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Transcriptome analysis of bovine day 16 conceptus derived after transfer of blastocyst from somatic cell nuclear transfer or in vitro production

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Dedicated to loving parents Betsha Weldenegodguad and Aster Mamo, carrying husband Tsegaw Seyoum and precious son Ebenezer Tsegaw

Transcriptome analysis of bovine day 16 conceptus derived after transfer of blastocyst from somatic cell nuclear transfer or in vitro production

In vitro embryo production (IVP) and somatic cell nuclear transfer (SCNT) have been used as tools of assisted reproductive technology to produce bovine pre-implantation embryos independent of the maternal environment. However, the embryonic and fetal losses after transfer of SCNT and IVP derived embryos is higher compared to the in vivo (AI) counterparts. This may be associated with the alterations in the molecular signatures and pathways at any stage of embryonic and /or fetal development. Therefore, to identify the molecular changes that could occur at day 16 SCNT and IVP derived embryos, large scale transcriptomic analysis was performed using Affymetrix-Bovine Genome Array. For this, day 7 blastocysts derived from SCNT, IVP and AI were transferred to oestrus synchronized Simmental heifers. Recipients were then slaughtered at day 16 of gestation and conceptuses were retrieved. Following morphological examination, filamentous embryos with visible embryonic disc were subjected to global tanscriptome analysis. The result demonstrated comparable in vivo development rate in SCNT (72.7%), IVP (62.2%) and AI (77.3%) embryo groups. However, considerable reduction in the trophoblast elongation size was observed in SCNT (93.3mm) compared to IVP (186.6mm) and AI (196.3mm) derived embryos. In addition, more than 20% of SCNT (10.7 mm ± 1.08) and IVP (20.1 mm ± 0.15) conceptuses had tubular shape, suggesting a delay in recapitulating filamentous morphology. Gene expression profiling analysis revealed that the transcript levels of 477 genes, which are involved in various pathways including arginine and proline, glycerolipid and fatty acid metabolism, were significantly altered in SCNT embryos compared to AI. Similarly, 365 genes were differentially expressed in IVP embryos compared to AI. Thus, several canonical pathways including TNRF-1 and tight junction signalling pathways were affected in IVP derived conceptuses. To predict whether the altered transcripts were associated with pre-elongation in vitro culture environment or errors in transcriptional reprogramming, unique or commonly differentially expressed genes were analyzed in SCNT and IVP embryos compared to AI or donor cells (fibroblast). Accordingly, 71 transcripts including (FOLR1, MYO1B, RCN2, H2AFJ, HSPB1 and GATM) were found to be not transcriptionally reprogrammed as their expression resembled more the donor cells than AI embryos. The remaining transcripts were either partially or incompletely reprogrammed. In addition, quantitative real time PCR (qPCR) based expression profiling of candidate transcripts in developmentally delayed SCNT or IVP embryos showed low mRNA levels of IFNt, FGFR2, CLDN1 and ARHGEF2 in developmentally lagging IVP and SCNT embryos compared to their respective elongated counterparts. In conclusion, the present study identified deviation in elongation size, gene expression and the corresponding molecular pathways in day 16 SCNT and IVP conceptuses compared to their AI counterparts which may subsequently be associated with fetal development.

Transkriptom-analyse von bovinen 16 Tage alten Embryonen, gewonnen durch den Transfer von Blastozysten aus klonierten somatischen Zellen sowie der in vitro Produktion

In vitro Embryo Produktion (IVP) und somatischer Kerntransfer (SCNT) sind Werkzeuge der assistierten Reproduktionstechnologien und finden ihren Einsatz um bovine Präimplantations-embryonen unabhängig von der mütterlichen Umwelt zu erzeugen. Allerdings sind embryonale und fetale Verluste nach dem Transfer von SCNT und IVP gewonnenen Embryonen höher im Vergleich zu in vivo (AI) erzeugten Embryonen. Dies kann mit den Veränderungen der molekularen Signaturen sowie Signalwegen in den unterschiedlichen Stadien der embryonalen und/oder fetalen Entwicklung zusammenhängen. Um molekulare Veränderungen zu identifizieren, die am Tag 16 von SCNT und IVP gewonnene Embryonen auftreten können, wurde mit Affymetrix-Bovine Genome Arrays eine Transkriptomanalyse durchgeführt. Hierzu wurden Tag 7 Blastozysten von SCNT, IVP und AI erzeugten Embryonen in Östrus synchronisierte Fleckviehfärsen übertragen. Am Tag 16 der Trächtigkeit wurden die Rezipienten geschlachtet und die Embryonen entnommen. Nach morphologischen Untersuchungen wurden filamentöse Embryonen mit sichtbarer Keimscheibe einer globale Tanskriptomanalyse unterzogen. Das Ergebnis zeigte in den verschiedenen Embryogruppen SCNT (72,7%), IVP (62,2%) und AI (77,3%) eine vergleichbare in vivo Entwicklung. Allerdings konnte eine erhebliche Verringerung in der Größe der Trophoblasten Elongation in SCNT (93,3 mm) im Vergleich zu IVP (186,6 mm) und AI (196,3 mm) Embryonen beobachtet werden. Darüber hinaus wiesen mehr als 20% der SCNT (10,7 mm ± 1,08) und IVP (20,1 mm ± 0,15) Embryonen eine Röhrenform auf, was auf eine verzögerte rekapitulierte filamentöse Morphologie hindeutet. Die Auswertung der Transkriptomanalyse zeigte beim Vergleich von SCNT mit AI 477 signifikant unterschiedlich expremierte Gene, die in verschiedenen Signalwegen beteiligt sind, einschließlich Arginin und Prolin, Glycerolipid und Fettsäure-Metabolismus. Des Weiteren wurden 365 signifikant unterschiedlich exprimierte Gene beim Vergleich von IVP Embryonen mit AI Embryonen identifiziert. Relevante Signalwege dieser Gene waren unter anderem TNRF-1 und Tight-Junction Signalisierung. Um festzustellen, ob die veränderten Transkripte mit der in in vitro Kultur bedingten Präelongation oder mit Fehlern der transkriptionellen Reprogrammierung assoziiert sind, wurden einzigartige oder häufig unterschiedlich exprimierte Gene in SCNT und IVP Embryonen gegenüber AI oder Donorzellen (Fibroblasten) analysiert. Dementsprechend zeigten 71 Transkripte einschließlich FOLR1, MYO1B, RCN2, H2AFJ, HSPB1 und GATM keine transkriptionelle Reprogrammierung, da deren Expressionprofil mehr dem der Donorzellen als dem der AI Embryonen ähnelte. Die restlichen Transkripte waren entweder teilweise oder vollständig reprogrammiert. Zusätzlich, zeigten auf quantitative Real Time PCR (qPCR) basierende Kandidatengenexpressionsprofile in entwicklungsverzögerten SCNT oder IVP Embryonen niedrigere mRNA Spiegel in IFNtau, FGFR2, CLDN1 und ARHGEF2 im Vergleich zu ihren elongierten Gegenstücken. Schlussfolgernd konnten mit dieser Studie Abweichungen in den Elongationsgrößen, den Expressionsprofilen und den entsprechenden molekularen Signalwegen in Tag 16 SCNT und IVP produzierten Embryonen im Vergleich zu AI produzierten Embryonen beobachtet werden. Diese Ergebnisse könnten in Zusammenhang mit den weiteren fötalen Entwicklung gebracht werden.

Contents

List of abbreviations

A	Adenine
ACC. No	Gene bank accession number
Affy	Affymetrix
AI	Artificial insemination
Annotate	Annotation
aRNA	Amplified ribonucleic acid
ATP	Adenosine tri phosphate
BLAST	Basic local alignment search
BSA	Bovine serum albumin
cDNA	complementary deoxy ribonucleic acid
CHX	Cycloheximide
CL	Corpus luteum
COCs	Cumulus oocyte complex
CR1	Charles Rosenkrans medium
cRNA	Complementary ribonucleic acid
DE	Differentially expressed genes
DEPC	Diethylpyrocarbonate
DMAP	dimethylaminopurine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EF	Electro-fusion
EGA	Embryonic genome activation
ES	Embryonic stem cell
FBs	Fibroblast cells
FDR	False discovery rate
FSH	Follicle stimulating hormone
GCRMA	Guanine cytokine multi array
GEO	Gene Expression Omnibus

GO	Gene ontology
GOStats	Gene ontology statistics
GnRH	Gonadotropin-releasing hormone
GTP	Guanosine triphosphate
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
ICI	Intra-cytoplasmic injection
ICM	Inner cell mass
IPA	Ingenuity pathway analysis
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISG	IFNt-stimulated genes
IVF	In vitro fertilization
IVP	In vitro production
iPS	Induced pluripotent stem cells
IVT	In vitro transcription
KO	Knock out
LE	Luminal epithelium
LH	Luteinizing hormone
LIMMA	Linear models for microarray data
LOS	Large offspring syndrome
MEM	Minimum essential medium
mRNA	Messenger ribonucleic acid
NCBI	National center for biotechnological information
OCS	Oestrus cow serum
OZT	Oocyte-to-zygote transition
P4	Progesterone
PBS	Phosphate buffer saline
PCA	Principal component analysis
PCC	Premature chromosme condensation
PCR	Polymerase chain reaction
PGF2α	Prostaglandin F2α
qPCR	Quantitative polymerase chain reaction
RIN	Ribonucleic acid integrity number
RNA	Ribonucleic acid

RNase	Ribonuclease
RNasin	Ribonuclease inhibitor
RNAi	RNA interference
rpm	Revolution per minute
SCNT	Somatic nuclear transfer
SLS	Sample loading solution
SSC	Sodium chloride sodium citrate
TE	trophectoderm
tRNA	Transfer ribonucleic acid
ZGA	Zygotic genome activation
ZP	Zona pelluciada

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1. Introduction

The possibility of in vitro production of fertilized or cloned embryos has improved our understanding the molecular and biochemical mechanisms involved in mammalian embryogenesis, nuclear reprogramming and cell fate determination as well as the effect of embryo manipulation and in vitro culture on pre-implantation embryo development. The lessons accumulated so far showed that, despite their autonomy, pre-implantation embryos from IVP and SCNT are under the influence of confounding factors of nuclear transfer and /or culture environment, which subsequently affect their pre- and post-hatching development.

Bovine embryo development begins with unification of differentiated gamet as to form a single united and embryonic totipotent genome. Within that genome the correct temporal pattern of gene expression that will lead to the appropriate differentiative and formative events are expected to happen (Latham 2005). In mammals, the maternal RNA and the proteins present in the oocyte's cytosol are responsible for early embryonic development. These maternal components govern the first embryo cleavages and as they drop by degradation or usage, the zygote nuclei start transcription and taking control of embryonic development (Memili and First 1999, Minami et al. 2007). This process is called embryonic genome activation (EGA). For mice, bovine and human, major EGA takes place at the 2-cell, 8-cell and 4- to 8-cell stage, respectively (Telford et al. 1990). In the case of SCNT the oocyte executes its normal functions, but with an alternative substrate, the donor somatic cell nucleus, in place of the normal sperm and egg genomes (Latham 2005). Thus, SCNT embryos have additional challenges of dedifferentiating the differentiated donor somatic cell to a totipotent embryonic state (Yang et al. 2007). This requires the stage specific activation of transcripts important for early development and at the same time silencing of genes associated with differentiation (Hochedlinger and Jaenisch 2003). In other words, the donor nucleus is expected to reprogram to a state equivalent to the zygotic one, which entails expression of the correct genes at the right times as it occurs during normal embryogenesis (Kanka et al. 1991). Several studies (Beyhan et al. 2007, Niemann et al. 2010, Rodriguez-Osorio et al. 2009, Smith et al. 2005) have reported the occurrence of global transcriptional reprogramming at the bovine blastocyst stage by comparing the SCNT blastocyst gene expression with that of the AI or IVP origin and donor cells. In addition to the efficient reprograming at the balstocyst stage, the blastocyst formation rate from cloned bovine oocytes ranges from 20% to 60% (Choi et al. 2002, Forsberg et al. 2002, Liu et al. 2001, Rodriguez-Alvarez et al. 2010b). However, to term development after SCNT does not exceed 10% in cattle (Heyman 2005,

Yang et al. 2006), suggesting no direct association between the development of reprogrammed blastocyst and full-term developmental capacity.

On the other hand several studies have shown deregulation of developmentally important genes in SCNT embryos (Dean et al. 2001, Enright et al. 2003, Han et al. 2003, Li et al. 2006) which may be associated with the reported lower success rate of pregnancy establishment, as well as postnatal abnormalities. Failure to express the normal array of embryonic genes may also result from culture condition of the embryo prior to transfer to the uterus of the surrogate mother. For instance, the common placental pathologies which are collectively designated as large offspring syndrome LOS in SCNT and IVP embryo pregnancies are believed to be a result of suboptimal embryo culture environment (Farin and Farin 1995, Kruip and Den Daas 1997, Walker et al. 1996, Yang et al. 2005). Nevertheless, the extent and magnitude of abnormal phenotypes such as congenital anomalies and perinatal death (Heyman et al. 2002a) in SCNT derived pregnancies can not only be associated with in vitro culture condition (Chavatte-Palmer et al. 2012). In support of this, recently Salilew-Wondim et al. (2012) showed that more than 90% affected transcripts in day 50 SCNT placenta weren't significantly altered in IVP placenta compared to AI implicating the presence of additional contributing factors associated with SCNT. One of these factors could be errors in nuclear reprogramming, in which small reprogramming errors accrued up to the blastocyst stage may be magnified in subsequent developments (Smith et al. 2005) especially during the peri-implantation period, where the majority of embryo losses occurres (Clemente et al. 2011, Grealy et al. 1996).

During the period of peri-implantation (between day 15 and 17 of pregnancy), the bovine blastocyst undergoes a process of elongation and the trophectoderm occupies sufficient area for uptake and secretion of protein, allowing for communication with or modification of the maternal system by changing the morphology and biochemical activity of the uterine epithelium (Godkin et al. 1982, Spencer and Bazer 2004). The trophectoderm of the conceptus then begin to produce IFNt that prolongs the lifespan of the corpus luteum (CL) to maintain the secretion of progesterone necessary for the maintenance of pregnancy (Mann et al. 1999, Song et al. 2011, Spencer and Bazer 2004, Thatcher et al. 2001). Likewise, the endometrium fine-tunes its physiological response to the presence of the embryo (Bauersachs et al. 2009, Forde et al. 2011, Mansouri-Attia et al. 2009) and these responses can reflect the type of conceptus present. Altered immune responses of the endometrium were observed for the SCNT-derived conceptus compared to in vitro produced counterparts as early as day 18 (Bauersachs et al. 2009). Similarly, numerous biological functions and several canonical

pathways with a major impact on metabolism and immune function are found to be significantly altered in caruncles and intercaruncular areas of the endometrium with SCNT as compared to AI pregnancies at the onset of implantation (Mansouri-Attia et al. 2009). Such responses of the maternal environment may depend on the intrinsic characteristics of the embryos themselves whether they are in vivo, in vitro or SCNT derived and consequently result in differences in the expression of sets of genes that modulate the uterine response.
In this regard except analysis of few markers of trophoblast or innercell mass, insulin-like growth factor (IGF)-related genes (Arnold et al. 2006a, Fujii et al. 2010, Sawai 2009, Smith et al. 2010) and a single homologous custom bovine array based (Rodriguez-Alvarez et al. 2010b) study on day 17 of IVF and SCNT embryos, data on large scale gene expression analysis of cloned embryos at peri- implantation stage is scarce.

Therefore, the aim of this study was

I) To investigate the inherent molecular characteristics of day 16 embryos derived from SCNT, IVP and AI pregnancies.
II) To determine the transcriptional reprogramming status of a donor genome in SCNT derived elongated embryos.
III) To characterize the expression of selected candidate genes with respect to elongation size of embryos from various sources.

2. Literature review

2.1 Bovine pre-implantation embryo development

Fertilization marks the beginning of pre-implantation development that corresponds to the cleavage of the zygote into smaller and smaller blastomeres (Dard et al. 2008). Following fertilization, the embryo goes through stages of division, modelling and cellular compaction (morula stage). This is the first critical stage of embryo development and is an essential step for differentiation and blastocyst formation (Larue et al. 1994). Then "blastocyst stage" occurs when the embryo develops a fluid-filled central cavity, the "blastocoel", which is surrounded by a single layer of cells, the "trophectoderm" (Schlafer et al. 2000). Before it reaches to blastocyst stage (at day 7), and despite cell division, the overall diameter of the embryo remains virtually unchanged from the zygote until blastocyst expansion, estimated to be 150 to 190 µm including a zona pellucida thickness of approximately 12 to 15 µm (Lindner and Wright 1983). At the blastocyst stage the true growth commences, with rapid cell division and differentiation. The process gives rise to two different cell types, namely the inner cell mass (ICM) and the trophoblast cells. The trophoblast cells (TE) give rise to the tissues of the fetal placenta and associated extraembryonic membranes, (Schlafer et al. 2000), whereas the ICM develops into the three germ layers of the developing embryo (endoderm, mesoderm and ectoderm) (Schlafer et al. 2006).

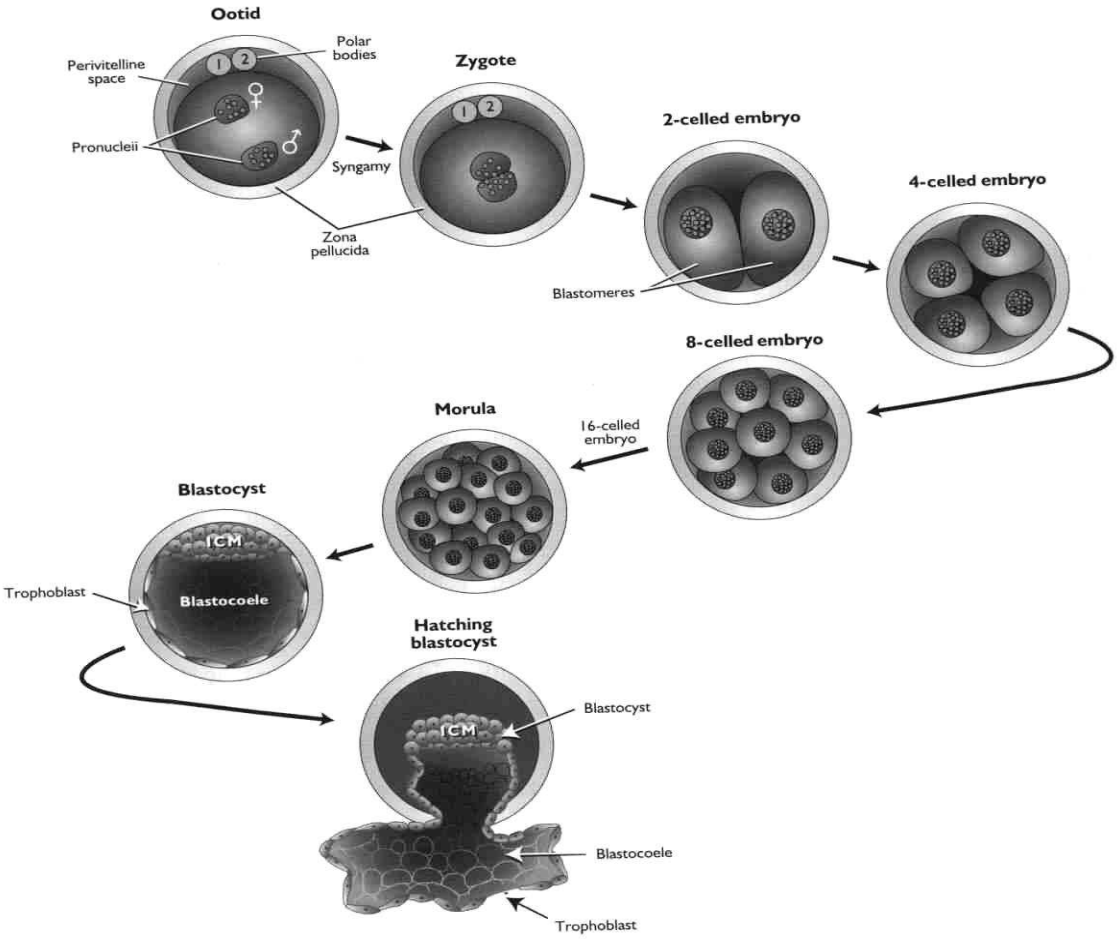


Figure 1. Development of pre-implantation embryo within the zona pellucida. The single-celled embryo (zygote) undergoes cleavage (mitotic division) to give rise to two daughter cells called blastomeres. Mitotic divisions continue until a morula is formed. The morula develops into a blastocyst consisting of an inner cell mass (ICM), a blastocoele cavity and a trophoblast. Finally, the rapidly growing blastocyst "hatches" from the zona. Adapted from http://www.animal.ufl.edu/ans3319/Notes/Chpt9_ANS3319CFetalplacentaLab_10.pdf.

2.2 Post-hatching progresses /embryo elongation

Before the blastocyst can expand and implant, it needs to hatch from the zona pellucida (ZP). Hatching involves the embryonic production of proteases that will digest the ZP (Sathananthan et al. 2003). At about day 8 the zona pellucida begins to fragment and the blastocyst 'hatches. Once the blastocysts have escaped from the ZP, the hatched blastocyst has re-expanded, the inner cell mass bulges to the outside of the sphere while still confined within the zona pellucida until about day 12 (Betteridge et al. 1980) (Figure 1). Soon after

hatching the ICM of the blastocyst forms the hypoblast (sometimes referred to as the primitive endoderm) (Maddox-Hyttel et al. 2003) that extends to cover the inside of both the ICM and the trophoblast (Cremonesi et al. 2011, Maddox-Hyttel et al. 2003, Vejlsted et al. 2006). After formation of the hypoblast is completed, the remaining cells of the former ICM are referred to as the epiblast (Cremonesi et al. 2011, Vejlsted et al. 2006). The shape of hatched blastocysts is then transformed from spherical to ovoid during a transitory phase preceding the obvious elongation that usually begins after day 12 (Betteridge et al. 1980). Following that blastocyst undergoes rapid elongation increasing from less than 1 cm on day 12 to more than 10 cm by day 16 (Robinson et al. 2006).

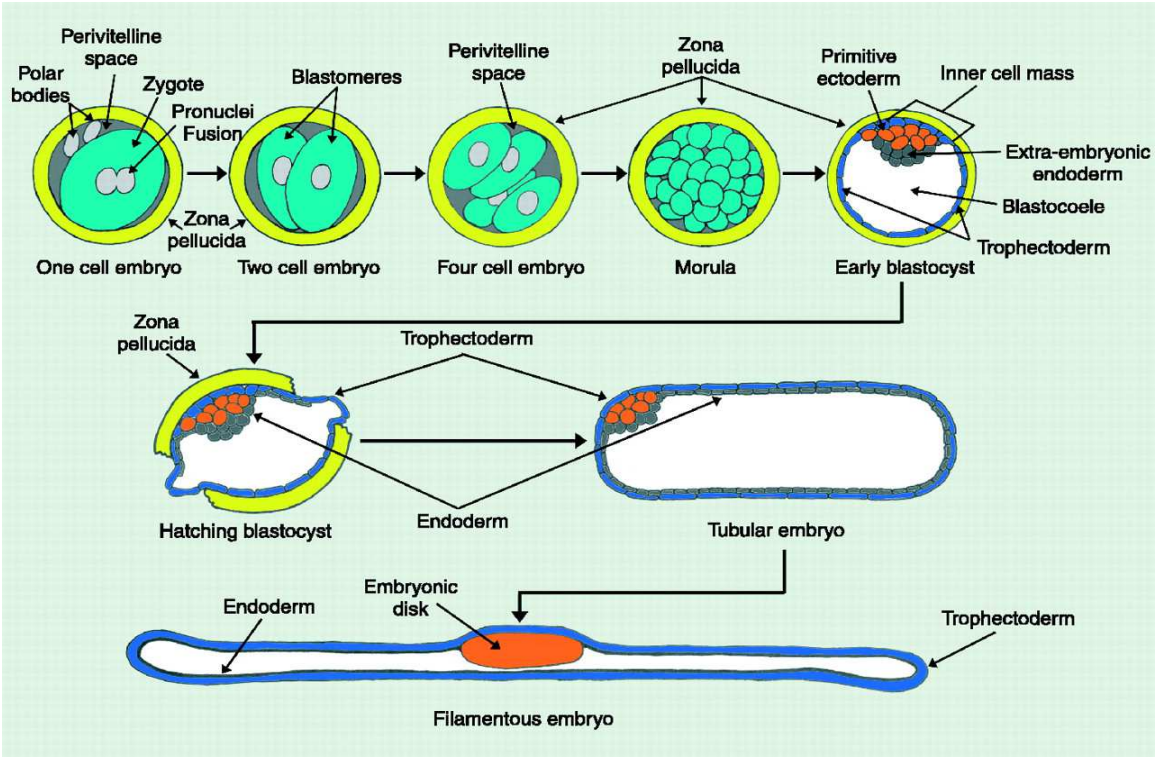


Figure 2. Schematic view of embryo elongation from spherical blastocysts to a tubular and then a filamentous form due to rapid elongation of trophectoderm. Adapted from (Bazer et al. 2009).

2.3 Physiological significance of embryo elongation

Expansion of conceptus from spherical to a tubular and then filamentous form allows the embryo to acquire sufficient trophoblast tissue needed to establish intimate embryo-maternal

contact. By day 16, the embryo is sufficiently developed to signal its presence to the maternal system and recognized through its secretion of interferon tau (IFNt) (Imakawa et al. 1987), which is the pregnancy recognition signal, that prevents development of the endometrial luteolytic mechanism. Interferon tau is secreted by embryonic trophoblast cells and acts in the uterus and prevents luteolysis by inhibiting PGF2_ release, resulting in the maintenance of CL function (Robinson et al. 2008). This antiluteolytic effect of IFNt results in the maintenance of a functional CL and, hence, secretion of progesterone (P4) that is essential to maintain a uterine environment that supports events critical to successful development of the conceptus to term (Spencer and Bazer 2004). This period is therefore defined as the maternal recognition period for maintenance of CL (Spencer and Bazer 2004). It can be defined as the physiological process whereby the conceptus signals its presence to the maternal system and prolongs lifespan of the CL and stimulate the endometrial functions necessary to the survival of the conceptuses and suppress the immune rejection of the conceptus by the uterus at implantation (Hansen 2011).

In addition to the embryo capacity to direct the maternal system to produce a local environment that serves the needs of the embryo, as long as embryo development played out in the reproductive tract the mother has a major impact on embryonic survival.

2.4 Uterine environment

Prior to the blastocyst stage, the embryo is relatively autonomous (i.e., the embryo may not be entirely dependent upon the uterine environment) as evidenced by the fact that blastocysts can be successfully developed in vitro in large numbers using in vitro fertilization (IVF) technology and transferred to synchronized recipients (Clemente et al. 2011). However, after hatching embryo survival and elongation depend on maternal environment and uterine glands' secretions (Gray et al. 2002). The endometrial glands synthesize, secrete and／or transport a complex mixture of amino acids, ions, glucose, transport proteins and growth factor called histotroph (Bazer 1975). These secretions are essential for the development of the blastocyst, which is free living during the pre-attachment period.

Ovarian steroids, especially progesterone (P4) is a key player in regulating endometrial secretions (Robinson et al. 2008) for conducive attachment and survival of the conceptus (Garrett et al. 1988). Evidence from cattle studies has demonstrated a correlation between progesterone concentrations in milk and blood during the first few days after conception and the likelihood of embryo survival (Butler et al. 1996, Green et al. 2005, Mann and Lamming

1995, McNeill et al. 2006). Elevated P4 concentrations lead to an increase in interferon-tau production (Mann and Lamming 2001) resulting in enhanced conceptus elongation (Carter et al. 2008, Satterfield et al. 2006). On the other hand, lower consentrations of circulating P4 are also associated with reduced embryo/conceptus development and survival (Diskin and Morris 2008). It was suggested that the most likely route by which P4 affects embryo survival is through an indirect effect on the uterus (Morris and Diskin 2008). This raises the possibility that P4 can modulate events including changes in gene expression in the tissues of the uterus (Forde et al. 2009) resulting in changes in the composition of histroph to which the developing embryo is exposed. Therefore, it is important to understand the precise relationship between maternal hormone environment and embryo development. In an attempt to answer this question experiments were done on embryos cultivated in vitro with or with out exogenous P4 supplementation and in the presence and absence of oviduct epithelial cells. Following blastocyst transfer, the effect of in vitro P4 supplementation on in vivo embryo survival and elongation was measured (Clemente et al. 2009). The results showed that addition of P4 to culture medium affected neither the blastocyst rate nor conceptus elongation following transfer to synchronized recipient heifers. The next question was whether P4 induced changes on endometrium enhanced embryo development. The same authors showed that a modified uterine environment induced by artificially elevated circulating P_4 is capable of advancing the post-hatching elongation of day 7 blastocysts (Clemente et al. 2009). What is clear from this study is that progestrone modulated endometrium promotes conceptus elongation.

2.5 Early embryonic development and transcription

Maternal mRNAs and proteins which oocyte synthesize and accumulate during oogenesis, implement basic biosynthetic processes in the early embryo, direct the first mitotic divisions, and specify initial cell fate and patterning. As development proceeds, different processes are triggered that together form the maternal-to-zygotic transition (MZT): i) subset of the maternal mRNAs degradation ii) beginning of transcription of the zygotic genome iii) rising of novel embryo-specific transcripts (Minami et al. 2007).

2.5.1 Maternal mRNAs store and its utilization

It is likely that oocytes of all animals contain an abundance of dormant or masked mRNA that is translated only when the cell re-enters the meiotic divisions (oocyte maturation) or after fertilization (Richter 2007). In mammals, these maternal mRNAs are degraded shortly after fertilization and can't direct more than the first few cell divisions. However, in drosophila and xenopus, mRNAs stored in an oocyte are stable after fertilization and regulate many aspects of embryonic development (Akam 1987, Duval et al. 1990). Translation control of maternal transcripts in these organisms during the time of transcriptional quiescence is, it is because the maternal RNA is stored using a specific configuration. The mRNA is de-polyadenylated at the 3' end and capped at the 5' end (Gebauer and Richter 1996). The dominant hypothesis is that cap ribose methylation is facilitated during the process of polyadenylation, and the interaction between the modification machinery at the 3'- and 5'-UTRs of these transcripts underlies translational control at this time in development (Kuge and Richter 1995).

The translational potential of a maternal mRNA transcript is determined by the length of the poly (A) tail. Accordingly an increase in translation is associated with poly(A) tail elongation (80–150 and longer), whereas translational repression correlates with shortening of the poly tail (A) (~20–40 nucleotides long) (Richter 1999). Just as polyadenylation is important for the translation of mRNAs, removal of the poly (A) tail is a key step in mRNA degradation. Shortening of the mRNA 3′ poly (A) tail, deadenylation is often a rate-limiting step for mRNA degradation and translational silencing. In the 3'-to-5' mRNA decay pathway, the deadenylation facilitates degradation of the mRNA from the 3' end by enzyme exosome (Mitchell and Tollervey 2000, van Hoof and Parker 1999). Removal of the 5′-monomethyl guanosine cap (decapping) renders mRNA susceptible to the 5′→3′ degradation pathway by exposing them to exonucleases (e.g., XRN1) that rapidly degrade the mRNA from the 5′ end (Barckmann and Simonelig 2013, Chen and Shyu 2011). Studies of xenopus oocyte and embryonic development revealed what molecular entities are involved in the regulation of the polyadenylation and deadenylation in the cytoplasm of oocytes. One such protein is maskin, which associates with the cytoplasmic polyadenylation element binding protein (CPEB) located in the 3' UTR region on mRNAs that contains a cytoplasmic polyadenylation element (CPE). This complex represses translation through the inhibitory action of maskinelF4E located at the 5' end of the RNA (Richter 2007). Translation of specific mRNAs then proceeds according to a combination of cytoplasmic codes acting on RNA associated proteins interacting with the 3' UTR sequence of the stored RNAs (Piccioni et al. 2005).

Role of miRNAs in maternal genome degradation

Recent progress in the regulation of mRNA stability concerns the role of small non-coding RNAs. MicroRNAs (miRNAs) are about 22-nucleotide noncoding RNAs, which in general are known to regulate gene expression by targeting their 3' untranslated regions (3 UTRs). MicroRNAs biogenesis starts from transcription of miRNA genes by either RNA polymerase II or RNA polymerase III to primary miRNA transcripts (pri-miRNA). The pri-miRNA is next endonucleolytically cleaved by the nuclear microprocessor complex formed by the RNase III enzyme Drosha (RNASEN) and the DGCR8 (DiGeorge critical region 8) which liberates a 60–70nt stem loop intermediate, known as the miRNA precursor, or the pre-miRNA. This pre-miRNA is actively transported from the nucleus to the cytoplasm. The pre-miRNA is further chopped by Dicer to produce the double-stranded miRNA duplex (Bartel 2004, Winter et al. 2009, Zamore and Haley 2005). The duplex is unwound by a helicase and the mature strand is incorporated into the RNA-induced silencing complex (RISC). Depending on miRNA complementarity to a target mRNA, the RISC mediates down-regulation of gene expression by either translational repression or deadenylation and subsequent mRNA degradation (Chekulaeva and Filipowicz 2009, Filipowicz et al. 2008). The role of miRNA as a control mechanism in the degradation of maternal mRNAs has been implicated in recent studies. MicroRNA-dependent mRNA decay was first identified in zebrafish, where miR-430 expressed in the embryo at the onset of zygotic transcription was shown to mediate deadenylation and clearance of hundreds of maternal mRNAs (Giraldez et al. 2006). Taking the advantage of this temporal expression pattern, of miR-430, Bazzini et al. (2012) addressed repression of maternal mRNAs by miR-430. The results revealed that both translational repression and deadenylation are induced by miR-430, as the cause and effect of miR-430 mediated gene silencing during early fish development. A similar temporal regulation by miRNAs exists in *Xenopus* where the miR-430 ortholog miR-427 leads to the de-adenylation and distruction of hundreds of maternal mRNAs in the frog embryo (Lund et al. 2009). In mammals, the roles of miRNA in maternal RNA degradation are not well understood. In addition, the findings have been equivocal with some studies, reporting the possible role of miRNA, as dicer null mouse oocytes and embryos are unable to precede development and arrested at the first meiosis and die before gastrulating at day 7.5 (Bernstein et al. 2003; Harfe et al. 2005) yet others reporting minimal or no impact of miRNA as evidenced by defective spindle phenotype of $Dcr1^{-/-}$ and $Ago2^{-/-}$ oocytes, which is absent in $Dgcr8^{-/-}$ oocytes (Ma et al. 2010). The later group suggested RNA interference (RNAi) as

the dominant RNA silencing pathway essential for oocyte-to-zygote transition OZT (Ma et al. 2010) and the lethal phenotype observed by Dicer KO could be explained by deregulation of the biogenesis of siRNAs (Bernstein et al. 2003, Ma et al. 2010).

2.5.2 Onset of embryonic genome activation

The oocyte-to-zygote transition entails coordinate removal of the maternal transcriptome and its replacement with a zygotic transcriptom (Ma et al. 2012). Timing of the major embryonic genome activation (EGA) is somewhat different from species to species. In mice, EGA occurs rapidly (in late one–cell embryos), whereas in bovine and ovine, EGA is more delayed (eight - to 16 - cell stage) (Telford et al. 1990). During this time the differentiated oocyte and sperm nuclei become reprogrammed to be the active genome of the now totipotent embryo (Oh et al. 2000). Genome activation initiates transcriptional activity within the embryonic nucleus, and subsequent development is dependent upon newly synthesized mRNA and protein. To understand the onset of embryonic transcription, generally, two approaches have been used: (1) comparing transcript profiles of oocytes and two- to four-cell stage embryos with blastocyst profiles and (2) blocking transcription by addition of α-amanitin to the culture medium. Alpha - amanitin is a specific inhibitor of RNA polymerase II and blocks *de novo* mRNA synthesis (Goddard and Pratt 1983, Golbus et al. 1973). The later approach in combination with large scale gene expression profiling of oocytes (MII) and 8-cell-stage embryos using Affymetrix Bovine Genome Array revealed a set of 258 up regulated genes in 8 cell embryos as compared to MII (Misirlioglu et al. 2006). Ontology analysis of these genes identified regulators of transcription (NFYA , USF2), cell adhesion (DSC2 , COL12A1), signal transduction (PTGER4 , ADRBK1), transporters (CRABP1), metabolism - related genes, and immune response - related genes. In another study using the same array platform a total of 12,000 to 14,500 transcripts were identified in bovine oocytes and early embryos including 1-cell, 2-cell, 8-cell, morula and blastocyst (Kues et al. 2008). Of these, a total of 9,263 genes which met a criterion of $P \leq 0.05$ and a minimum cut off of 2-fold between the maternal and embryonic stages, were subjected to K means clustering method and clustered into different groups based on their expression pattern. The first group comprised the maternal transcript and contained 4,173 genes and was characterized by a drop of transcription levels between the 4- and 8-cell stages. A second group showed embryonic transcription and represented 3,505 genes. Significantly increased transcript levels were found from the 4-cell

to 8-cell stage onwards, indicating genes (ID2, ZO3, CLDN4, TP53, etc.) transcribed during major embryonic activation.

In addition, in recent years, investigative attention has turned to analyzing profiles of small, non-coding RNAs including miRNAs (microRNA), during the MZT (Ma et al. 2010, Suh et al. 2010, Tang et al. 2007). In the mouse, the expression patterns of miRNAs are divided into three classes: maternal, maternal-to-zygote and zygote patterns (Tang et al. 2007). *De novo* synthesis of miRNAs commences at the two-cell stage. This includes expression of miR-290 to miR-295, which are the first embryonic miRNAs to be detected. Very few studies have investigated the role of miRNAs during the bovine maternal-to-zygotic transition. Quantification of miR-21 and miR-130a in early bovine development showed a significant rise in expression level from zygote until the 8-cell stage (Yamada et al. 2009). Similarly quantification of miR-10 and miR-424 in early bovine development showed a steady expression level from the GV oocyte until the 16-cell. In addition, increased expression level of miR-127 and miR-145 was also detected at 4-cell and 8-cell stages during pre-implantation bovine embryo development (Tesfaye et al. 2009). These studies suggested the possible role of miRNAs in maternal transcript turn over and maternal-to-zygotic transition.

2.5.3 Novel embryo-specific transcripts

In bovine pre-implantation embryos development two major transient ‘waves of *de novo* transcription’ occur in two phases. The first wave during the 2- to 4-cell stage corresponds to minor genome activation (minor ZGA) and the second wave, during the 4- to 8-cell stage, known as major genome activation (major ZGA) (Barnes and First 1991, Memili and First 1999, Viuff et al. 1996). ZGA promotes a dramatic reprogramming of gene expression pattern, coupled with the generation of novel transcripts that are not expressed in oocytes (Kanka 2003). This delineates the totipotent state of each blastomere at the cleavage stage of embryogenesis, and these steps are prerequisite for future cell lineage commitments and differentiation (Yamada et al. 2009). In the mouse transcriptome analysis of late zygotes indicated that approximately 60% of *de novo* transcripts are novel for the embryos, (Hamatani et al. 2004). This is again reflected by more than 37% increment in protein synthesis through out the 2-cell stage while another 6 % undergo transient increases at the mid 2-cell stage (Latham et al. 1991). It is thought that the first period of transcription is to direct the synthesis of these mid 2-cell stage proteins, which could promote the transcription of other genes, leading to the second burst of transcription (Latham et al. 1991). Consistent with this notion

using suppression subtractive hybridization (SSH) technique, 310 unique transcripts were expressed *de novo* in bovine late eight cell embryos (Vigneault et al. 2009). A very high proportion of these genes have a demonstrated involvement in gene transcription or RNA processing. Using qPCR expression profiling, many of these transcripts were shown to be expressed in the six and very early eight-cell-stage embryos. This implied that these early transcripts in cattle may play a key role in the activation of the major transcriptional burst detected at the 8- to 16-cell stage. This is likely to be the case for genes such as HNRNPA2B1, RBMX, KLF10, ZNF41, DDX5, and DDX39 (Frei et al. 1989, Natale et al. 2000, Telford et al. 1990) which are related to transcription, either directly by binding DNA or indirectly through RNA processing (Carson et al. 2001, Franze et al. 1991).

As soon as the embryo reaches the major EGA, the embryonic program designed to bring it to the blastocyst stage is launched (Wang et al. 2004). For instance, at 8- to 16-cell stages, CDX2 is detectable at various levels in the nuclei of most blastomeres, regardless of their external or internal location. However, in the mouse during the transition from 16- to 32-cell stage, the level of CDX2 becomes stronger in the external blastomeres and weaker in the internal blastomeres, eventually establishing the TE-specific expression at the blastocyst stage (Dietrich and Hiiragi 2007). In contrast, the pluripotency markers OCT4 and NANOG become restricted to influence ICM fate (Yamanaka et al. 2006).

The establishment of the pluripotent lineage proceeds differently in different species. NANOG protein is found to be ICM specific in bovine blastocysts (Kuijk et al. 2008) while OCT4 is expressed in the ICM and TE of bovine and porcine blastocysts (Berg et al. 2011, Shi et al. 2003). In addition the transcripts of OCT4 arise from both the maternal and embryonic genomes, while NANOG is synthesized by the embryo alone (Khan et al. 2012). This suggested that NANOG could be a likely candidate for pluripotent lineage specification in the bovine species.

2.5.4 Zygotic gene activation in nuclear transferred embryos

Somatic cell nuclear reprogramming in cloning experiments represents an interesting tool to study the important events during embryonic development. In nuclear transplantation (cloning), a differentiated somatic cell nucleus is transformed into an undifferentiated totipotent (capable of developing into a whole individual) state when inserted in an enucleated oocyte (Hochedlinger and Jaenisch 2006). This requires huge epigenetic changes that result in a transition from a somatic to an embryonic gene expression pattern and is referred to as

“nuclear reprogramming”. Nuclear reprogramming in cloning experiments is equivalent to ZGA in a context of natural fertilization insofar as both result in establishment of totipotency (Suzuki et al. 2006, Zhao et al. 2010). Therefore, nuclear transplantation could potentially be used to study the factors involved in and the permanence of the switch from maternal to embryonic control of development (Telford et al. 1990).

It has been widely assumed that the nuclear reprogramming that makes cloning possible occurs during the period immediately following nuclear transfer (Latham 2004). This means that the donor nucleus requires to shut down the donor's cell-type-specific RNA transcription pattern and begin to transcribe embryo-specific genes in a fashion that resembles EGA (Wang et al. 2011). However, cloned mice probed for the occurrence of this event are demonstrated to be defective in recapitulating the correct stage-specific gene expression. The first transiently induced genes transcribed from the embryonic genome were absent or greatly reduced in cloned two-cell embryos (Vassena et al. 2007). Similarly qPCR based expression profiling of selected zygotically activated genes at 2-cell stage of mouse cloned embryos showed repression of 35-65% analyzed genes in SCNT compared to IVP embryos (Suzuki et al. 2006). Another study showed punctual onset of embryonic gene expression in cloned mouse 1-cell, 2-cell, 4-cell, morulae, and blastocysts with high degree of transcript abundance variability as compared to non manipulated control groups (Sebastiano et al. 2005). In bovine, quantification of a total of 13 maternally and embryonically expressed genes in SCNT 8-cell embryos constructed from two donor cell lines namely cumulus and fibroblast showed differences in mRNA levels of Cx43, GLUT-1, IGF-1R and E-cad between the two types of NT embryos (Amarnath et al. 2007). However, these differences decreased and the expression levels did not differ from their in-vitro produced counterparts at the blastocyst stage. Given these observations, it appears likely that nuclear reprogramming is a slow, ongoing process in the early cloned embryos and does not occur within the hours immediately following SCNT, but occurs progressively during cleavage, and likely continues after implantation (Campbell et al. 1996a, Latham 2004).

2.6 Maternal environment transcriptom landscape

Establishment of pregnancy in ruminants requires blastocyst growth to form an elongated conceptus that produces interferon tau (IFNt), the pregnancy recognition signal, and initiates implantation. In ruminants, conceptus growth and elongation clearly depend on the uterine milieu, because hatched blastocysts and trophoblastic vesicles do not elongate in vitro

(Flechon et al. 1986, Heyman et al. 1984) or fails to occur in vivo in the absence of uterine glands (Gray et al. 2002). For example, ewes that lack uterine glands and histotroph failed to maintain pregnancy beyond day 14. The implication is that any disruption in the physiological regulation of uterine function, either due to intrinsic errors (Hansen 2002) or abnormal embryo–maternal communication during the peri-implantation period (Bauersachs et al. 2009) compromises development of embryos. Nearly 40% of pregnancy losses in cattle is associated with a failure of maternal recognition of pregnancy before day 18 of gestation (Farin et al. 2001). Those embryos lagging in development fail to signal to the mother in sufficient time or in a suitably robust manner (Roberts et al. 1996) are likely destined for loss (Farin et al. 2004).

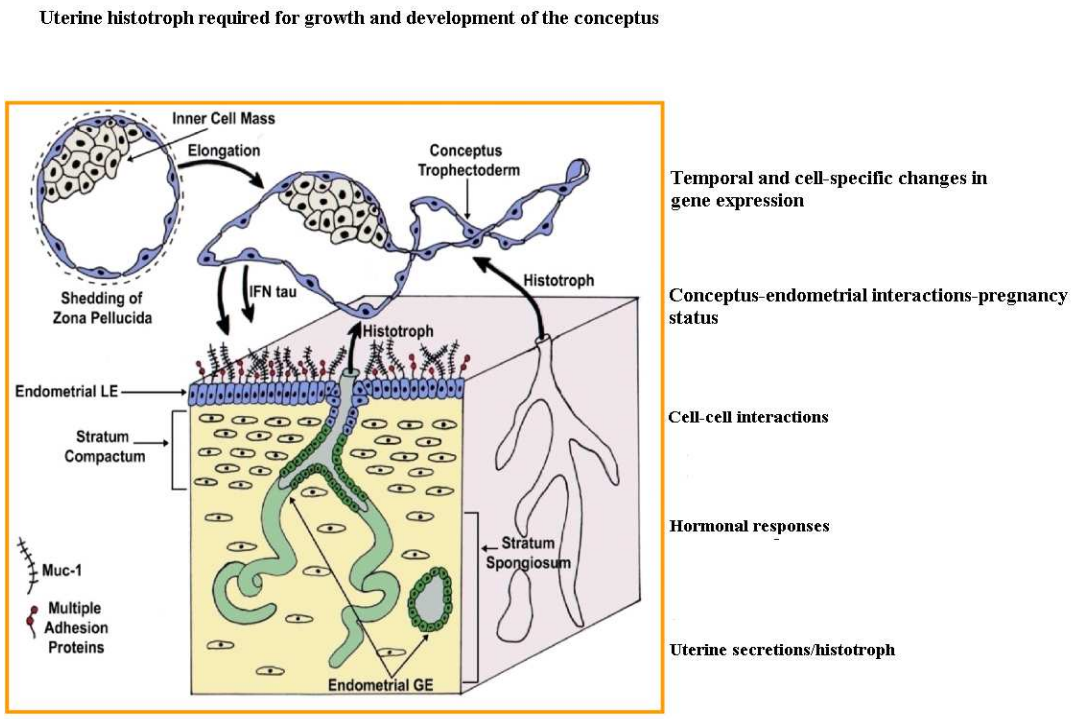


Figure 3. Histotroph includes molecules secreated or transported in to the uterine lumen to stimulate growth and development of the conceptus during the peri-implantation period. Adapted from (Bazer et al. 2012).

In addition to signalling pregnancy recognition in ruminants, IFNt alone or in concert with P4 regulates uterine gene expression. It is an established fact that the process of elongation is exclusively maternally driven (Gray et al. 2002). Particular emphasis is on P4 modulated genes that are expressed in the epithelia and could contribute to uterine luminal fluid when conceptus elongation begins after day 12 (Forde et al. 2012). Several studies in sheep and

cattle showed how IFNt - stimulated genes (ISGs), (Hansen et al. 1999, Spencer et al. 2004, Spencer et al. 2007) and the interaction between IFNt and progesterone regulates uterine gene expression (Gray et al. 2004, Song et al. 2005, Song et al. 2006). The majority of studies examining the molecular mechanisms of conceptus-endometrial interactions carried out during the peri-implantation period of pregnancy have focused on the maternal side, describing changes in the transcriptome of the endometrium (Bauersachs et al. 2006, Forde et al. 2009). Recently, Forde et al. (2011) showed how the presence of an embryo contributed for differences in the endometrial transcriptome between pregnant and cyclic heifers during the period of pregnancy recognition. The authors compared endometrial gene expression profiles of pregnant and non-inseminated cyclic controls at day 5, 7, 13 and 16 using the Affymetrix array platform. The main finding of the study was, only at pregnancy recognition, the hormonal environment and the endocrine mechanisms regulating endometrial gene expression, differs between pregnant and cyclic heifers. This suggests that the transcriptomic alteration that occur in the endometrium as time from estrous to the luteal phase progresses occur in a similar manner irrespective of whether an embryo is present (Bauersachs et al. 2012, Forde and Lonergan 2012).

Transcriptomic analysis of the endometrial response of pregnancy has not only been used to unravel the molecular events surrounding the process of pregnancy recognition but can reflect the type of conceptus present. The recipient's endometrium reactions for cloned and fertilized embryos were different as early as day 18 (Bauersachs et al. 2009). Such differences even aggravated at day 20 at caruncles and intercaruncular areas of the endometrium approximately 5 days after pregnancy signalling commences (Mansouri-Attia et al. 2009). These evidences showed that the endometrium is sensitive to manipulations that occur prior to embryo transfer.

2.7 Nuclear transfer technology

One of the most fundamental questions in developmental biology is the control of cellular differentiation. In the majority of species, the product of sexual reproduction is the fertilized egg or zygote. This single cell inherits a single maternal and a single paternal copy of the genome. From this genetic material and the maternally inherited organelles, proteins, RNAs, etc., found in the unfertilized egg, an embryo, fetus, and finally an adult animal develop (Campbell 1999). During this developmental process, cellular differentiation results in the production of all of the tissues and organs that make up the mature adult. The long standing question was whether the process of development and cell differentiation requires a loss or

stable change in the genetic constitution of cells. Up until the 1950s, it was thought possible that genes could become lost or permanently inactivated in those cells that follow different lineages in which certain genes would never normally be required. The original reason for wishing to carry out nuclear transfer to eggs was to test the hypothesis whether the genome of somatic cell is complete in the sense of containing copies of all genes in the genome (Campbell 1999, Colman 1999, Gurdon and Wilmut 2011).

2.7.1 Brief history of cloning

It has now been more than five decades since Briggs and King reported successful production of Northern Leopard Frog *Rana pipiens,* tadpoles via NT (King and Briggs 1956). This study was the first to prove that during cell differentiation, inactive nuclear genes were not lost or permanently inactivated; in other words, the nuclei retained totipotency. In an extension of this experiment, Gurdon (1966) successfully produce male and female fertile xenopus from the intestinal epithelium of feeding tadpoles. However, success with NT in mammals was not reported until 1980s'. Historically, a primary difficulty in performing somatic cell nuclear transfer in mammals has been the small size of the mammalian egg (McGrath and Solter 1983). The mammalian egg (in second meiotic metaphase) is <0.1% the volume of an amphibian egg. Hence, before nuclear transfer could succeed in mammals, micromanipulation techniques were required that could handle, enucleate, and fuse a very small mammalian egg with a single somatic cell (Gurdon and Byrne 2003). The first report of mammalian nuclear transfer was on rabbit morula cell nuclei into enucleated rabbit eggs (Bromhall 1975). These experiment produced embryos that arrested during cleavage, with a low percentage reaching the morula stage but not yet an entire organism. The first live birth was reported by using somatic cell nuclei was in 1981. Microinjection of inner cell mass (ICM) nuclei into enucleated zygotes resulted in successful production of three cloned mice (one male and two females) (Illmensee and Hoppe 1981) whereas the transfer of trophectodermal nuclei failed to support development. However, the techniques and the results haven't been reproduced (Colman 1999) and modified (McGrath and Solter 1983). Two years later Steen Willadsen published a description of the first mammalian clones that resulted from the transfer of the nuclei of 8- or 16-cell-stage sheep embryos into an enucleated unfertilized eggs (Willadsen 1986). The lesson learned from Willadsen that the enucleated oocyte is a better recipient than a zygote because it allows more time for the donor nucleus to adapt and change within the egg cytoplasm before having to support the developmental processes (Solter 2000), may have

contributed to the change to using oocytes as recipient cells (Cheong et al. 1993, Kono et al. 1991). Following that, progress in large animal cloning accelerated rapidly and in the first half of the 1990s larger animals became the models of choice for nuclear transfer. However, in all cases, embryonic cells were used as nuclear donors. The conceptual breakthrough came with the generation of Dolly, the cloned sheep (Wilmut et al. 1997). In this instance, the nuclear donor was not an early embryonic cell but a fully differentiated mammary gland cell. Dolly was the living proof that the nuclei of a fully differentiated cell still contain all of the information required for the development of a full organism if placed in the proper environment or "reprogrammed" properly (Piedrahita et al. 2004). Subsequently, the first surviving cloned mouse, Cumulina, was also born in the following year (took her name from the cumulus cell nucleus from which she was derived) (Wakayama et al. 1998). Dolly and Cumulina didn't remain the only mammals cloned from adult somatic cell for long, with in a year Kato et al. (1998) confirmed that cloning using adult somatic cells was indeed possible in cattle. To date sheep, cattle, mice, rabbit, goat, cat, pig, dogs, buffalo, camel and monkey are not the only species to join the list of the adult somatic cell cloned.

2.7.2 Technical procedures of SCNT

Somatic cell cloning (cloning or nuclear transfer) is a technique in which the nucleus of a metaphase-II oocyte is replaced by the nucleus of a somatic cell for the generation of a new individual, genetically identical to the somatic cell donor (Tian et al. 2003). The basic procedure, by which a living cell nucleus is transplanted to an egg or oocyte, was established by King and Briggs (1952). They used *Rana pipiens* and sucked a blastula cell into a micropipette so that the cell wall was broken but the nucleus remained intact and covered by cytoplasm. The whole cell was injected into an unfertilized egg in second meiotic metaphase. The egg was enucleated manually by removing the metaphase spindle with its chromosomes from the surface of the egg.

Currently mainly three different SCNT protocols have been developed for the production of cloned offspring. The most widely used protocols are the classical cloning method employing micromanipulators (Wilmut et al. 1997) and the handmade cloning (Vajta et al. 2001), where all steps are performed manually without the aid of micromanipulators. The main difference between the two SCNT protocols is that the handmade cloning procedure involves the removal of the zona pellucida. Zona pellucida free reconstructed embryos are to be cultured under specific in vitro conditions up to the blastocyst stage prior to transfer to recipients.

Technical steps of cloning protocol

Enucleation of the recipient oocytes

Enucleation of the MII oocyte may be achieved by a number of techniques; the most popular is capillary incision of the zona pellucida, using a micromanipulator followed by removal of the polar body and adjacent metaphase chromosomes by suction into a glass pipette (Hosaka et al. 2000). Additional methods include enucleation by centrifugation (Tatham et al. 1995) and bisection of the oocyte followed by removal of fragment containing the nuclear material (Vajta et al. 2001, Vajta et al. 2003) (the so-called “handmade” cloning method). Although this technique has the advantage of simplicity, it does remove more oocyte cytoplasm and therefore, it may reduce the amounts of proteins needed for reprogramming and early embryonic development.

Donor cell preparation and fusion

A biopsy of tissue taken from the selected donor animal can be cultured in vitro in order to multiply and store at the frozen state. Each individual donor cell isolated from the culture plate is inserted into the perivitelline space of the enucleated recipient oocyte by micromanipulation and then introduced into the oocyte cytoplasm. Two methods are currently employed for somatic nuclear transfer in mammals: electro-fusion (EF) and intra-cytoplasmic injection (ICI). The basic differences between ICI and EF is, ICI takes a nucleus isolated from the donor cell and introduces it with part of the surrounding cytosol into the recipient cytoplast, whereas EF introduces the entire donor cell into the recipient oocyte. Comparison of the remodelling pattern of donor nuclei after nuclear transfer by injection or fusion showed that a high rate of premature chromosme condensation (PCC) occurred in both cases (Kurome et al. 2003). However, observation 1 h after nuclear transfer showed that the resultant nuclear configuration using the two transfer methods is different in that for injection the majority showed condensed chromosomes while for fusion the majority showed metaphase-like chromosomes (Kurome et al. 2003). The authors suggested that using the fusion method produces a more rapid formation of the spindle to prematurely condensed chromosomes.

Activation of the reconstructed embryo

Activation of the reconstructed complexes can either be achieved by short electrical pulses or by brief exposure to chemical substances that regulate the calcium influx into the complexes and/or the cell cycle (Niemann and Lucas-Hahn 2012).

Temporary in vitro culture of reconstructed embryos

After fusion and activation, nuclear transfer embryos are developed in vitro up to the blastocyst stage using a variety of culture systems routinely used for bovine IVF embryos (Niemann and Lucas-Hahn 2012).

2. 8 Efficiency of mammalian cloning

As the ultimate goal of cloning is to obtain healthy fertile offspring, the efficiency of cloning should be assessed based on the proportion of healthy offspring produced. Dolly was just one cloned offspring that resulted after 277 attempts (Wilmut et al. 1997). The success rate of the first cloned mouse production from cumulus cell (2-8%) (Wakayama et al. 1998) seems nearly 6 times better than success rate of Dolly production (0.3%). Though, cloning technology improves the success rate hasn't shown dramatic change. After years similar inefficiencies are still being described for cloning adult animals. For example the proportion of reconstructed 1-cell cattle embryos that develop to transferable quality blastocysts after seven days of culture is comparable to that following *in vitro* embryo production (IVP) (i.e. in vitro matured, fertilised and cultured) with abattoir-derived oocytes (Wells 2005). However, the success rate (defined as the percentage of reconstructed embryos that develop to term) (Campbell 1999) was low in cloned embryos. Besides, those that survive to term are frequently defective (Cezar et al. 2003). Cloning by the transfer of nuclei from adult cells is still a hit-and-miss procedure (Solter 2000). In most mammalian species studied thus far, the survival rate to birth for cloned is only about 1%–5%, compared with a 30%–60% birth rate for IVF blastocysts (Yang et al. 2007). Numerous factors related to cloning procedures such as state and source of the donor cell, cytoplast source and quality, timing and methods of manipulation and activation and embryo culture conditions contribute to the death of clones, both in the embryonic and fetal periods as well as during neonatal life. The following parts will discuss some of these factors in detail.

2.8.1 Donor cell contribution

It has been generally believed that the type of nuclear donor cells is an important factor influencing cloning efficiency in mammals. To date several somatic cell types such as fetal and adult fibroblast, (Cibelli et al. 1998, Kubota et al. 2000) mammary gland cell, (Wilmut et al. 1997) cumulus cells, (Akagi et al. 2008) granulosa cells, (Bhojwani et al. 2005, Wells et al. 1999) blood leukocytes, (Galli et al. 1999) and oviduct cells (Goto et al. 1999) have been

used as a donor for production of SCNT animals. However, it is still unclear which somatic cell type is efficient for nuclear transfer (Oback and Wells 2002). As it has mentioned above, blastomeres were the first cells to be used in cloning amphibians (King and Briggs 1956). The concept of blastomere cloning was that these cells show little or no differentiation and should make better nuclear donors (Oback and Wells 2002). This idea is supported by earlier results of cloned mice derived from pluripotent mouse blastomeres. Mouse blastocysts derived from two-cell, four-cell or eight-cell mouse embryonic blastomeres were shown to develop to term at 29%, 22% and 18% efficiency, respectively (Cheong et al. 1993). In cattle however, the comparison of donor cells derived from different stages of development have generated controversial findings. The development of SCNT embryos both at blastocyst and post-implantation stage were similar when fetal, new born and adult female and male donor's cells were compared (Kato et al. 2000). Similarly, no detectable difference at the blastocyst stage was observed when somatic cell lines of various sources (fibroblast or granulosa cell) or different passages (7^{th} vs. 11^{th}) were used for cloning (Bhojwani et al. 2005). Yet, other groups have shown that blastocysts generated from cultured bovine fetal cells have higher success rates for both pregnancy and calving compared to those derived from cultured adult cells (Forsberg et al. 2002). On the other hand, comparison of bovine embryos reconstructed with different adult somatic cells showed that cumulus or ear fibroblast had better competence for blastocyst formation than embryos reconstructed with uterine or oviductal cells (Cho et al. 2002).

In most experiments involving cloned embryos fibroblasts have been used as donor cell (Heyman et al. 2002b). This includes cells isolated from skin, ear, testis and ovaries which are probably connective-tissue (stromal) fibroblasts rather than epidermal or germ cells, respectively (Wakayama and Yanagimachi 2001). Fibroblast cells are relatively easy to isolate culture and replicate *in vitro* (Iguma et al. 2005, Poehland et al. 2007). Besides, the use of adult somatic cells is more advisable than fetal cells, especially since this option allows selection of animals with increased production traits (high milk yield and growth rate) and valuable genetic merit, especially those in a transgenic program (Iguma et al. 2005).

2.8.2 Cytoplast source and quality

The quality and source of oocytes is a key factor in determining the proportion of oocytes developing to the blastocyst stage and the efficiency at which live offspring are produced (Lonergan et al. 2003, Rizos et al. 2002, Wells et al. 1997). Both in vivo- and in vitro-

matured oocytes have been used as recipients for production of cloned animals from differentiated cells. In cattle and pigs, in vitro oocyte maturation systems produce an abundant and stable supply of recipient oocytes because immature oocytes can be obtained from slaughtered animals. The oocyte maturation process is believed to be a crucial step influencing the subsequent developmental competence of oocytes (Sirard et al. 2006). In vivo-matured oocytes have a far higher developmental ability than IVM oocytes (Rizos et al. 2002). Nevertheless, the in vivo developmental potential of nuclear transfer embryos was not shown to be improved by using in vivo-matured oocytes (Akagi et al. 2008, Yang et al. 2008). However, owing to the smaller numbers of embryos used for transfer in these studies, further comparative studies are needed to fully evaluate the influence of oocyte source and maturation method on livestock cloning efficiency.

Another consideration is the source of oocytes, i.e., derived from prepuberal animals versus adult animals. It is accepted that the oocytes from prepuberal animals have reduced developmental competence compared with that of oocytes from adult animals, as indicated by the decreased blastocyst formation after in vitro fertilization (Marchal et al. 2001, Revel et al. 1995). In addition, in vitro developmental rates of nuclear transfer embryos involving adult cytoplasts were substantially faster than those of embryos produced from calf oocytes (Salamone et al. 2001).

In addition to the source of oocytes, the developmental competence (quality) of the recipient oocyte could also affect the cloning outcome, but only very little progress has been made about oocyte selection at present. Recently Su et al. (2012) showed the association of brilliant cresyl blue (BCB) staining of oocytes with SCNT efficiency. BCB staining has been used for selection of competent oocytes in several mammalian species and measured the activity of glucose-6-phosphate dehydrogenase (G6PDH), an enzyme which is expected to be high in immature oocyte as compared to mature. It was shown that BCB+ oocytes yielded increased in vitro and in vivo development rate of SCNT embryos (Su et al. 2012).

Zygote as a cytoplast source for cloning

The oocyte cytoplasts derived from methaphase II oocytes are almost exclusively used for SCNT. Alternatively recent studies focus is also shifted in re-exploring the ability of zygote as cytoplast source for SCNT (Egli et al. 2007, Fan et al. 2009, Mezzalira et al. 2011). A series of experiments in mice in the mid 1980s showed that donor nuclei could be successfully exchanged between fertilized eggs (zygote), with 90 percent reaching the blastocyst stage of embryonic development and beyond (McGrath and Solter 1983). Despite

nuclei recovered and transplanted from embryos at the two-cell stage could direct development to the blastocyst stage, enucleated zygote failed repeatedly to support development when later embryonic or somatic donors were used (Wakayama et al. 2000). These results led to the conclusion that mouse blastomere nuclei transferred into enucleated zygotes cannot support development in vitro (McGrath and Solter 1984). However, the notion that zygotes are poor nuclear recipients has been recently revised. Live born pups obtained from enucleated pronuclear zygotes reconstructed with interphase nuclei of 8-cell stage blastomeres were reported (Greda et al. 2006). Similarly early bovine zygotes were shown to support development of transferred somatic G1 phase nuclei when the maternal telophase II chromosomes and condensed sperm DNA were removed prior to pronuclei formation (Schurmann et al. 2006). These studies unequivocally demonstrated that zygotes retain the factors necessary to completely reprogram embryonic and somatic genomes. However, whether reprogramming factors in these cells are the same as the factors active in the oocytes remained to be elucidated (Oback 2008).

In addition to the cytoplast source, studies on mammalian oocyte highlighted the importance of the activation signal on cloned embryos subsequent development.

2.8.3 Method of egg activation

Nuclei of mature oocytes are arrested at a particular stage of meiosis that varies from species to species (Sagata 1996). Arrest is maintained by stabilisation of M-Phase promoting factor (MPF) and mitosis activating protein (MAP) (Haccard et al. 1993, Ruderman 1993). Fusion of a spermatozoon with a metaphase II (MII) arrested oocyte alleviate arrest, thereby allowing cell cycle progression, cell division, and embryogenesis to proceed (Raz and Shalgi 1998). The signal triggered by the sperm**,** referred to as activation (Horner and Wolfner 2008) and the molecular mechanism by which the activating signal is transmitted from the sperm to the oocyte is not fully understood. However, it is clear that this signal triggers a large increase in the concentration of intracellular free calcium, provoke a cortical reaction (to block polyspermy), reduce the levels of maturation-promoting factor (MPF) and mitogen-activated protein MAP kinase and releases the oocyte from meiotic arrest and permits resumption of meiosis (Ducibella et al. 2002, Moos et al. 1995, Von Stetina and Orr-Weaver 2011).

Activation can be initiated by fertilization or in the absence of sperm by artificial activating agents. Since sperm-mediated activation is absent in SCNT, an artificial activation is needed to initiate embryo development. These protocols mainly rely on raising the intracellular free

Ca^{2+} concentration in the ooplasm to mimic sperm-triggered events (Nakada and Mizuno 1998). Various mechanical, chemical and physical stumili have been used to elicit one or several Ca^{2+} transients in the oocyte. Chemical activation can be induced by exposure to the calcium ionophore A23187, (Eusebi and Siracusa 1983, Liu et al. 2002) 7% ethanol, (Eusebi and Siracusa 1983) and ionomycine (Loi et al. 1998, Silvestre et al. 2007). Alternatively, calcium stimulation followed by treatment with a protein synthesis inhibitor such as 6-dimethylaminopurine (DMAP) or phosphorylation inhibitor, cycloheximide (CHX) could also cause full activation of the newly matured oocytes (Liu et al. 1998, Soloy et al. 1997, Wells et al. 1999). Similarly, because most cell cycle regulators such as MPF and MAPKs are phosphorylation-dependent kinases, replacing a protein synthesis inhibitor with a phosphorylation inhibitor such as 6-DMAP in the combined treatment with a calcium stimulator such as A23187 also equally effective in inducing activation of oocytes (Liu et al. 1998, Susko-Parrish et al. 1994).

Electric stimulation is also another way of introducing Ca^{2+} in to oocyte cytoplasm by making the oocyte plasma membrane permeable in the presence of $Ca2^{+}$ with pulses of electricity. This method often referred to as electrical activation involves placing the oocyte between two electrodes typically 0.5 to 1 mm apart in a Ca^{2+} containing sugar based non- electrolyte solution of mannitol sucrose or glucose and delivering one or more DC pulses of electricity across the electrode (Collas et al. 1993; Rickords and White 1992). A single electric pulse has been shown to be enough to induce calcium transit in mouse (Rickords and White 1992), rabbit (Fissore and Robl 1992), cattle (Collas et al. 1993) and pig (Sun et al. 1992). The physical stimulus used for oocyte activation is the exposure of oocytes to room temperature before NT (Stice et al. 1994).

Oocyte activation is one of the crucial parameters determining the success of nuclear transfer and the subsequent development of cloned embryos (Bhak et al. 2006, Motlik et al. 2002). Improper oocyte activation may affect the level of nuclear reprogramming following SCNT (Ross et al. 2009).

Usually, activation treatment efficiency has been evaluated by pronuclear formation, cleavage and blastocyst rates, blastocyst cell number and ploidy (Van De Velde et al. 1999). However, huge variabilities are observed in the obtained results in comparative studies that involved different combined oocyte activating agents. For instance, Loi et al. (1998) reported that treatment of oocytes with ionomycin 6-DMAP resulted in the highest blastocyst and to term development rates. On the other hand, Shen et al. (2008) showed that electrical pulse, A23187 or ionomycin in combination with 6-DMAP could efficiently activate reconstructed bovine

oocytes but not to term development. The same authors showed that only oocytes activated by the A23187 and 6-DMAP combined treatment developed to term after transfer to the recipient cow (2008). Other groups reported that activation with ionomycin and DMAP enhanced the developmental rates in parthenotes and SCNT embryos compared to activation with ionomycin and CHX in time-dependent pattern (Bhak et al. 2006). The blastocyst rate of bovine reconstructed oocytes activated by ionomycin + 6-DMAP was similar to that of ionomycin + CHX activated oocytes, when the interval between fusion and activation was less than 1 h. However, a high blastocyst rate and quality in terms of total cell numbers of bovine reconstructed embryos was obtained by activation with ionomycin + 6-DMAP for a prolonged period of time (3.5-4 h) after fusion (Shin et al. 2001, Van De Velde et al. 1999). It has also been reported that a delay of incubation in 6-DMAP later than 6 h following treatment with calcium ionophore/ionomycin could induce abnormal ploidy development (Rho et al. 1998, Van De Velde et al. 1999). These results suggested that timing of activation of NT embryos affect development at the blastocyst stage (Im et al. 2007).

The timing of activation of recipient MII oocytes can be classified into two protocols as follows: (1) activation is performed immediately after fusion (simultaneous fusion and activation method, FA), (2) activation is performed several hours after fusion (delayed activation method, DA). Donor chromosomes are exposed to factors present in MII cytoplasm for only a short time in the FA method and for a long time in the DA method. In bovine NT using cultured somatic cells, the DA method has a high in vitro development rate compared with the FA method (Akagi et al. 2008, Shin et al. 2001, Wells et al. 1999). Prolonged exposure of incoming nuclei to a cytoplasm rich in metaphase-promoting factor (MPF) causes chromosome condensation (De Sousa et al. 2002). This condensation is believed to facilitate undefined nuclear changes that are essential for development (Campbell et al. 1996b).

2.9 Role of epigenetics during early embryogenesis and SCNT

All cells in an individual organism (with few exceptions) carry identical genetic information. Accordingly, functional specialization of cells during development is the outcome of differential transcriptional programs, not different genetic information. These programs are governed by the transcription/translation machinery, which in turn is guided and controlled by epigenetic (i.e., chemical) modifications of both DNA and chromatin (Bird 2007, Bonasio et al. 2010). Epigenetics is defined as nuclear inheritance that is not based on differences in DNA sequences (Holliday 1987). Epigenetics is believed to involve differential DNA

methylation, histone acetylation, chromatin configuration as well as other mechanisms (Tian 2004).

2.9.1 DNA methylation

In vertebrates, DNA methylation occurs on cytosines in a cytosine-phosphateguanine (CpG) context. CpG methylation is catalyzed by DNA methyltransferases (DNMTs), which convert S-adenosylmethionine to S-adenosylhomocysteine by the addition of a methyl group to the 5th position of a cytosine on DNA (Ostrup et al. 2012). CpG islands are frequently located within the promoter region of genes and methylation within the islands has been shown to be associated with transcriptional inactivation of the corresponding gene (Bird 1996).

Because pre-implantation reprogramming occurs after fertilization, and in the case of nuclear transfer, after fusion of the donor nucleus with the ooplast, it is important to understand changes in methylation following these events. Hours after fertilization, but prior to DNA replication and cleavage intensive demethylation of the paternal but not the maternal genome is observed in mice, rats, pigs and cattle (Beaujean et al. 2004, Dean et al. 2001, Fulka et al. 2004). In mice and cattle, the maternal genome retains its methylation markers during this period and the maternal genome is passively demethylated by a replication-dependent mechanism after the two-cell embryo stage (Fulka et al. 2008). In the mouse, the demethylation is gradual throughout the pre-implantation period up to the blastocyst stage (Dean et al. 2001) while in cattle a phase of remethylation occurs in the 8–16-cell stage (Santos et al. 2003).

In embryos derived via nuclear transfer, epigenetic modifications, such as the waves of demethylation and *de novo* methylation observed following fertilization also occurs, but is often disturbed in cloned embryos of many species and the level of DNA methylation remains much higher than in normal fertilized embryos in most cases (Beaujean et al. 2004, Dean et al. 2003, Kang et al. 2001a, Kang et al. 2001b). For instance, in bovine cloned embryos despite initial demethylation of the donor genome, passive demethylation does not occur to the level seen in normal embryos. Besides, the levels of methylation in the cells of the embryo are higher than normal at the four-cell and eight-cell stages (Dean et al. 2001). Moreover, the methylation status in pre-implantation cloned embryos, particularly at some repeat sequences, closely resembles the methylation levels in the donor cell genome (Kang et al. 2001a).

2.9.2 Histone modification

DNA in eukaryotic organisms is organized in a nucleoprotein complex called chromatin. The basic unit of chromatin is the nucleosome, which is comprised of 147 bp of DNA wrapped around an octamer of histones, formed by pairs of each of the four core histones (H2A, H2B, H3, and H4) (Davey et al. 2002, Luger et al. 1997). At the entry and exit sites located on the surface of the nucleosome core, the DNA is bound by a fifth histone, histone H1, known also as linker histone (Martins et al. 2012). Linker histone H1 stabilizes the higher-order folding of nucleosomes that defines distinct levels of chromatin organization and gene activity (Figure 4).

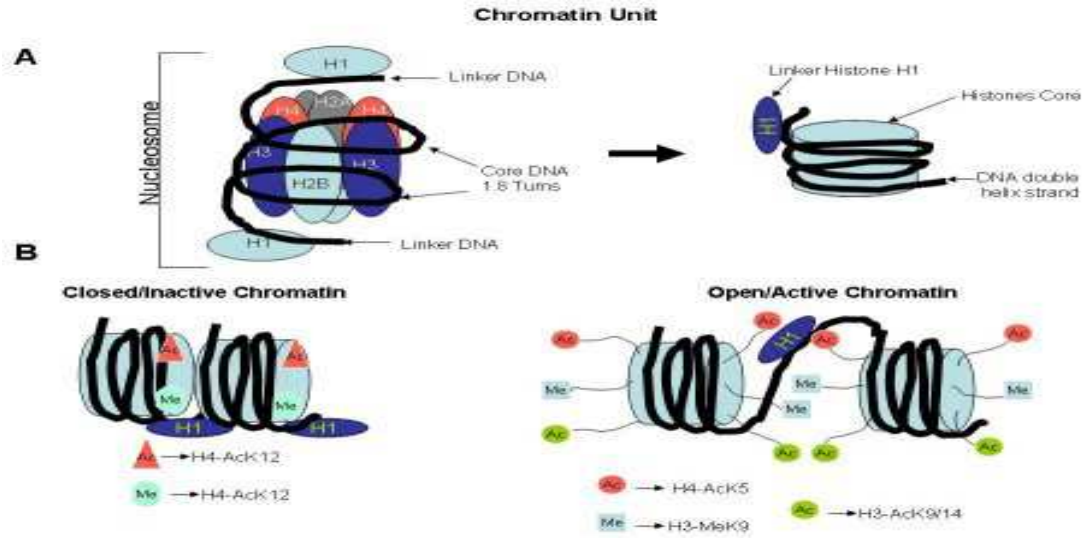


Figure 4. Open and condensed chromatin. A) The nucleosome structure B) A closed (suppressed) and an open (active) chromatin structure. Adapted from http://www.abcam.com/index.html?pageconfig=resource&rid=10189&pid=5#mod

The mammalian linker histone H1 family can be subdivided into somatic cell-specific subtypes, H1.0–H1.5 and H1x, and germ cell variants specific for sperm, H1t, H1T2 and HILS1, and oocytes, H1oo (Happel and Doenecke 2009). Changes in the chromatin histone variant composition have been suggested to be involved in genome reprogramming during gametogenesis and early embryonic development (Hajkova et al. 2008, Santenard and Torres-Padilla 2009).

During early embryo development, oocyte-specific linker histone transition occurs twice: first, shortly after fertilization or nuclear transfer ("H1foo replaces somatic H1); and second at around the time of the maternal-zygotic transition/midblastula transition (H1foo is replaced by somatic H1) (McGraw et al. 2006, Yun et al. 2012). In cattle linker histone H1 becomes undetectable in somatic nuclei within 60 min after injection into oocytes, and is completely replaced with the highly mobile oocyte-specific H1FOO linker histone variant (Teranishi et al. 2004). In contrast, core histones of somatic nuclei, especially H3 and H4, are not removed, but remain stably associated with somatic DNA (Misteli et al. 2000).

In addition to the exchange of proteins between the donor nucleus and oocyte cytoplasm, there are modifications to DNA and post-translationally to proteins that occur after SCNT (Whitworth and Prather 2010). In chromatin, the extruding tails of the histone proteins are the preferential sites for posttranslational modifications. Histone tails are subjected to a wide range of postranslational modifications, including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, glycosylation, biotinylation and carbonylation (Strahl and Allis 2000). The detection of different covalent histone modifications is also often used as an indicator of remodeling of the somatic cell nucleus. Histone acetylation results in loss of the positive charges on lysine residues located in the core histone N termini, which would weaken the association between histone N-terminal domains and DNA favoring transcription factor binding (Zhang et al. 2002). Addition and removal of acetylation at histone residues are catalyzed by histone acetyltransferase (HAT) and histone deacetylase (HDAC), respectively (Figure 5). Thus, HAT is implicated in creating an "open" chromatin environment and HDAC a "closed" one (Hayashi and Masukata 2011). Studies on the acetylation of histones in bovine cloned embryos have found aberrancies (Enright et al. 2005, Santos et al. 2003). The histone deacetylase (HDAC) inhibitor trichostatin A (TSA) alproic acid, scriptaid, and sodium butyrate treatment of reconstructed embryos or donor cell increases cloning efficiency including live births (Kishigami et al. 2006). This suggests that hypoacetylation may be one limiting factor for the development of cloned embryos (Rybouchkin et al. 2006).

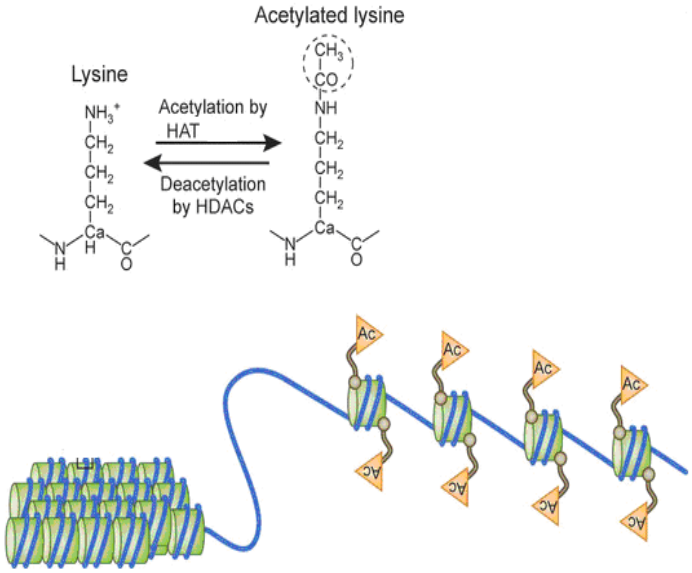


Figure 5. Hypermethylated DNA recruits silencing transcription chromatin remodeling complexes with histone deacetylases (HDACs) and promotes chromatin condensation. Hypomethylated DNA unfolds into a 'beads-on-a-string' structure in which histones are accessible for chromatin remodeling factors such as histone acetyltransferase (HAT). Adapted from (Korzus 2010).

2.9.3 Imprinting

Most autosomal genes are normally expressed from both paternal and maternal alleles, whereas imprinted genes are expressed predominantly, or exclusively, from either the maternal or paternal allele. Thus, the expression of imprinted genes does not follow a Mendelian pattern of inheritance (which indicates the equal participation of the alleles derived from both parents) (Tycko and Morison 2002) but instead depends on the parent-of-origin to dictate its expression (Ferguson-Smith and Surani 2001). That means genomic imprinting results in only one inherited copy of the relevant imprinted gene being expressed in an embryo. For paternally imprinted genes, the paternal allele is epigenetically modified to prevent transcription, ensuring that the embryo has only mono-allelic expression from the maternally inherited copy or, conversely, for maternal imprinted genes the paternal allele is expressed while the maternal allele is silenced (Choufani et al. 2010). Genomic imprinting could be either maternal or paternal, with the phenotypic consequences manifested relatively late in development or even into adulthood (Reik et al. 1993).

Imprinting is an epigenetic modulation of transcription and it is mainly due to the differential methylation of cytosine residues in CpG nucleotides in specific differentially methylated regions (DMRs) (Neumann et al. 1995). DMRs can have different properties: some are methylated in the silenced allele, whereas others are methylated on the active copy.

Imprinted genes account for only 0.1–0.5% of the genome but have a disproportionately important influence on early mammalian development (Constancia et al. 2004, Tycko and Morison 2002). Many imprinted genes are involved in the control of fetal growth and development in mammals (Reik et al. 2003) and dysregulation of their expression by deletion, inappropriate silencing or loss of imprinting has been implicated in several pathologies in the placenta and fetus, including sporadic, inherited and environmentally induced growth disorder (Lim and Ferguson-Smith 2010, Miozzo and Simoni 2002).

Many of the developmental defects observed in cloned bovine foetuses suggest the involvement of growth regulating genes, particularly those known to be imprinted. Defects such as placental and fetal overgrowth and perinatal death may result from deregulation of imprinted genes in fetus and placenta (Eggenschwiler et al. 1997). Fetal tissue seems less vulnerable to this type of imprinted problem as compared to placenta. In cloned mice several imprinted genes show abnormally low expression in the placenta, whereas no differences are seen in the fetus (Inoue et al. 2002). Similarly abnormal allelic expression pattern of the imprinted IGF2R gene was observed in placentas but not in the organs of cloned bovine calves (Yang et al. 2005). Moreover, evaluation of methylation patterns of imprinted genes following SCNT indicates that methylation errors at imprinted loci are common (Mann et al. 2003). In the mouse, only 4% of the SCNT-derived pre-implantation stage embryos recapitulate the expression of the imprinted genes H19, MEG3, IGF2R, ASCL2 and SNRPN relative to in-vivo-derived blastocysts (Mann et al. 2003).

Apart from somatic cell transfer, imprinted genes are responsive to a range of environmental cues, such as in vitro fertilization and maturation. In vitro environment results in the loss of genomic imprinting and biallelic expression (Dean et al. 2001, Dean et al. 2003, Doherty et al. 2000, Mann et al. 2004) which are subsequently associated with alterations in placental growth and development. Imprinting also varies dynamically across gestation in human, mouse and bovine placentas (Arnold et al. 2006a, Guillemot et al. 1995, Pozharny et al. 2010). In cattle, the imprinted MASH2/ASCL2 gene is biallelically expressed in trophoblast before implantation but is paternally silenced thereafter (Arnold et al. 2006a).

2.9.4 X chromosome inactivation

Sex chromosome dimorphism leads to a genetic imbalance between the homogametic (XX) and heterogametic sexes (XY), which mammals compensate by inactivating one of the two X chromosomes during female development in a process called X chromosome inactivation (XCI) (Lyon 1961). This epigenetic phenomenon involves non-coding RNAs (ncRNAs), antisense transcription, histone modifications, and DNA methylation to distinguish two genetically identical X chromosomes as active and silent entities within the same nucleus (Erwin and Lee 2008). In mice, the best-studied species two forms of XCI are observed: imprinted and random. Imprinted XCI inactivates the paternally inherited X chromosome (Xp) and occurs in the extra-embryonic lineages (Takagi and Sasaki 1975, Takagi et al. 1982). Random XCI occurs in the embryonic lineages in which the X chromosome of either parental origin (Xp or maternal X chromosome Xm) can be stochastically chosen for inactivation (Okamoto et al. 2005). This pattern persists in the trophectoderm lineage, such that only the maternal X chromosome is expressed in the placenta. In the ICM, however, the paternal X chromosome is reactivated, after which the paternal and maternal chromosomes are subject to random inactivation in the developing embryo (Okamoto et al. 2005).

In somatic cloning through nuclear transfer, the cloned zygotes receive one active (Xa) and one inactive (Xi) X chromosome from the donor cells. The introduction of a somatic cell nucleus containing one inactive X chromosome into oocyte cytoplasm creates an unusual epigenetic situation different from that in naturally fertilized female zygotes, in which both X chromosomes are active (Tian 2004). Using somatic donor cells containing genetically marked X chromosomes, it was shown that X inactivation was random in the epiblast lineage of cloned mice (Eggan et al. 2000). This means that the epigenetic marks that distinguish the active X from the inactive X are removed in somatic cells and reestablished on either X in the embryonic lineage after NT (Eggan et al. 2000, Rideout et al. 2001). However, in bovine clones aberrant patterns of X chromosome inactivation as well as transcript levels of X-linked genes, including XIST, have been found among different tissues (Wrenzycki et al. 2002, Xue et al. 2002). Besides, placental samples exhibited random X inactivation as opposed to the non-random preferential paternal X inactivation seen in normal controls and healthy SCNT calves (Xue et al. 2002).

2.10 Differences between cloned and fertilized embryos with respect to the early developmental events

In mammals, oocytes are ovulated at metaphase II and remain arrested at this stage until fertilization. The oocyte remains arrested at this stage for months (mouse) or even years (cattle) (Hyttel et al. 1997) and decades (humans) (Kishimoto 2003, Whitaker 1996) until a preovulatory hormonal surge. Once fertilized, the oocyte completes meiosis with the extrusion of the second polar body and the formation of male and female pronuclei. Shortly after their formation, DNA replication is initiated in the pronuclei. After DNA replication, equal segregation of the genetic material occurs by mitosis and the zygote cleaves to form two daughter blastomeres (Campbell et al. 1996a).

As part of the NT procedure the maternal chromosomes are removed to produce an enucleated oocyte. Following donor cell fusion or nuclear microinjection into the enucleated oocyte (cytoplast), the donor nucleus undergoes numerous subsequent events. In summary these include 1) nuclear envelop break-down (NEBD) followed by 2) premature chromosome condensation (PCC) 3) dispersal of nucleoli, 4) reformation of the nuclear envelope, and 5) nuclear swelling (Campbell et al. 1993). Nuclear swelling is indicative of extensive exchanges of proteins between the cytoplasm and the transferred nuclei and considered as a morphological indication of nuclear remodelling in amphibians (Gurdon 1964) and mammals including cattle (Sung et al. 2007), rabbit (Stice and Robl 1988), pig (Prather et al. 1987) and mice (Czolowska et al. 1984). These series of ultrastructural and biochemical changes occuring in activated oocytes after nuclear transfer, result in the formation of pseudo pro nucleai which mimic cytophysiologically interphase nuclei which are formed after oocyte fertilization (Samiec and Skrzyszowska 2005).

Although the male and female pronuclei reside in the same ooplasm, several differences become established between them during the first cell cycle (McLay and Clarke 2003). The origin of the differences between maternal and paternal pronuclei seen in fertilized embryos is mechanistically linked to the unique origins and chromatin state of the oocyte- and sperm-derived chromosomes (Latham 2005). For instance, in mammals the maternal genome is pre-packaged with histones in the oocyte, and has a chromatin configuration containing high levels of H3 histones methylated on lysines 4, 9 and 27, and trimethlyated histone H4 lysine 20 (Liu et al. 2004, van der Heijden et al. 2005). Whereas during spermatogenesis spermatids completely lack histones repacked their DNA with protamines, which become rapidly replaced by maternally provided histones in the zygote (McLay and Clarke 2003,

Yanagimachi 2003). This protamine-histone replacement allows the paternal genome to acquire a chromatin state that is enriched in hyper-acetylated histones (Adenot et al. 1997). In addition to histone modification, the pronuclei appear first few hours after fertilization differs markedly in demethylation of their paternal DNA. Before fertilization both gametes are highly methylated, with sperm being more methylated than oocyte DNA (Howlett and Reik 1991). However, very rapid and active demethylation of the paternal pronucleus oocurs shortly after fertilization while the maternal DNA remains methylated during this time (Mayer et al. 2000). As mentioned above, normal fertilized embryos possess their two parental sets of chromosomes in two distinct pronuclei, and distinct differences have been documented between the pronuclei in various epigenetic modifications involving changes in DNA methylation and histone acetylation (Kono 1997, Latham 2005). Therefore, it is reasonable to assume that if an SCNT-generated embryo has to develop, the chromatin must, at least to some extent, recapitulate the normal events taking place during early embryogenesis. When SCNT embryos were probed for the presence of this modification, it was observed that the pseudo-pronucleus does exhibit characteristics typical neither for the maternal nor for the paternal pro-nucleus (Fulka et al. 2008). This might be due to the nuclease used in nuclear cloning comes from a somatic (body) cell that has not undergone the developmental events required to produce the egg and sperm (Rideout et al. 2001). For instance the somatic genome, already packaged with histones, is unlikely to undergo the massive histone replacement process observed with the paternal genome (Lorthongpanich et al. 2010). Besides the methylation patterns typical of biparental chromosomes are not reproduced, passive demethylation occurres with greatly reduced efficiency in cloned embryos (Bourc'his et al. 2001). Thus, somatic cell chromosomes would not exist in those same chromatin states, at least not with the same maternal–paternal dichotomy (Latham 2005). Whatever restrictions on gene expression may arise normally from differential modifications of maternal and paternal chromosomes in the fertilized zygote, these restrictions are probably lacking in cloned embryos (Latham 2005).

2.11 Transcriptional reprogramming

It is believed that complete reprogramming of a somatic cell nucleus by the recipient cytoplast would result in an embryo with a similar profile of gene transcription as that is seen in vivo. Expression profiles of genes provide a primary evaluation on the extent to which the donor genome is correctly reprogrammed. In order to address the question of the extent of

reprogramming of donor cells and the effects of SCNT on gene-expression patterns in blastocysts, Smith et al. (2005) compared gene-expression profiles in donor cells and day-7 blastocysts produced by artificial insemination (AI), in vitro fertilization (IVF), and SCNT using a 7872 element cDNA microarray representing 6300 unique genes. The result showed that the vast majority (84.2%) of genes represented on the array were differentially expressed in the fibroblast donor cells when compared with the SCNT embryos, with more than 1500 genes exhibiting more than twofold difference. Besides comparison of the gene-expression profiles of AI, IVF, and SCNT 7-day blastocysts revealed that, SCNT embryos had a gene-expression pattern that was more similar to the AI embryos than the AI were to IVF embryos. Though, the source of cDNA clones which Smith and colleagues used consisted predominantly of placenta and spleen cDNA libraries which lacked embryonic genes, the study was appreciated as the first dramatic demonstration of the extent of nuclear reprogramming that occurs during the SCNT process.

Following that information from multiple transcriptional analyses of transferable bovine blastocysts from various origins is starting to accumulate (Long et al. 2007, Pfister-Genskow et al. 2005, Somers et al. 2006, Zhou et al. 2008). One such study (Beyhan et al. 2007) examined the transcriptional reprogramming status of blastocysts constructed from two female adult ear skin fibroblast lines (LE: low efficiency and HE: high efficiency) that differed markedly in their culture characteristics, developmental potential and in the expression of over 3000 transcripts prior to NT. Interestingly, comparison of these two cell lines derived SCNT blastocysts with that of IVP blastocyst demonstrated indistinguishable gene expression profile. However, unlike their similar degree of transcriptional reprogramming at the blastocysts stage, the two cell lines showed significantly different cloning efficiency in terms of generating live birth. A possible explanation for this phenomenon is global transcriptional profiling at the blastocyst stage may not have a direct relationship with to term development capacity. An alternative explanation could be as long as there were differentially expressed genes between SCNT and AI blastocysts, it is possible that those transcripts may have a pronounced effect on downstream development and redifferentiation (Smith et al. 2005).

Once the step of somatic-to-embryonic nuclear reprogramming has been successfully overcome, the difficulty resides in the accurate re-differentiation of the reprogrammed nuclei (Yang et al. 2007). Thus, post-hatching development especially between day 7 to 21 SCNT conceptuses are therefore, of high interest to decipher how the embryo, the trophoblastic sac and the uterus depend on each other and to which extent they tolerate asynchronies (Hue et al.

2012). It is important to note that thus far, all expression profile studies have examined the whole blastocyst embryo, so it remains unclear whether the observed differences are in the inner cell mass (ICM), the trophectoderm or both lineages (Yang et al. 2007). It is noteworthy that 60–80% of cells in the blastocyst are committed to the extraembryonic tissues (Koo et al. 2002). Thus, aberrantly reprogrammed genes expressed in early trophoblast may not be detected at 7 days of development if the resulting differential expression is localized to a fractional population of cells fated to form the extra embryonic tissues (Rodriguez-Zas et al. 2008). Reports in cattle showed that SCNT derived embryos extraembryonic tissues were shorter than AI or IVP elongating controls at day 14 of pregnancy (Alexopoulos et al. 2008). In an effort to identify putative genes associated to SCNT embryo elonagation process, a recent review by Hue et al. (2012) summarized more than 60 genes altered (up- or down-regulated) in elongating tissue after SCNT as compared to controls (AI or IVP). The transcripts were identified (Arnold et al. 2006a, Fujii et al. 2010, Kato et al. 2007, Rodriguez-Alvarez et al. 2010a, Sawai 2009, Smith et al. 2010) using transcriptomic or a candidate gene approach and analysed *in silico*. Interestingly, 89% of the transcripts belonged to networks that perfectly matched some of the functions that were previously associated to the elongation process namely: “cell cycle” or “cellular development” (Table 1).

Table 1. Gene expression on bovine elongating conceptuses after SCNT: IPA analysis on 55 genes, from 7 studies: Arnold et al. (2006), Kato et al. (2007), Sawai (2009), Fujii et al. (2010), Rodriguez-Alvarez et al. (2010) and Smith et al. (2010). Gene names are provided according to the referenced HUGO terms.

ID	Molecules in network	Score	Focus molecules	Top functions
1	Actin, Alpha catenin, Alpha tubulin, **ASCL2**, **AURKA**, Caspase, **CCT2**, Cdc2, ERK1/2, Fgf, FSH, **GNB2L1**, hCG, Histone h3, IGF1R, Immunoglobulin, Insulin, **KRT8**, **KRT18**, Lh, **NCL**, **NPM1**, PP2A, **PTGS2**, Ribosomal 40s subunit, Rnr, **RPS3**, **RPS11**, **RPS12**, **RPS25**, Rsk, **TMPO**, **TPT1**, **TUBA4A**, Vegf	38	17	Cell death, cell cycle, DNA replication, recombination, and repair
2	Ap1, Caspase 3/7, **CDX2**, Collagen Alpha1, Collagen type I, Collagen type IV, Cyclin E, **ELF5**, **EOMES**, **FGF4**, **FURIN**, Growth hormone, **IGF1**, **IGF2**, Igf, **IGF2R**, **IGFBP2**, **IGFBP3**, Igfbp, **ILF2**, Laminin, LDL, N-cor, **NANOG**, NFkB (complex), PDGF BB, **POU5F1**, **SOX2**, SOX2-OCT4, SOX2-OCT4-NANOG, **SPARC**, Stat5 dimer, **TEAD4**, Tgf beta, **WEE1**	38	17	Cellular development, embryonic development, posttranslational modification
3	60S ribosomal subunit, Akt, Ck2, Creb, **ENO1**, ERK, G-protein beta, **GATA3**, Gsk3, **HAND1**, Histone h4, IgG, IL1, IL12 (complex), IL12 (family), Jnk, LOC342994, LOC646875, LOC653232, Mapk, P38 MAPK, p85 (pik3r), PI3K (complex), Pkc(s), Ras, RNA polymerase II, **RPL18**, **RPL23**, Rpl36, **RPL39**, **RPL10A**, **RPL35A**, Rpl38 (includes others), **RPLP2**, Ubiquitin	18	9	Gene expression, cellular development, hematological system development and function

Genes in bold are present in the input data list, adopted from Hue et al. (2012).

This suggests that the SCNT elongated embryos' transcriptome is not only informative because it reveales the prevailing earlier reprogramming errors or explained later placental defects but also delivers information on the elongation process (Hue et al. 2012). It is therefore critically important to determine the reprogramming status of transcripts differentially expressed in elongated SCNT embryos so as to define the role of those genes in subsequent embryo development.

2.12 Effect of culture medium on gene expression of early IVP and SCNT derived embryos

The in vitro production of embryo (IVP) is essentially a three-step process involving in vitro oocyte maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC). On the other hand, the standard SCNT procedure includes enucleation of ova, insertion of the donor cells (or nuclei) and activation of the reconstructed embryo and subsequently IVC of constructed embryos till blastocyst stage. Despite this autonomy the pre-implantation embryo is highly influenced by factors in the external environment such as those presented by embryo culture or nuclear transfer, which may contribute for their difference with their in vivo counterparts in many respects including having smaller embryonic discs (Bertolini et al. 2002), retarded embryonic development (Alexopoulos et al. 2008, Tveden-Nyborg et al. 2005), shorter trophoblast size (Alexopoulos et al. 2008) and high incidence of chromosomal abnormalities (Slimane et al. 2000). These adverse characteristics are even more pronounced in SCNT embryos (Alexopoulos et al. 2008, Booth et al. 2003) which have still unresolved unknown problems (De Sousa et al. 2002). In addition, the ability of the embryo to adapt to changing environmental conditions can exceed its own adaptive capacity, resulting in aberrant embryonic gene expression (Gao et al. 2003, Niemann and Wrenzycki 2000).

There is a large and continually increasing body of evidence supporting the hypothesis that the environment to which embryos are exposed in vitro can perturb gene expression in the developing embryo. Several studies have been performed to gauge the effect of the environment on gene expression during pre-implantation development. Most of the studies looked at the transcription of individual genes, often focusing on stress, imprinting and apoptosis related genes. In one such study, McElroy et al. (2008) showed the effect of culture conditions and SCNT on the expression of HSP70.2, integrin beta 1 (ITGB1), phosphoglycerate kinase 1 (PGK1), BAX and IGF2R in porcine pre-implantation embryos (McElroy et al. 2008). The amount of BAX mRNA was higher in IVP and SCNT blastocysts cultured in a medium with addition of 10% fetal bovine serum (FBS) on day 4 as compared with in vivo blastocysts, whereas the mRNA content was lower for HSP70.2, IGF2R and ITGB1 in IVP than in in vivo blastocysts. Given that HSP70.2 is a molecular chaperone that is generally up-regulated in response to stress, it is surprising that the transcription of HSP70.2 was lower in IVP embryos than in vivo porcine blastocysts (McElroy et al. 2008). However, the regulation of expression of heat shock proteins seems complex and sensitive to minor changes in environment and manipulation (Wrenzycki et al. 2001).

Various studies in cattle and sheep have shown that the production of embryos under specific culture environments resulted in not only altered gene expression but also altered conceptus and fetal development following transfer. For instance, LOS is often attributed to the culture system. LOS is identified by obvious abnormalities, such as increased incidence of oversize fetuses and calves, increased fetal myogenesis, dystocia, dysfunctional perinatal pulmonary activity, abnormalities in placental development and reduced pregnancy rates (Farin and Farin 1995, Kruip et al. 1997, Niemann and Wrenzycki 2000, Young et al. 1998). The incidence of these phenotypes evidenced the persistence of early molecular deviations and their correlation with developmental anomalies (Lazzari et al. 2002).

Numerous studies aimed to improve the culture medium for preimplantation of embryos that can produce cloned offspring after transfer to the recipient. In vitro optimization of embryo culture media has revealed that cloned embryos perform better in glucose containing media, thus suggesting a possible altered physiological metabolism (Chung et al. 2002) resembling that of somatic cells. In the mouse, studies have shown that optimal results are obtained when the reconstructed embryo is cultured in the media in which the donor cell was cultured in (Gao et al. 2003). With the aim to establish an efficient defined culture medium for bovine somatic cell nuclear transfer (SCNT) embryos, Wang et al. (2012a) recently compared various media. Modified synthetic oviductal fluid (mSOF) without bovine serum albumin (BSA) was used as the basic culture medium (BCM) and with (BSA) as a control. Defined culture media containing each polyvinyl alcohol (PVA), myo-inositol, a combination of insulin, transferrin and selenium (ITS), epidermal growth factor EGF or all were added to BCM and the effect in terms of blastocyst formation rate, blastocyst cell number and expression of transcripts H19, HSP70, BAX and IGF-2 was compared with that of control counterparts. No significant differences in expression levels of H19, HSP70 and BAX were found in blastocysts derived from optimized medium and undefined medium, although the relative expression abundance of IGF-2 was significantly decreased in the former.

Alternatively, supplementation of hormone such as 10 ng/ml leptin during bovine oocyte maturation improved blastocyst rate and reduced the proportion of apoptotic cells through transcriptional enhancement of the leptin receptor (LEPR), signal transducer and activator of transcription 3 (STAT3), and baculoviral inhibitor of apoptosis protein repeat - containing 4 (BIRC4) genes (Boelhauve et al. 2005). Similarly, in vitro culture (IVC) medium with 50 ng/ml leptin during pig partenogenic (PA) and SCNT embryo development showed a stage-dependent regulatory effect which its primary stimulator effect being observed at the 4-8-cell

stage (Wei et al. 2009). These results indicated a positive effect of leptin on pre-implantion embryo development.

Addition of growth factors such as vascular endothelial growth factor (VEGF) at the late porcine IVP and SCNT blastocyst development stage was found to have a beneficial effect on the blastocyst quality and development rate (Biswas et al. 2011). It was shown that VEGF mRNA along with fetal liver kinase-1/ kinase insert domain-containing receptor mRNA (flk-1/KDR) was detected in all pre-implantation embryos stages of IVF and SCNT embryos indicating that the activity was achieved through VEGF receptors. Similarly, culturing pig IVF and SCNT embryos with IGF-I significantly increased the number of total cells in blastocysts and decreased the number of apoptotic nuclei (Kim et al. 2006).

Improvement of somatic cell reprogramming ability was also reported when the donor cells or the reconstructed embryos were treated with trichostatin A, an inhibitor of histone deacetylases, before or after NT (Ding et al. 2008, Iager et al. 2008, Meng et al. 2009, Zhang et al. 2007). Unfortunately, the long-term effects of these reagents on embryo development and their safety in therapeutic cloning are not known. One essential difference between the IVF and SCNT embryos is that the IVF embryos were fertilized by sperm in vitro, whereas SCNT embryos received a diploid nucleus from cultured donor cells. As the underlying reprogramming processes could be very different between NT and in vitro fertilized embryos, different media systems may be warranted (Dai et al. 2009).

2.13 Application of somatic cell nuclear transfer

Although there are issues concerning the safety of food products derived from clones and their offspring and significant animal welfare concerns limiting the acceptability and applicability of the technology in its current form, the potential benefits of cloning in research, industry and agriculture are vast. The available literature (Bowring 2004, de Oliveira Junior and de Oliveira 2012, French et al. 2006, Revel 2000, Yang 2004, Yang et al. 2007) categorized these applications in two general areas: Therapeutic and reproductive. Less frequently mentioned possibility ‘basic researchs’ is also included in the recent review of Niemann and Lucas-Hahn (2012).

2.13.1 Reproductive cloning

Reproductive cloning is the formation of one or more animals from a body cell of another animal. Thus, the members of a clone are genetically identical to each other and also to the donor of the cell from which the clone is derived (Gurdon 2004).

Application of reproductive cloning for rapid multiplication of desired livestock

Nuclear transfer cloning, especially from somatic cell nuclei, could provide a means of expanding the number of chosen livestock. It has a potential to produce a genetic copy (a clone) of an already proven adult animal of exceptionally high genetic merit for commercial purposes, without recourse to time-consuming progeny testing (Basrur and King 2005). This would be particularly relevant in the sheep and beef industries, where cloned sires could be used in widespread natural mating to provide an effective means of disseminating their superior genetics. It could be used as a substitute for artificial insemination, which in these more extensive industries is often expensive and inconvenient (Wells 2005).

Conservation of endangered species

Cloning can be used along with other forms of assisted reproduction to help preserve indigenous breeds of livestock, which have production traits and adaptability to local environments that should not be lost from the global gene pool. Prominent examples are the birth of the Sardinian Mufflon which is threatened by extension. It was produced by transfer of cloned Sardinian mufflon embryos into domestic sheep foster mothers (Loi et al. 2001).

Cloning for transgenic applications

A transgene is a foreign deoxyribonucleic acid (DNA) construct containing a sequence that codes for a specific protein and a promoter region that confers gene expression in specific tissues, along with insulators and other regulatory sequences to protect, enable or enhance the expression of the introduced gene (Keefer 2004, Niemann et al. 2003). The so-called "transgenic animals" were first developed using mice, by microinjection of DNA into the nucleus of the egg (Gordon et al. 1980, Palmiter et al. 1982). Alternatively, transgenic offspring can be created by using genetically modified donor cells for nuclear transfer. It is possible to remove (knockout) as well as to add genes and facilitates precise modification of control regions or addition of genes to specific regions of the genome (knockin) (Campbell 2002). The biotechnological applications of producing genetically modified cloned offspring

are many fold and include those targeting reproductive performance, growth rate, carcass quality, milk production, milk composition and disease resistance (Wall 1996, Wheeler et al. 2003). As such, in the earlier days of domestication, the introduction of superior alleles for any of these traits into a new line would have necessitated continued genetic selection, cross-breeding (hybridisation) and repeated back-crossing to ensure the introgression of the introduced allele. Transgenesis offers a faster method of introducing new and desirable genes into domestic animals without recourse to cross-breeding (Wall 1996).

2.13.2 Therapeutic cloning

This term refers to the production of cells by nuclear transplantation or cloning so that they can be used for replacement of body cells that no longer function normally (Gurdon 2004).

Pharmaceutical and medical applications of cloning

SCNT has been proposed as an approach to generate patient specific pluripotent stem cells for potential therapeutic applications. Patients with particular diseases or disorders in tissues that neither repair nor replace themselves effectively (as occurs, for example, in insulin-dependent diabetes, muscular dystrophy, spinal cord injury, certain cancers and various neurological disorders, including Parkinson's disease) could potentially generate their own immunologically compatible cells for transplantation, which would offer lifelong treatment without tissue rejection (Wells 2005). The embryonic stem cells derived from the SCNT embryo, called ntES cells (embryonic stem cells by nuclear transfer), would be isogenic to the donor and pose no risk of immune rejection (Pan et al. 2012, Wells 2005). The generation of human embryos does (Thomson et al. 1998), however, raise ethical concerns. It is therefore important for those advocating the introduction of therapeutic cloning to examine every alternative that could achieve similar objectives. One of such breakthrough that could potentially resolve the ethical issues and rejection problems associated with the use of human ES cells in regenerative medicine (Liu et al. 2008) is forced expression of four transcription factors (OCT4, SOX2, NANOG, and LIN28) to reprogram, mouse embryonic fibroblasts to ES-like cells (Yamanaka et al. 2006). This technology has since been improved with additional factors (Takahashi and Yamanaka 2006, Takahashi et al. 2007) and microRNAs [termed induced pluripotent stem (iPS) cells](Anokye-Danso et al. 2011, Miyoshi et al. 2011). Though the iPS approach is more practical than SCNT (Dimos et al. 2008), several studies have raised concerns about the safety of the iPS cells (Hussein et al. 2011, Taapken et al.

2011, Zhao et al. 2011). These concerns renewed the interest in other alternatives such as SCNT. Recently, for the first time, human ntES cells were reported to be successfully derived from an SCNT embryo (Noggle et al. 2011). In the longer term, however, fundamental understanding of reprogramming may enable one cell type to be directly trans-differentiated into another cell type specifically required for cell-based therapy (Collas and Hakelien 2003).

3. Materials and methods

3.1 Materials

3.1.1 List of chemicals and kits

During this experiment, various chemicals, kits and culture media purchased from different manufacturers were used. Besides, during data analysis multifarious software packages, tools and databases were utilized.

Chemicals	Manufacturer/Supplier
10 x PCR buffer	Promega, WI, USA
2-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Munich, Germany
5 x First-Strand buffer	Invitrogen Life Technologies, Karlsruhe, Germany
Acetic acid	Roth, Karlsruhe, Germany
Agarose	Sigma-Aldrich Chemie GmbH, Munich, Germany
BME (essential amino acids)	Gibco BRL, life technologies, Karlsruhe, Germany
Bovine serum ablbumin (BSA)	Promega, Mannheim, 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

Ethylenediaminetetra acetic acid	Roth , Karlsruhe, Germany
Eukaryotic poly-A RNA control kit	Affymetrix, CA, USA
ExoSAP-IT	USB, Ohio, USA
FSH	Follitropin Vetrepharm, Canada
GeneChip® Bovine Genome Array	Affymetrix, CA, USA
GeneChip®3'hybridization, wash and stain kit	Affymetrix, CA, USA
GeneChip®3' IVT express kit	Affymetrix, CA, USA
Glycogen for sequencing	Beckman Coulter, Krefeld, Germany
GnRH	Fertagyl®; Intervet, Boxmeer, The Netherlands
Hemi-calcium lactate	Promega, WI, USA
Hepes	Sigma-Aldrich Chemie GmbH, Munich,Germany
Hoechst 33342	Invtrogen, Carlsbad, CA, USA
Hydrochloric acid	Roth, Karlsruhe, Germany
Hydroxylamine	Sigma-Aldrich Chemie GmbH, Munich, Germany
Hypotaurin	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Ionomycin	Sigma-Aldrich, Chemie GmbH, Munich, Germany
L-Glutamine	Sigma-Aldrich, Germany
Magnesium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
MEM (non essential amino acids)	Gico BRL, Life Technologies, Karlsruhe, Germany
Mineral oil	Sigma-Aldrich Chemie GmbH,

	Munich, Germany
Norgen RNA DNA & protein isolation kit	Norgen Bioteck Corporation, Ontario, Canada
Oligonucleotide primers	MWG Biotech, Eberberg, Germany
PGF2α	Estrumate®, Intervet, Munich, Germany
PicoPure™ RNA isolation kit	Arcturs, CA, USA.
Potassium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
Random primer	Promega, WI, USA
Ribo-nuclease inhibitor (RNasin)	Promega, WI, USA
RNAlater	Sigma-Aldrich, MI, USA
RNasin	Promega, WI, USA
RNA 6000 Nano LabChip® kit	Agilent Technologies Inc, CA, USA
RQ1 RNase-free DNase	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 bicarbonate	Sigma-Aldrich chemie, Steinheim, Germany
Sodium chloride	Roth , Karlsruhe, Germany
Sodium hydrogen sulphate	Sigma-Aldrich Inc, MO, USA
Sodium lactate solution (60%)	Sigma-Aldrich Inc, MO, USA
Sodium pyruvate	Sigma-Aldrich Inc, MO, USA
Trypsin-EDTA	Sigma-Aldrich, Chemie GmbH, Munich, Germany

Streptomycin	Sigma-Aldrich, Deisenhofen, Germany
Streptomycin sulphate	Sigma-Aldrich Inc, MO, USA
TCM199	Sigma, Taufkirchen, Germany
Superscript II reverse transcriptase	Invitrogen, CA, USA
iTaq SYBR Green Supermix with ROX	Bio-Rad Laboratories, Munich, Germany
Taq DNA polymerase	Sigma-Aldrich Inc, MO, USA

Reagents and media

Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH_2O added to	25 ml
3% BSA in PBS	BSA	30g
	10x PBS : added to	1,000.0 ml
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
DEPC-treated water (1000 ml)	DEPC	1 ml
	added to water	1000 ml
dNTP solution	dATP (100 mM)	10 µl
	dGTP (100 mM)	10 µl
	dTTP (100 mM)	10 µl

	ddH_2O added to	400 µl
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
3M Sodium acetate, pH 5.2	Sodium acetate	123.1 g
	ddH_2O added to	500 ml
	ddH_2O added to	1000 ml

3.1.2 Equipments

Equipment	Manufacturer
ABI PRISM® 7000 SDS	Applied Bio Systems
Affymetrix®GeneChip Hybridization oven 640	Affymetrix, CA, USA
Affymetrix®GeneChip Fluidics Station 450	Affymetrix, CA, USA
Agilent 2100 Bioanalyzer	Agilent Technologies , CA, USA
Affymetrix®GeneChip™3000 scanner	Affymetrix, CA, USA
ApoTome microscope	Carl Zeiss Microlmaging, Germany
Axon GenePix 4000B scanner	Axon Instruments, Foster City, CA, USA
Centrifuge	Hermel, Wehingen, Germany
CEQ™ 8000 Genetic Analysis	Beckman Coulter, Krefeld, Germany
CO2-incubator (MCO-17AI)	Sanyo, Japan
CH15 embryo flushing catheter	CH15, Wörrlein, Ansbach, Germany
Electrofusion machine CFA 400	Kruess Hamburg, Germany
Electrophoresis	BioRad, Munich, Germany

Embryo transfer syringe and sheath	IMV, L'Aigle, France
Nunc four well dishes	Thermo Fisher Scientific, Roskilde, Denmark
GAPSII	Corning, Amsterdam, Netherlands
Inverted fluorescence microscope DM IRB	Leica, Germany
Nanodrop 8000 spectrophotometer	Thermo Fisher Scientific, DE, USA
Memmert CO2 incubator	Fischer Scientific, Leicestershire, UK
Millipore apparatus	Millipore Corporation, USA
MyCycler Thermal Cycler	Bio-Rad Laboratories, CA, USA
MicroAmp® optical 96-well reaction plate with barcode	Applied Bio Systems
Stereomicroscope SMZ 645	Nikon, Japan
StepOnePlus real-time PCR system	Applied Bio Systems
Ultra-low freezer (-80^{o}C)	Labotect GmbH, Göttingen, Germany

3.1.3 List of software programs and statistical packages

Programs (software) and statistical packages	Source of the programs (software) and statistical packages
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)	
Ingenuity's pathway analysis	Ingenuity® Systems, www.ingenuity.com
BLAST program	http://blast.ncbi.nlm.nih.gov/Blast.cgi
EndNote X1	Thomoson

Entrez Gene	http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene
GeneChip® Operating System	Affymetrix, CA, USA
Primer Express ® software	Applied Biosystems, Foster City, CA, USA
Primer 3 (version 4)	http://frodo.wi.mit.edu/primer3/
Prism for windows (ver.5.0)	GraphPad software, Inc.
Principal component analyis (PCA)	http://folk.uio.no/ohammer/past
R statistical computing and graphics software	http://www.r-project.org/

3.2 Methods

3.2.1 Experimental animal handling and management

All experimental animals were heifers of the same breed, Simmental. Heifers were selected based on general clinical examination for in vivo embryo production, embryo transfer and slaughter. They were fed a total mixed ration and housed in a freestall (cubicles) barn with slotted floor lined with rubber mates. All experimental animals were handled and managed according to the rules and regulations of the German law of animal protection.

3.2.2 In vitro embryo production (IVP)

In vitro fertilization and in vitro culture was performed according to the protocol described elsewhere (Salilew-Wondim et al. 2010b). Briefly, bovine ovaries were obtained from a local slaughterhouse and transported in warm (30-35°C) physiological saline solution within 1-3 hours. Subsequently, cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm-diameter follicles using a 10-ml syringe loaded with an 18-gauge needle. COCs with evenly granulated oocyte cytoplasm surrounded by more than three compact layers of cumulus cells were selected under microscope. COCs were washed and incubated (in groups of 50) in four well dishes containing 400 µl of maturation medium that consisted of TCM-199 (Sigma, Taufkirchen, Germany) with Earle salts buffered, 4.43 mM Hepes and 33.9 mM sodium bicarbonate (Sigma-Aldrich chemie, Steinheim, Germany) supplemented with 10% estrous cow serum (OCS), 0.5 mM L-glutamine, 0.2 mM pyruvate, 50 mg/ml gentamycin sulphate and 10 µl/ml FSH (Follitropin, Vetrepharm, Canada) (Nunc, Roskilde, Denmark) at 38.7 °C and 5% CO2 in an humidified air. After maturation (22 h), all COCs were co-incubated with sperm of the same bull (1×10^6 spermatozoa/ml) in a fertilization medium consisting of Fert-TALP medium supplemented with 10 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/ml BSA, 1 µg/mL heparin, 10 µM hypotaurine, 20 µM penicillamine, and 2 µM epinephrine) at 38.7°C in 5% CO_2 in air. At the end of co-incubation about 18 h after fertilization, the presumable zygotes were denuded from cumulus cells. Cumulus-free presumptive zygotes were washed three times in CR1aa (Rosenkrans et al. 1993) supplemented with 10% OCS and then cultured in 400 µl of the same medium in four well dishes (Nunc, Roskilde, Denmark) under mineral oil at 38.7°C in 5% CO_2 in humidified air until blastocyst stage.

3.2.3 In vivo embryo production

In vivo derived day 7 blastocyst-stage embryos were collected from donor cows following superovulation and AI using a similar protocol as described in our previous study (Salilew-Wondim et al. 2010a). Briefly, Simmental cows were pre-synchronized by intramuscular administration of 500 mg of the prostaglandin $F_{2\alpha}$ (PGF2α) analogue cloprostenol (Estrumate, Munich, Germany) twice within 11 days. The cows then 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 order. Two $PGF_{2\alpha}$ treatments were performed at 60 and 72 h after the initial FSH injection. Finally, 48 h after the first $PGF_{2\alpha}$ application, ovulation was induced by administration of 0.02 mg of Buserelin. A total of three artificial inseminations were performed within a 12 h interval using the same bull as used for in vitro fertilization. At day 7 post insemination, embryos were flushed out by draining each uterine horn with 500 ml PBS using the CH15 embryo-flushing catheter (Wörrlein, Ansbach, Germany) via a three-way connector into an embryo filter (Immuno Systems Inc., WI, USA).

3.2.4 Derivation and preparation of donor cells

Bovine fibroblast cells (FB) were used as a donor cells for production of SCNT blastocysts. For this, primary fibroblast cell lines were established from biopsies taken from the ear of the bull whose semen was used for in vivo and in vitro embryo production. The biopsies were minced into 1-2 mm pieces, were washed several times, and were dispersed in T25 cell culture flask. The cells were then cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2 mM glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, Deisenhofen, Germany) and 10% fetal calf serum (Gibco, Karlsruhe, Germany) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. At 90% confluence, the cells were trypsinized (0.05% Trypsin/0.53 mM EDTA; Gibco), subpassaged, and frozen stored as aliquots in cell culture medium with 10% DMSO until use. Cells of passage 3 were cultured and induced to enter a period of quiescence (presumptive G0) by serum starvation (0.5%) for 7 days before nuclear transfer.

3.2.5 Somatic cell nuclear transfer (SCNT)

To generate SCNT blastocysts, oocytes were matured for 19 h. Metaphase II oocytes were enucleated by removal of the polar body and the attached cytoplasm with the metaphase plate utilizing a 25-µm beveled glass pipette. The absence of the metaphase plate was confirmed by minimum exposure to ultraviolet fluorescence. A single donor cell was placed into the perivitelline space of the oocyte in close contact with the oocyte membrane, utilizing a 30-µm beveled glass pipette. Fusion was induced within 2 h after complex reconstruction by a single electrical pulse of 25 V for 45 µs (Kruess electrofusion machine CFA 400, Hamburg, Germany) between two electrodes, with a spacing of 150 µm. Activation of reconstructed complexes was performed by incubation in 5 µM ionomycin for 4 minutes followed by incubation with 2 mM 6-dimethylaminopurine (DMAP) in CR1aa medim for 3.5 h. Subsequently, SCNT derived embryos were cultured in CR1aa medium under mineral oil at 38.7 °C in 5% CO_2 in humidified air until blastocyst stage.

3.2.6 Embryo transfer and day 16 embryo collection

Prior to embryo transfer, recipient cows were estrous synchronized using a similar protocol as described above for superovulation except recipient animals did not receive FSH and AI. Following this, good quality day 7 blastocysts produced in vitro (IVP), somatic cell nuclear transfer (SCNT) and in vivo (AI) were transferred nonsurgical into the uterus at day 7 of the estrus cycle. Then recipients of IVP embryos, SCNT embryos and AI were slaughtered 9 days later. Elongation stage embryos were flushed out of the uterus by cutting the corpus uteri and injection of PBS solution into the oviduct, they were washed twice in PBS and subjected to morphometric quality assessment. The entire conceptuse from each pregnancy group was measured. On the bases of the conceptus length and presence and abscence of embryonic disc, embryos were further classified in to two groups. Filamentous embryos with visible embryonic discs were considered as elongated while conceptuses having early tubular shape were regarded as developmentally delayed. All samples were stored immediately in -20 °C with RNAlater (Ambion Inc, Austin, TX, USA) for later use. Morphologically similar conceptuses were used for large scale gene expression analysis. Conceptuses identified as developmentally delayed from IVP and SCNT pregnancies were used for qPCR based candidate gene expression analysis.

3.2.7 RNA extraction and cDNA synthesis

Total RNA was isolated from each group of sample namely SCNT, IVP, AI and FBs, three biological replicates from each group, using DNA/RNA/protein purification kit (Norgen Biotek Corporation, Thorold, Canada) according to methods recommended by the manufacturer with slight modification. All centrifugation steps were performed in benchtop microcentrifuge at 14, 000 × g except noted. Precisely, individual embryos were lysed in 600 µl of lysis buffer and centrifuged for 2 min. After the supernatant was transferred to another RNase-free microcentrifuge tubes, 600 µl of 70% ethanol were applied and vortexed for 10 s. Lysate with ethanol was applied on the provided column and centrifuged for 2 min to retrieve the entire lysate volume. In parallel, fibroblast cells were lysed in 350 µl of lysis buffer and vortexed for 15 sec. Subsequently, 200 µl of 95% ethanol were applied before transfer to the column. Despite slight differences in lysate preparation of fibroblast cells and embryo samples, a similar procedure was carried out for RNA isolation. The column was reassembled with a new collection tube and washed twice with 400 µl RNA wash solution and placed in a fresh 1.7 ml elution tube provided with the kit. RNA was eluted with a total 150 µl of elution buffer and centrifuged for 2 min. Following precipitation of RNA, TURBO DNA-free Kit (Applied Biosytems) was used to remove carry over DNA. The quality of the resulting RNA was verified by the relative intensity of rRNA bands using Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip® Kit (Agilent Technologies Inc, CA, USA), RNA was quantified using a NanoDrop 8000 spectrophotometer. For reverse transcription, 1 µg of RNA was used in a 20 µl final reaction, containing 1 µl random primer (Promega), 1 µl Oligo dt , 10 mM each dNTP, 4 µl 5x first-strand buffer, RNasin, DTT 0.1 M and superscript II in the order of 0.3 µl, 2 µl and 0.7 µl, respectively. Cycling was performed at 25 °C for 5 min, 42 °C for 90 min and 70 °C for 15 min. The cDNAs were kept frozen at -20 °C until used in qRT- PCR experiments.

3.2.8 RNA amplification

RNA amplification, cDNA synthesis, labelling and hybridization was performed according to user manual of GeneChip®3' IVT Express Kit [P/N 702646 Rev. 7 (Affymetrix Inc)]. Briefly, total RNA isolated from three biological replicates of AI, IVP and SCNT-derived elongated embryos as well as donor fibroblast cells was subjected to global gene expression analysis using the GeneChip Bovine Genome Array (Affymetrix, CA, USA). A total of 250 ng RNA (to which 2 µl of diluted poly-A RNA controls were added) using T7 oligo (dT)

primer was used to generate first strand cDNA. Eukaryotic poly-A RNA control kit was used as a SPIKE-IN control to monitor the entire target labelling 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. Following this, the first strand cDNA was converted to double strand. The resulting double stranded cDNA was in vitro transcribed and biotin- labelled using IVT master mix at 37 ^{o}C for 16 h. The amplified RNA (aRNA) was then purified to remove unincorporated NTPs, salts, enzymes and inorganic phosphate using magnetic-beads. The quality of cRNA was assessed on the Agilent 2100 bioanalyzer, and 12 µg of this cRNA was fragmented using 5× fragmentation buffer in RNase-free water. The fragmentation reaction was carried out at 94 °C for 35 min to generate 35–200 base fragments for hybridization, and fragmented aRNA quality was also assessed using the Agilent bioanalyzer.

3.2.9 Affymetrix array hybridization, washing, staining and scanning

The GeneChip® Bovine Genome Array (Affymetrix, CA, USA) was used for hybridization. For this, a hybridization cocktail consisting of fragmented and labelled cRNA, control oligonucleotide B2 (3 nM), 20 x eukaryotic hybridization controls (bioB, bioC, bioD, cre) (Affymetrix, CA, USA), 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. Hybridization was performed for 16 h. The arrays were then washed and stained using the Fluidics Station 450/250 (Affymetrix, CA, USA) and scanned using the GeneChip™3000 laser confocal slide scanner (Affymetrix, CA, USA) integrated with GeneChip® Operating System (GCOS) as recommended in the GeneChip® expression wash, stain and scan user manual (P/N 702232 Rev.3) and .cel raw data files were generated.

3.2.10 Data analysis

Data analysis was carried out with “R”, an open-source-interpreted computer language for statistical computation and graphics and tools from the Bioconductor project (http://www.r-project.org) and (http://www. bioconductor.org) were used. Normalization and background correction was done using Guanine Cytosine Robust Multi-Array (GCRMA). During normalization, the CEL files were pre processed and converted into expression set using the GCRMA, considering probe sequence and the GC-content background correction. The CEL files and normalized data can be accessed in the Gene Expression Omnibus (GEO)

http://www.ncbi.nlm.nih.gov/geo/ with series entry GSE40101, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40101. For genes with more than one probe set mapped to it, the probe set with the largest inter quartile range of expression intensity was selected. Non-specific filtering was used to remove genes with low variance between arrays using a cutoff of 0.25 (Gregersen et al. 2010). This left 16,020 genes that were used for the following analysis. Genes with FDR ≤ 0.2, p≤0.05 and ≥FC ≥ 2 were taken as differentially expressed (DEG). Canonical Pathway Analyses were performed using Ingenuity Pathway Analysis ([IPA], Ingenuity Systems®, http://www.ingenuity.com) software utilizing gene transcripts attaining nominal levels of significance ($p < 0.05$).

3.2.11 PCR based sex determination assay

Due to the fact that we have not used sexed semen and the donor cell line was obtained from a bull, we took advantage of harvested DNA from each embryo sample during RNA isolation to use it for PCR based sex determination. The sex of elongated embryos was determined by using two sets of primers namely bovine gender-neutral and bovine male-specific primers. The sequence of the bovine gender-neutral primer was: upstream 5′-GCC CAA GTT GCT AAG CAC TC-3′ and downstream 5′-GCA GAA CTA GAC TTC GGA GC-3′ (Akyuz et al. 2010) was used to show the presence of DNA in all samples. The bovine Y-specific primer (Accession No. AC234853.4), upstream 5′-TGG ACA TTG CCA CAA CCA TT-3′ and downstream 5′-GCT GAA TGC ACT GAG AGA GA-3 was used to distinguish male and female DNA. The amplification was carried out for 40 cycles where each cycle consisted of template denaturation at 95 ^{o}C for 5 min followed by 95 ^{o}C for 30 sec, reannealing at 53 ^{o}C and 55 ^{o}C for bovine gender neutral and male-specific primers for 30 sec, respectively and primer extension for 72 ^{o}C for 1 min followed by a final extension at 72 ^{o}C for 10 min. A total of 10 µl of the PCR products was electrophoresed on a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. A single band at size of 102 bp was considered as female whereas the presence of two bands at length of 102 bp and 226 bp were referred to male embryo.

3. 2. 12 Selected genes for qPCR analysis

Based on the length of extra embryonic tissue, a total of 6 conceptuses which had disproportionally short trophoblast size ranging from 1-3 mm (n=3) IVP and 1.25 -5 mm

(n=3) SCNT day 16 embryos were identified and selected. RNA isolation was performed using the PicoPure™ RNA isolation kit (Arcturs, Munich, Germany) following the manufacturer's instruction. Genomic DNA contamination was eliminated by performing on-column DNA digestion using RNase-free DNase (Qiagen GmbH, Hilden, Germany). Candidate genes, trophoblast Kunitz domain protein 2 (TKDP2) and fibroblast growth factor receptor 2 (FGFR2) were selected on the bases of their common differential expression in both IVP and SCNT elongated embryos. Besides, genes claudin 1 (CLDN1), junctional adhesion molecule 2 (JAM2), Rho/Rac guanine nucleotide exchange factor 2 (ARHGEF2) and CCAAT/enhancer-binding protein alpha (CEBPA) were selected because these transcripts were associated with tight junction signaling pathway which is a common pathway affected in both IVP and SCNT embryos. Though interferon tau IFNt was found to be not differentially regulated in the three embryos comparison, the level of this transcript was quantified.

3.2.13 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (PCR) was performed to validate the microarray data and analyse the expression profile of candidate genes. For this up- and down-regulated genes which were randomly selected from each of the three comparisons (SCNT vs. AI, IVP vs. AI and IVP vs. SCNT) and candidate genes which were screened on the above mentioned criteria were used. All primers were designed using Primer 3 online software (http://frodo.wi.mit.edu/primer3/, accessed May 2011) and subsequently entered in the Basic local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed May 2011) to ensure specificity. An amplified PCR product of each primer was further validated by sequencing using CEQ 8000 Genetic Analysis (Beckman Coulter, Krefeld, Germany).

Each reaction consisted of cDNA, forward and reverse primers and 10 µl of SYBRgreen mastermix (Bio-Rad Laboratories, Munich, Germany) made up to a final reaction volume of 20 µl with RNase-and DNase-free water. The cycling conditions were 50 ^{o}C for 2 min, 95 ^{o}C for 10 min and 40 cycles of 95 ^{o}C for 15 sec followed by 60 ^{o}C for 1 min. A dissociation curve was included to ensure specifity of amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normaliser gene, as it was validated previously in pre- and peri-attachment conceptus development in sheep (GAPDH mRNA concentrations remained constant as elongation occurs) (Purcell et al. 2009). Quantitative real-time PCR was performed on the Step One plus Real-Time PCR System (Applied Biosytems). The comparative threshold cycle (CT) method was used to quantify the mRNA abundance.

Table 2. Primers and their sequences used for microarray validation

Number	Acc no	gene	Primer	Sequence (5'-3')
1	NM_001114522	SPINK4	Forward	gtcggcagaaaagttggttt
			Reverse	gtcagcatttcccatccttc
2	NM_001034270	TSPAN1	Forward	agaactgtgaagaggcgatg
			Reverse	cttaggggctctggaatagg
3	NM_175776	TSPO	Forward	ggtggatctcctgctgact
			Reverse	aggagcacctctggaactg
4	NM_001080358	TDGF1	Forward	ggctaagttgaagggcaagt
			Reverse	ttcccacttttactggacaga
5	NM_177521	SULT1A1	Forward	cataaaggaggaccccaaaa
			Reverse	catgaaggcagagatgctgt
6	NM_174076	GPX1	Forward	aagttccaggagacgtcgtt
			Reverse	atcaggaaaacgccaagaac
7	NM_001035103	ALAS2	Forward	ctgtgatctcctgctctcca
			Reverse	cctcagtccaggcttctagc
8	NM_175797	ARHGDIB	Forward	ccagtgatagccgaacaaga
			Reverse	cagcagtaaccaccaggaga
9	NM_201606	MRPL12	Forward	atccaagatgtcgggttgat
			Reverse	tgatcagcttcaccttgtcc

10	NM_001038558	CADM1	Forward	ttcactcatgaagccaaagg
			Reverse	aaatagggccagttggacac
11	NM_001076372	CCND2	Forward	cgacttcatcgaacacatcc
			Reverse	atctttgccaggagatccac
12	NM_001012683	TKDP2	Forward	gtagctcagctcctggaacc
			Reverse	gaaattccaccttggacacc
13	NM_001034034	GAPDH	Forward	gaaattccaccttggacacc
			Reverse	ctgcttcaccaccttcttga
14	NM_001075194	ADD3	Forward	agctttgccctcatgaagta
			Reverse	atgggaacccaacagttaca
15	NM_001205310.1	FGFR2	Forward	tcagatcagcctgcattctc
			Reverse	aacgaacaccatggcagtaa
16	NM_001001854.2	CLDN1	Forward	agccttatctcctttcctca
			Reverse	aggaatgctatctcccctca
17	NM_001083736.1	JAM2	Forward	cagctacatgcaccctctgt
			Reverse	gggtatgagacccattctgc
18	BC149006.1	CEBPA	Forward	ccagagggaccgaagttatg
			Reverse	agagcctcattctggcaagt
19	NM_001098881.2	ARHGEF2	Forward	cagcaaccatgacctgaaac
			Reverse	ctgtcctcatcaccagcatc

3.2.14 Statistical analysis

Real-time PCR data analysis was performed by comparing ΔCt values (cycle numbers at the threshold level of log-based fluorescence normalized to the GAPDH control gene) by student's t-test, with two-sided $P < 0.05$ indicating statistical significance. Mean differences in ΔC_t ($\Delta\Delta$Ct) were used to calculate fold differences in gene expression by the following formula: fold change= $2^{-\Delta\Delta Ct}$.

4. Results

4.1 In vivo development of blastocysts derived from SCNT, IVP and AI until day 16

Somatic cell nuclear transfer (SCNT), in vitro produced (IVP) and in vivo developed (AI) blastocysts were generated and transferred to synchronized recipients at day 7 of the oestrous cycle and retrieved at day 16 of gestation. The number of blastocysts transferred and their development in vivo until day 16 is indicated in Table 3. Results showed comparable in vivo development rate after SCNT, IVF and AI blastosysts transfer. On the bases of their trophoblast elongation size and morphology, filamentous embryos were considered as elongated, whereas early tubular shape embryos (<3 mm long) were classified as developmentally retarded or delayed. With regard to their size, the SCNT embryos exhibited shorter elongation size (93 mm) compared to IVP (186.6 mm) or AI (196.3 mm) embryos. In addition, when the elongation status of the SCNT and IVP embryos was compared to AI, none of AI embryos displayed a delay in development whereas SCNT (37.5%) and IVP (25%) did. Morphologically similar conceptuses with visible embryonic disc derived from SCNT, IVP and AI were used for gene expression analysis while impeded embryos were used for further characterization of selected candidate genes.

Table 3. Development of somatic cell nuclear transfer, in vitro and in vivo produced blastocysts until day 16

Group	Transferred	Recovered day 16 embryos				
		Recovery rate	Elongated embryos		Impeded growth	
			Filamentous	Length (mm)	Delayed	Length (mm)
	n	n (%)	n (%)	Mean ± SD	n (%)	Mean ± SD
AI	22	17 (77.3)	17 (100)	196.3 ± 49.9	-	-
IVP	45	28 (62.2)	21 (75)	186.6 ± 43.2	7 (25)	20.1 ± 0.15
SCNT	11	8 (72.7)	5 (62.5)	93.3 ± 16.3	3 (37.5)	10.7 ± 1.08

‘Delayed’ refers to early tubular embryos which were not elongated properly.

4.2 Transcriptome profile analysis of elongated embryos derived from SCNT, IVP and AI

To gain a comprehensive overview of the global wide gene expression in SCNT, IVP and AI embryos, the Affymetrix GeneChip® Bovine Genome Array representing more than 23,000 genes and 19,000 UniGene clusters was used. After removing uninformative probes that show little or no variability between arrays, a total of 16,020 probes representing 16,020 genes that passed the data filtering process were identified as being expressed. Then the level of gene expression in SCNT and IVP elongated embrys were compared with that of the AI counterparts. Using the criteria of a fold change ≥ 2, P-value ≤ 0.05 and false discovery rate (FDR) $\leq 20\%$, 477 transcripts were identified as being differentially expressed in SCNT conceptuses compared to AI. When the same criteria were applied for the IVP and AI embryos comparison, 365 genes were found to be diferentially expressed. In addition, 26 differentially expressed transcripts were found between SCNT and IVP embryo groups. Of these, more than 50% of the differentially regulated genes were down regulated in each comparison, namely: 315 transcripts in SCNT vs. AI, 258 transcripts in IVP vs. AI and 19 transcripts in SCNT and IVP. The overall expression profile comparison of the three embryo groups and number of differentially expressed genes is illustrated in Figure 6.

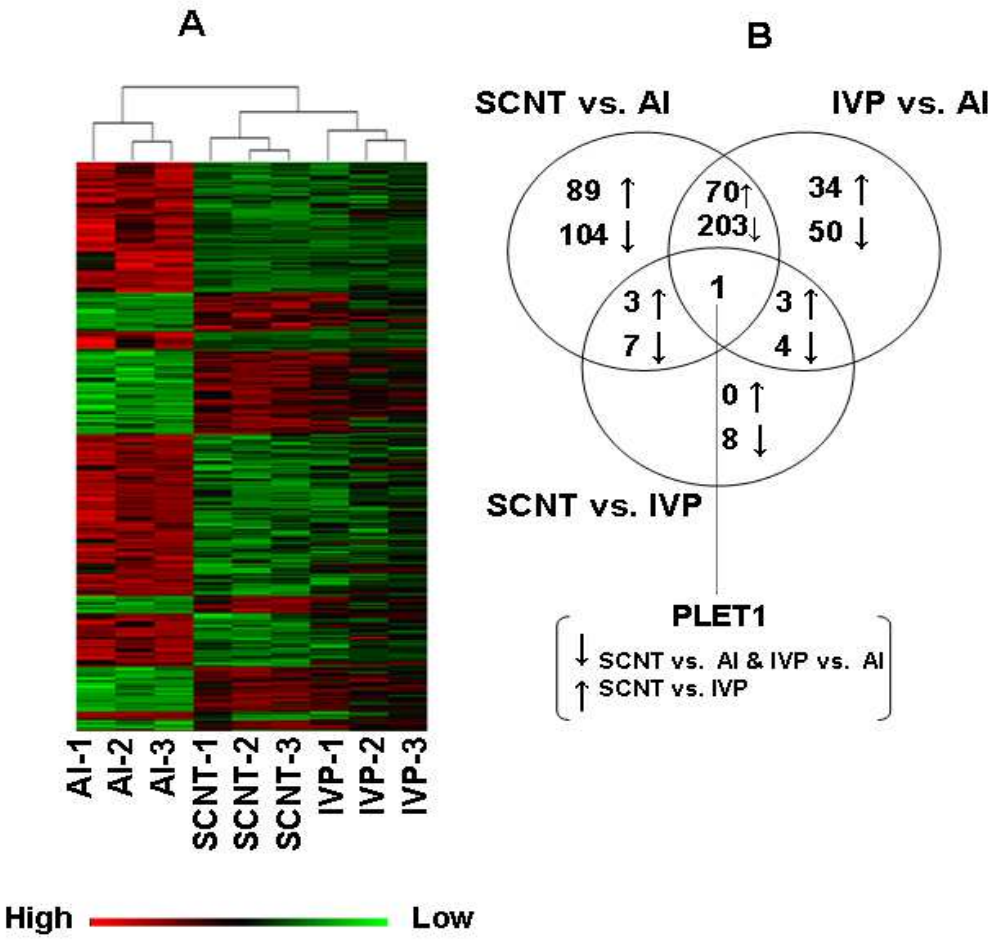


Figure 6. A) Heat map of differentially expressed genes in elongated embryos generated by artificial insemination (AI), somatic cell nuclear transfer (SCNT) and in vitro production (IVP). The numbers following each method of derivation indicate which of the three biological replicates is represented. The dendrogram on the top depicts the grouping of samples based on the similarity between them. Red colour signifies up-regulated genes, green signifies down- regulated genes and black represents intermediate expression as shown by the colour bar under the heat-map. B) Venn diagram characterizing differential gene expression between and specific to individual embryo types namely comparing SCNT vs. AI, IVP vs. AI and SCNT vs. IVP elongated embryos. Each circle represents the differential expression between the two indicated embryo types. Arrows represent the up-and down-regulated genes. Placenta-expressed transcript 1 (PLET1) gene is differentilly expressed in each embryo comparison with varying mRNA amount as indicated by the arrows.

4.3 Comparison of donor cell and embryo groups

Comparison of FB donor cells and SCNT embryos revealed differential expression of 3796 genes, of which nearly equal numbers of transcripts 1893 and 1903 were up- and down-regulated, respectively. The heatmap of differentially expressed genes in FB and three embryo samples revealed distinct clusters of the donor fibroblast cell in one group and the three embryo sources in another group, with expression profile of reproducible results among the biological replicates (Appendix 2). To visiualize the global distribution of expressed genes in each sample, principal component analysis (PCA) was performed. As shown in figure 7, the SCNT conceptuses samples were clustered away from donor fibroblast cells and situated near to IVP samples. This was in turn manifested by a 73.9% difference in gene expression between fibroblast donor cell and SCNT embryos.

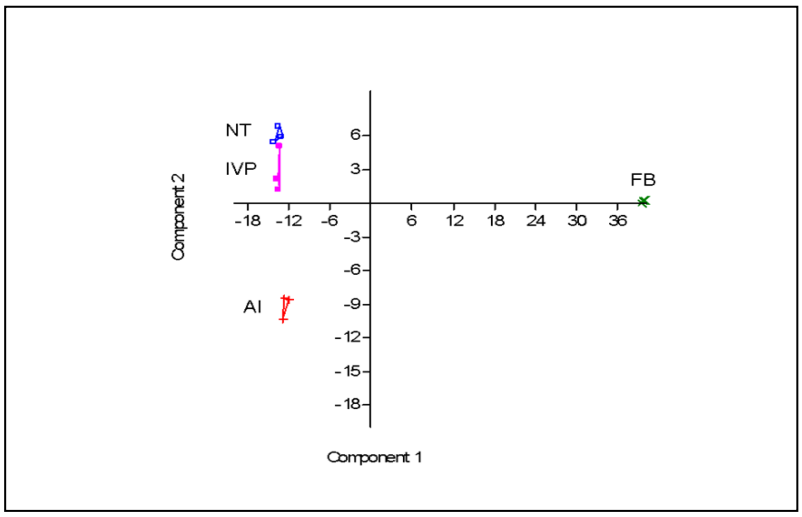


Figure 7. The PCA was performed based on all expressed genes in SCNT, IVP, AI and FB samples.

4.4 Chromosomal distribution of differentially expressed genes

The chromosomal distribution of differentially expressed genes in SCNT vs. AI, IVP vs. AI common to SCNT or IVP vs. AI comparison was investigated using GeneChip Bovine Genome Array annotation data (http://www.Affymetrix.com/Auth/analysis/downloads/na32/ivt/Bovine.na32.annot.csv.zip). Except some genes with unknown location (25 in SCNT vs. AI, 16 in IVP vs. AI and 13 common DE genes in SCNT and IVP) the majority of DE genes were distributed randomly across the chromosomes. However, a large number of differentially expressed genes in each

comparison were located on chromosome 1, 3, 5 and 19. Further inspection of the differentially expressed genes in these chromosme showed that most of the transcripts were down regulated.

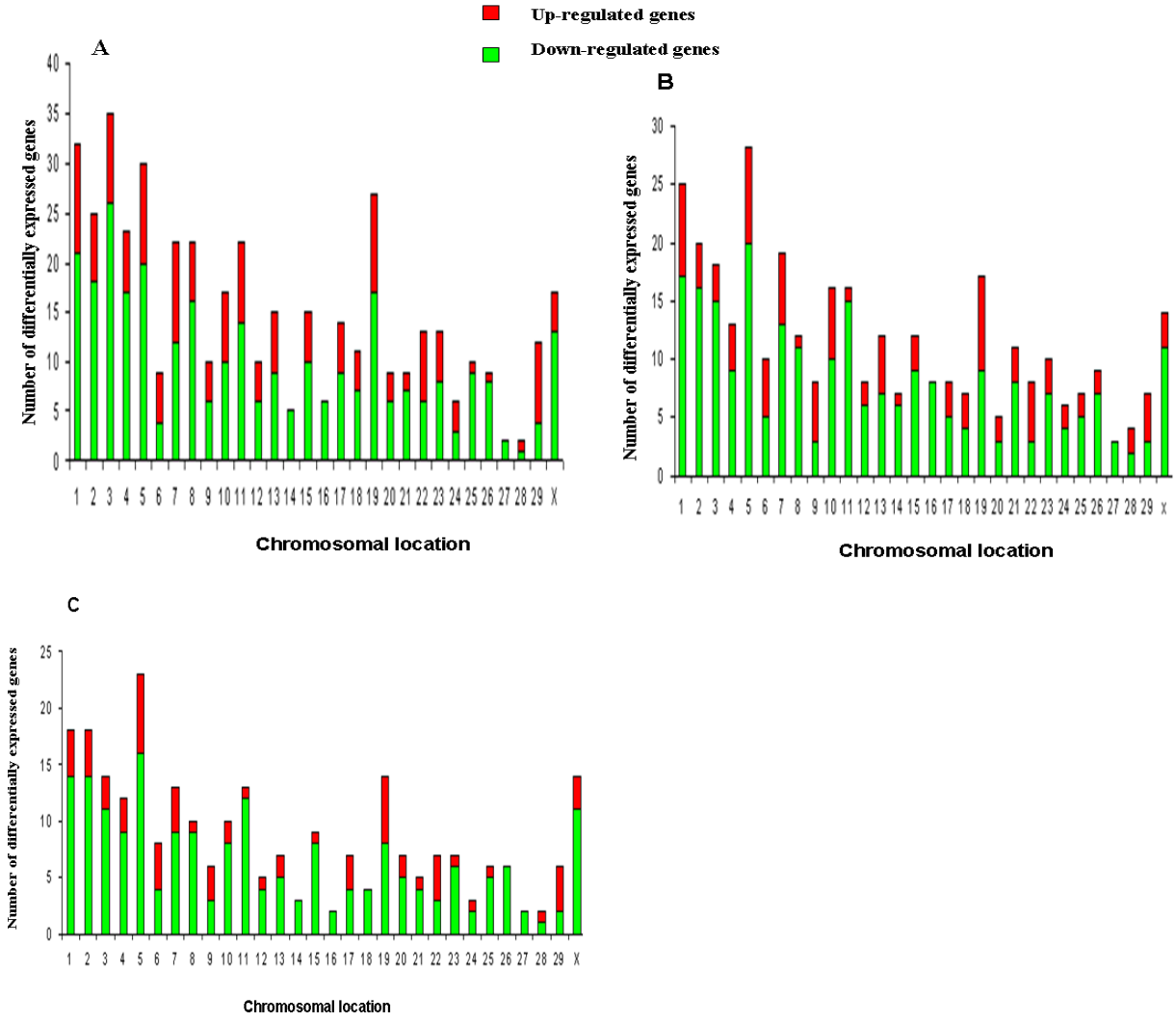


Figure 8. Differentially expressed genes and their chromosomal location. A) Differentially expressed genes and their chromosomal location in SCNT vs. AI comparison B) Differentially expressed genes and their chromosomal location in IVP vs. AI comparsion and C) Common differentially expressed genes and their chromosomal location in SCNT or IVP vs. AI comparison. The x-axis shows the number of chromosme including X chromosme and the y-axis shows the number of differentially expressed genes. Red and green represent, the up-and down-regualted genes, respectively.

4.5 Embryo sexing

As embryos obtained from SCNT were male in their sex, in order to elucidate a sex dependent expression differences between groups we have determined the sex of IVP and AI embryos used for gene expression analysis. Accordingly, one from each of IVP and AI groups was female while the other two were males (Figure 9). Looking in to the expression profile of all individual embryos we couldn not trace a sex dependent expression pattern.

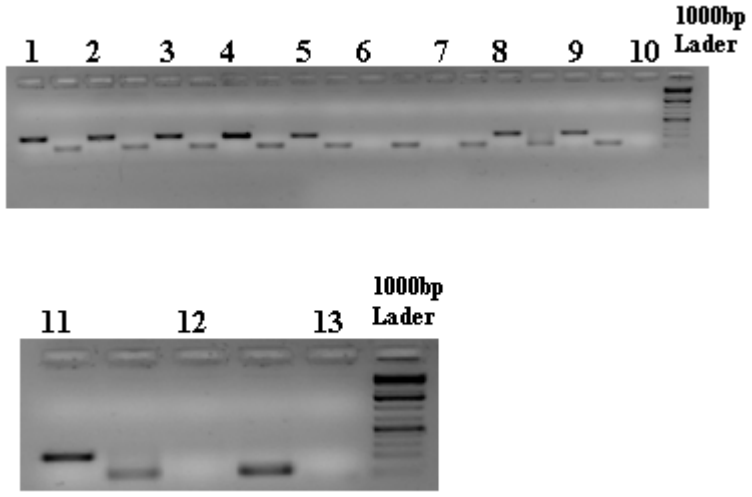


Figure 9. Sex determination assay using DNA isolated from SCNT, IVP and AI elongated embryos and bovine sex-neutral (102 bp) and male specific (226 bp) primers. Lane 1, 2 and 3 are SCNT, lane 4, 5 and 6 are IVP and lane 7, 8 and 9 are AI embryo DNA samples, individually. The numbered lanes denote the sex neutral product while the neighbouring lanes denote male specific product. All samples display 102 bp distinct products but only male embryos show 102 bp and 226 bp products. Lane 10 and 13 are negative controls. Lane 11 and 12 are DNA samples taken from known bull and cow, respectively.

4.6 Transcripts altered both in IVP and SCNT elongated embryos

To better understand pre-elongation culture effect on transcriptome alteration, those genes that were commonly altered in IVP and SCNT derived embryos (n=274) were examined further using the available bioinformatic tool and literature (Figure 10). Of these 274 genes, the expression level of 204 genes was lower in both IVP and SCNT embryos compared to their AI counterparts. These include the claudin family gene CLDN1, trophoblast specific gene, trophoblast Kunitz domain protein family (TKDP2), genes critical for trophoblast

differentiation heart and neural crest derivatives expressed 1 (HAND1), the gap junction protein genes known to play a role in uterine development and nutrient supply (GJB2, GJB4 and GJB5) and STSB and CTSL1 genes. On the other hand, the expression level of 70 transcripts including imprinted genes (IGF2R and MASH2) and CCND2, HSD17B1, TDGF1, SLC27A6, were found to be over represented in both IVP and SCNT embryos when both were compared to the AI counterparts.

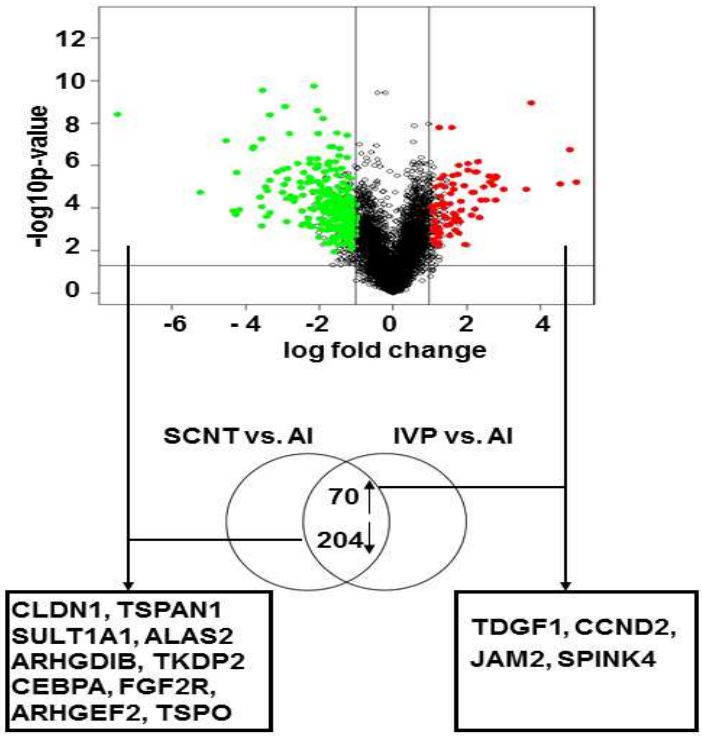


Figure 10. A volcano plot representation of common differentially expressed genes between SCNT and IVP elongated embryos. The significance cut-off was set to a FDR of (-log P-value ≥ 1), the absolute fold-change of all genes are represented by the circle, the three different colour codes used to represent insignificant genes (dark), differentially expressed genes being up-regulated (red) and differentially expressed genes being down-regulated (green). The list of genes on the left and right side of the volcano plot shows the qPCR validated up-and down-regulated genes, respectively.

4.7 Molecular pathways and gene networks affected in IVP and SCNT elongated embryos

Ingenuity pathway analysis (IPA) was used to characterize the function of differentially expressed transcripts in SCNT or IVP embryos, accordingly, the complete set of differentially regulated genes in SCNT vs. AI comparison was categorized in to various canonical pathways

(Table 4). The result showed several genes implicated in metabolic pathways (glycolysis, ascorbate and aldarate, arginine and proline, pyruvate, glycerolipid, fatty acid, gluthione, linoleic acid and glycerophospholipid metabolism) were significantly altered. On the other hand, most genes associated to blood coagulation (coagulation system and extrinsic prothrombin activation) were found to be down regulated in SCNT embryos compared to AI. Down regulated genes included those that encode coagulation factor, 2, 3 and 5. Similar significant down regulation was also observed for genes involved in TNFR1 signalling and agrin interaction at neuromuscular junction pathways. Besides, lPS/IL-1 mediated inhibition of RXR function, aryl hydrocarbon receptor signalling and immune response pathways like MIF regulation of innate immunity were disturbed in SCNT embryos as compared to the control AI embryos.

On the other hand, the top canonical pathways in IVP vs. AI comparisons showed only few metabolic pathways to be affected (pyruvate metabolism and arginine and proline metabolism) while genes participated in tight junction, agrin interaction at neuromuscular junction and TNRF-1 signalling were significantly down regulated (Table 5). Moreover, pathway analysis of genes commonly differentially expressed in IVP and SCNT when compared to AI revealed their involvement in tight junction signalling and glycolysis pathways (Table 6).

Table 4. Top canonical pathways affected in SCNT embryos

Cannonical pathways	-log (p-value)	Ratio	Molecules
LPS/IL-1 inhibition of RXR function	5.01	0.071	*ALDH4A1, ABCB1, ACSL3, SLC10A1, TNFRSF1A, GSTM3, ACOX1,ALDH9A1, ALDH1A1,JUN, FABP5, SCARB1, SULT1A1, CAT, SLC27A6, GSTP1*
Glycolysis	4.38	0.075	*ALDH4A1, ALDH2, ACSL3, ALDH1A1, DHRS9, ENO3, DLD, ALDH9A1, GALM, ALDOC*
Ascorbate and aldarate metabolism	3.88	0.063	*ALDH4A1, ALDH2, ALDH1A1, PRHOXNB, ALDH9A1*
Arginine and proline metabolism	3.87	0.050	*ALDH4A1, ALDH2, ALDH1A1, SAT1, PRHOXNB, GOT1, GATM, ALDH9A1, ODC1*
Pyruvate metabolism	3.62	0.059	*AKR7A2, ALDH4A1, ALDH2, ACSL3, ALDH1A1,DLD, PRHOXNB, ALDH9A1*
Coagulation system	3.58	0.158	*F2R, PROS1, SERPINA5, F5, F3, FGG*
Glycerolipid metabolism	3.57	0.058	*AKR7A2, ALDH4A1, ALDH2, ALDH1A1, DHRS9, LPIN2, AGPAT3, MOGAT1, ALDH9A1*
Aryl hydrocarbon receptor signaling	3.12	0.069	*ALDH4A1, FOS, ALDH1A1, JUN, CCND2, GSTM3, CDKN1A, TGFB3, ALDH9A1, GSTP1, HSPB1*
Extrinsic prothrombin activation pathway	3.04	0.2	*PROS1, F5, F3, FGG*
Fatty acid metabolism	3.02	0.048	*ALDH4A1, ALDH2, ACSL3, ALDH1A1, DHRS9, ACOX1, SLC27A6, ALDH9A1, CYP51A1*
Glutathione metabolism	2.79	0.065	*GSTM3, GPX1, GGT1, GSS, ANPEP, GSTP1*
MIF regulation of innate immunity	2.61	0.1	*FOS, MIF, JUN, NFKBIA, PLA2G12A*
Agrin interactions at neuromuscular junction	2.39	0.087	*ITGB2, RAC2, JUN, PAK6, ITGA6, ERBB3*
Caveolar-mediated endocytosis signaling	2.34	0.082	*B2M, ITGB2, CD55, FLNC, ITGAV, ITGA6, CD48*
Linoleic acid metabolism	2.28	0.046	*PLA2G12A, PLA2R1, FADS2, CYP51A1, FADS3*
Glycerophospholipid metabolism	2.2	0.042	*PLA2G12A, GPD2, GPLD1, PLA2R1, GOT1, LPIN2, PLCL2, AGPAT3*
TNFR1 signaling	2.16	0.094	*FOS, JUN, NFKBIA, PAK6, TNFRSF1A*
Bile acid biosynthesis	2.16	0.047	*ALDH4A1, ALDH2, ALDH1A1, DHRS9, ALDH9A1*

The underlined and plain text genes respectively showed up-and down-regulation in each pathway.

Table 5. List of top canonical pathways affected in IVP embryos

Cannonical pathways	-log (p-value)	Ratio	Molecules
Pyruvate metabolism	2.873	0.044	*ACSL3*, *AKR7A2*, *AKR7A3*, *ALDH4A1*, *PCK1*, *PRHOXNB*
LPS/IL-1 inhibition of RXR function	2.788	0.045	*ABCB1*, *ACOX1*, *ACSL3*, *ALDH4A1*, *FABP5*, *SCARB1*, *SLC10A1*, *SLC27A6*, *SULT1A1*, *TNFRSF1A*
TNFR1 signaling	2.749	0.094	*CASP3*, *FOS*, *NFKBIA*, *PAK6*, *TNFRSF1A*
Tight junction signaling	2.546	0.055	*ARHGEF2*, *CEBPA*, *CLDN1*, *F2RL2*, *FOS*, *JAM2*, *PRKAG2*, *TGFB3*, *TNFRSF1A1*
Arginine and proline metabolism	2.518	0.034	*ALDH4A1*, *ARG2*, *ASS1*, *GOT1*, *ODC1*, *PRHOXNB*
Caveolar-mediated endocytosis signaling	2.297	0.08	*FOS*, *MIF*, *NFKBIA*, *PLA2G12A*
Agrin interactions at neuromuscular junction	2.293	0.072	*ERBB3*, *ITGA6*, *ITGB2*, *PAK6*, *RAC2*
Molecular mechanisms of cancer	2.090	0.034	*ARHGEF2*, *BMP7*, *CASP3*, *CCND2*, *CDKN1A*, *FOS*, *GNAI1*, *NFKBIA*, *PAK6*, *PRKAG2*, *RAC2*, *RHOB*, *TGFB3*
IL-10 signaling	2.089	0.064	*ARG2*, *BLVRB*, *FOS*, *IL6*, *NFKBIA*
Eicosanoid signaling	2.079	0.056	*DPEP1*, *PLA2G12A*, *PLA2R1*, *PTGES*
PTEN signaling	2.033	0.056	*CASP3*, *CDKN1A*, *FGFR2*, *FOXO4*, *IGF2R*, *MAGI1*, *RAC2*

The underlined and plain text genes respectively showed up- and down- regulation in each pathway.

Table 6. Common genes and their corresponding canonical pathways affected in IVP and SCNT embryos

Cannonical pathways	-log (p-value)	Ratio	Molecules
Tight junction signalling	3.30	0.055	*ARHGEF2, CEBPA, CLDN1, F2RL2, FOS, JAM2, PRKAG2, TGFB3, INFRSF1A*
LPS/IL-1 inhibition of RXR function	2.98	0.04	*ABCB1, ACOX1, ACSL3, ALDH4A1, SCARB1, SLC10A1, SLC27A6, SULT1A1, TNFRSF1A*
Caveolar-mediated endocytosis signalling	2.94	0.071	*B2M, CD48, CD55, ITGA6, ITGAV, ITGB2*
Agrin interactions at neuromuscular junction	2.76	0.072	*ERBB3, ITGA6, ITGB2, PAK6, RAC2*
MIF regulation of innate immunity	2.68	0.08	*FOS, MIF, NFKBIA, PLA2G12A*
Integrin signalling	2.46	0.043	*ARF4, ITGA6, ITGAV, ITGB2, PAK6, RAC2, RHOB, TSPAN1, TSPAN2*
Virus entry via endocytic pathways	2.43	0.06	*B2M, CD55, ITGA6, CXADR, ITGB2, RAC2*
Relaxin signalling	2.34	0.044	*FOS, GNAI1, GNB4, MPPE1, NFKBIA, PRKAG2, VEGFA*
TNFR1 signalling	2.31	0.075	*FOS, NFKBIA, PAK6, TNFRSF1A*
IL-8 signalling	2.29	0.041	*CCND2, GNAI, GNB4, GPLD1, ITGB2, RAC2, RHOB, VEGFA*
Germ cell-sertoli cell junction signalling	2.20	0.042	*ITGA6, PAK6, RAC2, RHOB, TGFB3, TNFRSF1A, TUBB6*
Glycolysis/gluconeogenesis	2.15	0.038	*ACSL3, ALDH4A1, DHRS9, ENO3, GALM*
MIF-mediated glucocorticoid regulation	2.13	0.071	*MIF, NFKBIA, PLA2G12A*
Molecular mechanisms of cancer	2.09	0.029	*ARHGEF2, CCND2, CDKN1A, FOS, GNAI1, NFKBIA, PAK6, PRKAG2, RAC2, RHOB, TGFB3*
PTEN signalling	1.99	0.048	*CDKN1A, FGFR2, FOXO4, IGF2R, MAGI1, RAC2*

The underlined and plain text genes respectively showed up- and down- regulation in each pathway.

4.8 Molecular networks affected in SCNT and IVP embryos

To characterize the functional consequences of gene expression changes associated with SCNT and IVP derived embryos, a molecular network analysis of differentially expressed genes, based on IPA data bases was performed. The results indicated that 21, 16 and 12 top scored gene networks to be affected in SCNT, IVP and common to both SCNT and IVP embryos respectively. These gene networks were selected based on the criteria of IPA score of 10 or higher and containing 9 or more genes. Figure 11, 12 and 13 summarized the top 4 gene networks found with IPA score greater than 25. Based on the identified gene networks, the top functional categories of transcripts in SCNT embryos are lipid metabolism, carbohydrate metabolism, DNA replication, recombination and repair and small molecules biochemistry. On the other hand, functional categories of transcripts in IVP embryos mostly related to cell and organ development, cellular movement, cellular growth and proliferation, small molecules biochemistry and carbohydrate metabolism while DNA replication recombination and repair were the commonly affected biological functions in both IVP and SCNT elongated embryos.

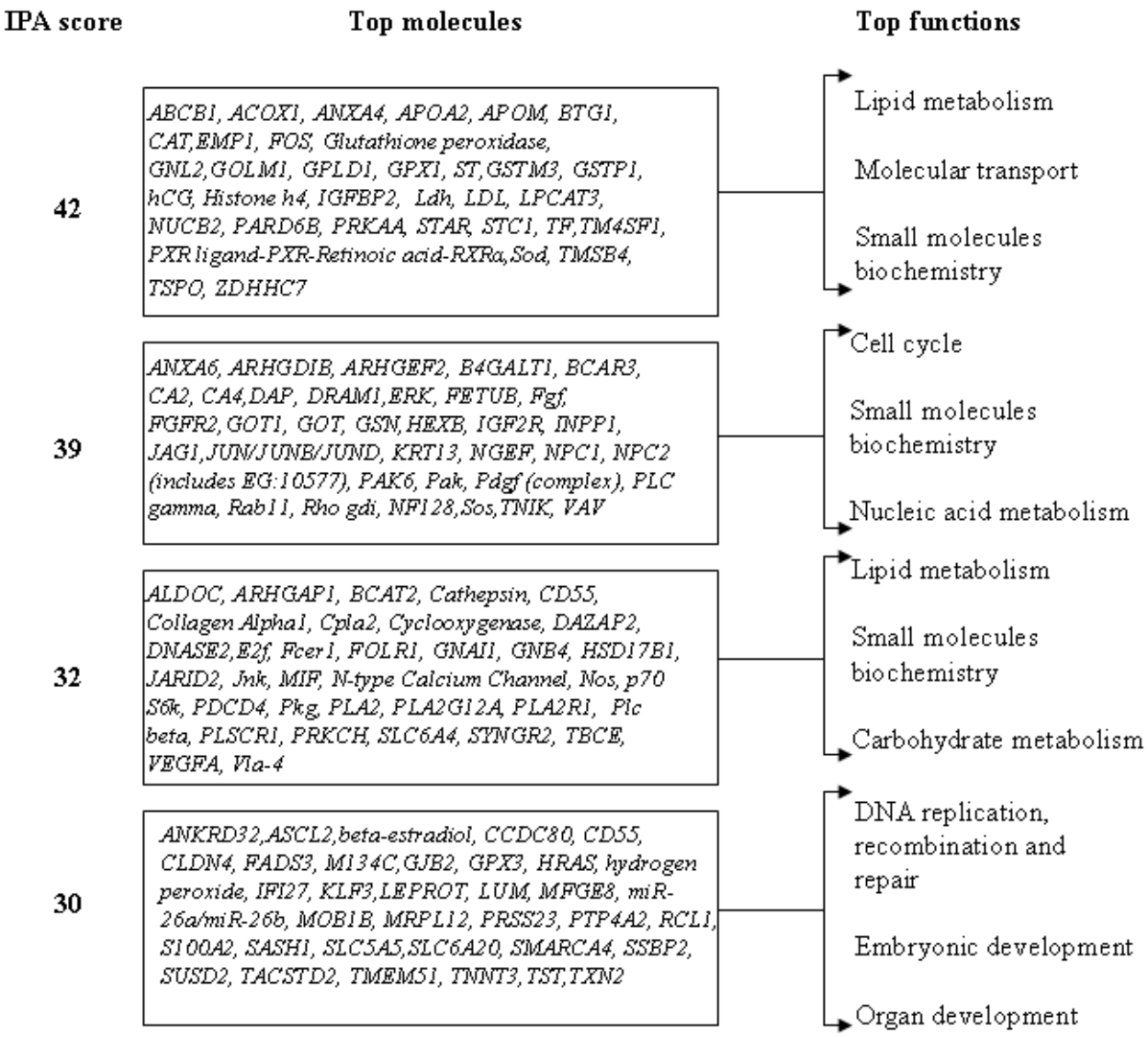


Figure 11. The top scored gene networks using differentially expressed transcripts in SCNT vs. AI elongated embryos. The IPA score = (-log (p-value)) is associated with the significance of the selected gene network. The higher the score the more reliable it is.

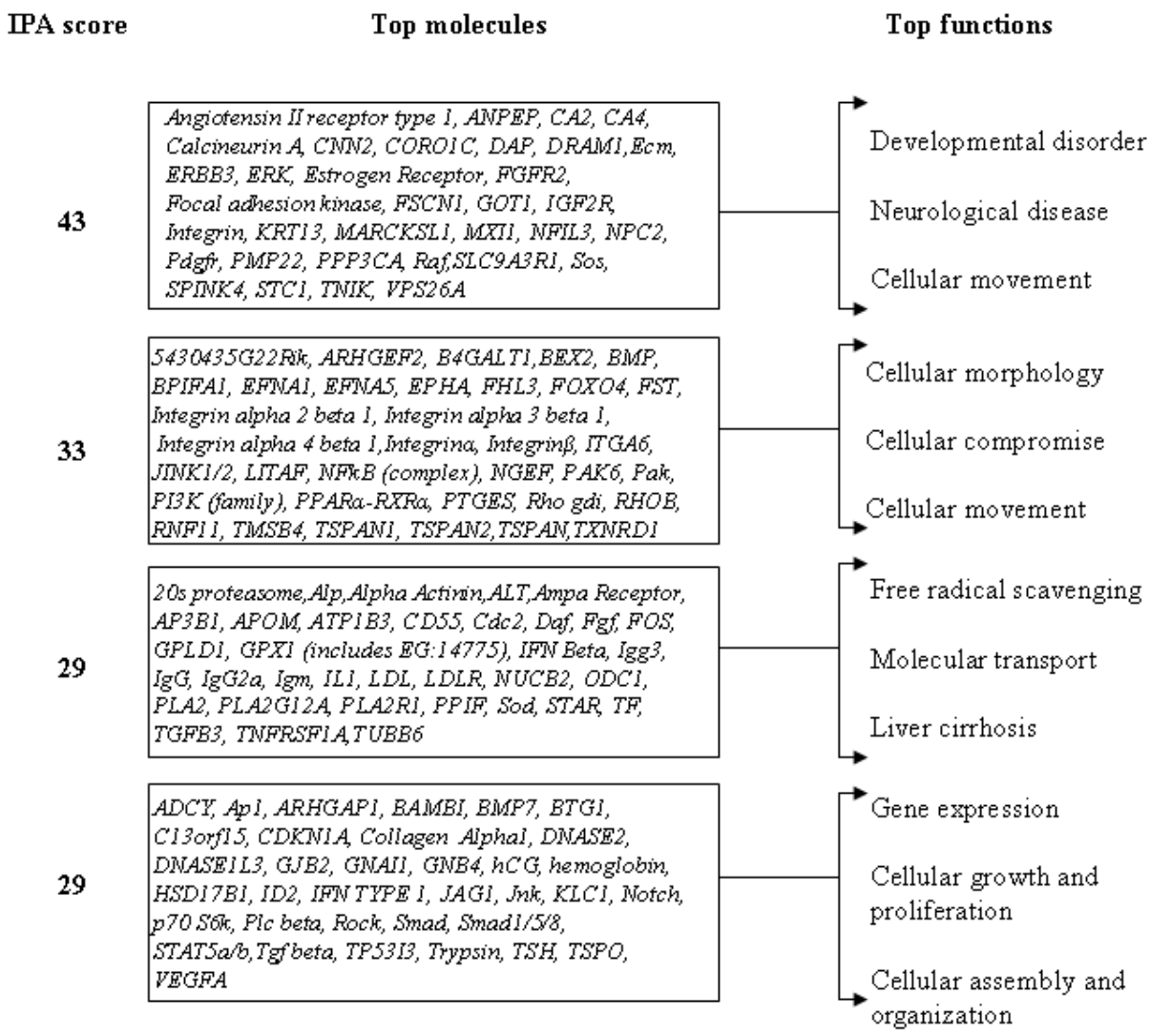


Figure 12. The top scored gene networks using differentially expressed transcripts in IVP vs. AI elongated embryos. The IPA score = (-log (p-value)) is associated with the significance of the selected gene network. The higher the score the more reliable it is.

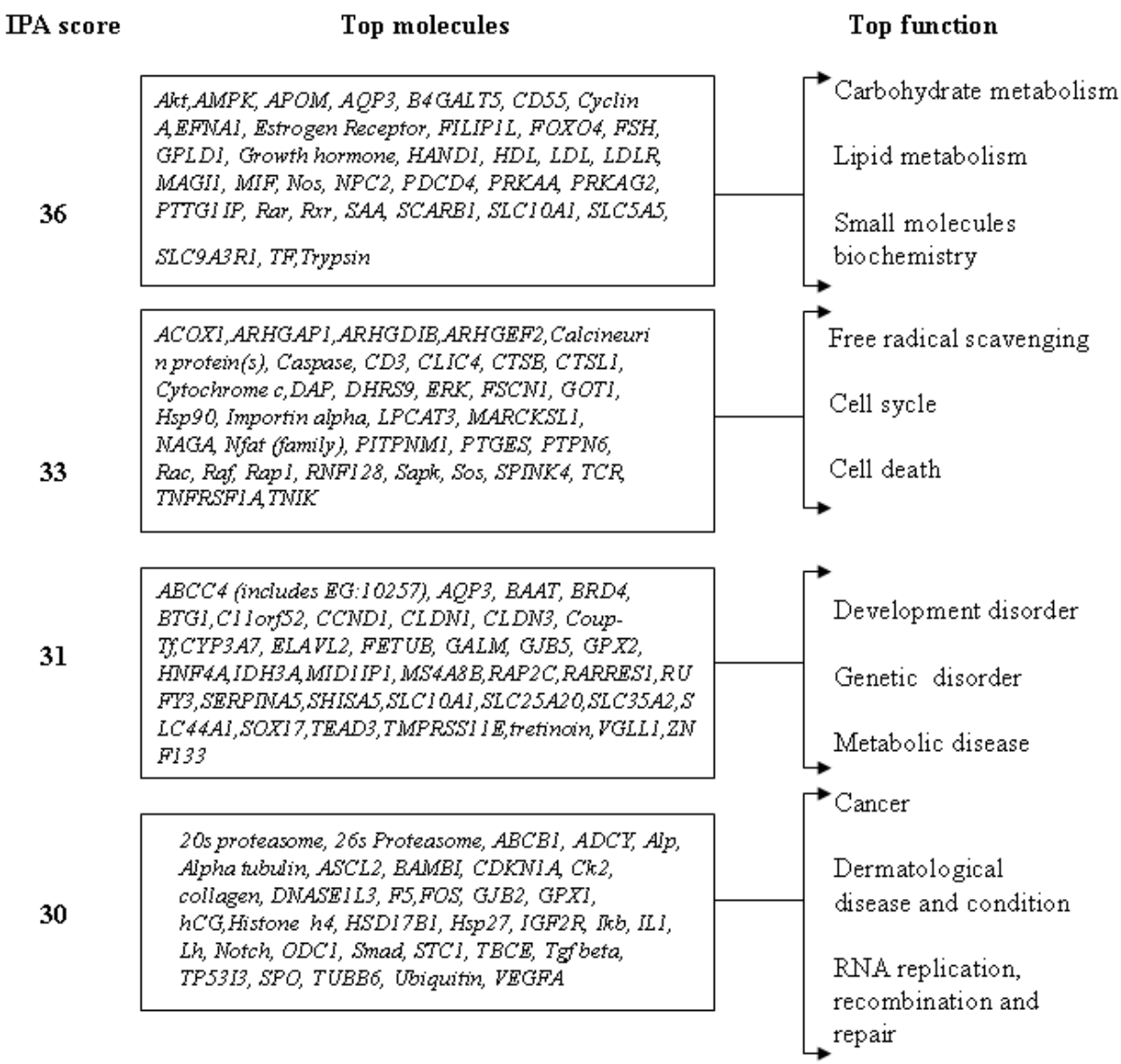


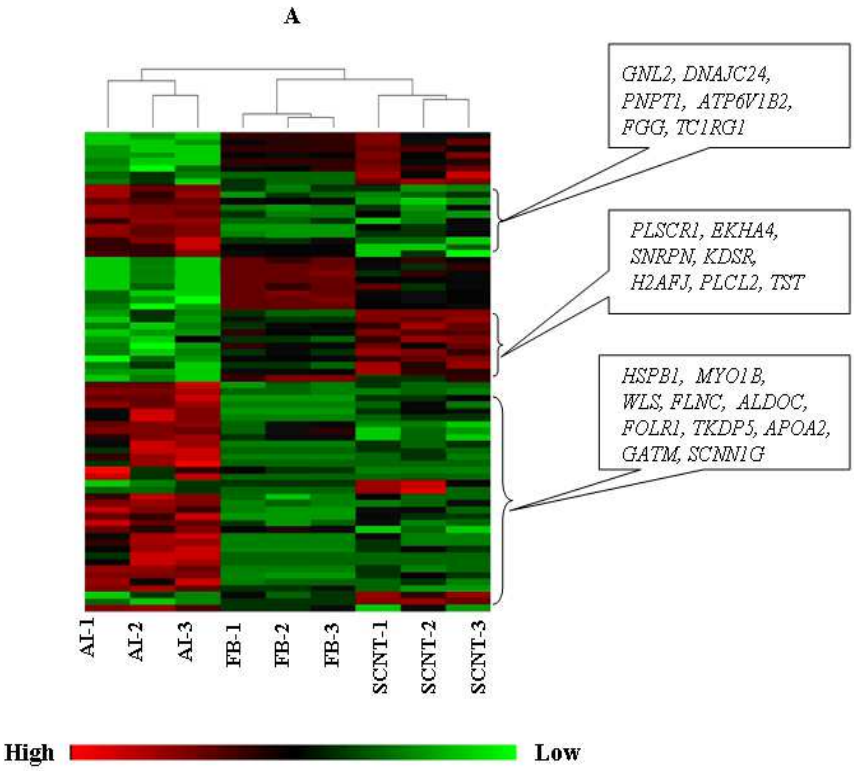
Figure 13. The top scored gene networks for common differentially expressed transcripts in IVP and SCNT elongated embryos. The IPA score = (-log (p-value)) is associated with the significance of the selected gene network. The higher the score the more reliable it is.

4.9 Identification of genes with different transcriptional reprogramming status

To evaluate the possible effect of somatic cell nuclear transfer on transcriptome alteration due to genome reprogramming, a list of 193 genes which are exclusively differentially expressed in SCNT vs. AI embryos were filtered and categorized in to various reprogramming status, based on their expression pattern namely: category 1: Transcriptionally not reprogrammed genes [genes which are differentially expressed between SCNT and AI conceptuses but not when SCNT is compared with FB (AI≠SCNT=FB)]. Category 2: Inappropriately reprogrammed genes [genes whose expressions resemble neither AI nor FB (AI≠SCNT≠FB)].

Category 3: Partially or intermediately reprogrammed genes [genes with intermediate expression in SCNT embryos when compared to both AI and FB].

Accordingly, out of 193 genes, 71 transcripts including GATM, HSPB1, FOLR1, GADD45B, MTFHD2, H2AFJ, TGFBR3, TSCNN1B, SCNN1G, CYP51A1 and RCN2 were found as transcriptionally not reprogrammed. Moreover, 91 transcripts including imprinted gene (CDKN1C), several genes associated with metabolism such as acyl-CoA thioesterase (ACOT4 and ACOT8), aldehyde dehydrogenase (ALDH2 and ALDH9A1), and solute carrier families (SLC10A, SLC16A3, SLC6A20) were incompletely reprogrammed. The remaining 31 transcripts which showed intermediate expression pattern in SCNT embryos including TKDP4, RBP4, RYPB, MYLCK, SLC2A5, SHMT2 and SUSD2 were classified as partially reprogrammed.



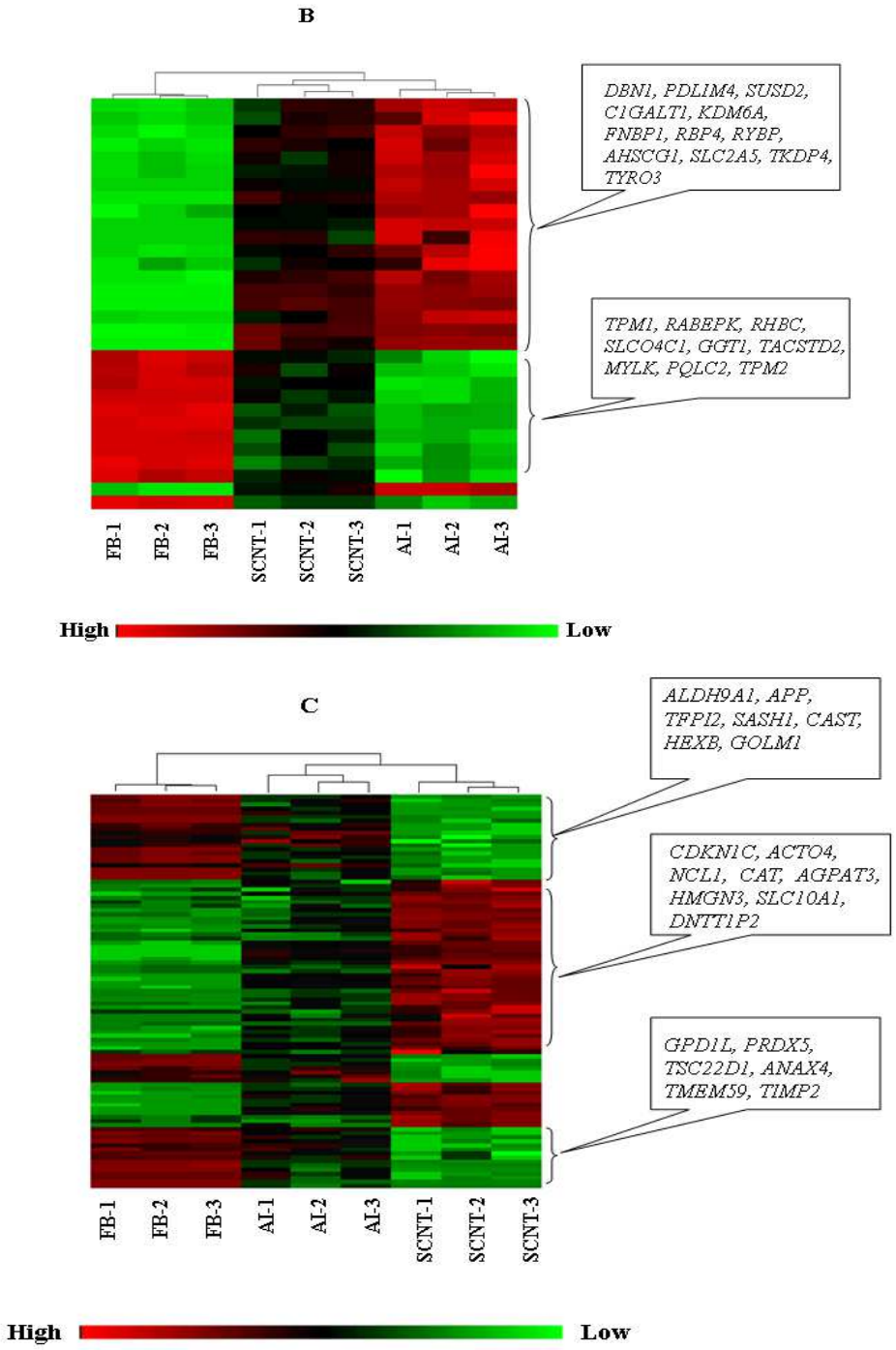


Figure 14. A) Heat-map representation of genes that are not transcriptionaly reprogrammed. The expression pattern of exclusively differentially expressed genes in SCNT elongated embryos that resemble donor fibroblasts (FBs) and differ from control (AI). B) Heat-map representation of intermediately reprogrammed genes whose expression in SCNT embryos is in between AI and FBs C) Heat-map representation of incompletely reprogrammed genes whose expressions neither resembled AI nor FBs. The dendrogram on the top depicts the

grouping of samples based on their similarity. Red represents high, green represents low and black represents intermediate gene expression levels as shown by the colour code at the bottom of the figure. Some representative transcripts from up-and down-regulated genes from each category are indicated in the respective boxes.

4.10 Pathways and gene networks affected due to incompletely reprogrammed transcripts

Because of the biological significance and their relative large number, the pathway and gene network analysis was performed for 91 incompletely reprogrammed genes. Among the canonical pathways appeared in the analysis of the set of incompletely/abruptly reprogrammed transcripts, 4 major pathways are related to metabolism. These include glycerolipid, methane, ascorbate and aldarate and arginine and proline metabolism (Figure 15). Moreover, 4 major networks were identified involving the 92 abruptly up- and down-regulated genes in SCNT embryos. Based on the identified gene networks with IPA score more than 25, the top functional categories affected are cell death, lipid metabolism, molecular transport, nucleic acid metabolism and cellular development (Figure 16).

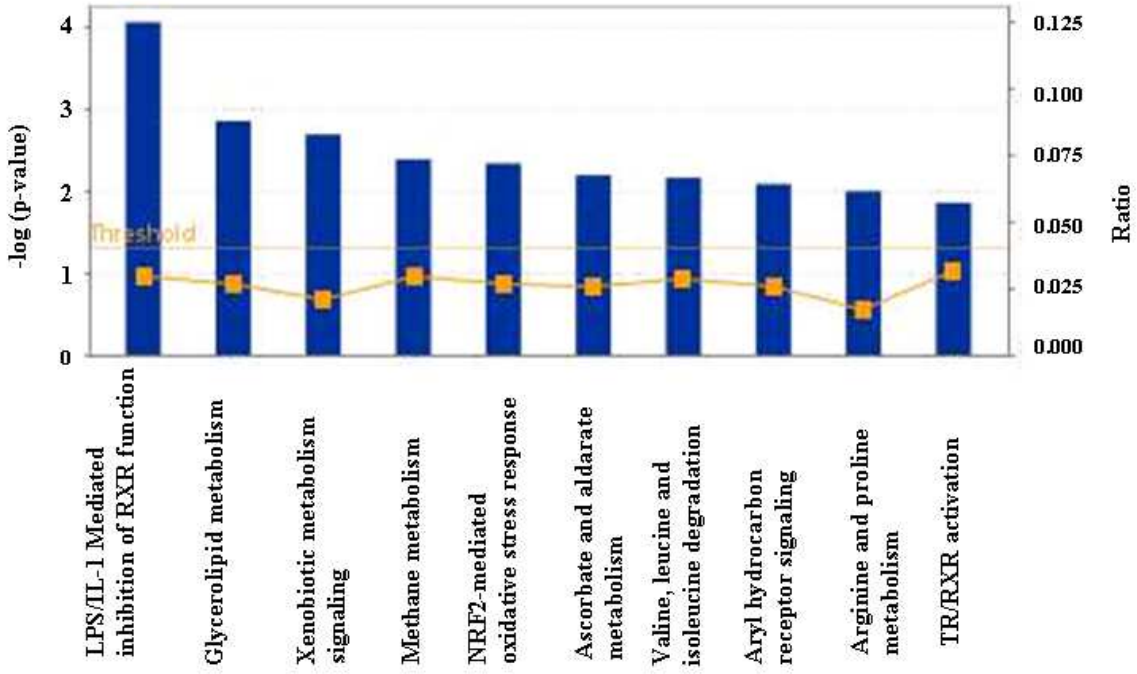


Figure 15. IPA-generated, top canonical pathways of 91 abruptly up- and down-regulated genes that are exclusively differentially expressed in SCNT elongated embryos. The line plot designates the ratio of the number of genes from our dataset that are available in the identified pathways divided by the total number of molecules that exist within the canonical pathway. Fischer's exact tests were used to calculate p-values (presented as $-\log_{10}$ P values).

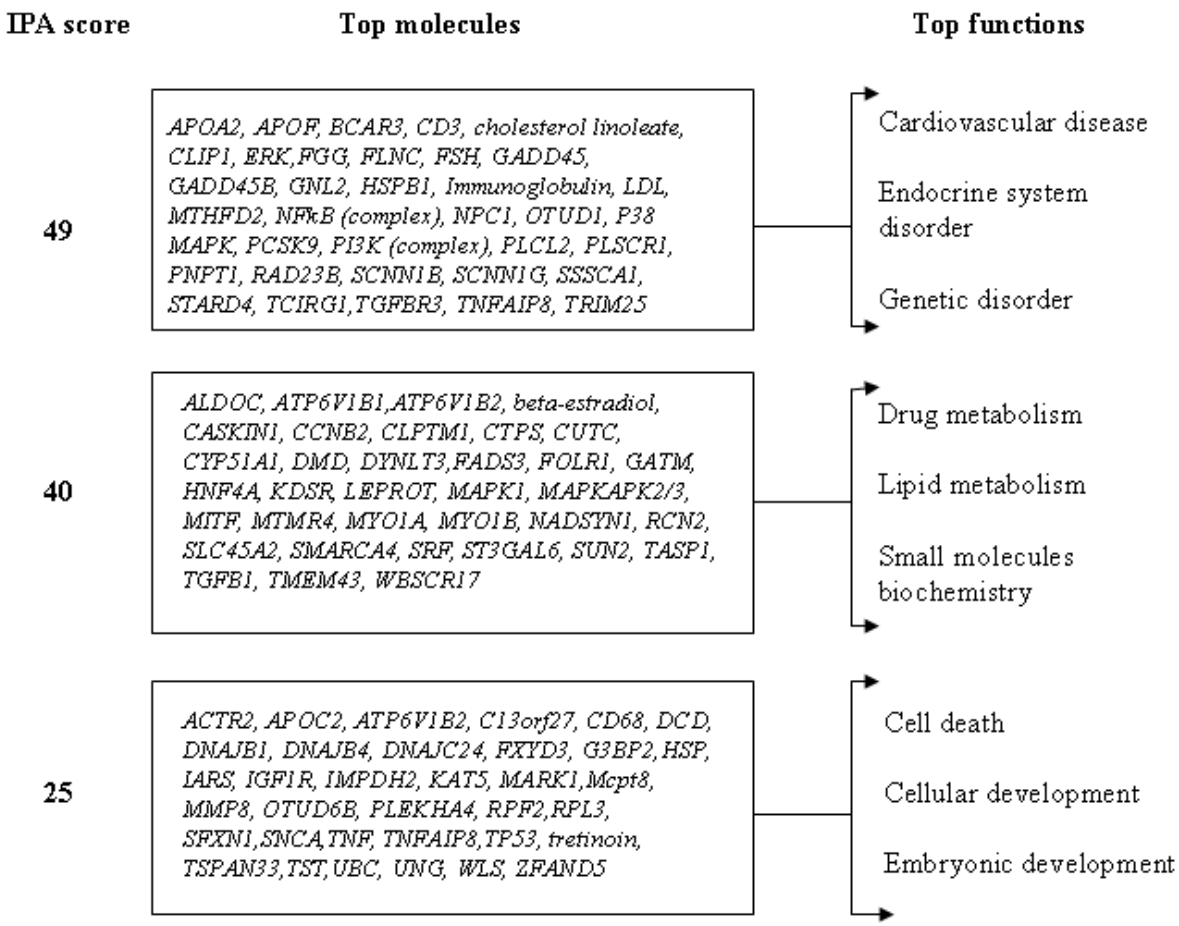


Figure 16. Top scored networks using IPA and not reprogrammed transcriptome of SCNT elongated embryos. The IPA score = (-log (p-value)), is associated with the significance of the selected gene network. The higher the score the more reliable it is.

4.11 Trophoblast elongation size dependent expression profile of selected genes in SCNT and IVP day 16 embryos

Even though the array analysis was performed using elongated embryos from SCNT, IVP and AI sources with comparable length, we were interested to check the expression of candidate genes in some of day 16 embryos with impeded growth from IVP and SCNT pregnancies. For this, we used disproportionally short size IVP (1-3 mm, n=3) and SCNT (1.25-5 mm, n=3) embryos, to compare the expression of candidate genes with their elongated counterparts. Candidate genes were selected based on their known function in relation to trophoblast development (FGFR2, TKDP2 and IFNtau) and their role in tight junction signaling pathway (CLDN1, CEBPA, ARHGEF2 and JAM2) which are hypothesized to be affected by pre-elongation culture manipulation of both IVP and SCNT embryos. Even though the microarray

data did not show significant differences in expression of IFNt, between the three groups, this gene was included as main player in maternal recognition of pregnancy and trophoblast elongation.

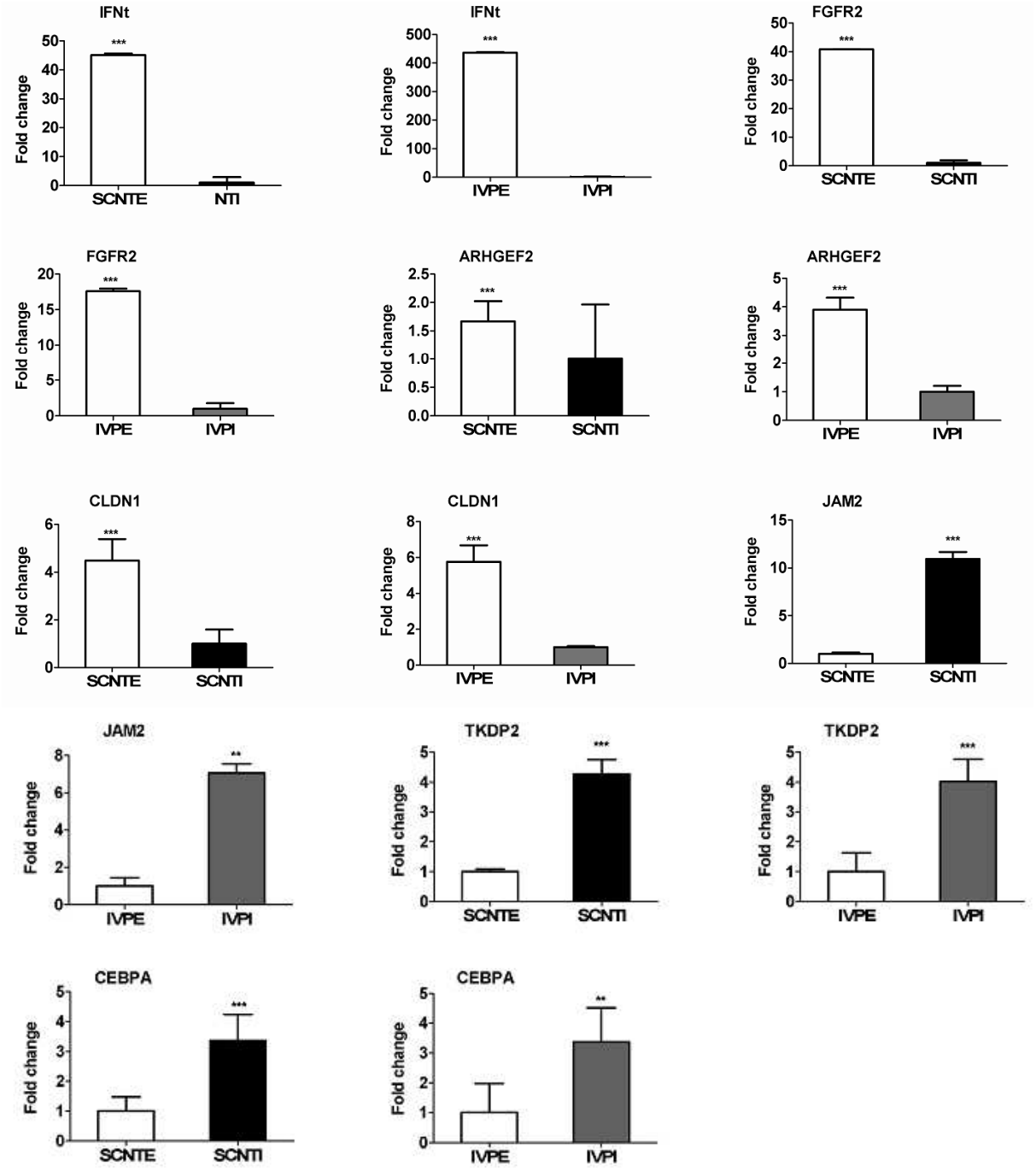


Figure 17. Quantitative real time PCR (qPCR) analysis of selected genes. Gene expression fold-change between SCNT-I (somatic cell nuclear transfer-impeded) embryos vs. SCNT-E (nuclear transfer-elongated) embryos, and gene expression fold-change of IVP-I (in vitro

produced-impeded) embryos vs. IVP-E (in vitro produced-elongated) embryos were compared. Stars above the bar denote if that gene showed significant differential expression by Student's t-test. **, -P< 0.01 and ***, -P < 0.001.

Quantitative real time PCR results demonstrated that transcript abundance of IFNt, FGFR2, CLDN1 and ARHGEF2 was significantly lower in both IVP and SCNT impeded embryos compared to their respective elongated counterparts. On the other hand, high mRNA abundance of TKDP2, JAM2, and CEBPA were noticed in IVP and SCNT impeded embryos compared to the elongated embryos (Figure 17).

In addition, the qPCR based gene expression comparison of all selected genes except IFNt showed no significant differences between IVP and SCNT impeded embryos (Figure 18).

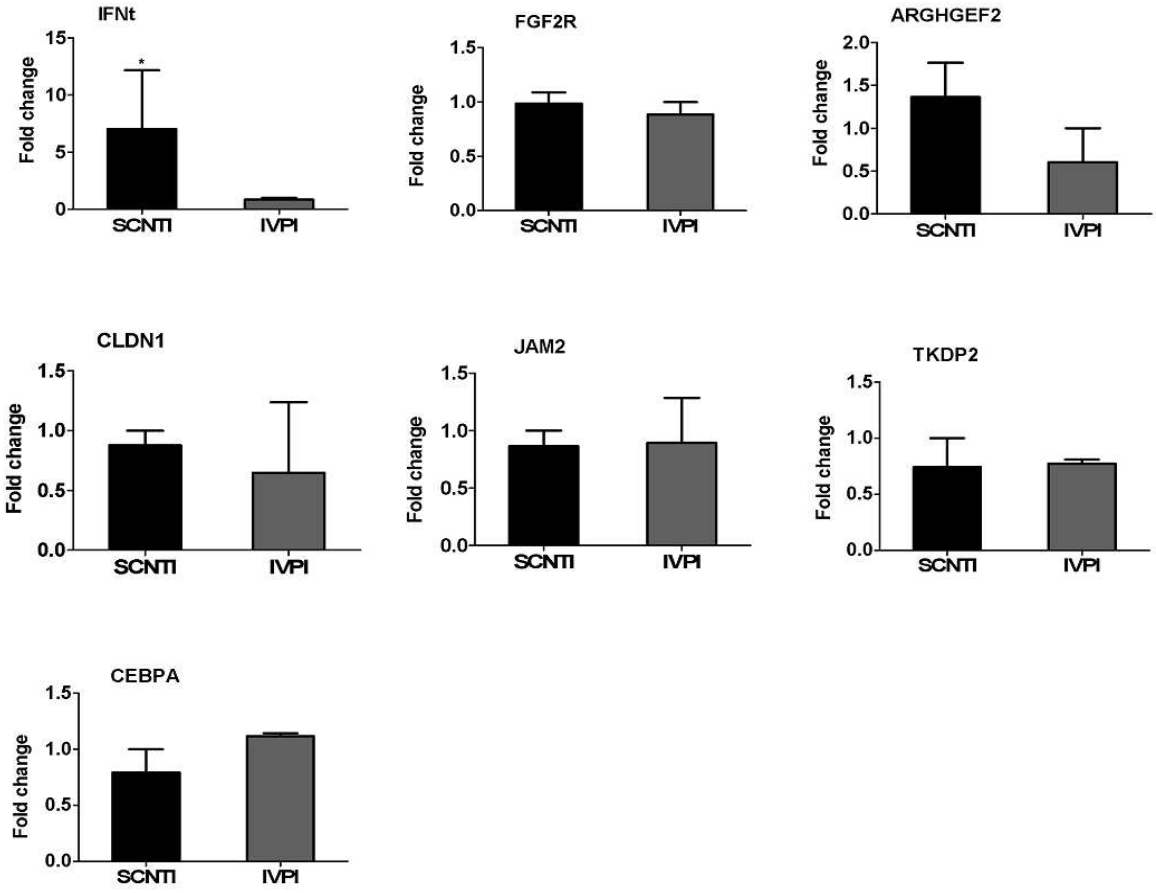


Figure 18. Quantitative real time PCR (qPCR) analysis of selected genes expression in IVP-I (in vitro produced-impeded: 1-3 mm) and SCNT-I (somatic cell nuclear transfer-impeded 1.25-5 mm) embryos. Gene expression fold-change between IVP-I and SCNT-I embryos were compared. Stars above the bar denote if that gene showed significant differential expression by Student's t-test. *,-P < 0.05.

4.12 Validation of microarray data

A total of 18 candidate transcripts were used for quantitative real time PCR analysis to validate the array results. Results revealed that all genes except ADD3 and CCND2 showed similar expression pattern with the microarray data.

Table 7. Confirmation of microarray data using qPCR

Gene name	Accession number	Comparison	Microarray		qPCR	
			FC	P	FC	P
SPINK4	NM_001114522	NT Vs. AI	-3.78	0.000000033	-104.04	0.002
TSPAN1	NM_001034270	IVP Vs. AI	-4.75	0.0000155	-235.1	0.01
TSPO	NM_175776	IVP Vs. AI	-3.05	0.0000008	-34.2	0.04
TDGF1	NM_001080358	IVP Vs. AI	2.61	0.000000065	11.70	0.04
SULT1A1	NM_177521	IVP Vs. AI	-2.15	0.00000116	-32.32	0.04
GPX1	NM_174076	NT Vs. AI	2.79	0.00000106	202.42	0.0004
ALAS2	NM_001035103	NT Vs. AI	-4.22	0.000000662	-153	0.02
ALAS2	NM_001035103	IVP Vs. AI	-4.18	0.000000735	-68.95	0.001
ARHGDIB	NM_175797	IVP Vs. AI	-2.22	0.00014	-9.39	0.04
MRPL12	NM_201606	NT Vs. AI	1.35	0.0000256	1.48	0.02
CADM1	NM_001038558	NT Vs. AI	-3.40	0.00000577	-37.75	0.02
CCND2	NM_001076372	IVP Vs. AI	3.07	0.0000000012	-5.42	0.05*
CCND2	NM_001076372	NT Vs. AI	3.75	0.0000000001	6.09	0.008
TKDP2	NM_001012683	IVP Vs. AI	-7.68	0.0000000005	-534.06	0.001
TKDP2	NM_001012683	NT Vs. AI	-7.47	0.0000000006	-535.28	0.001
ADD3	NM_001075194	NT Vs. AI	-2.66	0.000000006	0.94	0.27*
ADD3	NM_001075194	NT Vs. IVP	-2.69	0.0000000061	72.3	0.0004
FGF2R	NM_001205310.1	NT vs. AI	-2.02	0.0000067	-32.07	0.006
CLDN1	NM_001001854.2	NT vs. AI	-2.918	0.0000000003	-15.49	0.009
JAM2	NM_001083736.1	NT vs. AI	1.459	0.00035	2.02	0.008
CEBPA	BC149006.1	IVP vs. AI	-2.49	0.00035	6.02	0.016
ARHGEF2	NM_001098881.2	IVP vs. AI	-1.80	0.00001	-295.75	0.0003

FC, fold change, $p \leq 0.05$ is considered as significant, negative and positive values indicate up and down regulated genes respectively, '*' above p-value indicate genes whose expression are not validated by qPCR .

5. Discussion

Cloning and IVF technologies are important tools for production and manipulation of bovine pre-implanation embryos independent of the maternal environment. However, these technologies are limited by great inefficiency. The prominent aberration in IVP or SCNT derived embryos is that in vitro production systems and cloning technology, may lead to persistent alterations of gene expression patterns during embryonic and fetal development (Wrenzycki et al. 2004). In order to get a global overview of gene expression alteration induced by in vitro environment and somatic cell nuclear transfer, here we performed large scale gene expression analysis of day 16 conceptuses from SCNT, IVP and AI pregnancies as well as donor fibroblast cells.

Results revealed that the majority of the differentially expressed genes found in IVP (n= 258) and SCNT (n= 315) embryos were down regulated compared to AI. This alteration in gene expression may be originating from all steps of manipulation procedures including in vitro maturation, in vitro fertilization, and in vitro culture as well as failure in genome reprogramming of donor cell after SCNT. Exposure of embryos to in vitro culture is believed to affect both IVP and SCNT embryos while transcriptional reprogramming error is specific to SCNT embryos. To understand the effect of each component (culture environment and genome reprogramming), differentially expressed genes were categorized in two groups. The first category includes genes commonly differentially expressed between IVP and SCNT as both compared to their AI counterparts, are considered to be affected by pre-transfer in vitro culture environment. The second category includes DE transcripts unique to SCNT vs. AI elongated embryos comparison, which were subsequently examined for their transcriptional reprogramming status based on their expression in donor fibroblast cell.

5.1 Common differentially expressed transcripts found between IVP and SCNT elongated embryos revealed the effect of pre-elongation culture condition

In the last decade several transcriptome studies employing different array platforms were conducted in the blastocyst of various sources. Most of the comparisons which involved cloned embryos along with the respective donor cell used IVP as a control (Beyhan et al. 2007, de et al. 2005, Pfister-Genskow et al. 2005, Rodriguez-Osorio et al. 2009, Somers et al. 2006). However, both the IVP and SCNT embryos are derived from procedures that involve in vitro culture environment, which can significantly alter the transcriptome of the resulting

embryos. Identification of those genes may help to understand molecular mechanisms sensitive to the culture environment. Thus the present study identified 274 genes which were found to be commonly differentially expressed in IVP and SCNT compared to AI and those genes are believed to be sensitive to in vitro culture condition.

Several genes known to be associated with normal embryo and placenta development are found to be dysregulated in both IVP and SCNT elongated embryos. Among these, HAND1 is expressed in trophoblast lineage and plays an important role in trophoblast development (Arnold et al. 2006a). Moreover, gap junction protein 2 GJB2 (Cx26), which is known to be required for transplacental glucose uptake (Gabriel et al. 1998) and transcript which contribute to primitive endoderm differentiation and to the growth and maintenance of the inner cell mass (ICM) like fibroblast growth factor receptor 2 (FGFR2) are down regulated in both IVP and SCNT elongated embryos. Receptors for signalling molecules such as FGFR2 enable the embryo to communicate with the surrounding environment and activate downstream pathways (Vesterlund et al. 2011). Results from FGFR2 knock out experiments showed that homozygous embryos died a few hours after implantation evidencing an important role of FGFR2 during post-implantation development (Arman et al. 1998). Among subtypes of FGFRs, FGFR2b was suggested to play a role in regulation of IFNt expression because of its high expression in day 14 and 17 bovine conceptuses coincident with peak IFNt expression (Cooke et al. 2009). However, such correlation of FGFR2 with IFNt expression was not evident in our expression study. The non differential expression of IFNt between the three embryo groups in our study is in agreement with the report of Arnold et al. (2006a), in which no differential expression IFNt was found between AI, IVP and SCNT day 17 embryos. Trophoblast kunitz domain protein 2 (TKDP2) is down regulated in both IVP and SCNT groups. Similar to IFNt, trophoblast kunitz domain proteins are secreted by placenta in transient fashion, with maximal expression occurring during the time of apposition and adhesion of the trophoblast to the uterine luminal epithelium, a stage of pregnancy at which the mother shows uterine response to the presence of the adhering conceptus (MacLean et al. 2003).

The imprinted gene ASCL2 also known as MASH2, was found to be up regulated in both IVP and SCNT elongated embryos. MASH2 shows biallelic expression prior to implantation and maternal expression after implantation (Arnold et al. 2006b). Similar to our results, Arnold et al (2006a) showed an increased in relative amount of ASCL2 mRNA in day 17 bovine embryos produced by IVF or somatic cell nuclear transfer when compared with AI counterparts. Both in vitro culture and embryo transfer procedures affected genomic

imprinting in the extra embryonic tissues (Rivera et al. 2008). In mouse, ASCL2 could be one of the examples of this notion as it was expressed at a higher level in the placenta of the manipulated conceptuse than in the placenta of control conceptus (Rivera et al. 2008).

5.2 Common and distinct pathways affected in IVP and SCNT elongated embryos may show the inherent differences between the two embryo groups

Early embryo culture conditions likely affect the same pathways and gene networks in both SCNT and IVP conceptuses (Chavatte-Palmer et al. 2012). As shown in Table 4 and Figure 8, tight junction signalling pathways and the genes associated with this pathway and top gene networks which have impact on small molecules biochemistry, carbohydrate metabolism and DNA replication recombination and repair were commonly affected in both IVP and SCNT elongated embryos. The role of tight and adherens junctions in ovine endometrial luminal for blastocyst elongation and adherence of the trophectoderm during implantation were suggested by Satterfield et al. (2007). In the present study we identified 9 transcripts involved in tight junction signaling pathway (CLDN1, ARHGEF2, CEBPA, F2Rl2, FOS, JAM2, PRKAG2, TGFB3 and TNFRSF1A) and all genes except JAM2 were found to be down regulated in both IVP and SCNT embryos (Table 6). Based on these results it can be speculated that the development of both IVP and SCNT embryos was affected in a comparable manner during the elongation window. However, it can be suggested that both manipulated conceptuses may have different mechanism of compensation against the deleterious effect of pre-elongation culture.

Cloned embryos are more sensitive to culture environment as compared to IVF embryos (Yamanaka et al. 2009) as early as the one-cell stage (Chung et al. 2002). Cloned embryos also have higher energy demands than control embryos, a characteristic that may arise from aberrant expression of ATP-requiring processes expressed in the somatic donor cell type (Han et al. 2008). The results of the present study showed large number of genes to be differentially expressed in SCNT embryos which are known to be involved in 9 metabolic related pathways, as compared to the only 2 metabolic pathways appeared in IVP embryos (Table 4 and 5).

Similarly, lipid and carbohydrate metabolism, DNA replication, recombination and repair and small molecules biochemistry were among the functions altered in SCNT elongated embryos as compared to the functional disturbance of cell and organ development, cellular movement and cellular growth and proliferation in IVP embryos (Figure 11 and 12). These results

necessitate further optimization of in vitro culture environment for successful development of cloned embryos to generate healthy offspring after transfer to recipients.

5.3 Transcriptional reprogramming status of genes may compromise their functional contribution

Depending on their expression pattern between embryos derived from SCNT and AI as well as FB donor cell, transcriptional reprogramming statuses of genes were determined. Surprisingly, 73.9% of gene expression difference was observed between SCNT embryos and FB. Similar studies at the blastocyst stage reported 82.4% difference in gene expression of SCNT compared to donor fibroblast cell (Smith et al. 2005). This suggests the dynamism of the reprogramming process during the pre-and peri-implantation development period. Transcriptome analysis showed exclusive differential expression of 193 genes between SCNT and AI embryos, of which 71 transcripts were classified as transcriptionally not reprogrammed (Figure 14A). Some of these transcriptionally not reprogrammed genes identified in our study are known to be involved in maternal-fetal nutrient exchange, trophoblast elongation and embryogenesis. The folate receptor 1 (FOLR1) transcript which was down regulated in SCNT embryos is known to be involved in maternal–fetal folate transport. Deletion of FOLR1 in mice caused neural tube defects and death in utero at day 10 of gestation (Piedrahita et al. 1999). Likewise, epithelial sodium channel (ENaC) also called sodium channel, non-voltage-gated 1 participated in the maintenance of sodium transport and ionic homeostasis for both mother and fetus (del Monaco et al. 2008). This gene encodes three sub units (SCNN1 alpha, beta and gamma) (Meisler et al. 1994). Two sub units of this gene TSCNN1B and SCNN1G are found to be down regulated in SCNT embryos. Moreover, transcript cytochrome P450, family 51, subfamily A, polypeptide 1 (CYP51A1), which was down regulated in SCNT embryos in the present study is one of the critical gene at the initiation of bovine embryo elongation (Clemente et al. 2011).

Further inspection of genes in this category showed that down regulation of wntless homolog drosophila WLS gene. By using the mouse orthologue (GPR177), recently the function of this gene was investigated in mammalian tissues (Wang et al. 2012b). The result indicated that the expression of GPR177 mRNA in various tissues including cancer cells. Other studies demonstrated that mouse embryos with deficient GPR177 exhibit defects in establishment of the body axis, and homozygous (GPR177-/-) died during early embryogenesis (Fu et al. 2009, Fu et al. 2011), implicating the the role of wntless in embryo development.

Genes are assumed to be partially reprogrammed, when their transcript abundance is between normal fertilized and cloned embryos (Rodriguez-Osorio et al. 2009). A set of 31 transcripts was found to be partially reprogrammed as their expression pattern was found to be intermediate between donor fibroblast cell and AI (Figure 14B). For instance, a transcript of myosin light chain kinase (MYLK) was found to be down regulated in donor cells as compared to cloned embryos and remained relatively higher in cloned embryos as compared to AI. Such kind of partial reprogramming reflects inadequate inhibition of transcription factors associated with the somatic cell phenotype (Wilmut et al. 2011). One of the two forms of MYLK is embryonic in which its expression declines at birth to low or undetectable levels in most adult tissues. The second form smooth muscle MYLK, a predominant form found in adult tissues (Gallagher et al. 1995). Therefore, the lower expression of this gene in our donor cells was to be expected. However, the higher expression of this gene in cloned as compared to AI embryos may show the prevailing aberrant transcriptional reprogramming process. Additionally the mitochondrial enzyme serine hydroxymethyltransferase 2 (SHMT2) was found to be partially reprogrammed and showed up regulation in SCNT elongated embryos. On the other hand, genes such as RYBP required for early mouse embryo (Pirity et al. 2005) and retinal and lens development (Pirity et al. 2007), the metabolic related gene SLC2A5 and the placenta development and proliferation related gene TKDP4 were among the genes which were down regulated in SCNT embryos.

Inspection of the list of incompletely reprogrammed genes showed several genes associated with metabolism to be affected. This was further supported by IPA analysis which identified the consequence of such transcript alteration in glycerolipid metabolism, ascorbate metabolism, arginine and proline metabolism and methane metabolism (Figure 15). In vitro stress factor is proposed to include metabolic and substrate deficiencies (Leese 2002). The evidence that in vitro culture environment affects embryo metabolism comes from experiments showing increased glucose uptake in mouse cloned blastocyst cultured in glucose supplemented media (Chung et al. 2002, Han et al. 2008). This characteristic greatly distinguishes SCNT embryos from normal fertilized embryos and indicates a failure of complete early reprogramming of gene expression. Consistent with this observation, the data of the present study showed exclusive disturbance of metabolic pathways in SCNT embryos suggesting the need of possible intervention in improving culture environment that could suppot cloned embryos' pre-and post-hatching development.

Apart from its effect on embryo metabolism, in vitro environment is known to cause oxidative stress (Gad et al. 2012, Nasr-Esfahani and Johnson 1991). In human early pregnancy, an

increase in oxygen tension is associated with increased in mRNA of the antioxidant enzymes such as CAT within placental tissues (Myatt and Cui 2004). Detoxification enzymes, such as CAT have also been reported in human placental tissues of early pregnancy failure, suggesting that such an antioxidative mechanism might be developed and operate against possible oxidative damage in patients with susceptibility for miscarriage (Biri et al. 2006). In the current study heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) HSPA5 expression was higher in NT embryos accompanied by increased level of antioxidant enzyme catalase gene (CAT). Such response operated only in cloned embryos as compared to fertilized conceptuses suggesting possible defence response of CAT protein, probably because of abnormal placental function (Al-Gubory et al. 2010).

In addition, metallopeptidase inhibitor (TIMP2) protein was found to be aberrantly expressed in bovine and mouse cloned placentas (Kim et al. 2005, Kim et al. 2010). Recently, Ulbrich et al. (2011) indicated TIMP2 contribution in maternal recognition of pregnancy. Similarly, glycerol-3-phosphate dehydrogenase 1 (GPD1L) which was reported to be aberrantly reprogrammed in pig blastocyst (Whitworth et al. 2011) was found to be incompletely reprogrammed. The imprinted gene CDKN1C is among the set of few genes identified by Hori et al. (2010) aberrantly imprinted and suggested to be cause of large offspring syndrome (LOS) in both NT and IVF derived calves. CDKN1 was not differentially expressed in IVP embryos in the present comparison showed the possible effect of reprogramming error on the alteration of expression of this gene in cloned embryos. However, the different SCNT procedures used, the variation in stage of gestation at sample collection and the different micro/macroarrays platforms used, make it difficult to identify sets of genes that are consistently affected in SCNT pregnancies (Chavatte-Palmer et al. 2012).

5.4 Trophoblast elongation size dependent expression of candidates in IVP and SCNT elongated embryos

The process of elongation is required for conceptus survival in all ungulate species (Blomberg et al. 2008) and it is a prerequisite for successful implantation (Wilson et al. 2000). Studies probed SCNT embryos recapitulate elongation showed developmental retardation in SCNT embryos as compared to their equivalent in vivo or in vitro fertilized embryos (Alexopoulos et al. 2008, Rodriguez-Alvarez et al. 2010b). Similarly in the present study pronounced reduction in trophoblast size in day 16 SCNT embryos (93.3 mm) was observed as compared to IVP (186.6 mm) and AI (193.3 mm) embryos (Table 2). Though it is difficult to identify

putative genes associated with trophoblat size variation in different pregnancy groups in our study, we have investigated the expression of candidate genes in disproportionally short day 16 embryos from IVP and SCNT groups, to find out any association of the transcript abundance of those genes with the size of embryos.

Accordingly the expression of IFNt, FGFR2, CLDN1 and ARHGEF2 was significantly low in developmentally delayed IVP and SCNT embryos as compared their elongated counterparts (Figure 17). Higher expression of IFNt in the enlarged size trophoblast in the present study was in agreement with the previous observation in which expression of IFNt per cell was increased as the blastocyst enlarges and elongates (Farin et al. 1990, Kimura et al. 2004). This suggests that the lower expression of IFNt in disproportionally short embryos may result from retarded trophoblast development. Besides, the lower abundance of FGFR2, CLDN1 and ARHGEF2 in developmentally lagging embryos might indicate the importance of these genes during embryo elongation. However, further studies need to be done to determine their exact role in trophoblast development. Surprisingly higher expression of trophoblast kunitz domain protein (TKDP2) was noticed in both IVP and SCNT impeded embryos as compared to their respective elongated counterparts. Despite higher expression of TKDP family genes during bovine and ovine peri-implantation period (MacLean et al. 2003), the exact role of individual TKDPs has not yet been determined. Like IFNt, TKDPs are products of trophoblast mononuclear cells (Blomberg et al. 2008). Our result showed that unlike IFNt, the expression of TKDP-2 may not depend on trophoblast size.

Interestingly, junctional adhesion molecule 2 (JAM2) transcripts abundance showed an inverse relationship with trophoblast elongation size and was higher in both IVP and SCNT lagging conceptuses. The amount of JAM2 was initially higher in IVP and SCNT embryos as compared to AI (Figure 9). It can be suggested that an appropriate level of JAM2 mRNA might be important for better trophoblast elongation. Similarly, CCAAT/enhancer binding protein alpha (C/EBP) expression in developmentally delayed IVP and SCNT trophoblasts was significantly higher as compared to their respective elongated embryos. Experiments done in mice have suggested a crucial role for C/EBP-α and -β in embryogenesis, deletion of both genes resulted in mortality around embryonic day 10–11 due to gross failure in placental development (Begay et al. 2004). The same author showed that a single copy of either C/EBP-α in the absence of C/EBP-β, or C/EBP-β in the absence of C/EBP α could rescue embryogenesis. Furthermore, the mRNA levels of all selected genes except IFNt showed no variation in expression between IVP and SCNT impeded embryos (Figure 18), strengthening

our hypothesis that these common differentially expressed transcripts could be sensitive to pre-embryo transfer in vitro procedures.

6. Summary

In vitro produced embryos by either IVP or SCNT have been successfully used in many species to produce live offspring despite low efficency. The low efficiency of these technologies is ascribed to the profounding factors related to reprogramming of the donor genome and in vitro culture environment, which subsequently renders deviation in molecules and phenotypes as compared to non-manipulated control embryos. Although the specific effect of each component on transcriptome alteration is largely undefined, the use of various embryo groups' comparison at any stage of embryo or fetal development may offer insight on the key molecules and pathways altered. The aim of this study was to uncover the molecular changes in conceptuses derived from SCNT, IVP and AI pregnancies at the time of maternal recognition of pregnancy. For this, day 7 blastocysts derived from SCNT, IVP and AI were transfered to oestrus synchronized recipients. Conceptuses were then recovered from slaughtered cows at day 16. Despite similar embryo recovery rates among various groups of pregnancies, morphological analysis of conceptuses at this stage showed that 37.5% of SCNT and 25% IVP embryos were underdeveloped. In addition, those SCNT and IVP conceptuses that recapitulated filamentous morphology had shorter trophoblast size as compared to the AI counterparts. In order to gain a comprehensive overview of the transcriptome changes, RNA extracted from three filamentous embryos from each pregnancy group were subjected to global gene expression analysis by using GeneChip® Bovine Genome Array. Gene expression analysis revealed a large number of differentially expressed genes in SCNT vs. AI comparison (477) followed by IVP vs. AI (365) and SCNT vs. IVP (26). Interestingly, more than 50% of transcripts from each comparison were down-regulated. Thus, the affected mRNAs span a variety of functional categories, most notably but not limited to metabolism and tight junction signalling pathways in SCNT and IVP conceptuses, respectively. To investigate the SCNT and IVP induced gene alteration on elongated embryos, unique and common differentially expressed transcripts were taken into further analysis. Common differentially expressed transcripts were involved in tight junction signaling pathways while genes exclusively differentially expressed in SCNT elongated embryos were scrutinized for their reprogramming status. If reprogramming is highly efficient, most or all somatic genes are silenced and the appropriate array of embryonic genes are programmed for expression, then transcriptional activation should result in cloned embryos with characteristics much like those of normal fertilized embryos. Based on this scenario, various research groups have reported the occurrence of global reprogramming at the bovine blastocyst stage. The question

is if reprogramming occurs in bovine somatic cell cloning, why would only a very small proportion of embryos be enabled to develop to term and survive? The present study hypothesis was there must be multiple stages of reprogramming process which could be addressed through different approaches. The current study opted to use transcriptomic approach to elucidate the global transcriptional reprogramming status of SCNT embryos at the critical stage of maternal recognition of pregnancy. Thus, our results show various transcriptional reprogramming statuses of differentially expressed genes namely transcriptional not reprogrammed (FOLR1, GATM, APOA2, SCNN1G, SCNN1, TKDP5, ALDOC and WLS), incompletely reprogrammed (ACTO4, ACTO8, ALDH9A1, CAT, CDKN1, TIMP2 and GAR) and partially reprogrammed (MYLK, TKDP4, RYBP, AHSG and TYRO3). Furthermore, to identify the association of putative genes with embryo elongation size, qPCR based quantification of candidate genes (IFNt, IGF2R, ARHGEF2, CLDN1, JAM2, TKDP2 and CEBPA) in disproportionally short size SCNT and IVP embryos was performed. Accordingly, IFNt, FGFR2, CLDN1 and ARHGEF2 were significantly lower in developmentally delayed IVP and SCNT embryos as compared their elongated counterparts. On the other hand, the expression of TKDP2, JAM2, and CEBP-α showed inverse relationships with trophoblast size. The mRNA abundance of these genes was higher in developmentally lagging embryos as compared to their elongated counterparts. The result implicates that various molecules might be involved during the elongation process and that the transcript abundance of these genes might be a response or cause of trophoblast size variation during embryo elongation. In general, the current gene expression profiling of SCNT, IVP and AI elongated conceptuses presented a list of candidate genes which can be utilized in procedures that involve in vitro culture environment as well as reprogramming cell fate experiments. The remaining challenge is to understand the functional contribution of these genes to facilitate the possible technical or molecular intervention to enhance the production of healthy and fertile offspring from these techniques. In this regard, the present study contributed detailed information about altered molecular signature and pathways in SCNT and IVP conceptus during the peri-implantation period which may contribute to a better understanding of IVP and SCNT induced changes in embryos or fetuses obtained from such procedures.

7. Zusammenfassung

In vitro produzierte Embryonen durch IVP oder SCNT werden erfolgreich, trotz geringer Effizienzen, in unterschiedlichen Spezies eingesetzt um lebende Nachkommen zu erzeugen. Für die geringen Effizienzen dieser Technologien können viele tiefgründigere Faktoren verantwortlich sein. Diese Faktoren stehen im Zusammenhang mit der Reprogrammierung des Donor Genoms sowie mit dem in vitro Kulturmilieu und rufen anschließend Abweichungen in Molekülen und Phenotypen im Vergleich zu nicht manipulierten Kontroll-Embryonen hervor. Obwohl der spezifische Effekt jedes einzelnen Faktors auf die Veränderungen des Genoms weitgehend ungeklärt ist, bietet der Einsatz von unterschiedlichen Embryogruppen im Vergleich zwischen verschiedenen Entwicklungsstadien des Embryos oder des Fetus einen Einblick in Veränderungen von wichtigen Schlüsselmolekülen und Signalwegen. Das Ziel dieser Studie waren die molekularen Veränderungen im Embryo hergeleitet aus SCNT, IVP und AI Trächtigkeiten zum Zeitpunkt der ersten embryomaternalen Kommunikation aufzudecken. Hierzu wurden durch SCNT, IVP und AI Tag 7 erzeugte Blastozysten in Östrus synchronisierte Kühe übertragen. Am Tag 16 der Trächtigkeit wurden die Rezipienten geschlachtet und die Embryonen entnommen. Trotz ähnlicher Recovery Rate der Embryonen aus verschiedenen Trächtigkeitsgruppen wurden nach morphologischen Untersuchungen 37.5% der SCNT und 25% der IVP Embryonen als unterentwickelt eingestuft. Darüber hinaus zeigten SCNT und IVP Embryonen mit einer verzögerten rekapitulierten filamentösen Morphologie kleinere Trophoblasten im Vergleich zu AI erzeugten Embryonen. Um einen umfassenden Überblick über die Tanskriptomveränderungen zu erlangen, wurden drei filamentöse Embryonen aus jeder Trächtigkeitsgruppe für eine globale Genexpressionsanalyse mittels bovine GeneChip® Genome Array ausgewählt. Die Auswertung der Transkriptomanalyse zeigte viele unterschiedlich expremierte Gene bei SCNT vs. AI (477), gefolgt von IVP vs. AI (365) und SCNT vs. IVP (26). Interessanterweise waren mehr als 50% der Transkripte aus jedem Vergleich herunterreguliert. Folglich umfassen die betroffenen mRNAs eine Vielzahl von funktionellen Gruppen. SCNT und IVP Embryonen zeigten neben vielen anderen funktionellen Gruppen sich besonders in Stoffwechsel und Tight-Junction Signalwegen. Um herauszufinden ob SCNT und IVP eine Genexpressionsveränderung auf elongierte Embryonen induziert, wurden einzigartige oder häufig unterschiedlich exprimierte Gene für weitere Analysen verwendet. Häufig unterschiedlich exprimierte Transkripte die in Tight-Junction Signaling Wegen involviert waren, zeigten ausschließlich in SCNT elongierten

Embryonen eine unterschiedliche Expression. Aufgrund dieses Resultates wurden SCNT auf ihren Reprogrammierungsstatus überprüft. Wenn die Reprogrammierung sehr effizient ist, sind die meisten oder alle somatischen Gene abgeschaltet und die entsprechenden Bereiche der embryonalen Gene sind auf Expression programmiert. Demnach sollte sich die transkritptionelle Aktivierung der geklonten Embryonen charakteristisch der Transkription von normal fertilisierten Embryonen ähneln. Basierend auf dieser Gegebenheit haben bereits verschiedene Forschungsgruppen über Vorkommen von globaler Reprogrammierung in bovinen Blotozysten berichtet. Die Frage ist: Wenn Reprogrammierung bei Rindern aus somatischer Klonierung auftritt, warum ist dann nur ein sehr kleiner Anteil der Embryonen fähig sich für eine gewisse Zeit zu entwickeln und zu überleben? Die Hypothese der vorliegenden Studie war, dass mehrere Stufen der Reprogrammierung durch unterschiedliche Ansätze angesprochen werden könnten. In der vorliegenden Studie wurde der genomische Ansatz ausgewählt, um den Status der globalen transkriptionellen Reprogrammierung in SCNT erzeugten Embryonen in der kritischen Phase der ersten embryomaternalen Kommunikation aufzuklären. Das Ergebnis zeigte verschiedene transkriptionelle Reprogrammierungzustände von differentiell exprimierten Transkripten, nicht reprogrammierte (FOLR1, GATM, APOA2, SCNN1G, SCNN1, TKDP5, Aldoc und WLS) unvollständig programmierte (ACTO4, ACTO8, ALDH9A1, CAT, CDKN1, TIMP2 und GAR) und teilweise neu programmierte (MYLK, TKDP4, RYBP, AHSG und TYRO3). Darüber hinaus, um eine Assoziation von putativen Gene mit der Embryoelongationsgröße zu identifizieren, wurden qPCR Quantifizierungen von Kandidatengenen (IFNt, IGF2R, ARHGEF2, CLDN1, JAM2, TKDP2 und CEBPA) in entwicklungsverzögerten SCNT und IVP Embryonen durchgeführt. Dementsprechend waren IFNt, FGFR2, CLDN1 und ARHGEF2 signifikant niedriger expremiert in den entwicklungsverzögerten IVP und SCNT Embryonen gegenüber ihren elongierten Gegenspielern. Auf der anderen Seite zeigte die Expression von TKDP2, JAM2 und CEBP-α eine inverse Beziehung zur Trophoblstengröße. Die mRNA Expression dieser Gene war in entwicklungsverzögerten Embryonen höher im Vergleich zu ihren elongierten Gegenstücken. Das Ergebnis impliziert, dass verschiedene Moleküle in dem Prozess der Elongation involviert sein könnten und die Expression dieser Genen könnte eine Antwort oder Ursache für die Größenvariation der Trophobasten während der embryonalen Elongation sein. Im Allgemeinen hat die aktuelle Genexpressionsanalyse von SCNT, IVP und AI elongierten Embryonen eine Liste von Kandidatengene erbracht, welche für Verfahren die sowohl mit in-vitro Kulturbedingungen als auch Reprogrammierungen bei Zellkulturexperimenten verwendet werden können. Die

verbleibende Herausforderung ist es den funktionellen Beitrag dieser Gene zu verstehen, um mögliche technische oder molekulare Eingriffe zu erleichtern und dadurch die Produktion von gesunden und fruchtbaren Nachkommen aus dieser Techniken zu verbessern. In diesem Zusammenhang trug die vorliegende Studie dazu bei detaillierte Informationen über veränderte molekulare Signaturen und Signalwege in SCNT und IVP Embryonen während der peri-Implantationsperiode zusammeln, die zu einem besseren Verständnis der IVP und SCNT induzierten Veränderungen in Embryonen oder Feten aus solchen Verfahren beitragen können.

8. Conclusion

In conclusion, the common deviation in development, transcripts and molecular pathways both in SCNT and IVP consuptuses as compared to their AI conterparts might indicate the influence of the common pre-elongation culture environment. In addition, the distinct alteration in gene expression and pathways related to metabolism in SCNT embryos in this window demonstrated the need for optimization of in vitro culture condition to support better development of cloned embryos. Moreover, the slight enhancement in transcriptome similarity between cloned and donor fibroblast cells in our study as compared to the difference observed at the blastocyst stage (Smith et al. 2005) showed the dynamism of reprogramming process during pre-and peri-implantation period. Our results collectively suggested that transcriptome analysis of different development stages of cloned and in vitro fertilized embryos may yield understanding of the effect in vitro environment, dynamics of genes regulation and transcriptional reprogramming. Thus, subsequently facilitates possible interventions to enhance the efficiency of healthy offspring production.

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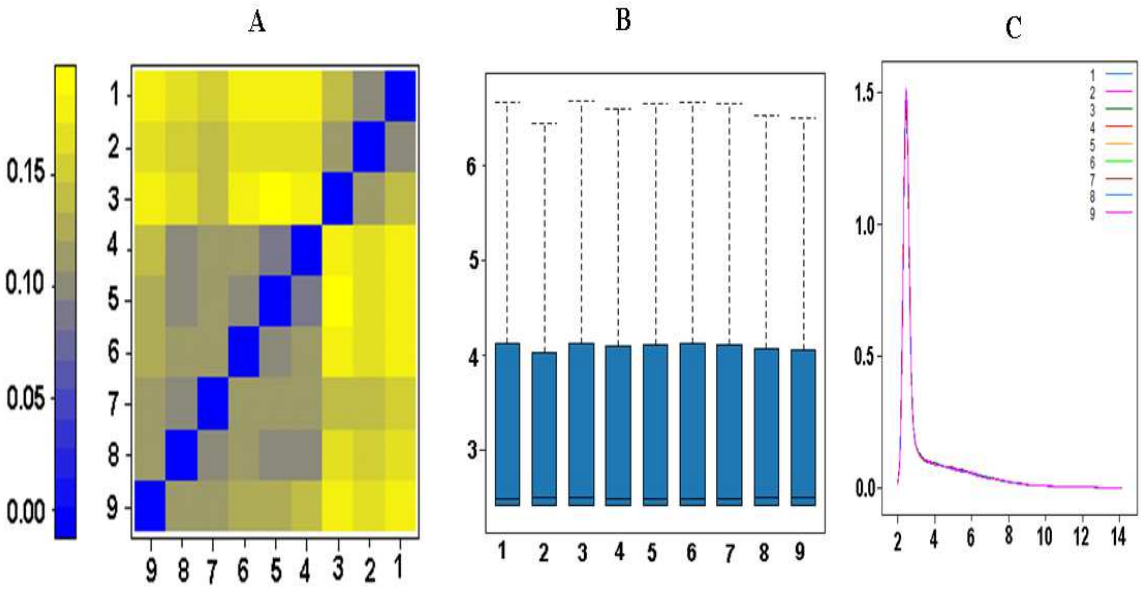
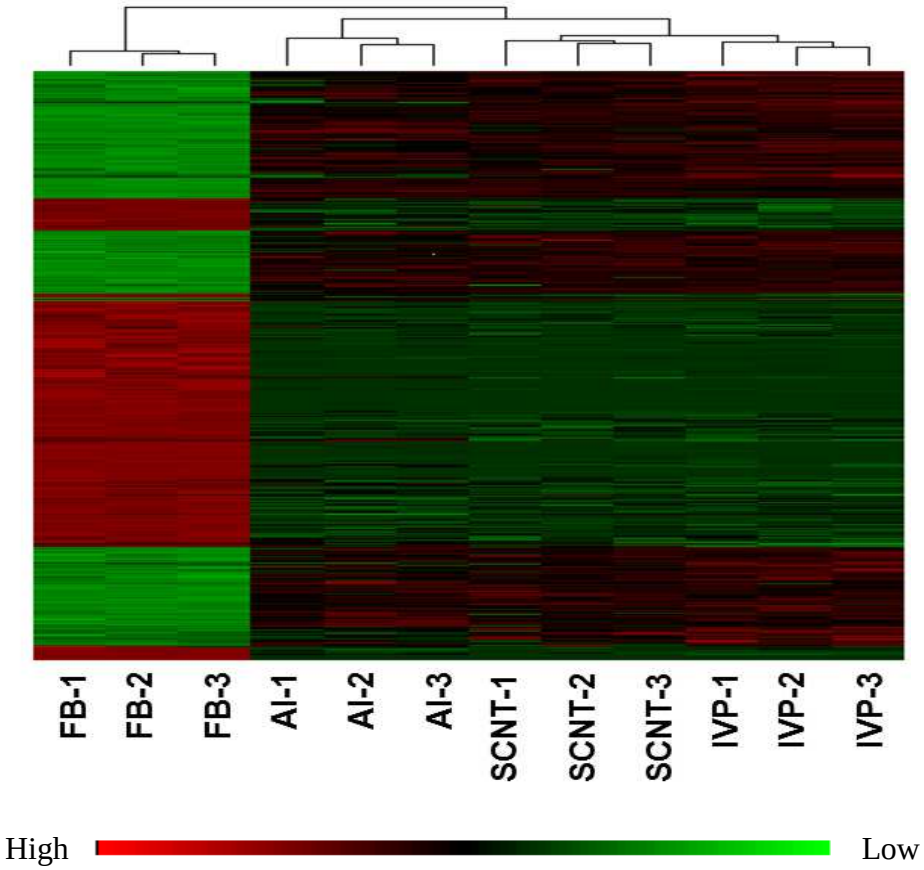
10. Appendices

Appendix 1: Review of the array quality assessments, array intensity distribution and between array comparisons after normalization. A) Heatmap presentation of the the correlation of arrays within and between biological replicates. The blue and greay clour represent high and low correlation, respectively. B) Box-plot summay 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-plot shows density estimates (smoothed histograms) of the data and the distribution of the arrays have similar shape and edges.



Appendix 2: Heat map illustration of gene expression difference between donor fibroblast cells (FBs) and the three embryo samples, namely artificial inseminated (AI), somatic cell nuclear transfer (SCNT) and in vitro producton (IVP). The number after each biological sample denotes their biological replicate. Red signifies high expression and green signifies low expression, and black signifies intermediate expression according to the colour bar shown below.



Appendix 3: List of the top differentially expressed genes between SCNT and AI derived elongated embryos. The positive and negative (FC) describes the up-and down - regulated genes respectively.

UniGene.ID	Gene.Title	Gene.Symbol	logFC	P.Value
Bt.22879	hydroxysteroid (17-beta) dehydrogenase 1	HSD17B1	4.8	4.59E-08
Bt.26921	solute carrier family 27, member 6	SLC27A6	4.5	2.59E-06
Bt.12809	immunoglobulin heavy constant mu	IGHM	4.4	0.00556389
Bt.4895	cyclin D2	CCND2	3.8	1.95E-10
Bt.44195	solute carrier family 9, member 3 regulator 1	SLC9A3R1	2.8	1.15E-06
Bt.102106	glutathione peroxidase 1	GPX1	2.8	1.06E-06
Bt.27824	vestigial like 1 (Drosophila)	VGLL1	2.8	1.36E-06
Bt.28476	monoacylglycerol O-acyltransferase 1	MOGAT1	2.6	1.04E-06
Bt.91427	glycerol-3-phosphate dehydrogenase 1-like	GPD1L	2.5	1.74E-05
Bt.856	deoxyribonuclease I-like 3	DNASE1L3	2.5	3.76E-06
Bt.33613	single-stranded DNA binding protein 2	SSBP2	2.4	0.00014173
Bt.22336	teratocarcinoma-derived growth factor 1	TDGF1	2.3	1.99E-07
Bt.9728	ATPase, Na+/K+ transporting, beta 3 polypeptide	ATP1B3	2.2	6.74E-07
Bt.4520	scavenger receptor class B, member 1	SCARB1	2.2	5.10E-05
Bt.555	carbonic anhydrase IV	CA4	2.1	0.00010599
Bt.4732	aldehyde dehydrogenase 1 family, member A1	ALDH1A1	2.0	0.00370295
Bt.89521	acyl-CoA synthetase long-chain family member 3	ACSL3	2.0	2.54E-07
Bt.64557	beta-2-microglobulin	B2M	2.0	0.00346596
Bt.23818	acyl-CoA oxidase 1, palmitoyl	ACOX1	1.8	5.85E-05
Bt.96910	phospholipid scramblase 1	PLSCR1	1.8	0.001411
Bt.8953	prostaglandin E synthase	PTGES	1.8	0.00089766
Bt.51814	solute carrier family 10, member 1	SLC10A1	1.8	3.22E-07
Bt.43859	acyl-CoA thioesterase 8	ACOT8	1.8	0.00031004
Bt.37893	acyl-CoA thioesterase 4-like	ACOT4	1.8	0.0001655
Bt.16630	transmembrane protein 144	TMEM144	1.8	0.00018509
Bt.25809	filamin A interacting protein 1-like	FILIP1L	1.8	9.28E-07
Bt.20330	protease, serine, 23	PRSS23	1.7	0.00020102
Bt.3891	fatty acid desaturase 2	FADS2	1.7	0.00060879
Bt.4804	cyclin-dependent kinase inhibitor 1C	CDKN1C	1.7	9.63E-05
Bt.48854	Dihydrolipoamide dehydrogenase	DLD	1.7	5.35E-08

Appendix: 3 Cont.

Bt.5534	thioredoxin reductase 1	TXNRD1	1.6	5.30E-06
Bt.25241	mucolipin 2	MCOLN2	1.6	8.82E-05
Bt.27262	pitrilysin metallopeptidase 1	PITRM1	1.6	2.73E-06
Bt.22589	phospholipase A2, group XIIA	PLA2G12A	1.6	1.05E-06
Bt.3898	isocitrate dehydrogenase 3 (NAD+) alpha	IDH3A	1.6	4.74E-09
Bt.46181	achaete-scute complex homolog 2	ASCL2	1.6	0.00026889
Bt.24154	transcription factor Dp-2	TFDP2	1.6	8.38E-06
Bt.23388	neuronal guanine nucleotide exchange factor	NGEF	1.6	2.80E-05
Bt.53492	tetraspanin 2	TSPAN2	1.5	0.00032153
Bt.103235	transforming growth factor, beta receptor III	TGFBR3	1.5	0.00102116
Bt.53077	tropomyosin 2 (beta)	TPM2	1.5	0.00265017
Bt.13245	MAP/microtubule affinity-regulating kinase 1	MARK1	1.5	3.49E-05
Bt.8643	reticulocalbin 2, EF-hand calcium binding domain	RCN2	1.5	1.88E-06
Bt.18203	junctional adhesion molecule 2	JAM2	1.5	0.00035879
Bt.3771	GAR1 ribonucleoprotein homolog (yeast)	GAR1	1.4	7.31E-05
Bt.26573	lysophosphatidylcholine acyltransferase 3	LPCAT3	1.4	8.49E-06
Bt.97115	stearoyl-CoA desaturase	SCD	1.4	3.95E-06
Bt.3254	tubulin folding cofactor E	TBCE	1.4	1.07E-06
Bt.60085	N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits	GNPTAB	1.4	4.54E-05
Bt.3248	aldehyde dehydrogenase 4 family, member A1	ALDH4A1	1.4	2.95E-05
Bt.8177	mitochondrial ribosomal protein L12	MRPL12	1.4	2.56E-05
Bt.97107	hypothetical protein LOC616423	MGC134282	1.3	4.73E-07
Bt.3415	chromosome 1 open reading frame 113 ortholog	C3H1orf113	1.3	0.00387299
Bt.56545	translocase of outer mitochondrial membrane 7 homolog (yeast)	TOMM7	1.3	1.52E-06
Bt.979	myosin IB	MYO1B	1.3	0.00014369
Bt.47903	ribosome production factor 2 homolog (S. cerevisiae)	RPF2	1.3	1.62E-05
Bt.557	solute carrier family 1 member 1	SLC1A1	1.3	3.23E-06
Bt.26665	pleckstrin homology domain containing, family A member 4	PLEKHA4	1.3	4.13E-05
Bt.44554	BCL2-associated athanogene 2	BAG2	1.3	1.55E-05
Bt.3562	low density lipoprotein receptor	LDLR	1.3	1.26E-05

Appendix 3: Cont

Bt.57506	ATPase, H+ transporting, lysosomal V0 subunit a4	ATP6V0A4	1.3	0.00091373
Bt.16175	caprin family member 2	CAPRIN2	1.3	0.00054616
Bt.59278	zinc finger, HIT-type containing 3	ZNHIT3	1.3	9.63E-05
Bt.22534	peripheral myelin protein 22	PMP22	1.3	5.79E-09
Bt.5174	inositol polyphosphate-1-phosphatase	INPP1	1.3	0.00028457
Bt.4167	nucleobindin 2	NUCB2	1.2	0.00075405
Bt.6685	methylenetetrahydrofolate dehydrogenase 2, methenyl tetrahydrofolate cyclohydrolase	MTHFD2	1.2	0.00010207
Bt.44002	taspase, threonine aspartase, 1	TASP1	1.2	1.43E-05
Bt.56241	CAP-GLY domain containing linker protein 1	CLIP1	1.2	0.0011491
Bt.22969	serpin peptidase inhibitor, clade G member 1	SERPING1	1.2	0.00064208
Bt.45288	src kinase associated phosphoprotein 1	SKAP1	1.2	0.00087323
Bt.27485	frizzled homolog 10 (Drosophila)	FZD10	1.2	0.00222668
Bt.15528	macrophage migration inhibitory factor	MIF	1.2	0.00265086
Bt.65105	pregnancy-associated glycoprotein 11-like	LOC788843	1.2	3.38E-05
Bt.5267	annexin A6	ANXA6	1.2	7.57E-05
Bt.405	follistatin	FST	1.2	0.00381559
Bt.42564	zinc finger, DHHC-type containing 7	ZDHHC7	1.2	3.20E-05
Bt.49065	ribonuclease/angiogenin inhibitor 1	RNH1	1.2	4.10E-06
Bt.53256	phospholipase C-like 2	PLCL2	1.2	0.00029052
Bt.4138	vascular endothelial growth factor A	VEGFA	1.2	0.00026708
Bt.91283	RUN and FYVE domain containing 3	RUFY3	1.2	0.00042659
Bt.13913	1-acylglycerol-3-phosphate O-acyltransferase 3	AGPAT3	1.1	0.00048793

Appendix 3: Cont.

UniGene.ID	Gene.Title	Gene symbol	logFC	P.Value
Bt.28030	trophoblast Kunitz domain protein 2	TKDP2	-7.5	6.98E-10
Bt.13362	tetraspanin 1	TSPAN1	-5.2	7.00E-06
Bt.23250	alpha-2-HS-glycoprotein	AHSG	-4.7	7.62E-06
Bt.103200	gap junction protein, beta 4, 30.3kDa	GJB4	-4.5	1.62E-08
Bt.63143	integrin, beta 2	ITGB2	-4.3	6.43E-05
Bt.49467	aminolevulinate, delta-, synthase 2	ALAS2	-4.2	6.62E-07
Bt.4946	ras-related C3 botulinum toxin substrate 2	RAC2	-4.2	5.53E-05
Bt.390	S100 calcium binding protein G	S100G	-3.8	3.97E-08
Bt.9625	serine peptidase inhibitor, Kazal type 4	SPINK4	-3.8	3.18E-08
Bt.14198	hypothetical protein LOC100270756	LOC100270756	-3.6	1.34E-08
Bt.28194	glutathione S-transferase omega 1	GSTO1	-3.6	0.00037272
Bt.89770	lysozyme (renal amyloidosis)	LYZ1	-3.5	3.94E-05
Bt.57922	CD48 molecule	CD48	-3.5	3.61E-06
Bt.49311	family with sequence similarity 84, member A	FAM84A	-3.4	0.00012258
Bt.41664	cell adhesion molecule 1	CADM1	-3.4	5.77E-06
Bt.63969	guanine nucleotide binding protein (G protein), beta polypeptide 4	GNB4	-3.3	8.06E-10
Bt.87242	steroidogenic acute regulatory protein	STAR	-3.3	1.69E-06
Bt.49341	translocator protein (18kDa)	TSPO	-3.1	6.17E-07
Bt.12805	phospholipase B domain containing 1	PLBD1	-3.0	4.70E-07
Bt.91089	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	CD55	-3.0	7.18E-06
Bt.11088	CD97 molecule	CD97	-3.0	2.81E-06
Bt.49689	claudin 1	CLDN1	-2.9	3.22E-10
Bt.49713	creatine kinase, mitochondrial 1 (ubiquitous)	CKMT1	-2.9	0.00023121
Bt.7145	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	GZMB	-2.9	0.00101952
Bt.3435	fin bud initiation factor homolog (zebrafish)	FIBIN	-2.9	4.33E-07
Bt.5970	S100 calcium binding protein A2	S100A2	-2.8	1.45E-06
Bt.23917	fibrinogen beta chain	FGB	-2.8	2.66E-05
Bt.29416	ring finger protein 128	RNF128	-2.8	7.15E-09
Bt.2046	sushi-repeat-containing protein, X-linked	SRPX	-2.8	1.07E-05
Bt.88701	adducin 3 (gamma)	ADD3	-2.7	6.83E-09

Appendix 3: Cont.

Bt.49700	allograft inflammatory factor 1	AIF1	-2.7	1.38E-05
Bt.48905	fibrinogen gamma chain	FGG	-2.6	0.00077552
Bt.3537	sulfotransferase family, cytosolic, 1A,	SULT1A1	-2.6	2.40E-07
Bt.57034	5'-nucleotidase, ecto (CD73)	NT5E	-2.5	0.00293654
Bt.4757	Rho GDP dissociation inhibitor (GDI) beta	ARHGDIB	-2.5	6.25E-05
Bt.4332	CCAAT/enhancer binding protein (C/EBP), alpha	CEBPA	-2.5	0.00035306
Bt.32520	brain expressed X-linked 2	BEX2	-2.4	0.00029245
Bt.452	glycosylphosphatidylinositol specific phospholipase D1	GPLD1	-2.4	1.03E-05
Bt.53163	Similar to trophoblast Kunitz domain protein 2	LOC515917	-2.4	0.00318993
Bt.12764	dehydrogenase/reductase (SDR family) member 9	DHRS9	-2.3	0.0002557
Bt.52974	TIMP metallopeptidase inhibitor 2	TIMP2	-2.3	0.00013575
Bt.17819	Sodium channel, nonvoltage-gated 1, gamma	SCNN1G	-2.3	0.00015025
Bt.2159	transmembrane protein 45A	TMEM45A	-2.3	2.49E-07
Bt.24447	coagulation factor II (thrombin) receptor-like 2	F2RL2	-2.3	1.39E-07
Bt.12327	thioredoxin interacting protein	TXNIP	-2.3	1.30E-06
Bt.48365	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	GPD2	-2.2	0.00041119
Bt.48881	galactose mutarotase (aldose 1-epimerase)	GALM	-2.2	4.68E-05
Bt.3890	Tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A	-2.2	0.00038944
Bt.21759	butyrobetaine (gamma), 2-oxoglutarate dioxygenase	BBOX1	-2.2	6.03E-06
Bt.17182	gap junction protein, beta 5, 31.1kDa	GJB5	-2.1	4.28E-11
Bt.16382	calcitonin receptor-like	CALCRL	-2.1	5.66E-05
Bt.52605	FBJ murine osteosarcoma viral oncogene homolog	FOS	-2.1	7.37E-06
Bt.1537	N-myc downstream regulated 1	NDRG1	-2.1	2.90E-05
Bt.7873	basal cell adhesion molecule (Lutheran blood group)	BCAM	-2.1	4.36E-07
Bt.5044	apolipoprotein H (beta-2-glycoprotein I)	APOH	-2.1	0.00038253
Bt.8247	parahox cluster neighbor	PRHOXNB	-2.1	6.16E-10
Bt.8856	Rh family, B glycoprotein (gene/pseudogene)	RHBG	-2.1	4.00E-07
Bt.49731	carbonic anhydrase II	CA2	-2.0	1.46E-07
Bt.64701	gap junction protein, beta 2, 26kDa	GJB2	-2.0	2.06E-06
Bt.8088	fibroblast growth factor receptor 2	FGFR2	-2.0	6.71E-06
Bt.23268	Niemann-Pick disease, type C2	NPC2	-2.0	2.82E-07
Bt.26851	DNA-damage regulated autophagy modulator 1	DRAM1	-2.0	1.34E-05
Bt.1907	hypothetical LOC614490	LOC614490	-2.0	6.34E-06
Bt.49475	enolase 3 (beta, muscle)	ENO3	-2.0	0.00139207
Bt.11748	similar to family with sequence similarity 20, member C	LOC534672	-2.0	1.49E-05

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Bt.51689	similar to Formin-like protein 2 (Formin homology 2 domain-containing protein 2)	LOC788312	-2.0	4.23E-05
Bt.16830	leucine zipper, down-regulated in cancer 1	LDOC1	-2.0	0.00057688
Bt.4622	mannosidase, alpha, class 2B, member 1	MAN2B1	-2.0	3.01E-06
Bt.97059	phospholipase A2 receptor 1, 180kDa	PLA2R1	-2.0	7.29E-06
Bt.29568	ELL associated factor 2	EAF2	-1.9	1.58E-09
Bt.10281	trophoblast Kunitz domain protein 5	TKDP5	-1.9	0.00332136
Bt.393	cathepsin B	CTSB	-1.9	5.40E-06
Bt.26628	RAP2C, member of RAS oncogene family	RAP2C	-1.9	6.48E-06
Bt.5336	transferrin	TF	-1.9	0.00027372
Bt.16137	aldehyde dehydrogenase 9 family, member A1	ALDH9A1	-1.9	0.00158163
Bt.44383	cannabinoid receptor 2 (macrophage)	CNR2	-1.9	6.79E-07
Bt.5250	milk fat globule-EGF factor 8 protein	MFGE8	-1.8	7.66E-05
Bt.10272	stanniocalcin 1	STC1	-1.8	8.15E-05
Bt.49570	ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1	-1.8	1.31E-06
Bt.13676	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	SERPINE2	-1.8	0.00011831
Bt.2712	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5	SERPINA5	-1.8	0.00298173
Bt.49336	ras homolog gene family, member B	RHOB	-1.8	1.84E-06
Bt.43926	Rho/Rac guanine nucleotide exchange factor (GEF) 2	ARHGEF2	-1.8	1.02E-05
Bt.2749	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-1.8	1.41E-06
Bt.27351	solute carrier family 5 (sodium iodide symporter), member 5	SLC5A5	-1.8	0.00062041
Bt.64779	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	ERBB3	-1.8	4.72E-06
Bt.55961	hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1	HERC1	-1.8	2.87E-07
Bt.3750	S100 calcium binding protein A11	S100A11	-1.8	0.00023127
Bt.47778	MAX interactor 1	MXI1	-1.8	0.00023303
Bt.7826	pituitary tumor-transforming 1 interacting protein	PTTG1IP	-1.8	2.16E-05
Bt.11861	solute carrier family 25 (mitochondrial carrier, Aralar), member 12	SLC25A12	-1.7	2.16E-07
Bt.102113	MARCKS-like 1	MARCKSL1	-1.7	4.86E-06
Bt.53829	retinoic acid receptor responder (tazarotene induced) 1	RARRES1	-1.7	0.00294362
Bt.48977	RAS, dexamethasone-induced 1	RASD1	-1.7	3.84E-08
Bt.11770	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	SLC25A20	-1.7	6.75E-05
Bt.91163	ephrin-A1	EFNA1	-1.7	1.07E-05
Bt.87081	similar to poliovirus receptor-related 3	LOC534360	-1.7	5.21E-07
Bt.6087	transmembrane 4 L six family member 1	TM4SF1	-1.7	0.00059482
Bt.5141	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	B4GALT1	-1.7	3.19E-05
Bt.46230	heart and neural crest derivatives expressed 1	HAND1	-1.7	0.00190195

Appendix 4: List of the top differentially expressed genes between IVP and AI derived elongated embryos. The positive and negative (FC) describes the up-and down - regulated genes respectively.

UniGene.ID	Gene.Title	Gene symbol	logFC	P.Value
Bt.26921	solute carrier family 27, member 6	SLC27A6	4.7	1.94E-06
Bt.22879	hydroxysteroid (17-beta) dehydrogenase 1	HSD17B1	4.4	1.04E-07
Bt.4895	cyclin D2	CCND2	3.1	1.25E-09
Bt.856	deoxyribonuclease I-like 3	DNASE1L3	2.6	2.07E-06
Bt.22336	teratocarcinoma-derived growth factor 1	TDGF1	2.6	6.50E-08
Bt.27824	vestigial like 1 (Drosophila)	VGLL1	2.5	3.03E-06
Bt.64557	beta-2-microglobulin	B2M	2.4	0.00101053
Bt.44195	solute carrier family 9, member 3 regulator 1	SLC9A3R1	2.3	5.77E-06
Bt.91427	glycerol-3-phosphate dehydrogenase 1-like	GPD1L	2.1	7.03E-05
Bt.102106	glutathione peroxidase 1	GPX1	2.1	1.30E-05
Bt.4520	scavenger receptor class B, member 1	SCARB1	2.1	9.03E-05
Bt.33613	single-stranded DNA binding protein 2	SSBP2	2.1	0.00039469
Bt.28476	monoacylglycerol O-acyltransferase 1	MOGAT1	2.0	1.12E-05
Bt.25809	filamin A interacting protein 1-like	FILIP1L	1.9	4.40E-07
Bt.12768	phosphoenolpyruvate carboxykinase 1 (soluble)	PCK1	1.8	0.00032497
Bt.9728	ATPase, Na+/K+ transporting, beta 3 polypeptide	ATP1B3	1.8	4.13E-06
Bt.8953	prostaglandin E synthase	PTGES	1.7	0.00113962
Bt.65578	BCL2-associated athanogene 3	BAG3	1.7	0.0001303
Bt.555	carbonic anhydrase IV	CA4	1.7	0.00059341
Bt.89521	acyl-CoA synthetase long-chain family member 3	ACSL3	1.7	1.27E-06
Bt.3891	fatty acid desaturase 2	FADS2	1.6	0.00082384
Bt.62616	transporter 2, ATP-binding cassette, sub-family B)	TAP2	1.6	0.00147768
Bt.54006	similar to RGC-32	MGC148992	1.6	0.00035373
Bt.3562	low density lipoprotein receptor	LDLR	1.6	1.86E-06
Bt.53492	tetraspanin 2	TSPAN2	1.6	0.00025097
Bt.17734	zinc finger, AN1-type domain 2A	ZFAND2A	1.5	5.98E-07
Bt.3248	aldehyde dehydrogenase 4 family, member A1	ALDH4A1	1.5	1.35E-05
Bt.16175	Caprin family member 2	CAPRIN2	1.4	0.0002091
Bt.18203	junctional adhesion molecule 2	JAM2	1.4	0.00039628
Bt.23388	neuronal guanine nucleotide exchange factor	NGEF	1.4	5.68E-05
Bt.16630	transmembrane protein 144	TMEM144	1.4	0.00110671
Bt.6775	annexin A3	ANXA3	1.4	0.00031995
Bt.46181	achaete-scute complex homolog 2 (Drosophila)	ASCL2	1.4	0.00076924
Bt.92178	transmembrane protein 88	TMEM88	1.4	0.00017692
Bt.91186	Cysteine/tyrosine-rich 1	CYYR1	1.3	0.00098
Bt.20330	protease, serine, 23	PRSS23	1.3	0.0014221
Bt.3254	Tubulin folding cofactor E	TBCE	1.3	1.60E-06
Bt.49580	brain protein 44-like	BRP44L	1.3	0.00015827
Bt.4138	vascular endothelial growth factor A	VEGFA	1.3	1.68E-06

Appendix 4: Cont.

Bt.15528	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	MIF	1.3	0.00185713
Bt.3898	isocitrate dehydrogenase 3 (NAD+) alpha	IDH3A	1.3	3.52E-08
Bt.1548	gamma-inducible protein 30	IFI30	1.3	0.00549349
Bt.23818	acyl-CoA oxidase 1, palmitoyl	ACOX1	1.3	0.00101928
Bt.29621	glutathione peroxidase 2	GPX2	1.3	0.00213918
Bt.633	sideroflexin 1	SFXN1	1.2	4.84E-06
Bt.7490	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	MTHFD1L	1.2	9.50E-05
Bt.405	Follistatin	FST	1.2	0.00347891
Bt.5534	thioredoxin reductase 1	TXNRD1	1.2	6.13E-05
Bt.32740	laminin, alpha 1	LAMA1	1.2	0.00016033
Bt.11195	palate, lung and nasal epithelium associated	PLUNC	1.2	3.02E-07
Bt.22534	peripheral myelin protein 22	PMP22	1.2	9.06E-09
Bt.48892	gametocyte specific factor 1	GTSF1	1.2	0.00135376
Bt.60085	N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits	GNPTAB	1.2	0.00015139
Bt.97115	Stearoyl-CoA desaturase (delta-9-desaturase)	SCD	1.2	1.57E-05
Bt.57506	ATPase, H+ transporting, lysosomal V0 subunit a4	ATP6V0A4	1.2	0.00162894
Bt.22399	Arginase, type II	ARG2	1.2	0.00011281
Bt.49065	ribonuclease/angiogenin inhibitor 1	RNH1	1.2	4.18E-06
Bt.29464	hypothetical protein LOC614047	LOC614047	1.2	3.75E-05
Bt.11942	Collagen, type XVIII, alpha 1	COL18A1	1.2	8.86E-08
Bt.17367	DnaJ (Hsp40) homolog, subfamily B, member 1	DNAJB1	1.1	0.00061839

Appendix 4: Cont.

UniGene.ID	Gene.Title	Gene symbol	logFC	P.Value
Bt.28030	trophoblast Kunitz domain protein 2	TKDP2	-7.7	5.39E-10
Bt.262	trophoblast Kunitz domain protein 3	TKDP3	-5.1	0.00190812
Bt.13362	tetraspanin 1	TSPAN1	-4.8	1.55E-05
Bt.6410	Placenta-expressed transcript 1 protein	PLET1	-4.3	8.10E-07
Bt.103200	gap junction protein, beta 4, 30.3kDa	GJB4	-4.3	2.37E-08
Bt.49467	aminolevulinate, delta-, synthase 2	ALAS2	-4.2	7.35E-07
Bt.390	S100 calcium binding protein G	S100G	-3.9	3.32E-08
Bt.9625	serine peptidase inhibitor, Kazal type 4	SPINK4	-3.9	2.55E-08
Bt.63143	Integrin, beta 2	ITGB2	-3.8	0.00017652
Bt.14198	hypothetical protein LOC100270756	LOC100270756	-3.5	1.41E-08
Bt.57922	CD48 molecule	CD48	-3.5	3.59E-06
Bt.87242	Steroidogenic acute regulatory protein	STAR	-3.4	1.47E-06
Bt.49713	Creatine kinase, mitochondrial 1 (ubiquitous)	CKMT1	-3.3	7.60E-05
Bt.28194	glutathione S-transferase omega 1	GSTO1	-3.3	0.00061362
Bt.4946	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	RAC2	-3.2	0.00037114
Bt.89770	lysozyme (renal amyloidosis)	LYZ1	-3.2	9.03E-05
Bt.11088	CD97 molecule	CD97	-3.1	1.78E-06
Bt.49341	translocator protein (18kDa)	TSPO	-3.0	8.00E-07
Bt.49689	claudin 1	CLDN1	-2.9	3.16E-10
Bt.63969	Guanine nucleotide binding protein (G protein), beta polypeptide 4	GNB4	-2.8	4.13E-09
Bt.41664	cell adhesion molecule 1	CADM1	-2.7	3.57E-05
Bt.49311	family with sequence similarity 84, member A	FAM84A	-2.7	0.0007314
Bt.3435	fin bud initiation factor homolog (zebrafish)	FIBIN	-2.7	7.62E-07
Bt.4332	CCAAT/enhancer binding protein (C/EBP), alpha	CEBPA	-2.6	0.00023508
Bt.29416	ring finger protein 128	RNF128	-2.6	1.55E-08
Bt.452	glycosylphosphatidylinositol specific phospholipase D1	GPLD1	-2.4	1.00E-05
Bt.12764	dehydrogenase/reductase (SDR family) member 9	DHRS9	-2.4	0.00017846
Bt.89090	pregnancy-associated glycoprotein 12-like /// pregnancy-associated glycoprotein 12	LOC782451 /// PAG12	-2.3	0.00497919
Bt.2046	sushi-repeat-containing protein, X-linked	SRPX	-2.2	6.12E-05

Appendix 4: Cont.

Bt.4757	Rho GDP dissociation inhibitor (GDI) beta	ARHGDIB	-2.2	0.00014305
Bt.16382	calcitonin receptor-like	CALCRL	-2.2	4.40E-05
Bt.21759	butyrobetaine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1	BBOX1	-2.2	5.95E-06
Bt.3537	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	SULT1A1	-2.2	1.16E-06
Bt.49700	allograft inflammatory factor 1	AIF1	-2.1	8.01E-05
Bt.17182	gap junction protein, beta 5, 31.1kDa	GJB5	-2.1	4.63E-11
Bt.64701	gap junction protein, beta 2, 26kDa	GJB2	-2.1	1.61E-06
Bt.27351	Solute carrier family 5 (sodium iodide symporter), member 5	SLC5A5	-2.1	0.00020141
Bt.91089	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	CD55	-2.1	0.00014018
Bt.26851	DNA-damage regulated autophagy modulator 1	DRAM1	-2.0	1.21E-05
Bt.48881	galactose mutarotase (aldose 1-epimerase)	GALM	-2.0	9.44E-05
Bt.12327	thioredoxin interacting protein	TXNIP	-2.0	3.37E-06
Bt.12805	phospholipase B domain containing 1	PLBD1	-2.0	1.50E-05
Bt.3890	tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A	-2.0	0.0006944
Bt.1240	deoxyribonuclease II, lysosomal	DNASE2	-2.0	1.66E-06
Bt.4622	mannosidase, alpha, class 2B, member 1	MAN2B1	-2.0	2.84E-06
Bt.16830	leucine zipper, down-regulated in cancer 1	LDOC1	-2.0	0.00057876
Bt.8088	fibroblast growth factor receptor 2	FGFR2	-2.0	9.20E-06
Bt.5970	S100 calcium binding protein A2	S100A2	-2.0	3.44E-05
Bt.8247	parahox cluster neighbor	PRHOXNB	-1.9	1.02E-09
Bt.53829	retinoic acid receptor responder 1	RARRES1	-1.9	0.0014012
Bt.24447	coagulation factor II (thrombin) receptor-like 2	F2RL2	-1.9	6.42E-07
Bt.1907	hypothetical LOC614490	LOC614490	-1.9	1.07E-05
Bt.2712	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5	SERPINA5	-1.9	0.00253421
Bt.9791	peptidylprolyl isomerase F	PPIF	-1.9	3.25E-05
Bt.7873	basal cell adhesion molecule	BCAM	-1.9	1.37E-06
Bt.393	cathepsin B	CTSB	-1.8	7.10E-06
Bt.29568	ELL associated factor 2	EAF2	-1.8	2.48E-09
Bt.48977	RAS, dexamethasone-induced 1	RASD1	-1.8	2.28E-08
Bt.10814	coagulation factor II (thrombin) receptor	F2R	-1.8	1.66E-05
Bt.49475	enolase 3 (beta, muscle)	ENO3	-1.8	0.00280978
Bt.10272	stanniocalcin 1	STC1	-1.8	0.00010617
Bt.44383	cannabinoid receptor 2	CNR2	-1.8	1.00E-06
Bt.46230	heart and neural crest derivatives expressed 1	HAND1	-1.8	0.00129096

Appendix 4: Cont.

Bt.5546	guanine nucleotide binding protein, alpha inhibiting activity polypeptide 1	GNAI1	-1.8	5.57E-06
Bt.61173	histone cluster 2, H2be	HIST2H2BE	-1.8	0.00019048
Bt.11861	solute carrier family 25, member 12	SLC25A12	-1.8	1.96E-07
Bt.4725	B-cell translocation gene 1, anti-proliferative	BTG1	-1.7	9.80E-09
Bt.5336	transferrin	TF	-1.7	0.00047952
Bt.6803	TRAF2 and NCK interacting kinase	TNIK	-1.7	1.93E-05
Bt.1655	inositol(myo)-1(or 4)-monophosphatase 2	IMPA2	-1.7	0.00146599
Bt.22869	fatty acid binding protein 5 (psoriasis-associated)	FABP5	-1.7	3.21E-05
Bt.61846	tumor protein p53 inducible protein 3	TP53I3	-1.7	0.00024078
Bt.13162	Keratin 5	KRT5	-1.7	0.00013112
Bt.51689	similar to Formin-like protein 2	LOC788312	-1.7	0.00014394
Bt.11770	solute carrier family 25, member 20	SLC25A20	-1.7	8.16E-05
Bt.49731	carbonic anhydrase II	CA2	-1.7	9.01E-07
Bt.4125	pregnancy-associated glycoprotein 8	PAG8	-1.6	0.00083399
Bt.32520	brain expressed X-linked 2	BEX2	-1.6	0.00422438
Bt.38271	Motile sperm domain containing 1	MOSPD1	-1.6	1.61E-05
Bt.97059	phospholipase A2 receptor 1, 180kDa	PLA2R1	-1.6	3.76E-05
Bt.23268	Niemann-Pick disease, type C2	NPC2	-1.6	2.16E-06
Bt.43926	Rho/Rac guanine nucleotide exchange factor (GEF) 2	ARHGEF2	-1.6	2.86E-05
Bt.7826	pituitary tumor-transforming 1 interacting protein	PTTG1IP	-1.6	4.76E-05
Bt.48365	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	GPD2	-1.6	0.0042225
Bt.8856	Rh family, B glycoprotein (gene/pseudogene)	RHBG	-1.6	4.04E-06
Bt.2159	transmembrane protein 45A	TMEM45A	-1.6	6.01E-06
Bt.61523	similar to hCG27535	LOC539805	-1.5	0.00171722
Bt.5250	milk fat globule-EGF factor 8 protein	MFGE8	-1.5	0.00030725
Bt.57867	N-acetylgalactosaminidase, alpha-	NAGA	-1.5	0.0001616
Bt.30711	hypothetical LOC789163	LOC789163	-1.5	0.0002886
Bt.65686	Jagged 1	JAG1	-1.5	0.00221115
Bt.17819	Sodium channel, nonvoltage-gated 1, gamma	SCNN1G	-1.5	0.00253486
Bt.52086	solute carrier family 13 (sodium/sulfate symporters), member 4	SLC13A4	-1.5	0.00011492
Bt.11748	similar to family with sequence similarity 20, member C	LOC534672	-1.5	0.00016924
Bt.26241	tripartite motif-containing 36	TRIM36	-1.4	1.47E-08
Bt.21732	ribonuclease T2	RNASET2	-1.4	0.00073685
Bt.52605	FBJ murine osteosarcoma viral oncogene homolog	FOS	-1.4	0.00019644

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Curriculum vitae

4. Publications

Betsha S, Hoelker M, Salilew-Wondim D, Held E, Rings F, Große-Brinkhause C, Cinar MU, Havlicek V, Besenfelder U, Tholen E, Looft C, Schellander K, Tesfaye D (2013): Transcriptome profile of bovine elongated conceptus obtained from SCNT and IVP pregnancies. Mol Reprod Dev 80, 315–333

Betsha S, Salilew-Wondim D, Havlicek V**,** Besenfelder U, Rings F, Hoelker M, Schellander K, Tesfaye D (2011): Global gene expression analysis of elongated embryos produced by somatic cell nuclear transfer and in vitro fertilization. Reprod Fertil Dev 24, (Abstr)

Betsha S, Salilew-Wondim D, Havlicek V, Besenfelder U, Hölker M, Rings F, Cinar MU, Schellander K, Tesfaye D (2011): Global transcriptome analysis of elongated embryos produced by somatic cell nuclear transfer and in vitro fertilization. Deutschen Gesellschaft für Züchtungskunde e.V. und der Gesellschaft für Tierzuchtwissenschaften e.V., September 6-7, 2011 Munich, Germany (Abstr)

Betsha S, Salilew-Wondim D, Hölker M, Havlicek V, Besenfelder U, Schellander K, Tesfaye D (2011): Large scale gene expression analysis of elongated embryos derived from nuclear transfer, artificial insemination and in vitro pregnancies. 3rd Embryo genomics meeting, September 20-22, 2011 Bonn, Germany (Abstr)

Betsha S, Melaku S (2009): Supplementations of Hyparrhenia rufa-dominated hay with groundnut cake- wheat bran mix: effects on feed intake, digestibility and nitrogen balance of Somali goats. Trop Anim Health Prod 41, 927-933

Melaku S, **Betsha S** (2008): Bodyweight and carcass characteristics of Somali goats fed hay supplemented with graded levels of peanut cake and wheat bran mixture. Trop Anim Health Prod 40, 553-560

5. Training

R-program and its applications in statistics, July 25-29, 2011. Bonn University, Bonn, Germany

Curriculum design, program and cource development and revision, January 11-12, 2008. Awassa, Ethiopia

Meat quality assessment, March 7-8, 2008. Debre Zeit, Ethiopia

6. Award

Special jury award winner at Ethiopia-Sudan development marketplace, 26-28 February, 2003. Addis Ababa, Ethiopia