was found to be high in 18-day mouse embryo but gradually declined with postnatal age (Khalyfa et al. 2001). A decrease in EF1A1 mRNA level was also observed from fetal (20-day old) to adult rat brain (Lee et al. 1992). The agreement and

contrary of the expression pattern of this gene between the present study and in the previous studies could predict different roles in bovine preimplantation development which need further investigation.

The bovine MSX1 (formerly Hox-7.1) was found to be upregulated in biopsies derived from blastocysts resulting in no pregnancy by 2.4 fold compared to those resulting in calf delivery. Our result is in accordance with the resulted obtained by Pavlova et al. (1994) who have reported that MSX1 is expressed at high levels in uterine epithelial cells of non pregnant mice, and these cells undergo pronounced changes in morphology in response to embryo implantation and show a concomitant decrease in MSX1 levels. Furthermore MSX1 was found to be expressed at sites where cellular proliferation and programmed cell death occur suggesting its participation in programmed cell death (PCD) (Marazzi et al. 1997, Tribulo et al. 2004). This transcript is detected in several developing organs in vertebrates, including the facial primordia, particularly at the sites where epithelial–mesenchymal interactions occur during organogenesis (Liu et al. 2004). Overexpression of MSX1 suppressed cell growth and cell cycle progression in human ovarian cancer cell line as found by Park et al. (2001). So far no reports are available on the role of MSX1 in mammalian preimplantation development. Our immunohistochemistry results suggest the potential involvement of this protein in bovine embryogenesis. Further investigation will be necessary to identify the regulatory mechanism of this gene in bovine preimplantation embryo.

Pituitary tumor transforming gene (PTTG1), is a novel oncogene whil is expressed at low level in normal human adult and fetal tissues (Zhang et al. 1999) and abundantly in most tumors, including those of the pituitary (Yu and Melmed 2001), ovary and testis (Puri et al. 2001), kidney, liver and endometrial tissue (Kakar 1998). PTTG1 mRNA is found to be expressed in a stage-specific manner in spermatocytes and spermatides during rat spermatogenesis (Pei 1999). PTTG1 is important for maintaining chromosome stability, cell cycle progression, and appropriate cell division as found by Wang et al. (2001) who reported that PTTG1 -/- mouse embryo fibroblasts exhibited aberrant cell cycle progression with prolonged G2-M phase and binucleated and multinucleated nuclei with increased

aneuploidy. Overexpression of PTTG1 induces cellular transformation and promotes tumor formation in nude mice and stimulates expression of the Bax gene which induces apoptosis in a human embryonic kidney cell line (Hamid and Kakar 2004). In accordance with this, PTTG1 was found to be up-regulated in embryos resulting in no pregnancy by nearly 4 fold change compared to those resulted in calf delivery.

CD9 gene which expressed in blastocysts and endometrium epithelial cells in man and in bovines (Xang and Maclaren 2002) was found to play a role in inhibiting embryo implantation (Liu et al. 2006). This is in agreement with our results showing the up-regulation of CD9 in biopsies derived from blastocyst resulting in no pregnancy. Given the fact of striking similarities between embryogenesis and biology of cancer cells, especially in the process of invasion, CD9 might be involved in embryo invasive behaviours (Liu et al. 2006). The glycoprotein CD9 is a widely expressed member of the transmembrane-4-superfamily (TM4SF; also called tetraspanians) as found by Maecker et al. (1997). Several TM4SF members, including CD9, CD81, CD82 and CD63 are involved in cell proliferation and differentiation, adhesion, motility and cancer. CD9 is considered to be critical in fertilization, because CD9 null mice showed complete sterility due to the deficiency in egg-sperm fusion (Kaji et al. 2000, Miyado et al. 2000).

5.2 Genes up-regulated in biopsies of blastocysts resulting in no pregnancy (G1) and resorption (G2) compared to calf delivery (G3)

The aldose reductase (AKR1B1) gene which is known for its 20α-Hydroxysteriod dehydrogenase activity was found to be up-regulated in both biopsies derived from blastocysts resulting in no pregnancy and resorption. The aldose reductase gene was found to be strongly expressed in the endometrium at the time of luteolysis in bovine (Madore et al. 2003), suggesting its potential involvement in pregnancy failure. The enzyme of this gene is known to have two different activities namely; metabolising progesterone, which is found to be important to implantation (Jurisicova and Acton 2004) and synthesizing PGF2α and subsequently terminating pregnancy. Aldose reductase is also known to cause apoptosis in some type of cells like cardiomyocyte being induced by sorbitol as a response

to hyperosmotic pressure (Galvez et al. 2003). On contrary high glucose in culture media could lead to up-regulation of aldose reductase and subsequent accumulation of sorbitol in cytoplasm and activate apoptotic pathways (Wirtu et al. 2004). This hypothesis is in agreement with the result reported by Pampfer et al. (1997) that nuclear fragmentation in rat blastocysts exposed to high glucose and TNF

Phosphoglycerate kinase (PGK1), a key enzyme in glycolysis and encoded from the X chromosome is found to be upregulated in G1 and G2 biopsies compared to the G3 group. High level glucose concentration which triggers apoptosis during preimplantation in murine embryos (Riley et al. 2004) has led to the overexpression of this gene (Mohan et al. 2002). This is in accordance with what was found by Pampfer et al. (1997) who reported that exposure of embryo to high glucose level lead to nuclear fragmentation in blastocyst as mentioned above.

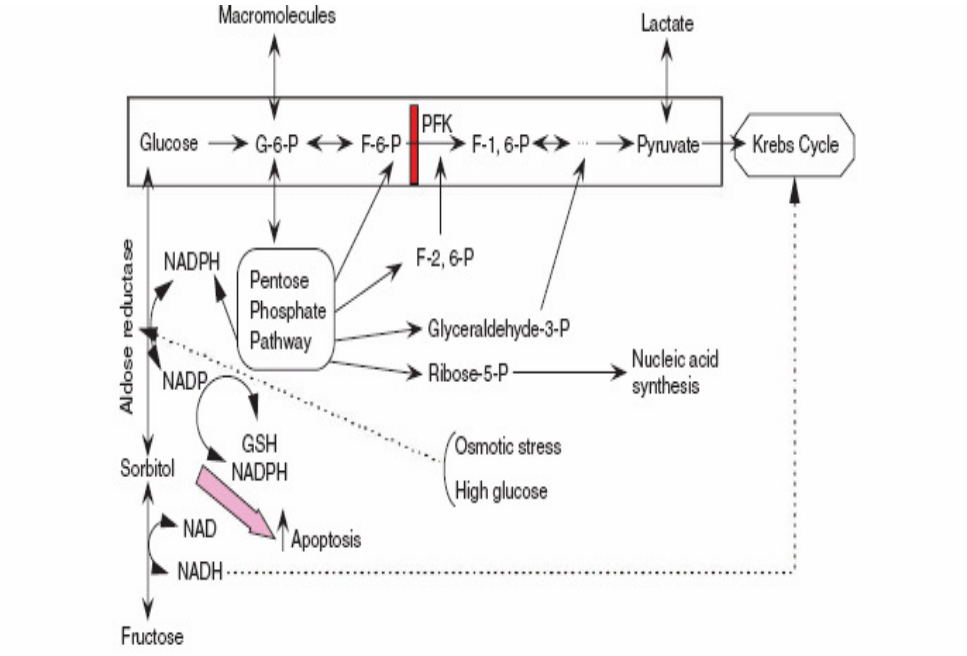


Figure 19: Pathways of glucose metabolism and related factors influencing embryonic development (Wirtu et al. 2003).

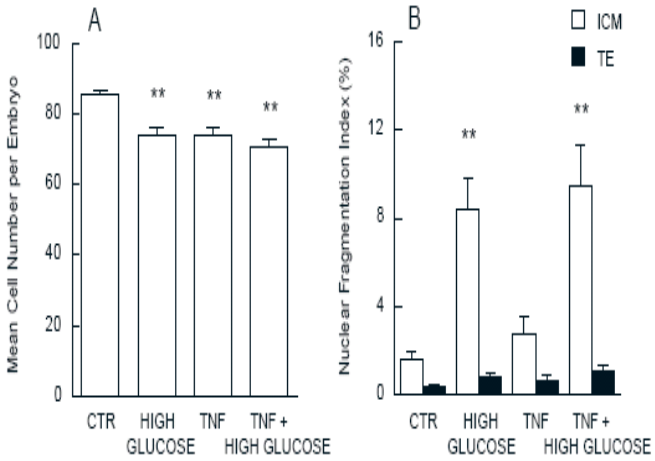


Figure 20: Nuclear fragmentation in blastocysts exposed to high glucose and TNF (Pampfer et al. 1997)

One of the genes showing unclear trend is cytokeratin A 8 (KRT8). This gene had shown high expression in biopsies resulting in resorption compared to those ended with calf delivery. On the other hand it showed low expression in biopsies derived from blastocysts resulting in no pregnancy compared to calf delivery one. This gene belongs to the cytokeratins which constitute the main group of intermediate filaments. They assemble as many as 30 different proteins (Moll et al. 1982). It has been shown that the expression of different cytokeratin polypeptides changes in many vertebrates during development, adulthood, regeneration and hyper-proliferation, suggesting that each one plays a distinct role in cell life (Kallionen et al. 1995). Cytokeratin 8 is the early and fundamental keratin expressed together with keratin 18 during development of many vertebrates (Jackson et al. 1980, 1981), and the main keratin present in hyperproliferative human cells (Moll et al. 1982). Cytokeratin 8 plays a fundamental role in natural morphogenetic movement such as gastrulation (Torpey et al. 1992). Neverthless there is evidence that in some vertebrates such as in mice, a genetic deficiency of cytokeratin 8 allows a perfect gastrulation, but leads to high lethality at the neonatal stage (Brock et al. 1996). It is also known that cytokeratin 8 deficient mice develop a severe disease of the gastrointestinal tract mainly characterised by colorectal hyperplasia and inflammation (Loranger et al. 1997).

Similarly in the present study, the polyubiquitin gene which has been shown to be upregulated in tumor cells (Llovera et al. 1994), was found to be up-regulated in both, the biopsies resulting in no pregnancy and those ending in resorption compared to those derived from blastocysts resulting in calf delivery. During development, a large number of cells die in a predicted spatial and temporal of programmed cell death (PCD), or apoptosis. This process is crucial for differentiation and involves programmed regulation of gene expression. One of the first genes known to be involved in PCD is the polyubiquitin gene that is up-regulated during the metamorphosis of the hawk moth (Herskko and Ciechanover 1998). In the same study it has been shown that the ubiquitin system is implicated in the immune response, development and PCD. Abnormalities in ubiquitin-mediated processes have been shown to cause pathological conditions, including malignant transformation. Interestingly, it has been found that elevated concentration of TNF triggers the enhanced the expression of ubiquitin gene as found by Costelli et al. (1993) and Garcia-Martinez et al. (1993).

Protease serine 23 (PRSS23) gene transcription was upregulated 4.7 and 5 fold change in G1 and G2 respectively compared to G3 group. This gene encodes a member of the trypsin family of serine proteases which are the largest class of mammalian proteases; many of these enzymes exert their reaction on matrix degradation (Salamonsen 1999). Even Protease serine 23 belongs to this family but its specific function is still unknown as reported by NCBI (http://www.ncbi.nlm.nih.gov/entrez/).

5.3 Genes up-regulated in biopsies from blastocysts resulting in calf delivery (G3) compared to resorption (G2)

Prostaglandins (PGs) which are involved in the process of blastocyst implantation (Psychoyos et al. 1995, Lim et al. 1997) by increasing endometrial vascular permeability and subsequent decidualization (Kennedy 1994), are known to be produced by both the endometrium and the blastocyst, where the former is thought to be the major source of the prostaglandins involved in implantation (Snabes and Harper 1984, Wang et al. 2002, Huang et al. 2004). PGs produced by embryos may be involved in other functions during the

preimplantation period such as modulation of the endometrial implantation site (Wakuda et al. 1999). This may support our findings in which higher cyclooxygenase-2 (COX-2) which converts arachidonic acid into prostaglandins was detected in biopsies derived from blastocysts resulting in successful pregnancy and calf delivery. COX-2 deficiencies in mice showed multiple female reproductive failures, including implantation defects (Lim et al. 1997)

Study by Charpigny et al. (1997) showed that COX-2 protein was localized in trophoblast cells than in the inner cell mass. This may suggest that this gene is necessary for the elongation process that is the result of an intense proliferation of trophoblastic cells and subsequent implantation (Psychoyos et al. 1995). Moreover, higher expression of COX-2 during the time of the implantation window suggested an important role for the prostaglandins released by the embryo in mediating interactions with the uterus (Charpigny et al. 1997, Wang et al. 2002).

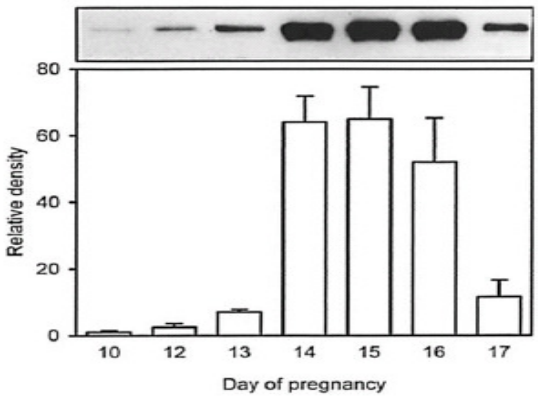


Figure 21: Cox-2 immunodetection in 10-17 days old ovine embryos (Charpigny et al. 1997).

In addition to the time of implantation, the expression of COX-2 was studied in preimplantation periods of various species. In bovine embryos, COX-2 expression appears transient and is associated with the first cleavages, since a decrease in the concentration occurs at the morula stage (Gurevich and Shemesh 1994). Similar study in mouse embryos found that COX-2 was at a low level at the zygote stage and was maintained at a high level from 2-cell to blastocyst stage (Tan et al. 2005).

The homeobox-containing gene family plays a pivotal role in regulating, patterning and axial morphogenesis in the developing embryo. The caudal-related homeobox protein CDX2 is a transcriptional regulator essential for trophoblast lineage, functioning as early as implantation. The CDX2 gene is the earliest trophoblast-specific transcription factor reported to date (Roberts et al. 2004, Tolkunova et al. 2006). CDX2 is required for proper trophectoderm in mice (Strumpf et al. 2005). At the blastocyst stage, CDX2 mRNA was confined to trophectoderm (TE) and absent from inner cell mass (ICM) cells in mouse embryos (Deb et al. 2006). An earlier gene targeting approach has demonstrated that CDX2 null embryos fail to implant, suggestive of a major defect in TE development (Chawengsaksophak et al. 1997, Rossant 2001). The same authors show that the implantation failure was due to a loss of TE epithelial integrity and/or increased incidence of apoptosis in TE cells. Therefore, CDX2 is one of the genes crucial for placental development, by which its aberrations in embryo can result in implantation or placental defect (Hall et al. 2005). Similarly, CDX2 was found at higher level in biopsies derived from blastocysts resulting in calf delivery (G3) compared to those ended with resorption.

Ornithine decarboxylase (ODC1), an enzyme which converts ornithine to putrescine, plays an important role in diverse biological processes, including cell growth, differentiation, transformation and apoptosis (Pendeville et al. 2001). In the present study ODC1 gene product was found to be lower in biopsies from blastocysts resulting in resorption compared to those resulting in calf delivery. This result in complete agreement with what was found by Pendeville et al. (2001) who have shown that mice embryos lacking ODC1 develop normally to the blastocyst stage and implant but die shortly thereafter, before the onset of gastrulation. Also it has been found that scheduled administration of difluoromethylornithine DFMO (a potent inhibitor of ODC1) during pregnancy in mice induces resorption of embryos when introduced at gestational days 7 and 8 (Fozard et al. 1980). This means that eliminating ODC1 function by gene targeting compromises early mouse embryonic development (Pendeville et al. 2001). These studies together with our result lead us to speculate that this gene plays an important role during pregnancy establishment.

The Plasminogen activators (PAs) are serine proteases which convert the inactive plasminogen to the active protease plasmin (Aflalo et al. 2004). PAs are thought to play a role in regulating extracellular proteolysis associated with gametogenesis, fertilization, and early embryonic development (Strickland et al. 1976). Plasminogen and its activators and inhibitors participate in the implantation process in human (Khamsi et al. 1996) and in rat (Aflalo et al. 2004). Plasminogen activators (PLAU) was found to be localized in mouse ovary and implanting embryo (Sappino et al. 1989). The activity of PLAU was lowest in two-cell-stage rat embryos and increased as embryos developed into blastocysts (Harvey et al. 1995, Aflalo et al. 2004). This gene provides trophoblast cells with an efficient mechanism to trigger the localized production of plasmin; a protease that can catalyze the degradation of all components of the extracellular matrix, such as is needed for endometrial disruption accompanying implantation and early growth of the embryo (Sappino et al. 1989). In our study we found that PLAU was up-regulated in biopsies derived from blastocysts resulting in calf delivery by more than 4 fold compared to those biopsies from blastocysts ended with resorption. This in accordance with other studies in mouse and rat (Sappino et al. 1989, Harvey et al. 1995, Aflalo et al. 2004) and cow (Whiteside et al. 2001) which have shown the involvement and importance of PLAU in implantation, particularly in the invasion process, where the reduction of this gene is associated with implantation failure in mice (Axelrod 1985). It has been reported that mouse blastocyst and trophoblast produce PAs activity at a time that corresponds to the period of trophoblastic invasiveness (Strickland et al. 1976). These findings support the assumption that blastocyst PLAU activity is important for proper implantation and subsequent pregnancy establishment.

Plac8 gene which is known as invasion-specific gene was found to be up-regulated in blastocysts resulting in calf delivery by more than 26-fold compared to those resulting in resorption. Similar studies in bovine have reported that Plac8 is highly expressed in endometrium of pregnant cows compare to non-pregnant ones (Galaviz-Hernandez et al. 2003, Klein et al. 2006), suggesting its potential role in placenta development and fetus maternal interface. This gene has shown a ratio of 15-fold change in placenta to embryo in microarray assays (Tanaka et al. 2000).

5.4 Genes up-regulated in biopsies derived from blastocysts resulting in calf delivery (G3) compared to no pregnancy (G1) and resorption (G2)

Thioredoxin (TXN) is a ubiquitous protein disulfide reductase, and known to be involved in the implantation of mouse embryos (Bing et al. 2003). Moreover, it was found to be a response to oxidative stress to protect the in vitro embryo development in bovine (Bing et al. 2003) and mouse (Nonogaki et al. 1991). TXN is believed to be early pregnancy factor (EPF) (Cavanagh and Morton 1994), as it is expressed in the preimplantation embryo (Clarke 1997). Others suggest that TXN is part of the components that are required for the expression of EPF (Di Trapani et al. 1997).

The heterogeneous nuclear RNA-binding proteins (HNRNP) constitute a family of over 20 proteins (Dreyfuss et al. 1993). The RNP proteins are thought to be involved in the processing reactions required to generate mature mRNA and they also play an important role in export of mRNA from nucleus (Michael et al. 1995). The association of hnRNP proteins with RNA begins as the nascent pre-mRNA emerges from the RNA polymerase II transcription machinery and remains through processing and export of mRNA (Dreyfuss et al. 1993). In particular HNRPA1, one of the most abundant nuclear proteins, revealed a number of properties that suggest its involvement in many aspects of RNA metabolism (Weighardt et al. 1995). In our study HNRPA1 gene was abundant at 2.7 and fold more in biopsies resulting in calf delivery compared to those resulting in resorption. Previous study in our lab (El-Halawany et al. 2004) which employed a quantitative real-time PCR to profile the HNRNP1 gene in bovine pre-implantation embryos showed that the highest expression is at the 2-cell stage and further down regulated until morula stage with a slight increase at blastocyst stage. Vautier et al. (2001) reported that following transcription activation of one-cell mouse embryos HNRPA1 protein concentrates in the pro-nuclei, making use of carrier-mediated transport pathway suggesting that HNRPA1 transport appears to be coupled to transcription dependent modification of the protein.

5.5 Comparison of expression levels obtained from microarray experiment and real time PCR

Overall the differential expression detected by the two methods was comparable (Figure 14 and 15). However among the differentially expressed genes confirmed by real-time PCR some genes showed notable differences of expression between the two methods (AKR1B1, TXN, PLAC8 and COX-2), as it has been reported by other investigators (Rajeevan et al. 2001). This may be due to the fact that array results can be influenced by each step of the complex assay, from array manufacturing to sample preparation. Furthermore, the mode of background subtraction and normalisation of the raw data could result in normalized values that are too low or too high (Rajeevan et al. 2001**,** Bauersachs et al. 2004).

In conclusion, we have identified genes with recognized and potential roles in pregnancy and genes whose functions yet need to be defined in this event. Several of these genes have been implicated in previous reports as being associated with embryo loss or survival during preimplantation period. Factors discussed in this study may be explained individually, but the simultaneous expression and interactions of these molecules may be important for elucidating how embryo death occurres and subsequently embryo loss. The identification of unique genetic markers for the onset of pregnancy signifies that genome-wide analysis coupled with functional assays is a promising approach to resolve the molecular pathways required for successful pregnancy. Normalizing the expression patterns of these genes may improve full term survivability of IVP cattle embryos. Future research to optimize establishment and maintenance of pregnancy in cattle should focus on cross-talk between the endometrial cells and conceptus and improve placentation to ultimately result in successful pregnancy past the embryonic period, when pregnancy loss is likely to occur.

6 Summary

The present study was carried out, (i) to compare transcriptional activity of embryo biopsies derived from blastocysts resulting in different pregnancy phenotypes after transfer to recipients, (ii) to identify differentially regulated genes between three biopsy groups and (iii) to do further functional analysis for the selected candidate gene at protein level.

For microarray experiments, biopsies (30-40% of the intact embryo) containing both inner cell mass and trophectoderm cells were taken from IVP day 7 blastocysts (n=118) and 60-70% part were transferred to recipients after re-expansion. Based on the success of pregnancy, biopsies were pooled in three groups, namely; those resulting in no pregnancy (G1), in resorption (G2) as in calf delivery (G3). Transcriptional analysis of these groups of biopsies (with three biological and three technical replicates for each group) was performed using two different cDNA arrays. The first is BlueChip cDNA array which contained 4928 spots in two sub-arrays per slide. Each sub-array is composed of 2304 randomly selected clones obtained from four different suppression substractive hybridizations (SSH). First SSH: GV oocytes subtracted from somatic tissues, second SSH: GV oocytes subtracted from Day-8 blastocyst, third SSH: Day-8 blastocyst subtracted from GV oocytes and fourth SSH: Day-8 blastocyst subtracted from somatic tissues.

The second array is a home-made bovine preimplantation specific custom cDNA array (219 clones). The majority of the clones is generated from different developmental stages of bovine preimplantation embryos using stage specific cDNA library construction, differential display and suppressive substractive hybridisation. In addition to these, some specific clones that are known to be expressed during embryo pre-implantation development were also amplified with gene specific primers and included in the array. The genes in this array have been classified based on the available information for the bovine, human and mouse species in the gene data bank. Therefore, they were classified functionally into 20 major categories. The majority of the known genes was comprised of genes in protein binding, transcription factor activity, DNA and nucleotide binding, transporters activity and ion binding categories. About 15.9 % of the genes and ESTs have not been classified yet for their function either in the bovine or other species.

For array hybridization, approximately 3µg of amplified RNA from pools of biopsies (each 10 biopsies) was used as a template in reverse transcription reactions incorporating amino-modified dUTPs into cDNA using CyScribe^{TM} post-labelling kit (Amersham Bioscience, Freiburg, Germany). The synthesized cDNAs from the G1, G2 and G3 group were differentially labelled using N-hydroxysuccinate-derived Cy3 and Cy5 dyes. Slides were scanned by a GenePix 4000B scanner and images were analysed using GenePix Pro Version 4.0 software. Data were normalized and finally analysed to obtain the differentially expressed genes. The above experiments were then repeated with reverse-labelled cDNA samples as dye-swaps.

A cDNA array analysis between G1 and G3 biopsy groups revealed that a total of 46 and 6 clones were up- and down-regulated, respectively in G1 group compared with G3 (41 from BlueChip and 11 from home made array) ($1.5 \leq$ fold change ≤ 4.7). These differentially regulated genes represent genes with known function (77%), ESTs (11.5%) and novel transcripts (11.5%). The genes with known function include those which are involved in protein binding (25%), protein biosynthesis (15%), metabolism (12.5%), transcription (5%), calcium binding (5%) and those with other function (37.5%). On the other hand a comparison between G2 and G3 groups of biopsies showed that 37 and 21 clones were up- and down-regulated, respectively, in G2 compared with G3 (44 from BlueChip and 14 from home made array) ($1.6 \leq$ fold change ≤ 6). These differentially regulated genes represent those transcripts with known function (79%), ESTs (5%) and novel transcripts (16%) Genes with known function include those genes involved in protein binding (20%), protein biosynthesis (20%), metabolism (13%), transcription (9%), growth factor (5%) and those with other function (34%).

All differentially regulated genes were classified based on their functions according to criteria of gene ontology consortium classifications. The expression of those unknown and novel ESTs showed profiles similar to one or the other of the annotated genes, as determined by tree hierarchical clustering analyses. Quantitative real-time PCR was used to confirm the results of microarray experiments. A total of 15 selected genes (AKR1B1, ANXA2, ATP5A1, CD9, COX-2, EEF1A1, HNRPA1, KRT8, MSX1, ODC1, PLAC8, PLAU, PTTG1, TNF, TXN), was quantified in independent biopsy samples from the three groups using quantitative real-time PCR (qPCR) to verify the results obtained by array

hybridization. In general, the quantitative real-time PCR confirmed the expression of 87% (13/15) of the genes generated from the array hybridization while the expression profile of 3% (2/15) (ANXA2 and ATP5A1) was not in agreement with the microarray results. Four genes namely PTTG1, EEF1A1, TNF and CD9 have shown 3.9, 3.2, 1.2 and 2.2 fold more abundant, respectively in biopsies resulting in no pregnancy group compared to those resulting in calve delivery. Interestingly, MSX1 was not detected at all in the calf delivery group. Similarly four genes COX-2, PLAC8, PLAU and ODC1 were found to be 9.6, 26.1, 4.3 and 3.1 times respectively more abundant in biopsies resulted in calf delivery compared to those from blastocysts resulted in resorption. Four transcripts (AKR1B1, TXN, HNRPA1 and KRT8) which were found to be differentially regulated in both experiments were also validated with real time PCR. The AKR1B1 transcript was found to be abundant at 13.6 and 2.1 fold more in biopsies resulting in no pregnancy and resorption, respectively compared to calf delivery. On the other hand, TXN transcript was abundant at 19.2 and 3.3 fold in biopsies derived from blastocysts resulting in calf delivery and no pregnancy respectively compared to those ended up in resorption. The same trend was observed for HNRPA1 gene which was abundant at 2.7 and 2.1 fold more in biopsies resulting in calf delivery and no pregnancy respectively compared to those resulting in resorbed embryos. Finally KRT8 has been shown expression of 3 and 2 fold changes in resorption and calf delivery groups, respectively compared to the no pregnancy group. Overall the differential expression detected by the two methods was comparable. However, among the differentially expressed genes confirmed by real-time PCR some genes showed notable expression difference between the two methods (AKR1B1, TXN, PLAC 8 and COX-2).

The transcription factor gene MSX1 transcript was further investigated at the protein level using immunohistochemistry throughout preimplantation stage embryos. The protein product was found dispersed in the cytoplasm in immature and mature oocytes stages, with a reduced fluorescence signal after maturation. Starting from late zygote stage until the blastocyst stage the protein is found to be localized around the nucleus. At the blastocyst stage, more intensive staining was detected in the trophectoderm cells compared to the inner cell mass cells.

In conclusion, we generated direct candidates of blastocyst specific genes which may play an important role in determining the fate of the embryo after transfer.

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