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Bovine pre-transfer endometrium and embryo transcriptome fingerprints as predictors of pregnancy success after embryo transfer

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Dedicated to my father Salilew Wondim, my mother Bizunesh Adela and my sister Emebet Salilew Wondim

Genexprssionsprofile von vor dem Transfer biopsierten bovinen Endometrien und Embryonen als Prädikator für den Trächtigkeitserfolg beim Rind.

Abweichende Genexpressionen sowohl des Embryos als auch des Endometriums sind ein Grund für verminderte Trächtigkeitsraten nach dem Embryotransfer beim Rind. Die Selektion der Embryonen einerseits und der Empfängertiere an Hand des Endometriums andererseits basierend auf den Genexpressionsmustern, stellt eine große Herausforderung dar. Um herauszufinden, ob Korrelationen zwischen der Genexpression des Endometriums vor dem Transfer und der des Embryos auf der einen Seite und einer erfolgreichen Trächtigkeit auf der anderen Seite, vorliegen, wurde eine globale Transkriptionsanalyse der Biopsien des Endometriums und des Embryos mittels des GeneChip® Bovine Genome Array und einem präimplantations spezifischen cDNA Arrays durchgeführt. Die Biopsieproben des Endometriums wurden an Tag 7 und Tag 14 des Vorzykluses der Versuchstiere (Simmental Färsen) genommen. Des Weitern wurden in vivo Blastozysten am Tag 7 gespült, eine Biopsieprobe genommen (ca. 30-40%) und die Embryonen auf die Empfängertiere übertragen. Diese Biopsieproben wurden nach der Trächtigkeitsuntersuchung den Gruppen der trächtigen Färsen (rezeptives Endometrium) und der nicht-trächtigen Färsen (nicht-rezeptives Endometrium) zugeordnet. Insgesamt wurden 1126 unterschiedlich exprimierte Gene zwischen den rezeptiven und nicht-rezeptiven Endometrien an Tag 7 detektiert. Des Weiteren zeigten sich qualitative und quantitative Veränderungen in bedeutenden biologischen Prozessen und molekularen Pathways, wie in der zellulären Anordnung, posttranskriptionalen Modifikation, Signaltransduktion, Apoptose, im Zellzyklus und in der Imunantwort. Konträr zu diesen Ergebnissen waren an Tag 14 nur 14 Gene zwischen den Endometrien unterschiedlich reguliert. Im Bereich der Transkriptionsdynamik waren zwischen den Gruppen an Tag 7 1867 Gene und am Tag 14 254 Gene unterschiedlich exprimiert. Diese relativ große Anzahl unterschiedlich regulierter Gene zeigt die Transkriptomplastizität des rezeptiven Endometriums. Zwischen den Biopsieproben der Embryonen, die zu einer Trächtigkeit führten und denen die keine Trächtigkeit induzierten zeigten 70 Gene eine unterschiedliche Expression. In der ersten Gruppe wurden 32 Gene, darunter SPAG17, PF6, UBE2D3P, DFNB31, AMD1, DTNBP1 und ARL8B expremiert und in der zweiten Gruppe 38 Gene darunter SGK1, GBF1, KRT8, DTX2, RNF34, ARL8B, RYBP und EEF1. Die vorliegende Arbeit zeigt demzufolge, dass das Genexpressionsprofil des Endometriums und der Embryonen vor dem Transfer als Vorhersage für den Trächtigkeitserfolg dienen kann.

Bovine pre-transfer endometrium and embryo transcriptome fingerprints as predictors of pregnancy success after embryo transfer

Aberrant endometrial and embryonic gene expression is one of the causes of pregnancy failure in cattle. However, selecting cows with adequate endometrial receptivity and embryos of higher developmental competence based on the gene expression pattern has been a greater challenge. To investigate whether the pre-transfer endometrial and embryonic gene expression pattern has a direct relation with upcoming pregnancy success, a global endometrial and embryonic transcriptome analysis was carried out in endometrial and embryo biopsy samples using GeneChip® Bovine Genome Array and preimplantation specific cDNA array, respectively. For this, endometrium biopsies were taken at days 7 and 14 of the estrous cycle in Simmental heifers during the pre-transfer period. In the next cycle, in vivo produced day 7 blastocysts were transferred to the recipients at day 7 of the estrous cycle after taking 30-40% parts of the blastocyst as a biopsy for transcriptome analysis. After pregnancy diagnosis, the heifers were classified as calf delivery (receptive endometrium) and no pregnancy (non-receptive endometrium) groups. Subsequently, the endometrial biopsies (taken at days 7 and 14 of the estrous cycle) and the embryo biopsies were categorized as calf delivery or no pregnancy groups. The results revealed 1126 genes were differentially expressed between receptive and non-receptive endometrium at day 7 of the estrous cycle. These differences were accompanied by qualitative and quantitative alteration of major biological process and molecular pathways including cellular localization, post-transcriptional modification, signal transduction, apoptosis, cell cycle and immune response. However, only 14 genes were differentially expressed between receptive and non-receptive endometrium at day 14 of the estrous cycle. Furthermore, the transcriptome dynamics of receptive and non-receptive endometrium between day 7 and 14 of the estrous cycle revealed 1867 and 254 differentially expressed genes, respectively. The higher number of differentially expressed genes and functional categories between day 7 and 14 of the estrous cycle in receptive compared to non-receptive endometrium revealed the transcriptome plasticity of receptive endometrium. In addition, the gene expression profile in embryos biopsies resulted in calf delivery and those resulted in no pregnancy revealed 70 genes to be differentially expressed between the two embryo groups. Among those, 32 genes including SPAG17, PF6, UBE2D3P, DFNB31, AMD1, DTNBP1 and ARL8B were elevated in calf delivery groups and 38 genes including SGK1, GBF1, KRT8, DTX2, RNF34, ARL8B, RYBP and EEF1 were elevated in no pregnancy embryo groups. Therefore, the present study highlights the potential of pre-transfer endometrial and embryonic gene expression patterns as predictors of pregnancy success in cattle.

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List of abbreviations

A	Adenine
ACC. No	Gene bank accession number
aRNA	Amplified ribonucleic acid
dUTP	2'-Deoxyuridine 5'-Triphosphate
Affy	Affymetrix
AI	Artificial insemination
Annotate	Annotation
ATP	Adenosine tri phosphate
bIFN-τ	Bovine interferon tau
BLAST	Basic local alignment search
BME	Basal medium eagle
BSA	Bovine serum albumin
bTP-1	Bovine trophoblast protein-1
CD	Cluster of differentiation
CDd14	calf delivery day 14
CDd7	calf delivery day 7
cDNA	complementary deoxy ribonucleic acid
COCs	Cumulus oocyte complex
CR1	Charles Rosenkrans medium
cRNA	Complementary ribonucleic acid
Cy3	Cyanine 3
Cy5	Cyanine 5
DAPI	4’,6-Diamidin-2’-phenylindoldihydrochlorid
DAVID	Database for annotation, visualization and integrated discovery
DE	Differentially expressed genes
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNTP	Deoxynucleotide triphosphate
DOP	Degenerated oligonucleotide primer

DP	Days postpartum
dscDNA	Double stranded complementary deoxyribonucleic acid
DTCS	Dye terminator cycle sequencing
DTT	Dithiothreitol
E.coli	Escherichia coli
E2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
ESTs	Expressed sequence tags
FDR	False discovery rate
FITC	Fluoresceinisothiocyanat
FSH	Follicle stimulating hormone
GCRMA	Guanine cytokine multi array
GE	Glandular epithelium
GEO	Gene Expression Omnibus
GFP	Green florescent protein
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
GOStats	Gene ontology statistics
GTP	Guanosine triphosphate
GV	Germinal vesicle
h	Hour
hCG	Human chorionic gonadotropin
ICM	Inner cell mass
IPA	Ingenuity pathway analysis
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVT	*In vitro* transcription
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria broth or Luria-Bertani broth
LE	Luminal epithelium
LH	Luteinizing hormone
LIMMA	Linear models for microarray data
LOWESS	Locally weighted scatter plot smoothing
Marray	Microarray

MEM	Minimum essential medium
MHz	Mega Hertz
MIAME	Minimum information about microarray experiment
min	Minute
m	Milli
MP	Milk production
M	Molar
mRNA	Messenger ribonucleic acid
NAOAc	Sodium oxaloacetic acid
NCBI	National center for biotechnological information
NPd14	Non pregnant day 14
NPd7	Non pregnant day 7
^{o}C	Degree centigrade
oIFN-τ	Ovine interferon tau
oTP-1	Ovine trophoblast protein-1
P	Pregnant
P4	Progesterone
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PGF2α	Prostaglandin F2α
Pmole	Pico mole
qPCR	Quantitative polymerase chain reaction
r	Correlation coefficient
RIN	Ribonucleic acid integrity number
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RQ	RNA qualified
S	Stroma
s	Seconds
SAS	Statistical Analysis System
SCNT	Somatic nuclear transfer
SDS	Sodium dodecyl sulfate

s.e.m	Standard error of mean
SH	Standing heat
SLC	Solute carrier
SLS	Sample loading solution
SSC	Sodium chloride sodium citrate
ST	Standing time
TAE	Tris acetate ethylendiamin tetra acetat
TE	Tris-ethylendiamin-tetra acetat
TIFF	Tagged image file format
tRNA	Transfer ribonucleic acid
US	Ultasonography
UTP	Uracil triphosphate
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
µ	Micro
l	Litter
g	Gram
α	Alpha
β	Beta

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1 Introduction

Despite the wider application of embryo transfer technology in modern cattle breeding, several transfers end up without pregnancy establishment. This pregnancy failure has enormous economic implications by retarding genetic progress and increasing the cost of maintaining recipients.

Pregnancy failure can be attributed to incompetency of the embryo and/or inadequate endometrial receptivity. The implantation incompetency of the embryo can be caused by poor quality oocytes associated with inadequate mRNA and protein storage during oocyte maturation, inappropriate expression of genetic information derived from the embryonic genome during embryonic genome activation or inability of the embryo to produce sufficient pregnancy signals and secretion during perimplantation period (Bettegowda et al. 2008, Kanka 2003, Latham and Schultz 2001, Schultz et al. 1999, Gandolfi et al. 2005, Senger 2003). Thus, abnormal genetic constitutions of the blastocysts or unsuccessful transitions from maternal to embryonic transcription could account for many failures of early embryonic growth and implantation. Hence, for successful implantation to occur in the receptive endometrium, the blastocysts must attain implantation competency through accumulation of transcripts and secretions essential to control cell differentiation and maternal recognition of pregnancy (Spencer et al. 2008). Successful pregnancy is therefore the result of coordinated and synchronized crosstalk between the conceptus and the receptive endometrium in a temporal and cell specific manner (Carson et al. 2000, Gonzalez et al. 2000, Lim et al. 2002, Simon et al. 2001, Spell et al. 2001, Wolf et al. 2003).

Endometrial receptivity is the status of the endometrium to be ready to accept the embryo for implantation (Swierz and Giudence 1997) and it comprises temporary and unique sequence of events that makes the embryo to adapt to the new environment (Bergh and Navot 1992). Thus, assessment of endometrial receptivity is required to identify endometrium that will be suitable for assisted reproduction technology. Similar to implantation competent embryos, the endometrial receptivity is also known to be affected by certain genes whose expression is induced or reduced overtime. In this regard, different sets of genes were reported to be expressed or suppressed during the window implantation in human endometrium (Giudice et al. 2002, Giudice 2004, Kao et al. 2002, Lessey et al. 1996, Martin et al. 2002, Riesewijk et al. 2003). Thus, aberrant endometrial genes expression during critical period of pregnancy establishment could result in implantation

failure and infertility (Tabibzadeh and Babaknia 1995, Tapia et al. 2008). Therefore, selecting cows having adequate endometrial receptivity and developmentally competent embryos based on the gene expression pattern may increase the number of calves borne following embryo transfer. Hence, investigating the endometrial and embryonic gene expression during the time of embryo transfer and relating this information to pregnancy outcome may provide a unique opportunity to generate molecular marker that are related to endometrial receptivity and embryo implantation.

Despite enormous efforts in characterizing bovine endometrial gene expression during the estrous cycle and early pregnancy (Bauersachs et al. 2005, Bauersachs et al. 2006, Bauersachs et al. 2009, Mansouri-Attia et al. 2009, Mitko et al. 2008), the recipient endometrial gene expression patterns during the estrous cycle and the subsequent effect on the upcoming pregnancy outcome is not investigated. With regard to the embryo, previously it has been shown the possibility of establishing a direct connection between transcript abundance of *in vitro* produced bovine blastocyst biopsy and pregnancy success after transfer of embryos (El-Sayed et al. 2006). However, no information is available on the relationship between in vivo produced bovine embryo transcriptome profile and pregnancy success. Thus, investigating the gene expression of the endometrium and in vivo derived transferable embryos in relation to the pregnancy success can be useful in developing molecular markers associated with establishment of successful pregnancy. Therefore, this experiment was aimed at establishing a direct link between the pre-transfer endometrial or in vivo derived embryo gene expression and pregnancy outcome using endometrium and embryo biopsy technology in conjunction with the pregnancy outcome information. To achieve the main objective, the specific objectives were

1. To identify pre-transfer cycle endometrial transcriptome profile changes and transcriptomic functional and molecular signaling alterations in heifers that resulted in calf delivery and heifers that resulted in no pregnancy.
2. To investigate the diestrus endometrial transcriptome dynamics of heifers that result in calf delivery or heifers that resulted in no pregnancy.
3. Characterization of selected candidate genes during the estrous cycle and early pregnancy period.
4. To identify transcriptome profile and molecular signaling alteration in in vivo derived embryos that resulted in calf delivery and embryos resulted in no pregnancy.

2 Literature review

2.1 Oocyte quality and its effect on pregnancy success

Oocytes development is one of the main consequences of coordinated and complex events of physiological events during the estrous cycle. Oocyte is a key regulator of multiple aspects of female fertility, including ovarian follicular development and early embryogenesis (Matzuk et al. 2002). Unlike spermatozoa, which are generated continuously from puberty onwards, the population oocyte is limited during the life times. Therefore, the number and developmental competence of oocytes reaching the ovulation phase determine the number of offsprings the female animal produces during the reproductive lifetime (Gandolfi et al. 2005). Thus, developmental competence of oocytes is acquired when the oocyte achieves nuclear, plasma membrane and cytoplasmic changes during the follicular phases of the estrous cycle (Albertini et al. 2003, Brackett 1985, Eppig et al. 1996, Krisher 2004).

In vitro, the oocyte developmental process is dependant on many factors including maturation or culture media (Fouladi Nashta et al. 1998). For instance, different add-ins in the maturation and culture medium including fetal bovine serum, purified bovine LH hormones, amino acid and retinoic acid were found to increase the developmental competence of oocytes (Brackett et al. 1989, Hashimoto et al. 2002, Mohan et al. 2003, Watson et al. 2000). Amino acid supplementation of oocyte maturation media may serve as osmolytes, energy substrates, pH regulators and chelators of heavy metals and as precursors for protein synthesis (Gardner 1994, Gardner et al. 1996, Lee and Fukui 1996). In addition, embryonic development was reported from bovine oocytes aspirated from follicles greater than 2-8 mm in diameter (Blondin and Sirard 1995, Kruip et al. 2000, Lonergan et al. 1994, Yang et al. 1998). Similar studies in buffalo evidenced that the fertilization rate, cleavage and embryo development were significantly higher in COC's aspirated from large follicles, followed by medium and small-sized normal follicles (Raghu et al. 2002). Moreover, higher proportion of goat oocytes aspired from larger follicles reached to MII stage and oocytes from small and medium follicles yielded significantly lower proportion of hatched blastocysts than large follicles (Crozet et al. 1995). Thus, low developmental rates of oocytes from small follicles may be associated with their inability to reach complete meiotic and/or cytoplasmic competence, or they are from follicles already undergoing atresia.

Unlike to the *in vitro* counterparts, in vivo developmental competence of oocytes depends on the dietary system, ambient temperature, breed, parity and age of the animal. Alterations in the quantity of food consumed or the composition of the diet imposed solely during the pre-mating period can adversely affect oocyte maturity, blastocyst yield, prenatal survival and the number of offspring born alive (Ashworth et al. 2009). For instance, animals fed on rumen inert fat exhibited higher proportion of cleaved embryos than soya or linseed fed animals (Fouladi-Nashta et al. 2007). However, increased protein and urea intake may affect blastocyst development by elevating ammonia concentration in the follicular fluid (Adamiak et al. 2005, McEvoy et al. 1997a, Powell et al. 2006). This may adversely affect the oocyte developmental competence by inhibiting the growth and metabolism of the oocyte-supporting granulosa cells (Rooke et al. 2001). Similarly, long chain n-6 polyunsaturated fatty acid high density lipoprotein found to be resulted in reduced embryo development in ewes (Wonnacott et al. 2010). Moreover, high fat feeding can improve significantly the blastocyst production, total inner cell mass and trophectoderm cells than the low-fat group (Fouladi-Nashta et al. 2007). Similarly, (Mantovani et al. 1993) reported that the yield of transferable embryos after superovulation in beef cattle was significantly reduced when heifers had access to concentrates ad libitum compared with restricted concentrates. In addition, the dietary energy levels were found to be affected both the morphology and the number follicle in ewes and heifers (Boland et al. 2001, O'Callaghan et al. 2000). The effect of feeding level on oocyte quality was reported to be dependent on the body condition of the animal, with the high level of feeding being beneficial to oocytes from animals of low body condition, but detrimental to oocytes from animals of moderately high body condition (Adamiak et al. 2005).

Short time feed restriction and level of feeding can affect the oocyte quality and embryo survival. For instance, restricting energy intake before slaughter can enhance subsequent in-vitro development of the oocytes from small follicles (McEvoy et al. 1997b) and short-term restrictions in dietary intake before mating increased subsequent pregnancy rates in cattle (Dunne et al. 1997). Furthermore, restriction feed intake was found to reduce the expression of glucose transporter 3 (SLC2A3), sodium/glucose co-transporter 1 (SLC5A1) and Na^+/K^+ ATPase mRNA in oocytes, while expression of PTGS2, HAS2 and the leptin receptor in granulosa cells was increased (Schmidt et al. 2009). Similarly, (Pisani et al. 2008) indicated that feeding ewes with 0.5 above maintenance requirements was found to alter the relative abundance of transcripts involved in oocyte metabolic activity. This can suggest that changes to the immediate environment surrounding oocytes and embryos can

alter the pattern of genes expressed, or epigenetic modifications that is heritable changes in gene function that occur without an alteration in DNA sequence in oocytes and embryos which in turn contribute to altered developmental potential (Burdge et al. 2007).

The effect of season on oocytes developmental competence has been documented. The oocyte or embryonic developmental competence found to be increased during the cold seasons compared to the hot season (Rocha et al. 1998, Ryan et al. 1992, Zeron et al. 2001). This may be associated with modifications of the physical and chemical properties of the membranes. For instance, heat stress induces apoptosis in bovine and rabbit embryos increased the expression of heat shock proteins in porcine and mouse embryos, disrupts the microtubule and microfilaments and alters the methylation status of imprinted genes histocompatibility 9 (H19) and insulin-like growth factor 2 receptor (Igf2r) in mouse embryos (Bernardini et al. 2004, Kim et al. 2002, Makarevich et al. 2007, Paula-Lopes and Hansen 2002, Rivera et al. 2004, Zhu et al. 2008). Similarly, the heat stress during El Nino periods was found to be associated with reduction in the average number of embryos recovered from donor cows, the proportion of live embryos and the quality of live embryos (Benyei et al. 2003). From this, it can be possible to summarize that inherent nature of the animal (genetic makeup), the nutritional status, the feeding level and type of nutrient as well as the seasonal variation in temperature can affect the developmental competence of the oocyte, embryo survival or pregnancy rate in cattle and other domestic farm animals.

2.2 The role of oocyte mRNAs stores on oocyte quality

Although the environmental conditions and the type of breed significantly influence the oocyte quality, the earliest stages of embryogenesis in mammals are regulated by maternally-inherited RNAs and proteins stored within the oocyte (Bachvarova 1985). Transcription and storage of maternal mRNA occurs during follicular development in which the oocyte synthesizes and accumulates large stores of the total RNAs and protein. These stored mRNAs and proteins are essential for completion of the meiotic cell cycle, the establishment of an embryonic genome and regulation of preimplantation embryo development (Bachvarova 1985, Bachvarova et al. 1985, Bachvarova 1992, Betts and King 2001, De Sousa et al. 1998, Eichenlaub-Ritter and Peschke 2002, Kastrop et al. 1991, Levesque and Sirard 1995, Lonergan et al. 2003, Piccioni et al. 2005)

The accumulated mRNA and protein by growing oocyte is followed by massive destruction during oocyte maturation. For instance, an estimated 85 pg of mRNA is

believed to be available in a GV-stage mouse oocyte, but polyadenylated mRNA declines during oocyte maturation by about 50 pg and about 30% of the total mRNA undergoes degradation (Paynton 1998). The polyadenylation may be associated with translational activation, whereas deadenylation is associated with translational silencing (Piccioni et al. 2005). Further losses of maternal mRNA occur during early cleavage stages (Brevini-Gandolfi et al. 1999, Brevini-Gandolfi et al. 2001, Piccioni et al. 2005).

Although, the RNA transcribed during the period of cytoplasm maturation is very stable, the fates of different mRNA species are controlled at several levels (Wassarman 1996). Different species of mRNA are polyadenylated in different degrees. Those mRNA transcripts with long poly-A tails of ~150 A residues are utilized for immediate use and those with shorter poly-A tails of 90 A are used only after elongation of the poly-A tail (Bachvarova 1992). Therefore, the oocyte transcriptome is expected to reflect its potential to develop into an embryo (Thelie et al. 2009). Thus, the oocytes intrinsic quality and developmental competence is mainly influenced by the presence or absence of those transcripts in the oocyte (Bilodeau-Goeseels 2003). This in turn can affect the ability of the embryos further development and survival.

2.3 Oocyte specific transcripts and their effect on embryonic development

The stability of oocyte mRNA is crucial for normal development and any disturbance of this process can negatively affect oocyte developmental competence and cause blockage of further embryonic survival (Gandolfi et al. 2005). Although, the ability of an oocyte to develop into embryo depends on the accumulation of specific information and molecules, because of their importance role, more emphasis has been given to oocyte-specific transcripts (Eichenlaub-Ritter and Peschke 2002). Oocyte specific transcripts may differ between species and stage of pre-implantation development. Oocyte mRNAs with short poly (A) tails are translationally inactive and are activated upon extension of the tail during specific stages of embryo development (Brevini-Gandolfi et al. 2001). Many oocyte-specific factors have been recognized to influence the acquisition of developmental competence. Maternal effect genes produce transcripts that form a pool of mRNA in the oocyte cytoplasm that can sustain post-fertilization development until the embryonic genome is activated (Memili and First 1999).

The mammalian oocyte provides not only proteins that are essential for initial cell divisions in the embryo but also proteins and RNAs that are implicated in regulating the

paternal genome. Developmentally competent oocytes accumulate large pools of RNA in the cytoplasm and nucleus which stops when the oocyte reaches of diameter of 110 µm (Bachvarova 1992, Fair et al. 1996). The study in mouse oocyte indicated that mature oocytes with the diameter of 100-120 µm have an estimated amount of 2.4 ng total RNA and 40-60 pg mRNA (Bilodeau-Goeseels and Schultz 1997).

The genetic programs within the oocyte play an important role in orchestrating the multiple events required for successful folliculogenesis, fertilization and early development (Dean 2002). In addition, oocyte specific genes are important for the initial cleavage divisions of embryonic development (Yao et al. 2004).

The mammalian oocyte expresses several genes specific to oocytes that can play a key role in regulation of fertility in mammals (Bettegowda et al. 2007). Some of those genes include growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), bone morphogenetic protein 6 (BMP6), zona pellucida (ZP) genes, MLF1-interacting protein (MLF1IP), factor in the germline alpha (FIGα), B-cell translocation gene 4 (BTG4) and phosphotyrosine-binding protein (xPTB), Mater and JY-1 (Elvin et al. 2000, Tong et al. 2000, (Vallee et al. 2005). Among those, (FIGα) is required for expression of the zona pellucida genes that encode ZP1, ZP2 and ZP3 (Liang et al. 2007). Mater is found to be essential for embryonic development beyond the two-cell stage and females that lack the maternal effect gene Mater are sterile (Tong et al. 2000).

In oocytes, some transcripts are required for oocyte-specific processes and metabolism, while others are presumably stored for use during early embryonic development, prior to the activation of zygotic expression (Li et al. 2006). For instance, the zona pellucida genes (ZP1, ZP2 and ZP3) are required during folliculogenesis, species-specific fertilization and passage of the early embryo down the oviduct (Rankin et al. 1996). Knockout mice lacking oocyte-derived zona pellucida protein 3 (ZP3) or ZP2 display defects in early antral and preovulatory follicle development, cumulus-oocyte complex formation, ovulation and unable to form a zona matrix despite synthesis of ZP1 and ZP2 (Rankin et al. 2001, Zhao and Dean 2002). Moreover, ZP1 null mice form zona pellucida with only ZP2 and ZP3, but the matrix is structurally flawed and some growing follicles have ectopic granulosa cells lodged between the oolemma and zona pellucida (Dean 2002). Later in folliculogenesis, Zp1 null follicles can develop accentuated perivitelline space prior to ovulation. Although, Zp1 null females are fertile, they have decreased fecundity due to precocious hatching of early embryos from the structurally compromised zona matrix (Rankin et al. 1999). Blastocysts derived from *in vitro* maturation and fertilization of eggs

from Zp2 or Zp3 knockout females are not capable of completing development after transfer to wild-type pseudo pregnant recipients (Zhao and Dean 2002).

Other group of oocyte specific genes is the transforming growth factor beta (TGF-beta) members which are potent regulators of cell proliferation and differentiation. Three members of TGF- beta, growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and BMP6 are expressed by the oocyte and may mediate effects attributed to the oocyte. GDF9 is absolutely required for pre-antral follicle development, alters the periovulatory expression of granulosa cell genes and stimulates cumulus (Elvin et al. 2000). For example, in the mouse, growth differentiation factor (GDF9), is expressed only in oocytes and is required for early folliculogenesis and plays role in follicular development after the primary follicle stage (Dong et al. 1996). Moreover, BMP15, functions in a cooperative manner with GDF9 to govern ovulation and fertilization rates (Yan et al. 2001). For instance, animals homozygous for the BMP15 or GDF9 mutations are sterile due to arrested follicular development from the primary stage of growth. In sheep, GDF9 mRNA is present in germ cells before and after ovarian follicular formation as well as throughout follicular growth, whereas BMP15 mRNA is found in oocytes only from the primary stage of growth (McNatty et al. 2003).

2.4 Embryogenesis and gene expression

2.4.1 Preimplantation embryonic development

The process by which fertilized oocytes divide and ultimately result in specialized tissues and organs of a mature organism is one of the greatest mysteries of life. To achieve this, the oocytes must first attain the cytoplasmic and nuclear maturation and reach to the metaphase II stage. Once the oocyte is matured, the process of fertilization starts when the sperm cell binds to the zona pellucida and fuse with the cell membrane of the oocyte. However, actual fertilization occurs when the sperm enter the oocyte cytoplasm and fuse its pronucleus with the oocyte pronucleus restoring the number of chromosomes that is typical of a given species. Since the resulting zygote is a large cell that has low nuclear to cytoplasmic ratio, the zygote divides without an increase in cell mass. Thus, in the process of its development to the blastocysts, the mammalian preimplantation embryo passes through distinct metabolic phases including changes in protein synthesis, changes in energy requirements and amino acid uptake. Apart from this, the preimplantation embryo undergoes morphological changes including compaction, cavitation and hatching (Crosier

et al. 2000, Crosier et al. 2001). Compaction is the first step in the process of differentiation and is fundamentally important for the formation of viable blastocysts.

In cattle, early cleavage occurs on day 2 after fertilization and between day 3 and 4 the embryo reaches to 8-16 cells stage (Morris et al. 2001). The embryo reaches compacted morula or blastocyst stage 5-6 days after fertilization. One of the major event during preimplantation development is the formation of blastocyst which later differentiated in to two distinct cell lineages, the trophectoderm cells (TE) and the inner cell mass (ICM) (Watson 1992). The ICM gives rise to the embryo, whereas the TE forms the placenta (Goossens et al. 2007). The TE, the first differentiated cell type of development, is a specialized tissue that initiates implantation or attachment and is the progenitor of the placenta. The ICM is the pluripotent progenitor of the embryo proper (Rossant 2004, Yamanaka et al. 2006). A blastocyst is therefore characterized by the presence of fluid filled sac called blastocoels. Therefore, the body plan of the embryo proper is established when the pluripotent epiblast gives rise to the germ layers ectoderm, mesoderm and endoderm (Russ et al. 2000). Compaction is the first step in the process of differentiation and is fundamentally important for the formation of viable blastocysts.

2.4.2 Preimplantation embryonic losses

Embryonic mortality significantly limits the success of establishment and maintenance of pregnancy in cattle and it is a major impediment to the adoption of a range of assisted reproductive technology in the cattle breeding industry. The higher proportion of embryo loss occurs in the first 8-19 days after artificial insemination (AI) when early embryo is completely dependent on the oviduct and uterine environment for its survival (Morris and Diskin 2008).

Understanding the root causes of preimplantation and postimplantation fetal losses is required to design strategies towards improving the efficiency of *in vitro* fertilization and preimplantation genetic diagnosis. Embryo losses can be caused by several factors including the maternal environment (hormonal, gene expression, production performance), the intrinsic quality of the embryo including chromosomal abnormality and other environmental factors. These factors can be grouped as those of genetic, physiological, endocrine and environmental origin (Ashworth 1994 , Bazer 1994). Among these, chromosomal abnormalities causes marked alterations in the genetic make up or genome of the embryo and interferes with the course of embryo development during the embryo

and the maternal communication. For instance, it is believed that about 50% of postimplantation embryo losses are due to chromosomal abnormalities and that cytogenetic abnormalities account for 23-71% of preimplantation losses (Plachot et al. 1988). In addition, the progesterone level is one of the determinants of embryo survival and development. The level of progesterone has been implicated in embryonic deaths during four phases of pregnancy, during the early postovulatory period (before day 6 after mating), during days 4 to day 9 after mating, during maternal recognition of pregnancy (day 14 to 17) and during the late embryonic period (day 28 to 42) when placentation and attachment are in progress (Inskeep 2004). It has been suggested that the proportion of pregnancy failure in cattle is due to failure of the embryo to produces adequate amounts of interferon-τ (IFN-τ) to block uterine prostaglandin F2α production (Thatcher et al. 1989).

Table 1: Cattle embryo losses during different gestation periods

	Fertilization	Cumulated loss (%) by gestation week					
		1	2	3	4	5-8	Reference
Beef heifers	AI	13	4		-	47	Diskin and Sreenan 1980
Beef heifers	AI	-	6	21	19	-	Roche et al. 1981
Beef heifers	AI	24	16	-	-	-	Maurer and Chenault 1983
Beef cows	AI	16	3	-	-	-	Maurer and Chenault 1983

2.4.3 Embryonic genome activation and embryonic development

Fertilization brings together the haploid genomes of two highly differentiated cells into the oocyte cytoplasm. After fertilization, the genome of the newly formed embryo is first transcriptionally inactive and the embryo depends on the stored RNA and protein during oocyte maturation (Jeanblanc et al. 2008b). Studies in cattle and mouse have shown that cattle and mouse embryos are able to survive until 8-cell and 2-cell stage embryos, respectively despite inhibition of polymerase dependent transcription by alpha-amanitin during the earliest stages of embryo development (Memili and First 2000, Natale et al. 2000, Plante et al. 1994, Telford et al. 1990). However, after some stage of development, the embryo depends on the newly synthesized RNA or protein as the maternal mRNA is

rapidly degraded and < 30% of the original mRNA could be available until 2-cell stage embryo (Clegg and Piko 1983b, Clegg and Piko 1983a). Hence, the embryo is dependent on stored maternal mRNA until the maternal-zygotic transition. After this time, the new genes are expressed by overcoming the transcriptional repression and embryonic developmental program is guided by a new program (Gandolfi and Gandolfi 2001, Meirelles et al. 2004). Thus, failure to initiate the embryonic genome transcription may result in lower embryonic development. Study by Salilew-Wondim et al. (2007) have also indicated that bovine zygotes exhibited better developmental competence to the blastocyst stage were found to be enriched with AURKA, DDX10, NASP, DNMT1 and SMARCA5 genes compared to those that have lower developmental potential. This may suggest lower genomic activation in lower developing groups compared to those exhibiting higher developmental potential. The embryo then need to reprogram the newly formed embryonic genome to a totipotent state (Duranthon et al. 2008). Hence, preimplantation embryo development and differentiation, which results in the formation of a blastocyst, requires the activation of the embryonic genome to become implantation competent (Dey et al. 2004). The key pre-implantation embryonic developmental process, compaction and differentiation of the morula into the blastocyst stage are the results of embryonic genome activation (Schultz et al. 1999).

Embryonic genome activation can be classified into as minor and major genome activation depending on the species. During minor genomic activation, there is a reduced transcriptional activity and it does not require any specific transcription factor. Unlike to the minor genome activation, major genome activation is marked by rapid transcriptional activity and newly synthesized RNA/proteins which are essential for further embryonic development (Jeanblanc et al. 2008a). Moreover, major activation promotes a dramatic reprogramming of gene expression pattern coupled with the generation of novel transcripts that are not expressed in oocytes (Vallee et al. 2005). Therefore, as development proceeds after fertilization, embryogenesis depends on the expression of genetic information derived from the embryonic genome (Bettegowda et al. 2008, Kanka 2003, Latham and Schultz 2001, Schultz et al. 1999). During this time, several maternal mRNAs are degraded and degradation eliminates gene products that might hinder further embryonic development. Elimination and silencing of some gene can be processed by means of chromatin-mediated repression, by limiting the transcription machinery and transcriptional repression (Schier 2007).

Genome activation may occur in an orderly manner in which some genes being transcribed before the beginning of major genome activation and other being transcribed after the occurrence of major genome activation. The changes in chromatin protein content, particularly histone proteins, chromatin structure and cytoplasmic macromolecular may regulate the genome transcription and specificity of transcription (Latham 1999, Latham and Schultz 2001).

The time of embryonic genome activation is species dependant (Misirlioglu et al. 2006). For instance, it occurs at the 2-cell stage in mice (Kidder and McLachlin 1985), 4-cell stage in humans, rats, pigs and 8- to 16-cell stage in cattle and sheep (Whitworth et al. 2004). Minor genome activation is initiated as early as the 1-cell zygotic stage in bovine (Tesarik et al. 1987, Tesarik J 1987). It occurs at the 6- to 8-cell stage in monkeys (Schramm and Bavister 1999) and in 4000 to 8000-cell embryos in Xenopus laevis (Etkin and Balcells 1985).

In the absence of proper genome activation, the majority of bovine embryos stop developing within a few cell cycles after initiating cleavage. This developmental block arises in the bovine embryo at the eight-cell-stage (Whitworth et al. 2004).

Embryos that are able to transcribe their own genome are able to differentiate and implant in the uterus due to its ability to overcome chromatin repression and/or ability to activate transcription of important developmental genes (Betts and King 2001). For instance, genes that are required for regulation of transcription, chromatin-structure, cell adhesion and signal transduction were increased at the 8-cell stage compared with 8-cell embryos treated with α-amanitin and MII oocytes (Misirlioglu et al. 2006). The situation of embryonic genome activation in embryos produced by somatic cell nuclear transfer (SCNT) may be different from those embryos produced by fertilization. In nuclear transfer embryos, the somatic nucleus has to be reprogrammed it self to restart and continue the developmental process. It is believed that the somatic nucleus substitute somatic gene expression program with embryonic gene expression necessary for normal embryo development (Han et al. 2003). Generally, the early preimplantation bovine embryonic development (zygote to 8-cell stage) largely depends on maternally derived mRNAs and proteins. However, when the embryo reaches to the 8-cell stage, the embryo starts to rely on transcripts derived from genome activation, afterwards the bovine embryo produces several transcripts that can be required for further development and establishment of implantation (Memili et al. 1998, Memili and First 1998, Memili and First 1999, Memili and First 2000, Telford et al. 1990). Therefore, the pre-implantation period of embryonic development is manifested by

dynamic quantitative and qualitative changes of mRNA and protein contents (Paynton 1998, Temeles et al. 1984). However, those embryos that are unable to initiate gene transcription will fail to be implanted in the uterus and end up to demise.

2.5 Transcripts required during blastocyst formation and differentiation

Blastocyst formation is associated with trophectoderm cell differentiation, which arises through fundamental biological processes that are related to establishment of cell polarity. Blastocyst formation is required for implantation to occur and it is the most important morphological determinant of embryo quality prior to embryo transfer.

Several physiological events and specific gene families are operating to regulate blastocyst formation (Watson et al. 1999). Genes expressed in the blastocyst stage are therefore can be candidates for controlling regulation processes that take place at the beginning of cellular differentiation (Ponsuksili et al. 2002). Identification of those genes participating in blastocyst formation and the analysis of their expression patterns may help to understand the mechanisms that control blastocyst formation (Goossens et al. 2007). Those gene families may include the E-cadherin-catenin cell adhesions, tight junction gene, Na^+/K^+-ATPase gene family, the aquaporin gene families, gap junction (connexin 43), transforming growth factor alpha (TGF-alpha) and epidermal growth factor (EGF) (Watson 1992, Watson and Barcroft 2001, Watson et al. 2004). These genes may encode different cellular activities such as cell polarity, active transport of Na^+ and K^+, cell junction, cytoskeletal, ion transporter and water channel gene that could be required by embryos during blastocyst formation (Watson et al. 2004).

Genes encoding for transcriptional factor activity are also expressed during blastocyst formation. The expression of transcriptional factor genes may be required to during embryos differentiation to the inner cell mass (ICM) and trophectoderm (Tr). Numerous transcriptional factors including RNA exonuclease 1 (Rex1), GATA-binding protein 3 (GATA3), T-Box (eomesodermin), caudal type homeobox (CDX2), activating protein 2 gamma (AP2γ), basic helix loop-helix (bHLH) and Mash2 are expressed in trophectoderm (Imakawa et al. 2004). Among those, the importance of eomesodermin (T-box) during mouse embryo differentiation into trophoblast has been described in Russ et al. (2000).

Stage dependant expression of pattern of caudal type homeobox 2 (CDX2), OCT4, NANOG homeobox (NANOG), GATA binding protein 3 (GATA3) and GATA binding protein 6 (GATA6) also found to be essential during the mouse embryonic development. For

instance, OCT4 is expressed in all stages of embryos before the late morula and (NANOG) is specifically induced during late morulae. Similar to NANOG, CDX*2* is expressed in the late morulae and is required for the repression of OCT4 and NANOG in the trophectoderm of the blastocyst. On the other hand, GATA6 is expressed in the primitive endoderm of the late blastocyst, when the expression of OCT4 and NANOG are repressed (Wang and Dey 2006). This may suggest the preimplantation embryo development and blastocyst formation are triggered by induction and reduction of the expression pattern of certain genes at temporal fashion. Furthermore, (Ho et al. 1995) also described the expression Na^+/K^+-ATPase, Sp1 (Sp1 transcription factor), TATA box-binding protein (TBP), IGFI, IGFII, IGFI receptor and IGFII receptor which are associated with blastocyst development, blastocyst hatching. Apart from these, the importance of STA3 (Takeda et al. 1997), DNA polymerase delta (Uchimura et al. 2009), Ped (Warner et al. 1987, Warner et al. 1988).

Gene targeted experiments in bovine embryo revealed several genes to be essential for blastocyst formation. For instance, targeted knockdown of baculoviral inhibitors of apoptosis repeat-containing 6 (BIRC6), survivin (BIRC5), KRT18, OCT4, MSX1 and E-cadherin were associated with reduced blastocyst formation *in vitro* (Goossens et al. 2010, Nganvongpanit et al. 2006, Salilew-Wondim et al. 2010, Tesfaye et al. 2010).

Genes that are required in blastocyst formation can vary depending on the culture condition and the source of the embryo *in vitro*. For instance, nuclear transfer and chromatin transfer are enriched with genes assumed to be involved in metabolism, signaling and apoptosis compared to blastocysts derived from in vitro fertilized embryos IVF (Zhou et al. 2008). In an attempt to investigate abnormal gene expression patterns in embryos of SCNT blastocysts, a global gene expression analysis showed that 28 genes to be differentially expressed between SCNT and AI blastocysts (Aston et al. 2009). Furthermore, several studies demonstrated that in cloned bovine blastocysts, the expression levels of specific genes including, DNA (cytosine-5)-methyltransferase 1 (DNMT1), fibroblast growth factor 4 (FGF4), fibroblast growth factor 2 (FGF2), Interleukin 6 (IL6), heat shock protein (HSP) and interferon tau IFN τ are different from in vivo developed embryos. This difference is mainly influenced by donor cells type (Daniels et al. 2001) and cell fusion methods (Niemann et al. 2002, Wrenzycki et al. 2001).

2.6 Estrous cycle and pregnancy establishment

2.6.1 Physiology of the estrous cycle and its implication in pregnancy success

Although the transcripts stored during oocyte maturation, the status of embryo genome activation, the type and nature gene expressed during blastocyst formation influence embryo survival and implantation, the physiological phenomena that occur during the estrous cycle is the backbone pregnancy establishment.

The average length of the bovine estrous cycle is 21 days for mature cows and 20 days for heifers. According to the typical characteristics of uterine structure and function, the estrous cycle can be divided into four stages: estrus, metestrus and diestrus, proestrus (Tan et al. 2003). During estrus, the estrogen and FSH are declining accompanied by LH surges during standing estrus. During this time, the theca cells start producing progesterone to inhibit LH and FSH release and ovulation occurs 12-18 hours after the end of estrus. Estrus phase is followed by metestrus which is the time of luteal development. The FSH surge that occurs during this time may recruit the first follicular wave for the next cycle. Diestrus is the longest phase of the estrous cycle. It lasts from days 5-17 of the estrous cycle and is marked by the presence of the mature corpus luteum. Progesterone is produced by large and small luteal cells. Corpus luteum is composed of small and large luteal cells. The large luteal cells are derived from granulosa cells and the small luteal cells are derived from the thecal cells. The large luteal cells produce most of the progesterone and have the prostaglandin receptors, while the small luteal cells have LH receptors. Nearly at the end of late luteal phase (day 17-18), in the absence of pregnancy recognition signal, prostaglandin is released and causes luteal regression. With the loss of progesterone inhibition, GnRH rises and causes stimulation of LH and FSH. The follicles mature by the influence of FSH. The growing follicle produces estrogen in the granulosa cells to induce signs of estrus in the cow. Inhibin is also produced by the growing follicle and prevents other smaller follicles from growing. This inhibition of smaller follicles assures selection of a single follicle to ovulate. The estrogen causes progesterone receptor synthesis, which allows LH binding to luteal cells. Therefore during these different phases of the estrous cycle, gonadotropins influence folliculogenesis, ovulation, corpus luteum function and steroidogenesis in the ovary (Kojima 2003). The physiological and hormonal changes that occur during the estrous cycle prepare the reproductive tract of the female animal for the period of sexual receptivity, ovulation and implantation. Therefore, understanding the biological mechanisms, the physiology, hormonal regulation and molecular events of the

bovine estrous cycle is essential for increasing the pregnancy rates of farm animals. Thus, estrous cycle has a direct impact on the reproductive performance of the cattle or other farm animals as it gives repeated opportunities for female animal to become pregnant throughout its productive lifespan.

The estrous cycle is marked by morphological, histological and hormonal changes of the reproductive tracts. Among which, the uterine histology and morphological changes during the estrous cycle has special association with pregnancy success. These may include changes in the height of the surface epithelium, the height of the glandular epithelium, the size of the gland lumen, the development of the gland, secretion in the gland lumen and stromal edema (Marinov and Lovell 1968). The histological characteristic of the uterus varies depending on the fertility status of the animal. For instance, the presence of uterine histological differences between the normal cyclic cows and repeat breeders have been described (Ohtani and Okuda 1995). The authors have indicated that on day 1 of the estrous cycle, the uterus of repeat breeder exhibited glandular secretions and supranuclear vacuolation that were not were not observed in the normal cows. Moreover, the stromal indices, the stromal mitoses and pseudodecidual reaction were absent in the repeat breeders during day 8 of the estrous cycle. Similarly, the histological changes of the uterus during the estrous cycle including, the presence of metrorrhagia on days 0 to 1, mitoses in glandular epithelium on day 5, basal vacuolation in the surface epithelium between day 5 to 6 and stromal mitoses on days 9 to 12 has been described (Ohtani et al. 1993). This may suggest that the uterine morphology during the estrous cycle to be an indicator of fertility. In addition to changes in histology and morphology, neutrophil concentration changes have been observed in the uterus of dairy cows during the estrous cycle (Subandrio et al. 2000).

Estrous cycle irregularities and inadequate estrus detection has been one of the major cause of reproductive wastage in cattle. In dairy cows, estrus detection is showing a declining trend due to directional selection (Figure 1). A decrease in estrous detection rates in Holstein and Jersey dairy herds has been reported (Harrison et al. 1989, Harrison et al. 1990, Washburn et al. 2002). Another study showed that higher yielding cows exhibit weaker signs of estrus than lower yielding cows consequently resulted in longer calving interval (Harrison et al. 1990). The physiological mechanism for negative correlation between high milk production and estrus duration traits is not clear. However, the hypothesis can goes to the relationship between feed consumption, energy allocation and hormonal regulation. For instance, cows selected for high milk yield are more often prone

to negative energy balance as they spend a relatively large proportion of the available nutrients on milk production, which can cause fertility problems during a period of negative energy balance (Chagas et al. 2007). Thus, decrease estrus duration in high producing cows may be related to the altered energy metabolism that can disturb the endocrine signaling and circulating concentrations of estrogen that ultimately resulted in inhibiting estrous behavior (Lopez et al. 2004, Roche 2006). Moreover, changes in reproductive physiology that are associated with high milk production may in part be explained by elevated progesterone and estrogen clearance rates he liver of a lactating cow due to increased feed intake which can lead to an increased liver blood flow and metabolic activity (Boer et al. 2010).

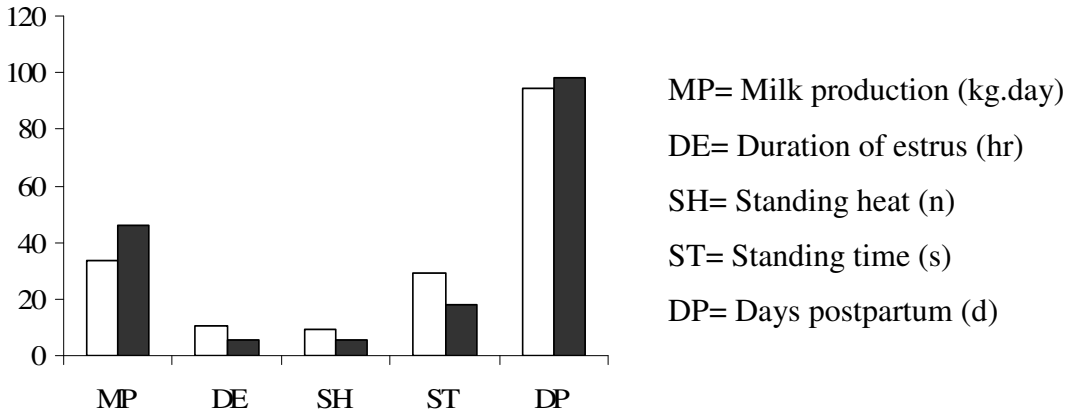

Figure 1: Characteristics of estrus in high (black bar) and low milk producing cows (white bar). Compiled from Lope et al. (2004).

2.6.2 Hormonal events during the estrous cycle and its effect on pregnancy establishment

The physiology of estrous cycle uncovers coordinated functional activities of different segments of reproductive system, the nervous and endocrine systems (Jainudeen and Hafez 2000). The type and level of hormones participating in the regulation of the estrous cycle and pregnancy has been indentified and characterized for several years. The measurements of hormone levels in different parts of the uterine and ovarian vasculature revealed the presence of regulating mechanism in the estrus cycle and pregnancy (Koziorowski and Stanisława 2007).

The concentration and regulation of the ovarian and uterine hormones changes depending on the phase of the estrous cycle via feed back mechanism of luteinizing hormone and

follicle-stimulating hormone from the gonadotrophins of the anterior pituitary (Hewitt et al. 2003, Kojima 2003, Richardson et al. 2006). For instance, on the onset of preovulatory gonadotrophin surge, the number of LH and estrogen receptors increase in the theca and granulosa cells accompanied reduction of FSH receptors. Thus, between days 3 and 7 of the estrous cycle, a single estrogen-active follicle develops and peak estrogen can be observed in plasma around this time (Glencross et al. 1973).

Estrogen is believed to increase the vascular growth of the endometrium and plays a major role in triggering the gonadotropin surge. This increased level of estrogen maintains higher estrogen/progesterone ratio and greater magnitude of uterine blood flow during the estrous cycle (Ford et al. 1979, Ford 1982, Roman-Ponce et al. 1983). Progesterone is the major hormone secreted by the corpus luteum in a pulsatile manner, pulses coinciding during the luteal phase with those of follicle stimulating hormone (Walters et al. 1984). The level of progesterone start to increase at day 5 and reach peak at day 14 of the estrous cycle (Diaz et al. 1986) and high progesterone concentrations during the luteal phase inhibit the estradiol-induced gonadotropin surge by reducing pituitary responsiveness to gonadotropin releasing hormone (Attardi et al. 2007, Richter et al. 2005). Moreover, the presence and the amplitude of progestrone in the luteal phase influences the time interval between rise of estrogen levels and the induction of estrous behavior or the LH surge (Skinner et al. 2000). On the other hand, the level of progesterone decreases to basal concentrations starting on days 17 to 18 (Schams et al. 1977). The decrease progestrone is associated with the loss of progesterone receptor and increases in epithelial estrogen receptor and oxytocin receptor in the endometrial cells (Goff 2004). Oxytocin from the pituitary and corpus luteum, stimulates the pulsatile release of the prostaglandin F2α ($PGF_{2\alpha)}$ via the oxytocin receptor in the luminal epithelium of the endometrium, resulting in regression of the corpus luteum (McCracken et al. 1999). On the other hand, during the time of low progesterone, there is a pulse increase of estrogen concentration (Fata et al. 2001, Schedin et al. 2000). On the other hand, PGF2α secreted from the uterus or particularly in the endometrial luminal and superficial glandular epithelium resulted in the demise of the corpus luteum (LaFrance and Goff 1990).

The trans-action of hormone of the ovary and uterine origin limits the number of off springs the female animal can produce during its productive lifetime. However, it is necessary to understand the mechanism how the hormones of the ovarian origin act on the uterus and hormones of uterine origin acts on the ovary. As reviewed by (Koziorowski and Stanisława 2007), in cow, the mesovarium venous vessels runs in a closer apposition to the

ovarian artery. The majority of uterine and ovarian arteries and arterioles running in the area of mesovarium are enmeshed by a dense net of venous vessels forming veno-venous network. Moreover, the presence of lymphatic system structures in the mesovarium and mesometrium may participate in the local hormones exchange and the estrous cycle regulation. The concept of transfer of uterine PGF from the uterine vein to the ipsilateral artery by a counter current exchange mechanism was also suggested (Peters 1985).

In uterus, the estrogen and progesterone receptor systems maintain the level of those hormones and transmit information into timely relevant developmental responses. The coordinated action of those entities resulted in follicular growth and development, follicular atresia, estrous cycle functioning and ovulation, corpus luteum functioning or pregnancy establishment. Thus, a better understanding of the physiology and endocrinology of the estrous cycle will improve reproductive management of dairy cows and facilitates the successful application of estrous synchronization and embryo transfer technologies.

2.6.3 Endometrial molecular events during the estrous cycle

The bovine uterus walls consist of the endometrium tunica muscularis or myometrium and the tunica serosa or perimetrium. The endometrium is the inner lining of the uterus consisting of the luminal epithelium, uterine glands and connective tissue. The endometrium is subdivided in caruncular and intercaruncular parts. The endometrium prevents the adhesions of the opposite walls of the myometrium by maintaining the patency of the uterine cavity. It is the main organ playing a crucial role in early embryo-maternal communication and pregnancy establishment (Bauersachs et al. 2005).

During the estrous cycle, the endometrium exhibits substantial gene expression changes that are associated with different functions. On this regard, the gene profile study indicated that at late estrus of the estrous cycle, the bovine endometrium is enriched with transcripts whose function is associated with cell-to-cell adhesion, cell motility and extracellular matrix. However, at day 12 of the estrous cycle (luteal phase), the endometrium is enriched with genes whose function is associated with transport proteins and ion (Bauersachs et al. 2005). Similar study also indicated that genes which are highly expressed at estrus are related to extracellular matrix remodelling, transport and cell growth and morphogenesis, whereas immune response and metabolic pathways were overrepresented in genes increased at diestrus (Mitko et al. 2008). The study in human endometrium (Kao et al.

2002) also revealed that those genes whose expression were increased during the secratory phase were related to cholestrerol trafficking, signal transduction but genes which are down regulated in proliferative phase are related to transcription factor activity and immune modulating. Moreover, the endometrial gene expression between secratory and proliferative phase of Rhesus monkey also indicated that the expression level of certain genes including PLK, SAT2, SLPI and MT1G to be higher in proliferative phase, where as the mRNA level of TGFBI or BIGH3, PENK, CSRP2, COL7A1, SFRP4, PGRMC1, CXCL12 and BGN to be higher in secratory phase (Ace and Okulicz 2004). These transcriptome abundance differences can be attributed to differences in the level of progesterone or estrogen hormones. Estrogen is known to affect the gene repression and gene stimulation by acting through its nuclear receptors. For instance estrogen has been found to down regulate the expression of cyclin G2 (CCNG2) resulting in the recruitment of the co-repressor N-CoR (NCOR1) and histone deacetylases leading to a hypo acetylated state of the chromatin (Stossi et al. 2006). Similarly, others (Hewitt et al. 2003, Hong et al. 2004, Moggs et al. 2004, Watanabe et al. 2003), reported that cyclin-dependent kinase inhibitor 1B (CDKN1B) and growth arrest specific 1 (GAS1) were down regulated during the first 1–8 h after estrogen administration.

Despite the accumulated evidences that many endometrial or ovarian gene expression changes during the estrous cycle are mainly regulated by progesterone and estrogen levels, the progesterone level can be in turn regulated by other genes. For instance, the Booroola (FecB) gene found to control the progesterone level during the estrous cycle and across pregnancy (Xia et al. 2003). The Booroola phenotype is associated with a point mutation in the kinase domain of the bone morphogenetic protein receptor 1 B (BMPR1B) and is known to involve in differentiation of ovarian follicles leading to the production of large numbers of ovulatory follicles that are smaller in diameter than wild-type follicles (Souza et al. 2003).

2.7 Endometrial receptivity towards embryo implantation

Uterine or endometrial receptivity refers the status of the uterus or endometrium ready to accept the blastocyst for implantation (Swierz and Giudence 1997). It comprises temporary and unique sequence of events that makes the embryo to adapt to the new environment (Bergh and Navot 1992).

Assessment of endometrial receptivity is required to identify endometrium that will be suitable for assisted reproduction technology. Therefore, identification of the receptive endometrium may help to correlate the changing endocrine, biochemical and morphological endometrial parameters with endometrial receptivity. Thus, understanding the significance of endometrial receptivity helps to achieve a better understanding of its relationship to fertility. Moreover, for optimal results in assisted reproductive technology, it is critical to recognize the time of embryo transfer that would best corresponds with the implantation time. However, the mechanism of uterine receptivity is a biological mystery that remains unsolved despite enormous advancements in understanding its physiology, structural, biochemical and molecular aspects associated with development and function (Makker and Singh 2006).

In humans, in addition to Pinopodes, several non invasive assessment of endometrial receptivity including high resolution transvaginal ultrasonography (US), three-dimensional US, Doppler US, three-dimensional power Doppler US, magnetic resonance imaging and endometrial tissue blood flow has been used to determine the endometrial receptivity (Elnashar and Gamal 2004). Moreover, the dominant features of the receptive phase endometrium can be marked by its plasma membrane transformation of luminal epithelium, glandular secretion, stromal decidualization and changes of the immune cell populations.

2.7.1 Signaling pathways and molecules triggering endometrial receptivity

Understanding the molecular changes that occur during the period of endometrial receptivity may help to develop molecular markers associated with endometrial receptivity and pregnancy success. The qualitative and quantitative measurement of endometrial receptivity is lacking in cattle and other ruminants. However, the information from mice with delayed implantation, (Reese et al. 2001), uterine gland knockout ewe (Gray et al. 2002, Spencer et al. 1999) and several gene targeted studies have contributed substantial information to get insight into potential markers of uterine receptivity and molecular mechanisms occurring during embryonic implantation into the maternal endometrium.

The receptive state of the endometrium may be characterized by the expression of genes that permits endometrium to respond to the embryo and allow the attachment (Sharkey and Smith 2003). The use of global gene expression approach such as microarrays and proteomic methods in the endometrium provided enormous information regard to the

endometrial receptivity at the molecular level. Recently, several molecules have been reported whose function is essential for uterine receptivity in rodents and primates. Differential expression of genes by uterine epithelial and stromal cells in response to progesterone may influence uterine receptivity to implantation in mammals (Bazer et al. 2009b). From this standpoint, several human endometrial gene expression profiles have performed to have a clue about the molecular mechanism endometrium during the secratory or luteal phase. Wang and Dey (2006) have summarized the gene profile from different experiment (table 2) and several commonly differentially expressed genes that believed to involve in human endometrial receptivity. However, the higher progesterone does not necessary indicate the endometrium is at receptive stage.

Table 2: Differentially expressed genes between high and low progesterone peaks in human endometrium

	Comparison of five independent studies				
Gene	LH+(8–10) vs. LH-(4–6)	LH+(7–9) vs. LH+(2–4)	LH+7 vs. LH+26	LH+(6–8) vs. LH-(3–5)	LH+8 vs. LH+3
ANXA4	+		+		+
APOD	+	+	+	+	
C1R	+			+	+
DAF	+		+	+	+
DF	+		+	+	
GBP3		+	+	+	
IL15	+	+	+		+
MAP3K5		+	+	+	+
SERPING1			+	+	+
SPP1	+	+	+	+	+
TGFB	+	+		+	
CCNB	+	+	+		
FRPHE	+	+		+	
GATA2	+			+	+
MSX1	+		+		+
MSX2	+	+	+		

Upregulated genes during the time of high progesterone (Italics) and genes down regulated in low progesterone (Times New Roman)

Temporal and spatial expression of specific endometrial genes may contribute for the establishment uterine receptivity by making endometrium hospitable to the implanting blastocyst (Lessey 1998). Many factors involve in preparation of endometrium to accept the embryo. Among these, many genetic factors are likely to be involved in the success or failure of implantation. The study from mouse model indicated that the biochemical markers of endometrial receptivity may include endometrial adhesion molecules, endometrial anti-adhesion molecules, endometrial cytokines, endometrial growth factors, endometrial immune markers and others (Wang and Dey 2006). Therefore, the next section of this review focuses on various biochemical and molecular events that occur in the endometrium during its preparation for implantation.

2.7.1.1 The role of endometrial adhesion molecules in endometrial receptivity

Endometrial receptivity involves molecules that facilitate embryo adhesion to initiate the window of receptivity and anti-adhesion molecules that close the widow implantation (Tabibzadeh 1998). Several adhesion molecules include cadherins, selectins, members of the immunoglobulin super family and integrins have been reviewed (Lessey and Young 1997). Among these, little is known about the role of cadherins or selectins in endometrial receptivity.

The integrin family of cell adhesion molecules are a major class of receptors for the extra cellular matrix and participate in cell-cell and cell-substratum interaction (Albelda and Buck 1990) and they bind ligands found in the extracellular matrix (Lessey 1997). Integrins are a family of transmembrane glycoproteins, formed by the association of two different, non-covalently linked, α and ß subunits. Both α and β subunits contain two separate tails, both of which penetrate the plasma membrane and possess small cytoplasmic domains (Humphries 2000). The integrins including α1β1, α4β1and αvβ3 are expressed during the secratory phase of human endometrium. Integrins are believed to be involved in embryo-endometrial interaction at the time of implantation (Lessey 1998).

The spatial and temporal distribution of integrins in bovine endometrium showed that, the luminal epithelium was found to express integrin subunits β1, α3, α6 and integrin $\alpha_v\beta3$. Of these, α6 and $\alpha_v\beta3$ exhibited estrous cycle-dependent and strongest expression in the luminal epithelium, basement membrane region and underlying sub epithelial stromal cells at proestrus, estrus and metestrus. However, α3 and α4 exhibit estrous independent expression distribution (Kimmins and MacLaren 1999).

Integrins are largely accepted as markers of uterine receptivity in humans (Lessey 1998). For instance biopsies from women with unexplained infertility had reduced significantly beta 3 expression suggesting abnormal endometrial integrin expression can be associated with unexplained infertility (Lessey et al. 1995). In the rabbit, the alpha (v) beta integrin is expressed in the embryo and trophoblast and it may be involved in early embryo-maternal interaction (Illera et al. 2003). In addition, the $\alpha_v\beta_3$ integrin appears implicated in placental invasion into the maternal vasculature (Zhou et al. 1997).

2.7.1.2 The role of cytokines in endometrial receptivity

Early embryo signaling as the result of maternal hormonal or cytokine-mediated preparation may be the step forward to permit adequate proliferation of the stroma for successful implantation (Ledee et al. 2007). Cytokines comprise a group of proteins that separately or in concert modulate a variety of cellular functions, such as cellular proliferation and differentiation (Achache and Revel 2006). They are implicated in critical reproductive events such as ovulation and implantation. Since implantation failure is a major causes of pregnancy failure, a better understanding of the cytokine-receptor in relation to pregnancy success may help to improve the diagnosis and treatment of infertility (Kauma 2000). Cytokines are small glycoprotein that involve in several activities including mediating communication between cells. Cytokines were discovered and investigated in the immune and hematopoietic systems and they are produced and act on in several cells of diverse lineages. Both pleiotropy and redundancy may exist within the cytokine families, but all cytokines act by binding to specific receptors belonging to a variety of classes that utilize different signal transduction pathways (Ledee et al. 2007, Salamonsen et al. 2000).

The uterine glandular or luminal epithelium or in the decidualized stromal cells is believed to be an important site for cytokines and cytokine receptors (Salamonsen et al. 2000). Numerous cytokines and their receptors are implicated in mammalian implantation embryo implantation (Lim et al. 2002). Among these, leukaemia inhibitory factor (LIF), interleukin 1 (IL1), interleukin-2 (IL2) interleukin 6 (IL6) interleukin 12 (IL12), interleukin 15 (IL15), interleukin18 (IL18) hepatocyte growth factor (HGF), stem cell factor (SCF), macrophage colony-stimulating factor (MCSF), colony stimulating factor 1 (CSF1), granulocyte-macrophage colony-stimulating-factor (GMCSF) and insulin-like growth factors (IGF) play a major role during implantation (Kauma 2000). Cytokines such as CSF1, GMCSF,

IL1 and IL6 were found to be important for increasing the numbers of implantation sites or litter sizes (Salamonsen et al. 2000). On the other hand, the expression of some specific cytokines in the endometrium including IL12, IL15 and IL18 reported to be resulted in abnormal uterine receptivity (Ledee et al. 2007).

Among the cytokine family, gene targeting, reciprocal embryo transfer and expression studies show the essential role of maternal LIF in uterine preparation and blastocyst attachment in mice (Lim et al. 2002, Stewart et al. 1992). (Stewart et al. 1992) using a mouse model indicated that mouse lacking functional LIF protein appeared to be fertile, but their blastocysts failed to implant (Lim et al. 2002). Inactivation of gp130, a signaling partner for the LIF receptor, also results in implantation failure (Salamonsen et al. 2000). Leukaemia inhibitory factor (LIF) is produced by the receptive phase endometrium (Chen et al. 1995) and its expression can be reduced by antiprogestin, mifepristone (Danielsson et al. 1997). The effect of LIF on rabbit endometrial receptivity and embryo implantation also indicated that transfer of embryos to LIF treated rabbit recipients significantly increased pregnancy rate compared to the control and significantly decreased when embryo transfer was done to LIF and mifepristone-treated recipients (Liu et al. 2001). The mechanism by which LIF participates in the endometrial receptivity and implantation events was addressed using LIF-deficient mice (Song et al. 2000). The authors reported that although the normal expression of genes related to uterine cell-specific proliferation, the epidermal growth factor (EGF) including amphiregulin (Ar), heparin-binding EGF-like growth factor (HBEGF) and epiregulin, were not expressed in LIF homozygous null uteri before and during the time of implantation. This may suggest that dysregulation of specific EGF-like growth factors in the uterus contributes to implantation failure in mice lacking LIF.

2.7.1.3 The role of growth factors in endometrial receptivity

The temporal and cell-specific manner expression of various growth factors and their receptors in the uterus are important for implantation (Carson et al. 2000, Cross et al. 1994, Lim et al. 2002, Norwitz et al. 2001, Paria et al. 2001a). The epidermal growth factor (EGF) family of growth factors comprises several proteins including transforming growth factor alpha (TGFα), heparin-binding EGF-like growth factor (HBEGF), amphiregulin, betacellulin, epiregulin and neuregulins (Das et al. 1997, Lim et al. 1998). Among these, HBEGF was found to be expressed in the uterus at the implantation sites in mice before the time of implantation (Das et al. 1994) and the expression of betacellulin, epiregulin,

neuregulin-1 and cyclooxygenase 2 (COX-2) were found to be detected around the time of the attachment reaction of the implantation process (Chakraborty et al. 1996, Das et al. 1997, Lim et al. 1998). Furthermore, HBEGF influences blastocyst activities for implantation in paracrine and juxtacrine manners (Raab et al. 1996).

2.7.2 The role of hormones in endometrial receptivity

Several endometrial factors controlled by ovarian steroid hormones, estrogen and progesterone are known to regulate uterine receptivity resulting in successful implantation. In rodents and primates, the implantation window is established when estrogen affects the endometrium that has been under the influence of progesterone (Imakawa et al. 2004). However, the requirement of ovarian hormones for endometrial receptivity may be species dependant. For instance, estrogen is required for preparation of the progesterone-primed uterus for implantation in mice and rats, but in other species estrogen alone does not lead to the maturity of the endometrium(Yang et al. 2001). In addition, the study by (Levi et al. 2001) indicated that exposure of developing endometrium to higher level of estrogen may result in loss of on endometrial receptivity. Similarly, excessive administration of estrogen post ovulation found to hinder implantation (Morris and Van Wagenen 1973). Thus, the low endometrial receptivity and embryo implantation in part may be caused by elevated level of estrogen (altered estrogen to progesterone ratio) (Forman and Estilow 1988).

Unlike to estrogen, progesterone is believed to initiate implantation in hamster, guinea pig, pig and rabbit (Paria et al. 2001a). Exogenous progesterone has been shown to exert utero-relaxing effects and it has been hypothesized that progesterone supplementation before embryo transfer may improve receptivity in IVF (de Ziegler et al. 1998) and progesterone is one of the factor which influences the probability of success or failure. Lower concentrations of progesterone 6 days after mating was associated with failed pregnancy in cows (Henricks et al. 1971). The report of (Lamming and Darwash 1998) also demonstrated increased progesterone level in cows that get pregnant compared to those failed to be pregnant.

Not only the concentration but also the time of progesterone peak affects the pregnancy success in cattle. Because delayed progesterone rise facilitates luteolysis at the normal time resulting in a reduced length of the period of progesterone secretion and lower conception rate in cattle. Lamming and Darwash (1995) reported that, after day 5 every one day delay

in the time of the progesterone rise there is a 0.7 day shortening of the subsequent luteal phase.

Progesterone, acts on the uterus to stimulate blastocyst preimplantation growth and elongation and also regulates several genes (Mann and Lamming 2001). The study by (Forde et al. 2009) indicated that P4 supplementation found to be altered the expression of fatty acid binding protein (FABP), diacylglycerol O-acyltransferase 2 (DGAT2), myostatin (MSTN) and crystalline gamma S (CRYGS) that are associated with triglyceride synthesis, glucose transport that may contribute to the composition of histotroph. Moreover, other study has also revealed that early P4 treatment increased blastocyst growth on days 9 and 12 accompanied by changes in the expression of genes involved in endometrial WNT system (Satterfield et al. 2008). Although the effect of progesterone on the endometrium is antagonistic to those of estrogen, both estrogen and progesterone are required to maintain acceptable level of estrogen to progesterone ratio for embryo implantation may required to maintain the adaptation of embryo in the new environment as deviations from ideal values of this ratio might affect the chances for successful embryo implantation. Therefore uterine differentiation to support embryo development and implantation is coordinated by progesterone and estrogen in a spatiotemporal manner (Huet-Hudson et al. 1989).

In addition to the ovarian hormones, the gonadotropin hormones do have substantial influence on endometrial or uterine preparation for receptivity. The increase in the uterine LH receptors and LH level in the perimplantation period has been explained (Bonnamy et al. 1993) suggesting the importance of LH in determining endometrial receptivity for implantation and subsequent decidualization.

2.8 Embryo competency for implantation

Following embryonic genome activation, the embryo grows faster to form a blastocyst. In bovine, blastocyst formation occurs seven days after fertilization (Guillomot 1995). By the time the embryo reaches to the blastocyst stage, it sheds its zona pellucidae to gain implantation competency. The differentiated and expanded blastocyst is composed the outer polarized epithelial trophectoderm, the primitive endoderm and the pluripotent inner cell mass which is the future cell lineages for the embryo proper (Dey et al. 2004). Within two weeks after fertilization, trophoblast elongates from 150 µm to 300 mm (Guillomot 1995) and placentation is initiated with apposition to the uterus (Bertolini et al. 2002,

Viebahn 1999). The process of trophoblast expansion depends on cell multiplication, cell growth and cell shape remodeling and cellular differentiation (Wintenberger-Torres and Flechon 1974). The formation of the trophectoderm and its subsequent development into trophoblast tissue are crucial steps for the initiation of implantation and the establishment of pregnancy. Trophoblast cells produce a variety of growth factors, cytokines and hormones that influence the conceptus and maternal physiology in an autocrine, paracrine and/or juxtacrine manner (Petraglia et al. 1998, Roberts et al. 1999). The blastocyst intrinsic characteristics such as its genetic constitution, morphology and hatching ability are one of the major components successful implantation. Abnormal genetic constitutions or unsuccessful transitions from maternal to embryonic transcription could account for many failures of early embryonic growth and implantation. Thus, successful implantation to occur in the receptive endometrium, the blastocyst must attain implantation competency and/or the blastocysts must be activated. For this, the blastocyst need to be enriched with transcripts and molecular signaling required for controlling cell differentiation and maternal recognition of pregnancy (Spencer et al. 2008). To this end, a global gene expression study showed that implantation competent blastocysts could be molecularly distinguishable from those which can not be activated for implantation. For instance, increased expression level of Hgf1 (which encodes heparin-binding EGF-like growth factor (HBEGF), ErbB1 and ErbB4 were found to be enriched in activated blastocysts compared to the dormant mouse blastocysts (Wang and Dey 2006). In addition, the gene expression analysis on elongated bovine blastocyst at day 17 after fertilization also indicated that the expression level of OCT4, NANOG, IFN-τ, EOMES, FGF4, SOX2 and CDX2 were found to be expressed in IVP embryos. However, NANOG, EOMES and FGF4 were reduced and IFN-τ was found to be over expressed in cloned embryos (Rodriguez-Alvarez et al. 2010). Similarly, TXN2 deficient mice embryos exhibited markedly increased apoptosis and die during mid gestation around E10.5 (Yamamoto et al. 2003). Further more homozygous mutants for thioredoxin die shortly after implantation and the conception were resorbed prior to gastrulation is associated with a dramatically reduced proliferation of inner mass cells (Matsui et al. 1996). This may suggest that cytosolic (TXN1) and mitochondrial (TXN2) thioredoxins are found to be essential during embryonic development. Apart from those, disruption of p97, proteins involved in vesicle and organelle biogenesis, cell-cycle regulators and transcription factors resulted in death embryo at peri-implantation stage (Muller et al. 2007). Moreover, the study on phospholipid hydroperoxide glutathione peroxidase (PHGPx), an intracellular antioxidant

enzyme that can directly reduce lipid hydroperoxide in membrane, indicated that mouse embryos homozygous for PHGPx-null die between 7.5 and 8.5 days post coitum suggesting the importance of PHGPx in early gastrulation stage and for normal mouse development (Imai et al. 2003). Similarly, the coordinated expression of uterine polyamine-related genes may also be important for embryo implantation. The study by (Zhao et al. 2008) showed that embryo implantation was significantly inhibited by α-difluoromethylornithine, an ODC inhibitor due to by the up-regulation of S-adenosylmethionine decarboxylase gene expression. In addition, in an attempt to investigate the gene expression pattern of blastocyst that develop to terms, (El-Sayed et al. 2006), identified some genes including COX2, CDX2, ALOX15, BMP15, PLAU and PLAC8 to be associated with embryos resulted in calf delivery. On the other hand, genes including KRT8, OCLN, PGK1 and AKR1B1 were enriched in embryos resulted in resorption and EEF1A1, MSX1, PTTG1, PGK1, AKR1B1 and CD9 were found to be enriched in embryos resulted in no pregnancy at all.

In addition to several molecules, the role of endocannabinoids, a group of lipid mediators that are ligands for the cannabinoid receptors CB1 and CB2 and N-arachidonoylethanolamine (anandamide) are also required during the mammalian embryo implantation process (Mechoulam et al. 1998). The reduced level of endometrial anandamide and embryo/blastocyst CB1 levels during the commencement of implantation may suggest its role in regulating implantation process (Lim et al. 2002). Therefore, low levels of anandamide are stimulatory and high levels of anandamide inhibit blastocyst growth and induce spontaneous abortions (Lim et al. 2002, Paria et al. 2001b). The blastocyst competency for implantation can be facilitated by its own secretions. Those secretions are species dependant and they are required to establish maternal recognition of the presence of the embryo the secretion (Table 3).

Table 3: Pregnancy recognition factor in cattle and other species

Species	Pregnancy recognition factor	Critical days after ovulation	Time of attachment (days after ovulation)
Cow	bIFN-τ (bTP-1)	15-16	18-22
Ewe	oIFN-τ (oTP-1)	13-14	15-18
Sow	Estradiol (E2)	11-12	14-18
Woman	hCG	7-12	9-12
Bitch	None needed	-	-

Adapted from Senger 2003

IFN-τ promotes uterine implantation by increasing protein synthesis in glands (Imakawa et al. 1987, Spencer et al. 1996). For instance, the ovine blastocyst begins to secrete oFN-τ immediately after hatching, continues during the elongation phase and reaches peak when the conceptus attachment to the uterus is initiated on day 16 (Imakawa et al. 2004). These temporal and spatial expression patterns indicate the existence of an early, trophoblast specific, regulatory mechanism for IFN-τ gene expression. In cattle and other ruminants, interferon tau (IFN-τ), is the conceptus cytokine secreted by the trophoblast cells into the uterine lumen (Roberts 2007). It is maintains pregnancy and facilitates the process of maternal recognition of pregnancy by decreasing endometrial oxytocin receptors, ultimately reducing the stimulation of PGF2 alpha synthesis and to preventing luteolysis (Imakawa et al. 2004, Spencer et al. 1996).

If the pregnancy signal [interferon tau (IFN tau)] or also called bovine trophoblastic protein 1 (bTP-1) is not received by the corpus luteum, then the prostaglandin that has been synthesized by the uterus is transferred to the ipsilateral corpus luteum by a local utero-ovarian countercurrent exchange mechanism. The prostaglandin then binds to the large luteal cells and causes luteal death via either direct action or a vascular constriction. The prostaglandin also causes release of oxytocin from the large luteal cells, which causes the uterus to release more prostaglandin (Figure 2). Estrogen from the dominant follicle is important in that it induces the uterine prostaglandin synthesis and the uterine oxytocin receptors. IFN-*τ* induces signal transduction through the JAK-STAT pathway (Darnell et al. 1994). Implantation involves a complex sequence of signalling events, consisting in the acquisition of adhesion ligands together with the loss of inhibitory components, which are crucial to the establishment of pregnancy.

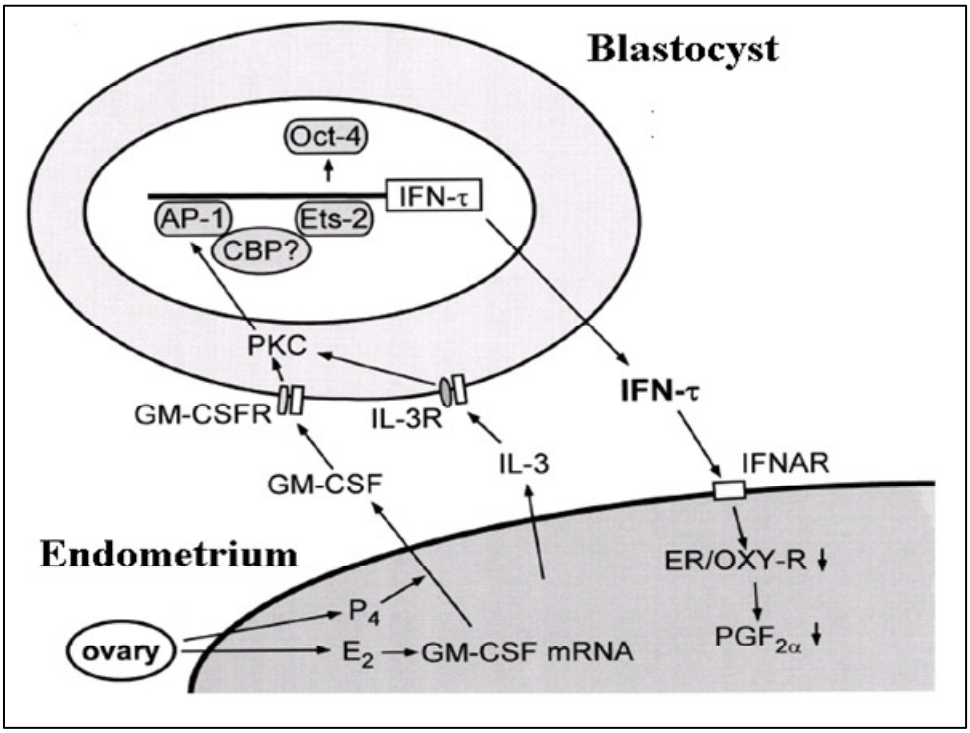

Figure 2: Interferon tau production and maternal conceptus communication. IL3 and GMCSF exhibited increased expression by estrogen and progesterone, respectively. As the result, these cytokines increase the expression of interferon tau through their respective receptors by intra cellular signaling pathway through the protein kinase C system (PKC). Adapted from Imakawa et al. (2004)

2.9 Process of embryo implantation

The blastocyst stage embryo and uterine differentiation to the receptive phase are the major requirement for successful establishment of the embryo-uterine communication during embryo implantation processes (Paria et al. 2001a). The bidirectional interaction between embryo and endometrium is mediated by paracrine signals that pass from endometrial tissue to the embryo and vice versa. Therefore, successful implantation requires a receptive endometrium, implantation competent blastocyst and a synchronized dialogue between maternal and embryonic tissues (Aplin 2000). During this period, various cellular activities like elongation of embryonic tissues, cell-cell contact between the mother and the embryo and various molecular changes take place (Ushizawa et al.

2004). Implantation therefore, involves a series of steps leading to an effective reciprocal signaling between the blastocyst and the uterus. It requires the trophoblast attachment via its apical plasma membrane to the apical plasma membrane of the uterine epithelium (Denker 1993).

The implantation processes is variable depending on the species and this difference is one of the causes to formulate a unifying theme for the molecular basis of embryo implantation. The process and phases of embryo implantation in ruminants and other animal is described by several authors (Aplin 2000, Bazer et al. 2009a, Spencer et al. 1996, Spencer et al. 2007). According to these authors, the process of implantation has 5 phases. In phase 1, the blastocyst sheds its zona pellucida and the spherical blastocyst continues to expand. Afterwards, the elongated blastocyst migrates and changes its from a spherical to tubular and filamentous form (ruminants) or remain spherical prior to implantation. In phase 2, the blastocysts migrate and undergo orientation without apparent cellular contact between the trophectoderm and endometrial epithelia. Phase 3 is the apposition phase during which the conceptus trophectoderm associates closely with endometrial luminal epithelium for unstable adhesion. In ruminants, the conceptus develops papillae that extend into the superficial ducts of the uterine glands for adhesion and absorption of histotroph (Figure 3). Phase 4 is the adhesion phase characterized by the trophectoderm becoming firmly adhered to endometrial luminal epithelium. In ruminants, this is the period of inter digitation of trophectoderm and endometrial luminal epithelium. During this phase, several molecules secreted by the endometrial epithelia, including glycosylated cell adhesion molecule (GLYCAM1), galectin 15 (LGALS15) and secreted phosphoprotein 1 (SPP1 or osteopontin), interact with receptors (integrins and glycoconjugates) on the apical surface of trophectoderm and LE to facilitate adhesion. In cattle implantation is non-invasive and exhibits an extended preimplantation period and during this time the endometrium secretes histotroph (proteins, carbohydrates, sugars, lipids and ions (Burghardt et al. 2002). During phase 5, trophoblast giant binucleate cells are formed that begin to differentiate from mononuclear trophoblast cells and then migrate to and fuse with the luminal epithelium to form multinucleated syncytial plaques.

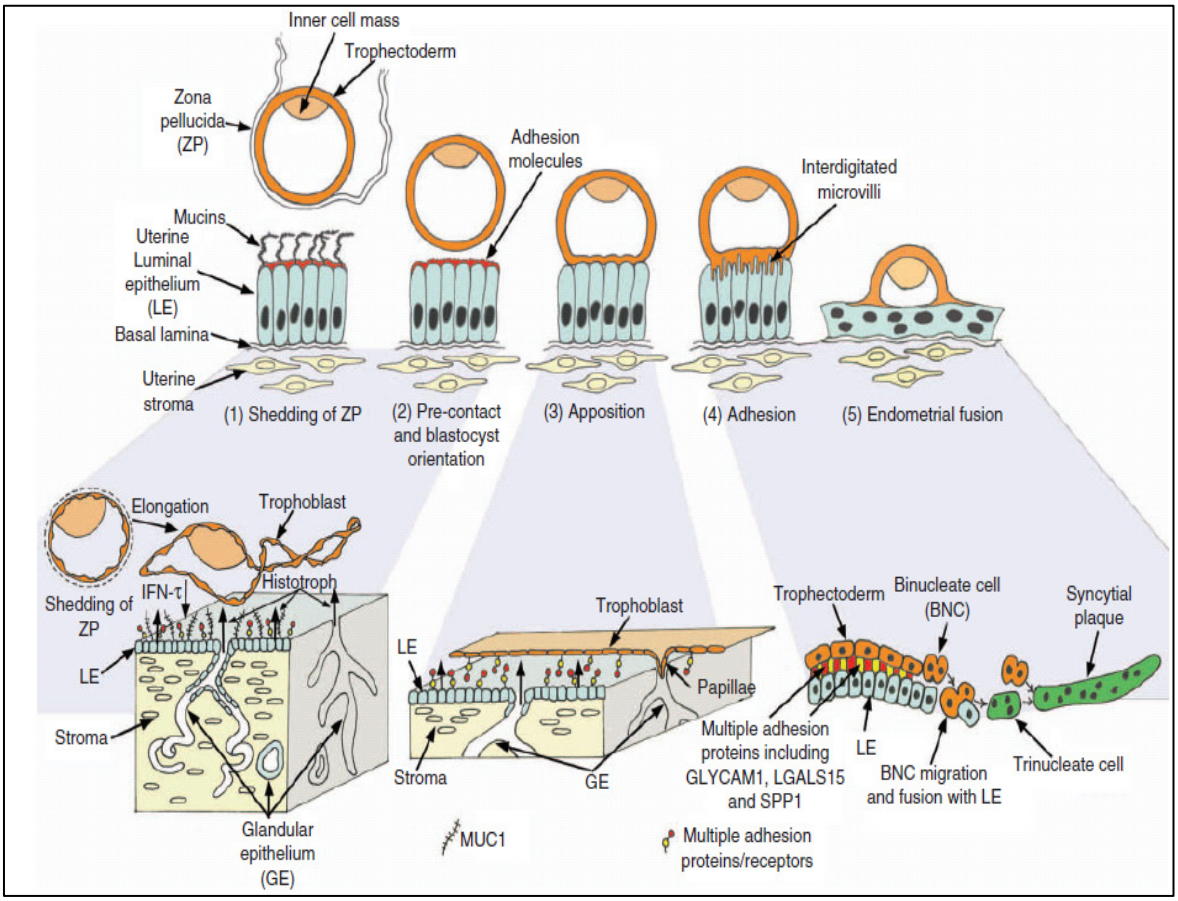

Figure 3: Phases of blastocyst implantation. Pre-attachment blastocysts undergo shedding of the zona pellucida (Phase 1) and precontact orientation (Phase 2). Phase 3 includes apposition and transient attachment. Phase 4 is marked by adhesion of trophectoderm to endometrial LE. Phase 5 involves formation of trophoblast giant binucleate cells that begin to differentiate from mononuclear trophoblast cells and then migrate to and fuse with the LE to form multinucleated syncytial plaques. Adapted from Spencer et al. (2007).

3 Materials and methods

3.1 Materials

3.1.1 Animals

During the experimental period, 54 Simmental cyclic heifers were used for endometrial sample collection and embryo transfer. Ten Simmental cows were used for superovulation and in vivo embryo production. Eighteen cyclic Simmental heifers were slaughtered at different days of the estrous cycle and three heifers were slaughtered at day 50 of gestation period. All animals were selected based on general clinical examination and normal ovarian cyclicity and housed in a free-stall barn with slotted floors and cubicles, lined with rubber mats and they were fed a total mixed ration. Animal handling and management was adhered to the rules and regulations of the German law of animal protection

3.1.2 Embryos and endometrial biopsies

Day 7 blastocysts produced by superovulation and the endometrial biopsies collected at day 7 and 14 of the estrous cycle were used for the study.

3.1.3 Materials for laboratory analysis

3.1.3.1 Chemicals, kits, biological and other materials

During this experiment, the following chemicals and biological materials from various manufacturers were used.

Chemicals or biological materials	Manufacturer/Supplier
4', 6'-diamidino-2-phenylindole hydrochloride (DAPI)	Burlingame, CA, USA
10x PCR buffer	Promega, WI, USA
2x rapid ligation buffer	Promega, WI, USA
2-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Munich, Germany
E. coli competent cells	Stratagene, Amsterdam, The

	Neatherlands
5x First-Stand buffer	Invitrogen Life Technologies, Karlsruhe
Acetic acid	Roth, Karlsruhe, Germany
Agar-Agar	Roth, Karlsruhe, Germany
Agarose	Sigma-Aldrich Chemie GmbH, Munich
Ampicillin	Roth , Karlsruhe
BME (essential amino acids)	Gibco BRL, life technologies, Karlsruhe
Boric acid	Roth , Karlsruhe, Germany
Bovine serum ablbumin (BSA)	Promega, Mannheim, Germany
Calcium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
Cy3 conjugated Goat anti-mouse lgG + lgM (H+L)	Jackson ImmunoResearch Laboratories, PA, USA
Chloroform	Roth , Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Roth , Karlsruhe, Germany
dNTPs	Roth , Karlsruhe, Germany
DTT	Invitrogen Life Technologies, Karlsruhe, Germany
Dye terminator cycle sequencing (DTCS)	Beckman Coulter, Krefeld, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
ExoSAP-IT	USB, Ohio, USA
Ethylenediaminetetra acetic acid	Roth , Karlsruhe, Germany

Formaldehyde	Sigma-Aldrich Chemie GmbH, Munich, Germany
Fluorescein isothiocyanate (FITC) Conjugated goat anti-rabbit secondary antibody	Lifespan Biosciences, WA, USA
Glycogen for sequencing	Beckman Coulter, Krefeld, Germany
Hemi-calcium lactate	Promega, WI, USA
Hydrochloric acid	Roth, Karlsruhe
Hepes	Sigma-Aldrich Chemie GmbH, Munich
Hydroxylamine	Sigma-Aldrich Chemie GmbH, Munich, Germany
Hypotaurin	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Isopropyl -D-thiogalactoside (IPTG)	Roth, Karlsruhe, Germany
Igepal	Roth, Karlsruhe, Germany
Isopropyl β-D-thiogalactoside (IPTG)	Roth, Karlsruhe, Germany
L-Glutamine	Sigma-Aldrich, Germany
Magnesium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
MEM (non essential amino acids)	Gico BRL, life technologies, Karlsruhe
Mineral oil	Sigma-Aldrich Chemie GmbH, Munich, Germany
Mous anti-human monoclonal antibody against TIMP3	LifeSpan biosciences inc, WA, USA
Oligonucleotide primers	MWG Biotech, Eberberg, Germany

Pepton	Roth , Karlsruhe, Germany
Penicillin	Sigma-Aldrich Chemie GmbH, Taufkirchen
pGEM®-T vector	Promega, WI, USA
Phenol red solution (5% in D-PBS)	Sigma-Aldrich Chemie GmbH , Munich
Potassium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
Rabbit polyclonal primary antibody against IRF6, ITPR1, PTGS2 or MMP2	Santa Cruz Biotechnologies Inc., CA., USA
Random primer	Promega, WI, USA
Ribo-nuclease inhibitor (RNasin)	Promega, WI, USA
RNA later	Sigma-Aldrich, MI, USA
RQ1 RNase-free DNase	Promega, WI, USA
RNasin	Promega, WI, USA
Sample loading solution (SLS)	Beckman Coulter, Krefeld, Germany
Sequagel XR Sequencing Gel	Beckman Coulter, Krefeld, Germany
Sodium acetate	Roth , Karlsruhe, Germany
Sodium chloride	Roth , Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Inc, MO, USA
Sodium hydrogen sulphate	Sigma-Aldrich Inc, MO, USA
Sodium lactate solution (60%)	Sigma-Aldrich Inc, MO, USA
Sodium pyruvate	Sigma-Aldrich Inc, MO, USA
Streptomycin sulphate	Sigma-Aldrich Inc, MO, USA
Superscript II reverse transcriptase	Invitrogen, CA, USA

iTaq SYBR Green Supermix with ROX	Bio-Rad laboratories, Munich, Germany
T4 DNA ligase	Promega, WI, USA
Taq DNA polymerase	Sigma-Aldrich Inc, MO, USA
Tris	Roth ,Karlsruhe, Germany
Triton X-100	Roche Diagnostics GmbH, Mannheim, Germany
Yeast extract	Roth, Karlsruhe, Germany
yeast tRNA	Sigma-Aldrich Chemie GmbH, Munich
X-Gal (5-bromo-4-chloro-3-indolylbeta-D-galactopyranoside)	Roth, Karlsruhe, Germany
RNeasy mini kit	Qiagen, Hiden, Germany
RNA 6000 Nano LabChip® Kit	Agilent Technologies Inc, CA, USA
MEGAscript® T7 Kit	Applied Biosystems, CA, USA
Eukaryotic poly-A RNA control kit	Affymetrix, CA, USA
AmpliScribe T7 transcription kit	Epicentre technologies, WI, USA
CyScribeTM GFXTM Purification kit	Amersham Biosciences, Freiburg, Germany
Amersham post-labelling kit	Amersham Bioscience, Freiburg, Germany
CyScribeTM GFXTM Purification kit	Amersham Biosciences, Freiburg, Germany
(DOP)-PCR master kit	Roche Diagnostics GmbH, Mannheim, Germany
QIAquick PCR Purification	Quigen, Hiden, Germany
PicoPure^{TM} RNA isolation kit	Arcturs, CA, USA.

3.1.3.2 Reagents and media

During this experiment, the following reagents and media formulation were used.

CR1-aa culture medium (50 ml)	Hemi-calcium lactate	0.0273 g
	Streptomycin sulphate	0.0039 g
	Penicillin G	0.0019 g
	Sodium chloride	0.3156 g
	Potassium chloride	0.0112 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium pyruvate	0.0022 g
	L-Glutamine	0.0073 g
	Phenol red solution (5% in D-PBS)	100 µl
	Sodium hydrogen carbonate	0.080 g
Modified parker medium	HEPES	0.140 g
	Sodium pyruvate	0.025 g
	L-Glutamin	0.010 g
	Gentamicin	500 µl
	Medium 199	99 ml
	Hemi calcium lactate	0.06 g
	added to water	110 ml
Prehybridization	BSA	0.5 g
	10 % SDS	0.5 ml
	20 % SSC	7.5 ml
	Add water	till 50 ml
Hybridization buffer	Deionized formamide	20 m
	50 X Denhardt's solution	5 ml
	20X SSC	12.5 ml
	Sodium pyruvate	22 mg
	Tris-HCL (pH 7.5	2.5 ml
	10% SDS	0.5 ml
	Millipore water until	50 ml

LB-agar	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Agar-Agar	12.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH_2O added to	800.0 ml
LB-broth	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH_2O added to	800.0 ml
BSA (3%)	Bovine serum albumin	0.15 g
	added to PBS+PVA	5 ml
DEPC-treated water (1000 ml)	DEPC	1 ml
	added to water	1000 ml
Lysis buffer (100 µl)	Igepal (0.8%)	0.8 µl
	RNasin	5 µl
	DTT	5 µl
	added to water	100 µl
TAE (50x) buffer, pH 8.0	Tris	242.0 mg
	Acetic acid	57.1 ml
	EDTA (0.5 M)	100.0 ml
	ddH_2O added to	1000.0 ml
TE (1x) buffer	Tris (1 M)	10.0 ml
	EDTA (0.5 M)	2.0 ml
X-gal	ddH_2O added to	1000.0 ml
	X-gal	50.0 mg
(16%) Para formaldehyde (10 ml)	N, N'-dimethylformamide	1.0 ml
	Para formaldehyde	1.6 g
	added to water	10 ml
PBS + PVA (50 ml)	Polyvinyl alcohol (PVA)	300 mg
	PBS added to	50 ml
Permeabilizing solution (10 ml)	Triton X-100	5 µl

	Glycine + PBS added	10 ml
Physiological saline solution	Sodium chloride	9 g
	added to water	1000 ml
Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH_2O added to	25 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
	ddH_2O added to	400.0 µl
IPTG solution	IPTG	1.2 g
	ddH_2O added to	10.0 µl
3M Sodium Acetate, pH 5.2	Sodium Acetate	123.1 g
	ddH_2O added to	500 ml
1M EDTA, pH 8.0	EDTA	37.3 g
	ddH_2O added to	1000 ml
Phenol Chloroform	Phenol : Chloroform	1 : 1 (v/v)
0.2% Triton-X100:	Triton	2 ml
	10x PBS : added to	1,000.0ml
0.3% BSA in PBS	BSA	3 g
	10x PBS : added to	1,000.0 ml
3% BSA in PBS	BSA	30g
	10x PBS : added to	1,000.0 ml

3.1.4 Equipements

During this experiment, the following reagents and media formulation were used.

Equipment	Manufacturer
Ultrasonography (Pie Medical, 5 MHz)	Pie Medical
Bovine preimplantation embryo specific cDNA array	Laval University
ApoTome microscope	Carl Zeiss MicroImaging, Germany
Cytobrush	Gynobrush, Hamburg, Germany
CH15 embryo flushing Cather	Immuno Systems
Inverted fluorescence microscope DM IRB	Leica, Germany
Stereomicroscope SMZ 645	Nikon, Japan
Centrifuge	Hermel, Wehing
Electrophoresis	BoRad, Munich
My Cycler Thermal cycler	Bio-RadLaboratories, CA, USA
SHKE6000-8CE refrigerated Stackable Shaker	Thermoscinentific, IWA, USA
CEQ™ 8000 Genetic Analysis	BeckmanCoulter,Krefeld, Germany
ABI PRISM® 7000 SDS	Applied Bio systems
Affymetrix®GeneChip™3000 scanner	Affymetrix, CA, USA
Affymetrix®GeneChip Fluidics Station 450	Affymetrix, CA, USA
Affymetrix®GeneChip Hybridization oven 640	Affymetrix, CA, USA
Agilent 2100 bioanalyzer	Agilent Technologies , CA, USA
GAPSII	Corning (Amsterdam)
Nanodrop 8000 Spectrophotometer	Thermo Fisher Scientific, DE, USA
Axon GenePix 4000B scanner	Axon Instruments, Foster City, CA

Four well dishes	Thermo Fisher Scientific, Nunc, Roskilde, Denmark
Rigid thin wall 96 X 0.2 ml skirted microplates for real-time PCR	STARLAB GmbH (Ahrensburg)
GeneChip® Bovine Genome Array	Affymetrix, CA, USA

3.1.5 Programs (soft wares) and statistical packages used

Programs (soft wares) and statistical packages	Source of the programs (soft wares) and statistical packages
GeneChip® Operating System	Affymetrix, CA, USA
R statistical computing and graphics software	http://www.r-project.org/
Bioconductor packages	
Library (affy), Library (marray)	
Library (GCRMA), Library (LIMMA)	
Library (sma), Library (anotate)	http://www.bioconductor.org/
Library (gostats), Library (Go) Library(qualityMetrix)	
Library(gplots)	
GNU Image Manipulation Program (GIMP)	Spencer Kimball, Peter Mattis und das GIMP-Entwicklerteam
SAS (version 9.2)	SAS Institute Inc., NC, USA
GenePix Pro (ver. 4.0)	Axon Instruments, Foster City, CA
KEGG pathway	http://www.genome.jp/kegg/pathway.html
Ingenuity's pathway analysis	Ingenuity® Systems, www.ingenuity.com
Entrez Gene	http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene

AxioVision	Carl Zeiss International
EndNote X1	Thomoson
Primer Express ® software	Applied Biosystems, Foster city, CA, USA
Primer 3 (version 4)	http://frodo.wi.mit.edu/primer3/
BLAST program	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Prism for windows (ver.5.0)	GraphPad software, Inc.

3.2 Methods

3.2.1 Pre-transfer endometrial gene expression in relation to pregnancy outcome

3.2.1.1 Endometrial biopsy collection

The design and experimental setup of the present study is indicated in Figure 4. Prior to endometrial biopsy collection, experimental heifers were estrous synchronized by intra muscular administration of 500 mg of the prostaglandin $F_{2\alpha}$ (PGF2α) analogue cloprostenol (Estrumate, Munich, Germany) twice within 11 days. Two days after each of the PGF2α treatments, animals received 0.02 mg GnRH-analogue buserelin (Receptal) (Intervet, Boxmeer, the Netherlands). Two PGF2α treatments were performed on day 14 and 17 followed by administration of 0.02 mg GnRH at day 18 to induce ovulation. Common signs of estrus were monitored by visual observation three times per day followed by careful palpation of ovaries to check the presence of corpus luteum. Following this, endometrial biopsies were collected from experimental animals using a cytobrush technique. Cytobrush technique is a consistent and reliable method for obtaining endometrial samples for cystologic examination and gene expression analysis from dairy cows (Gabler et al. 2009, Kasimanickam et al. 2005, Kaufmann et al. 2009). For collecting the endometrial samples, 20 mm in length and 6 mm in diameter brush (Gynobrush, Heinz Herenz, Hamburg, Germany) protected by a one-way catheter was inserted via the cervix into the uterine body. Inside the uterus, the brush was pushed gently out of the catheter and rolled along the uterine wall. Thereafter, the brush was retracted into the catheter to protect it from contamination during the passage through the genital tract. For each animal, endometrial samples were collected at days 7 and 14 of the estrous cycle during the pre-

transfer cycle (one cycle prior to embryo transfer). The endometrial samples were stored in RNA later (Sigma-Aldrich, Mi, USA) until further use. Day 7 and 14 of the estrous cycle were chosen for two main reasons. Firstly, most often embryo transfer in cattle is performed using blastocyst stage embryo at day 7 of the estrous cycle (Bauersachs et al. 2009, Hill et al. 2000, Mansouri-Attia et al. 2009, Rodriguez-Alvarez et al., Rodriguez-Alvarez et al. 2010) or in case of artificial insemination, most of the embryos reach to the uterus and develop to the blastocyst stage at day 7 of the estrous cycle. Moreover, day 7 of the estrous cycle is marked by formation of corpus luteum. Secondly, in the absence of embryo in the uterus, the uterine prostaglandin F2α (PGF2α) increases at day 14 of the estrous cycle leading to corpus luteum regression (Shemesh and Hansel 1975).

3.2.1.2 Superovulation and artificial insemination

Pre-synchronization was performed by i.m. administration of 500 mg of the prostaglandin $F_{2\alpha}$ (PGF2α) analogue cloprostenol (Estrumate; Munich, Germany) twice within 11 days. Two days after each of the $PGF_{2\alpha}$ treatments, animals received 0.02 mg of GnRH-analogue buserelin (Receptal) (Intervet, Boxmeer, The Netherlands). Twelve days after the last GnRH injection, cows received the first of eight consecutive FSH-injections over 4 days in decreasing dose. Two $PGF_{2\alpha}$ treatments were performed 60 and 72 h after the initial FSH administration. Finally, 48 h after the first $PGF_{2\alpha}$ application, ovulation was induced by administration of 0.02 mg of buserelin and three artificial inseminations were performed within 12 h interval. The time of the second insemination (60 h after the first $PGF_{2\alpha}$ application) was defined as Day 0.

3.2.1.3 Blastocyst recovery and embryo transfer

Blastocysts embryos were flushed 7 days after insemination by draining each uterine horn with 500 ml phosphate buffer saline solution (PBS) using CH15 embryo-flushing catheter (Wörrlein, Ansbach, Germany) connected to an embryo filter (Immuno Systems Inc., WI, USA). Good quality blastocysts were subjected to biopsying using a beaver micro blade (Minitüb, Tiefenbach, Germany) fixed to a micromanipulator under inverse microscope (Leica Camera Inc., Solms, Germany). Following biopsying, 60-70% portion of the blastocyst containing both inner cell mass (ICM) and trophectoderm cells (TE) was cultured *in vitro* in CR1 medium supplemented with amino acid for 2 h and transferred to

54 synchronized Simmental heifers as indicated above. The remaining 30-40% of the embryo biopsy containing both ICM and TE was washed twice in PBS and snap frozen in cryo-tubes, containing minimal amounts of lysis buffer [0.8% Igepal (Sigma-Aldrich, MO, USA), 40 U ml^{-1} RNasin (Promega, WI, USA), 5 mM dithiothreitol (DTT) (Promega, WI, USA)]. All frozen embryos were stored at -80 ^{o}C until RNA isolation.

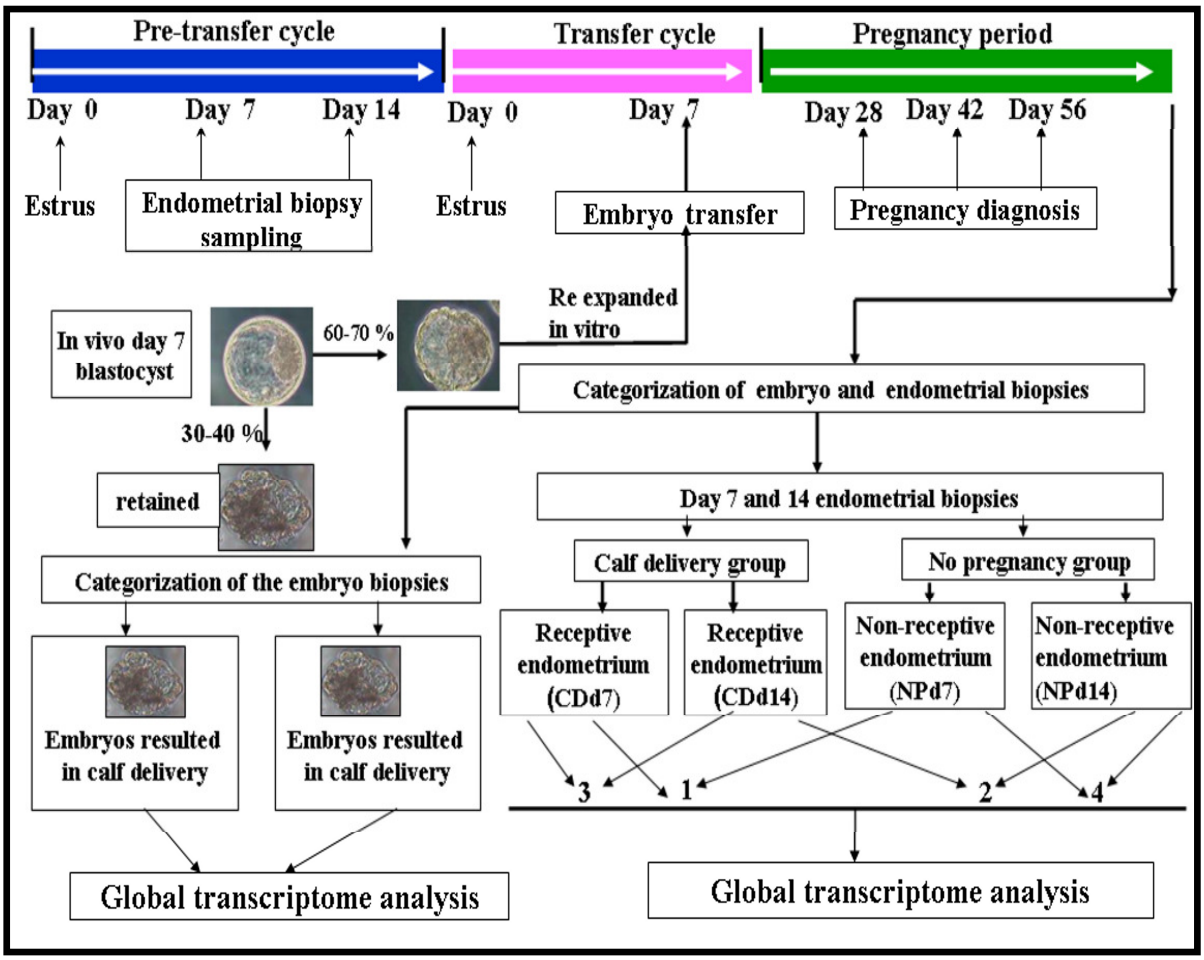

Figure 4: The overall experimental design applied in the study. Endometrial biopsies were taken during the pre transfer period. In the next cycle, blastocyst stage embryos were transferred to the recipients after part of the embryo as a biopsy. Following pregnancy diagnosis, the endometrial and embryo biopsies were classified according to the pregnancy outcome. Global transcription analysis was performed from embryo and endometrial biopsies. Numbers 1 and 2 represent the transcriptome analysis between receptive and non-receptive endometrium at day 7 and 14 of the estrous cycle whereas 3 and 4 represent the transcriptome dynamic analysis of receptive and non-receptive endometrium.

3.2.1.4 Pregnancy diagnosis and categorization of endometrial and embryo biopsies

Pregnancy diagnosis was performed at days 28 and 42 using ultrasonography (Pie Medical, 5 MHz) and at day 56 by rectal palpation. Those heifers returning to heat at day 21 were categorized as non pregnant or considered as non-receptive endometrium. Those heifers resulted in calf delivery were categorized as the calf delivery group or considered as receptive endometrium. At the end of gestation, the endometrial and embryo biopsies collected during the pre-transfer period were classified according to the pregnancy outcome information (Figure 4). Those endometrial biopsies collected at days 7 and 14 of the estrous cycle from calf delivery groups (receptive endometrium) were designated as CDd7 and CDd14, respectively. Endometrial biopsies taken at days 7 and 14 of the estrous cycle from those subsequently resulted in no pregnancy (non-receptive endometrium) were designated as NPd7 and NPd14, respectively. Similarly, the embryo biopsies were classified according to the pregnancy outcome information. Each embryo biopsied from blastocysts transferred to the recipients was snap frozen and stored separately. Following pregnancy diagnosis, the embryo biopsies were classified as those embryo biopsies resulted in calf delivery or resulted in no pregnancy group (Figure 4). During RNA isolation those embryo biopsies resulted in calf delivery were pooled in three replicates and those embryo biopsies resulted in no pregnancy were pooled in three other replicates.

3.2.1.5 Total RNA isolation from endometrial biopsies

Total RNA was extracted from three pools of endometrial sample from each category using RNeasy mini kit (Qiagen, Hilden, Germany) with slight modification. Briefly, three pools of endometrial cytobrush samples from each group, namely: CDd7, CDd14, NPd7 or NPd14 were transferred in 2 ml Eppendorf tube filled with 500 µl of RLT buffer containing 10% 14.3 M βeta mercaptoethanol (β-ME) (Thermo Fisher Scientific Inc., IL, USA). The samples were then vortexed for 10 minutes. Complete retrieval of the cytobrush contents were performed by three consecutive washings in 500 µl of RLT- β-ME buffer. The samples were then centrifuged at 12575 rpm. The clear lysate was then retrieved to which 1 volume of 70% ethanol was added. After mixing, 700 µl of the sample was then transferred to RNeasy spin column and centrifuged in refrigerated universal centrifuge Z233MK (Hermle Labortechnik, Wehingen, Germany) for 15 s at 12575 rpm. The supernatant was discarded. Since the sample volume was exceeding 700

µl, successive aliquots were centrifuged in the same spin column. Following this, 700 µl RW1 buffer was added to the samples in the spin column and centrifuged for 15 s at 12575 rpm. After removing the supernatant, 500 µl RPE buffer was added to the spin column followed by 15 s centrifugation 12575 rpm. This step was repeated for the second time and centrifuged at the same speed for 2 min. The spin column was then transferred to a new 2 ml collection tube and centrifuged at full speed for 1 min to eliminate any possible carryover of RPE buffer and residual flow-through remained on the spin column. Following this, the RNeasy spin column was transferred to a new 1.5 ml collection tube and 40 µl RNase-free water was added directly to the spin column membrane. The spin column membrane was then centrifuged for 1 min at 12575 rpm to elute the RNA. One micro litter of the RNA was used to measure the concentration and quality using Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific Inc, DE, USA).

3.2.1.6 DNase treatment of total RNA samples

Prior to subsequent application, the RNA samples were freed from any DNA contamination using RNA-qualified (RQ1) RNase-free DNase I (Promega, WI, USA). One unit of RQ1 RNase-free DNase and 1µl RQ1 RNase-free DNase 10x reaction buffers were used per 1 µg of total RNA. To this, RNase-free water was added to reach the required volume and incubated at 37 ^{o}C for 1 h. At the end of the incubation time, the reaction was purified using RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacture's recommendation. Briefly, the samples were first adjusted to 100 µl with RNase-free water and to this, 350 µl RLT buffer and 250 µl 100% ethanol were added. The samples were then transferred to RNeasy mini spin column placed in a 2 ml and centrifuged at 12575 rpm for 15 s. After decanting the flow-through, 500 µl RPE buffer was added to the samples and centrifuged at 12575 rpm for 15 s. After the final wash with 500 µl RPE for 2 min at 12575 rpm, any possible carryover of RPE buffer and residual flow-through remained on the RNeasy spin column was eliminated by centrifugation the spin column for 2 min at full speed. Afterwards, 30 µl RNase-free water was directly added to the spin column membrane fixed in a new RNase free 2 ml collection tube and centrifuged at 12575 rpm for 1 min. The RNA concentration and quality was analyzed using Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific Inc, DE, USA). RNA integrity was evaluated using Agilent 2100 bioanalyzer with RNA 6000 Nano LabChip® Kit (Agilent

Technologies Inc, CA, USA). The ribosomal RNA ratio (28S to 18S) of the RNA samples was between 1.9 and 2.1 and the RNA integrity number (RIN) was between 7.5 and 8.4.

3.2.1.7 RNA amplification from endometrial biopsies

3.2.1.7.1 First cycle first and second strand cDNA synthesis

Two cycles eukaryotic target labeling assay was performed following the recommendation of the GeneChip® expression analysis technical manual (P/N 702232). For this, 100 ng of total RNA isolated from endometrial biopsies resulted in calf delivery or no pregnancy was used as a starting material. The eukaryotic poly-A RNA control kit was used as a SPIKE-IN control to monitor the entire target labeling process, The controls were then amplified and labelled together with the samples as each eukaryotic GeneChip® probe array contains probesets for several B. subtilis genes (lys, phe, thr and dap) that are absent in eukaryotic samples. Prior to first strand cDNA synthesis, 2 µl of 50 µM T7-Oligo (dT) primer and 2 µl diluted eukaryotic poly–A RNA controls were mixed and 16 µl of RNase free water was added to this mix to final volume 20 µl. From this, 2 µl was taken and added to the 100 ng total RNA and RNase free water was added to final 5 µl volumes. This mix was incubated for 6 min at 70 ^{o}C. After the end of the incubation period, 5 µl mix (containing 2 µl 5x 1st strand reaction mix, 1 µl 0.1M DTT, 0.5 µl RNase inhibitor, 0.5µl 10 mM dNTP, 1 µl superscript II) was added to the reaction and incubated for 1 h at 42 ^{o}C. Afterwards, the enzyme was deactivated by heating the reaction at 70 ^{o}C for 10 min followed by cooling at 4 ^{o}C for 2 min. Following the first cycle first strand cDNA synthesis, second strand cDNA was synthesized from first strand cDNA. For this, a 10 µl first cycle second strand master mix (4.8 µl RNase free water, 4 µl 17.5mM diluted $MgCl_2$, 0.4 µl 10mM dNTP, 0.6 µl E.coliDNA polymerase and 0.2 µl RNase H) was added to this 10 µl first stranded cDNA. The reaction was incubated for 120 min at 16 ^{o}C and for 10 min at 75 ^{o}C.

3.2.1.7.2 First cycle, IVT amplification of cRNA

In vitro transcription (IVT) and RNA amplification was performed using MEGAscript® T7 Kit (Applied Biosystems, CA, USA) using dsCDNA as a template. For this a 30 µ IVT mix (5 µl each 10X reaction buffer, ATP solution, CTP solution, UTP solution GTP solution and enzyme) was added to the double stranded cDNA. The reaction was incubated at 37 ^{o}C for 16 h. At the end of the incubation period, the complementary RNA (cRNA)

was purified using GeneChip® IVT cRNA cleanup kit (Affymetrix, CA, USA). At this step, the concentration and quality of cRNA was determined using Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific Inc, DE, USA).

3.2.1.7.3 Second-cycle, first strand and double cDNA synthesis

Second –cycle, first strand cDNA synthesis was performed using 600 ng cRNA resulted in first cycle of IVT amplification. For this, first 2 µl random primers (final concentration 0.2 µg/µl) was added to the cRNA and RNase free water was added to final volume of 11 µl. This reaction was incubated for 10 min at 70 ^{O}C. Afterwards, 9 µl second–cycle, first-strand master mix (containing 4 µl 5x 1st strand reaction buffer, 2 µl 0.1M DTT, 1 µl RNase inhibitor, µl 10mM dNTP, µl superscript 11) was added to each sample and incubated for 1 h at 42 ^{O}C. After cooling at 4 ^{O}C for 2 min, 1 µl of RNase H enzyme was added to each sample. The reaction was incubated for 20 min at 37 ^{O}C followed by heating at 95 ^{O}C for 5 min. Subsequently, the T7-Oligo (dT) promoter primer was used in the second-strand cDNA synthesis to generate double-stranded cDNA template containing T7 promoter sequences. For this T7-oligo (dT) primer (5 µM of final concentration) was added to the reaction and incubated for 6 min at 70 ^{O}C. After cooling at 4 ^{O}C for 2 min, 125 µl second cycle second strand master mix (containing 30 µl 5x 2nd strand reaction mix, 3 µl of 10 mM dNTP, 4 µl E.coli/DNA polymerase I) was added and incubated for 2 h at 16 ^{O}C. The reaction was terminated after incubating the sample with 2 µl T4 DNA polymerase at the same temperature for 10 min. The samples were then purified using the sample clean up module and the double-stranded cDNA was eluted in 12 µl RNase free water.

3.2.1.7.4 Biotin labeling and fragmentation of cRNA

The resulting double-stranded cDNA was then in vitro transcribed and labeled using a 40 µl biotinylated nucleotide analog/ribonucleotide mix (4 µl 10X IVT labeling buffer, 12 µl IVT labeling NTP mix, 4 µl IVT labeling enzyme mix, 8 µl RNase free water) in the second IVT reaction using GeneChip IVT labeling kit (Affymetrix, CA, USA). The reaction was incubated at 37 ^{O}C for 16 h. After the end of incubation period, the biotin labeled cRNA was purified and eluted with 11 µl RNAse free water. The concentration and quality of biotin labeled cRNA was determined using Nanodrop 8000

Spectrophotometer (Thermo Fisher Scientific Inc, DE, USA). Fifteen microgram biotin labeled cRNA was then fragmented at 94 ^{O}C for 35 min using 5x fragmentation buffer. The size distribution of fragmented biotin labeled cRNA samples were analyzed using Agilent 2100 bioanalyzer with RNA 6000 Nano LabChip® Kit (Agilent Technologies Inc, CA, USA).

3.2.1.8 Target hybridization

The GeneChip® Bovine Genome Array (Affymetrix, CA, USA) was used for hybridization. For this, a hybridization cocktail consisting of 10 µg fragmented and labeled cRNA, control oligonucleotide B2 (3 nM), 20x eukaryotic hybridization controls (bioB, bioC, bioD, cre) (Affymetrix, CA, USA), 2X hybridization mix, DMSO and RNAse free water were mixed to a final volume of 200 µl. The mix was then heated at 99°C for 5 min followed by 5 min incubation at 45°C. Meanwhile, the probe arrays were wetted with 1x hybridization buffer and incubated at 45 °C for 10 min in hybridization oven with 60 rpm rotation. After removing the hybridization buffer from each of the probe arrays, 130 µl hybridization cocktail was filled to each probe array and placed in the hybridization oven at 45 ^{o}C with 60 rpm rotation. Hybridization was performed for 16 h. Three biotin labeled cRNA hybridizations were performed for endometrial samples of each category (CDd7, CDd14, NPd7 and NPd14).

3.2.1.9 Washing and staining probe arrays

The probe arrays were washed and stained using the Fluidics Station 450/250 integrated with GeneChip® Operating System as recommended in GeneChip® expression wash, stain and scan user manual (P/N 702731 (Affymetrix) as recommended in GeneChip® expression wash, stain and scan user manual (P/N 702731). Briefly, after 16 h of hybridization, the hybridization cocktail was removed and the probe array cartridge was filled with 160 µl wash buffer A. The probe arrays were then inserted in designated module of the fluidic station. Meanwhile, each of the vials containing 600 µl stain cocktail 1 and 600 µl stain cocktail 2 were was placed in sample holder 1 and 2 of the Fluidics Station 450/250, respectively. Similarly, a vial containing 800 µl array holding buffer was placed in sample holder 3 in the fluidic station. Afterwards, the needle lever was pressed down and the needles were snapped into position to start washing.

3.2.1.10 Scanning the probe arrays

After washing, the probe arrays were scanned using affymetrix®GeneChip™3000 confocal slide scanner (Affymetrix, CA, USA) integrated with GeneChip® Operating System as recommended in GeneChip® expression wash, stain and scan user manual (P/N 702731).

3.2.1.11 Affymetrix array data analysis

The microarray data normalization and background correction was performed using guanine cytosine robust multi-array analysis (GCRMA) (Wu and Irizarry 2004). For this, we used R software, (www.r-project.org) and bioconductor packages (www.bioconductor.org). During normalization, the CEL files were first converted into expression set using the guanine cytosine robust multi-array average (GCRMA) considering probe sequence and the GC-content background correction. Starting with the probe-level data from a set of GeneChips, the perfect-match values were background-corrected, normalized and finally summarized resulting in a set of expression measures. The raw and normalized data are available at Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/geo/ with accession number GSE21049 or GSE20974. The intensity distribution and uniformities between the arrays were evaluated by assessing Pearson correlation and heatmap, MA plots and density plots (Appendix 1). Differentially expressed (DE) genes were obtained using linear models for microarray data (LIMMA) which quires two matrices namely the design matrix which provides a representation of the different RNA targets which have been hybridized to the arrays and the contrast matrix which allows the coefficients defined by the design matrix to be combined into contrasts of interest (Smyth 2005). Genes were considered differentially expressed (DE) when the $p < 0.05$ and fold change ≥ 2, FDR ≤ 0.27. p-values were adjusted using the Benjamini–Hochberg procedure that controls the false discovery rate (Benjamini and Hochberg 1995). Differentially expressed genes were further classified according to their gene ontology (GO) of molecular functions and biological processes using GO statistical analyses (GOstats) (Falcon and Gentleman 2007). Moreover, molecular pathways participated by DE genes were identified from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). The molecular networks involved by the

differentially expresses genes was analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

3.2.2 Expression profile of candidate genes during the estrous cycle and early pregnancy

Following identification of differentially expressed genes between the receptive and non-receptive endometrium, the expression pattern of selected candidate genes were examined at different phases of the estrous cycle and early gestation period. Candidate genes were randomly selected among those whose expression was increased in receptive endometrium [interferon regulatory factor 6 (IRF6), prostaglandin E receptor 4 (PTGER4), Inositol 1,4,5-trisphosphate receptor type 1 (ITPR1), adenosine A2B receptor (ADORA2B), prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase (PTGS2/ COX-2), tissue inhibitor of metalloproteinase 3 (TIMP3), cell division cycle 20 homolog (CDC20), wingless-type MMTV integration site family, member 11 (WNT11] and 3 other genes whose expression was increased in non-receptive endometrium [signal transducer and activator of transcription 5A (STAT5A), matrix metalloproteinase 2 (MMP2) and angiotensin II receptor, type 1 (AGTR1)]. This was aimed at evaluating the pattern of those differentially expressed genes at different stage of the estrous cycle and to assess whether the genes increased or reduced in receptive endometrium during the estrous cycle will continue or decay during early gestation period. For this, endometrial samples was collected from 18 normal cyclic heifers slaughtered at days 0, 3, 7, 14 and 19 of the estrous cycle following standard synchronization protocol and 3 animals were slaughtered at day 50 of the gestation period. Endometrial samples were collected from seven different positions of the uterus by scraping, namely ipsilateral cranial, ipsilateral middle, ipsilateral caudal, contralateral cranial, contralateral middle, contralateral caudal and corpus (Bauersachs et al. 2005). Blood samples were collected from each animal before slaughtering by jugular venipuncture. The blood samples were refrigerated at 4°C for 12-24 hrs before being centrifuged at 1500 g at 4°C for 20 min. Serum was separated and stored at -20°C until assayed to determine progesterone concentration by time-resolved fluoroimmunoassay using an AutoDELFIA™ Progesterone kit (Perkin Elmer, Wallac Oy, Turku, Finland).

3.2.3 Immunohistochemical localization of candidate genes

The protein expressions of IRF6, ITPR1, PTGS2, MMP2 and TIMP3 were localized in the ipsilateral middle part of the uterine tissue section at different days of the estrous cycle and at day 50 of the gestation period. For this, the sections were washed in PBS and fixed in 4% (w/v) para formaldehyde overnight at 4°C. The fixed specimens were permeabilized in 0.5% (v/v) Triton-X100 (Sigma) in phosphate buffer saline solution (PBS) and blocked in 3% (w/v) bovine serum albumin (BSA) (Roche Diagnostics, Basel, Switzerland) in PBS. The samples were incubated overnight using rabbit polyclonal primary antibody specific IRF6, ITPR1, PTGS2 or MMP2 and mouse anti-human monoclonal antibody specific to TIMP3. Rabbit polyclonal primary antibody (Santa Cruz Biotechnologies Inc., CA., USA) against IRF6 and PTGS2 was diluted at 1:100 and rabbit polyclonal primary antibody (Lifespan Biosciences, USA) against ITPR1 and MMP2 was diluted at 1:50 in blocking solution. Mouse anti-human monoclonal antibody (LifeSpan biosciences, Inc, WA, USA) against TIMP3 was diluted 1:100 in blocking solution. Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit secondary antibody (Lifespan Biosciences, WA, USA) diluted at 1:100 was used in all cases except TIMP3. Cy3 conjugated Goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc. PA, USA), diluted at 1:100 used for TIMP3. Counterstaining was performed using 4', 6'-diamidino-2-phenylindole hydrochloride (DAPI) (Burlingame, CA, USA). After the final wash in PBS, the sections were mounted on glass slides and visualized on an ApoTome microscope (Carl Zeiss MicroImaging Inc., Germany).

3.2.4 Pre-transfer embryonic gene expression in relation to pregnancy outcome

3.2.4.1 RNA isolation from embryo biopsies

Total RNA was extracted from 15 embryos that resulted in calf delivery or no pregnancy in three pools each consisting of five embryos using PicoPure™ RNA isolation kit (Arcturs, CA, USA). For this, 20 µl extraction buffer was added to each embryo biopsy followed by incubation at 42° for 30 min. At the end of the incubation time, embryos from the same treatment group were pooled together and 1 volume 70% ethanol was added to the pooled samples. The samples were transferred to the pre conditioned purification column. The RNA was then bind to the column by centrifugation for 2 min at 1057 rpm, immediately followed by a centrifugation at 13500 rpm for 30 s. After washing with 100

µl wash buffer 1 at 9475 for 1 min, on column DNase treatment was performed for 15 min using RNase-fee DNase (Qiagen, CA, USA). Following this, the samples were washed for 15 s with 40 µl wash buffer followed 100 µl wash buffer 2 for one min at 9457 rpm. After the second wash with 100 µl wash buffer 2 at 13500 rpm for 2 min, the purification column was transferred to a new 0.5 ml microcentrifuge tube. The RNA was then eluted using 12 µl elution buffers. One micro litter of the RNA was used to determine the concentration and quality using Nanodrop 8000 Spectrophotometer.

3.2.4.2 RNA amplification from embryo biopsies

3.2.4.2.1 First and second stranded cDNA synthesis

First strand cDNA was synthesized from total RNA samples of embryo resulted in calf delivery and no pregnancy group using T7 promoter attached oligo $(dT)_{21}$, superscript reverse transcriptase II (Invitrogen, CA, USA) and random hexamers as described previously (El-Sayed et al. 2006, Ghanem et al. 2007, Salilew-Wondim et al. 2007). Second strand cDNA was then synthesized using degenerated oligonucleotide primer (DOP)-PCR master kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendation. Briefly, 1 µl DOP primer, 1 µl T7 primer, 40 µl 2xDOP master mix and 16 µl RNasin were added to 20 µl first cDNA and incubated at 95 ^{o}C for 5 min, 94 ^{o}C for 1min and 30 ^{o}C for 2 min. Subsequently the temperature was increased at the rate of 0.2 ˚C /s until it reaches 72 ˚C and incubated for 3 min at this temperature. Afterwards, the global PCR amplification was continued for the rest of 10 cycles at 94 ˚C for 30 s, 60 ˚C for 30 sec, 72 ˚C for 3 min. The reaction was terminated after final extension at 72 ^{o}C for 7 min. Following this, the double stranded DNA (dscDNA) was purified using phenol-chloroform extraction method. For this, 80 µl phenol-chloroform was added to 80 µl dscDNA and centrifuged at 14000 rpm for 15 min. The aqua-phase was transferred to 2 ml tube to which 1 µl glycogen, 40 µl 5 M ammonium acetate, 200 µl 100% ethanol were added. The samples were then kept at -20 ^{o}C over nigh. In the next day, the samples were centrifuged using refrigerated universal centrifuge Z233MK (Hermle Labortechnik, Wehingen, Germany) at 14000 rpm for 30 min. After removing the supernatant, the dsCDNA pellets were washed in 500 µl 70% ethanol. After removing all traces of ethanol, the pellets were dissolved in 10 µl of RNase free water.

3.2.4.2.2 In vitro transcription

The double strand cDNA was in *vitro* transcribed and amplified using AmpliScribe T7 transcription kit (Epicentre technologies, WI, USA). For this, a 10 µl in *vitro* transcription mix (2 µl 10x reaction buffer, 4µl dNTP mix [1 µl from each 0.1M ATP, 0.1M CTP, 0.1M GTP and 0.1M UTP), 2 µl DTT and 2 µl T7 RNA polymerase] was added to the 10 µl dscDNA. In vitro transcription and amplification was carried out at 42 ^{o}C for 3.5 h. Following DNase treatment at 37 ^{o}C for 30 min, amplified RNA was purified using RNeasy mini kit (Qiagen, Hilden, Germany). After the final wash, the aRNA was eluted in 30 µl RNase free water. The concentration and purity of the aRNA was evaluated using Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific Inc, DE, USA).

3.2.4.3 Aminoallyl labeling and dye coupling

Minimum information about microarray experiments (MIAME) guidelines (Brazma et al. 2001) was adhered to the experimental design. Three micrograms of amplified RNA from embryo resulted in calf delivery or no pregnancy was used as template in reverse transcription reaction. Aminoallyl-modified dUTPs were incorporated into the cDNA using the cyscribe post-labeling kit (Amersham Biosciences, Freiburg, Germany). The aRNA, anchored oligo (dT) and random nanomer primers were co incubated at 70 ˚C for 5 min followed by 10 min incubation at room temperature. Following this, 10 µl reaction mix (containing 4 µl of 5x first strand buffer, 2 µl of 0.1 M DTT, 1.5 µl of dNTP mix, 1.5 µl aminoallyl dUTP and 1µl CyScript reverse transcriptase) was added and incubated at 42 ˚C for 90 min. At the end of this reaction, 2 µl of 2.5 M NaOH was added to hydrolyse any rest of mRNA and incubated at 37 ˚C for 15 min. After adding 10 µl of 2 M HEPES, aminoallyl labelled cDNA samples were purified using CyScribe^{TM} GFX^{TM} Purification kit (Amersham Biosciences, Freiburg, Germany). The purified aminoallyl labelled cDNA samples were then eluted in 60 µl 0.1 M sodium bicarbonate and immediately labelled by Cy^{TM} fluorescent dyes using Amersham post-labeling kit (Amersham Bioscience, Freiburg, Germany). For this, purified aminoallyl cDNA in (0.1M sodium carbonate) was directly added into aliquot of Cy^{TM} fluorescent dyes using the cyscribe post-labeling kit (Amersham Biosciences, Freiburg, Germany), dissolved in DMSO. Those aminoallyl cDNA derived from those embryos resulted in calf delivery were labelled with N-hydroxysuccinate-derived Cy5 dye and those samples of embryo resulted in no pregnancy

were labelled with Cy3 dye. To avoid variation due to dye coupling, aminoallyl labelled cDNA samples of the calf delivery group were labelled with Cy3 and no pregnancy groups were labeled with Cy5 dyes. The dye labelled samples was then incubated for 15 min at room temperature in dark. At the end of incubation, non reacting dyes were quenched by adding 15 µl of 4 M hydroxylamine solution (Sigma-Aldrich Inc, MO, USA) and incubated for 15 min at room temperature in dark. The reaction was then purified with CyScribe™ GFX™ Purification kit (Amersham Biosciences, Freiburg, Germany) and finally eluted in 60 µl elution buffer.

3.2.4.4 Hybridization to the target probes

3.2.4.4.1 Target clones

Ready made bovine cDNA array (BlueChip version 3, kindly provided by Prof. Marc-André Sirard, (Laval University, Quebec, Canada) was used for hybridization. The BlueChip array consisting of more than 3000 probesets (6766 spots) and about 2300 targets genes derived from subtraction suppressive hybridization of bovine embryo and tissues. In addition to the target probesets, the BlueChip array consists of control clones. The name of control clones include, alien1 (530 spots), alien2 (540 spots), GFP (4 spots), Doublets (96), GFP (348 spots), GFP½ (30 spots), GFP1 (30 spots), GFP¼ (6 spots), GFP1/8 (6 spots), GFP 1/16 (6 spots) and H_2O/DMSO (380), Negative (6 spots), plant (540 spots), vide (96 spots), tubulin (8 spots), ubiquitin (8 spots) and actin (12 spots).

3.2.4.4.2 Probe preparation and hybridization

Before hybridizing, the arrayed slides were placed in a corning GAPS II slide container containing 50 ml pre-hybridization buffer [0.5 g bovine serum albumin (Roche Diagnostics, Basel, Switzerland) + 0.5 ml 10% SDS + 7.5 ml 20% SSC + 42 ml sterile water] and incubated for 20 min at 55 ^{o}C. After the end of incubation, the slides were sequentially washed with boiled and cold water and isopropanol. The slides were then centrifuged at 2000 rpm for 2 min. Immediately before hybridization, dye labelled probes were dissolved in 55 µl formamid based buffer (15 µl hybridization buffer + 30 µl 100% formamid + 10 µl distilled water) to which 2.5 µl yeast tRNA and 2.5 µl human cot-DNA (Invitrogen, Karlsruhe, Germany) were added to avoid non specific hybridization. After denaturing at 95 ^{o}C for 5 min, the mix was then hybridized to the array and covered with

cover slips (ROTH, Karlsruhe, Germany). A total of six hybridizations (three biological and three technical replicates as dyeswap) were carried out between the two sample groups. The hybridized array slide was then placed in the hybridization chamber (GFL, Dülmen, Germany) and incubated at 42 ^{o}C for 20 h in dark. At the end of the incubation, the slides were sequentially washed for 10 min with 2x SSC plus 0.1% SDS, 5 min each with 0.2x SSC and 0.1% SSC buffers, 1 min each with water and isopropanol and centrifuged at 2000 rpm for 2 min.

3.2.4.5 Image capture and quantification

Microarray slides were scanned using Axon GenePix 4000B scanner (Axon Instruments, Foster City, CA). The images of the spots were quantified to create reports of spot intensity data using GenePix Pro analysis software (version 4.0) (Axon Instruments, Foster City, CA). The images were saved as a multiple channel image and as a single image for both channels (Cy3 and Cy5) in 16-bit tagged image file format (TIFF).

3.2.4.6 Microarray data analysis from embryo biopsies

The normal expression and offset method was used to correct the background (Ritchie et al. 2007). LOESS normalization was applied to normalize within array variations (Smyth and Speed 2003, Yang et al. 2002) and scale-normalization was used to normalize differences between arrays due to changes in the photomultiplier tube setting of the scanner. A mean $\log_2$ transformed value of (Cy5/Cy3) was calculated from three replicates to obtain one value per clone. The raw and normalized data are available at Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/geo/ with accession number GSE21047 or GSE21049. Differentially expressed genes were obtained using linear models for microarray data (LIMMA) (Smyth 2005). Those genes whose average expression value $\geq$ 1.5 fold change and $p < 0.05$ were considered as differentially expressed genes. The heatmap of differentially expressed genes was drawn using the heatmap function of bioconductor.

3.3 Validation of differentially expressed genes using quantitative real time polymerase chain reaction (qPCR)

3.3.1 Primer design and gene specific PCR amplification

In the current study, qPCR was used to validate differentially expressed genes and to quantify some candidate genes across the estrous cycle. For this, sequence specific primers were designed using primer express v 2.0 (Applied Biosystems, Foster city, CA) or primer 3.0 online primer design tool (http://frodo.wi.mit.edu/primer3/) for candidate genes that were differentially expressed in endometrial biopsies (Table1) and embryo biopsies (Table 2). All primers were purchased from Eurofins MWG synthesis GmbH (MWG Biotech, Eberberg, Germany) and diluted at 100 pm stock solution. The PCR reaction was carried out for each primer in 20µl reaction volume using 2 µl cDNA template, 4 µl of 10x PCR buffer (Sigma-Aldrich), 0.5 µl of dNTP, 0.5 µl of each specific primer (10 pmole forward and reverse), 0.5 µl of Taq polymerase (Sigma-Aldrich) and millipore water was added to complete the rest of the volume. After the end of the reaction, 5 µl of the PCR product was loaded onto ethidium bromide stained 2 % agarose gel run in 1xTAE buffer. The presence and specificity of DNA bands were observed under BioRad Chemidoc XRS Gel Documentation System (Biorad, Munchen, Germany). After confirmation of specific DNA bands, the amplified PCR product was sequenced to verify the identity of the gene. For this, the sequence reaction was carried out by using 2µl 10 pm reverse primer or forward primer to 5 µl PCR product. Subsequently, 4 µl master mix (DTCS) and 9 µl Millipore water was added to final volume of 20 µl. Afterwards, the sequence reaction was carried out for 30 cycles at 96 ^{o}C for 20 s, 50 ^{o}C for 20 s and 60 ^{o}C for 4 min. Afterwards, the sequencing reaction was transferred to a 5µl stop solution [1µl glycogen, 2 µl 3M NAOAc (PH 5.2), 2 µl 0.1M EDTA (PH 8)]. The mixed was homogenized by vortexing. After adding 60 µl 100% ethanol, the samples were centrifuged at 14000 rpm for 15 min at 4 ^{o}C in refrigerated universal centrifuge Z233MK (Hermle Labortechnik, Wehingen, Germany). After this, the supernatant was removed and again the samples were washed two times in 200 µl 70 % ethanol and dried in room temperature. Finally, the pellets were dissolved in 40 µl SLS and transferred to the sequencing plate, covered with mineral oil (Beckman Coulter, Krefeld, Germany) and immediately loaded to CEQ^{TM} 8000 Genetic Analysis (Beckman Coulter, Krefeld, Germany) sequencing machine. The similarity of the sequence result to the original sequence was verified using the NCBI/BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 4: Primers used for validation of differentially expressed genes between endometrial biopsies

Gene name	ACC. No	5-3 sequence	BP
S100B	NM_001034555	F: GTGAGCTGTGATGTTTGCCTA R: ATAAAATTCCCTGGCTTCACC	366
S100A13	NM_205800	F: CTGAGACACGGATACAGCAAA R: ACTGCTGAGTGACCAGTTCCT	325
TLR6	NM_001001159	F: GTTTGGGAAAATGTTGCTGAT R: GCTCTAGAGTGCAGGCTCAGT	244
PTGER4	NM_174589	F: AGGACAACCTAACGTGCTGAT R: AAGGCTAATCCTTCCACACCT	261
PLK4	AC146966	F: GGAGAGACCCCATTTTTGAAT R: GTTGTTTGACCCTCTGGTGAT	230
MYC	NM_001046074	F: AGAGCAGCAAAAGCTCAAGTC R: TAAAGATCCAGCCAAGGTTGT	227
IRF6	NM_001076934	F: CAAGGATAACATCGTTGCTCA R: GAAAGGCTTGAAAGCTGGTTA	200
IRF5	NM_001035465	F: ATTGTGGAGGGAAGGAACTCT R: GACTGTGAACCCAAGGATTGT	248
IL18	NM_174091	F: CATCAGCTTTGTGGAAATGAA R: GGGGTGCATTATCTGAACAGT	208
MMP2	BC149404	F: TTGGGAGCCAACAGAGACTAC R: GGAAATGCAATGTCATAGTG	209
AGTR1	NM_174233	F: AAGAGCTTAAGACTGCCATGC R: AACATGACTACCTGGGACTGG	293
PLCB1	NM_174817	F: GCCCCTCAGCTACCATATCTT R: GTCAAGTCTGGAGATGGCTTC	208
SLC25A5	NM_174659	F: CCAGATCCCAAGAACACTCAT R: CATACAGGACAAGCACAAACG	271
ADORA2B	NM_001046333	F: ATAAACACAGCCATCCTCCTG R: TTACTGAGCCTTCTCCTGGAA	205

ACC. No = Gene bank accession number, BP= number of base pairs amplified

Table 4: Cont.

Gene name	ACC. No	5-3 sequence	BP
CAMK2D	NM_001046333	F: TGCCTGCATAGCGTACATTAG R: AGGACCTTCACAACTTCATGC	229
FUT8	NM_177501	F: CTTGGTCAGCTGGACTACACA R: GTTCGAGGTTCGTGAGGATAA	252
B3GNT2	NM_001102497	F: AGTGACTGCTTTCCTGTTTCAG R: TCCCCCAAAAGAGAACAGAT	216
ALG8	NM_001076125	F: CGTACTGATTGACCCTCCTGT R: TATGGAGGACAAGTGGCTTCT	231
CDC2	NM_174016	F: TTTGGGCACTCCCAATAAT R: AACACAGGGAAACTGTCAGA	242
CCNB2	NM_174264	F: TGGAGCTGACTCTCGTTGAC R: CTGAGGAATCGTGCTGATCT	253
NGF	NM_001099362	F: GGGAGAGGTGAACATCAAC R: TCTTCCTGCTGAGCACAC	233
RPS6KA5	XM_580522	F: GCTTTTCCTCCTGTAGTCA R: ACTTCCTCTCTTGCATAGG	251
MAPK12	NM_001098953	F: ATGGAATCTCTCTTGGAAGC R: TCCTCAGCACAGTTCAG	241
MAP4K2	XM_869702	F: GCTCACAGCAACCTCTACAT R: TGACAGCAGCTTCTTCTGGT	264
SFRP1	NM_174460	F: CTCCCTAAGGCATCTTGTCTG R: CAGGAATCTCGTCTGTGTCC	213
PRKACB	BC149047	F: GTACGTCTTTGGCTGTCGAGT R: ATCAGCATGTTCTTGGGTCT	218
WNT11	NM_001082456	F: TTGTGCTTTGCCTCCACT R: ACAGAACATTCCGTTGAGGAC	266
NGF	NM_001099362	F: GGGAGAGGTGAACATCAAC R: TCTTCCTGCTGAGCACAC	233
WNT11	NM_001082456	F: TTGTGCTTTGCCTCCACT R: ACAGAACATTCCGTTGAGGAC	266
AURKB	NM_183084	F: TTTCTGCTCTGGTGAGGATTT R: TCCAGTGGTTAGGACTCCAAG	181

ACC. No = Gene bank accession number, BP= number of base pairs amplified

Table 4: Cont.

Gene name	ACC. No	5-3 sequence	BP
TIMP3	NM_174473	F: AGCCAGCAGATAGACTCAAGG R: GCTTGCTCCAGACTCAGAAAC	193
CLD23	XM_592516	F: GGAGAATGAAGGGACTAATGCT R: AACAGGTTCCATTTTGTTCCA	102
STAT5A	AJ237937	F: TGTCTCCAACCTTGCTCACTA R: AAGAGGCCCTATTCCAAGTTC	230
COLA2	NM_174520	F: TTGAAGGAGTAACCACCAAGG R: TGTCCAAAGGTGCAATATCAA	320
MAPK12	NM_001098953	F: ATGGAATCTCTCTTGGAAGC R: TCCTCAGCACAGTTCAG	241
MAP4K2	XM_869702	F: GCTCACAGCAACCTCTACAT R: TGACAGCAGCTTCTTCTGGT	264
SFRP1	NM_174460	F: CTCCCTAAGGCATCTTGTCTG R: CAGGAATCTCGTCTGTGTCC	213
ITPR1	NM_174841	F: GCGCTAAATTAGAGGCTCAGA R: GTAGCATGTTGGTTCCGATG	218
MYLK	NM_176636	F: AGCCCCATGTAAAACCCTACT R: GGCTTCTCCAAGACTGTTGAC	262
F2R	NM_001103097	F: GACCAACATATGGTGTGGACAA R: CACAGGAAGCAGTAACCCAAA	135
PRKACB	BC149047	F: GTACGTCTTTGGCTGTCGAGT R: ATCAGCATGTTCTTGGGTCT	218
RPS6KA5	XM_580522	F: GCTTTTCCTCCTGTAGTCA R: ACTTCCTCTCTTGCATAGG	251

ACC. No = Gene bank accession number, BP= number of base pairs amplified

Table 5: Primers used for validation of DE genes between embryo biopsies resulted in calf delivery and embryo biopsies resulted in no pregnancy.

Gene name	ACC. No	5'-3' sequence	BP
SGK1	NM_001102033	F: CCCTTTTATAGCCGAAACACA R: TGGGTTAAAAGGGGGAGTAAT	236
ARL8B	NM_001046071	F: TCCTAATGCCTTGGATGAGA R: GGGATTAGGAAGGACCGTATT	257
RNF34	NM_001014858	F: GATTGATACCTGTCGGGAGAA R: CGTCTTCTGTGTTTGCTGAAG	271
ARL6IP1	NM_001078157	F: AGACTGCAAGTCTGGAAGAGC R: GAACAAGGTAGTCAGCCAAGC	209
UBE2D3P	NM_001075135	F: CCAGAGATTGCACGGATCTAT R: GTGAATGAATGGAGGGAGGTA	140
AMD1	NM_173990	F: GAGTGGAATTCGTGACCTGAT R: GGCTTGAAGACTTCCACAACT	212
HSPA8	NM_174345	F: GCTGGAACTATTGCTGGTCTC R: TCCACCCAAGTGAGTATCTCC	209
STX8	NM_001078037	F: GGTGGAGAATACCGATGAGAA R: CTCGGCTTTACTCCATCTCTG	288
GAPDH	NM_001034034	F: AATGGAGCCATCACCATC R: GTGGTTCACGCCCATCACA	240

ACC. No = Gene bank accession number, BP= number of base pairs amplified

3.3.2 Gene cloning and Transformation

Once the specificity of each of the primers were confirmed by sequencing, the plasmid DNA containing the specific PCR product was prepared to be used as a serial dilution for a standard curve. For this, the PCR product was purified using QIAquick PCR Purification (Qiagen, Hilden, Germany) and ligated to pGEM®-T easy vectors (Promega, WI, USA) that contains 3'-T overhangs. The ligation reaction was performed in 5 µl reaction mix containing of 3 µl of ligation buffer, 0.5 µl pGEM®-T vectors, 0.5 DNA ligase enzyme and 2 µl PCR product. The ligation reaction was incubated at 20 ^{o}C for 2 h. At the end of the ligation reaction, 3µl of the ligation product was transferred to 15 ml tube to which 100

µl competent (E.coli, JM109 strain) was added. The competent cells and the ligation product co incubated for 20 min in ice followed by 90 s heat shock at 42 ^{o}C and then 2 min cold shock in ice. Afterwards, 700 µl of Luria-Bertani (LB) broth was added to the cells and cultured at 37 ^{o}C in SHKE6000-8CE refrigerated Stackable Shaker (Thermoscinentific, IWA, USA) for 90 min at 110 rpm speed. After 70 min, 20 µl of IPTG and 20 µl of X-gal were uniformly distributed on the LB agar/ampicillin plate. After the incubation period, the bacterial culture was plated onto pGEM®-T easy vectors LB agar/ampicillin/IPTG/X-gal plate and incubated overnight at 37 ^{o}C.

3.3.2.1 Blue/white screening of recombinants and colony picking

Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on LB agar/ampicillin/IPTG/X-gal plate. Therefore, successful cloning of an insert into pGEM®-T Easy vector interrupts the coding sequence of β-galactosidase resulting recombinants in white colony formation. Following this, four independent white colonies assumed to contain inserts were picked and inoculated to the 30 µl 1xPCR buffer and the same colony was inoculated to the 600 µl LB broth continuing ampicillin. In addition, two independent blue colonies were picked and inoculated into the PCR strip containing 30 µl 1xPCR to be used as a control. The inoculums in 600 µl LB broth were incubated at 37 ^{o}C in SHKE6000-8CE refrigerated Stackable Shaker (Thermoscinentific, IWA, USA) until the result of M13 product was known. The bacterial suspension in the 30 µl 1xPCR was heated for 15 min at 95 ^{o}C. From this, 10 µl this lysate was used as a template for M13 PCR reaction to which a mix consisting of 0.5 µl dNTP, 0.5 µl M13 forward primer (TTGTAAACGCGGCCAGT), 0.5 µl M13 reverse primer (CAGGAAACAGCTATGACC), 2 µl 10x PCR reaction buffer was added. M13 PCR reaction was carried out first by denaturing the at 95 ^{o}C for 3 min followed by 35 cycles that repeated at 95 ^{o}C for 30 s, 59 ^{o}C for 30 s, 72 ^{o}C for 1 min. M13 PCR reaction was termed after extension at 72 ^{o}C for 10 min. At the end of the reaction, 5µl of the M13 product mixed with 2 µl loading buffer was loaded to 2% agarose gel stained with ethidium bromide. The colonies that contain PCR fragments (white colonies) were identified depending the distance travelled by DNA fragment in 2% agarose gel electrophoresis. The blue colonies were at lower position compared to the white colonies. Following this, the colonies confirmed for the presence of PCR fragment were transferred to 15 ml sterile tube and additional 5 LB broth/ampicillin was added. The bacterial

suspension was further cultured over night at 37 ^{o}C to increase numbers and therefore the amount of DNA.

3.3.2.2 Plasmid isolation

The plasmid was isolated using GenElute plasmid mini prep kit (Sigma-Aldreich, St.Lous, USA) according to the manufacture's recommendation. Briefly, overnight cultured competent cells were pelleted by centrifuging at 13000 rpm for 1 min. The medium was removed and the pellets were lysed with 200 µl lysis solution. Afterwards, the lysate was incubated for 5 min at room temperature. Afterwards the cell debris were Precipitated by adding 350 µl neutralization/binding solution and centrifuging at maximum speed for 10 minutes. Following this, the GenElute Miniprep binding column was inserted into a provided microcentrifuge tube and 500 µl of the column preparation solution was added followed by centrifugation at 13000 rpm for 30 s. The cleared lysate was then transferred to the column and centrifuge at 13000 rpm for 30 s. The filtrate was decanted and 750 µl of the diluted wash solution to was added to the column followed by centrifugation at 13000 for 30 s. The flow-through liquid was decanted and the column was centrifuged again at maximum speed for 2 minutes without any additional wash Solution. Finally, the spin column was transferred into a collection tube to which 50 µl of Millipore water was added to the centre of the spin column membrane and centrifuged at speed of 13000 rpm for 1 min. To confirm the presence of the plasmid DNA, 5 µl of the plasmid DNA was analyzed in ethidium bromide stained 2% agarose gel electrophoresis running in 1x TAE buffer. The concentration of the plasmid was determined using Nanodrop 8000 Spectrophotometer.

3.3.2.3 Plasmid or M13 sequencing

Although insertional inactivation of the α-peptide allows identification of recombinants with on LB agar/ampicillin/IPTG/X-gal plate, the specificity of gene cloning was further validated by sequencing of M13 PCR product or plasmids DNA isolated from overnight cultured recombinant bacteria. A 15 µl mix consisting 2 µl M13 (1.6 pmol) reverse primer or forward primer, 4 µl master mix (DTCS) and Millipore water was added to final volume 15 µl. The thermocyler program, post reaction clean and loading of the samples to CEQ^{TM}

8000 Genetic Analysis sequencing machine was performed as indicated above for sequencing PCR products.

3.3.2.4 Preparing serial dilution from plasmids

The serial dilution consisting of 10^1 to 10^9 copy number of molecules was prepared from plasmid DNA using the online software, (http://molbiol.edu.ru/eng/scripts/h01_07.html), which converts known DNA concentration to copy number of molecules. Briefly, to determine the number of molecules, the plasmid concentration (ng/µl) and the nucleic acid size (the size of the pGEM®-T easy vectors + PCR fragment of each gene) were submitted to the software. The dilution that contains 10^9 molecules was then determined in 50 µl volume based on the number of molecules obtained in 1 µl plasmid DNA. Following this 10^8 dilution was determined using 5 µl of 10^9 dilution and 45 µl Milipore water. The remaining 10^7-10^1 dilutions were determined in the similar way using 5 µl of the higher dilution and 45 µl of Millipore water.

3.3.3 Transcript level quantification

The minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines were employed during measuring gene expression level using quantitative real time PCR (Bustin et al. 2009). According to the guideline, qPCR was used as abbreviation of quantitative real time PCR through the text. Following this, the cDNA samples used for qPCR were synthesized by reverse transcription of total RNA endometrial and embryo biopsy samples as described previously in our lab (El-Sayed et al. 2006, Ghanem et al. 2007, Salilew-Wondim et al. 2007). Prior to qPCR, the concentration of the cDNA samples from each treatment group was determined using the Nanodrop 8000 Spectrophotometer (Biotechnology GMBH, Erlangen, Germany). Similar amount of cDNA samples were used to compare samples from different treatment groups. The qPCR was performed in 20 µl reaction volume containing iTaq SYBR Green Supermix with ROX (Bio-Rad laboratories, Munich, Germany), the cDNA samples, the specific forward and reverse primer in ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster city, CA). The qPCR thermal cycling parameter was set as 95 ^oC for 3 min, 40 cycles of 15 sec at 95 ^oC and 45 sec at 60 ^oC. At the end of the qPCR reaction, the specificity of amplification for each gene was evaluated by monitoring the dissociation

(melting) curve. Transcript abundance of the samples was determined using the relative standard curve method using a serial dilution of 10^1-10^9 copy numbers prepared from plasmid DNA. The data generated was considered for further analysis provided that the slope and the regression line (R^2) of the standard curve were between (-3.2 to – 3.6) and $\geq$ 0.99, respectively. Following this, the copy numbers of the target genes were normalized against the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The expression of GAPDH was not significantly different between the samples to be compared. The normal distribution was tested by the Kolmogorow–Smirnov method and those skewed expression values were $\log_2$ transformed prior to statistical analysis. The Student's t-test or least significant difference test procedures of SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) was used to determine mRNA differences between the samples. Differences with $p < 0.05$ were considered as significant.

4 Results

4.1 Embryo biopsy transfer and success rate

From 54 biopsied embryos transfers, 59% heifers returned to heat at day 21 and those heifers were designated as non pregnant or non-receptive endometrium. The remaining 41% heifers were pregnant until day 56 and 27 % ended up with successful calf delivery. Those heifers ended up with successful pregnancy and calf delivery were designated as calf delivery group or receptive endometrium. Here the underline question was why some transfers resulted in calf delivery and others not despite similarities in breed, age and synchronization protocol. Thus, it was hypothesized that those two groups of heifers and the corresponding transferred embryos may have different molecular enrichments that can affect the embryo maternal communications for establishment pregnancy success. Therefore, to investigate the molecular mechanism associated with the endometrial receptivity and embryos implantation potential, the pre-transfer endometrial and embryonic gene expression was investigated in relation to the pregnancy outcome using endometrial and embryo biopsies techniques. The results of these comparisons are presented as follows.

4.2 Pre-transfer gene expression in receptive and non-receptive endometrium

4.2.1 Pre–transfer gene expression in receptive and non-receptive endometrium at day 7 of the estrous cycle

Using the GeneChip® Bovine Genome Array, consisting of over 23,000 bovine transcripts, the gene expression differences were uncovered between the endometrium of heifers that resulted in calf delivery and heifers resulted in no pregnancy at day 7 of the estrous cycle during the pre-transfer period. For this, the gene expression profile of biopsies taken at day 7 the estrus cycle from those subsequently resulted in calf delivery [receptive endometrium (CDd7)] were compared to those endometrial samples taken at day 7 of the estrous cycle from those subsequently resulted in no pregnancy [non-receptive endometrium (NPd7)] following embryo transfer. The result revealed that from 8605 detected genes in both receptive (CDd7) and non-receptive endometrium (NPd7), a total of 1126 differentially expressed (DE) genes were identified between the two groups.

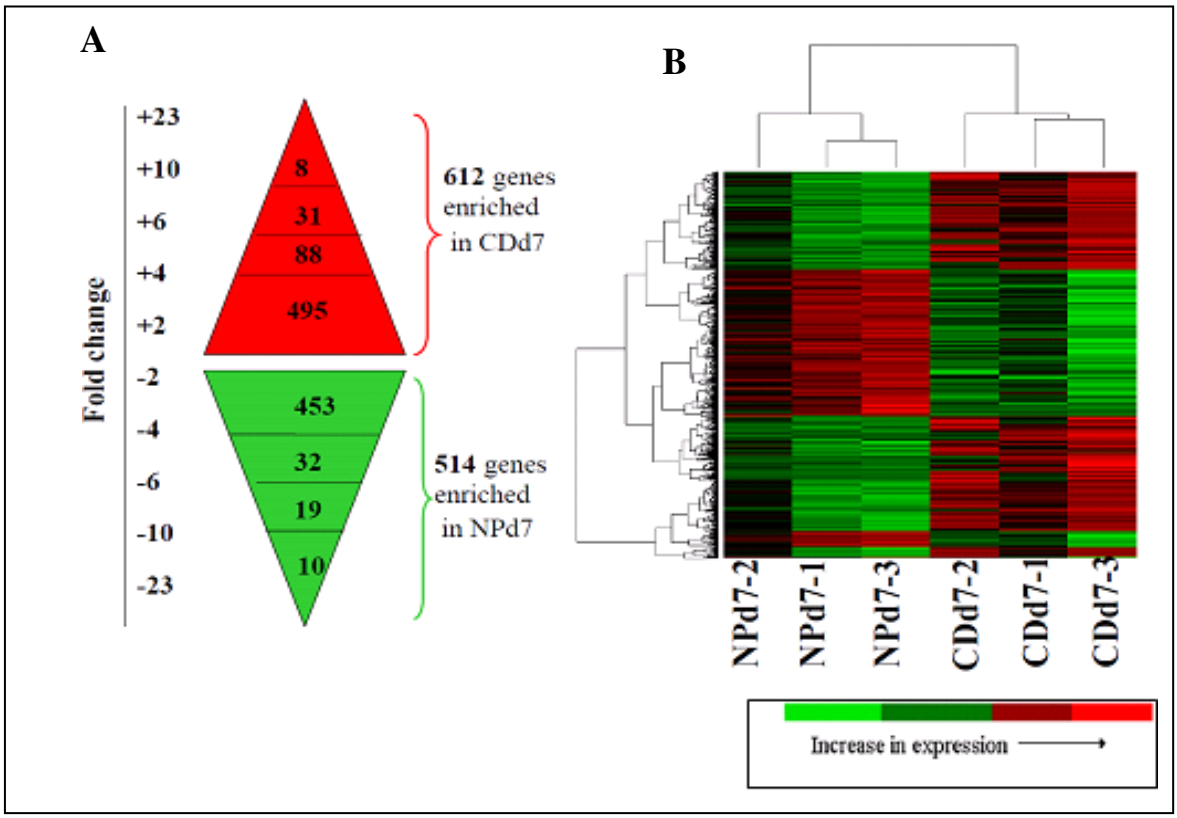

Figure 5: Pre-transfer endometrial transcriptome alteration between receptive (CDd7) and non-receptive endometrium (NPd7) at day 7 of the estrous cycle. (A): The number and fold change distribution of genes elevated in CDd7 or NPd7. (B): The heatmap and hierarchical clustering showing the expression pattern of DE genes in CDd7 and NPd7 endometrial samples. Labels in the horizontal axis of the heatmap, CDd7-1, CDd7-2 and CDd7-3 represent independent biological replicates in receptive endometrium and NPd7-1, NPd7-2 and NPd7-3 represent independent biological replicates from non-receptive endometrium groups. The horizontal dendrogram indicate the expression pattern of transcripts in CDd7 and NPd7. The vertical dendrogram elucidates the similarities or differences of transcript expression within the biological replicates and between CDd7 and NPd7 endometrial samples. Red and green colors in (B) indicate the high and low expression level, respectively.

From the total 1126 DE genes, the expression level of 612 genes was abundantly expressed in receptive endometrium (CDd7) in which 81% exhibited 2 to 4 fold changes increase and 29% of them exhibited 4.1 to 23 fold change increased compared to NPd7 (Figure 5A). The top abundantly expressed genes in CDd7 compared to NPd7 include CDC20, GCLC,

PLLP, COL4A4, TMEM163, LOC510320, MTTP, NETO2, CDC2, COX6B2 and PPP2R5A (Figure 6). On the other hand, the expression level of 514 DE genes was reduced in CDd7, but abundantly expressed in NPd7. From those 88.3 % exhibited 2 to 4 and 10.7% showed 4.1 to 23 fold changes increases in NPd7 compared to CDd7 (Figure 5A).The top abundantly expressed genes in NPd7 compared to CDd7 include HOXA9, COL1A2, NTRK2, COL3A1, GJA1, COL21A1, LHFP, IGJ, SPOP, OGN, COL6A1 and PARC (Figure 6)

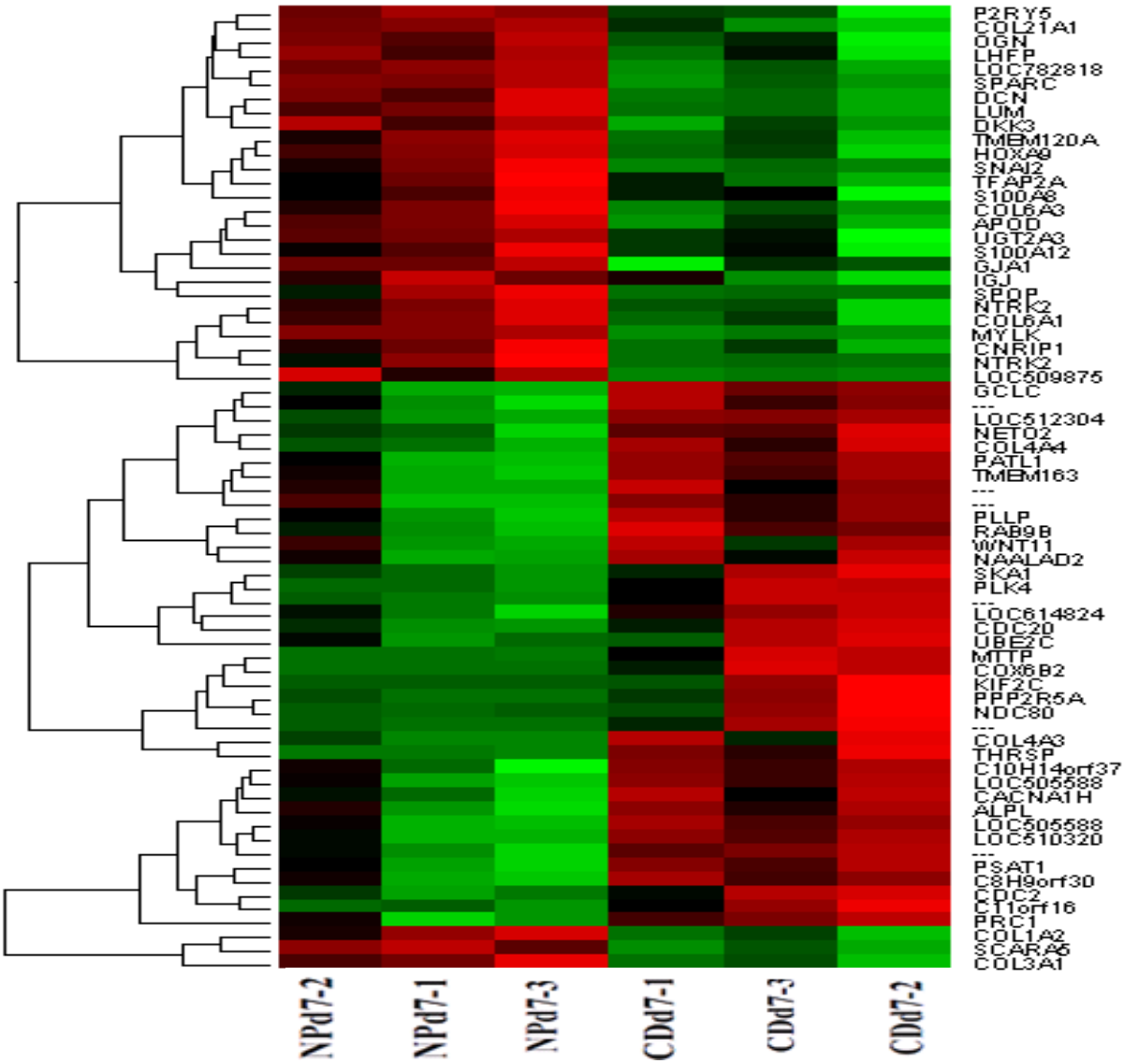

Figure 6: The expression pattern of DE genes exhibited ≥ 5 fold difference between CDd7 and NPd7. Labels in the horizontal axis of the heatmap, CDd7-1, CDd7-2 and CDd7-3 represent independent biological replicates from receptive endometrium and NPd7-1, NPd7-2 and NPd7-3 represent independent biological replicates from non-receptive endometrium groups. The red and green colors indicate high and low expression pattern, respectively.

4.2.1.1 Differentially expressed gene clusters between receptive and non receptive endometrium at day 7 of the estrous cycle

From the 1126, DE genes identified between receptive (CDd7) and non-receptive (NPd7) endometrium, there were several gene clusters whose expression was elevated either in CDd7 or in NPd7. Among those, the expression levels of solute carrier genes, cell division or cell cycle related genes, adaptor related genes, kinesin genes, leucine rich repeats, coiled domains, ATPases, protein kinases and phosphatase gene clusters, transmembrane proteins, junctional adhesion molecules, integrins and WD repeats were higher in CDd7 (Figure 7). On the other hand, the expression levels of cluster of genes including cluster of differentiation molecules (CD), chemokine molecules, homeobox genes, collagen families, S100 calcium binding protein and zinc finger proteins were increased in (NPd7) (Figure 8). Apart from these, the fold changes, the UniGene ID, the gene symbol and full name of the first 300 DE genes are described in appendix 2.

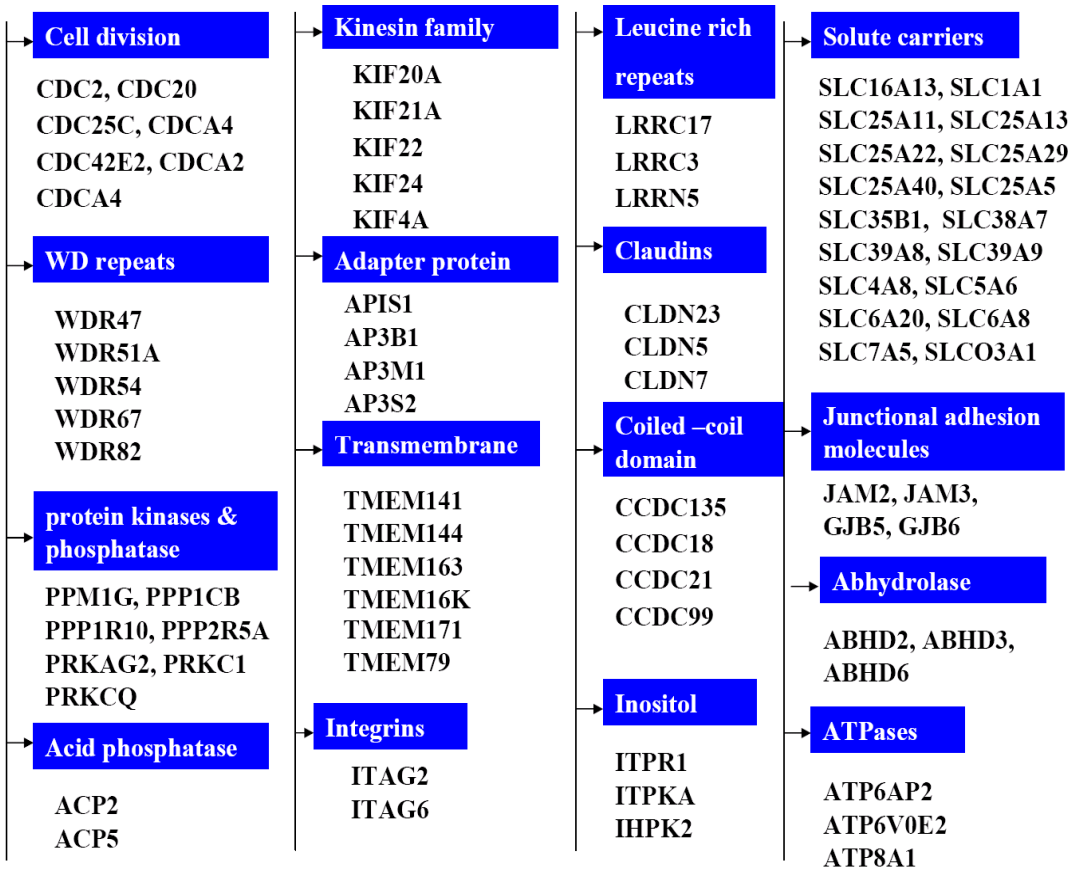

Figure 7: Genes clusters exhibited increased expression level in receptive (CDd7) compared to non-receptive endometrium (NPd7) at day 7 of the estrous cycle.

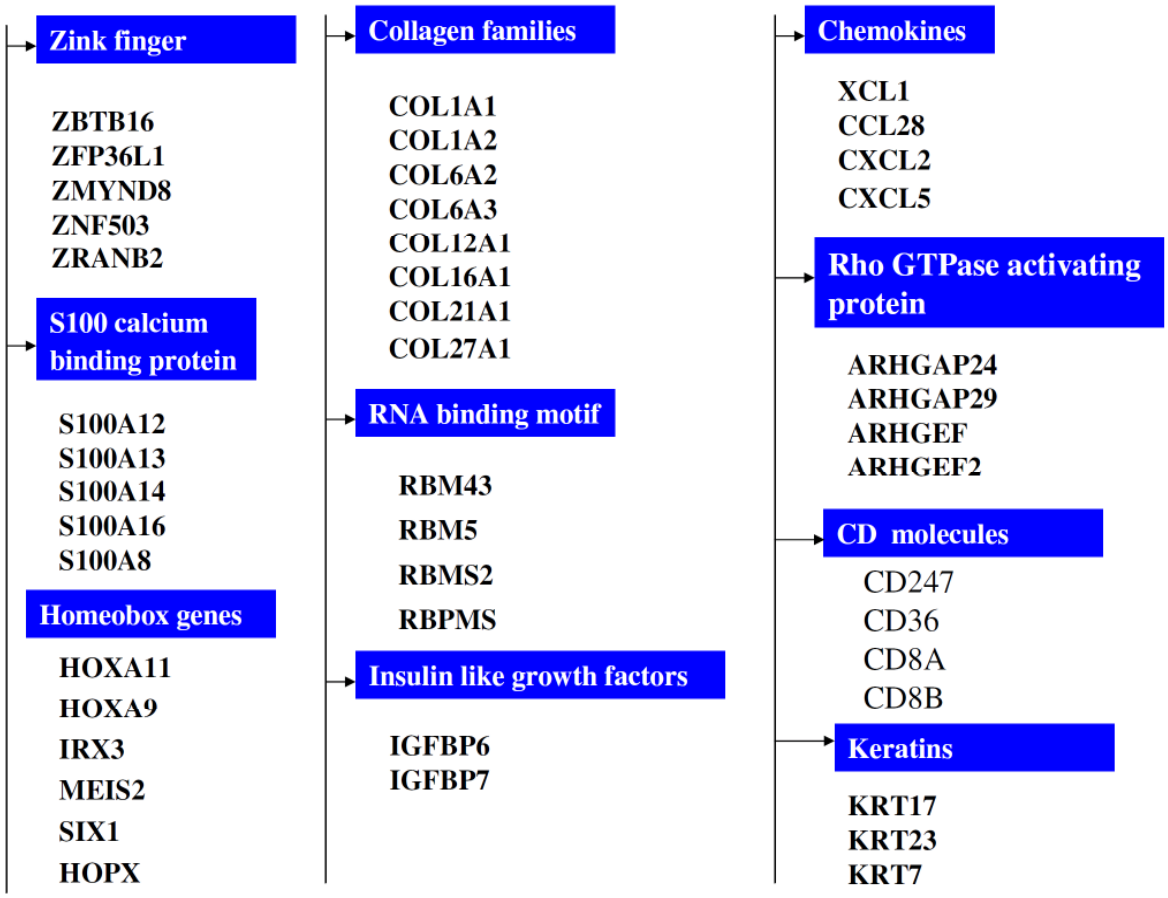

Figure 8: Gene clusters exhibited reduced expression level in receptive endometrium (CDd7) but increased in non-receptive endometrium (NPd7) at day 7 of the estrous cycle.

4.2.1.2 Transcriptomic functional alteration in receptive and non-receptive endometrium at day 7 of the estrous cycle

To investigate the transcriptomic functional changes that have been occurred in receptive and non-receptive endometrium at day 7 of the estrous cycle, the gene ontology (GO) enrichment of biological process and molecular function was analyzed for those genes differentially expressed between CDd7 and NPd7. Thus, 15 and 13 biological processes were found to be overrepresented in those DE genes that were elevated in CDd7 and NPd7, respectively (Figure 9). The biological process overrepresented in CDd7 include, macro molecule localization or cellular material transport (FABP5, solute carrier genes, ITPR1, VDAC1, adapter related proteins, KIF20A, KIF4A), regulation of transcription

(PPARA, MYB, NR2F1, PPARGC1A, HEY1, TFDP2, ASF1B, MED27, IRF6), signal transduction (PTGER4, F2RL1, ADORA2B, SPHK1, ADAM10, ITGA2, PLCB1, RPS6KA5, RAB9B, WNT11), post translational modification (BMPR2, MAPK6, PLK1, MARK1, PPM1G, PTPN3, RNF144B, UBE2S), cell cycle or cell division related process (CDC2, CCNA2, CCNB2, AURKB, UBE2C, PTTG1, CDCA2, NUSAP1, CKS2, BIRC5) and apoptosis (BCL2, CDC2, BIRC5, GCLC). The representative biological processes of GO terms and the expression pattern of DE genes enriched in CDd7 is indicated in figure 10. On the other hand, cellular metabolic processes, response to stimuli, immune response and induction of apoptosis and chemokine activity were among the biological process overrepresented in those genes whose transcript level was elevated in NPd7 compared to CDd7 (Figure 9B). The representative GO terms and the expression patterns of DE genes enriched in NPd7 are indicated in figure 12.

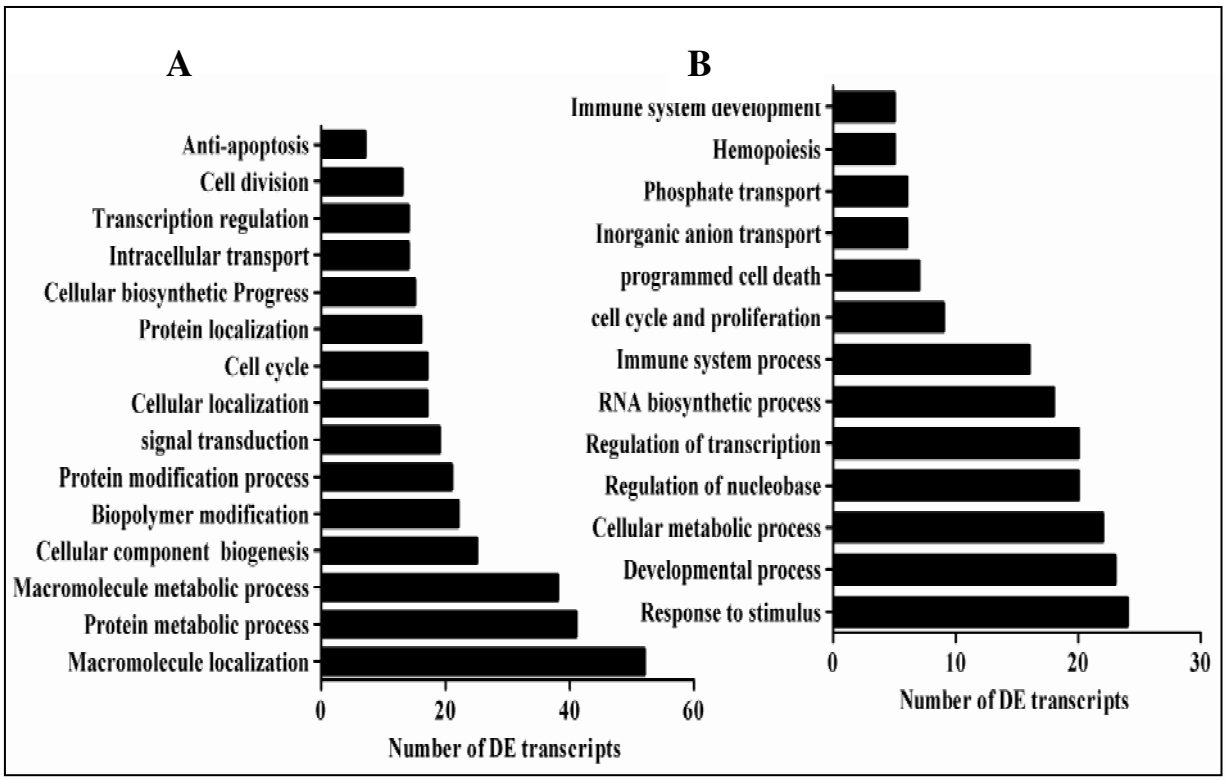

Figure 9: Ontological classification of DE genes that were elevated in CDd7 or NPd7 according to their biological process. (A): Biological processes overrepresented in genes elevated in CDd7. (B): Biological process of overrepresented in genes elevated in NPd7. The vertical axis indicates biological processes and the horizontal axis indicate the number of DE genes involved in a specified biological process. DE = differentially expressed.

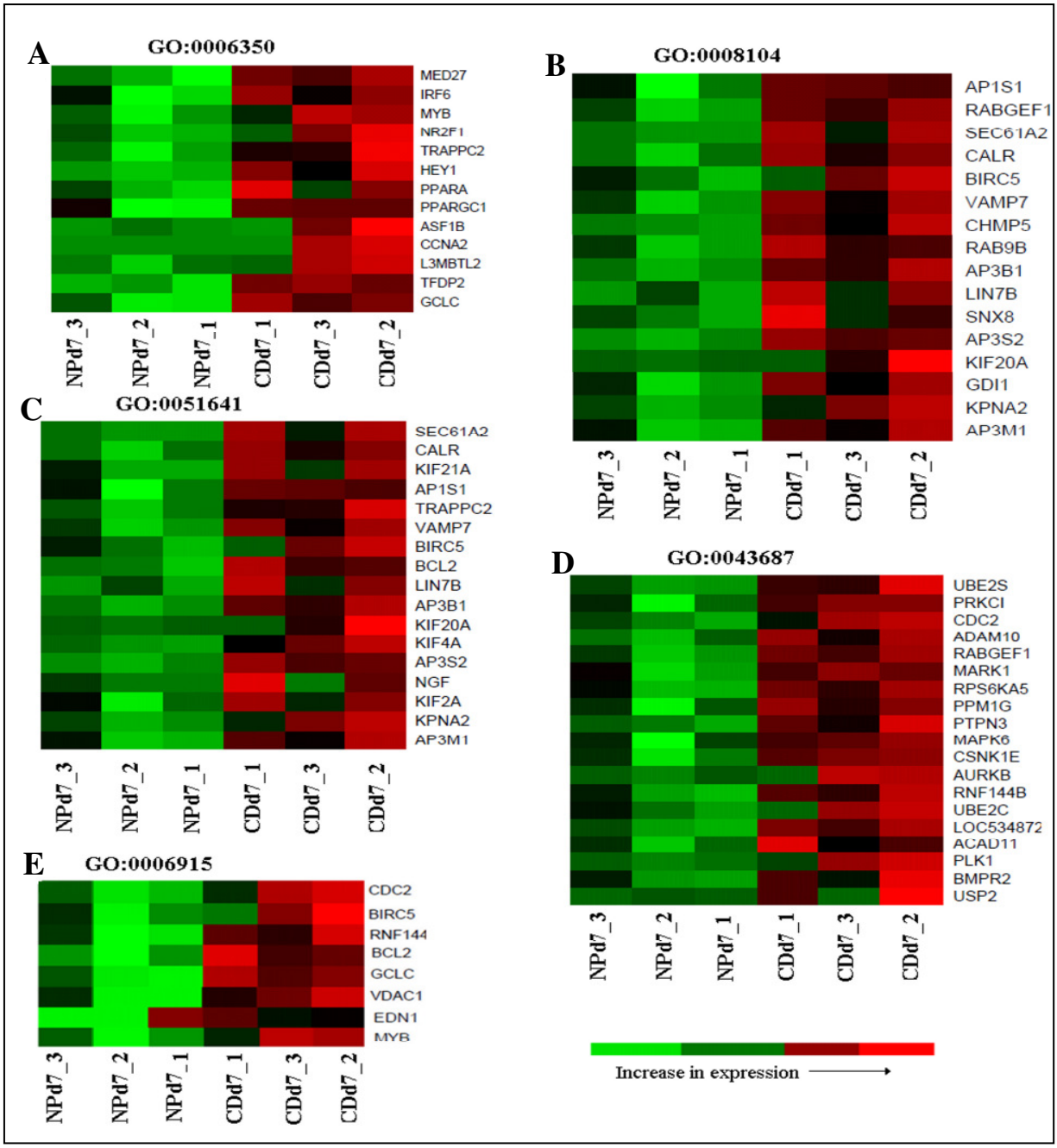

Figure 10: Representative biological processes overrepresented in DE genes increased in receptive (CDd7) compared to non-receptive (NPd7) endometrium. (A): Genes involved in gene transcription. (B): Genes involved in protein localization or transport. (C): Genes involved in cellular localization. (D): Genes involved post-translational modification. (E): Genes involved in anti-apoptosis. The number on top of each heatmap indicates the gene ontology accession number. Red and green colors reflect high and low gene expression levels, respectively.

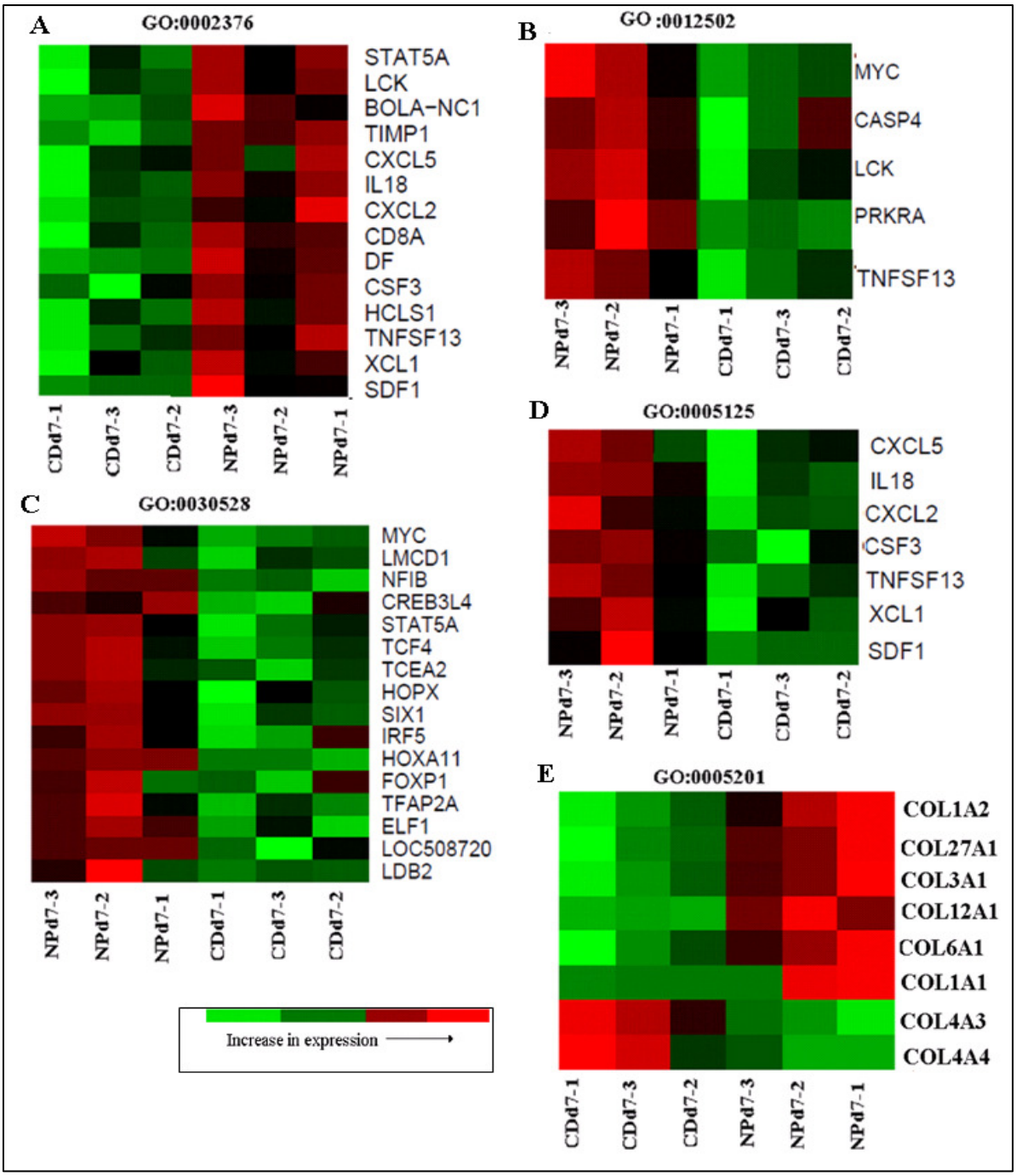

Figure 11: Biological processes or molecular functions overrepresented in genes exhibited increased transcript level in non-receptive (NPd7) compared to receptive endometrium (CDd7) at day 7 of the estrous cycle. (A): Genes involved in immune system. (B): Genes involved in induction of apoptosis. (C): Genes involved in transcription regulation. (D): Genes involved in chemokine or cytokine activity. (E): Genes involved in extracellular matrix. The number on top of each heatmap indicates the gene ontology accession number. Red and green colors reflect the high and low expression, respectively.

In addition to the biological process, the DE genes were classified according to molecular functions describing different activities that occurred at the molecular level. Thus, several significant molecular functions were overrepresented in those genes elevated in CDd7 or NPd7 (Figure 12). Some of molecular function overrepresented by those genes reduced in CDd7 but elevated in NPd7 include protein binding, transcription factor activity (CREB3L4, STAT5A, ELF1, FLI1, NFIB, MYC, IRF5, SIX1, HOPX and HOXA11), kemokine activity (CXCL2, CXCL1, CXCL5, XCL1) (Figure 11D) extracellular matrix structural constituent (COL1A1, COL1A2, COL3A1, COL27A1) (Figure 11E). On the other hand, several molecular functions include binding activities (protein, ion, nucleic acid and ATP binding), transferase activity, transmembrane transport (ITPR1, solute carriers, transmembrane proteins, VDAC1), metal peptidase activities, symporter activity and kinase activity were overrepresented in genes elevated in CDd7 compared to NPd7 (Figure 13).

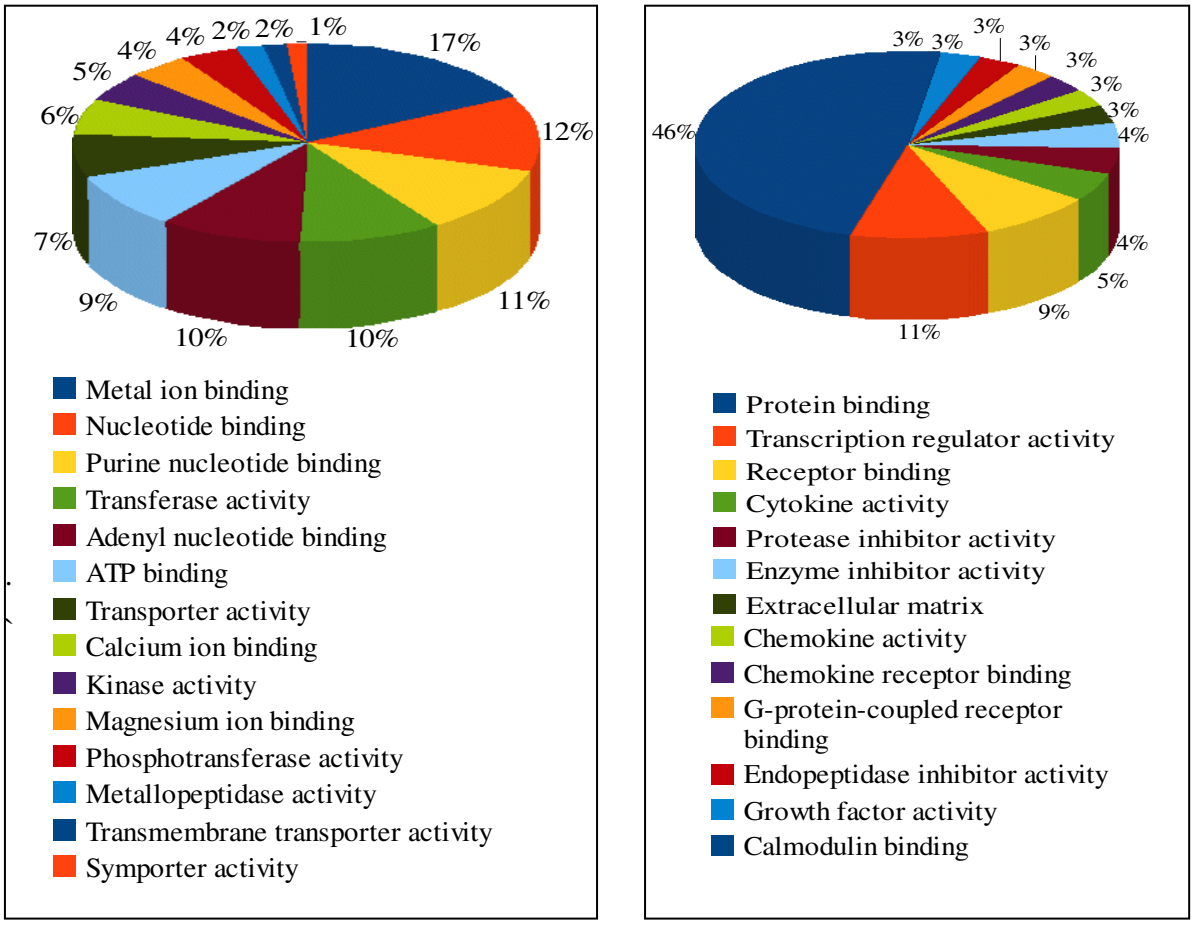

Figure 12: Molecular function overrepresented in genes enriched in receptive (CDd7) (left) and non-receptive endometrium (NPd7) (right) at day 7 of the estrous cycle.

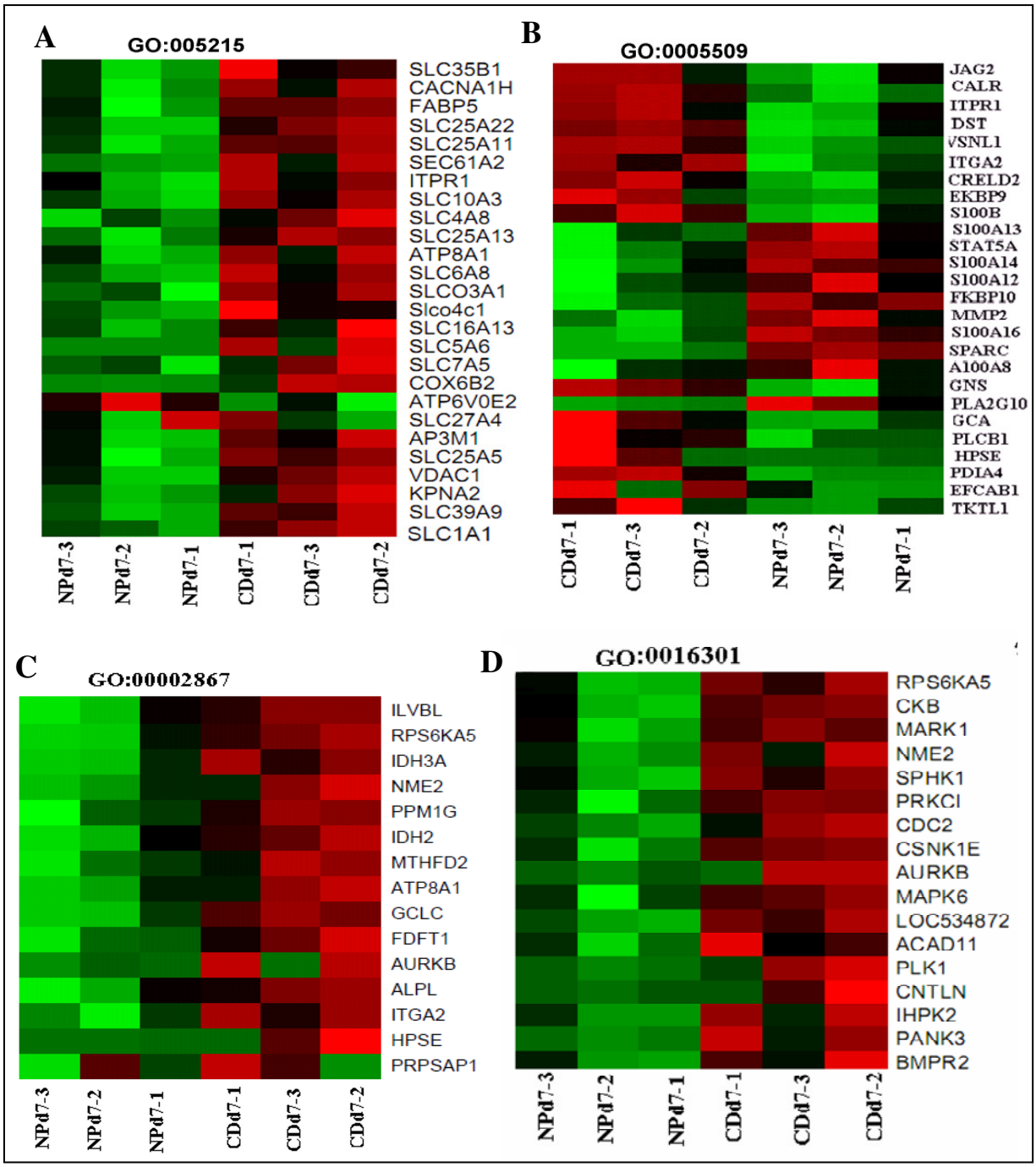

Figure 13: Representative molecular functions overrepresented in genes enriched in CDd7 compared to NPd7. (A): Genes involved in transporter activity. (B): Genes involved in calcium ion binding activity. (C): Genes involved in magnesium ion binding. (D): Genes involved in kinase activity. The number on top of each heatmap indicates the gene ontology accession number. Red and green colors reflect high and low expression, respectively

4.2.1.3 Molecular pathway identified in differentially expressed genes between receptive and non-receptive endometrium at day 7 of the estrous cycle

To understand transcriptomic signaling and metabolic alteration in receptive and non-receptive endometrium, the most important pathways involved by DE genes were identified from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Accordingly, those genes that were differentially expressed between CDd7 and NPd7 were found to be involved in 25 molecular pathways (Table 6). Functionally, those pathways were classified broadly into 8 groups namely, cell communication (Integrin Signaling, tight junction and focal adhesion), signaling molecules and interaction (ECM-receptor interaction and cytokine-cytokine receptor interaction), signal transduction (MAPK signaling pathway and calcium signaling pathway), cell growth and death (p53 signaling pathway, cell cycle, apoptosis), nervous system (long-term potentiation), genetic information processing (ubiquitin mediated proteolysis) and immune system (Toll receptor pathway, hematopoietic cell lineage, antigen processing and presentation and T cell receptor) and endocrine system (insulin signaling pathway, PPAR signaling pathway and GnRH signaling pathway.

From these, the tight junction, integrin signalling, inositol phosphate metabolism, calcium signalling pathway, glycan structures biosynthesis 1, focal adhesion, apoptosis, cell cycle, vascular endothelial growth factor (VEGF), long term potentiation and PPAR signalling pathways were enriched by genes increased in CDd7. The graphical illustration and the number of DE genes involved in one of those with highest number of DE genes, tight junction pathway is indicated in figure 14. Similarly, T cell receptor pathway, cytokine-cytokine receptor, p53 signalling pathway, Jak-STAT signaling pathway and cell adhesion pathways were dominated by DE genes whose expression was elevated in NPd7.

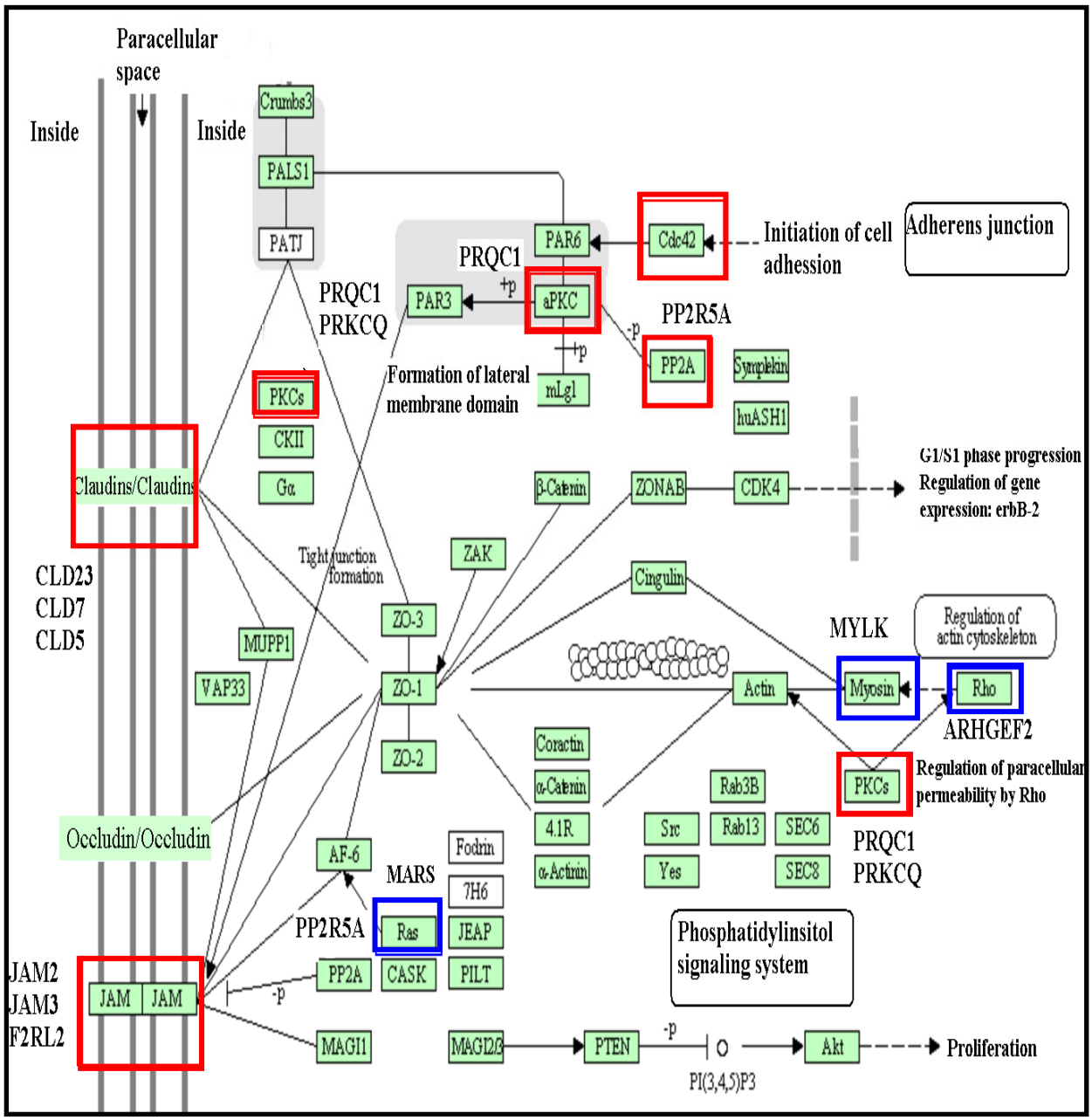

Figure 14: Differentially expressed genes between CDd7 and NPd7 involved in tight junction pathway. The claudin and junctional adhesion molecules (JAMs), which makeup the transmembrane protein of this pathway, were increased in CDd7. In the cytoplasmic plaque, aPRC, PP2A, PRKC, MYOSIN and RHO contains differentially expressed genes. Genes elevated in CDd7 are marked by red background and those elevated in NPd7 are marked with blue background.

Table 6: Molecular pathways containing genes elevated in receptive (CDd7) and non-receptive endometrium (NPd7)

Name of molecular pathways	Differentially expressed genes	Pathway category
Tight junction	**TIAM1, F2RL2, PRKCI, CLDN23, CLDN5, JAM3, JAM2**, **CLDN7,** ARHGEF2, PRKCQ, MRAS, MYLK	Cell communication
Focal adhesion	**ITGA2**, **ITGA6**, **VEGFB**, **COL4A4**, **COL6A2, COL6A3, PPP1CC**, **PPP1CB** ,MYLK, COL1A1, COL1A2	Cell communication
Integrin Signaling	**ROCK1, PPP1CC**, **TSPAN7**, **DIRAS3, ITGA2, MRAS, ITGA6**, **PPP1CB**, RND3, MYLK, BCAR	Cell communication
Inositol Phosphate metabolism	**PRKCQ, SGK1**, **MAPK6**, **CDK**6**, PLCB1**, **PLK1**, **IHPK2, ITPKA**, **PLCL2**, **CDC2,** INPP1	Metabolism
ECM-receptor interaction	**CD36**, **HMMR**, **ITGA2**, **COL4A4**, **COL6A2, COL6A3,** AGRN,COL1A1, COL1A2,	Signaling molecules
Cytokine-cytokine receptor interaction	**VEGFB**, **BMPR2**, CSF3, IL18, TNFSF13, SDF1, CXCL2, CXCL1, CXCL6, XCL1	Signaling molecules
MAPK signaling pathway	**NGF, RPS6KA5**, **MAPK12**, **MAPK6**, NTRK2, MYC MRAS, MAPK13, PTPRR	Signal Transduction
Apoptosis	**NGF, CDC2, CCNB2**, **DDB2**, **CDK6, BIRC5, FFA, BCL-2,** TP53I3, SNAI2	Cell growth and death
Calcium signaling pathway	**SLC25A5, PLCB1, ITPR1**, MYLK, **F2R, ADORA2B,** CAMK2D, AGTR1	Signal Transduction
Ubiquitin mediated proteolysis	**UBE2C, SYVN1, CDC20, DDB2, PIAS2, UBE2S**, UBE2L6, UBE3B	Genetic information processing
T cell receptor	**MAPK1**, **PRKCQ**, LAT, CD247, CD8A, CD8B, LCK, MAPK13**2**	Immune system
p53 signaling pathway	**CDC2, CCNB2**, **DDB2, TIMP3**, **CDK6**, TP53I3, SNAI2, SERPINB5	Cell growth and death
Neuroactive ligand-receptor interaction	**PTGER4, F2RL2, ADORA2B**, **F2R** F2RL1**,** CALCRL, AGTR1	Signaling molecules
GnRH signaling pathway	**PLCB1, ITPR1, MAPK12**, **ITGA6,** CAMK2D, MAPK13, MMP2	Endocrine system
Cell cycle	**CDC2, CCNA2, CCNB2, CDC20 , PLK1,CDK6**	Cell growth and death
PPAR signaling pathway	**FABP5**, **PPARA**, **PLTP**, **SLC27A4,** ACOX2, CD36,	Endocrine system
Insulin signaling pathway	**PPP1CC, PPARGC1A**, **PRKCI**, **PPP1CB**, SOCS2	Endocrine system
Long-term potentiation	**PPP1CC, PLCB1, ITPR1**, **PPP1CB**, CAMK2D	Nervous System
Glycan structures biosynthesis	**FUT8**, **B4GALT4**, **B3GNT2**, **ALG8**, **ALG11**	Metabolism
Hematopoietic cell lineage	**ITGA2**, **ANPEP**, CD8B, CD36, CD8A, CSF3	Immune system
Antigen processing and presentation	**HSPA2**, CD8B, LOC508720, CALR, CD8A	Immune system
Jak-STAT signaling pathway	**PIAS2,** CSF3, STAT5A, SOCS2, MYC	Signal Transduction
Cell adhesion molecules	**JAM2**, PDCD1LG2, CD8A, CD8B	Signaling molecules
VEGF signaling pathway	V**EGFB, MAPK12**, **PTGS2,** MAPK13	Signal Transduction
Toll receptor pathway	**MAPK12**, IKBKE, MAPK13, IRF5	Immune System

Genes elevated in receptive (CDd7) are indicated in bold face and those enriched in non-receptive endometrium (NPd7) are lightfaced

4.2.1.4 Molecular networks represented in differentially expressed genes between receptive and non receptive endometrium at day 7 of the estrous cycle

In addition to ontological classification and KEGG pathway analysis, the biological relationships between differentially expressed genes were also assessed using Ingenuity Pathway Analysis (www.ingenuity.com). For this, differentially expressed genes were uploaded into the program. The biologic relationship of uploaded genes was established with IPA software into networks according to the published literature in the database. A score was assigned to each network in the data set to estimate the relevance of the network to the uploaded gene list. Accordingly, network eligible genes were found to be involved in 34 different molecular networks (Appendix 3). The top molecular networks consisting of several differentially expressed genes were the cell cycle, cell death or reproductive system disease network, genetic disorder network, hematological system development and function network, cell cycle, DNA replication, recombination and repair network, post-translational modification network, endocrine system development and function network, antigen presentation network, cell-to-cell signaling network and immune cell trafficking network. The two molecular networks with highest score are described in figure 15 and 16. The cycle, cell death or reproductive system disease network (Figure 15) consisted of 31 focus genes, out of which 22 genes were enriched in CDd7 and the central gene, v-myc myelocytomatosis viral oncogene homolog (MYC), was reduced in CDd7. This network involves two transcriptional regulators (MYC and SUPT3H) which were down regulated in CDd7, but increased in NPd7. Apart from this, the enzyme molecules including GART, IDH3A, UBE2C, IDH2 have a direct relation to MYC. In addition, this network showed that reduced expression level of MYC in CDd7 was associated with reduction in the expression of SUPTH3, TP53I3, PLA1A, PMP2, SFRP1 and TNFSF13. On the other hand, a reduction of MYC gene expression was associated with increased expression of a number of genes including IDH3A, UBE2C, IDH2, ITAG6, GPC1, CSK2, EMP1 and PSAT1 (Figure 15).

Similarly, the genetic disorder, lymphoid tissue structure and development network (Figure 16) was the second high score molecular network identified in differentially expressed genes between CDd7 and NPd7. Genes including TIMP1, AP3B1, AP3S2, DST, VAMP7 are located in the cytoplasmic vesicle of the cell and VDAC1 is located in mitochondrial nucleoid and pore complex. Furthermore, AP3B1, AP3S2, AGTR1, VDAC1 and COL18A1 were the central genes of this network.

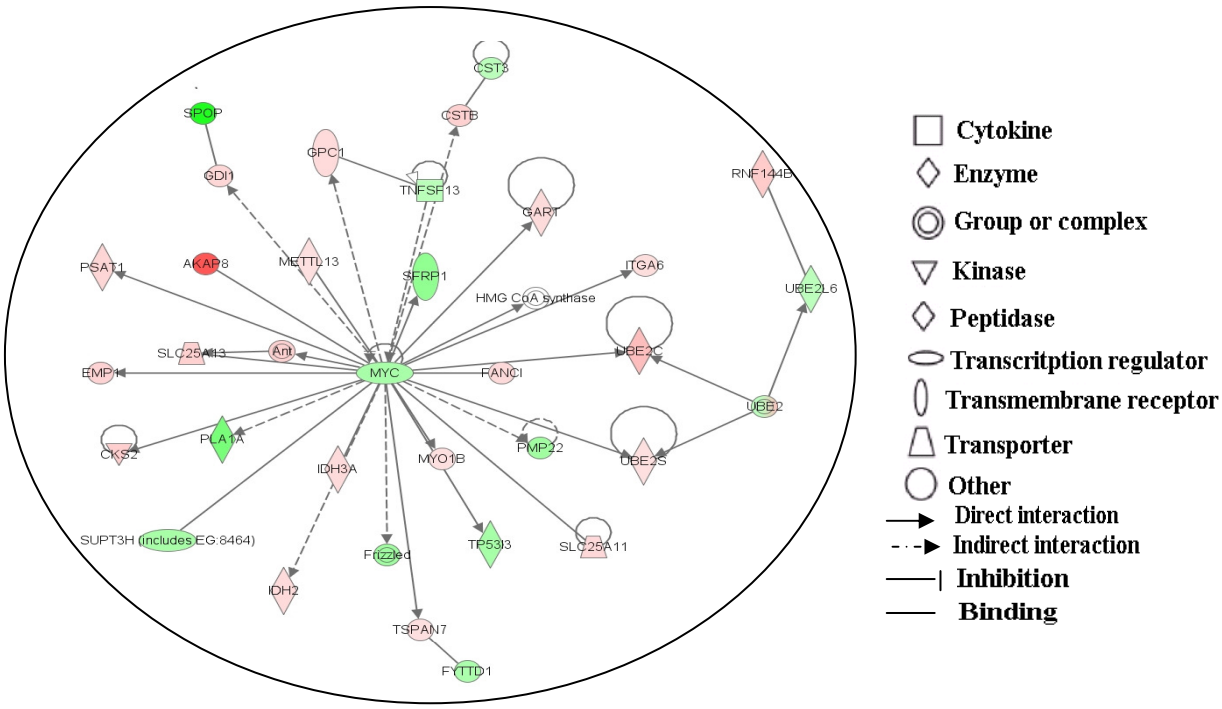

Figure 15: Cell cycle, cell death, reproductive system disease network. Genes in red and green background indicated the genes enriched in CDd7 and NPd7, respectively

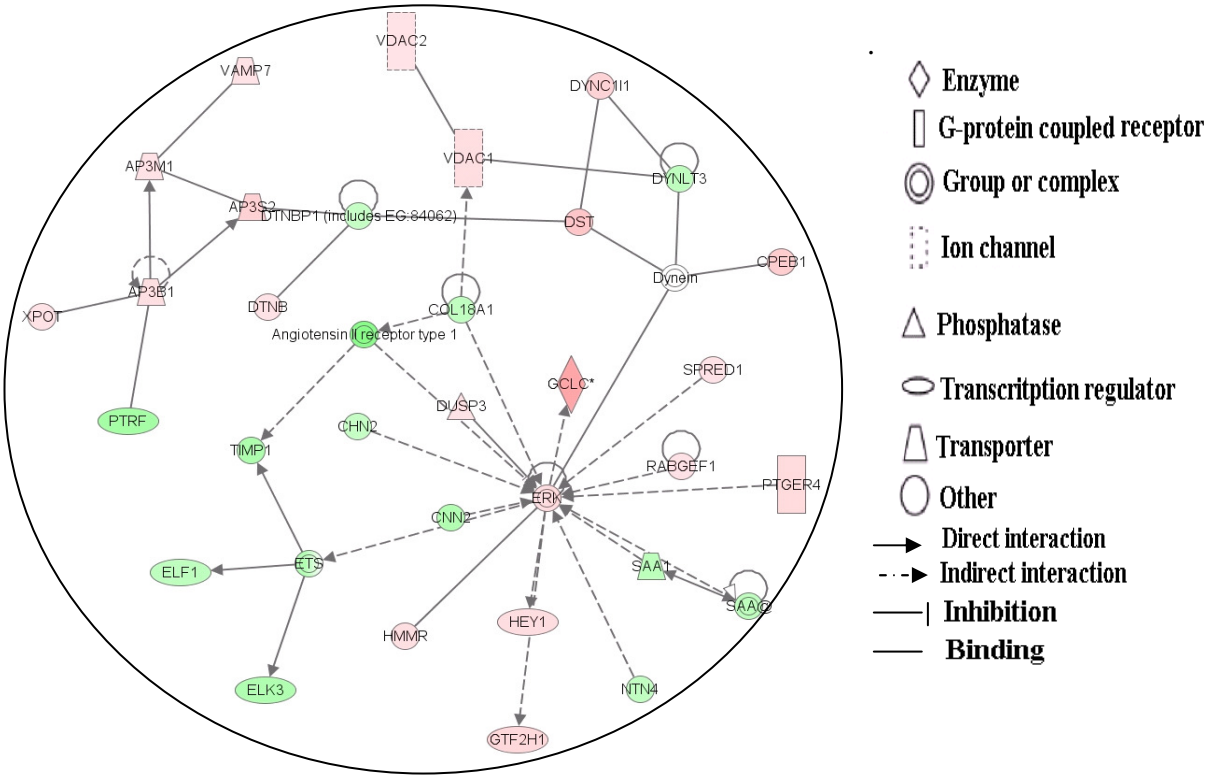

Figure 16: The genetic disorder, lymphoid tissue structure and development network. Red and green backgrounds indicate the genes enriched in CDd7 and NPd7, respectively.

4.2.2 Pre-transfer gene expression of receptive and non-receptive endometrium at day 14 of the estrous cycle

In addition to day 7, the pre-transfer gene expression of receptive and non-receptive endometrium was investigated at day 14 of the estrus cycle using endometrial samples collected at day 14 of the estrous cycle from heifers resulted in calf delivery (CDd14) and from those resulted in no pregnancy (NPd14). The results revealed that including 5 ESTs, only 14 genes were differentially expressed between CDd14 and NPd14 (Figure 17).

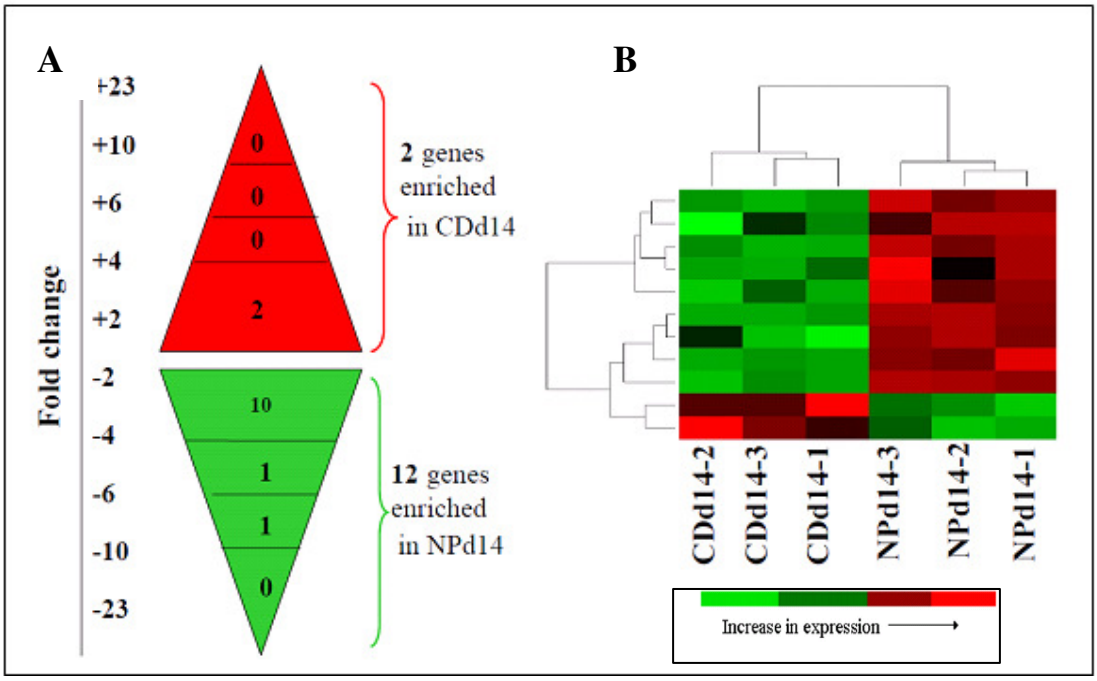

Figure 17: Pre-transfer transcriptome alteration between receptive (CDd14) and non-receptive endometrium (NPd14) at day 14 of the estrous cycle. (A): The number and fold change distribution of DE genes that were increased in CDd14 or NPd14. (B): The heatmap and hierarchical clustering describing the expressional pattern of DE genes in CDd14 and NPd14. Labels on the horizontal axis of the heatmap, CDd14-1, CDd14-2 and CDd14-3 represent independent biological replicates in receptive endometrium groups and NPd14-1, NPd14-2 and NPd14-3 represent independent biological replicates from non-receptive endometrium groups at day 14 of the estrous cycle. The horizontal dendrogram indicates the expression patterns of transcripts in CDd14 and NPd14. Red and green colors reflect the high and low expression, respectively.

From a total of 14 DE genes, 12 genes exhibited 2 to 4 fold change and 2 genes showed above 10 fold change difference between CDd14 and NPd14 (Figure 10A). Among these, the expression of C20ORF54 and frizzled homolog 8 (FZD8) was higher in CDd14, while the expression of LOC786821, bromodomain and WD repeat domain containing 1 (BRWD1), ankyrin repeat domain 11 (ANKRD11), solute carrier family 25 (SLC25A12), gap junction protein alpha 1 (GJA1), scavenger receptor class A member 5 (SCARA5), immunoglobulin lambda-like polypeptide (IIGLL1) and other 5 ESTs was higher in NPd14.

Ontological classification the DE genes showed that those genes were found to be involved in G-protein coupled receptor protein signaling pathway and Wnt receptor signaling pathway (FZD8), in utero embryonic development (BRWD1 and GJA1), transition metal ion transport (SCARA5) and transmembrane transport (SLC25A12). However, the number of differentially expressed and the transcriptomic function alteration detected between CDd14 and NPd14 was significantly lower compared to the number of differentially expressed genes and transcriptomic functional alteration that has been detected between CDd7 and NPd7 (Figure 5). This difference indicates that remarkable molecular alteration between the receptive and non-receptive endometrium at day 7 of the estrous cycle than day 14 of the estrous cycle. This result also indicates the receptive and non-receptive endometriums are molecularly distinguishable during day 7 than day 14 of the estrous cycle.

4.3 Diestrus transcriptome dynamics of receptive and non-receptive endometrium

4.3.1 Diestrus transcriptome dynamics of receptive endometrium

To identify transcriptome profile changes that has been occurred in the receptive endometrium between day 7 and day 14 of the estrous cycle, the gene expression pattern was analyzed using CDd7 and CDd14 endometrial samples. The result indicated that from 17,368 detected transcripts both in CDd7 and CDd14, 1867 genes were differentially expressed between the two groups. Of these, the transcript levels of 1014 genes were higher in CDd7 and the transcript level of 853 were increased in CDd14 (Figure 11, Appendix 4). The genes including, DGKI, EDN3, SVS8, SPC24, PRODH, PDZK1, PLLP, CCNB1, LPL and GDPD1 exhibited a higher fold increase (54 to 397 fold) in CDd7, but the transcript level of SLC39A2, DEFB1, TINAGL1, C15H11ORF34, LOC286871, IL8, GPLD1, LYZ1 and IGFBP1 was found to be higher (45 to 418 fold) in CDd14. Therefore, the data

indicates that the expression profile of receptive endometrium changes as the endometrium proceeds from day 7 to day 14 of the estrous cycle.

4.3.1.1 Genes clusters identified between day 7 and day 14 of the estrous cycle in receptive endometrium

From a total of 1867 differentially expressed genes identified between CDd7 and CDd14, several groups of genes were elevated either in CDd7 or in CDd14. The gene clusters elevated in CDd7 compared to CDd14 include the solute carriers (SLC10A3, SLC12A2, SLC16A1, SLC22A5, SLC25A1, SLC25A11, SLC27A2, SLC29A1, SLC2A1, SLC30A5, SLC31A2, SLC35B1 and others), family with sequence similarity (FAM117A, FAM3A, FAM55C, FAM62A, FAM81B, FAM84A, FAM92A1), ectonucleotide pyrophosphatase (ENPP1, ENPP2, ENPP3, ENPP4, ENTPD5), fbox genes (FBXL20, FBXO2, FBXO31, FBXO31, FBXO36), mitochondrial ribosomal protein (MRPL38, MRPS25, MRPS36, MRPS36, MRPS6), nudixes (NUDT12, NUDT22, NUDT5, NUDT5), ring finger proteins (RNF144B, RNF149, RNF183, RNF185, ubiquitin specific peptidase (USP12, USP2, USP33, USP46, USP7), collagens (COL4A1, COL4A3, COL4A4), the adaptor related proteins (AP1S1, AP3B1, AP3M1, AP3S2), eukaryotic translation initiation factor (EIF2C2, EIF4G3, EIF4G3), inositol triphosphate (ITPKA, ITPR1, ITPR3). Similarly, gene clusters increased in CDd14 were the ankryls (ANKRD22, ANKRD50, ANKS6), Rho proteins (ARHGAP17, HGAP21, ARHGAP24, ARHGAP26, ARHGDIB), ATpases (ATP11B, ATP13A4, ATP6V0A2, ATP6V0A4), CD molecules (CD14, CD36, CD48, CD53, CD59, CD69, CD83), cyline dependant kinases (CDK6, CDK7, CDK8, CDKN1A, CDKN2C), interlukins (IL17RC, IL18, IL18BP, IL1RN, IL20RA, IL8), Krupple like factors (KLF10, KLF11,KLF15) and phosphodiesters (PDE3B, PDE4B, PDE4DIP, PDE6D).

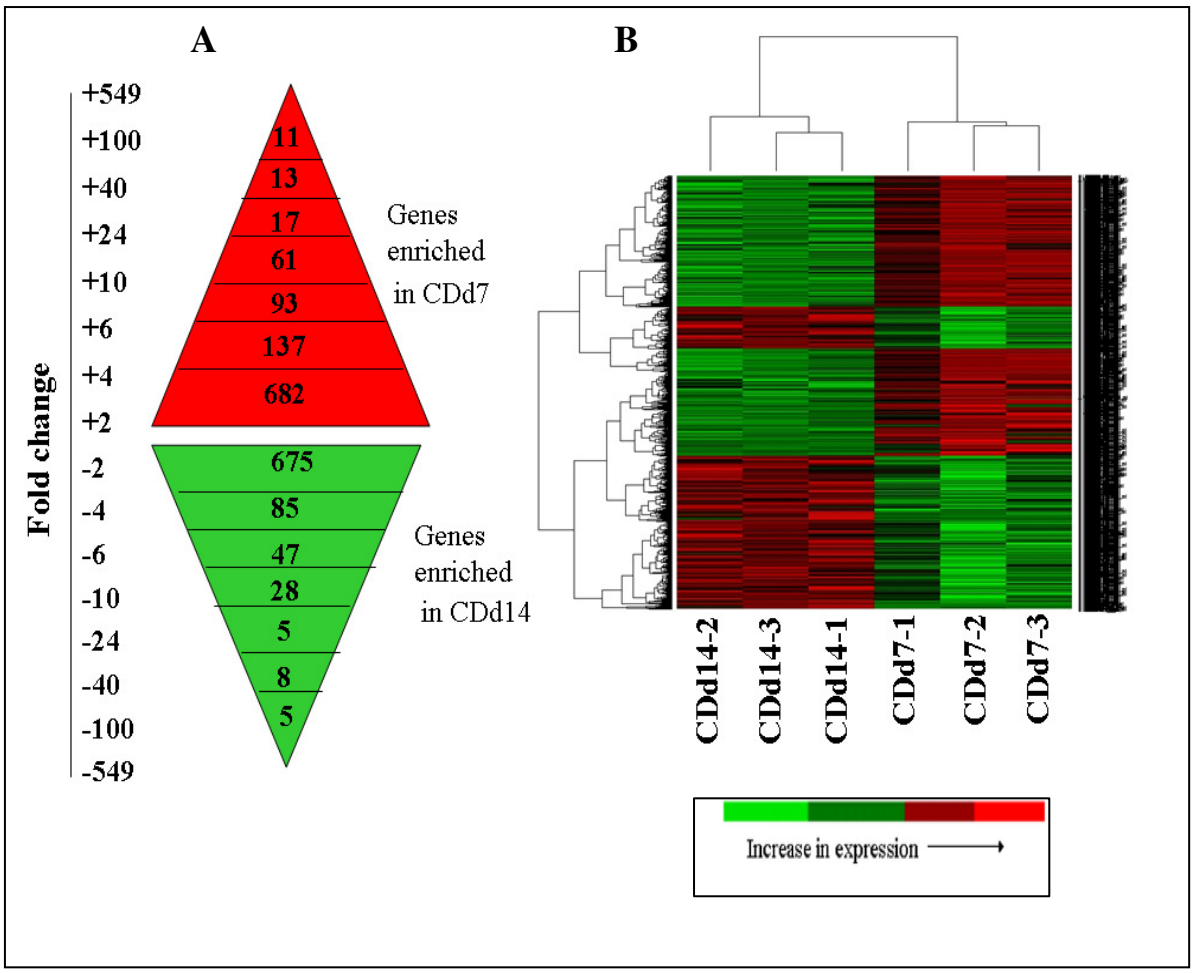

Figure 18: Pre-transfer transcriptome dynamics of the receptive endometrium between day 7 and 14 of the estrous cycle. (A): The number and fold change distribution of DE genes between CDd7 and CDd14. (B): The heatmap describing the expression pattern of DE genes between CDd7 and CDd14. Labels on the horizontal axis of the heatmap, CDd7-1, CDd7-2 and CDd7-3 represent independent biological replicates from receptive endometrium groups at day 7 of the estrous cycle. CDd14-1, CDd14-2 and CDd14-3 represent independent biological replicates from receptive endometrium groups at day 14 of the estrous. Red and green colors reflect high and low expression, respectively.

4.3.1.2 Transcriptomic functional alteration between day 7 and 14 of the estrous cycle in receptive endometrium

The functional contribution of these genes that has been altered between day 7 and 14 of the estrous cycle in receptive endometrium was assessed using the gene ontology database. Thus, the differentially expressed genes that were higher in CDd7 and CDd14 were found

to be involved in different functions (Figure 19). Those abundantly expressed in CDd7 were found to be involved in various biological processes including, cellular material transport (solute carriers, AP1S1, AP3B1, AP3M1, AP3S2, FDX1, FUT8, GDI1, ITPR1, ITPR3, KIF21A, KPNA2), metabolic process (PRODH, LPL, GDPD1, BDH1, PFKFB2, PHGDH), phosphorylation, post-translational protein modification (USP2,UBL4A, UBE2S, UBE2D4, UBE2A, SRPK2). On the other hand, immune responses (STAT5A, IL8, LOC512486, IL1RN, C5, NDRG1, CXCL5, TLR5, HCLS1, SCIN, LCK, IL18, apoptotic process (CASP4,LCK, MYC), signal transductions (KDR, RAB3B, F2RL2, NMB, PLAT, IFI6, PLEK, ITGB5) were among the main biological processes overrepresented in those genes whose transcript level was increased in CDd14.

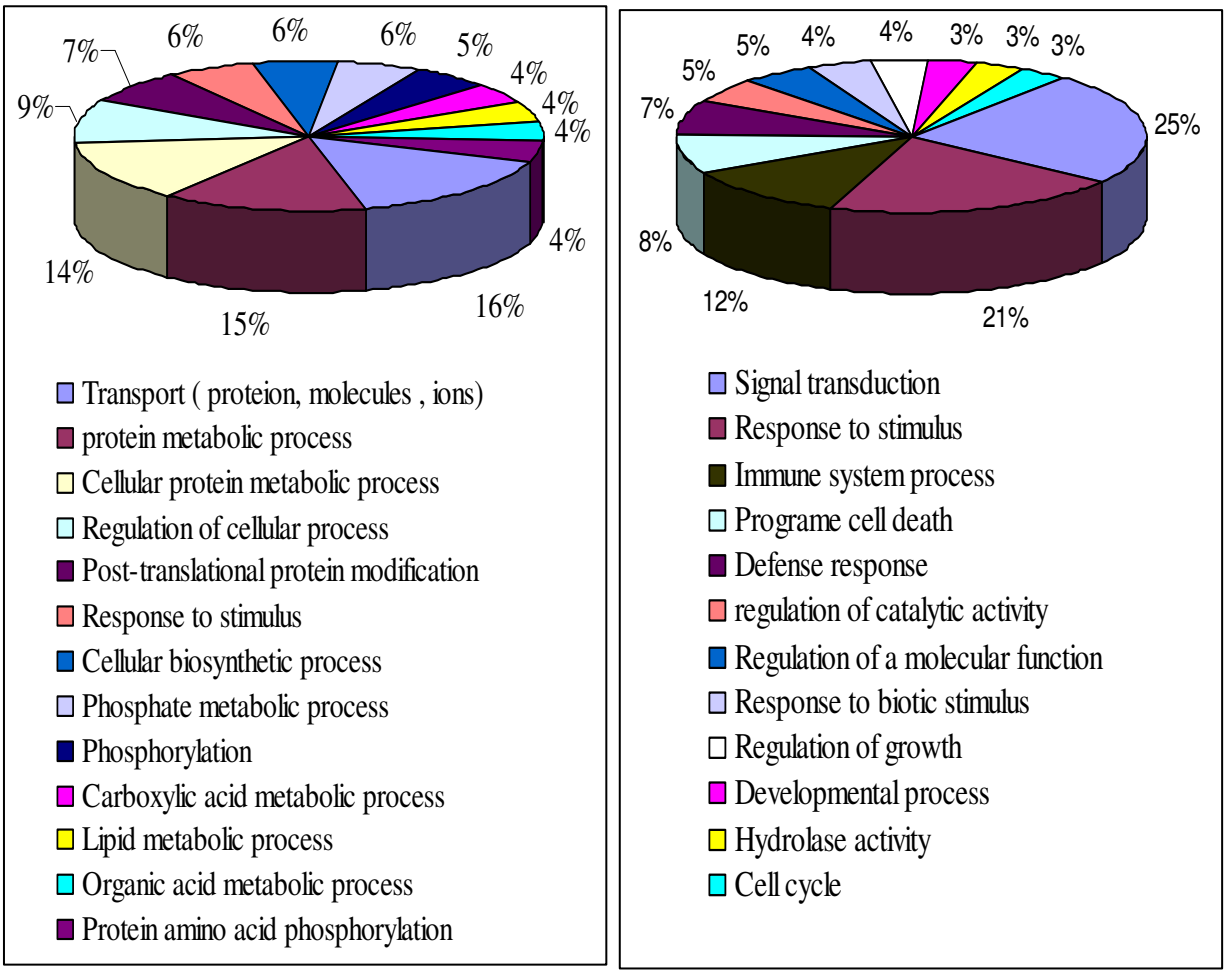

Figure 19: Biological processes overrepresented in DE genes enriched in CDd7 (left) and CDd14 (right) as the receptive endometrium proceeds from day 7 to day 14 of the estrous cycle.

4.3.1.3 Molecular pathway identified in differentially expressed genes between day 7 and 14 of the estrous cycle in receptive endometrium

Those differentially expressed genes between CDd7 and CDd14 were found to be involved in 21 molecular pathways (Table 7). Among these, the MAPK signaling, Toll-like receptor signalling, adipocytokine signaling, cell adhesion molecules and extra cellular matrix receptor interaction pathways were enriched by those genes whose expression levels was higher in CDd14. On the other hand, PPAR signaling pathway, calcium-signaling pathway, neuroactive ligand-receptor interaction, GnRH signaling pathway, glycine, serine and threonine metabolism were enriched by those genes whose transcript level was increased in CDd7. Among those, the graphical representation of GnRH signaling pathway and the number DE genes involved in this pathway is described in figure 20.

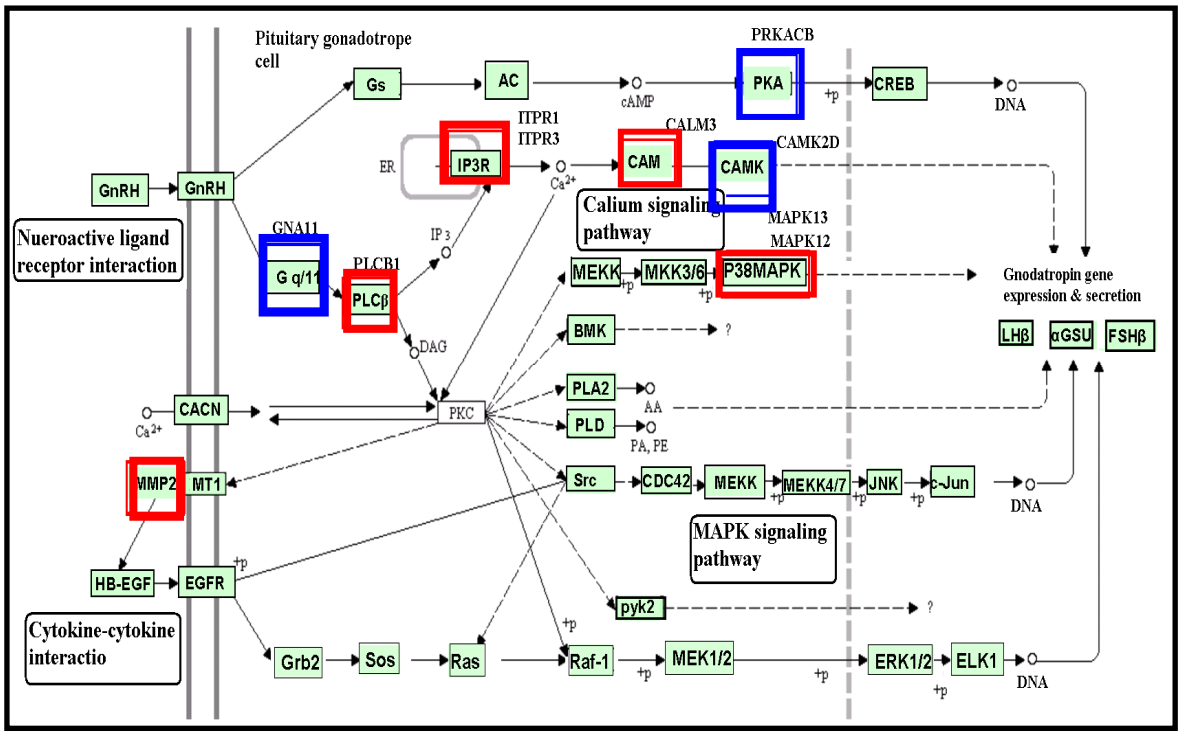

Figure 20: The DE genes between CDd7 and CDd14 are found to be involved in Gonadotropin-releasing hormone (GnRH) signaling events. The DE genes (MMP2), G/q11 (GNAI1), PLCB, IP3R (ITPR1, ITPR3), CAMK (CAMK2D), P38MAPK (MAPK13, MAPK12) are located in the critical streams of the path indicating the change in the activity of the GnRH pathway as the endometrium proceeds from day 7 to 14 of the estrous cycle. Genes elevated in CDd7 are marked by red background and those elevated in CDd14 are marked with blue background.

Table 7: List of molecular pathways containing DE genes enriched in CDd7 and/or CDd14

Pathway list	Differentially expressed genes
MAPK signaling pathway	**FLNA, MAPK12, ,NGF, RPS6KA5,**CD14, ARRB1, FOS, PRKACB, PDGFB, PTPRR, MYC, NFKB1, MAPK13, IKBKB, MAP3K8, TGFBR2
Toll-like receptor signalling	**NFKBIA, IKBKE, MAPK12,** IL8, CD14, IRF5, FOS, TLR5, NFKB1,MAPK13, IKBKB, TLR2, MAP3K8
Glycan structures- biosynthesis 1	**B3GNT2, FUT8, ALG11, ALG8, B4GALT4, CSGALNACT2,** MGAT4A CHST1, C1GALT1, HS6ST1
Adipocytokine signaling pathway	**ADIPOR2, PPARGC1A, NFKBIA, PPARA,** ACSL5, IKBKB, NFKBIE, NFKB1, PRKAA2, CD36, SLC2A1
Insulin signaling pathway	**PPARGC1A, PRKAR2B, ARAF, PPP1CB, PFKM,** IKBKB, PRKACB, PRKAA2, SOCS2
Calcium signaling pathway	**ITPR1, F2R, HRH1, SLC25A5, ITPR3, ADORA2B.** CAMK2D, PRKACB, ADRB2
Regulation of actin cytoskeleton	**ITGA2, F2R, ARAF, LIMK2, WASF2,** ARHGEF6, PDGFB, SCIN, CD14, ITGB5
Cell adhesion molecules (CAMs)	**VCAM1, JAM2, CLDN1,** SDC1, BOLA-DMB, PVR, DCD1LG2, SDC2
Gap junction	**ITPR1, CDC2, ITPR3, GUCY1A3,** TUBB6, GNAI1, PDGFB, PRKACB
Neuroactive ligand-receptor interaction	**PTGER4, F2RL1, F2R, HRH1, ADORA2B,** PTGER2, ADRB2, F2RL2
GnRH signaling pathway	**ITPR1, MAPK12, MMP2, ITPR3**, CAMK2D, MAPK13,PRKACB
Tryptophan metabolism	**ALDH3A2, HSD17B10, HSD17B4, WARS,** WARS2, CYP1A1
PPAR signaling pathway	**LPL, SLC27A6, PLTP, FABP5, PPARA,** ACSL5, CD36, SLC27A2
Focal adhesion	**ITGA2, VEGFB, FLNA, PPP1CB,** PDGFB, ITGB5, MET, KDR
Jak-STAT signaling pathway	**IL6ST,** IL20RA, STAT5A, SPRY2, MYC, PIM1, SOCS2
Glycan structures - biosynthesis 2	**B4GALT4, FUT1, B3GNT2,** UGCG, 3GALT2, ST3GAL6
Arginine and proline metabolism	**PRODH, ARG2, CKB, ASL, GLUD1,** GATM, ASS1
ECM-receptor interaction	**ITGA2,** HMMR, SDC1, AGRN, SDC2, CD36, ITGB5
Cytokine-cytokine receptor interaction	**IL6ST,** TGFBR2, IL20RA, IL18, MET, KDR, IL8
Glycine, serine and threonine metabolism	**PHGDH, PSAT1, PEMT, CBS,** GATM, PSPH
Urea cycle and metabolism of amino acid	**ARG2, ALDH3A2, SMS, ASL,** GATM, ASS1

DE genes enriched in CDd7 are indicated in bold face and genes enriched in CDd14 are indicated in light face

4.3.2 Diestrus transcriptome dynamics of non-receptive endometrium

The transcriptome dynamics of non-receptive endometrium between day 7 and 14 of the estrous cycle was investigated by comparing the gene expression of endometrial samples collected at day 7 (NPd7) and day 14 of the estrous cycle (NPd14) from heifers resulted in no pregnancy. The result revealed that from 17,361 detected transcripts both in NPd7 and NPd14, 254 genes were differentially expressed between the two groups (Figure 21). Compared to the transcriptome dynamics of receptive endometrium (CDd7 vs. CDd14) presented in figure 18, the number of differentially expressed genes detected between NPd7 and NPd14 was lower by 1613 genes revealing the transcriptome plasticity of the receptive endometrium during the diestrus phase of the estrous cycle. The most top elevated genes in NPd7 compared to NPd14 includes, DGKI, SVS8, SPC24, SPOP, COL6A1, TXNDC6, NTRK2, APOD and some of top genes down regulated in NPd7, but increased in NPd14 include (PLEK, FMNL3, TLN2, CCL11, NPNT, SPP1, BNBD4, SDS, IL8, SLC39A2). The description of 100 top differentially expressed genes is described in appendix 5.

4.3.2.1 Transcriptomic functional alteration between day 7 and 14 of the estrous cycle in non-receptive endometrium

The differentially expressed genes between NPd7 and NPd14 were found to be involved in different biological and molecular functions. For instance, those which showed an increase expression level in NPD7 compared to NPd14 were found to be involved in several biological process including ion transport (CHRNE, KCNMA1, COL1A1, COL1A2, COL3A1, COL6A1), cell cycle process (CDK5, SPC24, MLF1, Sep-04), nervous system development (DPYSL2, CDK5, SERPINF1), phosphorelation (CDK5, PRKAR2B, MYLK, RPS6KA5, MAPK10), transferase activity (NTRK2, DGKI, TXNDC6, RPS6KA5, MAPK10, MYLK, CDK5). On the other hand, immune system (TLR2, IL8, IL1RN, GBP5), cytokine activity (SPP1, IL1RN, IL8), transcriptional regulator (FOS. ELF1, BHLHB2, ATF3), pyrophosphate activity (MX1, TUBB2A, GBP5, CFT, RNF213) were among the biological or molecular functions overrepresented in genes elevated in NPd14.

The KEGG pathway analysis also indicated only three pathways to be significantly affected by those genes. The DE genes that were increased in NPd7 compared to NPd14 were found to be involved in focal adhesion (COL1A2, MYLK, MAPK10 and COL1A1),

MAPK signaling pathway (NTRK2, MAPK10 and RPS6KA5) and cell communication pathway (COL1A2 and COL1A1). On the other hand, those genes that increased in NPd14 were found to involve in toll-like receptor signaling pathway (FOS, TLR2, SPP1 and IL8).

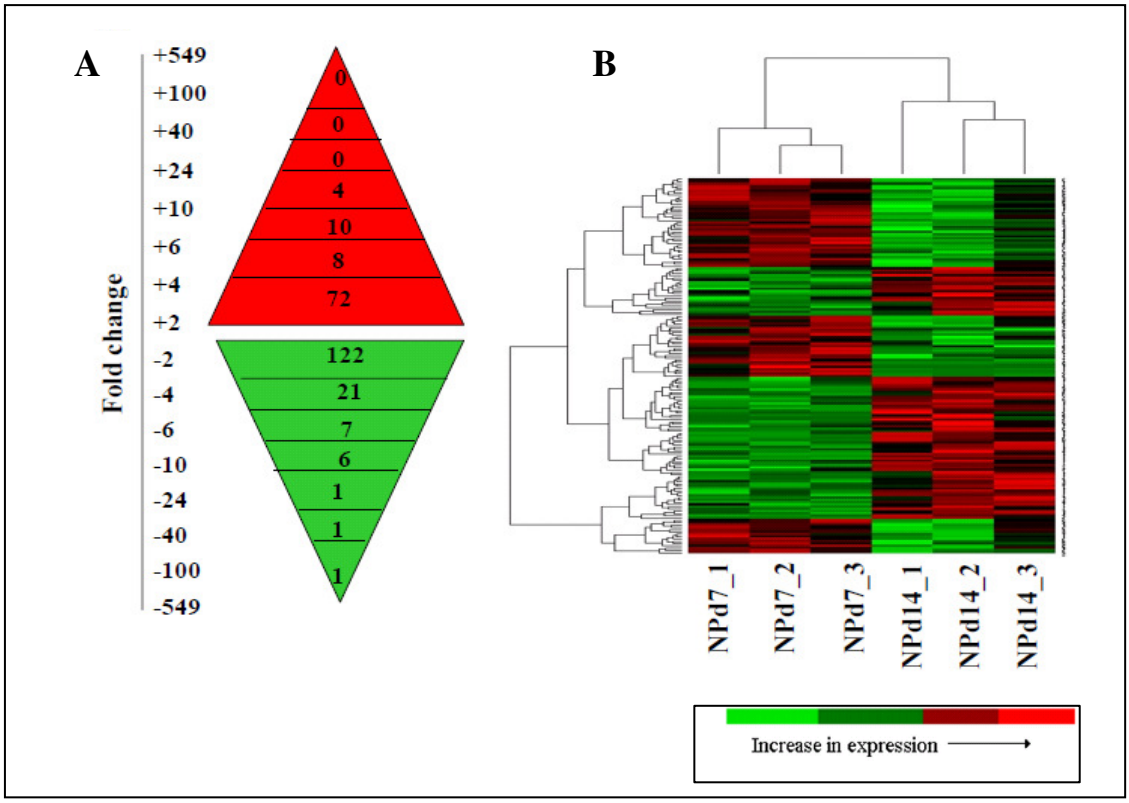

Figure 21: Pre-transfer transcriptome dynamics in non-receptive endometrium between day 7 and 14 of the estrous cycle. (A): The number and fold change distribution of DE genes identified between NPd7 and NPd14. (B): The heatmap describing the expression pattern of DE genes between NPd7 and NPd14. Labels on the horizontal axis of the heatmap, NPd7-1, NPd7-2 and NPd7-3 represent independent biological replicates from non-receptive endometrium groups at day 7 of the estrous cycle. NPd14-1, NPd14-2 and NPd14-3 represent independent biological replicates in non-receptive endometrium groups at day 14 of the estrous. Red and green colors reflect high and low expression, respectively.

4.3.3 Endometrial genes elevated at day 7 or day 14 of the estrous cycle irrespective of endometrial receptivity

After investigating the transcriptome dynamics of receptive and non-receptive endometrium between day 7 and 14 of the estrous cycle, the common genes that were

elevated at day 7 or 14 of the estrous cycle in both endometrial statuses were identified. The result revealed that 121 genes were differentially expressed both in CDd7 vs. CDd14 and NPd7 vs. NPd14. Among those, 86 genes elevated at day 14 and 35 genes were elevated at day 7 of the estrous (Figure 22). This result shows that the expression pattern of 121 genes between day 7 and 14 of the estrous cycle is independent of the endometrial receptivity. Those genes that were elevated at day 7 were found to be involved in regulation of transferase activity, protein kinase activity, phosphorylation, protein metabolic process and phosphate metabolic process. However, those increased in day 14 of the estrous cycle were found to be involved mainly in immune response, cytoskeleton organization and biological adhesion.

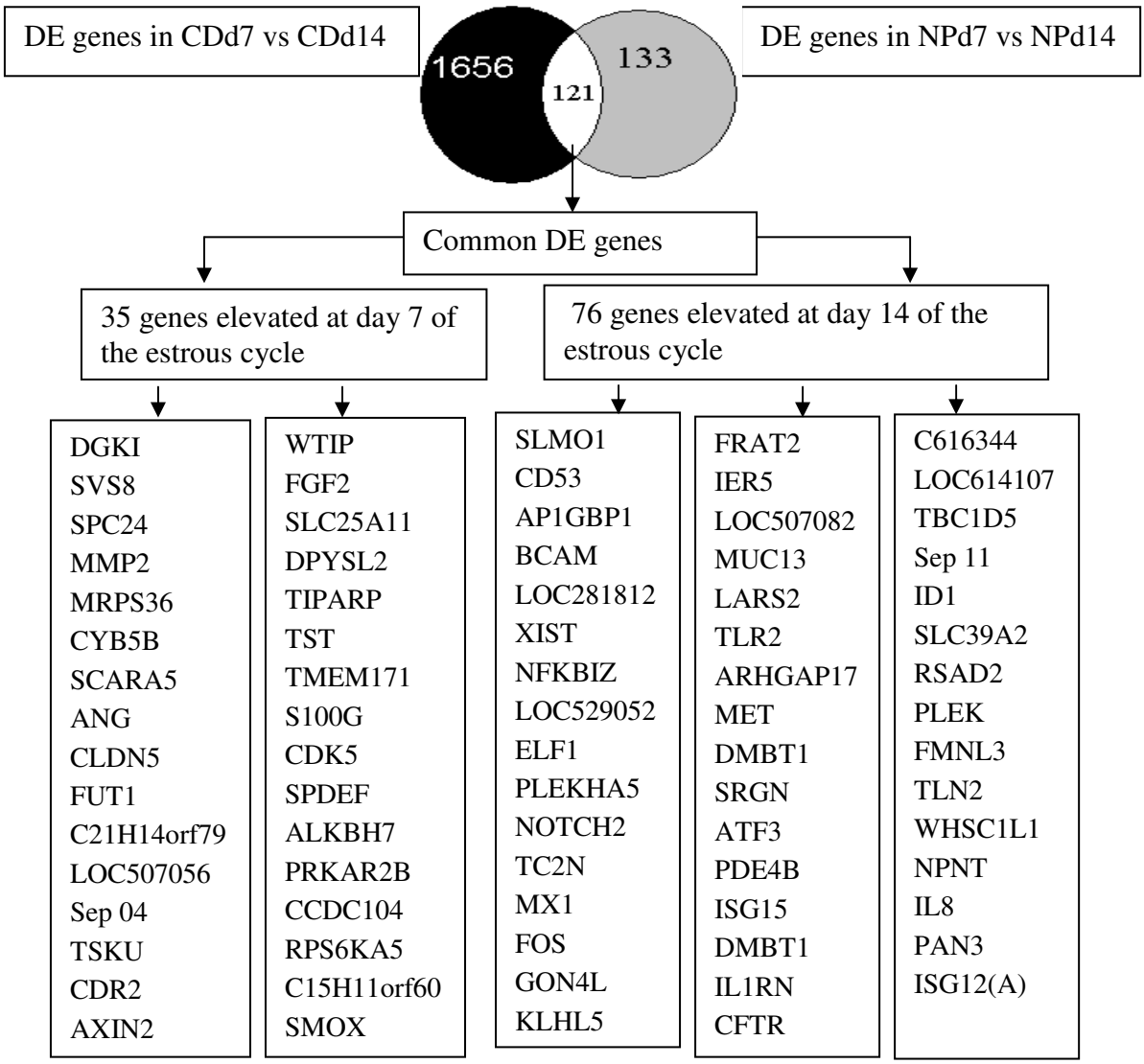

Figure 22: Genes elevated at day 7 or 14 of the estrous cycle both in receptive and non receptive endometrium.

4.4 Expression profile of selected candidate genes across the estrous cycle and early gestation period

Following detection of differentially expressed genes between the receptive (CDd7) and non-receptive (NPd7) endometrium, the genes involved in signal transduction (PTGER4, AGTR1, ITPR1, ADORA2B and WNT11), metalloendopeptidase activity (MMP2 and TIMP3), transcriptional factor activity (IRF6 and STAT5A), cell cycle (CDC20) and prostaglandin-endoperoxide synthase activity (PTGS2/COX-2) were analyzed in the endometrial samples collected from heifers slaughtered at different days of the estrous cycle and at day 50 of gestation period. The expression profile of those genes across the estrous cycle revealed that, the transcript level of IRF6, STAT5A, PTGER4, AGTR1, ITPR1, ADORA2B, WNT11, MMP2 and TIMP3 was found to be higher ($p < 0.05$) at day 7 and 14, but was lower ($p < 0.05$) at day 19 and 0 of the estrous cycle (Figure 23). Unlike other candidate genes, the expression pattern of PTGS2/COX-2 exhibited a dramatic increased ($p < 0.05$) from day 19 to day 0 followed by a decrease at day 3 and after wards it reached peak at day 7 and 14 of the estrous cycle (Figure 23). On the other hand the expression of CDC-20 was higher ($p < 0.05$) a day 3 of the estrous cycle. When the expression profile was extended until day 50 gestation period, the IRF6, ITPR1, STAT5A and AGTR1 exhibited an increase trend from proestrus to diestrus and reached peak in pregnant endometrium, but the expression of PTGER4, PTGS2/COX-2, TIMP3 and ADORAB2B was reduced in pregnant endometrium compared to the expression detected at day 7 and 14 of the estrous cycle of cyclic heifers (Figure 23). This shows that those genes exhibited higher or lower expression level during the estrous cycle can be further enhanced or reduced following embryo implantation.

The spatio-temporal expression of those candidate genes at different region of the endometrium also revealed lower expression ($p < 0.05$) in the corpus region compared to other endometrial region during the estrous cycle (Appendix 6). In addition, the majority of the candidate genes showed no significant difference between the ispislateral and contra lateral position of the endometrium at each day of the estrous cycle and early pregnancy (Appendix 6).

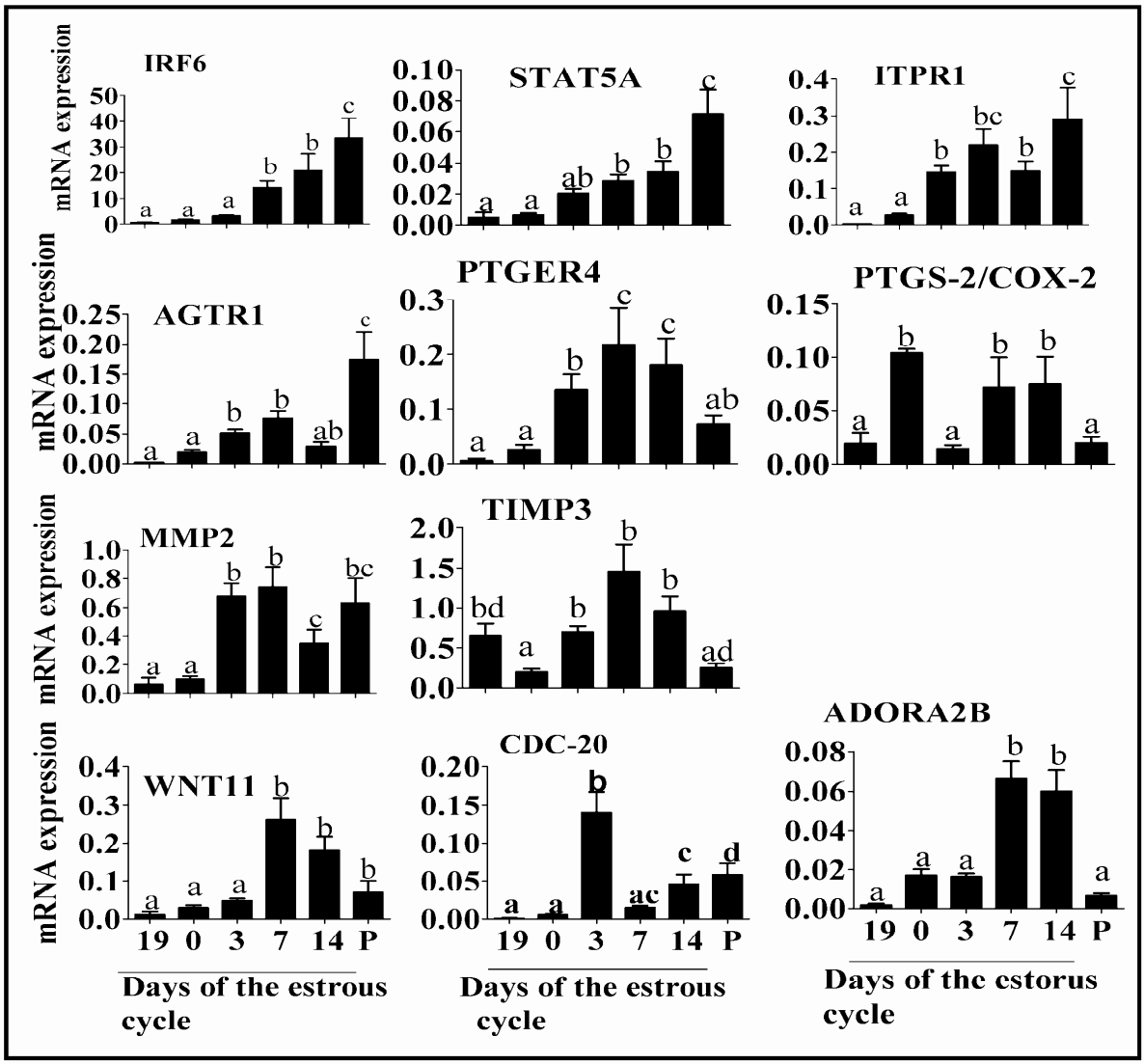

Figure 23: The expression pattern of selected candidate genes across the estrous cycle and at day 50 gestation period (P). The vertical axis indicates the relative mRNA expression level of candidate genes normalized to the mRNA level of GAPDH. Bars show mean ± s.e.m. Bars with different letters are statistically significant ($p < 0.05$).

4.5 Protein expression of selected candidate genes

Parallel to the mRNA quantification, the immunohistochemical localization of IRF6, ITPR1, PTGS2, MMP2 and TIMP3 was performed in the tissue sections taken at the ipsilateral middle part of the uterus. The result revealed the presence of spatio-temporal differences in protein signal intensities of those gens during the estrous cycle and early pregnancy (Figure 24). For instance strong intensity of ITPR1 protein was detected in day

3, 7, 14 and pregnant endometrium. The PTGS2 protein revealed strong signals in luminal epithelium (LE) and glandular epithelium (GE) at day 0 and 3. However, it was lower in LE and higher in GE at day 7, 14 and pregnant endometrium. Similarly, stronger signal MMP2 protein signal was detected in GE at day 0, 14 and pregnant endometrium. However, at day 7 and 19 the MMP2 protein was stronger in LE and stroma cells of the endometrium suggesting variation in expression in different layers of the endometrium. Lower protein signals of TIMP3 were detected at day 0 and pregnant endometrium. Moreover, except at day 0 and pregnant endometrium, strong signal was detected in LE, GE and stroma cells of the endometrium (Figure 24).

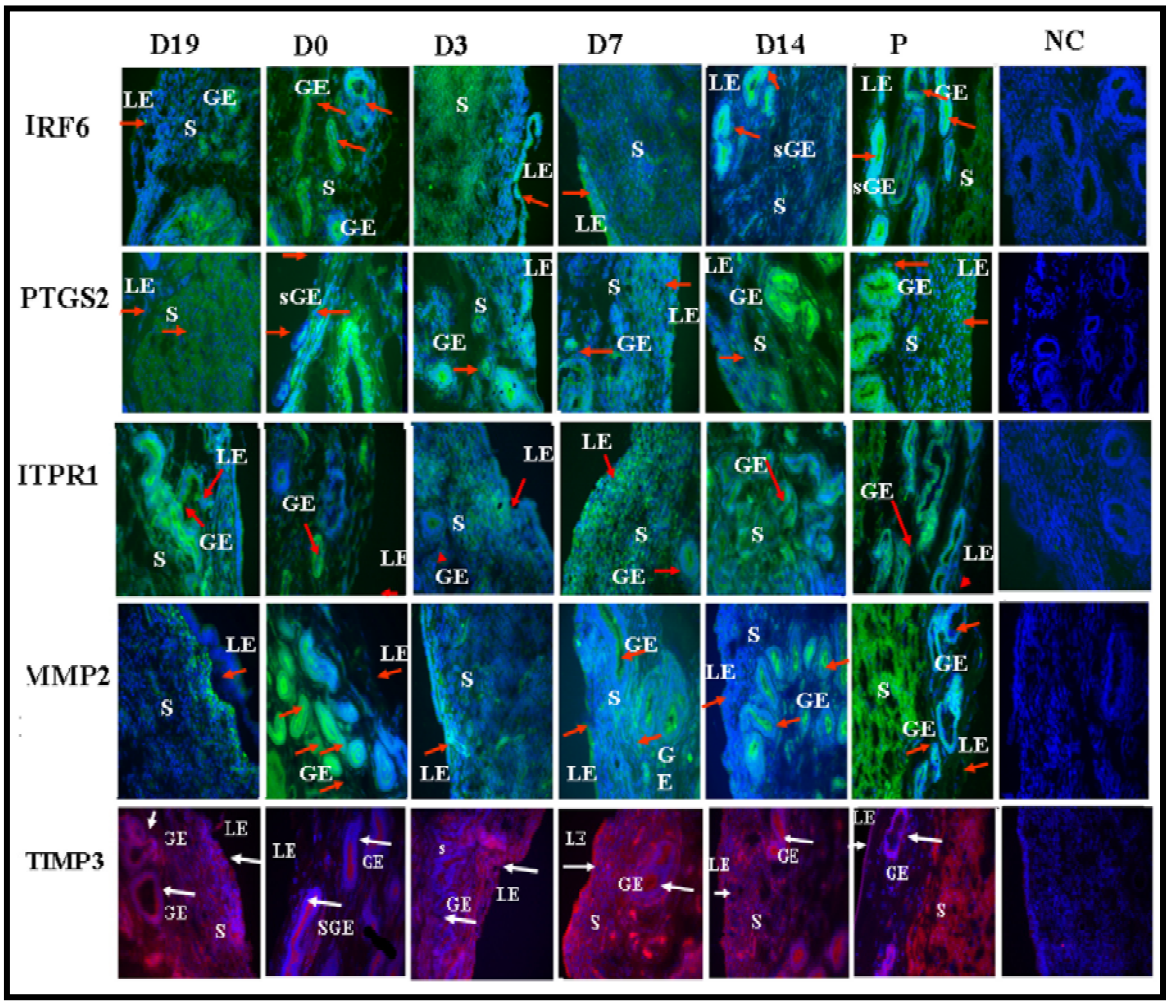

Figure 24: Immunohistochemical localization of selected candidate genes across the estrous cycle and day 50 gestation period (P). The green and red colors indicate the protein signal and the blue colors describe nuclear staining, respectively. NC = Negative control, sGE = Superficial glandular epithelium, GE = Glandular epithelium, LE = Luminal epithelium, S = stroma cells, D = day.

4.6 Progestrone level and mRNA expression of differentially expressed genes

Serum progesterone was assessed at each day of the estrous cycle to evaluate whether the expression of those differentially expressed genes were influenced by the level of progesterone. Following this, a correlation analysis was performed between the progesterone level and the mRNA expression of selected candidate genes (Table, 9). Among those, the STAT5A mRNA expression exhibited a significant positive correlation with progestrone level. However, STAT5A was down regulated in receptive endometrium and conversely upregulated in non-receptive endometrium. Other genes including IRF6, PTGER4 and ITPR1 showed a higher correlation coefficient but the correlation was not reached to 5% significant level (Table 8).

Table 8: Correlation between progestrone level and mRNA expression of DE genes during the estrus cycle

Gene	Correlation coefficient (r)	p value
IRF6	0.85	0.06
STAT5A	0.98	0.002
AGTR1	0.58	0.29
PTGR4	0.86	0.06
ITPR1	0.86	0.06
ADORA2B	0.79	0.11
WNT11	0.73	0.15
COX-2	0.01	0.98
MMP2	0.72	0.17
TIMP3	0.67	0.20
CD20	0.48	0.40

4.7 Gene expression difference between embryo biopsies that resulted in calf delivery and no pregnancy

In addition to endometrial gene expression, the transcriptome abundance difference between embryo biopsies that resulted in calf delivery or no pregnancy was determined using embryo biopsies that were retained prior to embryo transfer. For this, the transcriptional differences of embryo biopsies resulted in calf delivery were compared with those embryos biopsies resulted in no pregnancy using preimplantation embryo specific cDNA array in a series of six hybridization experiments (three biological replicates with dyeswap). The microarray data analysis using LIMMA revealed that including 9 ESTs, a total of 70 genes were differentially expressed between the two embryo groups. The average linkage clustering analysis (Figure 25) reveals that there were many subgroups within the up or down regulated gene (or clusters) sharing similar expression patterns. Such co-expressed genes may possibly sharing common biological function. Furthermore, the expression of each gene in each biological replication remains uniform indicating the reproducibility of the data (Figure 25).

The magnitude of differentially expressed (DE) genes showed that 20% of the of them exhibited two or more fold change difference and 80 % showed 1.5-2 fold change difference between the two embryo groups. Furthermore, the transcript level of 32 genes including SPAG17 (PF6), UBE2D3P, DFNB31, AMD1, DTNBP1NUP35, GDE1 (MIR16), ARL6IP, NUDT2, FAM161A, LGMN,LEO1, CCL16AP2B1, PTDSS1 and HSPA8 was abundant in those embryos resulted in calf delivery (Figure 25). On the other hand, the transcript level of 38 genes including SGK1, GBF1, FADS1, KRT8, RIF1, GART, DTX2, WDR26, RNF34, KPNA4, ARL8B, RYBP and WDR13 was higher in embryos resulted in no pregnancy. This differences indicated that the embryos that resulted in calf delivery are molecularly distinguishable from those resulted in no pregnancy.

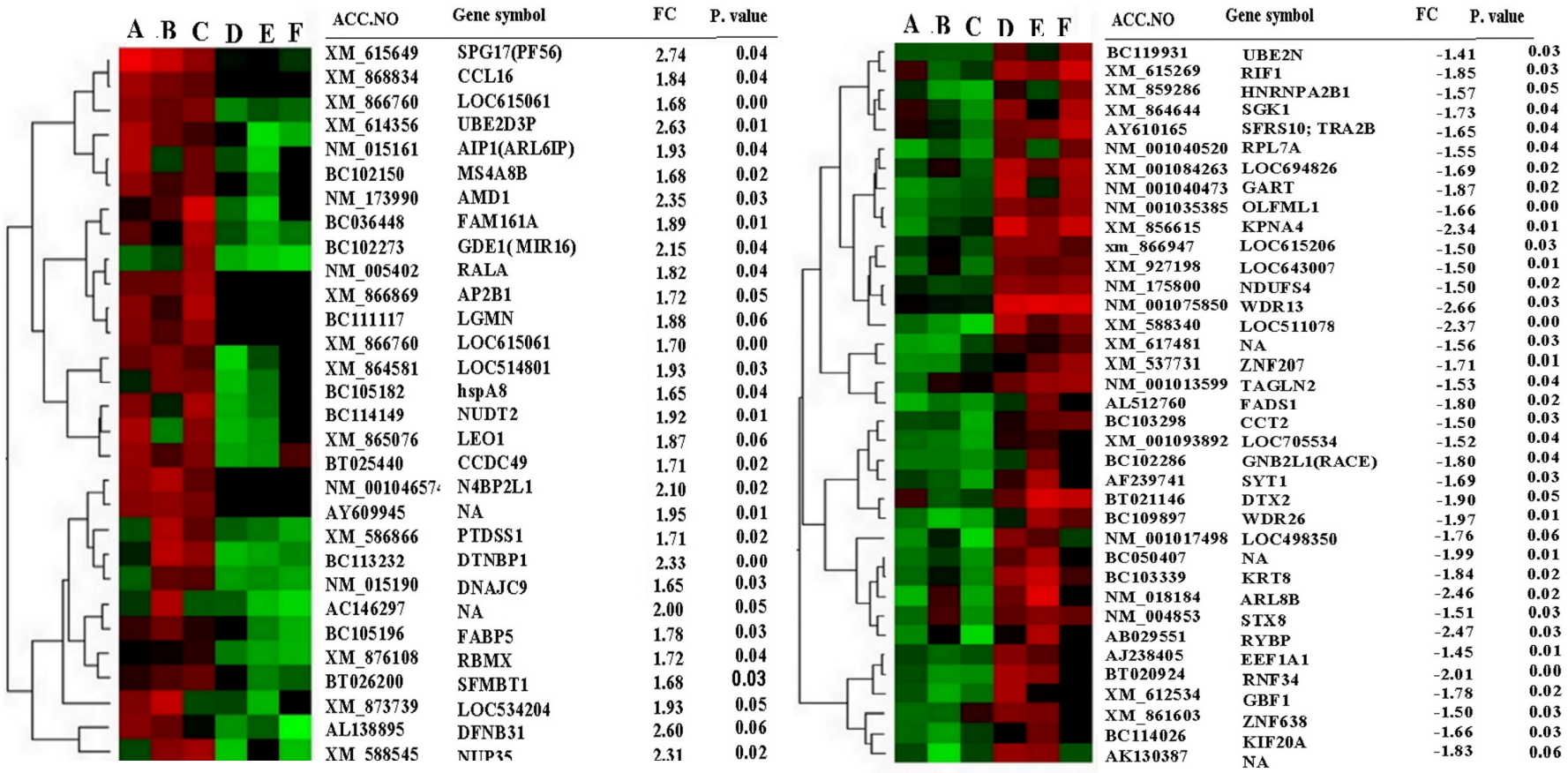

Figure 25: Hierarchical clustering of differentially expressed genes between embryo biopsies that resulted in calf delivery and no pregnancy. Embryo biopsies resulted in calf delivery were clustered in one group (left), while the embryo resulted in no pregnancy were clustered in the second group (left). A, B and C on the top of the heatmaps represent three biological replicates during hybridization and D, E and F describe the dyeswap hybridization for A, B and C replicates. Positive and negative numbers indicate genes increased in embryo biopsies resulted in calf delivery and no pregnancy groups, respectively. FC = Fold change, ACC. No = Gene bank accession numbers.

4.7.1 Functional classification of differentially expressed genes between embryo biopsies resulted in calf delivery and no pregnancy

The functions and relevance of the differentially expressed genes between the two embryo groups were assessed using the public databases, the gene ontology http://www.geneontology.org/ and DAVID functional annotation bioinformatics microarray analysis (http://david.abcc.ncifcrf.gov/). Thus, only 45 genes have known ontological function. Those gene were found to be involved in different biological and molecular functions (Table 9) including, protein binding, transferase activity, chemokine activity and cell to cell signalling, signal transduction, physiological response to stimulus, protein/mRNA transport, regulation of transcription, adenosylmethionine decarboxylase activity and apoptosis. Moreover, 17 DE genes were mapped to the known KEGG pathways (Figure 26).

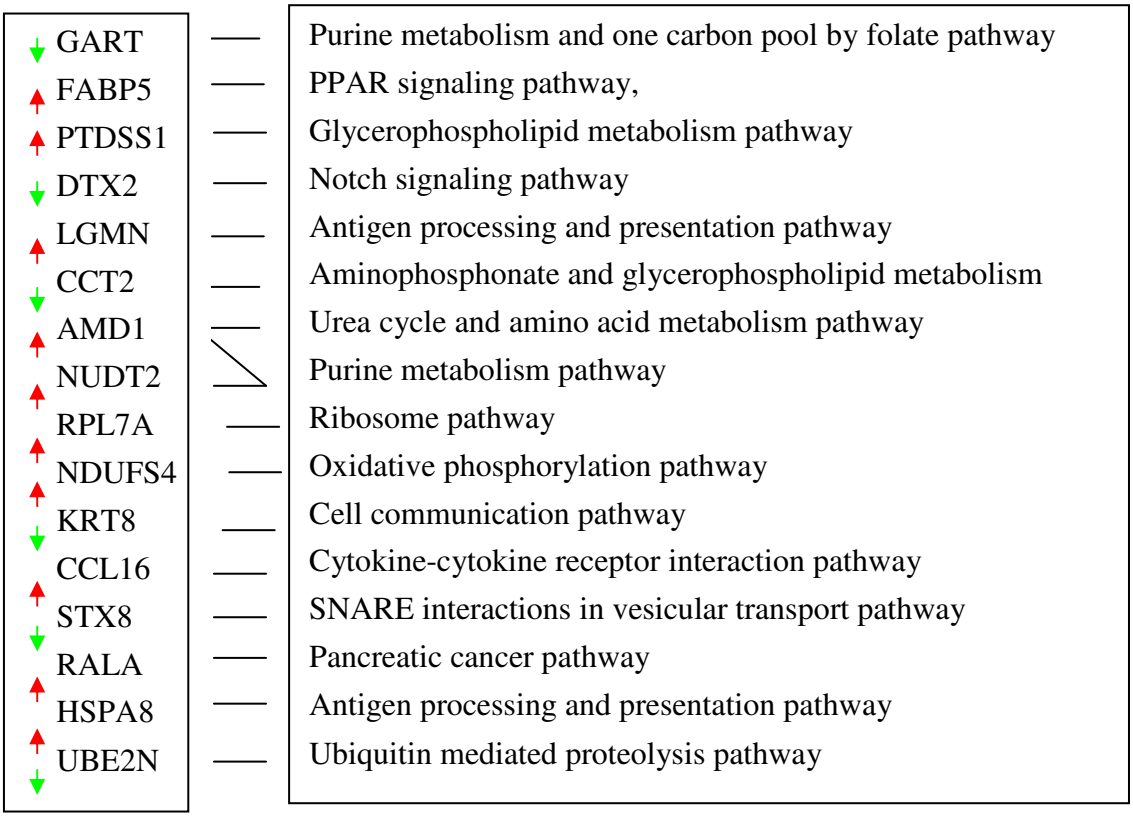

Figure 26: List of molecular signaling and metabolic pathways involved by DE genes between embryo biopsies resulted in calf delivery and no pregnancy. Red and green arrows indicated genes enriched and reduced, respectively in embryo biopsies resulted in calf delivery compared to embryo resulted in no pregnancy.

Table 9: Molecular function and biological process of DE genes between embryo biopsies resulted in calf delivery and no pregnancy

Acc. No	Gene symbol	Gene bank name	Molecular function	Biological process
XM_615649	SPAG17	Sperm associated antigen 17	Protein binding	-
AL138895	DFNB31	Deafness, autosomal recessive 31	Protein binding	Response to stimulus
NM_173990	AMD1	S-adenosylmethionine decarboxylase 1	Adenosylmethionine decarboxylase activity	Spermidine biosynthetic process
BC113232	DTNBP1	Dystrobrevin binding protein 1	Identical protein binding	Response to stimulus
XM_588545	NUP35	Nucleoporin 35	-	mRNA and protein transport
BC102273	GDE1	Glycerophosphodiester phosphodiesterase 1	Glycerophosphodiester	Glycerol metabolic process
NM_015161	ARL6IP	Adp-ribosylation factor-like 6 interacting protein	Protein binding	Co-translational protein targeting to membrane
BC114149	NUDT2	Nucleoside diphosphate linked moiety X	GTP binding	Induction of apoptosis
BC111117	LGMN	Legumain	Protein serine/threonine kinase activity	Proteolysis
XM_865076	LEO1	Leo1, paf1/RNA polymerase II complex component, homolog	Protein binding	Regulation of transcription
XM_868834	CCL16	Chemokine (C-C motif) ligand 16	Chemokine activity, cell-cell signaling	Immune response inflammatory response
NM_005402	RALA	Ras-related protein	GTP binding, signal transduction	Chemotaxis
BC105196	FABP5	Fatty acid binding protein 5	Fatty acid binding, protein binding, transporter activity	Lipid metabolic process transport

Table 9: Cont.

Acc. No	Gene symbol	Gene bank name	Molecular function	Biological process
XM_866869	AP2B1	Adaptor-related protein complex 2, beta 1 subunit	protein transporter activity	-
XM_586866	PTDSS1	Phosphatidylserine synthase 1	Transferase activity	-
BC102150	MS4A8B	Membrane-spanning 4-domains, subfamily a, member 8B	Receptor activity	-
BC105182	HSPA8	Heat shock 70kd protein 8	ATP and protein binding	Protein folding
NM_015190	DNAJC9	DNAJ (HSP40) homolog, subfamily c, member 9	Heat shock protein binding	-
XM_876108	RBMX	RNA binding motif protein, X-linked	RNA and protein binding, RNA splicing	Nuclear mRNA splicing
XM_861603	ZNF638	Zinc finger protein 638	DNA binding	RNA splicing
BC102286	GNB2L1	Guanine nucleotide binding protein (G-protein), beta polypeptide 2-like 1	Receptor binding	-
NM_004853	STX8	Syntaxin 8	-	Transport
BC103298	CCT2	Chaperonin containing tcp1, subunit 2	ATP and unfolded protein binding	Protein folding
NM_001013599	TAGLN2	Transgelin 2	Protein binding	-
NM_175800	NDUFS4	NADH dehydrogenase (ubiquinone) fe-s protein	NADH dehydrogenase activity	Electron transport
NM_001040520	RPL7A	Ribosomal protein l7A	RNA binidng	Ribosome biogenesis translational elongation
XM_859286	HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1	RNA splicing factor activity	RNA splicing and transport
AY610165	SFRS10	Splicing factor, arginine/serine-rich 10	RNA splicing factor activity	RNA splicing
AF239741	SYT1	Synaptotagmin 1	Calcium and calmodulin binding	Synaptic transmission

Table 9: Cont.

Acc. No	Gene symbol	Gene bank name	Molecular function	Biological process
BC114026	KIF20A	Kinesin family member 20A	ATP and nucleotide binding	Protein transport
NM_001035385	OLFML1	Olfactomedin-like 1		Regulation of cell proliferation
XM_537731	ZNF207	Zinc finger protein 207	Transcription factor activity	Transcription regulation
BC050407	ZNF83	Zinc finger protein 83	Transcription factor activity	Transcription regulation
XM_864644	SGK1	Serum/glucocorticoid regulated kinase1	Protein serine/threonine kinase activity, transferase activity	Apoptosis sodium ion transport
XM_612534	GBF1	Golgi brefeldin a resistant guanine nucleotide exchange factor 1	ARF guanyl-nucleotide exchange factor activity	Regulation of ARF protein signal transduction
AL512760	FADS1	Fatty acid desaturase 1	Oxireductase activity	Cell to cell signaling
BC103339	KRT8	Keratin 8	Protein binding	Apoptosis
XM_615269	RIF1	Rap1 interacting factor 1 homolog	Binding	Cell cycle
NM_001040473	GART	Phosphoribosylglycinamide formyltransferase	Transferase activity	Biosynthetic process
BT021146	DTX2	Deltex homolog 2	Metal and protein ion binding	Notch signaling pathway
BC109897	WDR26	Wd repeat domain 26	-	Cell signal transduction
BT020924	RNF34	Ring finger protein 34	Ligase activity, protein and metal ion binding	Apoptosis
XM_856615	KPNA4	Karyopherin alpha 4	Protein transporter activity	intracellular protein transport
NM_018184	ARL8B	Adp-ribosylation factor-like 8B	Nucleotide binding	Small GTPase mediated signal transduction
AB029551	RYBP	RING1 and YY1 binding protein	Apoptosis	Regulation of transcription

4.8 Validation of microarray data using real time quantitative PCR (qPCR)

4.8.1 Validation of differentially expressed genes between receptive and non receptive endometrium at day 7 of the estrous cycle

A total of 36 DE genes between CDd7 and NPd7 were randomly selected. Hence, the qPCR data revealed that 32 out of 36 DE genes (89%) to be according to the microarray results (Table 10, Appendix 7). However, CAMK2D exhibited a reverse trend and NGF and MYLK were not different between the two groups.

Table 10: Validation of DE genes between receptive (CDd7) and non-receptive (NPd7) endometrium at day 7 of the estrous cycle using qPCR

Gene name	ACC. No	Microarray results		qPCR results	
		FC	p value	FC	p value
ADORA2B	NM_001075925	2.17	0.007	18.81	0.03
AGTR1	NM_174233	-4.0	0.004	-18.4	0.05
ALG8	NM_001076125	2.55	0.002	9.34	0.03
CNB2	NM_174264	4.88	0.02	6.94	0.02
CDC2	NM_174016	7.42	0.0017	23.84	0.01
COL1A2	NM_174520	9.18	0.001	5.24	0.09
FUT8	NM_177501	2.15	0.03	5.85	0.006
MAPK12	NM_001098953	2.97	0.03	11.17	0.04
PLCB1	NM_174817	3.24	0.017	8.61	0.05
PTGER4	NM_174589	2.72	0.01	4.94	0.05
WNT11	NM_001082456	5.99	0.04	176	0.02
IL18	NM_174091	-2.49	0.002	-2.95	0.12
TIMP3	NM_174473	5.32	0.015	5.3	0.03
MMP2	NM_174745	-2.58	0.014	-7.1	0.12
ITPR1	NM_174841	5.35	0.01	3.12	0.05
IRF6	NM_001076934	3.39	0.04	80	0.03

FC= Fold change, p value $\leq$ 0.05 considered as significant, positive and negative values indicate genes elevated in CDd7 and NPd7, respectively.

4.8.2 Validation of differentially expressed genes between day 7 and 14 of the estrous cycle in receptive endometrium

A total of 28 DE genes between day 7 (CDd7) and day 14 (CDd14) in receptive endometrium were selected. These genes were also differentially expressed between CDd7 and NPd7. The qPCR data revealed 24 genes from 28 genes (85%) followed a similar trend to microarray results, but four genes, namely IL18, S100A13, TLR6 and PLK4 showed no fold change difference between the two groups (Table 11).

Table 11: Validation of DE genes between CDd7 and CDd14 in receptive endometrium using qPCR

Gene symbol	ACC. No	Microarray results		qPCR results	
		FC	p value	FC	p value
S100B	NM_001034555	3.14	0.009	14.5	0.05
MMP2	NM_174745	2.82	0.009	43.9	0.09
PTGS2	NM_174445	14.13	0.01	3.2	0.06
WNT11	NM_001082456	14.5	0.007	93.0	0.007
IFR6	NM_001076934	3.53	0.035	31.2	0.03
TIMP3	NM_174473	15.0	0.009	6.72	0.02
PTGER4	NM_174589	6.0	0.0003	4.0	0.05
CDC20	NM_001082436	16.0	0.005	130.0	0.01
ADORA2B	NM_001075925	2.2	0.007	12.78	0.07
ALG8	NM_001076125	2.7	0.001	44.0	0.03
CDC2	NM_174016	2.88	0.007	38.3	0.04
FUT8	NM_177501	2.0	0.04	21.67	0.09
ITPR1	NM_174841	16.0	0.009	23.0	0.07
STAT5A	NM_001012673	-2.59	0.02	-2.48	0.07
IL18	NM_174091	-2.45	0.0003	1.09	0.96
S100A3	NM_205800	-2.16	0.23	-1.16	0.84
TLR6	NM_001001159	-2.3	0.023	-1.15	0.94

FC= Fold change, p value ≤ 0.05 considered as significant, positive and negative values indicate genes elevated in CDd7 and CDd14, respectively

4.8.3 Validation of differentially expressed genes between embryo biopsies resulted in calf delivery and no pregnancy

Eight differentially expressed genes between the embryo biopsies resulted in calf delivery and no pregnancy, namely AMD1, HSPA8, STX8, SGK1, DTX2, RNF34, ARL8B and UBE3D2P were validated using qPCR and the results indicated that the microarray result and the qPCR was fitting to each other (Table 12).

Table 12: Validation of DE genes between embryo biopsies resulted in calf delivery and no pregnancy using qPCR

		Microarray results		qPCR results	
Gene symbol	ACC. No	FC	p value	FC	p value
AMD1	NM_173990	2.35	0.03	3.22	0.02
HSPA8	BC105182	1.65	0.03	2.93	0.2
STX8	NM_004853	-1.5	0.02	-57.8	0.02
SGK1	XM_864644	-1.7	0.04	-2.59	0.05
DTX2	BT021146	-1.9	0.05	-1501	< 0.0001
RNF34	BT020924	-2.0	0.002	-55.1	0.02
ARL8B	NM_018184	-2.5	0.028	-7.61	0.25
UBE3D2P	XM_614356	2.6	0.01	83.7	0.4

FC= Fold change, p value ≤ 0.05 was considered as significantly different. Positive and negative values indicate genes enriched in embryos resulted in calf delivery and embryos resulted in no pregnancy, respectively.

5 Discussion

5 .1 Transcriptome difference between receptive and non-receptive bovine endometrium

Although embryo transfer has been widely used to utilize genetically superior cows and circumvent infertility, several recipients end up with no pregnancy after embryo transfer. This pregnancy failure has been exerting a challenge on selecting and maintaining suitable recipients for embryo transfer. Successful pregnancy is the result of coordinated and synchronized crosstalk between the blastocyst and the endometrium in a temporal and cell specific manner (Bowen and Burghardt 2000, Simon et al. 2000, Spell et al. 2001). The dynamic interaction between the embryo and endometrium can be stimulated provided both the endometrium and embryos are enriched with key molecular mediators and signaling pathways at the time of arrival of the embryo into the uterus. Thus, understanding the molecular changes that occur in endometrium and embryo during the time of embryo transfer is fundamental to understand the molecular mechanisms related establishment of pregnancy. Therefore, in the present study a direct connection was established between the bovine pre-transfer endometrial and embryonic gene expression and the pregnancy outcome to identifying maternal and embryonic genes that could presumably associated with pregnancy success. For this, the pre-transfer endometrial samples collected at day 7 and 14 of the estrous cycle were used to investigate the endometrial genes associated with pregnancy establishment and the transcriptome of the embryo biopsies retained from transferred embryos were analyzed to identify embryonic genes associated with pregnancy establishment.

The gene expression from pre-transfer endometrial samples evidenced noticeable endometrial gene expression differences between heifers that resulted in calf delivery (receptive endometrium) and those resulted in no pregnancy (non-receptive endometrium) at day 7 of the estrous cycle. Detailed bioinformatics and literature mining approach was applied to characterize those differentially expressed genes with respect to bovine endometrial receptivity and embryo implantation. Thus, KEGG pathway analysis, biological processes and molecular functions analysis of gene ontology and molecular network analysis using IPA evidenced the presence of transcriptomic functional alteration between receptive and non-receptive endometrium at day 7 of the estrous cycle (Figures 9, 10, 11, 12, 13, Table 6, Appendix 3). However, the two groups of the endometrium tended to exhibit minimum transcriptome expression profile and transcriptomic functional

alteration at day 14 of the estrous cycle (Figure 17). Here the fundamental question to be raised is what contributes for higher transcriptome profile and transcriptomic function alteration in receptive and non receptive endometrium at day 7 than day 14 of the estrous cycle. Although further experimentation is necessary to answer this question, the higher gene expression differences between receptive and non-receptive endometrium at day 7 than day 14 of the estrous cycle may be attributed to the degree of endometrial response to the circulating progesterone. The progressively increasing level of progestrone may induce the expression of two sets of genes in receptive endometrium. One set to be those genes triggered in the expectation of embryo and the second groups to be those associated with induction of follicular waves. Therefore, unlike the non-receptive endometrium, at day 7 of the estrous cycle, the receptive endometrium may be enriched with several molecules that are associated with the establishment of pregnancy. However, from day 14 onwards, the progesterone that regulates the uterine environment loses its ability to block formation of oxytocin (Senger 2003). The endometrial $PGF_2\alpha$ then starts to increase on day 14 and reaches its peak from day 15 to day 17 of the estrous cycle consequently resulting in luteolysis of corpus luteum (Shemesh and Hansel 1975). Therefore, those groups of genes induced or reduced in receptive endometrium in the expectation of the incoming embryo during day 7 of the estrous may gradually decline during day 14 of the estrous cycle. This may consequently result in little or no gene expression differences between receptive and non-receptive endometrium at day 14 of the estrous cycle. Thus, this result may suggest that the receptive and non-receptive endometrium can be molecularly distinguishable at day 7 than day 14 of the estrous cycle. Therefore, the discussion part of this finding mainly focuses on transcriptional difference between the receptive and non-receptive endometrium that has been occurred at day 7 of the estrous cycle where 1126 genes and uncharacterized expresses sequences tags were exhibited difference in transcriptome abundances between the two statuses of the endometrium. Therefore, in the following section, the biological relevance of differentially expressed gene clusters in relation to endometrial receptivity and embryo implantation is presented.

5.1.1 Differentially expressed gene clusters between receptive and non-receptive endometrium at day 7 of the estrous cycle

Once the embryo reaches the uterus for implantation, the molecular dialogue between the implanting conceptus and the endometrium is mediated by several molecules of

endometrial and/or embryo origin (Dey et al. 2004, Paria et al. 2002, Tabibzadeh and Babaknia 1995, Wang and Dey 2006, Wolf et al. 2003). Identifying those molecules in endometrium and embryo prior to embryo transfer may help to select receptive endometrium and embryos with higher implantation potential. In line to this, in the present study 1126 genes consisting of several gene clusters were differentially expressed between the receptive and non-receptive endometrium at day 7 of the estrous cycle (Figures 5, 6, 7 and 8, Appendix 2). This may suggest that the occurrence of different molecular events in the endometrium whose expression is either induced or turned off in the expectation of blastocysts arrival into the uterus.

5.1.1.1 The solute carriers and transmembrane proteins

In the current study, 18 solute carrier (SLC) genes (SLC10A3, SLC16A13, SLC1A1, SLC25A11, SLC25A13, SLC25A22, SLC25A29, SLC25A40, SLC25A5, SLC35B1, SLC38A7, SLC39A8, SLC39A9, SLC4A8, SLC5A6, SLC6A20 ,SLC6A8, SLC7A5 and SLCO3A1) were among several gene clusters that has been increased in receptive endometrium (CPd7) compared to non-receptive endometrium (NPd7) (Figure 7). Among those, SLC1A1 was found to be higher in pregnant compared to non-pregnant heifers (Bauersachs et al. 2006). Moreover, SLC1A1 and SLC6A8 were reported to be up regulated in receptive human endometrium (Riesewijk et al. 2003) and during window human implantation (Kao et al. 2002), respectively.

The gene ontological classification showed that the solute carriers (SLC) are involved in transport of cellular materials across the cell membrane (Fig 13A). Furthermore, the SLC genes are generally believed to be involved in transport of cellular materials across the cell membrane (Ishida and Kawakita 2004, Kanai and Hediger 2004, McKie and Barlow 2004, Palmieri 2004). This result may suggest the increased solute and protein transportation across the cell membrane of the receptive endometrium compared to non-receptive endometrium. In addition, other gene cluster with similar function to SLC, including transmembrane proteins (TMEM141, TMEM144, TMEM163, TMEM16K, TMEM171 and TMEM79) and the voltage- dependant anion channel 1 (VDAC1) were enriched in receptive endometrium. The report of (Abu-Hamad et al. 2006, Abu-Hamad et al. 2009) also indicated that cells with low levels of VDAC1 were found to exhibit reduced ATP-synthesis capacity and limited metabolite exchange between mitochondria and cytosol. This in turn suggests increased level of solute carriers or transmembrane proteins or gatekeepers genes are required to efficient removal or entrance of molecule from the cell.

Therefore, the enrichment of solute carrier genes, transmembrane and other mitochondrial gatekeeper genes in receptive compared to the non-receptive endometrium at day 7 of the estrous cycle may suggest the presence of effective metabolite exchange between mitochondria and cytoplasm in receptive endometrium.

5.1.1.2 Cargo protein sorters, transporter and neuro transmitter gene clusters

In addition to the SLC, transmembrane gene clusters, the transcript level of adaptor related protein and their subunits (AP1-S1, AP3-B1, AP3-M1 and AP3-S2) were increased in the receptive compared to the non-receptive endometrium (Figure 7). Among the four adapter related proteins (AP1, AP3, AP2 and AP4) known in mammalian cells (Boehm and Bonifacino 2001, Boehm and Bonifacino 2002), the current study identified the first two to be higher in receptive compared to non-receptive bovine endometrium at day 7 of the estrous cycle. Although their function contribution is not yet known in bovine endometrial receptivity, ontological classification indicated that adaptor related protein and their subunits were found to be involved in protein localization (transport) and cellular localization (Figure 10B and C) and also they are central foucs genes in genetic disorder, lymphoid tissue and development molecular network (Figure 16). Similarly, other authors (Boehm and Bonifacino 2001, Newell-Litwa et al. 2007) indicated that adaptor related proteins are required for protein cargo sorting, trafficking of integral membrane proteins and neurotransmission. For instance, adapter related protein 1 (AP1) is reported to be involved in the organization and transport of proteins within the cell (Montpetit et al. 2008). In addition, the AP-1 is associated with the trans-Golgi network for transport proteins to the endosomal/lysosomal system and to the cell surface (Kim and Hersh 2004). Furthermore, it also generates vesicles from the trans-Golgi network for transporting cargoes to late-endosome and lysosome compartments, whereas AP-1 adaptors found in endosomes generate vesicles transporting to the cell surface or back to the Golgi complex (Newell-Litwa et al. 2007). Similarly, adapter related protein 3 (AP3) is believed to be essential for neurotransmitting and ion transport (Newell-Litwa et al. 2007), trafficking proteins to lysosomes and formation of lysosome-related organelles (Kantheti et al. 1998, Newell-Litwa et al. 2007, Simpson et al. 1997). In addition, AP-3 is important in neuronal protein trafficking and functions in the formation of synaptic vesicles from endosomes (Kim and Hersh 2004).

Similarly, the expression level of seven kinesin genes (KIF20A/MKLP2, KIF21A, KIF24, KIF4A, KIF22/KID, KIF2A and KIF2C/MCAK) which have similar function to adapter related proteins were found to be higher in receptive compared to non-receptive endometrium (Figure 7). According to gene ontological classification, those kinesin gene clusters were found to be involved in cellular localization (Figure 10C). Kinesins are motor proteins required to regulate cargo transporting of intra cellular components, vesicles and several organelles including mitochondria (Fisher and Kolomeisky 2001, Hirokawa et al. 2009). For instance, kinesin family member 20A (KIF20A) is required to control the intra-Golgi transport. Kinesin family member 4A (KIF4A) and kinesin family member C2 (KIFC2) are implicated in neuron functioning in which the former is required for transporting cell adhesion molecule required in axonal elongation (Peretti et al. 2000) and the later may involve in transporting of multi vesicular body like (mvb-like) organelles in dendrites (Saito et al. 1997). Therefore, it is possible to speculate that the increased expression level of adapter related proteins and kinesin in receptive may required to enhance neurotransmission or to increase accumulation and transduction of energy by trafficking of integral membrane proteins and cellular materials including adhesion molecule, organelles and vesicles. Apart from this, the increased level of adapter related protein in receptive bovine endometrium might be associated with increased number of lysosomes that could be required for uterine receptivity and embryo implantation.

5.1.1.3 The cell cycle, anti and pro apoptosis genes

In the current study, the cell cycle gene clusters (CDC2, CDC20, CDC25C, CDC42SE2, CDCA2 and CDCA4) and other cell cycle related genes CCNA2, CCNB2, AURKB, CDCA2, NUSAP1, CKS2 and JAG2 were increased in receptive endometrium compared to non receptive endometrium. The involvement of these genes during cell cycle has been reported by different authors (Amabile et al. 2009, Borysov and Guadagno 2008, Ge et al. 2009, Katsuno et al. 2009, Turowski et al. 2003, Wolthuis et al. 2008, Yoon et al. 2004). Therefore, the increased expression of cell cycle related genes in receptive endometrium could be associated with increase in the size and development of luminal and glandular epithelium of the receptive endometrium that may be required for preparing the uterus for implantation.

In the current study, the increased level of cell cycle related genes was accompanied by increased expression level of anti apoptosis genes (BCL2, VEGFB, EDN1, MYB, CDC2, BIRC5, VDAC1 and GCLC) and reduced expression level of the pro-apoptotic genes (PRKRA, CASP4, PYCARD, LCK, TP53I3/PIG3 and MYC). Apoptosis normally involves several biochemical events resulting in morphological changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. Although, the balance between cell death and proliferation is not well known during the receptive phase of bovine endometrium just before embryo implantation, the increase level of anti-apoptotic and reduced level of pro-apoptotic genes in receptive endometrium may provide a specific advantage for the epithelial cell of the receptive endometrium to escape the apoptosis induced signals and consequently can exhibit lower frequency of apoptosis accompanied by proliferation. For example, (Daikoku 1998) indicated that the BCL2 deficient mice exhibited many apoptotic. Similarly, strong expression of BCL2 protein in the endometrium glands was also reported (Gompel et al. 1994). Moreover, the BCL2 may be an essential gene product for the survival of endometrial glandular cells (Tabibzadeh et al. 1995). Similarly, survivin (BIRC5) is required fore endometriotic implant growth promotion (Tarkowski et al. 2001). Contradictory reports are available on the importance of survivin during pregnancy and implantation. In one report, it has been described that high survivin expression in abortion cases than the normal pregnancy in rats (Garcia et al. 2007). However, (Fest et al. 2008) reported similar survivin expression pattern between non pregnant and aborted counter parts. Furthermore, these authors indicated a 5-fold increase in the expression of survivin in pregnant compared to non-pregnant rat suggesting the involvement of survivin during implantation. The vascular growth factor B (VGEFB), which shares a high degree of sequence homology with vascular growth factor (VEGF) and phosphatidylinositol glycan anchor biosynthesis class F (PIGF), is a potent apoptosis inhibition by suppressing the expression of BH3 protein (Li et al. 2008). Increased level of VEGFB stimulates endothelial cell growth and proliferation (Olofsson et al. 1998).

The pro-apoptotic genes may influence the preparation of the uterus for receptivity and implantation by facilitating the death of cells than they divide ultimately leading to loss of endometrial epithelial cells. This may in turn resulted in loss of endometrial receptivity for implantation. Hence, in the current finding, the down-regulation of pro-apoptotic genes in receptive endometrium may suggest faster proliferation than cell death in endometrial cells. For instance, in the current result, one of the pro-apoptotic gene v-myc

myelocytomatosis viral oncogene homolog (v-MYC) was down regulated in receptive endometrium compared to non receptive endometrium. This gene is believed control several genes in embryonic stem cells that function in biological processes (Kidder et al. 2008). Similarly, in the current study, MYC was found to be the central gene influencing in the number genes involving in cell cycle, cell death and reproductive system diseases network (Figure 15). Furthermore, in appropriate expression of MYC leads to enhanced apoptosis or inappropriate expression is correlated with a wide array of human malignancies (Askew et al. 1991, Evan et al. 1992, O'Connell et al. 2003, Shi et al. 1992) due to accumulation of p53 (Tabibzadeh et al. 1995). Moreover, over-expression and localization of the myc product may have an important role in the initiation, differentiation and progression of endometrial carcinoma (Bai et al. 1994). Furthermore, elevated levels of MYC gene expression in neoplastic cells from all seven bovine leukemia viruses induced bovine tumors (Gupta et al. 1986). This may suggest that the reduced level of v-MYC including other proapoptotic genes and increased level of anti-apoptotic genes in receptive endometrium may suggest faster cellular proliferation and differentiation to prepare the endometrium for substantial remodelling.

5.1.1.4 The protein phosphatases and protein kinases

The protein phosphatases (PPM1G/PP2CG, PPP1CB, PPP1R10/PNUT3 and PPP2R5A) and protein kinases (PRKCQ, PRKCI, PRKAG2) (Figure 7) were among several clusters of genes increased in receptive compared to endometrium. The functional contribution of protein phosphatases and protein kinases in preparing mammalian endometrium for receptivity and embryo implantation is not yet well documented. The protein phosphatases and protein kinases gene clusters are believed to function together in a coordinated manner to dictate the level of protein phosphorylation in the cell. The protein phosphatases involves in protein phosphorylation and posttranslational modification and protein kinases acts as effectors of phosphorylation (Eyster et al. 2007). The coordinated activity of protein kinase C (PRKC) and protein phosphatases during the pregnancy period of rat have been also documented by several authors (Eyster 1993, Eyster et al. 1998, Gibori et al. 1988). For example, the protein phosphatase gamma (PPM1G) regulates protein stability (Suh et al. 2009) and smooth muscle cell survival (Tchivilev et al. 2008). On the other hand, protein phosphatase 1 regulatory subunit 10 (PPP1R10) controls the expression of genes that are critical for certain physiological and pathological processes including Rb-

phosphatase activity and apoptosis (De Leon et al. 2008, Lee et al. 2009). Similarly, among the protein kinases, kinase AMP-activated gamma 2 (PRKAG2) regulates chemical pathways in restoring the balance of ATP during chemical reactions that require ATP and protein kinase C (PKC) is known to be against apoptosis and required for cell proliferation (Grossoni et al. 2007). Apart from this, those protein kinases and phosphatases enriched in receptive endometrium were found to be the main constituent of the tight junction pathway (Figure 14, table 6). Tight junction pathway involves in various signaling events including controlling cell-to-cell-adhesion, cell signaling and membrane trafficking (Kohler and Zahraoui 2005). Therefore, in figure 14, it has been implicated that the protein kinases (aPKC) and protein phosphatases form the main components of the cytoplasmic plaque of the pathway towards cell proliferation, regulation of actin cytoskeleton and regulation of gene regulation. From this it is possible to speculate that although exact information is lacking on the mechanism how those genes function in the endometrium during the bovine implantation period, the simultaneous upregulation of those genes in receptive endometrium may be useful in maintaining the balance of protein phosphorylation and dephosphorylation in receptive endometrium for a wide range of cellular processes.

5.1.1.5 Cluster of differentiation (CD) and chemokine molecules gene clusters

The embryo during implantation and throughout pregnancy is considered as foreign and unlikely to avoid the scrutiny of immune cells that continually infiltrate the endometrium and scavenge the uterine lumen (Roberts et al. 1996). The high rate of early embryonic mortality in cattle and other animals may be maternal immunological attack against conceptus antigens of paternal origin (Thatcher et al. 1984). Therefore, reduced level of immune response of the maternal environment may be required to maintain the conceptus during the entire gestation period. In the present study, cluster of differentiation molecules (CD247/CD3Z, CD36, CD8A, CD8B), chemokine molecules (XCL1, CCL28, CXCL2, CXCL12, CXCL5), (Figure 11D) were found to be reduced in receptive compared to non-receptive endometrium. Several authors (Patel et al. 2007, Walter and Santamaria 2005, Whitcomb et al. 2007) have described the functional contribution of CD molecules in immune system. For instance, CD36 has been found to be involved in innate immune response in mouse (Patel et al. 2007, Silverstein and Febbraio 2009), it interacts with the Toll-like receptors (TLR) to process the inflammatory responses (Erdman et al. 2009). Lower level of CD247/CD3-zeta expression in maternal serum has been demonstrated in

normal pregnancies (Lam et al. 2003). Similarly, CD8 believed to contribute to initiation, progression and regulation of autoimmune responses (Walter and Santamaria 2005). In addition, CD8A, CD8B and CD247 were found to be involved in T-receptor signaling pathway (Table 6) and CD8A and CD8B were found to be involved in antigen presentation network (Appendix 3). The importance of chemokine genes during immune response has been previously described (Ogawa et al. 2004, Whitcomb et al. 2007). In addition, all differentially expressed chemokine genes were found to be involved in cytokine-cytokine receptor interaction pathway (table 6) suggesting their involvement in immune system. Among them, elevated concentrations of CXCL5 had been associated with higher risk of miscarriage in human (Whitcomb et al 2007).

Apart from those CD and kemokine molecules several DE genes related to the immune system, colony stimulating factor 3 (CSF3), IL18, non-classical MHC class I antigen (BOLA-NC1), tumor necrosis factor super family member 13 (TNFSF13), complement factor D (CFD/DF) were reduced in the receptive endometrium. Previous reports also indicated lower IL18 mRNA expression in pregnant compared with non-pregnant women and reduced pregnancy rate was reported in IL18 positive women compared to IL18 negative group (Kruse et al. 2000, Ledee-Bataille et al. 2004). Moreover, detection of IL18 is suggested to be used as non-invasive and simple method to predicted inadequate uterine receptivity, independent of embryo quality (Ledee-Bataille et al. 2004). Therefore, the reduced level of CD molecules, kemokines and other immune related genes in receptive compared to non- receptive endometrium may suggest that the receptive endometrium can adjust the immune system to create conducive environment for immune tolerance of the incoming embryo.

5.1.1.6 S100 gene clusters and endometrial receptivity

With the exception of S100B, the expression pattern of the S100 proteins including S100A12, S100A13, S100A14, S100A16 and S100A8 was reduced in receptive endometrium compared to the non-receptive endometrium. The contribution of S100 genes in bovine endometrial receptivity and embryo implantation is not known. However, some evidences indicate that S100 genes may be function in the immune system similar to chemokine or CD molecules. For instance, S100 genes including S100A8, S100A9, S100A12 and S100A13 are believed to function as a cytokine-like manner (Foell et al. 2007, Sedaghat and Notopoulos 2008). Similarly, the expression of S100A12 and S100A8

were found to be higher in inflamed tissue, where neutrophils and monocytes belong to the most abundant cell types (Foell et al. 2007). Moreover, 100% of homozygous null for S100A8 gene in rats caused rapid and synchronous embryo resorption at day 9.5 embryo development (Passey et al. 1999). This may suggest that the S100AB null embryos had lost the immune system to overcome the mother immune scrutiny. Some of S100 proteins down regulated in receptive endometrium (S100A13, S100A8, S100A14, S100A16) may associated with endometriosis, tumorigenesis and progression and can be used as a marker of actively progressing endometriotic process (Hayrabedyan et al. 2005, Yao et al. 2007a, Yao et al. 2007b). Therefore, the reduced level of S100 those genes in receptive endometrium may have two fold advantage, for one thing it may be associated with suppression of tumerigenesis and secondly it may required for modulation of the maternal immune system in the endometrium to be hospitable for the incoming embryo.

5.1.1.8 The extracellular matrix (ECM): The Collagens, TIMP3, TIMP1 and MMP2

The collagens, tissue inhibitors of matrix metalloproteinase (TIMP) and matrix metalloproteinase (MMP) are among the main components of ECM and abnormalities in those ECMs can be associated with implantation defects (Iwahashi et al. 1996b, Jokimaa et al. 2002, Skinner et al. 1999). In current study, several collagens including, collagen type I (COL1A1,COL1A2), collagen type VI (COL6A1), collagen type XII (COL12A1), collagen type IVIII (COL18A1), collagen type XXI (COL21A1) and collagen type XXVII (COL27A1) were reduced and collagen type IV alpha 3 and alpha 4 (COL4A3, COL4A4) were increased in receptive compared to non-receptive endometrium (Figure 8). According to the gene ontological classification, those DE collagens were involved in extra matrix remodeling (Figure 11E). Moreover, COL1A1, COL1A2, COL4A4, COL6A2, COL6A3 were found to be involved in focal adhesion and ECM receptor interaction pathway (Table 6). Among these, collagen type I, IV, VI and collagen type XVIII have been detected in human and bovine endometrium (Aplin et al. 1988, Boos 2000, Gaide Chevronnay et al. 2009, Iwahashi et al. 1996, Stovall et al. 1992). Similar to the current study, other report also indicated very low collagen type VI in the decidual stroma indicating loss of stromal type VI collagen contributes to the remodelling of the maternal extracellular matrix of pregnancy (Mylona et al. 1995). Moreover, the expression level of collagen type I (COL1A1, COL1A2) and collagen type VI (COL6A1) were reported to be higher in non pregnant cow compared to pregnant cow (Bauersachs et al. 2006). The reduced level of

collagen type I (COL1A1) may be associated with its minor role in the maintenance of uterine tensile strength of the cyclic cow (Boos 2000).

Unlike other collagen families, the expression of collagen type IV alpha 3 (COL4A3) and collagen type IV alpha 4 (COL4A4) were elevated in receptive compared to non- receptive endometrium (Figure 11E). Collagen IV is a common constituent of basement membrane (Boos 2000) and it has been suggested to enhance trophoblast invasion by providing a substratum for cell migration (Aplin 1996, Irving et al. 1995). Besides collagen IV was found to be detected in stromal cells and the basement membrane of glands and blood vessels in fertile women and it was absent in women suffering from unexplained infertility (Bilalis et al. 1996).

In addition to collagens abundances, the ECM is regulated by two groups of enzymes, matrix metalloproteinases and their inhibitors, tissue inhibitors of metalloproteinases (TIMP) and cystatins respectively (Jokimaa et al. 2002). Inline to this, the current study identified the expression level of matrix metalloproteinase (MMP2) and tissue inhibitors of metalloproteinases 1 (TIMP1) to be lower and tissue inhibitors of metalloproteinases 3 (TIMP3) to be higher in receptive compared to non- receptive endometrium. Higher mRNA levels of TIMP1 and MMP2 were associated with unexplained infertility and recurrent miscarriages in humans (Jokimaa et al. 2002). Therefore, higher expression of those genes may be associated with infertility in both humans and cattle. Apart from this, higher TIMP3 expression level can be indicative of normal functioning endometrium while lower level of TIMP3 was observed in endometriosis cases (Jokimaa et al. 2002). The expression of TIMP3 may play an important role in regulating the depth of trophoblast invasion during embryo implantation (Li et al. 2000). Moreover, higher expression of TIMP3 in maternal cells surrounding invading embryonic tissue and absence of TIMP1 or TIMP2 in the embryo proper, trophoblasts, or the area of the uterine decidual reaction was demonstrated during mouse embryo implantation indicating the importance of TIMP3 in implantation process (Reponen et al. 1995). Therefore, the increased or reduced expression of collagens, TIMPs and MMP2 in the receptive endometrium may be associated with maintenance of endometrial integrity by maintaining normal turnover of extracellular matrix proteins.

5.1.1.9 Junctional adhesion, gap junction or connexin in receptive endometrium

Junctional adhesion, gap junction or connexin expression are a biological marker for endometrial receptivity in human (Grummer and Winterhager 1998). In this study, the

mRNA level of junctional adhesion molecules 2 and 3 (JAM2/JAMB and JAM3/JAM-C) was found to be increased in receptive compared to the non-receptive endometrium. The higher expression of JAM3/JAM-C can be associated with increased in the number of uterine natural killer cells that are required to enhance blood flow to the implantation site, which may be required in the development of the placenta during implantation (Hanna et al. 2006, Herington and Bany 2007, Herington and Bany 2006). Moreover, those genes are believed to enhance cell-to-cell adhesion and control the flow of solutes and water between cells of epithelium (Aurrand-Lions et al. 2000, Aurrand-Lions et al. 2001, Ueki et al. 2008). Moreover, the junctional genes along with claudins are the main components of the transmembrane proteins of tight junctional pathway (Figure 14).

Among the gap junctional proteins, gap junction protein beta 5 (GJB5) or connexin-31 (CX31.1) and gap junction protein beta 6 (GJB6) or connexin-30 (CX30) were elevated but gap junction protein alpha-1 or connexin 43 (GJA1/CX43) was reduced in receptive compared to non- receptive endometrium. Among these, GJB5/CX31.1 is believed to be required for normal placental development (Zheng-Fischhofer et al. 2006). GJB6/CX30 is found to be abundant in late pregnant epithelium (Locke et al. 2007). Unlike other connexins, connexin 43 (GJA1/CX43) was expressed at lower level in receptive endometrium compared to the non- receptive endometrium. Similar to our result, the expression of CX43 was found to be reduced in the receptive phase of the endometrium. A decrease in CX43 gene expression in endometrium at the time of implantation may be required to reduce cell-to-cell communication and to facilitate the invasion of trophectoderm through the stroma cells (Granot et al. 2000). This may suggest that low level of CX43 is required during the receptive period of the endometrium.

Other families of cell adhesion molecules which were increased in receptive compared to non-receptive endometrium were the alpha integrin 2 (ITGA2) and alpha 6 integrin (ITGA6). Similar reports in humans have shown increased expression of ITGA2 and ITGA6 during window of implantation (Bischof et al. 1993, Lanteri et al. 1998). Moreover, the up regulation of ITGA6 expression was speculated to be related to its involvement in the attachment of cells to the extracellular matrix and induction of cell migration and invasion (Vernet-Tomas Mdel et al. 2006). Therefore, the increased level of these integrin in receptive endometrium compared to non-receptive endometrium may suggest the importance of appropriate integrin expression for establishment of uterine receptivity and embryo implantation.

5.2 Diestrus dynamics of receptive and non- receptive endometrium

In addition to investigating transcriptome difference between receptive endometrium and non-receptive endometrium, in the current study, the diestrus period transcriptome dynamics of receptive or non-receptive endometrium was addressed to get insight into the temporal transcriptome accumulation or degradation of the receptive and non-receptive endometrium. Therefore, the transcriptome dynamics of receptive or non-receptive endometrium was compiled between endometrial biopsies collected at day 7 and 14 post estrus. The data showed that more than 1800 gene transcripts were differentially expressed between day 7 and 14 in receptive endometrium, but only 254 transcripts were differentially expressed between day 7 and 14 in non-receptive endometrium suggesting mRNA dynamics (degradation or induction) is seven times higher in receptive endometrium than the non-receptive endometrium. This can be related to the fact that, the non-receptive endometrium has few or no genes that support the incoming embryo at any stage of the estrous cycle. However, in receptive endometrium the expression level of several genes that reached peak or reduced at day 7 of the estrous cycle in the expectation of the incoming embryo alter their expression pattern at day 14 of the estrous cycle. Therefore, unlike the non-receptive endometrium, the transcriptome abundance of those genes that are associated with endometrial receptivity can be changed at day 14 post estrus resulting in higher transcriptome abundance difference between day 7 and 14 in receptive compared to non-receptive endometrium.

To understand the functional alteration changes that occured in the receptive endometrium between day 7 and 14 of the estrous cycle, those genes which changed their expression between the two time points were subjected to gene ontology for gene enrichment analysis. Accordingly, the receptive endometrium at day 7 of the estrous cycle is mostly associated with expression of genes that are responsible for cellular material transport, metabolic process, phosphorylation, post-translational protein modification. However, as it reaches to day 14 of the estrous cycle, the receptive endometrium starts to be enriched by genes, which are responsible for the immune responses, apoptotic process and signal transductions (Figure 9). Although no report is available on the transcriptome dynamics of the receptive endometrium, the existing data on bovine endometrial gene expression also indicated transcriptomic alteration changes of bovine, human and rabbit endometrium during the different phases of the estrous cycle (Bauersachs et al. 2005, Kao et al. 2002, Mitko et al. 2008). Moreover, changes in transcriptome alteration of the bovine receptive

endometrium were also accompanied by changes in metabolic, cytokine and signaling pathways. The enrichment of those molecular pathways varies whether the endometrium is at day 7 or 14 of the estrous cycle (Table 7). For instance, the gonadotropin releasing hormone pathway was among the 21 molecular pathways involved by those genes whose expression level is variable as receptive endometrium is forwarding from day 7 to day 14 of the estrous cycle (Figure 20). This pathway is associated with in activation of transcription factors gonadotropin expression and secretion that can regulate the production and release of gonadotropins, LH and FSH. Moreover, this pathway has slots towards other 3 signaling pathways, namely MAPK and cytokine-cytokine signaling pathways (dominated by genes enriched in day 14) and nueroactive ligand receptor interaction pathway (dominated by genes enriched at day 7 of the estrous cycle). This may suggest that as the receptive endometrium proceeds from day 7 to day 14 of the estrous cycle, the molecular pathways change towards the direction of transcription of genes required for production of hormones, cell proliferation and differentiation.

Following global gene expression profiling between receptive and non-receptive endometrium, or between day 7 and 14 of the estrous cycle in receptive or non-receptive endometrium, the expression patterns of selected DE genes was determined across the estrous cycle and at 50 days gestation period (P). These was aimed at investigating whether up or down regulating genes in receptive endometrium during the estrous cycle will continue or decay during early gestation period. The data on selected genes showed that IRF6, ITPR1, STAT5A and AGTR1 exhibited an increase trend from proestrus to diestrus and reached peak in pregnant endometrium (Figure 23), whereas the expressions of PTGER4, PTGS2/COX-2, TIMP3 and ADORAB2B were reduced in during early pregnancy period compared to day 7 and 14 of the estrous cycle. This may suggest that those genes which were increased in receptive endometrium during the receptive phase of the endometrium can be increased further or reduce their expression after embryo implantation. For instance, interferon regulatory factor 6 (IRF6) was among the genes increased during pregnancy. This gene is believed to play critical roles in endometrial gene expression, conceptus trophectoderm growth and differentiation, regulator of keratinocyte proliferation and differentiation during embryogenesis (Fleming et al. 2009). Therefore, the increased level of IRF6 in pregnant endometrium may suggest the requirement of IRF6 during per and postimplantation periods. Similarly, the expression profile of STAT5A also indicated an increase level in pregnant endometrium even if it was down regulated in receptive endometrium. During early pregnancy in cattle and other ruminants,

progesterone (P4) from the corpus luteum and interferon tau (IFNτ) from the conceptus act on the endometrium regulate genes important uterine receptivity and conceptus growth (Gray et al. 2006). STAT5A is one of the genes increased by IFNτ (Gray et al. 2006). The increased level of STAT5A at pregnant endometrium might be then driven by activation of IFNT on STAT5 gene during the implantation. Therefore, the down regulation of STAT5A in receptive endometrium during the estrous cycle may be compensated by the time embryo starts to produce IFNτ. On the other hand, cyclooxygenase-2 or PTGS2 was upregulated in receptive endometrium and exhibited lower expression in pregnant endometrium compared to the expression detected at day 7 and 14 of the estrous cycle suggesting reduction of its expression after implantation. This can be also associated with effect IFNτ. As described in several reports (Binelli et al. 2000, Pru et al. 2001, Xiao et al. 1999), the expression level of PTGS2 is reduced following IFNτ exposure. For instance, the expression level of PTGS2 down-regulated at day 14 of pregnancy in sheep and by treatment of ewes with IFNτ suggesting the effect of IFNτ on the reduction of PTGS2 gene during pregnancy (Binelli et al. 2000, Chen et al. 2006, Xiao et al. 1999). This together with the current result can suggest that the up-regulation of PTGS2 during the receptive phase may be maintained to lower level by the effect of the embryo following during implantation.

5.3 Transcriptome alternation in embryos resulted in calf delivery and no pregnancy

Embryo competency for implantation and uterine receptivity are two distinct processes that are equally important for successful implantation (Carson et al. 2000, Dey et al. 2004, Wang and Dey 2006). Thus the embryo can functions as an active unit with its own molecular program of cell growth and differentiation (Carson et al. 2000, Dey et al. 2004). Therefore, in the current study, in addition to endometrial gene expression, the transcriptome differences between embryo biopsies resulted in calf delivery and biopsies resulted in no pregnancy was analyzed to generate candidate genes associated with those embryo implantation potential. Subsequently, significantly differentially expressed genes between the two groups of embryo that were involved in several biological and molecular functions have been identified (Figure 25, Figure 26, Table 9). This shows that embryos that result in calf delivery can be molecularly distinguishable from embryos ended up in no pregnancy. However, the number of differentially expressed genes and molecular signatures derived from comparing the two groups of embryo biopsies was by far fewer

compared to the differentially expressed genes obtained from comparing the endometrial biopsies resulted in calf delivery and no pregnancy. This does not imply that the endometrium or the maternal environment is more important than the embryo during embryo pregnancy establishment. However, this was indeed related to differences in the microarray platform utilized in the two experiments.

Among the DE genes that were identified between the two embryo groups, CXCL16, AMD1, SPAG17, PF6, UBE2D3P, DFNB31, DTNBP1 and NUP35 were found to be higher in embryo biopsies resulted in calf delivery compared to those resulted in no pregnancy. Among these, reports have also shown the importance of CXCL16 and AMD1 in cytotrophoblast invasion, placentation, cell migration and mouse embryo development (Huang 2006, Lin et al. 2009, Nishimura et al. 2002). On the other hand, eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), was found to be higher in embryo biopsies derived from blastocysts resulted in no pregnancy. Similar study in our lab (El-Sayed et al. 2006)**,** showed higher abundance of EEF1A1 expression in embryo biopsies derived from in vitro produced blastocysts that resulted in no pregnancy. This shows the presence of common genes related to the progressive development of embryo derived either from in vivo or *in vitro* condition.

Collectively, the present data uncovered the pattern of the bovine pre-transfer endometrial and embryo gene expression in relation to pregnancy establishment. To that end, endometrium that resulted in calf delivery displayed different transcriptome profile compared to the endometrium that resulted in no pregnancy at day 7 of the estrous cycle. Those differences were accompanied by qualitative and quantitative alteration of major biological processes and molecular pathways. Along with the endometrial gene expression, the embryo that developed to term displayed different patterns of gene expression compared to those embryos failed to establish pregnancy suggesting the potential contribution of embryo transcripts during pregnancy establishment. Therefore, our data provide a global perspective of the endometrium and embryo transcriptome in relation to the possible pregnancy outcomes. The data generated in the present study can be utilized for selecting candidate genes for further investigation aiming at developing molecular markers related to endometrial receptivity and embryo competency. However, future functional studies will be necessary to understand the key functional contribution of differentially expressed genes with respect to pregnancy success. Apart from this, in-depth investigation may be required to fully understand to what extent endometrial state can repeat it self with regard to its molecular signatures during consecutive estrous cycles.

6 Summary

The intrinsic factors associated the aberrant gene expression in the uterine endometrium and embryo has been one of the major causes of pregnancy failure in cattle. Therefore, selecting cows with adequate endometrial receptivity and implantation competent embryos based on gene expression pattern may increase the number of calves borne following embryo transfer. Hence, investigating the endometrial and embryonic gene expression before embryo transfer and relating this information to the pregnancy outcome provides a unique opportunity for generating molecular marker that are associated with endometrial receptivity and embryo implantation. Therefore, in the current study, a direct connection has been established between pre-transfer endometrial and embryo gene expression and pregnancy outcome using endometrial and embryo biopsy techniques. For this, 54 Simmental cyclic heifers were estrus synchronized and endometrium biopsies were taken at day 7 and 14 of the estrous cycle during the pre-transfer period. In the next cycle, in vivo produced day 7 blastocysts stage embryos were transferred to the recipients at day 7 of the estrous cycle after taking 30-40% parts of the blastocyst as a biopsy for transcriptome analysis. After pregnancy diagnosis and at the end of gestation period, heifers were grouped according to the pregnancy outcome. Those heifers returned to heat after 21 days were classified as no pregnancy group (non-receptive endometrium) and those heifers ended up with successful pregnancy were classified as calf delivery group (receptive endometrium). The embryos that were biopsied from blastocysts transferred to the recipients and endometrial biopsies collected during the pre-transfer period were then categorized based on the pregnancy success. The embryo biopsies were classified as those biopsies resulted in successful calf delivery and those resulted in no pregnancy. Similarly, the endometrial biopsies taken at days 7 and 14 of the estrous cycle during the pre-transferred period from heifers resulted in successful calf delivery were designated as CDd7 and CDd14, respectively. Similarly, endometrial samples collected at days 7 and 14 of the estrous cycle from heifers resulted in no pregnancy groups were designated as NPd7 and NPd14, respectively. Following this, the gene expression difference between receptive and endometrium during day 7 and 14 of the estrous cycle, the transcriptome dynamics of receptive endometrium and non-receptive endometrium were established using GeneChip® Bovine Genome Array. Furthermore, the transcriptome abundance difference between the embryo biopsies resulted in calf delivery and those resulted in no pregnancy were investigated using the bovine preimplantation specific cDNA array.

The pre-transfer endometrial gene expression difference between heifers resulted in calf delivery (receptive endometrium) and heifers resulted no pregnancy (endometrium) at day 7 of the estrous cycle was investigated by comparing the transcriptome abundance of CDd7 and NPd7. The result revealed that 1126 genes were differentially expressed between the two groups of endometrium of which 612 genes were elevated in receptive endometrium (CDd7) and 514 were elevated in non-receptive endometrium (NPd7). From those, CDC20, GCLC, PLLP, COL4A4, TMEM163, MTTP, NETO2, CDC2, COX6B2 and PPP2R5A were strongly expressed in CDd7 whereas five or more folds in NPd7 elevated HOXA9, COL1A2, NTRK2, COL3A1, GJA1, COL21A1, LHFP, IGJ, SPOP, OGN, COL6A1 and SPARC. In addition to individual genes, several gene clusters were found to be differentially expressed between the two groups of endometrium. Among those, the solute carrier gene cluster, cell division cycle clusters, adaptor related protein complex, kinesin clusters, leucine rich repeats, coiled domains, ATPases, protein kinases and phosphatase clusters, transmembrane proteins, junctional adhesion molecules and integrins were abundantly expressed in receptive endometrium (CDd7) groups. However, gene clusters including, cluster of differentiation molecules, chemokine molecules, homeobox genes, collagen families, S100 calcium binding protein and zinc finger proteins were enriched in endometrium (NPd7). Functional classification of the differentially expressed genes indicated that those genes whose expression level was elevated in CDd7 groups were found to be involved several functions including macromolecule localization, protein/cellular localization, transmembrane transport, post transcriptional modification, binding activities (nucleotide, ion, ATP), signal transduction, metabolic process, anti-apoptosis, gene transcription and cell cycle. However, the genes elevated in NPd7 were mainly involved in cellular metabolic processes, responding to stimuli, immune response, chemokine and cytokine activity and induction of apoptosis. Furthermore, the regulator pathway annotation analysis performed based the Kyoto encyclopedia of genes and genomes database revealed that the genes enriched in CDd7 mainly involve the tight junction, integrin signalling, inositol phosphate metabolism, calcium signalling pathway, focal adhesion, apoptosis, GnRH, VEGFB and PPAR signalling pathways. On the other hand, the genes abundantly expressed in NPd7 were mainly found to involve in T cell receptor pathway, cytokine–cytokine receptor, p53 signalling pathway and focal adhesion pathways. Moreover, the Ingenuity Pathway Analysis revealed that, the differentially expressed genes between CDd7 and NPd7 were grouped into a set of 34 different molecular networks. The relevant functions that repeatedly appear in the different

molecular networks were cell cycle network, cell death, reproductive system disease, genetic disorder, cellular movement, DNA replication recombination and repair network, lipid metabolism, molecular transport network, antigen presentation network, post-translational modification, cellular assembly and organization, cell signaling, cellular movement, immune cell trafficking, neurological disease, RNA post-transcriptional modification. Therefore, the differences in regulatory pathways and biological processes and molecular functions evidenced the presence of transcriptomic functional and molecular signaling alteration between receptive and non-receptive endometrium at day 7 of the estrous cycle during the pre-transfer period.

In addition to day 7 of the estrous cycle, the pre-transfer gene expression difference between receptive endometrium and endometrium was investigated at day 14 of the estrous cycle using endometrial biopsies taken during day 14 of the estrous from those eventually resulted in calf delivery and from those resulted in no pregnancy groups. For this, the transcriptome abundance of CDd14 and NPd14 was compared. The result showed that only 14 DE genes between the two groups of the endometrium in which the expression level of 2 genes (C20ORF54 and FZD8) to be higher in CDd14, while the expression of 12 genes (LOC786821, BRWD1, ANKRD11, SLC25A12, GJA1, SCARA5, IIGLL1 and other 5 ESTs) to be higher in NPd14. Therefore, this result showed that the number of genes and molecular signals altered between CDd14 and NPd14 was significantly lower compared to the number of genes and molecular signals altered between CDd7 and NPd7. This shows that minimum difference in transcriptomic functional alteration between receptive and non-receptive endometrium at day 14 of the estrous cycle and pronounced difference in day 7 of the estrous cycle. This remarkable difference in transcriptomic functional alteration between receptive and non-receptive endometrium at day 7 of the estrous cycle indicates the endometrium that eventually result in calf delivery and endometrium result in no pregnancy can be molecularly distinguishable at day 7 than day 14 of the estrous cycle.

Following investigating the transcriptome difference between the receptive and non-receptive endometrium at day 7 and 14 of the estrous cycle, the temporal transcriptome dynamics of the receptive endometrium was examined to understand the transcriptome changes that undergoes as the receptive endometrium proceeds from day 7 to day 14 of the estrous cycle. For this, the endometrial gene expression was analyzed using day 7 endometrial samples (CDd7) and day 14 endometrial samples (CDd14) collected from heifers resulted in calf delivery. The result indicated that 1867 genes were differentially

expressed between the CDd7 and CDd14. Of these the transcript level of 1014 genes was elevated in CDd7 while the transcript level of 853 were increased in CDd14 indicating pronounced transcriptome changes of the receptive endometrium between day 7 and 14 of the estrous cycle. The functional classification also revealed that those genes elevated in CDd7 were involved in various functions including, cellular material transport, phosphorylation, post-translational protein modification. On the other hand, the immune responses, apoptotic process and signal transductions were among the main biological processes in those genes whose transcript level was increased in CDd14 compared to CDd7. Moreover, 21 molecular pathways were affected as endometrium progresses from day 7 to day 14 of the estrous cycle. The MAPK signaling, Toll-like receptor signalling, adipocytokine signaling, cell adhesion molecules (CAMs) and extra cellular matrix (ECM)-receptor interaction pathways were enriched by the genes abundantly expressed in CDd7. On the other hand, the PPAR signaling, calcium signaling, neuroactive ligand-receptor interaction, arginine and proline metabolism, GnRH signaling, urea cycle and metabolism of amino acid, tryptophan metabolism, Glycine, serine and threonine metabolism pathways were enriched by the genes abundantly expressed in CDd14.

Similar to the receptive endometrium, the transcriptome changes of the non-receptive between day 7 and 14 of the estrous cycle was investigated by comparing NPd7 and NPd14 endometrial biopsy samples. From this analysis, it was evidenced that 254 genes to be differentially expressed between NPd7 and NPd14 groups. Compared to the transcriptome dynamics of the receptive endometrium (CDd7 vs. CDd14), the number of differentially expressed genes and functional categories identified in NPd7 vs. NPd14 was lower by 1613 genes indicating the transcriptome plasticity of receptive endometrium during the day 7 and 14 of the estrous cycle.

Once the pre-transfer endometrial gene expression of receptive and non-receptive endometrium was uncovered, the mRNA and protein expression pattern of some selected candidate genes were assessed at five different days of the estrous cycle (at days 19, 0 (21) 3, 7 and 14 of the estrous cycle and day 50 of the gestation period using endometrium samples collected from targeted slaughtered heifers. For this, candidate genes that exhibited an elevated expression in receptive endometrium (CDd7) namely, (IRF6, PTGER-4, ITPR1, ADORA2B, PTGS2/ COX-2, WNT11, CDC20 and TIMP3) and others (STAT5A, MMP2 and AGTR1) which were increased in non-receptive endometrium (NPd7) were selected. The majority of those candidate genes showed higher expression levels at day 7 and 14 of the estrous cycle. In addition, the expression pattern of IRF6,

ITPR1, STAT5A and AGTR1 exhibited an increase trend from proestus to diestrus and reached peak in pregnant endometrium. However, the expression of PTGER4, PTGS2/COX-2, TIMP3 and ADORAB2B was reduced in pregnant endometrium compared to the expression observed at day 7 and 14 of the estrous cycle. In addition, immunohistochemical localization of IRF6, ITPR1, PTGS2 and MMP2 revealed differences in the protein signal intensities during the estrous cycle and early pregnancy in superficial, luminal and glandular epithelial and stroma cells suggesting temporal and special expression of those genes. Therefore, this may indicate those genes exhibited higher or lower expression during the estrous cycle may induce or switch off their expression following implantation.

The blastocyst competency for implantation is equally important as endometrial receptivity to determine the pregnancy outcome. Thus, parallel to transcriptome analysis from the endometrial biopsies, the transcriptome abundance differences between embryo biopsies resulted in calf delivery and biopsies resulted in no pregnancy was analyzed to generate candidate genes that are associated with the blastocysts competency for implantation. The microarray data analysis revealed a total of 70 differentially expressed genes between the two embryo groups involving in different function categories including gene expression, cell signaling and cell communication, transport of macromolecules, transferase activity and chemokine activity. Compared to the transcriptomic alteration difference that has been occurred between receptive and non-receptive endometrium, the numbers of genes that were differentially expressed and molecular signatures generated while between embryo biopsies resulted in successful calf delivery and no pregnancy were by far fewer. However, this difference does not guarantee that the endometrium or the maternal environment is more important than the embryo for pregnancy establishment. This difference was indeed related to differences in the microarray plat form used.

In conclusion, the present study provides significant mechanistic insight about pre-transfer cattle endometrial and embryo gene expression in relation to the pregnancy success. The data highlights the potential of pre-transfer endometrial and embryo biopsy transcripts for predicting pregnancy success in cattle which is a step forward in developing molecular markers for selecting embryos that are capable of developing further and recipient cows that are presumably become pregnant. Apart from this, the results provided a long list of several developmentally important genes that can be utilized for selecting candidate genes for further investigation aiming at developing molecular markers for predicting pregnancy success in cattle. However, future functional studies will be required to understand the

functional contribution of differentially expressed genes with respect to pregnancy success. In addition, in-depth investigation may be essential to fully understand to what extent endometrial state can repeat it self with regard to its molecular signatures during consecutive cycles. Taken together, this data contributes substantial information towards increasing pregnancy success in cattle using gene expression as predictor tool.

7 Zusammenfassung

Die Hauptgründe für die Trächtigkeitsverluste nach Embryotransfer beim Rind stellen inhärente Faktoren verbunden mit der abberanten Genexpression im Endometrium und Embryo dar. Durch die Selektion adäquater endometrialen Rezeptivität und Embryonen mit hohem Entwicklungspotntial basierend auf dem Genexpressionsmuster, könnten die Trächtigkeitsraten vermutlich gesteigert werden. Infolgedessen bietet die Analyse der endometrialen und embryonalen Genexpression vor dem Transfer bezogen auf den Trächtigkeitserfolg, eine einzigartige Möglichkeit zur Identfizierung molekularer Marker für die Rezeptivität des Endometriums und der embryonalen Entwicklungskompetenz.

In der vorliegenden Arbeit wurde eine direkte Abhängigkeit der Expressionsprofile von Endometrium und Embryo vor dem Transfer zum Trächtigkeitserfolg hergestellt. In diesem Versuch wurden 54 Simmeltaler Färsen synchronisiert und deren Endometrium an Tag 7 und 14 des Vorzykluses biopsiert. Im folgenden Zyklus wurden in vivo Blastozysten an Tag 7 gespült und auf diese Empfängertiere übertragen, nachdem ca. 30 – 40% der Blastozysten als Biopsieprobe für die Transkriptomanalyse entnommen wurden. Nach der Trächtigkeitsuntersuchung und am Ende des Gestationszyklus wurden die Tiere bezüglich des Trächtigkeitserfolgs klassifiziert. Färsen, die nach 21 Tagen wieder im Östrus waren wurden in die Gruppe nicht Trächtiger (non-rezeptives Endometrium) eingeteilt, während Färsen mit einer erfolgreichen Trächtigkeit der Kalb Liefernden Gruppe (rezeptives Endometrium) zugeteilt. Die Embryonen wurden ebenfall basierend auf dem Trächtigkeitserfolg kategorisiert. Die Biopsieproben der Endometrien gliederten sich nochmals innerhalb der Gruppen bezüglich des Zeitpunktes der Probennahme in Tag 7 und Tag 14 (CDd7 und CDd14 für die trächtigen Färsen und NPd7 und NPd14 für die nicht-trächtigen Färsen). Der GeneChip® Bovine Genome Array diente dem Nachweis unterschiedlicher Genexpressionen zwischen und innerhalb der Gruppen. Mittels eines präimplantations spezifischen cDNA Arrays wurden Unterschiede in der Menge der Transkriptome der Embryonen in Abhängigkeit des Trächtigkeitserfolgs untersucht. Der Expressionsunterschied der pre-Transfer endometrial Gene zwischen der rezeptive Endometrium Gruppe und der nicht-rezeptive Endrometrium Gruppe am Tag 7 des Östrus-Zykluses wurde durch den Vergleich der Transcriptome von CDd7 und NPd7 untersucht.

Die Ergebnisse der Genexpressionsanalyse der Endometrien zeigen, dass insgesamt 1126 Gene unterschiedlich expremiert wurden, wobei 612 Gene im rezeptivem Endrometrium (CDd7) und 517 im nicht – rezeptivem Endrometrium (NPd7) hoch reguliert waren. Von

diesen waren CDC20, GCLC, PLLP, COL4A4, TMEM163, MTTP, NETO2, CDC2, COX6B2 und PPP2R5A sehr stark expremiert in CDd7, wohingegen in NPd7 die Gene HOXA9, COL1A2, NTRK2, COL3A1, GJA1, COL21A1, LHFP, IGJ, SPOP, OGN, COL6A1 und SPARC fünffach oder mehr hochreguliert waren. Zusätzlich zu den einzelnen Genen fanden sich auch Unterschiede in einigen Genclustern, von denen der Solute Carrier Gene Cluster**,** Zellteilungszyklus Cluster, Adaptor related Protein Komplex**,** Kinesin Cluster, leucinreiche Repeats, coiled domains**,** ATPasen, Proteinkinasen und Phosphatasen Cluster, Transmebrnproteine, junktionale Adhäsionsmoleküle, intergrins in CDd7 hochexpremiert waren. Im Gegensatz dazu waren Gencluster, wie Cluster of differentiation molecules**,** chemokine Moleküle, Homeobox Gene, Kollagenfamilien, S100 Calcium binding Protein und das Zinc finger Protein vermehrt in Endometrium von NPd7 angereichert. Betrachtet man die unterschiedlichen Expressionsmuster bezüglich einer funktionalen Klassifizierung wird deutlich dass die hoch regulierten Gene der CDd7-Gruppe in verschiedene Prozesse involviert sind, wie Lokalisierung von Makromolekülen, Protein/ zelluläre Lokalisation, Transmembrantransport, posttranskriptionale Modifikation, Anheftungsaktivitäten (Nukleotide, Ione, ATP), Signaltransduktion, metabolische Prozesse, Anti-Apoptose, Gentranskription und Zellzyklus. Die hochregulierten Gene der NPd7 Gruppe hingegen sind hauptsächlich an zellulären, metabolischen Prozessen, Stimuliantwort, Immunantwort chemokine und cytokine Aktivitäten und Induktion der Apoptose beteiligt.

Des Weiteren zeigte die *Regulator Pathway Annotation Analyse*, basierend auf dem *Kyoto Encyclopedia of genes and genomes database,* dass die hochregulierten Gene in CDd7 haupsächlich in thight junction, Integrin Signalisierung, Inositol Phosphat Metabolismus, Calcium signalling Pathway, fokale Adhäsion, Apoptose, GnRH, VEGFB und PPAR signalling pathway, involviert ist. Die hochregulierten Gene in NPd7 waren größtenteils am T cell Rezeptor Pathway, Cytokin-Cytokin Rezeptor, p53 signalling Pathway und an fokalen Adhäsionspathways beteiligt. Darüber hinaus brachte die *Ingenuity Pathway Analyse (Meirelles et al.)* hervor, dass die unterschiedlich expremierten Gene zwischen CDd7 und NPd7 in eine Reihe von 34 verschiedenen molekularen Netzwerken gruppiert werden konnten. Zu den relevanten Funktionen, die immer wieder in diesen Netzwerken auftraten, gehören unter anderem, das Zellzyklus Netzwerk, Apoptose, Krankheiten im Reproduktionssystem, genetische Fehlsteuerungen, zelluläre Bewegung, DNA-replikations, rekombinations und repair Netzwerk, Fettstoffwechsel, molekulare Transport Netzwerk, Antigen Presentation Netzwerk, posttranslationale Modifikationen, zelluläre

Anordnung und Organisation, Zellsignalisierung, Immunzellenaustausch, neurologische Erkrankungen und RNA posttranskriptionale Modifikationen. Demzufolge bewiesen die Unterschiede in regulatorischen Pathways, biologischen Prozessen und molekularen Funktionen das Auftreten von transkriptomisch funktionalen und molekularen Veränderungen zwischen einem rezeptiven und einem nicht-rezeptiven Endometrium an Tag 7 des Zykluses in der Periode vor dem Embryotransfer. In einem weiteren Versuch wurde auf gleiche Weise, die Genexpressionsprofile des Endometriums an Tag 14 untersucht. Hier waren konträr zu Tag 7 nur 14 Gene zwischen den Gruppen CDd14 und NPd14 unterschiedlich expremiert. Zwei Gene（C20ORF54 und FZD8) waren in CDd14 hochreguliert, in NPd14 waren jedoch insgesamt 12 Gene hochreguliert, unter anderem LOC786821, BRWD1, ANKRD11, SLC25A12, GJA1, SCARA5, IIGLL1 fünf weitere ESTs. Insgesamt sind die Änderungen der Genexpressionsprofile zwischen CDd14 und NPd14 signifikant geringer als die Unterschiede an Tag 7. Daraus lässt sich schließen, dass an Hand der transkriptomisch funktionalen Änderungen an Tag 7 zwischen rezeptiven und nicht – rezeptiven Endometrien der Trächtigkeitserfolg deutlich besser absehbar ist als an Tag 14 des Östrus. Im Folgenden wurden die Unterschiede im Transkriptom der rezeptiven und nicht-rezeptiven Endometrien an Tag 7 und 14 untersucht, wobei besonders die temporäre Transkriptomdynamik des rezeptiven Endometiums betrachtet wurde, um die Veränderungen im Endometrium, die zwischen Tag 7 und Tag 14 ablaufen, besser verstehen zu können. Hierfür wurden die Genexpressionsprofile von CDd7 und CDd14 gegenübergestellt. Insgesamt wurden 1867 Gene unterschiedlich expremiert, davon waren 1014 Gene in CDd7 hochreguliert und 853 in CDd14. Eine funktionale Klassifikation der hochregulierten Gene von CDd7 zeigt mannigfaltige Funktionen, wie Zellmaterialtransport, Phosphorilierung und posttranslationale Proteinodifikationen. Auf der anderen Seite konnten die Gene, die an Tag 14 hochreguliert waren mit Immunantwort, Apoptose und Signaltransduktionen in Verbindung gebracht werden. Des Weiteren wurden 21 molekulare Pathways von den Veränderungen im Endometrium von Tag 7 zu Tag 14 beeinflusst. Gene der MAPK Signalisierung, Toll-like Rezeptor Signalisierung, Adipocytokine Signalisierung, Zell Adhesion Moleküle (CAMs) und extra Zellulare Matrix Rezeptoren waren stark bei der Expression im CDd7 Stadium vertreten. Auf der anderen Seite, Gene der PPAR Signalisierung, der Kalzium Signalisierung, der neuroactiven Ligand-Rezeptor Interaktion, des Arginin und Proline Metabolismus, der GnRH Signalisierung, des Urea Zykluses und Metabolismus von Aminosäuren, des Tryptophan Metabolismus, Glycine und des Serine und Threonine Metabolismus pathway,

waren am häufigsten in der CDd14 Gruppe vertreten. In ähnlicher Weise konnten Veränderungen des Transcriptoms im nicht- rezeptiven Endometrium zwischen Tag 7 und Tag 14 des Östrus, bei der vergleichenden Untersuchung von NPd7 und NPd14 Endumetrium Proben, festgestellt werden. Insgesamt wurden 254 Gene unterschiedlich zwischen der NPd7 und der NPd14 Gruppe reguliert. Im Vergleich zur Transkriptomdynamik der rezeptiven Endrometrium (CDd7 vs. CDd14), war die Anzahl der unterschiedlich exprimierten gene und der funktionallen Kategorien die in NPd7 vs. NPd14 identifiziert wurden mit 1613 Genen niedriger unter der Angabe der transkriptomen Plastizität des rezeptivem Endrometrium während der Tage 7 und 14 des Östrus. Nachdem die Genexpressionsprofile der rezeptiven und nicht- rezeptiven Endometrien entschlüsselt waren, wurden die mRNA- und Proteinexpressionsprofile von einigen Kandidatengenen zu fünf verschiedenen Zeitpunkten des Östruses (Tag 0, 3, 7, 14, 19) sowie an Tag 50 der Trächtigkeit, von geschlachteten Färsen, gemessen. Dafür wurden sowohl Kadidatengene die im rezeptivem Endrometrium (CDd7) hochreguliert waren (IRF6, PTGER-4, ITPR1, ADORA2B, PTGS2/ COX-2, WNT11, CDC20 and TIMP3) sowie Gene (STAT5A, MMP2 and AGTR1) die eine erhöhte Expression im nicht – rezeptivem Endrometrium zeigten selektiert. Die Mehrheit dieser selektierten Kandidatengene wurde an Tag 7 und 14 des Östruszykluses hochreguliert. Zusätzlich wurden die Gene IRF6, ITPR1, STAT5A und AGTR1 vom Proöstrus zum Diöstrus zunehmend stärker expremiert und erreichten ihren Höhepunkt zur Zeit der Trächtigkeit. Im Gegensatz dazu nahm die Expression der Gene PTGER4, PTGS2/COX-2, TIMP3 und ADORAB2B, die an Tag 7 und 14 hochreguliert waren, mit der Trächtigkeit ab. Des Weiteren zeigte die immunhistologische Lokalisation von IRF6, ITPR1, PTGS2 und MMP2 Unterschiede in den Proteinsignalintensitäten an der Oberfläche im Verlauf des Östruszykluses und der frühen Trächtigkeit. Die Luminal- und Drüsenepithelien sowie die Stromazellen lassen eine temporäre und räumliche Expression dieser Gene vermuten. Demzufolge ist es möglich, dass Gene, die im Verlauf des Östruszykluses stark oder schwach expremiert werden, die Implantation induzieren oder währenddessen abgeschaltet werden. Die Entwicklungskompetenz der Blastozysten ist in gleichem Maße entscheidend für den Trächtigkeitserfolg wie die Rezeptivität des Endometriums. Neben der Analyse des Endometriums wurden ebenfalls die Unterschiede in der Transkriptommenge der Embryobiopsien gemessen, gegliedert in Embryonen, die eine Trächtigkeit induzierten und die, die nicht zu einer Trächtigkeit führten. Dadurch konnten geeignete Kandidatengene gefunden werden die mit der Entwicklungskompetenz der Blastocysten assoziiert werden können. Mittels der Microarray Data Analyse konnten

70 Gene detektiert werden, die in den beiden Gruppen unterschiedlich expremiert wurden. Die Gene sind in verschieden funktionellen Kategorien wie, Genexpression, Zellsignalisierung und Zellkommunikation, Transport von Macromolekülen, Transferase und Chemokine Aktivität, involviert. Im Vergleich zur Expressionsanalyse der Endometrien, waren die Unterschiede jedoch zwischen den Gruppen der Embryonen deutlich geringer. Daraus lässt sich allerdings nicht schließen, dass das Endometrium und die maternale Umgebung einen größeren Einfluss auf den Trächtigkeitserfolg haben, als der Embryo selbst. Die Unterschiede resultieren vermutlich aus den verschiedenen Microarray Platformen.

In der vorliegenden Arbeit wird ein deutlicher Zusammenhang zwischen den Genexpressionprofilen des Endometriums und der Blastozysten zum Trächtigkeitserfolg gezeigt. Die Daten heben besonders das Potential der endometrialen und embryonalen Transkripte hervor, die als Prediktoren für eine erfolgreiche Trächtigkeit dienen und eine Vorstufe für die Entwicklung von Kandidatengenen sind, die eine Selektion von optimalen Empfängertieren und potenten Embryonen möglich machen. Es sind jedoch weitere Untersuchungen erforderlich, um die exakte funktionale Wirkung der unterschiedlich expremierten Gene, die eine Trächtigkeit induzieren, zu verstehen. Zusätzliche Untersuchungen sind nötig, um zu verstehen, inwiefern sich die molekulare Signatur in aufeinanderfolgenden Zyklen gleicht. Insgesamt bringen diese Daten beachtliche Informationen hervor um die Trächtigkeitserfolge beim Rind mittels Genexpressionsanalysen vorherzusagen.

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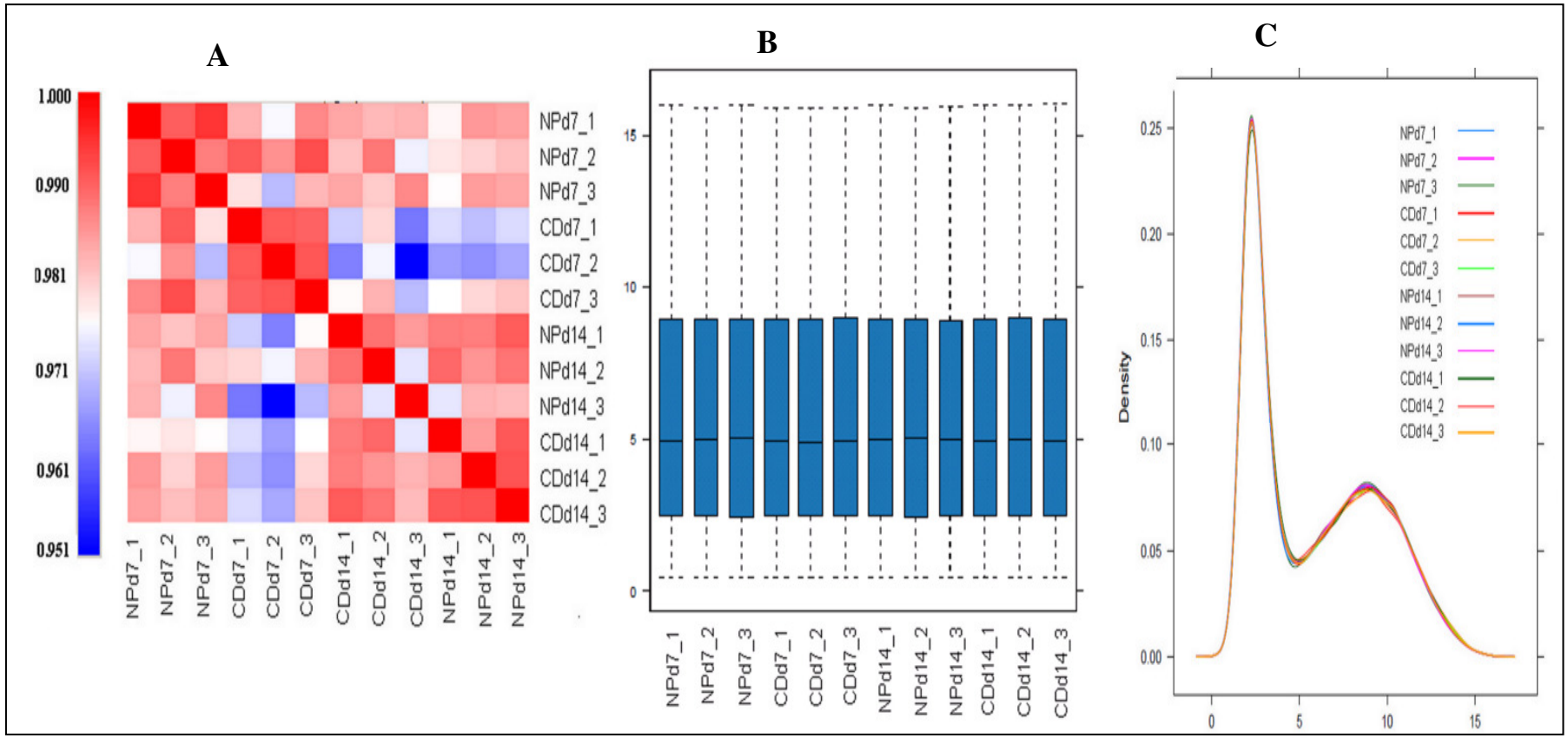
9 Appendices

Appendix 1: The intensity distribution and uniformities between the arrays after normalization. (A): Pearson correlation of the arrays within and between the biological replicates. The red and blue colors represent higher and lower correlation, respectively. (B): Box-plots indicating simple summary of the distribution of probe intensities across all arrays. Each box corresponds to one array. The boxes have similar size and Y position (median). (C): Density estimates (smoothed histograms) of the data showing similarities in shapes and ranges of the arrays



Appendix 2: List of top differentially expressed genes between receptive (CDd7) and non-receptive (NPd7) endometrium at day 7 of the estrous cycle. Negative and positive fold changes (FC) describe genes increased in CDd7 and NPd7, respectively.

UniGene ID	Gen title	Gene symbol	FC	p value
Bt.639	Cell division cycle 20 homolog	CDC20	22.7	0.003
Bt.1522	Similar to hexokinase domain containing 1	LOC614824	17.2	0.022
Bt.54026	Glutamate-cysteine ligase, catalytic subunit	GCLC	11.0	0.000
Bt.60335	Plasma membrane proteolipid (plasmolipin)	PLLP	10.3	0.004
Bt.91476	Collagen, type iv, alpha 4	COL4A4	9.8	0.000
Bt.48435	Transmembrane protein 163	TMEM163	9.5	0.002
Bt.40997	Hypothetical protein loc510320	LOC510320	9.3	0.001
Bt.64166	Microsomal triglyceride transfer protein	MTTP	9.3	0.001
Bt.25546	Neuropilin (nrp) and tolloid (tll)-like 2	NETO2	9.1	0.003
Bt.91771	Cell division cycle 2, g1 to s and g2 to m	CDC2	7.4	0.002
Bt.29868	Cytochrome c oxidase subunit vib testes-specific	COX6B2	7.4	0.002
Bt.47953	Protein phosphatase 2	PPP2R5A	7.3	0.009
Bt.24664	N-acetylated alpha-linked acidic dipeptidase 2	NAALAD2	7.2	0.012
Bt.101388	Polo-like kinase 4	PLK4	7.2	0.000
Bt.92053	Similar to cordon-bleu homolog (mouse)	LOC505588	6.8	0.020
Bt.13670	Alkaline phosphatase, liver/bone/kidney	ALPL	6.7	0.043
Bt.25034	Rab9b, member ras oncogene family	RAB9B	6.5	0.000
Bt.12794	Collagen, type iv, alpha 3 (goodpasture antigen)	COL4A3	6.5	0.003
Bt.20428	Protein regulator of cytokinesis 1	PRC1	6.3	0.002
Bt.15730	Kinesin family member 2c	KIF2C	6.2	0.019
Bt.52359	Chromosome 9 open reading frame 30 ortholog	C8H9orf30	6.2	0.025
Bt.18700	Chromosome 11 open reading frame 16	C11orf16	6.1	0.019
Bt.51579	Chromosome 14 open reading frame 37 ortholog	C10H14orf37	6.1	0.015
Bt.21876	Wingless-type mmtv integration site family, member 11	WNT11	5.9	0.045
Bt.13057	Calcium channel, voltage-dependent,	CACNA1H	5.	0.013

Appendix 2: Cont.

UniGene ID	Gen title	Gene symbol	FC	p value
Bt.23453	Spindle and kt associated 1	SKA1	5.8	0.007
Bt.65598	Thyroid hormone responsive	THRSP	5.8	0.000
Bt.6129	Ndc80 homolog, kinetochore complex component (s. Cerevisiae)	NDC80	5.7	0.014
Bt.53123	Ubiquitin-conjugating enzyme e2c	UBE2C	5.6	0.032
Bt.28359	Hypothetical loc616971	LOC616971	5.5	0.000
Bt.5578	Cytoskeleton associated protein 2-like	CKAP2L	5.5	0.017
Bt.64294	Aldo-keto reductase family 1, member c4	AKR1C4	5.5	0.047
Bt.21924	Similar to dermatan/chondroitin sulfate 2-sulfotransferase	LOC535975	5.5	0.022
Bt.26822	Similar to scl/tal1 interrupting locus	LOC510012	5.4	0.001
Bt.22305	Inositol 1,4,5-triphosphate receptor, type 1	ITPR1	5.3	0.017
Bt.20340	Similar to myotonic dystrophy kinase	LOC513675	5.3	0.046
Bt.10882	Timp metallopeptidase inhibitor 3 (sorsby fundus dystrophy, pseudoinflammatory)	TIMP3	5.3	0.016
Bt.7037	Sphingosine kinase 1	SPHK1	5.2	0.002
Bt.16037	Globoside alpha-1,3-n-acetylgalactosaminyltransferase 1	GBGT1	5.2	0.013
Bt.58521	Claudin 5 (transmembrane protein deleted in velocardiofacial syndrome)	CLDN5	5.2	0.001
Bt.54858	Synovial sarcoma, x breakpoint 2 interacting protein	SSX2IP	5.2	0.028
Bt.91910	Sorbin and sh3 domain containing 2	SORBS2	5.2	0.007
Bt.66560	Similar to map/microtubule affinity-regulating kinase 3	LOC534872	5.2	0.000
Bt.48572	Guanine nucleotide binding protein (g protein), beta 5	GNB5	5.1	0.000
Bt.27994	Alanyl (membrane) aminopeptidase (aminopeptidase n, aminopeptidase m, microsomal aminopeptidase, cd13, p150)	ANPEP	5.1	0.000
Bt.21924	Similar to dermatan/chondroitin sulfate 2-sulfotransferase	LOC535975	5.0	0.000
Bt.12757	Nerve growth factor (beta polypeptide)	NGF	5.0	0.042

Appendix 2: Cont.

UniGene ID	Gen title	Gene symbol	FC	p value
Bt.101478	Granzyme k (granzyme 3; tryptase ii)	GZMK	4.9	0.012
Bt.38424	Dystonin	DST	4.9	0.001
Bt.28324	Nucleolar and spindle associated protein 1	NUSAP1	4.9	0.017
Bt.41	Cyclin b2	CCNB2	4.9	0.023
Bt.27135	Cysteine conjugate-beta lyase	CCBL1	4.8	0.034
Bt.16830	Leucine zipper, down-regulated in cancer 1	LDOC1	4.8	0.045
Bt.24872	Kinesin family member 4a	KIF4A	4.8	0.000
Bt.64525	Similar to elongation factor 1 alpha	EEF1A1	4.8	0.004
Bt.44919	Solute carrier family 6, member 20	SLC6A20	4.8	0.037
Bt.17189	Protein kinase c, theta	PRKCQ	4.7	0.005
Bt.22869	Fatty acid binding protein 5	FABP5	4.7	0.005
Bt.35560	Glycine n-methyltransferase	GNMT	4.6	0.038
Bt.34944	Phospholipid transfer protein	PLTP	4.6	0.007
Bt.59609	Junctional adhesion molecule 3	JAM3	4.6	0.000
Bt.42379	Baculoviral iap repeat-containing 5 (survivin)	BIRC5	4.5	0.028
Bt.43706	Actin binding lim protein 1	ABLIM1	4.5	0.001
Bt.42997	Grancalcin, ef-hand calcium binding protein	GCA	4.5	0.003
Bt.12781	V-myb myeloblastosis viral oncogene homolog	MYB	4.5	0.012
Bt.12761	Integrin, alpha 2 (cd49b, alpha 2 subunit of vla-2	ITGA2	4.5	0.014
Bt.7142	Similar to aim1	LOC526200	4.5	0.000
Bt.37590	Transketolase-like 1	TKTL1	4.5	0.010
Bt.74758	Similar to chromosome 1 open reading frame	LOC520588	4.5	0.009
Bt.53189	Tudor and kh domain containing	TDRKH	4.4	0.001
Bt.53256	Phospholipase c-like 2	PLCL2	4.4	0.017
Bt.35935	Gap junction protein, beta 6, 30kda	GJB6	4.4	0.039
Bt.27397	Centrosomal protein 72kda	CEP72	4.4	0.006
Bt.54995	Synovial apoptosis inhibitor 1, synoviolin	SYVN1	4.3	0.008
Bt.10814	Coagulation factor ii (thrombin) receptor	F2R	4.3	0.002
Bt.38709	Rev3-like, catalytic subunit of dna polymerase	REV3L	4.3	0.001

Appendix 2: Cont.

UniGene ID	Gen title	Gene symbol	FC	p value
Bt.49510	Inositol hexaphosphate kinase 2	IHPK2	4.3	0.003
Bt.13798	Hypothetical loc615685	LOC615685	4.3	0.008
Bt.19592	Aquaporin 9	AQP9	4.3	0.011
---	Rho-associated, coiled-coil containing protein kinase 1	ROCK1	4.3	0.000
Bt.21912	Eukaryotic translation initiation factor 2c, 2	EIF2C2	4.3	0.001
Bt.30182	Chromosome 13 open reading frame 3 ortholog	H12C13ORF3	4.2	0.007
Bt.5091	Diacylglycerol kinase, alpha 80kda	DGKA	4.2	0.008
Bt.76624	Cytoplasmic polyadenylation element binding protein 1	CPEB1	4.1	0.014
Bt.20920	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	PPARGC1A	4.1	0.013
Bt.76544	Tumor necrosis factor receptor superfamily, member 6b, decoy	TNFRSF6B	4.1	0.043
Bt.39403	Kn motif and ankyrin repeat domains 2	KANK2	4.1	0.034
Bt.100379	Non-smc condensin i complex, subunit g	NCAPG	4.1	0.021
Bt.46419	Six transmembrane epithelial antigen of the prostate 2	STEAP2	4.0	0.028
Bt.22046	Alpha-methylacyl-coa racemase	AMACR	4.0	0.034
Bt.7331	Aurora kinase b	AURKB	4.0	0.026
Bt.53247	Sushi domain containing 2	SUSD2	3.9	0.017
Bt.56648	Similar to piggybac transposable element derived 5	LOC616498	3.9	0.037
Bt.66635	Ring finger 144b	RNF144B	3.9	0.004
Bt.64036	Guanylate binding protein 4	GBP4	3.9	0.018
Bt.22054	Chromosome 20 open reading frame 7 ortholog	C13H20ORF7	3.82	0.004
Bt.29462	Cell division cycle associated 2	CDCA2	3.8	0.010
Bt.100721	Multiple c2 domains, transmembrane 1	MCTP1	3.8	0.022
Bt.4747	Nucleoporin 88kda	NUP88	3.8	0.021
Bt.7490	Methylenetetrahydrofolate dehydrogenase	MTHFD1L	3.7	0.026
Bt.20369	Proline dehydrogenase (oxidase) 1	PRODH	3.7	0.044

Appendix 2: Cont.

UniGene ID	Gen title	Gene symbol	FC	p value
Bt.28617	Stomatin	STOM	3.7	0.009
Bt.101348	Ddhd domain containing 2	DDHD2	3.7	0.001
Bt.44935	Lipase, endothelial	LIPG	3.7	0.005
Bt.4084	Wd repeat domain 51a	WDR51A	3.7	0.003
Bt.91990	Rho-related btb domain containing 1	RHOBTB1	3.7	0.016
Bt.13573	Cdc28 protein kinase regulatory subunit 2	CKS2	3.6	0.003
Bt.25030	Transmembrane 9 superfamily member 2	TM9SF2	3.6	0.016
Bt.25834	Maternal embryonic leucine zipper kinase	MELK	3.6	0.018
Bt.16596	Leucine rich repeat containing 3	LRRC3	3.6	0.027
Bt.28443	Microtubule associated monoxygenase, calponin and lim domain containing 2	MICAL2	3.6	0.000
Bt.90915	Sulfotransferase family, cytosolic, 2b, member 1	SULT2B1	3.6	0.003
Bt.25541	Coiled-coil domain containing 135	CCDC135	3.6	0.003
Bt.14255	Farnesyl-diphosphate farnesyltransferase 1	FDFT1	3.6	0.039
Bt.9659	Ectonucleotide pyrophosphatase/phosphodiesterase 3	ENPP3	3.6	0.027
Bt.8135	Lecithin retinol acyltransferase	LRAT	3.6	0.017
Bt.25633	Regulator of g-protein signaling 22	RGS22	3.6	0.050
Bt.14403	Adaptor-related protein complex 3, sigma 2	AP3S2	3.6	0.000
Bt.8127	Heparanase	HPSE	3.5	0.023
Bt.65104	Heat shock 70kda protein 5 (glucose-regulated protein, 78kda)	HSPA5	3.5	0.001
Bt.58805	Pituitary tumor-transforming 1	PTTG1	3.5	0.039
Bt.28541	Shugoshin-like 1 (s. Pombe)	SGOL1	3.5	0.023
Bt.5487	Aldehyde dehydrogenase 3 family, member a2	ALDH3A2	3.5	0.007
Bt.39906	Hypoxia up-regulated 1	HYOU1	3.5	0.010
Bt.24640	Ilvb (bacterial acetolactate synthase)-like	ILVBL	3.5	0.015
Bt.87527	Potassium channel tetramerisation domain containing 17	KCTD17	3.5	0.037
Bt.65646	Chromosome 20 open reading frame 195 ortholog	C13H20orf195	3.4	0.025
Bt.16891	Serum/glucocorticoid regulated kinase 1	SGK1	3.4	0.002

Appendix 2: Cont.

UniGene ID	Gen title	Gene symbol	FC	p value
Bt.27878	Solute carrier family 25, member 13 (citrin)	SLC25A13	3.4	0.005
Bt.543	Nuclear receptor subfamily 2, group f, member 1	NR2F1	3.4	0.044
Bt.36484	Stefin c	LOC514170	3.4	0.032
Bt.101226	Sterol regulatory element binding transcription factor 1	SREBF1	3.4	0.008
Bt.5099	Calponin 3, acidic	CNN3	3.4	0.005
Bt.74139	T-cell lymphoma invasion and metastasis 1	TIAM1	3.4	0.001
Bt.49563	Rna binding motif (rnp1, rrm) protein 3	RBM3	3.4	0.010
Bt.28192	Interferon regulatory factor 6	IRF6	3.4	0.041
Bt.27973	Solute carrier organic anion transporter family, member 3a1	SLCO3A1	3.4	0.048
Bt.5959	Similar to retinal short chain dehydrogenase	LOC526726	3.4	0.029
Bt.3699	Dynein, cytoplasmic 1, intermediate chain 1	DYNC1I1	3.4	0.006
Bt.25924	Hypothetical protein loc540455	LOC540455	3.4	0.036
Bt.28839	Absent in melanoma 1-like	AIM1L	3.4	0.004
Bt.10412	Opa interacting protein 5	OIP5	3.4	0.015
Bt.85301	Histone cluster 1, h1c	HIST1H1C	3.3	0.021
Bt.43294	Acid phosphatase 2, lysosomal	ACP2	3.3	0.004
Bt.28185	Rac gtpase activating protein 1	RACGAP1	3.3	0.021
Bt.16050	Solute carrier family, member 22	SLC25A22	3.3	0.000
Bt.73560	Chromosome 10 open reading frame 119 ortholog	C26H10ORF119	3.3	0.011
Bt.28476	Monoacylglycerol o-acyltransferase 1	MOGAT1	3.3	0.035
Bt.44921	Denn/madd domain containing 2a	DENND2A	3.3	0.001
Bt.6685	Methylenetetrahydrofolate dehydrogenase	MTHFD2	3.3	0.010
Bt.65454	Cytoskeleton associated protein 2	CKAP2	3.3	0.036
Bt.49548	Mal, t-cell differentiation protein		3.3	0.017
Bt.448	Phospholipase c, beta 1		3.2	0.018
Bt.49367	Adaptor-related protein complex 1, sigma 1 subunit	AP1S1	3.2	0.026
Bt.3946	Hypothetical loc509875	LOC509875	-21.8	0.005
Bt.55266	Similar to procollagen alpha 2(v)	LOC782818	-20.0	0.001

Appendix 2: Cont.

UniGene ID	Gen title	Gene symbol	FC	p value
Bt.48850	Secreted protein, acidic, cysteine-rich	SPARC	-15.6	0.001
Bt.23508	Collagen, type vi, alpha 1	COL6A1	-11.4	0.000
Bt.5341	Osteoglycin	OGN	-11.4	0.002
Bt.22571	Speckle-type poz protein	SPOP	-11.37	0.002
Bt.101852	Immunoglobulin j chain	IGJ	-11.21	0.026
Bt.61894	Lipoma hmgic fusion partner	LHFP	-10.8	0.001
Bt.21896	Collagen, type xxi, alpha 1	COL21A1	-10.6	0.000
Bt.15972	Gap junction protein, alpha 1, 43kda	GJA1	-9.9	0.000
Bt.101209	Collagen, type iii, alpha 1	COL3A1	-9.8	0.003
Bt.64757	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2	-9.6	0.000
Bt.39894	Myosin light chain kinase	MYLK	-9.4	0.001
Bt.53485	Collagen, type i, alpha 2	COL1A2	-9.2	0.001
Bt.27144	Homeobox a9	HOXA9	-8.8	0.001
Bt.39050	Cannabinoid receptor interacting protein 1	CNRIP1	-8.6	0.001
Bt.2452	Lumican	LUM	-8.3	0.001
Bt.101209	Collagen, type iii, alpha 1	COL3A1	-7.7	0.001
Bt.4565	Snail homolog 2 (drosophila)	SNAI2	-7.3	0.002
Bt.4011	Transmembrane protein 120a	TMEM120A	-7.0	0.000
Bt.2750	Transcription factor ap-2 alpha	TFAP2A	-6.9	0.046
Bt.30575	Scavenger receptor class a, member 5	SCARA5	-6.8	0.000
Bt.42075	Udp glucuronosyltransferase 2 family	UGT2A3	-6.4	0.026
Bt.357	S100 calcium binding protein a12 (calgranulin c)	S100A12	-6.3	0.018
Bt.95150	Purinergic receptor p2y, g-protein coupled, 5	P2RY5	-6.3	0.000
Bt.68159	Collagen, type vi, alpha 3	COL6A3	-6.2	0.002
Bt.9360	S100 calcium binding protein a8	S100A8	-6.1	0.049
Bt.18710	Dickkopf homolog 3 (xenopus laevis)	DKK3	-6.0	0.005
Bt.64757	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2	-5.9	0.014
Bt.64625	Apolipoprotein d	APOD	-5.7	0.007
Bt.81912	Similar to ob-cadherin-1	CDH11	-5.5	0.001
Bt.23178	Decorin	DCN	-5.4	0.002

Appendix 2: Cont.

UniGene ID	Gen title	Gene symbol	FC	p value
Bt.31739	Abi gene family, member 3 (nesh) binding protein	ABI3BP	-5.4	0.005
Bt.2214	Insulin-like growth factor binding protein 7	IGFBP7	-5.1	0.017
Bt.11586	Pyd and card domain containing	PYCARD	-4.7	0.007
Bt.91101	Metallothionein 1e	MT1E	-4.7	0.004
Bt.8880	Sparc-like 1 (mast9, hevin)	SPARCL1	-4.7	0.001
Bt.92589	Roundabout, axon guidance receptor, homolog 1	ROBO1	-4.6	0.011
Bt.59000	Similar to calcium-activated chloride channel-2	LOC534256	-4.6	0.016
Bt.2524	Stromal cell-derived factor 1	SDF1	-4.5	0.009
Bt.89332	Fibroblast growth factor-binding protein (fgf-bp)	LOC281812	-4.5	0.000
Bt.51822	Collagen, type xvi, alpha 1	COL16A1	-4.3	0.020
Bt.5116	Hypothetical loc506831	LOC506831	-4.3	0.000
Bt.20385	Phospholipase a1 member a	PLA1A	-4.3	0.000
Bt.28406	Fc fragment of igg, low affinity iib, receptor	FCGR2B	-4.3	0.023
Bt.45949	Protein tyrosine phosphatase-like a domain containing 2	PTPLAD2	-4.3	0.047
Bt.13484	Protein phosphatase 1, regulatory	PPP1R3C	-4.2	0.000
Bt.64897	Insulin-like growth factor binding protein 3	IGFBP3	-4.1	0.014
Bt.19195	Coagulation factor xiii, a1 polypeptide	F13A1	-4.1	0.014
Bt.46205	Coiled-coil domain containing 68	CCDC68	-4.1	0.003
Bt.45446	Similar to nonclathrin coat protein zeta-cop	LOC616222	-3.9	0.001
Bt.48113	Doublecortin-like kinase 1	DCLK1	-3.9	0.001
Bt.4539	Angiotensin ii receptor, type 1	AGTR1	-3.9	0.000
Bt.6728	Necdin homolog (mouse)	NDN	-3.9	0.002
Bt.26788	Hypothetical loc535329	LOC535329	-3.9	0.001
Bt.4492	Collectin sub-family member 12	COLEC12	-3.8	0.001
Bt.27022	Homeobox a11	HOXA11	-3.8	0.003
Bt.8106	Nidogen 1	NID1	-3.7	0.052
Bt.43948	Cd36 molecule (thrombospondin receptor)	CD36	-3.6	0.010
Bt.9549	Hop homeobox	HOPX	-3.6	0.027

Appendix 2: Cont.

UniGene ID	Gen title	Gene symbol	FC	p value
Bt.991	Collagen, type xxvii, alpha 1	COL27A1	-3.5	0.001
Bt.2717	Fibroblast activation protein, alpha	FAP	-3.5	0.021
Bt.17819	Sodium channel, nonvoltage-gated 1, gamma	SCNN1G	-3.5	0.035
Bt.28237	Similar to family with sequence similarity 114,	LOC511583	-3.4	0.001
Bt.55899	Solute carrier family 15 (h+/peptide transporter	SLC15A2	-3.4	0.006
Bt.9675	Similar to extracellular proteinase inhibitor	LOC787253	-3.4	0.049
Bt.47030	Similar to polydom	LOC781282	-3.4	0.010
Bt.45145	Asporin	ASPN	-3.4	0.000
Bt.21035	S100 calcium binding protein a16	S100A16	-3.4	0.000
Bt.643	Similar to mpif-1	LOC508666	-3.3	0.013
Bt.29568	Ell associated factor 2	EAF2	-3.3	0.015
Bt.5226	Secreted frizzled-related protein 1	SFRP1	-3.3	0.008
Bt.48519	Rna binding protein with multiple splicing	RBPMS	-3.3	0.002
Bt.62191	Rab interacting lysosomal protein-like 2	RILPL2	-3.3	0.004
Bt.23618	Spondin 1, extracellular matrix protein	SPON1	-3.2	0.007
Bt.50	Cd8a molecule	CD8A	-3.2	0.003
Bt.91540	Keratin 17	KRT17	-3.2	0.010
Bt.4886	Leucine rich repeat containing 17	LRRC17	-3.2	0.022
Bt.49620	Tnfrsf16 associated protein 1	NGFRAP1	-3.2	0.014
Bt.36848	8kda amlexanox-binding protein	S100A13	-3.2	0.003
Bt.48875	Udp-gal:betaglcnac beta 1,3-galactosyltransferase	B3GALT2	-3.1	0.020
Bt.25507	Meis homeobox 2	MEIS2	-3.1	0.012
Bt.24447	Coagulation factor II recptor-like 2	F2RL2	-3.1	0.008
Bt.33171	Methionine sulfoxide reductase b3	MSRB3	-3.1	0.018
Bt.8222	Transmembrane protein 139	TMEM139	-3.1	0.002
Bt.29642	Nitric oxide synthase 1 (neuronal) adaptor protein	NOS1AP	-3.1	0.006
Bt.9958	Insulin-like growth factor binding protein 6	IGFBP6	-3.1	0.007
Bt.207	Transthyretin type I	TTR	-3.1	0.042
Bt.65440	Hypothetical protein loc781372	LOC781372	-3.0	0.018

Appendix 2: Cont.

UniGene ID	Gen title	Gene symbol	FC	p value
Bt.11286	Coiled-coil-helix-coiled-coil-helix domain containing 6	CHCHD6	-3.0	0.034
Bt.9573	Fxyd domain containing ion transport regulator 3	FXYD3	-3.0	0.001
Bt.78	Crystallin, beta b1	CRYBB1	-3.0	0.024
Bt.11446	Lim domain binding 2	LDB2	-3.0	0.032
Bt.62727	Transmembrane protein 204	TMEM204	-2.9	0.002
Bt.54851	Inhibitor of kappa light polypeptide gene enhancer in b-cells, kinase epsilon	IKBKE	-2.9	0.002
Bt.64122	Supervillin	SVIL	-2.9	0.013
Bt.45845	G protein-coupled receptor 77	GPR77	-2.9	0.002
Bt.14395	Hypothetical protein loc780785	C3orf57	-2.9	0.011
---	Frizzled homolog 2 (drosophila)	FZD2	-2.9	0.008
Bt.611	Chemokine (c-x-c motif) ligand 2	CXCL2	-2.9	0.020
Bt.22323	Ras association (ralgds/af-6) domain family	RASSF5	-2.9	0.017
Bt.1907	Similar to chromosome 11 open reading frame 52	LOC614490	-2.9	0.004
Bt.3213	Chordin-like 1	CHRDL1	-2.9	0.003
Bt.2047	Adrenomedullin	ADM	-2.9	0.026
Bt.66957	Fibroblast growth factor 2 (basic)	FGF2	-2.9	0.002
Bt.411	Neuregulin 1	NRG1	-2.8	0.031
Bt.8144	Chemokine (c motif) ligand 1	XCL1	-2.8	0.020
Bt.56229	T-cell receptor beta chain c region	LOC509513	-2.8	0.014
Bt.10343	Palmdelphin	PALMD	-2.8	0.030
Bt.28814	Similar to sulfatase 1	LOC535166	-2.8	0.047
Bt.12414	CD8b molecule	CD8B	-2.8	0.000
Bt.47753	Six homeobox 1	SIX1	-2.8	0.008
Bt.22534	Peripheral myelin protein 22	PMP22	-2.8	0.031
Bt.1579	Polymerase i and transcript release factor	PTRF	-2.7	0.000
Bt.41201	Poly polymerase family, member 16	PARP16	-2.7	0.002
Bt.20613	Breast cancer anti-estrogen resistance 3	BCAR3	-2.7	0.006
Bt.3216	Armadillo repeat containing, x-linked 1	ARMCX1	-2.7	0.001
Bt.16029	Fucosyltransferase 1	FUT1	-2.7	0.002

Appendix 2: Cont.

UniGene ID	Gen title	Gene symbol	FC	p value
Bt.36593	Fibromodulin	FMOD	-2.7	0.045
Bt.13273	Suppressor of cytokine signaling 2	SOCS2	-2.7	0.010
Bt.7031	Cytokine receptor-like factor 3	CRLF3	-2.7	0.002
Bt.18476	Interferon regulatory factor 5	IRF5	-2.7	0.049
Bt.92434	B-cell cll/lymphoma 11a (zinc finger protein)	BCL11A	-2.7	0.033
Bt.45633	Phosphoinositide-3-kinaseinteractingprotein1	PIK3IP1	-2.7	0.010
Bt.52435	Zinc finger, mynd-type containing 8	ZMYND8	-2.6	0.001
Bt.11149	Vimentin	VIM	-2.63	0.014
Bt.2638	Serpin peptidase inhibitor, clade f , member 1	SERPINF1	-2.6	0.042
Bt.41326	D component of complement (adipsin)	DF	-2.6	0.000
Bt.16738	Iroquois homeobox 3	IRX3	-2.6	0.004
Bt.41792	Chemokine (c-c motif) ligand 28	CCL28	-2.6	0.002
Bt.58299	Clmodulin-dependentprotein kinase II delta	CAMK2D	-2.6	0.002
Bt.5313	Matrix metallopeptidase 2	MMP2	-2.6	0.014
Bt.61846	Tumor protein p53 inducible protein 3	TP53I3	-2.6	0.015
Bt.8820	Hematopoietic cell-specific lyn substrate 1	HCLS1	-2.6	0.020
Bt.9832	Musculoskeletal, embryonic nuclear protein1	MUSTN1	-2.6	0.001
Bt.22381	Phospholipase a2, group x	PLA2G10	-2.6	0.001
Bt.100458	Suppressor of ty 3 homolog (s. Cerevisiae)	SUPT3H	-2.6	0.006
Bt.26426	Rna binding motif protein 43	RBM43	-2.6	0.005
Bt.9563	Ankyrin repeat, and basic leucine zipper 1	ASZ1	-2.6	0.037
Bt.2966	Dedicator of cytokinesis 11	DOCK11	-2.6	0.013
Bt.8795	Dysferlin	DYSF	-2.5	0.002
Bt.9102	Gtpase, imap family member	MGC137405	-2.5	0.014
Bt.2698	Fk506 binding protein 10, 65 kda	FKBP10	-2.5	0.001
Bt.27976	Timp metallopeptidase inhibitor 1	TIMP1	-2.5	0.000
Bt.25901	Denn/madd domain containing 4a	DENND4A	-2.5	0.007
Bt.11276	Zinc finger protein 503	ZNF503	-2.5	0.008
Bt.21164	V-myc myelocytomatosis viral oncogene	MYC	-2.5	0.001
Bt.28198	Intestine-specific transcript 1 protein	CIST1	-2.5	0.009
Bt.351	Coronin, actin binding protein, 1a	CORO1A	-2.5	0.050
Bt.234	Interleukin 18	IL18	-2.5	0.003

Appendix 3: List of molecular networks containing genes elevated (upward red arrow) or reduced (downward green arrow) in CDd7 compared to NPd7

	Differentially expressed genes	Net work name
1	↑AKAP8, Ant, ↑CKS2, ↓CST3, ↑CSTB, ↑EMP1, ↑FANCI, Frizzled, ↓FYTTD1, ↑GART, ↑GDI1, ↑GPC1, ↑IDH2, ↑IDH3A, Integrin alpha 3 beta 1, ↑ITGA6, ↑METTL13, ↓MYC, ↑MYO1B, ↓PLA1A, ↓PMP22, ↑PSAT1, ↑RNF144B, ↓SFRP1, ↑SLC25A11, ↑SLC25A13, ↓SPOP, ↓SUPT3H (includes EG:8464), ↓TNFSF13, ↓TP53I3, ↑TSPAN7, UBE2, ↑UBE2C, ↓UBE2L6, ↑UBE2S	Cell cycle Reproductive disease
2	Angiotensin II receptor type 1, ↑AP3B1, ↑AP3M1, ↑AP3S2, ↓CHN2, ↓CNN2, ↓COL18A1, ↑CPEB1, ↑DST, ↑DTNB, ↓DTNBP1 (includes EG:84062), ↑DUSP3, ↑DYNC1I1, Dynein, ↓DYNLT3, ↓ELF1, ↓ELK3, ERK, ETS, ↑GCLC*, ↑GTF2H1, ↑HEY1, ↑HMMR, ↓NTN4, ↑PTGER4, ↓PTRF, ↑RABGEF1, ↓SAA1, SAA@, ↑SPRED1, ↓TIMP1, ↑VAMP7, ↑VDAC1, ↑VDAC2, ↑XPOT	Genetic disorder Lymphoid tissue structure and development
3	Actin, ↓AGTR1, ↑AQP9, ↓CASP4, Caspase, ↑CNDP2, Cytochrome c, ↑DFFA, DNA-directed DNA polymerase, ↑EEF1A1, ↑EIF2C2, ↓EIF3F, ↓FOXA2, ↑GZMK, ↑H2AFX, ↑HIST1H1C, Insulin, ↑KIF2C, ↑MLPH, ↑PEPD, peptidase, ↓POLD4, ↑POLE2, ↑POLH, ↓RAB27B, ↑RAB8B, ↑REV3L, ↑SLC25A22, ↓SNAI2, ↑TARDBP, ↓TFAP2A, ↓TTR, ↑UNC13D, ↓VIM, ↑WARS	Cellular assembly lipid metabolism
4	APC, ↑ASF1B, ↑AURKB, ↑CCNA2, Cdc2, ↑CDC2, ↑CDC20, Cdc25B/C, ↑CDC25C, ↑CKAP2, Cyclin A, Cyclin B, Cyclin E, ↑DARS, ↑DYRK3, E2f, Histone h3, Histone h4, ↑JARID2*, ↑KIF4A, ↑LRAT, ↑NCAPG (includes EG:64151), ↑NUSAP1, ↑OIP5, ↑PLK1, ↑PRC1, Ras, Rb, ↑RCC2, ↑SFRS1, ↑SGOL1, ↓SMARCA2, ↓SMARCD3, ↑TYMS, tyrosine kinase	Cell cycle, DNA replication
5	↓ADAMTS5, ↑ADHFE1, Alpha catenin, ↑BCL2, ↑BIRC5, CAM, ↓CDH11, ↑CKB, ↑CLDN5, ↑CLDN7, ↑CLDN23, Creatine Kinase, ↑F2RL1, ↓FGF2, Glutathione peroxidase, Growth hormone, ↓GSTK1, hCG, Igfbp, ↓IGFBP6, ↓IGFBP7, ↓LUM, Mek, ↓MMP2, ↓OGN, Oxidoreductase, ↑OXNAD1, ↓PLAT, ↓RAB15, SERPINF, ↓SERPINF1, ↑TIMP3, Trypsin, Vegf, ↑VEGFB (includes EG:89811	Cell death, tissue development
6	↓ABCG1, ↑ADFP, ↑BRE, ↓CD36, ↓COL6A3, ↑ENPP1, HDL, HMG CoA synthase, ↑IP6K2, LXR ligand-LXR-Retinoic acid-RXRa,N-cor, NCOR-LXR-Oxysterol-RXR-9 cis RA, NFkB (complex), ↓NFKBIZ, Nr1h, ↑PELI1, PEPCK, ↑PIM2 (includes EG:11040), ↑PLTP, ↓PNRC1*, ↑PPARA, ↑PPARGC1A, ↓PYCARD, Rar, ↓RTKN, Rxr, ↓SLC25A20, ↑SREBF1, T3-TR-RXR, ↑THRSP, Thyroid hormone receptor, Tnf receptor, ↑TNFRSF6B, ↑TXNRD1, ↑VSNL1	Lipid metabolism Molecular transport
7	BCR, ↑BMPR2, C1q, CD8, ↓CD8A, ↓CD8B, ↓COL6A1, Collagen(s), Cpla2, ↓CRYAB, ↑CS, ↓DCN, ↓FAM3B, ↓FCGR2B, ↓FGFBP1, CFTR, ↑CNDP2, ↓F2RL2, ↓FAP, ↑GJB6, HEXDC, JAM, ↑JAM2, ↑JAM3, KCNMA1, LGMN, MIR181B2, MIRLET7E (includes EG:406887), P38 MAPK, ↓PLA2G10, ↑PLOD1, ↑RPS6KA5, ↑SEPT4, ↑SMPD3, SPHINGOMYELINASE	Antigen presentation
8	ALP, ↑ALPL, ↓BEND5, ↓C5ORF24, Calcineurin protein(s), Calmodulin, ↓CAMK2D, ↓CIITA, Creb, ↓CREB3L4, DUB, ↑EDN1, ERK1/2, Fgf, ↓FXYD5, ↑GCA, ↑LPCAT1, MHC Class I (complex), ↓MYLK, ↓NFIB, Nuclear factor 1, ↑OGDH, ↓PATZ1, PDGF BB, Pka, ↑PRKAG2, Rap1, ↑SLC7A7, ↓SORL1, ↑TRAPPC2, ↑USP2, ↑USP33, ↑USP46, ↑ZFP36, ↓ZFP36L1	Posttranscriptional modification
9	14-3-3, ↑ADAM10, ↑AGTPBP1, Ap1, ↑ASAP2, CD3, ↓CD247, ↑DGKA, ↓DKK3, ↑DLX4, ↓FYN, ↑GNS, ↓HCLS1, ↓HDAC7, IKK (complex), ↓LCK, LCK/Fyn, ↓LY9, ↑MARK3*, ↑MTHFD2*, NFAT (complex), Nfat (family), ↑NR2F1, ↑PACSIN3, ↑PRKCQ, ↑PTPN3, Ras homolog, ↓RORC, ↑SNX8, ↑SPHK1, SRC, ↓TCEA2, TCR, ↓TYROBP, ↑WDR47	Cell signaling
10	↓ADM, ↓AGPAT9, Akt, ↓CALCRL, Cyclin D, ↑EEF2K, ↓EIF4EBP1, ↓FOXO4, ↑HPSE, ↑HYOU1, Ige, ↓LAT, ↓MAPK13, ↑MTHFD1L, ↓NEK6, NGF, ↓NTRK2, p70 S6k, Pdgf, Pdgf Ab, PLC gamma, PP2A, ↑PTPN1, ↓S100A12, ↑S100B, Shc, ↑SLC1A1, ↑SNCB, ↑SORBS2 ↓SORBS3. Sos. ↓STK38. SYK/ZAP. ↓TFF3. VAV	Endocrine System development
11	↑ABLIM1, ↑ACP5, Alcohol group acceptor phosphotransferase, ↑ANPEP, ↓ASPN, ↓CALD1, Cbp/p300, ↑CCNB2, ↓CDK6, ↑CEP72, ↓COL1A1, ↓COL1A2, ↓COL3A1, ↑CSNK1E, Ctbp, ↑DDOST, ↑GATA2, ↑ITGA2, Laminin, ↑LDOC1, Mapk, ↑MAPK6, ↑MYB (includes EG:4602)*, ↓NID1, Pias, ↑PIAS2, Rsk, Smad, Smad2/3, Smad2/3-Smad4, ↓SPARC, Tgf beta, ↓TGFBR2, ↑TGIF1, ↓ZBTB16	Connective tissue Genetic disorder
12	26s Proteasome, ↑ANKIB1*, ↓AXL, ↑BAG3, ↑CAND1, ↑DDX58, ↑FAM81A, HLA-DR, Hsp27, Hsp70, ↑HSPA2, ↑HSPA5, Ifn, IFN Beta, IFNα/β, ↑IFT52, IRF, ↓IRF5, ↑IRF6, JAK, ↑KIF2A, ↑LY6E, MIR1, ↑NETO2, PI3K, ↓PIK3IP1, ↑PLEKHB2, ↓PRKRA, Proteasome, ↑PSMD14, ↑RADIL, RNA polymerase II, ↓SCNN1G, ↑SERPINB5, Ubiquitir	Cell death, cell-to-cell Signaling
13	↓ARHGEF3, BUB1B, ↑C15ORF39, ↑CCNB2, CD44, ↑CDC20, CDKN3, CMA1, ↓COL6A1, CYP4B1, dihydrotestosterone, ERBB2, FN1, ↓FXYD3, GPX3, ↑HYAL2, ↓IGFBP6, ↓IGJ, IL6, ↑NUP88, ↑PTGER4, RDH, RDH11, ↓RDH16, RPLP1, ↑SDR16C5, ↓SLC25A16, ↑SLC25A40, ↑SLC7A7, ↑SLCO3A1, ↓SNAI2, ↓SNX10, ↓SPARC, SQLE, ↓WBP5	Cellular growth and proliferation
14	↑ADFP, ADH1C (includes EG:126), ↓AGRN, ↓APOD, ↓ATAD4, beta-estradiol, CIDEC, CKS1B, ↓COLEC12, ↑EMP1, ↑EPHA1, FABP7, ↑GBGT1, GM2A, IDH1, IER2, ↑KIAA0922, LCAT, lipid, MAL, ↑MTTP, NR3C1, ↓PLEKHA4, ↓PNRC1*, ↓RBMS2, SIAH2, ↑SLC6A20, ↑SLC7A5, ↑SMS, SRM, ↓STAT5A, ↑SUSD2, TGTP, ↓TMEM44, ↑TRIM45	Lipid Metabolism Molecular tansport
15	↓CORO1A, ↓CSF3, ↓CXCL2, ↓CXCL5, F Actin, ↑FDFT1, FXR ligand-FXR-Retinoic acid-RXRa, ↓GLS, Hsp90, IL1, ↓IL18, IL12 (complex), ↑ITPKA, LDL, ↓LIPC, ↑LIPG, MHC Class II, Mlc, ↑MTTP, Myosin, ↑NGF, Nos, ↑PDIA4, ↓PIP5K1B, Pld, ↑PRKCI, ↓RND3, Rock, ↑ROCK2*, ↓SELL, ↑SEMA4D, ↓SOCS2, STAT, STAT5a/b, Tropomyosin	Immune cll trafficking lipid metabolism

Appendix 3: Cont

16	ACAT1, ACTN1, ADCYAP1, AFP, ↑**AMACR**, ↑**ARMC8**, ↓**C3ORF34**, C7ORF64, CP110, ↓**DDHD1**, ES22, ↑**FAM117A**, GAS1, GLA, ↓**GSTK1**, GSTM4, ↓**GSTO1**, GSTZ1 (includes EG:2954), ↑**GUSB**, HMGB2, HNF4A, ↑**KIF20A**, MGST3, ↑**MKRN1**, ORM2, ↑**PNMA1**, ↓**PPP1R3B**, PSMB7, ↑**RIOK1**, ↑**SLC39A9**, TFPT, ↑**TXNRD1**, ↓**UGT2A3**, ↑**ZNF318 (includes EG:24149)**, ↓**ZRANB2**	Antigen Presentation
17	CFTR, ↑**CNDP2**, ↓**F2RL2**, ↓**FAP**, ↑**GJB6**, HEXDC, JAM, ↑**JAM2**, ↑**JAM3**, KCNMA1, LGMN, MIR181B2, MIRLET7E (includes EG:406887), MMP24, ↑**NAALAD2**, NAALADL1, NAP1L4, ↓**NUDT4**, ↑**PATL1**, ↑**PEPD**, peptidase, PREP, ↑**RCN2**, ↓**RILPL2**, ↓**ROBO1**, ↑**SEC61A2**, SENP5 (includes EG:205564), ↑**SPG7**, TJP1, TJP3, ↑**TTLL4**, TUBB4, YME1L1, ↑**ZNF207**, ↓**ZNF503**	Protein degradation cell-to-cell signaling
18	↓**CHCHD6**, Ck2, ↑**CMTM6**, ↑**CYB5B**, ↓**DKK3**, DNAJC11, FKBP7, ↑**FKBP9**, ↓**FKBP10**, IMMT, ↑**ITPRIPL1**, ↑**KIF22**, ↑**KLHL26**, MIR300 (includes EG:723833), ↓**MSRB3 (includes EG:253827)**, MYCN, NOVA1, Peptidylprolyl isomerase, PIN1, ↑**PLLP**, PPIA (includes EG:268373), PPIB, PPIC, PPIF, PPIG, PPIL2, PPIL4, ↓**PTPLAD2**, ↓**S100A14**, ↓**S100A16**, ↓**SIX1**, ↓**TCOF1**, ↑**TWF2**, UBQLN4, ↑**YPEL1**	Developmental disorder tissue morphology
19	↓**ARHGAP24**, ↓**ARHGEF2**, ↑**CLSTN1**, ↑**CSRP2BP**, ↑**DIRAS3**, ENPEP, ↓**FAP**, Gα12/13, GLRX, ↑**GLUD1**, hydrogen peroxide, Integrin alpha 6 beta 1, ITGB1, ↑**JPH1**, L-glutamic acid, MAD2L2, MAPK8, ↓**MDFIC**, ↑**METAP1**, MGAT3, MIA, MIR124-1, MIRN101B, MTF1 MYO9B, N-acetyl-Asp-Glu, ↑**NAALAD2**, ↓**NAT1**, ↑**PANK3**, Ras homolog, ↓**S1PR2**, ↓**SNAI2**, ↑**SPAG9**, ↑**YEATS2***, ↑**ZZZ3**	Cell Death, RNA Post-transcriptional
20	↑**AKAP11**, Alpha Actinin, Calpain, CaMKII, ↑**CDCA2**, ↓**DCLK1**, FAK, ↓**FHL3**, Fibrinogen, ↓**FZD2**, Glycogen synthase, Gsk3, Integrin, Integrin alpha 2 beta 1, Integrinα, Integrinβ, ↓**ITGA10**, ITPR, ↑**ITPR3**, MAP2K1/2, Mlcp, Pkc(s), Pkg, PP1, PP1/PP2A, ↑**PPP1CB**, ↑**PPP1CC**, ↑**PPP1R10**, ↓**PPP1R3C**, ↓**PPP1R3D**, ↓**S100A8**, ↓**SKAP1**, ↑**SSX2IP**, ↓**TLN1**, ↑**WNT11**	Carbohydrate metabolism molecular transport
21	↑**ALOX5AP**, AMOTL2, AQP1, ↑**ASPM**, ↓**B3GALT2***, ↑**C20ORF195**, CALCOCO2, CCDC85B, ↑**CNN3**, ↓**COL16A1**, ↑**ESPL1**, ↓**F13A1**, ↓**KRT7**, KRT17, ↓**LMCD1**, MAD1L1, ↑**NDC80**, ↑**NDRG4**, NEDD4L, NEK2, ↑**NIPA2**, PDLIM7, PPID, ↑**PRC1**, RAD21, RHOD, SFTPC, ↑**SLC39A8**, SMURF2, ↓**ST3GAL5**, TGFB1, ↓**TMEM139**, TNNT1, TXNIP, ZWINT (includes EG:11130	DNA replication
22	AIP, AKR1B1, APOA1, ↑**ATG4D (includes EG:84971)**, ↓**C11ORF52**, ↑**C2ORF29**, ↑**C8ORF41**, D-glucose, ↑**DDX10**, ↑**EFCAB1**, ↑**FBXO31 (includes EG:79791)**, GOT1, HLA-B, HNF1A, HNF4A, IGF1, IL1B, INS1, ↑**INTS4**, ↑**MCEE**, MDH1, NME1, ONECUT1, ↓**PALMD**, ↑**PPARA**, retinoic acid, RPS27A, ↑**SLC35B1**, ↑**TMEM79**, ↓**TMEM120A**, UBE2N, ↓**VAMP5**, ↑**WDR51A**	Metabolism Molecular transport
23	↑**ACER3**, APP, Aryl Sulfatase, AXIN2, ↑**C13ORF27**, Ca2+, CCDC92 (includes EG:80212), ↓**CCL28**, CCR10, CPE, ERP44, F2RL3, FGF1, FGF6, ↓**FUT1**, ↓**FZD2**, ↓**GYPC**, ↓**LDB2**, LRP8, ↓**LRRC17**, MPP1, Nicotinic acetylcholine receptor, NRGN, PRSS3 (includes EG:5646), ↓**S100A8**, ↓**S100A13**, SELPLG, SNCA, ↑**SNCB**, ↓**SPARCL1**, ↓**SPON1**, ↑**SULF2**, ↑**TOR1A**, ↑**TOR1B**, ↑**XPNPEP1**	Cell signaling Molecular transport
24	↓**ABI3BP**, ↑**APPBP2**, ARRB1, ↓**C3ORF63**, CALB1, CASP3, ↑**CHMP5**, ↑**CHORDC1**, FOXD3, GBX2, ↑**GNMT**, ↓**HOPX**, HSP90AA1, ↓**IGFBP7**, ↑**IL17D**, ↑**KIAA1310**, KLK2, KTN1, LBP, MAD1L1, MT1A, MUL1, NIF3L1, ↑**PEX1**, POU5F1, ↑**PPM1G**, PTCH1, ↓**RBM5**, retinoic acid, RPL6, RPS27A, ↑**RTF1**, ↓**S100A8**, ↓**SFRP1**, TPM4	Cellular development
25	Adaptor protein 1, ↑**ANLN**, AP1G2, ↓**ARMCX1**, BANP, CALCR, ↑**DGKA**, ↑**ENPP3**, GPR44, HLX, IL4, ↑**ILF2 (includes EG:3608)**, ING2, ↓**INPP1**, ↑**LOC653653**, MRC1, PIGR, POLB, ↓**POLD4**, ↑**POLE2**, ↑**POLH**, POLM, PPP1R13L, ↑**PQLC3**, PRIM1, prostaglandin E2, Ptger, ↓**S100A8**, STAT4, ↓**SUPT3H (includes EG:8464)**, ↓**TAF1D**, ↑**TMEM131**, TP53, ↓**WHSC1L1**, XRCC5	DNA Replication, recombination
26	↑**ABHD2**, ↑**ACP2**, ↑**AFG3L2**, Aldehyde dehydrogenase (NAD), ALDH1B1, ↑**ALDH3A2**, ↓**ALDH3B1**, ATPase, BAT1, ↑**C1ORF77**, DDX1, ↑**DDX39**, DDX19B, DHX8, FANCC, ↑**FILIP1L**, FSH, GPR56, KIF1B, KIF20B, ↑**LETM1**, MYH1, MYO9A, MYO9B, ↓**PION**, PSG9, ↑**RHOBTB1** ↓**RNPC3**, SARNP, ↑**SGK1***, SMAD2, SMARCA4, ↓**THOC2**, VPS4B, ↓**WWTR1**	DNA replication, recombination
27	ADCY, ↑**ADORA2B**, AMPK, ↑**DOCK1**, ↑**F2R**, ↓**F2RL2**, G, G alphai, G protein beta gamma, G-protein beta, G-protein gamma, ↓**GNAI1**, ↑**GNB5**, ↓**GNG11**, Gpcr, GST, ↓**HOMER2**, ↑**ITPR1**, Jnk, K Channel, ↓**KNG1 (includes EG:3827)**, Mmp, ↑**MRAS**, ↓**MT1F**, ↑**NME2**, Par PLC, Plc beta, ↑**PLCB1**, ↑**PLCL2**, Rac, ↓**RGS19**, ↓**S1PR2**, Sapk, Tubulin	Cell signaling
28	↑**AFF1**, AMD1, ↑**C10ORF119**, C14ORF1, CBX7, ↑**CBX8**, ↑**CDC25C**, ↑**CDCA4**, CDKN2A, ↑**ENPP4**, ENTPD7, FAM49B, ↑**FLCN**, GMNN, GSPT1, HISTONE, ↑**HYLS1**, ↓**IKBKE**, ↑**KANK2**, ↑**L2HGDH**, MCM2, MECP2, ↑**MELK**, MLLT1, MYL12A, NFYB, ↑**NUDT5**, ↑**PPP1CB**, RAB27, RFC3, RPL3, SAE1, STK16, TK1, ↓**UBE3B**	Cellular development
29	↑**ALG11**, AMFR, ↓**APBB2**, ↓**ARHGAP24**, ATP5A1, ATP5D, ATP5O, ATP6V0C, ↑**ATP6V0E2**, ↑**B3GNT2**, ↑**B4GALT4**, B4GALT6, ↑**CDKN2C**, creatine, ↑**CS**, ↑**FICD**, Galactosyltransferase beta 1,4, ↓**GATM**, HTT, LDHC, MARCKS (includes EG:4082), ↓**MT1F**, N-acetyl-L-aspartic acid, NAB2, phosphocreatine, PTCRA, SIAH1, ↑**SLC6A8**, SP1, ↑**SYVN1**, ↓**TCEAL1**, TFAM, UBE2G1, UBE2G2, ↑**UBE2S**	Amino acid metabolism molecular transport
30	↑**ABHD3**, ↓**ALG13 (includes EG:79868)**, ATN1, ATP1A3, CTTNBP2, ↓**CTTNBP2NL**, DLG1, FAM40A, GRIN1, GRIN2C, KCNJ12, ↑**KLHDC2**, ↑**LIN7B**, ↑**LSM14A**, ↓**MARCH2**, MIR133A-1, MIR24-1 (includes EG:407012), MIR25 (includes EG:407014), MIR361 (includes EG:494323), NEFM, ↑**PDCD10**, ↓**PLEKHH3**, PRKAR2A, RERE, ↑**RICH2**, RP5-1000E10.4, RP6-213H19.1, ↑**RSPRY1**, ↑**SLC38A7**, SP4, STK25, STRN4, TRAF3IP3, ↑**TSGA14**, ↓**ZMYND8**	Cllular function and maintenance
31	ACSL, ACSL4, ACSL5, ↓**AGXT2L2**, ↑**ASL**, C5, ↑**CCBL1**, CCDC92 (includes EG:80212), ↓**CHRDL1**, ↑**CORO2A**, FOS, GBP1 (includes EG:14468), ↑**GBP4 (includes EG:115361)**, ↓**GPR77**, IFI44, IFNA2, IFNA7, IFNG, IL17RA, LARGE, MEFV, NIPSNAP1, ↓**P2RY5**, ↓**RTP4**, ↓**SELENBP1**, ↑**SEPT4**, ↑**SLC27A4**, SLC27A6, SP110, ↑**TACC3**, TRANSAMINASE, USP6NL, ↑**WARS**, YY1	Molecular transport
32	↑**ALDH3A2**, ARSA, ↑**ARSB**, ATP5A1, ATP5D, ATP5O, ↑**ATP6AP1**, ATP6V1E1, ↑**C11ORF16**, CBY1, CTNNB1, ↑**DCAKD**, ↓**DUSP23**, EWSR1, ↓**FRY**, ↑**LEO1**, LIMA1, ↓**MRPS26**, MTMR9, ↓**MXRA8**, ↑**PCCA**, PCCB, PKP2, PRPF19, PTPN14, ↑**RAB8B**, RAPSN, RPL8, RPS15A, SLC25A3, SLC2A4, ↑**SLC4A8**, SLC9A3R1, ↓**SVIL**, YWHAG	Genetic disorder, DNA replication
33	↑**BSPRY**, BYSL, ↓**CRYBB1**, CRYBB2, CSE1L, CTH, ↓**DENND4A**, ↓**EAF2**, ↑**FAM62A**, FPR2, ↓**HOXA9**, IARS, IKBKG, IPO7, IPO9, ↑**KIF21A***, KRT8, KRT18, LTBR, ↑**MARK1***, MIB1, ↑**NADK (includes EG:65220)**, PABPN1, PCTK2, ↓**PDLIM4**, PDXK, RBCK1, ↓**RBPMS***, RPLP0 (includes EG:6175), ↓**ST5**, TRO, ↑**TROAP**, VARS, YWHAZ	Cellular assembly and organization
34	↑**ABHD6**, ACLY, AHCYL1, ↑**ATP6AP2**, ↑**BTG3**, CAPN8, ↑**CCDC18**, ↑**CCDC99**, CPA1, EXOSC4, EXOSC5, farnesyl pyrophosphate, geranylgeranyl pyrophosphate, Hydrolase, IMPA2, LONP1, ↓**LRRC8D**, ↓**MDFIC**, MIR124, MIR124-2, ↑**MTMR6**, ↑**N4BP1**, Ndpk, ↑**NME2** NME3, ↑**NME4**, nucleoside diphosphates, ↓**PARP16**, PCSK6, POP7, PTPN7, RECQL5, ↑**SSFA2**, TMEM62, TMPRSS3	Amino acid metabolism, cell morphology

Appendix 4: List of top differentially expressed genes between day 7 and day 14 of the estrous cycle (CDd7 vs. CDd14) in receptive endometrium. Negative and positive fold changes (FC) describe genes increased in CDd7 and CDd14, respectively

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.22642	Diacylglycerol kinase, iota	DGKI	396.8	0.000
Bt.74713	Hypothetical protein mgc127538	MGC127538	271.6	0.001
Bt.26462	Endothelin 3	EDN3	208.2	0.001
Bt.20340	Similar to myotonic dystrophy kinase	LOC513675	158.7	0.000
---	Seminal vesicle secretion 8	SVS8	102.0	0.000
---	Similar to family with sequence similarity 43, member a	LOC539374	100.4	0.000
Bt.51579	Chromosome 14 open reading frame 37 ortholog	C10H14orf37	83.5	0.000
Bt.60923	Spc24, ndc80 kinetochore complex component, homolog	SPC24	82.9	0.000
Bt.20369	Proline dehydrogenase (oxidase) 1	PRODH	78.9	0.000
Bt.45257	Pdz domain containing 1	PDZK1	74.7	0.008
Bt.60335	Plasma membrane proteolipid (plasmolipin)	PLLP	72.0	0.000
Bt.15980	Cyclin b1	CCNB1	66.6	0.000
Bt.5387	Lipoprotein lipase	LPL	65.3	0.000
Bt.46878	Glycerophosphodiester phosphodiesterase domain containing 1	GDPD1	53.5	0.001
Bt.9602	Death associated protein-like 1	DAPL1	53.3	0.000
Bt.53247	Sushi domain containing 2	SUSD2	52.7	0.000
Bt.1539	Gm2 ganglioside activator	GM2A	38.9	0.000
Bt.24904	Adam metallopeptidase with thrombospondin type 1 motif, 6	ADAMTS6	38.6	0.002
Bt.44919	Solute carrier family 6 (proline imino transporter), member 20	SLC6A20	32.8	0.000
Bt.49607	3-hydroxybutyrate dehydrogenase, type 1	BDH1	31.5	0.002
Bt.60335	Plasma membrane proteolipid (plasmolipin)	PLLP	29.3	0.001

Appendix 4: Cont.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.1207	Solute carrier family 16, member 13	SLC16A13	28.4	0.002
Bt.16830	Leucine zipper, down-regulated in cancer 1	LDOC1	27.4	0.001
Bt.17189	Protein kinase c, theta	PRKCQ	26.9	0.000
Bt.3949	6-phosphofructo-2-kinase	PFKFB2	26.3	0.000
Bt.13588	Phosphoserine aminotransferase 1	PSAT1	26.1	0.001
Bt.35560	Glycine n-methyltransferase	GNMT	25.3	0.001
Bt.35560	Glycine n-methyltransferase	GNMT	25.1	0.001
Bt.5091	Diacylglycerol kinase, alpha 80kda	DGKA	25.1	0.000
Bt.26921	Solute carrier family 27 (fatty acid transporter), member 6	SLC27A6	23.9	0.004
Bt.22697	Solute carrier family 39 (zinc transporter	SLC39A8	23.4	0.000
Bt.7037	Sphingosine kinase 1	SPHK1	23.3	0.000
Bt.13057	Calcium channel, voltage-dependent, t type, alpha 1h subunit	CACNA1H	23.2	0.000
Bt.8135	Lecithin retinol acyltransferase (phosphatidylcholine--retinol o-acyltransferase)	LRAT	22.4	0.000
Bt.58521	Claudin 5 (transmembrane protein deleted in velocardiofacial syndrome)	CLDN5	22.2	0.000
Bt.24664	N-acetylated alpha-linked acidic dipeptidase 2	NAALAD2	21.4	0.001
Bt.16037	Globoside alpha-1,3-n-acetylgalactosaminyltransferase 1	GBGT1	21.3	0.000
Bt.27135	Cysteine conjugate-beta lyase loc781909	CCBL1	21.2	0.001
Bt.39860	Was interacting protein family, member 3	WIPF3	21.1	0.000
Bt.52359	Chromosome 9 open reading frame 30 ortholog	C8H9orf30	20.5	0.002
Bt.4844	Pleiotrophin (heparin binding growth factor 8	PTN	19.6	0.007
Bt.49548	Mal, t-cell differentiation protein /// similar to mal, t-cell differentiation protein	MAL	17.5	0.000
Bt.53026	Phosphoglycerate dehydrogenase	PHGDH	16.8	0.001
Bt.51909	Adiponectin receptor 2	ADIPOR2	16.7	0.002
Bt.22305	Inositol 1,4,5-triphosphate receptor, type 1	ITPR1	16.4	0.001

Appendix 4: Cont.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.639	Cell division cycle 20 homolog	CDC20	16.3	0.005
Bt.25235	Mitochondrial ribosomal protein s36	MRPS36	15.3	0.000
Bt.53600	Cytochrome b5 type b	CYB5B	15.2	0.000
Bt.10882	Timp metallopeptidase inhibitor 3 (sorsby fundus dystrophy, pseudoinflammatory)	TIMP3	15.2	0.001
Bt.46878	Glycerophosphodiester phosphodiesterase domain containing 1	GDPD1	15.2	0.002
Bt.46419	Six transmembrane epithelial antigen of the prostate	STEAP2	15.0	0.001
Bt.39403	Kn motif and ankyrin repeat domains 2	KANK2	14.8	0.001
Bt.40997	Hypothetical protein loc510320	LOC510320	14.7	0.000
Bt.51709	Septin 4	Sep 04	14.6	0.000
Bt.22399	Arginase, type ii	ARG2	14.6	0.002
Bt.21876	Wingless-type mmtv integration site family, member 11	WNT11	14.5	0.007
Bt.22336	Teratocarcinoma-derived growth factor 1	TDGF1	14.2	0.005
Bt.75644	Membrane bound o-acyltransferase domain containing 2	MBOAT2	14.0	0.016
Bt.52324	Nuclear protein 1	NUPR1	13.7	0.001
Bt.43717	Hydroxyprostaglandin dehydrogenase 15-(nad)	HPGD	13.5	0.006
Bt.5487	Aldehyde dehydrogenase 3 family, member a2	ALDH3A2	13.3	0.000
Bt.64525	Similar to elongation factor 1 alpha	EEF1A1	12.7	0.000
Bt.53600	Cytochrome b5 type b	CYB5B	12.3	0.000
Bt.91476	Collagen, type iv, alpha 4	COL4A4	11.6	0.000
Bt.54026	Glutamate-cysteine ligase, catalytic subunit	GCLC	11.6	0.000
Bt.45508	Mucolipin 3	MCOLN3	11.4	0.047
Bt.25034	Rab9b, member ras oncogene family	RAB9B	11.4	0.000
Bt.76544	Tumor necrosis factor receptor superfamily	TNFRSF6B	11.2	0.003
Bt.92053	Similar to cordon-bleu homolog (mouse)	LOC505588	11.0	0.006
Bt.48435	Transmembrane protein 163	TMEM163	10.6	0.002
Bt.38424	Dystonin	DST	10.6	0.000

Appendix 4: Cont.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.25525	Ectonucleotide phosphodiesterase 2	ENPP2	10.5	0.000
Bt.22046	Alpha-methylacyl-coa racemase	AMACR	10.4	0.002
Bt.5193	Acid phosphatase 5, tartrate resistant	ACP5	10.4	0.000
Bt.101236	Two pore segment channel 1	TPCN1	10.3	0.000
Bt.13798	Hypothetical loc615685	LOC615685	10.2	0.000
Bt.24640	Ilvb (bacterial acetolactate synthase)-like	ILVBL	10.1	0.000
Bt.25546	Neuropilin (nrp) and tolloid (tll)-like 2	NETO2	10.1	0.003
Bt.9659	Ectonucleotide pyrophosphatase/phosphodiesterase3	ENPP3	9.9	0.001
Bt.16596	Leucine rich repeat containing 3	LRRC3	9.9	0.001
Bt.64882	Dihydropyrimidinase-like 2	DPYSL2	9.8	0.000
Bt.7490	Methylenetetrahydrofolate dehydrogenase (nadp+ dependent) 1-like	MTHFD1L	9.7	0.001
Bt.90915	Sulfotransferase family, cytosolic, 2b, member 1	SULT2B1	9.5	0.000
Bt.87527	Potassium channel tetramerisation domain containing 17	KCTD17	9.5	0.002
Bt.49702	Visinin-like 1	VSNL1	9.5	0.000
Bt.64166	Microsomal triglyceride transfer protein	MTTP	9.3	0.001
Bt.92053	Similar to cordon-bleu homolog (mouse)	LOC505588	9.2	0.010
Bt.66560	Similar to map/microtubule affinity-regulating kinase 3	LOC534872	9.2	0.000
Bt.65258	Matrilin 2	MATN2	9.1	0.036
Bt.91589	Ero1-like	ERO1L	9.1	0.002
Bt.18700	Chromosome 11 open reading frame 16	C11orf16	9.1	0.007
Bt.25235	Mitochondrial ribosomal protein s36	MRPS36	9.1	0.001
Bt.49610	S100 calcium binding protein b	S100B	8.9	0.001
Bt.21924	Similar to dermatan/chondroitin sulfate 2-sulfotransferase	LOC535975	8.8	0.007
Bt.7142	Similar to aim1	LOC526200	8.8	0.000
Bt.22054	Chromosome 20 open reading frame 7	C13H20ORF7	8.7	0.000
Bt.13798	Hypothetical loc615685	LOC615685	8.7	0.004

Appendix 4: Cont.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.7043	Vascular cell adhesion molecule 1	VCAM1	8.6	0.007
Bt.101039	Similar to kiaa0672 gene product	LOC509257	8.7	0.005
Bt.4998	Procollagen-lysine oxoglutarate dioxygenase	PLOD1	8.5	0.000
Bt.28476	Monoacylglycerol o-acyltransferase 1	MOGAT1	8.5	0.002
Bt.2887	Nudix (nucleoside diphosphate linked moiety x)-type motif 5	NUDT5	8.3	0.000
Bt.42906	Phosphoribosyl pyrophosphate synthetase-associated protein 1	PRPSAP1	8.3	0.000
Bt.36484	Stefin c	LOC514170	8.3	0.002
Bt.62758	Glucuronidase, beta	GUSB	8.3	0.001
Bt.1207	Solute carrier family 16, member 13	SLC16A13	8.1	0.004
Bt.7142	Similar to aim1	LOC526200	8.0	0.000
Bt.51597	Thiosulfate sulfurtransferase (rhodanese)	TST	8.0	0.000
Bt.46406	Oxoglutarate (alpha-ketoglutarate) dehydrogenase	OGDH	8.0	0.000
Bt.91285	Gata binding protein 2	GATA2	7.9	0.000
Bt.41964	Methionyl aminopeptidase 1	METAP1	7.9	0.000
Bt.65743	Histamine n-methyltransferase	HNMT	7.8	0.000
Bt.12794	Collagen, type iv, alpha 3 (goodpasture antigen)	COL4A3	7.8	0.002
Bt.101388	Polo-like kinase 4	PLK4	7.8	0.000
Bt.3699	Dynein, cytoplasmic 1, intermediate chain 1	DYNC1I1	7.8	0.000
Bt.23641	Carbonic anhydrase xiii	CA13	7.8	0.003
Bt.17182	Gap junction protein, beta 5, 31.1kda	GJB5	7.7	0.000
Bt.88935	Creatine kinase, brain	CKB	7.7	0.003
Bt.91302	Similar to enpp5	LOC512304	7.7	0.000
Bt.21101	Aminocarboxymuconate semialdehyde decarboxylase	ACMSD	7.7	0.017
Bt.9850	Transmembrane protein 171	TMEM171	7.6	0.000
Bt.8091	Cell division cycle associated 4	CDCA4	7.6	0.000
Bt.13798	Hypothetical loc615685	LOC615685	7.6	0.002
Bt.9094	Unc-13 homolog d (c. Elegans)	UNC13D	7.5	0.002
Bt.29868	Cytochrome c oxidase subunit vib	COX6B2	7.4	0.002

Appendix 4: Cont.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.396	Cellular retinoic acid binding protein 1	CRABP1	7.3	0.029
Bt.4747	Nucleoporin 88kda	NUP88	7.3	0.002
Bt.53189	Tudor and kh domain containing	TDRKH	7.2	0.000
Bt.20428	Protein regulator of cytokinesis 1	PRC1	7.2	0.001
Bt.20920	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	PPARGC1A	7.1	0.000
Bt.87354	Six transmembrane epithelial antigen of the prostate 1	STEAP1	7.1	0.014
Bt.20013	Elovl family member 7, elongation of long chain fatty acids (yeast)	ELOVL7	7.1	0.006
Bt.43706	Actin binding lim protein 1	ABLIM1	7.1	0.000
Bt.19643	Potassium large conductance calcium-activated channel, subfamily, beta member 4	KCNMB4	6.8	0.005
Bt.13399	Beta-1,4-n-acetyl-galactosaminyl transferase 2	B4GALNT2	6.8	0.026
Bt.64734	Armadillo repeat containing 8	ARMC8	6.7	0.000
Bt.48867	Cytochrome p450, family 2, subfamily	CYP2C87	6.7	0.033
Bt.37565	Ribosomal protein s6 kinase, 90kda, polypeptide 5	RPS6KA5	6.7	0.000
Bt.57528	Similar to ikappabkinase complex-associated protein	LOC505465	6.6	0.000
Bt.33887	Surfactant, pulmonary-associated protein d	CL-46	6.6	0.023
Bt.9662	Prostaglandin e receptor 4 (subtype ep4)	PTGER4	6.5	0.000
Bt.24154	Transcription factor dp-2	TFDP2	6.4	0.000
Bt.34944	Phospholipid transfer protein	PLTP	6.4	0.002
Bt.13798	Hypothetical loc615685	LOC615685	6.4	0.001
Bt.27994	Alanyl (membrane) aminopeptidase	ANPEP	6.4	0.000
Bt.92594	Chromosome 4 open reading frame 34 ortholog	C6H4orf34	6.3	0.001
Bt.49270	Receptor accessory protein 4	REEP4	6.2	0.000
Bt.7142	Similar to aim1	LOC526200	6.2	0.000

Appendix 4: Cont.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.11180	Leucine rich repeat neuronal 5 precursor	LRRN5	6.2	0.001
Bt.12757	Nerve growth factor (beta polypeptide)	NGF	6.2	0.026
Bt.26607	Glypican 1	GPC1	6.2	0.000
Bt.15730	Kinesin family member 2c	KIF2C	6.2	0.020
Bt.44571	Similar to tumor necrosis factor, alpha-induced protein 2	LOC786672	6.1	0.006
Bt.38709	Rev3-like, catalytic subunit of DNA polymerase zeta	REV3L	6.1	0.000
Bt.12781	V-myb myeloblastosis viral oncogene	MYB	6.1	0.004
Bt.74139	T-cell lymphoma invasion and metastasis 1	TIAM1	6.1	0.000
Bt.37590	Transketolase-like 1	TKTL1	6.1	0.004
Bt.58988	Solute carrier family 31 (copper transporters), member 2	SLC31A2	6.1	0.002
Bt.7133	Ornithine decarboxylase 1	ODC1	6.1	0.000
Bt.41728	Solute carrier family 39 (zinc transporter), member 2	SLC39A2	-418.0	0.001
Bt.132	Defensin, beta 1	DEFB1	-160.3	0.000
Bt.1300	Tubulointerstitial nephritis antigen-like 1	TINAGL1	-157.1	0.004
Bt.6410	Chromosome 11 open reading frame 34 ortholog	C15H11ORF34	-130.6	0.007
Bt.594	Uterine milk protein precursor	LOC286871	-97.4	0.005
Bt.49740	Interleukin 8	IL8	-91.5	0.001
Bt.452	Glycosylphosphatidylinositol specific phospholipase D1	GPLD1	-91.3	0.003
Bt.67194	Lysozyme 1	LYZ1	-72.0	0.005
Bt.190	Insulin-like growth factor binding protein 1	IGFBP1	-45.2	0.006
Bt.594	Uterine milk protein precursor	LOC286871	-41.5	0.005
Bt.27007	Unc-45 homolog B (C. Elegans)	UNC45B	-40.5	0.016
Bt.7393	Nephronectin	NPNT	-38.3	0.001
Bt.13029	Hypocretin (orexin) receptor 1	HCRTR1	-36.5	0.006
Bt.40113	Solute carrier family 16, member 1	SLC16A1	-32.8	0.004
Bt.31841	Talin 2	TLN2	-28.0	0.000

Appendix 4: Cont.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.13772	Cytochrome P450, subfamily I (aromatic compound-inducible), polypeptide 1	CYP1A1	-23.0	0.017
Bt.13860	Kinase insert domain receptor (a type III receptor tyrosine kinase)	KDR	-22.1	0.005
Bt.23889	Microtubule-associated protein 2	MAP2	-20.2	0.011
Bt.6087	Transmembrane 4 L six family member 1	TM4SF1	-18.8	0.001
Bt.28230	Complement factor H-related 3	CFHR3	-17.5	0.011
Bt.6410	Chromosome 11 open reading frame 34 ortholog	H15C11ORF34	-17.4	0.031
Bt.49470	Solute carrier family 5	SLC5A11	-16.8	0.005
Bt.11462	Syndecan 2	SDC2	-15.9	0.001
Bt.27910	Chromosome 20 open reading frame 54	C20orf54	-14.8	0.000
Bt.468	Cystic fibrosis transmembrane conductance regulator	CFTR	-14.7	0.001
Bt.12967	Cadherin 16, KSP-cadherin	CDH16	-14.2	0.010
Bt.15758	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	-14.1	0.018
Bt.21896	Collagen, type XXI, alpha 1	COL21A1	-14.0	0.000
Bt.5318	Retinol binding protein 4, plasma	RBP4	-13.8	0.008
Bt.6087	Transmembrane 4 L six family member 1	TM4SF1	-13.4	0.000
Bt.9632	Deleted in malignant brain tumors 1	DMBT1	-12.8	0.001
Bt.4645	Similar to c1orf24	LOC504817	-12.6	0.001
Bt.13860	Kinase insert domain receptor (a type III receptor tyrosine kinase)	KDR	-12.0	0.004
Bt.89332	Fibroblast growth factor-binding protein	LOC281812	-12.0	0.000
Bt.26155	N-acetylneuraminate pyruvate lyase	NPL	-11.9	0.004
Bt.50127	Similar to RAD21-like 1 (S. Pombe)	LOC512408	-11.5	0.001
Bt.357	S100 calcium binding protein A12	S100A12	-11.4	0.004
Bt.28620	Solute carrier family 27	SLC27A2	-10.8	0.022
Bt.31739	ABI gene family, member 3 binding protein	ABI3BP	-10.2	0.001
Bt.49731	Carbonic anhydrase II	CA2	-10.0	0.029

Appendix 4: Cont.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.88771	Transmembrane protein 45B	TMEM45B	-9.9	0.006
Bt.60687	Formin-like 3	FMNL3	-9.5	0.004
Bt.20477	Raftlin, lipid raft linker 1	RFTN1	-9.1	0.017
Bt.388	RAB3B, member RAS oncogene family	RAB3B	-9.0	0.033
Bt.64122	Supervillin	SVIL	-8.9	0.000
Bt.24447	Coagulation factor II (thrombin) receptor-like	F2RL2	-8.8	0.000
Bt.49269	Myotubularin related protein 11	MTMR11	-8.7	0.002
Bt.42032	Cell division cycle associated 7	CDCA7	-8.7	0.041
Bt.62650	Ubiquitin D	UBD	-8.7	0.001
Bt.49579	Solute carrier family 29, member 1	SLC29A1	-8.5	0.018
Bt.9675	Similar to Extracellular proteinase inhibitor	LOC787253	-8.3	0.004
Bt.35472	Tetratricopeptide repeat domain 21B	SCN1A	-8.3	0.013
Bt.15782	CD59 molecule, complement regulatory protein	CD59	-8.2	0.015
Bt.5484	Very low density lipoprotein receptor	vldlr	-8.1	0.020
Bt.12140	Mucin 13, cell surface associated	MUC13	-8.0	0.000
Bt.23497	Myosin, light chain 3, alkali; ventricular, skeletal, slow	MYL3	-7.8	0.033
Bt.23889	Microtubule-associated protein 2	MAP2	-7.7	0.051
Bt.88773	Cystatin E/M	CST6	-7.7	0.014
Bt.8282	Dipeptidyl-peptidase 4 (CD26, adenosine	DPP4	-7.5	0.005
Bt.27973	Solute carrier organic anion transporter family, member 3A1	SLCO3A1	-7.4	0.005
Bt.43651	SLAM family member 7	SLAMF7	-7.4	0.037
Bt.25099	Phosphoserine phosphatase	PSPH	-7.2	0.028
Bt.46545	Similar to putative ISG12(a) protein	ISG12(A)	-7.1	0.002
Bt.63426	UDP glucuronosyltransferase 2 family, polypeptide B10	UGT2B10	-6.7	0.017
Bt.52393	Cathepsin H	CTSH	-6.6	0.007
Bt.25663	Copine VIII	CPNE8	-6.4	0.004
Bt.56845	Cysteine rich transmembrane BMP regulator1	CRIM1	-6.3	0.004
Bt.16087	Nidogen 2 (osteonidogen)	NID2	-6.2	0.024

Appendix 4: Cont.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.10630	Neuromedin B	NMB	-6.2	0.024
Bt.19932	Similar to cystin cilia-associated protein	LOC616812	-6.1	0.007
Bt.7724	Septin 11	Sep 11	-6.1	0.013
Bt.33922	Radical S-adenosyl methionine containing 2	RSAD2	-5.9	0.012
Bt.60918	Similar to Interferon-induced	LOC512486	-5.8	0.030
Bt.62067	Folate receptor 1 (adult)	FOLR1	-5.8	0.006
Bt.193	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A	MGAT4A	-5.6	0.007
Bt.25459	Atpase type 13A4	ATP13A4	-5.6	0.010
Bt.91165	Similar to ATP-binding cassette transporter	LOC617079	-5.5	0.008
Bt.12302	Plasminogen activator, tissue	PLAT	-5.5	0.000
Bt.25511	RAD51-like 1 (S. Cerevisiae)	RAD51L1	-5.5	0.004
Bt.22624	Leucine rich repeat containing 8 family, member D	LRRC8D	-5.4	0.000
Bt.62794	Transmembrane protein 117	TMEM117	-5.4	0.003
Bt.4646	Solute carrier family 2, member 1	SLC2A1	-5.3	0.024
Bt.39963	Neurochondrin	NCDN	-5.3	0.014
Bt.12967	Cadherin 16, KSP-cadherin	CDH16	-5.2	0.016
Bt.13456	Actin binding LIM protein family, member 3	ABLIM3	-5.1	0.028
Bt.95150	Purinergic receptor P2Y, G-protein coupled,5	P2RY5	-5.1	0.001
Bt.13084	Met proto-oncogene	MET	-5.0	0.000
Bt.53071	Phosphodiesterase 4B, camp-specific	PDE4B	-5.0	0.008
Bt.64706	Cold shock domain containing C2	CSDC2	-4.9	0.017
Bt.44795	Nuclear receptor coactivator 7	NCOA7	-4.9	0.001
Bt.92569	Solute carrier family 5, member 1	SLC5A1	-4.9	0.046
Bt.611	Chemokine (C-X-C motif) ligand 2	CXCL2	-4.8	0.002
Bt.1370	Argininosuccinate synthetase 1	ASS1	-4.8	0.018
Bt.411	Neuregulin 1	NRG1	-4.8	0.004
Bt.8544	CD69 molecule	CD69	-4.7	0.014
Bt.2801	Leprecan 1	LOC539976	-4.7	0.002
Bt.52082	Arrestin, beta 1	ARRB1	-4.6	0.006
Bt.4199	Interleukin 1 receptor antagonist	IL1RN	-4.6	0.007
Bt.16118	Similar to C-type lectin domain family 2,	LOC504309	-4.6	0.002

Appendix 5: List of top differentially expressed genes between day 7 and 14 of the estrous cycle (NPd7 vs. NPd14) in non-receptive endometrium. Negative and positive fold changes (FC) describe genes increased in NPd7 and NPd14, respectively.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.22642	Diacylglycerol kinase, iota	DGKI	13.80	0.014
---	Seminal vesicle secretion 8	SVS8	12.86	0.010
Bt.60923	SPC24, NDC80 kinetochore complex component, homolog (S. Cerevisiae)	SPC24	10.61	0.010
Bt.22571	Speckle-type POZ protein	SPOP	10.06	0.003
Bt.23508	Collagen, type VI, alpha 1	COL6A1	9.40	0.000
Bt.64594	Thioredoxin domain containing 6	TXNDC6	9.31	0.005
Bt.96859	Transcribed locus, moderately similar to NP_982279.1 apolipoprotein B mrna editing enzyme	---	8.81	0.004
Bt.64757	Neurotrophic tyrosine kinase, receptor,type2	NTRK2	8.23	0.005
Bt.64625	Apolipoprotein D	APOD	8.13	0.002
Bt.46762	Prune homolog 2 (Drosophila)	PRUNE2	8.08	0.001
Bt.55266	Similar to procollagen alpha 2(V)	LOC782818	7.70	0.012
Bt.20399	Hydroxysteroid (17-beta) dehydrogenase 13	HSD17B13	7.43	0.004
Bt.68159	Collagen, type VI, alpha 3	COL6A3	7.39	0.001
Bt.61691	PARK2 co-regulated	PACRG	6.17	0.011
Bt.5313	Matrix metallopeptidase 2	MMP2	5.13	0.001
Bt.54339	Sperm associated antigen 6	SPAG6	4.99	0.015
Bt.53485	Collagen, type I, alpha 2	COL1A2	4.90	0.010
Bt.2452	Lumican	LUM	4.71	0.007
Bt.2638	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin,	SERPINF1	4.70	0.004
Bt.27034	Tektin 1	TEKT1	4.60	0.014
Bt.20385	Phospholipase A1 member A	PLA1A	4.32	0.000
Bt.39050	Cannabinoid receptor interacting protein 1	CNRIP1	4.08	0.009
Bt.39894	Myosin light chain kinase	MYLK	3.98	0.000

Appendix 5: Cont.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.26488	Transcribed locus	---	3.97	0.009
Bt.39738	Transcribed locus	---	3.97	0.015
Bt.8880	SPARC-like 1 (mast9, hevin)	SPARCL1	3.88	0.003
Bt.53600	Cytochrome b5 type B	CYB5B	3.87	0.012
Bt.25235	Mitochondrial ribosomal protein S36	MRPS36	3.87	0.014
Bt.98978	Transcribed locus	---	3.87	0.007
Bt.4539	Angiotensin II receptor, type 1	AGTR1	3.85	0.000
Bt.101209	Collagen, type III, alpha 1	COL3A1	3.77	0.013
Bt.26656	Mitogen-activated protein kinase 10	MAPK10	3.75	0.011
Bt.21766	Myeloid leukemia factor 1	MLF1	3.67	0.011
Bt.4011	Transmembrane protein 120A	TMEM120A	3.61	0.007
Bt.68595	Transcribed locus	---	3.53	0.014
Bt.81912	Similar to OB-cadherin-1 /// cadherin 11, type 2, OB-cadherin	CDH11 /// LOC535363	3.47	0.008
Bt.45145	Asporin	ASPN	3.42	0.000
Bt.23178	Decorin	DCN	3.40	0.013
Bt.30575	Scavenger receptor class A, member 5	SCARA5	3.09	0.012
Bt.4210	Kininogen 1	KNG1	3.09	0.010
Bt.18339	Dynein, light chain, roadblock-type 2	DYNLRB2	3.06	0.015
Bt.23618	Spondin 1, extracellular matrix protein	SPON1	3.06	0.009
Bt.4492	Collectin sub-family member 12	COLEC12	3.03	0.002
Bt.68368	Transcribed locus	---	-2.86	0.001
Bt.22179	Similar to chromosome 1 open reading frame	LOC507126	-2.87	0.013
Bt.54463	Early growth response 1	EGR1	-2.90	0.006
Bt.92871	Transcribed locus	---	-2.94	0.001
Bt.1730	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	ID1	-3.00	0.005
Bt.93483	Transcribed locus	---	-3.05	0.000
Bt.24872	Kinesin family member 4A	KIF4A	-3.07	0.004
Bt.24645	Hypothetical LOC613372	LOC613372	-3.08	0.005
---	Frequently rearranged in advanced T-cell	FRAT2	-3.09	0.006

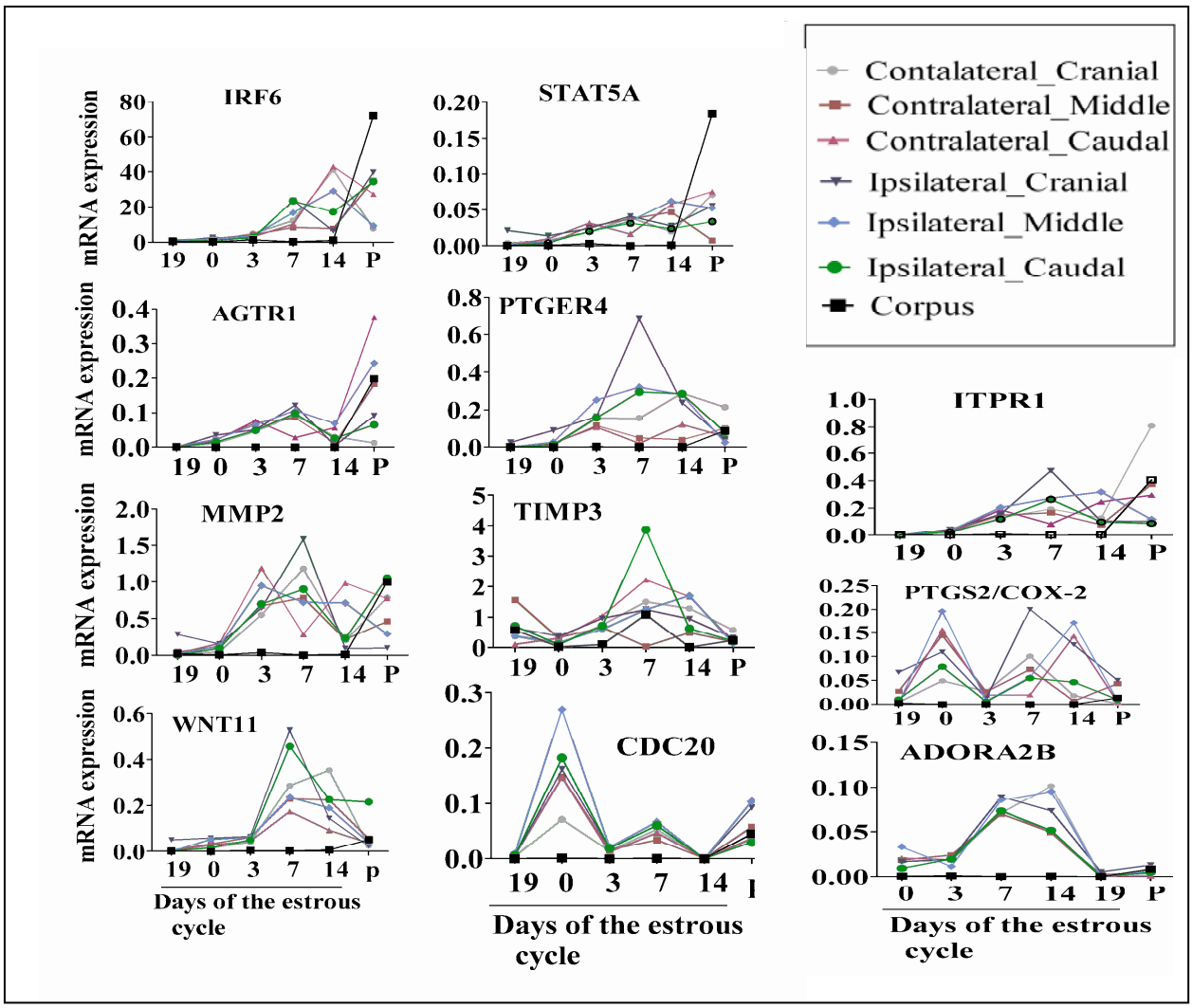
Appendix 5: Cont.

UniGene ID	Gene title	Gene symbol	FC	p value
Bt.78960	Transcribed locus	---	-3.10	0.000
Bt.16613	Transcribed locus	---	-3.11	0.006
Bt.1220	Transcribed locus	---	-3.12	0.012
Bt.9113	Immediate early response 5	IER5	-3.19	0.007
Bt.24515	Akt substrate AS250	LOC507082	-3.24	0.004
Bt.12140	Mucin 13, cell surface associated	MUC13	-3.30	0.014
Bt.96035	Transcribed locus	---	-3.34	0.001
Bt.59370	Leucyl-trna synthetase 2, mitochondrial	LARS2	-3.36	0.000
Bt.8945	Toll-like receptor 2	TLR2	-3.39	0.002
Bt.91648	Chondroadherin	CHAD	-3.45	0.015
Bt.93107	Transcribed locus	---	-3.51	0.000
Bt.93218	Transcribed locus	---	-3.55	0.000
Bt.9482	Rho gtpase activating protein 17	ARHGAP17	-3.61	0.000
Bt.91133	Ankyrin repeat domain 10	ANKRD10	-3.63	0.001
Bt.91073	Transcribed locus	---	-3.65	0.003
Bt.16892	Golgi-localized protein	GOLSYN	-3.69	0.012
Bt.13084	Met proto-oncogene	MET	-3.83	0.002
Bt.9632	Deleted in malignant brain tumors 1	DMBT1	-3.99	0.012
Bt.15784	Similar to squamous cell-specific protein	LOC786490	-4.03	0.011
Bt.10209	Serglycin	SRGN	-4.16	0.014
Bt.18533	Activating transcription factor 3	ATF3	-4.17	0.003
Bt.53071	Phosphodiesterase 4B, camp-specific	PDE4B	-4.35	0.012
Bt.96346	Transcribed locus	---	-4.38	0.011
Bt.101406	Transcribed locus	---	-4.39	0.015
Bt.367	Oxidized low density lipoprotein	OLR1	-4.69	0.002
Bt.12304	ISG15 ubiquitin-like modifier	ISG15	-4.82	0.009
Bt.32826	CD68 molecule	CD68	-5.24	0.004
Bt.9632	Deleted in malignant brain tumors 1	DMBT1	-5.24	0.012
Bt.61441	Transcribed locus	---	-5.27	0.005
Bt.4199	Interleukin 1 receptor antagonist	IL1RN	-5.34	0.004
Bt.468	Cystic fibrosis transmembrane conductance regulator	CFTR	-5.44	0.012

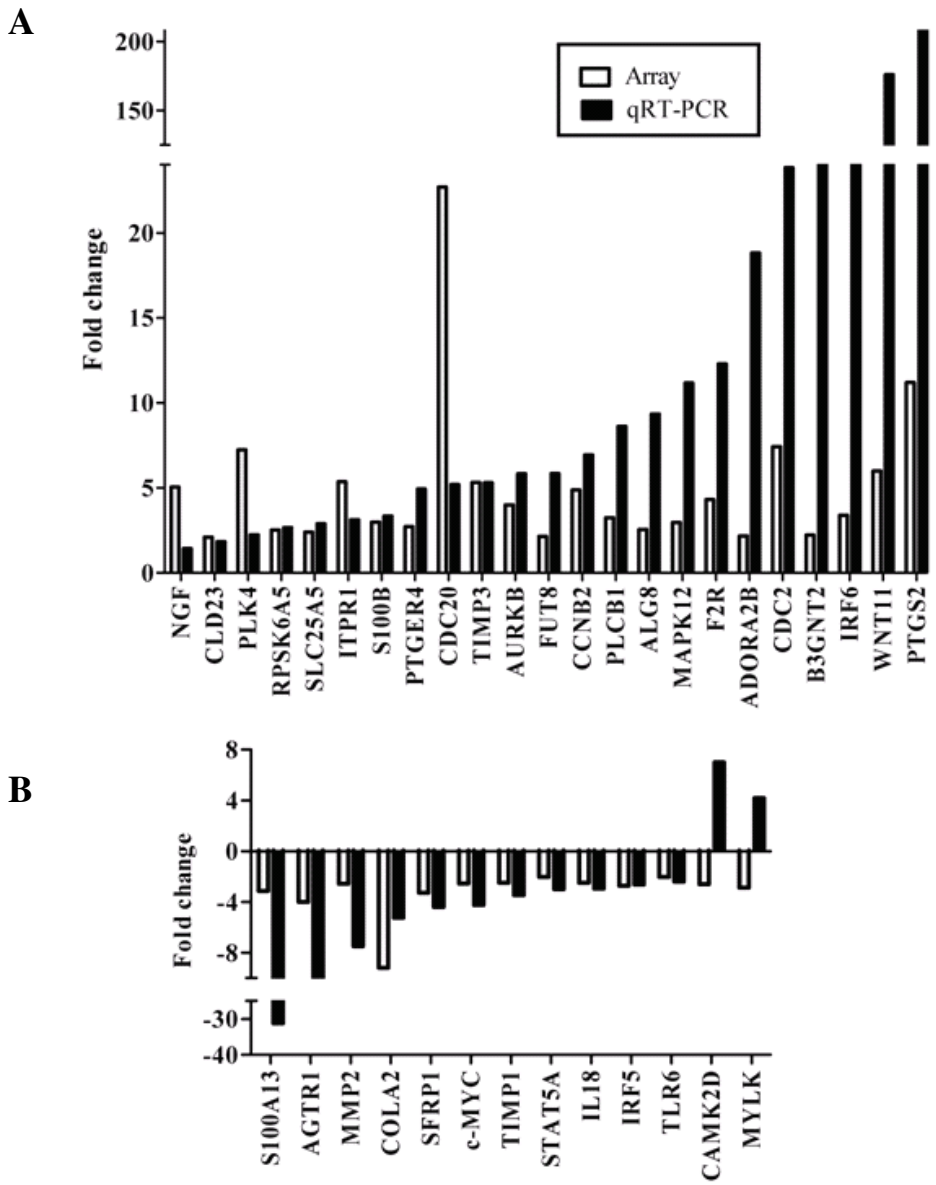
Appendix 5: Cont.

UniGene ID	Gene title	Gene symbol	FC	p. valu
Bt.87242	Steroidogenic acute regulatory protein	STAR	-5.87	0.010
Bt.33922	Radical S-adenosyl methionine domain containing 2	RSAD2	-6.18	0.010
Bt.46545	Similar to putative ISG12(a) protein	ISG12(A)	-5.65	0.005
Bt.65386	Pleckstrin	PLEK	-7.37	0.012
Bt.60687	Formin-like 3	FMNL3	-7.51	0.008
Bt.22477	Transcribed locus	---	-8.69	0.002
Bt.93099	Transcribed locus	---	-9.43	0.000
Bt.31841	Talin 2	TLN2	-11.6	0.002
Bt.2408	Chemokine (C-C motif) ligand 2	CCL11	-12.1	0.005
Bt.7393	Nephronectin	NPNT	-16.9	0.007
Bt.10536	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I,	SPP1	-21.2	0.006
Bt.13125	Neutrophil beta-defensin 4 /// defensin, beta 5 /// similar to neutrophil beta-defensin 4	BNBD-4	-22.2	0.002
Bt.5878	Serine dehydratase	SDS	-23.5	0.013
Bt.49740	Interleukin 8	IL8	-51.9	0.003
Bt.41728	Solute carrier family 39	SLC39A2	-112	0.005

Appendix 6: The spatio-temporal expression of selected candidate genes at different region of the endometrium during the estrous cycle and day 50 gestation period (P). The vertical axis indicates the relative mRNA expression level of each gene normalized GAPDH.



Appendix 7: Validation of differentially expressed genes between receptive (CDd7) and non- receptive (NPd7) endometrium at day 7 of the estrous cycle.
(A): Genes enriched in CDd7. (B): Genes enriched in NPd7



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3. Work experience

Aug.2001-Sept.2002:	Graduate Assistant at Alemaya University, Alemaya, Ethiopia.
8th July -Sept.5/2000:	Data collector for drought assessment and Evaluation at Harrerghe Catholic Secretariat (HCS), Dire Dawa, Ethiopia.
8th July-30th sept.2000:	Agricultural Development supervisor as a part time employee by Ministry of Agriculture, Western Gojjam Department of Agriculture, Bahirdar Zuria District Agricultural Office, Ethiopia.
June 1993-Sept.1998:	Agricultural Development Agent (DA), Ministry of Agriculture, Western Gojjam Department of agriculture. Bahirdar Zuria District Agricultural Office, Ethiopia.

4. Publication

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