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Molecular reprogramming in bovine embryos after serial somatic cell chromatin transfer

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MOLECULAR REPROGRAMMING IN BOVINE EMBRYOS AFTER SERIAL SOMATIC CELL CHROMATIN TRANSFER

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Somatic Cell Nuclear Transfer (SCNT), commonly known as cloning, is the transfer of a somatic nucleus into an enucleated oocyte to produce a clone. The chromatin structure of somatic cells permits the expression of certain genes, while silencing the rest of the genome. The cytoplasm of oocytes can reprogram a somatic nucleus by reactivating the genes necessary for embryonic development and silencing the somatic genes. However, the low efficiency of SCNT indicates that successful nuclear reprogramming is a rare event. The objectives of this study were determine the extent of transcriptional reprogramming in bovine blastocysts produced by serial rounds of chromatin transfer (from first and fourth generations), using blastocysts produced by *in vitro* fertilization (IVF) as controls, to identify cumulative errors in the transcriptome profile. Differentially expressed genes were studied further to determine their function in embryonic development. We identified a set of transcripts consistently misregulated in

blastocysts produced be chromatin transfer (CT), some of which had a more marked misregulation in the embryos produced by 4 successive rounds of cloning. Among the genes significantly upregulated in both CT groups compared to IVF blastocysts were both *de novo* DNA methylation enzymes DNMT3A and DNMT3B. Expression patterns, structural and functional analyses were performed for DNA methyltransferases. A high level of structural and functional conservation was observed for DNA methyltransferases among human, mouse, and bovine species. A set of genes that participate in early embryonic development, chromatin remodeling and DNA methylation were differentially regulated in cloned embryos and had not been fully annotated at the time of the analysis. We annotated those genes and submitted them to the Bovine Genome Sequencing Consortium database. These results have important implications for the selection of models for the study of DNA methylation during early development. The present study provides a valuable data set for identifying possible cumulative errors in somatic cell chromatin transfer that could hinder nuclear reprogramming, shedding light on the epigenetic role in reprogramming and cell plasticity.

Key Words: nuclear reprogramming, embryonic transcriptome, somatic cell nuclear transfer.

DEDICATION

To my son Samuel, my mom Alicia, and my sister Gloria who are my inspiration in everything I do and every choice I make.

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LIST OF ABBREVIATIONS

ATP

Adenosine 5'-triphosphate

BIT1 The Bcl-2 inhibitor of transcription

BSA Serum Albumin

cDNA Complementary DNA

cRNA Complementary RNA

CT Chromatin transfer

CT Chromatin transfer

DC Donor cell

DC Donor cell

DNA Deoxyribonucleic acid

DNase Deoxyribonuclease

DNMT1 DNA cytosine- 5-methyltransferase 1

DNMT1o Oocyte specific DNA cytosine- 5-methyltransferase 1

DNMT1s somatic DNA cytosine- 5-methyltransferase 1

DNMT2 DNA cytosine- 5-methyltransferase 2

DNMT3a DNA cytosine- 5-methyltransferase 3a

DNMT3b DNA cytosine- 5-methyltransferase 3b

DNMT3L DNA cytosine-like 5-methyltransferase

ES cells Embryonic stem cells

FBXO9 F-box protein 9

FCS Fetal Calf Serum

GAPDH

GATM glycine amidinotransferase

GNAI2 Guanine nucleotide binding protein alpha inhibiting activity polypeptide 2

GO Gene Ontology

GO Gene Ontology

H1 Histone 1

HAT Histone acetylase

HDAC Histone deacetylase

HDAC1 Histone deacetylase 1

HMG High mobility group proteins

HMGN High mobility group Nucleosomal proteins

HMGN3

HP1beta Heterochromatin protein 1 beta

hpm Hours post maturation

HSP70

HSPA1A

IGF2R Insulin-like growth factor 2 recepto

ISWI Imitation switch

IVF *In vitro* fertilization

K Lysine

Mash2

MBD Methyl Binding Domain proteins

miRNA micro RNAs

mRNA messenger RNA

NFYA Nuclear transcription factor Y alpha

NGDN Neuroguidin

NT Nuclear transfer

PALLD Paladin

PCR Polymerase Chain Reaction

PGR

PHD Plant Homeodomain

PLAC8

POU5F1 POU class 1 homeobox 1

RNA Ribonucleic acid

RNAse Ribonuclease

RT-PCR Reverse transcription PCR

SCCT Somatic cell chromatin transfer

SCNT Somatic cell nuclear transfer

STAT3 Signal transducer and activator of transcription 3

SWI/SNF SWItch/Sucrose NonFermentable

TIF1beta Transcriptional Intermediary Factor 1 beta

$tRNA^{Asp}$ Aspartic acid transfer RNA

TSA trichostatin A

XCI X-chromosome inactivation

CHAPTER 1

INTRODUCTION

The majority of cells in an organism differ both morphologically and functionally from one another (i.e. epithelial, muscle, connective, neural cells). However, they all originate from a single cell, the zygote, which through several cell divisions gives rise to all cell types. Once differentiated, each cell passes its specialized character on to the daughter cells ensuring the preservation of the appropriate tissue type. The genes that are transcriptionally active on the cells of a particular type are roughly the same, and differ from those expressed in a different type. The pattern of gene expression characteristic for a differentiated cell is “remembered” through subsequent cell divisions. The differences in gene expression among cell types are not genetic since, with very few exceptions, most cells in an organism contain exactly the same DNA sequence. These differences are epigenetic. The term “epigenetics” was introduced during the 1940’s by Conrad H. Waddington to describe "the events which lead to the unfolding of the genetic program" (Holliday, 2006). Epigenetics was applied forty years later to describe “the interactions

between genes and the cellular environment that produce a change in the cell phenotype" (Holliday, 1987).

As cells differentiate and specialize to become a particular cell type a "cellular memory" is established ensuring that only a specific set of genes will be transcribed and others will be silent (Eilertsen et al., 2007). The molecular mechanisms necessary to establish the cell memory include packaging unexpressed genes into more compacted forms of chromatin that are "marked" to repress the expression of the genes. DNA methylation, chromatin packaging, and remodeling of chromatin-associated proteins, such as linker histones, polycomb group, and nuclear scaffold proteins (Latham, 1999; Rideout et al., 2001) are some of the epigenetic mechanisms stably passed from cell to cell during cell division, ensuring the maintenance of distinctive cell types.

Although the epigenetic marks in somatic cells are stable, they can be removed, to a certain degree, and most cell types can be reprogrammed into becoming a different cell type. Furthermore, a somatic cell can be reprogrammed to develop into an embryo and become a new organism. One of the ways in which reprogramming of a differentiated cell can be achieved is Somatic Cell Nuclear Transfer (SCNT), commonly referred to as cloning. The somatic nucleus or even the whole somatic cell is transferred into an enucleated oocyte, from which its own genomic DNA has been removed (Campbell et al., 2001). After nuclear transfer, the oocyte is activated to start embryogenesis and finally generate a new organism (Campbell et al., 2007). Despite the technological advances in SCNT during the last decade, and its scientific and medical importance, the molecular processes involved in nuclear reprogramming remain largely unknown and the overall

efficiency of SCNT in mammals remains low. The efficiency of cloning, defined as the proportion of transferred embryos that result in viable offspring stands at 2-3% for all species. Cattle seem to be an exception with efficiency averaging 5–20% for this species. (Gurdon and Byrne, 2003; Sakai et al., 2005; Cibelli, 2007b; Oback and Wells, 2007b; Niemann et al., 2008). Failure to reprogram the donor genome is thought to be one of the main reasons for the low efficiency of cloning (Latham, 2005; Niemann et al., 2008).

One of the applications of SCNT is the production of human proteins in the milk of transgenic animals. Genetic modifications are performed on cultured cells, which are later used as nuclear donors to obtain transgenic animals by SCNT. Some of the transgenic phenotypes require multiple genetic modifications, but it is unlikely that somatic cells would divide for a sufficient length of time to allow for more than one genetic modification to be completed (Kasinathan et al., 2001a). It has been proposed that consecutive rounds of cloning, also referred to as "serial cloning", allow for rejuvenation and selection of transformed cultured cells (Hill et al., 2000; Hill et al., 2001; Liu et al., 2001; Kuroiwa et al., 2004) and that it may improve the efficiency of SCNT by increasing the reprogrammable potential of the somatic cells (Cho et al., 2007; Fujimura et al., 2008). Conversely, other reports suggest that epigenetic errors could accumulate in the embryos as a result of serial cloning and prolonged *in vitro* culture decreasing cloning efficiency. After serial cloning up to the sixth generation was performed in mice, cloning efficiency significantly decreased, although no signs of telomere shortening or premature ageing were observed (Wakayama et al., 2000). A greatly reduced in vitro and in vivo developmental capacity was reported for bovine embryos derived after several rounds of serial cloning (Peura et al., 2001; Kubota et al., 2004). It has been suggested that

extended culture associated with transfection and selection procedures may induce changes of somatic cells, which decrease the efficiency of nuclear transfer and that these changes cannot be reversed by recloning (Zakhartchenko et al., 2001).

Our central hypothesis is that improper molecular reprogramming (inefficient chromatin remodeling and DNA demethylation) in Somatic Cell Chromatin Transfer (SCCT) derived embryos is causative of alterations in gene expression, which are incremented by sequential rounds of cloning and negatively affect cloning efficiency. To test this hypothesis, our objectives were (1) to determine the extent of transcriptional reprogramming in blastocyst produced by SCCT, by comparing them to the transcriptome profiles of the somatic cells used as nuclear donors; (2) to identify cumulative errors in the transcriptome profile of bovine blastocysts produced by serial cloning (from the first and fourth generations), using blastocysts produced by *in vitro* fertilization (IVF) as controls; (3) to define the identities, roles, and expression patterns of important groups of genes in molecular reprogramming; (4) to identify the mechanism(s) of molecular reprogramming; (5) to determine the expression pattern of a panel of selected genes, in fibroblasts obtained from fetuses from zero to five rounds of serial cloning; (6) to describe the structural and functional conservation of DNA methyltransferases, and their expression patterns during early embryonic development; (7) to contribute in the annotation of genes involved in DNA methylation and chromatin remodeling.

CHAPTER 2

REVIEW OF PERTINENT LITERATURE

2.1 Somatic Cell Nuclear Transfer

The first successful nuclear transfer experiments were conducted by Briggs and King in 1952 using the frog *Rana pipiens*. When nuclei from blastula stage cells were transplanted into enucleated eggs, normal looking tadpoles were obtained. However, the cloned tadpoles never reached sexual maturity (Briggs and King, 1952). When nuclei from older, more differentiated endoderm cells were transplanted into eggs, the embryos failed to develop (Briggs and King, 1957). These findings suggested that as cell differentiation progresses, irreversible changes render the nucleus incapable of being reprogrammed. In 1962, biologist John Gurdon succeeded where Briggs and King had failed, performing nuclear transfer using fully differentiated cells from the intestine of a *Xenopus laevis* tadpole. Fertile adult frogs were obtained in this study (Gurdon et al., 1958) proving that the nucleus was able to be reprogrammed despite the differentiated status of the cell. Gurdon stated that "cell differentiation takes place without any stable changes to the genome". In the following years, Gurdon and his colleagues performed

several nuclear transfer experiments using somatic cells from a variety of tissues (Gurdon and Uehlinger, 1966; Gurdon et al., 1975; Gurdon, 2006).

In the 1980s several studies proved that, like amphibian cells, mammalian cells could also be reprogrammed. McGrath and Solter in 1983 successfully transplanted nuclei from embryonic cells of mice into enucleated zygotes (Illmensee and Hoppe, 1981; McGrath and Solter, 1983). Willadsen, a domestic animal embryologist at Cambridge University, repeated these experiments obtaining fully viable embryos using sheep blastomeres (from 8- and 16-cell embryos) as donor cells and enucleated oocytes as recipients (Willadsen, 1986). During the late 1980s and early 1990s, research conducted in Neal First's laboratory at the University of Wisconsin, resulted in cloned cattle and pigs obtained by using blastomeres (from 8- to 32-cell stage embryos) as nuclear donor cells (Prather et al., 1987; Robl et al., 1987). In 1994, the same group achieved, for the first time, the birth of normal calves derived from primary cultures of inner cell mass (ICM) cells that had been cultured for up to 27 days (Sims and First, 1994). In 1995, at the Roslin Institute in Edinburgh, two cloned lambs were born after nuclear transfer from an established embryonic cell line (from a day-9 embryo), which had been cultured for 6 to 13 passages (Campbell et al., 1996). One year later, the same group produced eight more cloned lambs. One of the lambs was "Dolly" the first mammal in history obtained by transfer of an adult somatic cell (mammary gland cell) nucleus (Wilmut et al., 1997). Since then, a wide range of somatic cells from several mammalian species has been used to perform SCNT (Cibelli et al., 1998; Wakayama et al., 1998; Baguisi et al., 1999; Polejaeva et al., 2000; Chesne et al., 2002; Shin et al., 2002; Galli et al., 2003; Woods et al., 2003; Zhou et al., 2003; Lee et al., 2005; Li et al.,

2006; Kim et al., 2007). The first reports of live offspring from thirteen mammalian species obtained by SCNT are summarized in Table 1.1.

Table 2.1 First reported offspring in different mammalian species obtained by somatic cell nuclear transfer from adult cells.

Year	Species	Donor cell type	Citation
1997	Sheep	Mammary epithelium	Wilmut et al.
1998	Cow	Fetal fibroblasts	Cibelli et al.
1998	Mouse	Cumulus cells	Wakayama et al.
1999	Goat	Fetal fibroblasts	Baguisi et al.
2000	Pig	Granulosa cells	Polejaeva et al.
2002	Rabbit	Cumulus cells	Chesne et al.
2002	Cat	Cumulus cells	Shin et al.
2003	Horse	Skin fibroblasts	Galli et al.
2003	Rat	Fetal fibroblasts	Zhou et al.
2003	Mule	Fetal fibroblasts	Woods et al.
2005	Dog	Skin fibroblasts	Lee et al.
2006	Ferret	Cumulus cells	Li et al.
2007	Wolf	Skin fibroblasts	Kim et al.

The overall efficiency of SCNT, defined as the proportion of transferred embryos that result in viable offspring, stands at 2-3% for all species (Gurdon and Byrne, 2003; Sakai et al., 2005; Cibelli, 2007b; Oback and Wells, 2007b). In cattle, SCNT has reached a greater efficiency, averaging 5-10% and with some reports of efficiency as high as 20% (Kato et al., 1998). Among the factors contributing to the greater success in cloning cattle may be the late embryonic genome activation specific for this species (Memili et al.,

1998; Memili and First, 2000; Misirlioglu et al., 2006) coupled with the optimization of reproductive technologies, such as *in vitro* embryo production and embryo transfer, brought about by the cattle industry (Dinnyes et al., 2002). Additionally, the efficiency of nuclear transfer technology in cattle may be enhanced by the fact that approximately half of all SCNT's worldwide are performed in this species (Oback and Wells, 2007a).

Failure to reprogram the donor genome is thought to be one of the main reasons for the low efficiency of cloning (Bourc'his et al., 2001; Dean et al., 2001; Rideout et al., 2001; Mann and Bartolomei, 2002). Various strategies have been employed to improve the success rate of SCNT, most of them focus on the donor cells including: a) use of different cell types as nuclear donors (Hill et al., 2000; Kato et al., 2000; Kato et al., 2004; Inoue et al., 2005); b) use of donor cells cultured for different number of passages (Zakhartchenko et al., 1999; Kubota et al., 2000; Jang et al., 2004); c) the importance of the cell cycle stage of the donor nucleus (Smith et al., 1996; Kasinathan et al., 2001a; Kasinathan et al., 2001b; Campbell and Alberio, 2003; Wells et al., 2003); and d) use of chemical agents and cellular extracts to modify the donor cell epigenetic marks (Jones et al., 2001; Enright et al., 2003; Enright et al., 2005). The influence of different oocyte enucleation, fusion, and activation methods on cloning efficiency has also been analyzed (Wang et al., 2001; Liu et al., 2002; Akagi et al., 2003).

2.2 Somatic Cell Chromatin Transfer

Somatic Cell Chromatin Transfer (SCCT) was first described in 2004 as a strategy to improve nuclear reprogramming (Sullivan et al., 2004). Somatic cells were exposed to an extract from mitotic cells prior to transfer into enucleated oocytes. Mitotic cell extracts induced condensation of somatic chromosomes and promoted removal of nuclear factors from the somatic nucleus. The overall efficiency of producing cloned calves by CT was similar to NT. Nevertheless, CT exhibited a trend toward enhanced survival of cloned calves after one month postpartum. Mitotic cell extracts elicit ATP-dependent condensation of chromosomes, and disassembly of the type A and B lamins from chromatin. Additionally, transcription factors such as the TATA-box binding protein (TBP) are removed from chromatin in the presence of mitotic cell extracts (Sullivan et al., 2004).

The nuclear lamina is a meshwork of protein filaments, which consists of major protein components including three major lamins, A, C, and B1 and a number of minor lamins (Goldman et al., 2002). Lamins are classified into A-type (A, C, AD10, and C2) and B-type lamins (B1, B2, and B3) and are involved in nuclear stability, chromatin structure and gene expression (Hall et al., 2005a). While the B-type lamin B2 is ubiquitously expressed in all cells, the lamins B1 and A/C are differentially regulated throughout development and in adult tissues (Broers et al., 1997; Gruenbaum et al., 2000). Expression of lamin B1 has been detected in the immature, germinal vesicle (GV) oocyte and throughout early embryogenesis in murine, bovine and porcine embryos (Constantinescu et al., 2006). Contrastingly, A-type lamins are primarily found in

differentiated cells. During mouse embryonic development, expression of A-type lamins is first detected on day 9 in extraembryonic tissues and on day 12 in the embryo itself (Prather et al., 1989).

In vitro and *in vivo* manipulations of nuclear lamina composition have shown that failure to assemble a correct set of lamins invariably leads to apoptosis (Steen and Collas, 2001). Inappropriate assembly of type A lamins has been detected in NT embryos, along with enhanced pronuclear TBP content, and increased resistance of DNA to DNAse I (Sullivan et al., 2004). Remodeling a somatic nucleus *in vitro* through condensation of chromosomes during interphase seems to alter the ‘‘memory’’ of chromatin organization in the somatic nucleus (Gerlich et al., 2003). The removal of these somatic factors from the donor nucleus could facilitate the incorporation of maternal chromatin remodeling factors. Although a recent study did not detect any significant differences in the global gene expression profiles of SCCT and SCNT embryos (Zhou et al., 2007), SCCT may represent a tool for studying nuclear reprogramming.

2.3 Serial Cloning

One of the applications of SCNT is the production of biopharmaceuticals such as human proteins in the milk of transgenic animals. Genetic modifications are performed on cultured cells, which are later used as nuclear donors to obtain transgenic animals by SCNT. Some of the transgenic phenotypes require multiple genetic modifications, but it is unlikely that somatic cells would divide for a sufficient length of time to allow for more than one genetic modification to be completed (Kasinathan et al., 2001a). It has

been proposed that consecutive rounds of cloning, also referred to as "serial cloning", allow for rejuvenation and selection of transformed cultured cells (Hill et al., 2000; Hill et al., 2001; Liu et al., 2001; Kuroiwa et al., 2004) and that it may improve the efficiency of SCNT by increasing the reprogramming potential of the somatic cells (Cho et al., 2007; Fujimura et al., 2008). Conversely, other reports suggest that developmental capacity is reduced for bovine embryos derived after several rounds of serial cloning (Peura et al., 2001; Kubota et al., 2004). Serially cloned mice, up to the sixth generation showed no signs of telomere shortening or premature ageing. However, cloning efficiency significantly decreased with increasing rounds of cloning (Wakayama et al., 2000). It has been suggested that extended *in vitro* culture associated with transfection and selection procedures may induce changes of somatic cells, which decrease the efficiency of nuclear transfer and that these changes cannot be reversed by recloning (Zakhartchenko et al., 2001).

Although the cellular and molecular events that take place during nuclear reprogramming are simultaneous and integrated they will be studied separately in the present review, for the sake of simplicity. First, the review focuses on the reprogramming machinery of the oocyte and the changes in chromatin structure that occur after fertilization and nuclear transfer. The second part of the review deals with epigenetic modifications including DNA methylation, gene imprinting, and X-chromosome inactivation, and their alterations after nuclear transfer. The expression patterns of genes that are crucial for embryonic development are discussed, focusing on the differences among embryos produces by fertilization and those produced by nuclear transfer. Finally,

the review describes the current strategies used for improving nuclear reprogramming and the future application of these to enhance cloning efficiency.

2.4 Reprogramming Factors in the Oocyte

Erasing the epigenetic marks of a somatic nucleus is a complex process that requires global changes in DNA methylation, chromatin structure, gene imprinting, X chromosome inactivation, and restoration of telomere length (Han et al., 2003b). A somatic cell cannot reprogram its own epigenome. However, the egg is a cell with "extensive experience" in reprogramming the genome of other cells. Once a sperm enters the oocyte during fertilization, its nucleus is surrounded by the oocyte machinery designed to reprogram the paternal genome. Although the entire process is not completely understood, it is known that sperm reprogramming involves remodeling of chromatin through removal of protamines and replacement by maternal histones. This event is closely followed by genome-wide demethylation to create the basis for proper gene regulation during embryogenesis (Reik et al., 2001; Santos et al., 2002; Santos et al., 2005). The oocyte genome is also subjected to demethylation and chromatin remodeling, but it happens after several cleavage divisions by a replication-dependent mechanism, based on the loss of maintenance methylase activity (Mayer et al., 2000). The analysis of methylation reprogramming in uniparental (parthenogenetic, gynogenetic, and androgenetic) embryos indicates that the reprogramming machinery in the egg cytoplasm treats the paternal and the maternal genomes in markedly different ways (Barton et al., 2001).

The same machinery that reprograms the sperm and oocyte genomes is the one responsible of erasing the "cellular memory" and reprogramming the donor nucleus after SCNT. However, since spermatozoa and somatic cells have such different chromatin structure and DNA methylation patterns, it is understandable that the oocyte may not reprogram a somatic nucleus with the same efficiency it reprograms the sperm DNA. Somatic nuclear reprogramming is delayed and incomplete when compared to sperm nuclear reprogramming (Latham, 2005). It can be argued that that the reprogramming of a somatic genome resembles the reprogramming of the maternal pronucleus undergoing a gradual replication dependent demethylation. The nuclear reprogramming event caused by SCNT could be considered a transdifferentiation process that implies the molecular dominance of one distinct cell type (the oocyte cytoplasm) over another (the somatic nucleus), resulting in transformation of the somatic nucleus into a totipotent nucleus (Western and Surani, 2002). The epigenetic marks in cloned embryos, fetuses, and adults from several species do not always correlate to those of their counterparts produced by fertilization. The low efficiency of cloning and the high levels of early and later embryonic lethality suggest that epigenetic reprogramming after SCNT is a complex process and its failure could result in fundamental and systematic errors (Dean et al., 2003; Jouneau and Renard, 2003).

Several different outcomes of SCNT have been observed ranging from embryos that fail to develop, up to the few cloned animals that have reached adulthood with no evident pathologies. Between these two distinct outcomes, there is a range of cloned animals that reach different stages. Some cloned embryos die during the earlier or later stages of pregnancy, while some make it all the way to term, but die during the perinatal

period. These outcomes could be the manifestation of different degrees of nuclear reprogramming. The first one could be a complete failure of the oocyte machinery to break down the nuclear membrane and reprogram the somatic chromatin, which would result in death of the NT embryo. A partial reprogramming of vital genes for initial development, would allow initial survival and development of the clone through the first developmental stages resulting later in an abnormal phenotype or lethality. Finally, a complete reprogramming would produce a normal animal (Rideout et al., 2001). The results from hundreds of SCNT experiments indicate that complete reprogramming happens only in a small proportion of the nuclear transfers (Panarace et al., 2007).

Oocytes are not the only cells capable of reprogramming the genome of other cells. Pluripotential embryonic stem (ES) cells, which are derived from the inner cell masses of blastocysts, have an intrinsic capacity for reprogramming nuclei of somatic cells. *In vitro* hybridization of somatic cells with ES cells leads to reprogramming of the somatic cells. The pluripotency of the ES-somatic hybrids has been proven as they contribute to all three primary germ layers of chimeric embryos (Beddington and Robertson, 1989; Nagy et al., 1993). The somatic pattern of DNA methylation is maintained in the hybrids, indicating that ES cells only have the capacity to reset certain aspects of the somatic cell epigenome (Tada et al., 2001; Tada et al., 2003). The use of ES cells will contribute to elucidating the mechanisms of epigenetic reprogramming involved in DNA and chromatin modifications (Tada and Tada, 2006). Individual oocyte and ES cell reprogramming factors are being used in cell-free reprogramming extracts. These and other agents that could improve the efficiency of nuclear reprogramming will be discussed later in the review.

2.5 Epigenetic Regulation of Development

2.5.1 Chromatin Remodeling in Early Embryonic Development

The basic unit of chromatin is the nucleosome, which is comprised of 147 base pairs of DNA wrapped around an octamer of histones, formed by pairs of each of the four core histones (H2A, H2B, H3, and H4). Each nucleosome is linked to the next by small segments of linker DNA. Chromatin is further condensed by winding in a polynucleosome fiber, which may be stabilized through the binding of histone H1 to each nucleosome and to the linker DNA (Wade and Kikyo, 2002). Enzymatic modifications of histones include phosphorylation, methylation, acetylation and ubiquitination, or the removal of these modifications (Nakao, 2001). These modifications are recognized by other structural proteins and enzymes, which together may stabilize the pattern of gene expression.

Little is known about the initial molecular events that ensure nuclear reprogramming in the mammalian oocyte. A significant proportion of the knowledge we have about nuclear reprogramming comes from the transfer of mammalian somatic cells into frog oocytes (Byrne et al., 2003) which, due to their size and availability, represent an appropriate system for the study of nuclear reprogramming. A number of structural proteins and enzymes that modify chromatin structure have been identified and are principal candidates for regulating early reprogramming events. Within an hour of the nuclear transfer, the mammalian somatic nuclear membrane breaks down, mimicking the breakdown of the sperm nuclear envelope after fertilization (Gao et al., 2004). The second event after SCNT appears to be condensation of the somatic cell chromosomes

upon exposure to the M-phase ooplasm, which directs the formation of a new spindle (Wakayama et al., 1998). In *Xenopus laevis*, somatic nuclei lose more than 85% of their own proteins when transferred into an enucleated oocyte, while simultaneously incorporating a substantial amount of protein from the cytoplasm (Gurdon et al., 1979). Oocyte activation leads to the formation of "pseudopronuclei" that resemble the pronuclei formed after fertilization but contain a random assortment of maternal and paternal chromosomes. Often two "pseudopronuclei" are formed but the formation of only one or more than two has been observed in mice (Latham et al., 2007). The successful union of the pseudopronuclei occurs at the first mitotic division, as it does in normal fertilized embryos (Latham, 2005). After SCNT, a global transcriptional silencing has been observed in mouse, cattle and rabbit clones (Latham et al., 1994; Kanka et al., 1996; Winger et al., 2000), followed by reappearance of the first signs of transcriptional activity by the two-cell stage, resembling embryonic genome activation after fertilization (Latham et al., 1994; Rideout et al., 2001).

The linker histone H1 may be involved in the regulation of gene expression during embryogenesis (Clarke et al., 1998). Somatic H1 is lost from most mouse nuclei soon after transfer. The rate of loss depends on the cell cycle stage of donor and recipient cells (Bordignon et al., 2001). Bovine linker histone H1 becomes undetectable in somatic nuclei within 60 minutes after injection into bovine oocytes, and is completely replaced with the highly mobile oocyte-specific linker histone variant H1FOO (Gao et al., 2004; Teranishi et al., 2004), suggesting an important role for linker exchange in nuclear chromatin remodeling. Histone 1 remains absent or in very low concentration in early cloned embryos, but becomes detectable at the 8- to 16-cell stage, when major

transcriptional activation of the embryonic genome occurs. At these stages, the oocyte molecules are replaced by the embryo derived H1 in a similar fashion to what happens in normally fertilized embryos (Bordignon et al., 1999). It seems that nucleoplasmin, along with other proteins in the oocyte, are involved in the H1 removal (Wade and Kikyo, 2002). In contrast, core histones of somatic nuclei, especially H3 and H4, are not removed, but are stably associated with the somatic DNA (Weisbrod et al., 1982; Misteli et al., 2000).

Histone tails are subjected to a wide range of postranslational modifications, including acetylation, phosphorylation, and methylation, which are also implicated in transcriptional silencing. Acetylated lysines on core histones (H3K9, H3K14, H4K16) of somatic cells have been observed to be quickly deacetylated following SCNT. Their reacetylation was observed following activation treatment in cloned mouse embryos. However, the acetylation of other lysine residues on core histones (H4K8, H4K12) persist in the genome of cloned embryos with only mild deacetylation occurring in the process of SCNT and activation treatment (Wang et al., 2007). In somatic cells, transcriptionally active 5S rRNA genes are packaged with hyperacetylated histone H4. In contrast, the silent oocyte 5S rRNA genes are associated with hypoacetylated histone H4, suggesting that hyperacetylation of histone H4 is necessary for transcriptional activity (Howe et al., 1998). It could be argued that after SCNT, the cloned embryos establish a histone acetylation pattern that partially resembles that of embryos produced by fertilization. The same has been reported for histone phosphorylation. Histone H3–S10 and H3–S28 were phosphorylated and dephosphorylated in the somatic chromatin in a manner paralleling the changes in oocyte chromosomes (Bui et al., 2006).

Histone acetylation and DNA methylation are tightly coupled through protein complexes containing DNA methyltransferase, DNMT1, and histone deacetylases, HDAC (Robertson et al., 2000) (Rountree et al., 2000). The role of the DNA cytosine-like 5-methyltransferase (DNMT3L) protein in the activation of Histone deacetylase 1 (HDAC1) (Deplus et al., 2002; Turek-Plewa and Jagodzinski, 2005) and targeting of unmethylated lysine on histone 3 tails (H3K4) (Ooi et al., 2007), provides another link between DNA methylation and histone acetylation that will be discussed later.

Along with histones, a number of non-histone nuclear proteins are also actively released from or incorporated into somatic chromatin after nuclear transfer (Kikyo et al., 2000). One such example is the basal transcription factor TATA binding protein (TBP) that is released from somatic chromatin by a chromatin remodeling protein complex (ISWI, a member of the SWI2/SNF2 super family) in the oocyte cytoplasm (Kikyo et al., 2000; Wade and Kikyo, 2002). The helicase activity of these multisubunit ATP-dependent enzymes unwinds DNA and redistributes nucleosomes in a tissue-specific manner (Nakao, 2001). The loss of a principal component of the basal transcriptional complex from somatic nuclei incubated in frog oocyte extract, provided the first indication that members of the SWI/SNF family of enzymes may have roles in the development of cloned embryos (Kikyo et al., 2000; Wilmut et al., 2002). Members of the high mobility group proteins (HMG), particularly those corresponding to the Nucleosomal subfamily (HMGN), are also actively removed from chromatin and later incorporated into it (Phair and Misteli, 2000; Shirakawa et al., 2000). These results suggest that the exchange of chromatin proteins between somatic nuclei and oocyte cytoplasm are similar to the physiological protein exchange that occurs after fertilization.

A schematic representation of the factors involved in nuclear reprogramming and chromatin remodeling molecules after nuclear is presented in Figure 2.1.

The report of shortened telomere length in Dolly the sheep lead the authors to conclude that "nuclear transfer does not restore telomere lengths" (Shiels et al., 1999). However, restoration or elongation of telomere length after SCNT has been documented in several species (Wakayama et al., 2000; Betts et al., 2001), even when senescent fibroblasts with drastically shortened telomeres were used as donor cells (Lanza et al., 2000). The length of telomeres, appears to be efficiently reprogrammed following NT suggesting that telomere shortening is not significantly impeding development of clones (Hochedlinger and Jaenisch, 2002). Serial cloning up to the sixth generation was performed in mice. Although no signs of telomere shortening or premature ageing were observed, cloning efficiency significantly decreased with increased rounds of cloning (Wakayama et al., 2000).

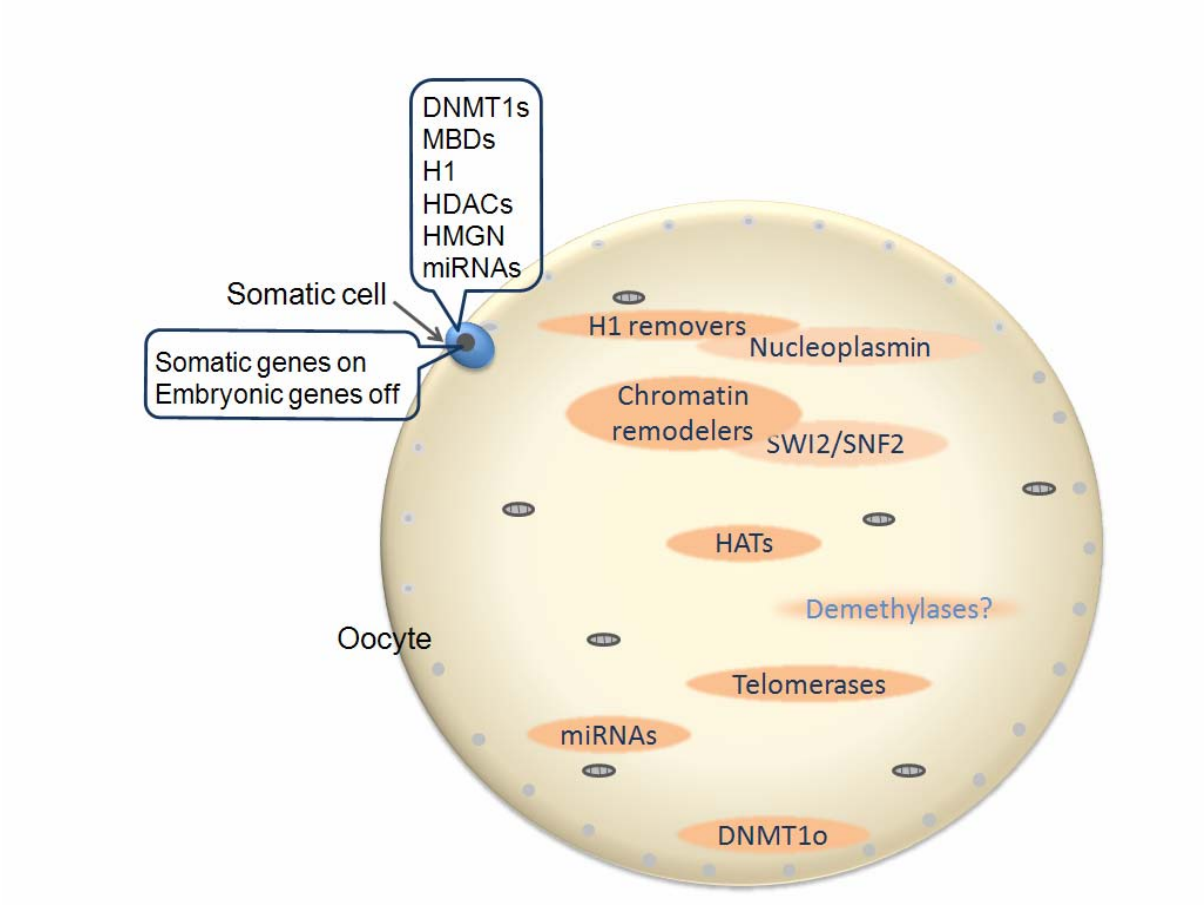

Figure 2.1 **Schematic representation of oocyte factors that participate in chromatin remodeling and reprogramming of the somatic nucleus.**

2.5.2 DNA Methylation in Early Embryonic Development

In mammalian cells, stable silencing of genes is frequently correlated with DNA methylation of promoter regions, along with specific modifications in the N-terminal tails of histones. DNA methylation is restricted to cytosine (C) residues in CG dinucleotides. DNA methylation is the most studied epigenetic mechanism used by the cell for the establishment and maintenance of a controlled pattern of gene expression (Quina et al., 2006). DNA methylation provides a genome-wide means of regulation, usually associated with the inheritance of lineage-specific gene silencing between cell generations (Robertson and Wolffe, 2000). The patterns of DNA methylation are distinct for each cell type and confer cell type identity (Szyf, 2005a). With few exceptions, unmethylated DNA is associated with an active chromatin configuration while methylated DNA is associated with inactive chromatin (Szyf, 2005b).

DNA methylation is accomplished by four DNA methyltransferases. The first DNA methyltransferase to be discovered, DNMT1, maintains the methylation pattern following DNA replication by using the parental DNA strand as a template to methylate the daughter DNA strand. This means that CG sequences paired with methylated CG dinucleotides are methylated by DNMT1 (Bestor et al., 1992; Pradhan et al., 1999). DNMT2, the smallest mammalian DNA methyltransferase, contains only the five conserved motifs of the C-terminal domain. Its function in DNA methylation has been enigmatic (Yoder and Bestor, 1998; Dong et al., 2001). While some studies report that DNMT2 has a role in DNA methylation (Kunert et al., 2003; Liu et al., 2003b; Tang et al., 2003), others have detected little DNA methylation activity for this enzyme

(Hermann et al., 2003; Rai et al., 2007). Recent research has demonstrated that DNMT2 methylates $tRNA^{Asp}$ in the cytoplasm (Goll et al., 2006; Jeltsch et al., 2006). Other two members of this protein family, DNMT3a and DNMT3b have been identified as *de novo* methyltransferases. These enzymes establish new DNA methylation patterns by adding methyl groups to unmethylated DNA, particularly during early embryonic development and gametogenesis (Okano et al., 1998; Okano et al., 1999).

Prior to fertilization, the genomes of both spermatozoa and oocytes are transcriptionally inactive and highly methylated (Reik et al., 2001). Within hours of fertilization, a dramatic genome-wide loss of DNA methylation has been reported in the male pronucleus (Mayer et al., 2000; Oswald et al., 2000). Several mechanisms have been suggested for the active demethylation of the paternal genome. Firstly, the removal of the methyl group from the cytosine, secondly, the removal of the methyl-cytosine base by glycosylation, and thirdly the removal of a number of nucleotides (excision repair) (Dean et al., 2003). The nature of the mechanisms involved in the active demethylation of the paternal genome remains known. After several cleavage divisions, the female pronucleus is also demethylated. However this process seems to be passively caused by a loss of methyl groups during each round of DNA replication due to the lack of DNMT1 (Mayer et al., 2000; Oswald et al., 2000). The only methylation marks preserved in the embryonic genome are thought to be the ones on the imprinted genes (Oswald et al., 2000; Reik et al., 2001; Young and Beaujean, 2004).

By the blastocyst stage, the embryo is hypomethylated (Monk et al., 1987). New methylation patterns are established, around the blastocyst stage, by the *de novo* DNA

methyltransferases, DNMT3a and DNMT3B, which add methyl groups to unmethylated CG dinucleotides. Once the new patterns of methylation are established, they can be propagated through rounds of DNA replication by DNMT1. Oocytes express an exclusive shorter isoform of DNMT1 called DNMT1o, which lacks 114 amino acids from the N-terminal domain since its translation initiation lies on exon 4 instead of exon 1 (Bestor, 2000). DNMT1o is stored in the cytoplasm of oocytes and early embryos (Ratnam et al., 2002) and only at the eight-cell stage, is transiently translocated to the nucleus (Kurihara et al., 2008). After implantation, maternal DNMT1o is soon replaced by the somatic DNMT1, expressed by the embryonic genome. The absence of DNMT1o from the nucleus during early embryonic development is in accordance with the global hypomethylation of the embryonic genome (Oliveri et al., 2007). Figure 2.2 is a schematic representation of the demethylation of paternal and maternal genomes after fertilization.

DNMT3L is a protein that has been associated with the DNA methyltransferase family although it lacks the methyltransferase motifs and therefore cannot methylate DNA. However, DNMT3L possesses a nuclear localization signal sequence (NLS) that enables its translocation to the nucleus and DNA binding. Recent results have shown that the C-terminal domain of DNMT3L interact with DNMT3A forming a dimer. The *de novo* methylation activity of DNMT3A, depends upon its dimerization with DNMT3L (Jia et al., 2007). Additionally, DNMT3L has a Plant Homeodomain (PHD) like motif that activates Histone deacetylase 1 (HDAC1) (Deplus et al., 2002; Turek-Plewa and Jagodzinski, 2005) and recognizes histone H3 tails that are unmethylated at lysine 4 (Ooi

et al., 2007). Thus, DNMT3L has a dual role in *de novo* DNA methylation, activating DNMT3A and interacting with unmethylated H3 (Jia et al., 2007).

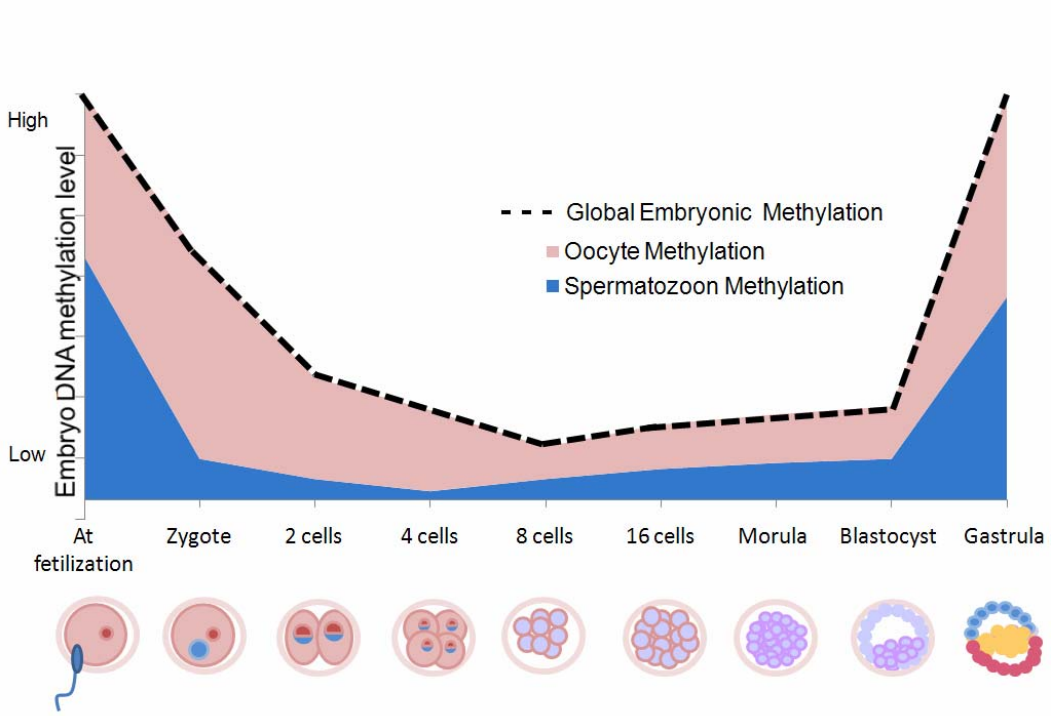

Figure 2.2 Schematic representation of the changes in DNA methylation in bovine mature gametes and early embryos.

Note: DNA methylation is shown as arbitrary units in the Y axis. The DNA methylation level of the preimplantation embryo is the sum of the spermatozoon and oocyte methylation. The sperm genome undergoes active demethylation, while the oocyte genome undergoes passive demethylation throughout several cell divisions. After the 8-cell stage a small wave of *de novo* methylation is observed. By the blastocyst stage, the DNA methylation level in the trophectoderm cells is markedly lower compared to cells of the inner cell mass ICM. At the peri-gastrulation stage DNA methylation is regained in the entire embryo.

The exact biological function of this dynamic reprogramming of DNA methylation in early development is unknown. Several studies demonstrate that DNA methylation is crucial for the establishment of gene expression during embryonic development (Eden and Cedar, 1994; Jones et al., 1998). However, other studies suggest that DNA methylation may only affect genes that are already silenced by other mechanisms in the embryo, indicating that DNA methylation could be a consequence rather than a cause of gene silencing during development (Nan et al., 1998; Walsh and Bestor, 1999; Bestor, 2000). Mutations in either the maintenance or the de novo methyltransferases result in early embryonic death in mice (Li et al., 1992; Young and Beaujean, 2004), indicating that the establishment and maintenance of appropriate methylation patterns are crucial for normal development. For many years, it was believed that the established methylation pattern was reliably and irreversibly maintained for the life of the organism (Szyf, 2005b). However, recent data suggests that DNA methylation is reversible and can change in response to intrinsic and environmental signals (Ramchandani and McConachie, 2005). Modulation of DNA methylation during early embryogenesis is a dynamic process that is developmentally regulated.

The study of DNA methylation after SCNT has shown that somatic cell chromatin undergoes only limited demethylation after SCNT (Fulka and Fulka, 2007). Embryos derived from nuclear transfer have an abnormal pattern of chromatin methylation, which in some cases resembles that of donor cells and is retained through several cell divisions in cloned embryos (Fairburn et al., 2002). The somatic-like methylation pattern maintained in cloned embryos up to the 4-cell stage indicates that active demethylation is

absent in nuclear transfer (Bourc'his et al., 2001). Other studies suggest that cloned embryos undergo active demethylation, but lack passive demethylation (Dean et al., 2001). It has also been reported that *de novo* DNA methylation starts precociously at the 4- to 8-cell stage in cloned embryos. By the 8 to 16-cell stage, cloned embryos showed a heterogeneous methylation pattern with some nuclei appearing hypomethylated and others hypermethylated. By the blastocyst stage, most nuclear transfer embryos seem to establish a global DNA methylation level comparable to that of embryos produced by fertilization. However, abnormally high methylation patterns are detected in some regions of the genome (Kang et al., 2001; Han et al., 2003b; Beaujean et al., 2004). Figure 2.3 is a schematic representation of the level of DNA demethylation after nuclear transfer as compared to the one occurring in embryos produced by fertilization.

It is not clear to what extent the DNA methylation pattern observed during normal development needs to be mimicked for cloning to succeed. Individual blastocysts display significant alterations in the methylation pattern. However, such aberrant reprogramming of DNA methylation does not seem to be lethal since several of the cloned embryos with hypermethylated DNA undergo development beyond the blastocyst stage (Bourc'his et al., 2001; Dean et al., 2001; Kang et al., 2001).

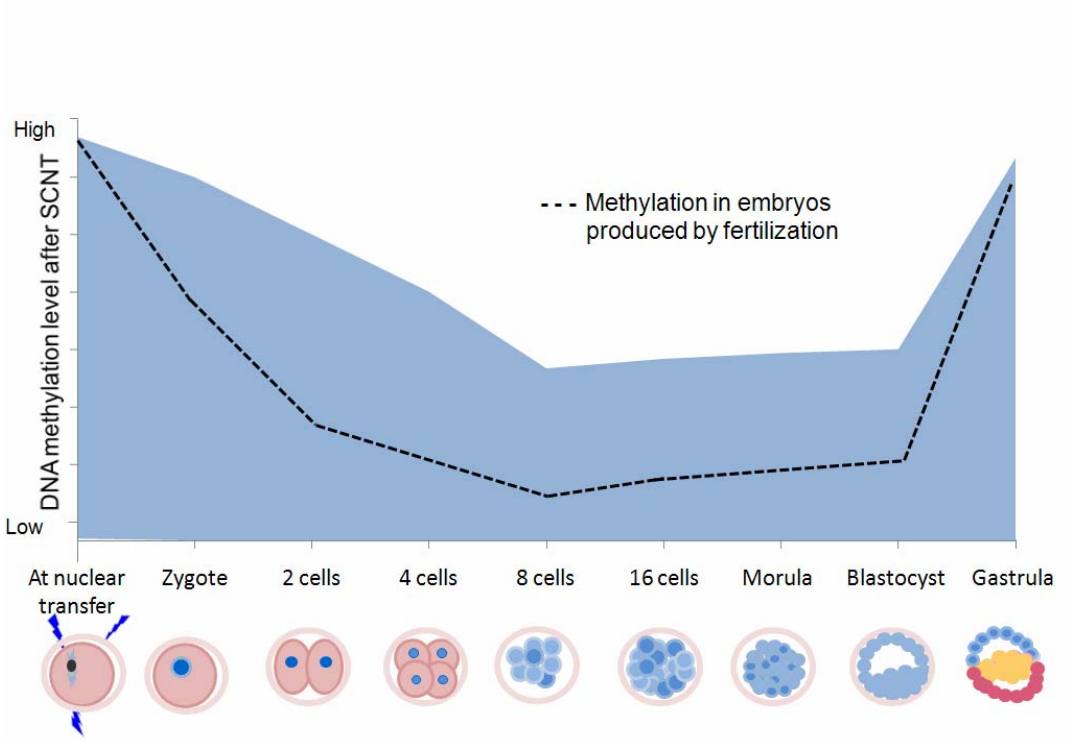

Figure 2.3. Schematic representation of the changes in DNA methylation in a somatic nucleus after nuclear transfer.

Note: DNA methylation is shown as arbitrary units in the Y axis. The extent of DNA demethylation of a somatic nucleus after SCNT is incomplete, compared to that of embryos produced by fertilization (dashed line). Although by the 8- to 16-cell stage the DNA methylation level of the cloned embryo has decreased considerably, the pattern of methylation is heterogeneous in the blastomeres. The trophectoderm and ICM cells of cloned blastocysts have similar methylation levels, unlike the differential methylation observed in embryos produced by fertilization.

Variation in imprinted gene expression has been observed in cloned mice. Interestingly, many of these animals survive to adulthood despite widespread gene misregulation, indicating that mammalian development may be rather tolerant to certain levels of epigenetic aberrations of the genome (Humpherys et al., 2001). These data imply that even apparently normal cloned animals may have subtle abnormalities in their DNA methylation pattern. Conversely, other studies have inversely correlated aberrant DNA methylation with the developmental potential of the cloned embryos (Santos et al., 2003).

In female mammalian embryos at about the morula stage, nearly all genes in one of the two X chromosomes are inactivated by a dosage compensation mechanism known as X-chromosome inactivation (XCI) (Lyon, 1961). In fetal tissues this inactivation is random; in some cells the inactivated X chromosome is paternal, while in others it is maternal. However, in the trophectodermal cells, the paternal X-chromosome seems to be the only inactivated one (Heard et al., 1997). Female embryos, obtained by nuclear transfer, receive a somatic nucleus, which already has one inactivated X chromosome. The recipient enucleated oocyte has to transiently activate the inactive X chromosome so that the embryo can later accomplish the random X chromosome inactivation that occurs in normal embryos. XCI has been monitored in cloned mouse embryos to study the reprogramming of a somatic female nucleus. Normal XCI patterns have been reported in cloned female tissues. Cloned female mice obtained from somatic cells with a transcriptionally 'inactive' paternal X-chromosome showed a random X-chromosome inactivation with an active paternal X-chromosome in some cells and an inactive one in

other cells (Yanagimachi, 2002). However, the trophectoderm cells maintained the inactivation of the X chromosome that was silent in the somatic cell, even when it was the maternal one (Eggan et al., 2000). Similar results have been reported for live bovine cloned calves. Additionally, aberrant XCI patterns have been detected in fetal and placental tissues from deceased cloned bovine and mice fetuses (Ohgane et al., 2001; Xue et al., 2002). Other studies have found significant failures in XCI in cloned mice and pigs (Nolen et al., 2005; Jiang et al., 2008). To date, the role of abnormal XCI in the low efficiency of cloning is not clear.

2.6 Gene Expression in SCNT derived Embryos

Differentiated cells have cell-specific gene expression. Genes transcriptionally active in one type of cell, for example, may be silenced in another cell type. There are genes, not all of them identified yet, whose activation means the difference between development and failure in a cloned embryo. These genes ensure blastocyst formation, implantation, and development to term, and their expression is the result of chromatin remodeling and DNA methylation modifications. These modifications not only ensure the activation of embryonic genes associated with a state of totipotency, but also the down-regulation of somatic genes that are not necessary and could even be detrimental for the embryo.

The global transcriptome profile of cloned embryos, relative to that of donor cells and embryos produced by fertilization has been studied using microarray technology.

Global alteration of gene expression has been reported in cloned embryos, which may present upregulation of donor cell-specific genes (Ng and Gurdon, 2005). Abnormal expression of genes playing important roles in early embryonic development, implantation and fetal development is of particular interest. The expression of imprinted genes was abnormal in cloned blastocyst at three levels: total transcript abundance, allele specificity of expression, and allelic DNA methylation. This study reported methylation and gene expression abnormalities for nearly all embryos and despite their morphological quality with considerable heterogeneity among individual embryos (Mann et al., 2003). These observations indicate that epigenetic marks associated with imprinted genes are not faithfully retained in the majority of cloned embryos. The low proportion of embryos exhibiting a comparatively normal pattern of imprinted gene expression at the blastocyst stage is consistent with the proportion of live-born clones.

Conversely, other studies have reported a significant reprogramming of SCNT embryos by the blastocyst stage and transcriptome profiles comparable to those of embryos produced *in vitro* or *in vivo*, suggesting that defects in gene expression for SCNT embryos may occur later during redifferentiation and/or organogenesis (Smith et al., 2005; Somers et al., 2006; Yu et al., 2007). Identifying key genes responsible for the general developmental failure in cloned embryos is not an easy task, since the alterations may be caused by a variety of factors including donor cell type, cell cycle stage, nuclear transfer protocol, source of the oocytes, embryo culture system, embryo transfer procedure, management of recipient cows, and operators' skills (Oback and Wells, 2007a). Consequently, there is a big variety of alterations that are not shared by all cloned embryos. The common thread uniting many of the SCNT failures can be traced to

epigenetic alterations, specifically failures in chromatin remodeling and DNA and histone methylation (Vignon et al., 2002; Santos et al., 2003; Suteevun et al., 2006).

POU5F1, the gene, encoding the transcriptional regulator Oct4, which is induced in somatic nuclei after nuclear transfer, has been one of the more studied markers of pluripotency (Nichols et al., 1998; Byrne et al., 2003; Westphal, 2005). Demethylation of the Oct4 promoter precedes reprogramming and is a prerequisite for its activation (Simonsson and Gurdon, 2004). Some studies have reported POU5F1 misregulation in SCNT embryos (Boiani et al., 2002; Beyhan et al., 2007a), while others report it at the expected concentration (Daniels et al., 2000; Smith et al., 2007). POU5F1 mRNA levels were comparable in bovine cloned embryos and embryos produced by *in vitro* fertilization (Rodriguez-Osorio et al., 2008). No significant difference in POU5F1 mRNA levels among cloned blastocysts and blastocysts produced by *in vitro* fertilization and artificial insemination were detected by microarray analysis and real-time PCR (Zhou et al., 2007). Another gene that has been extensively studied, due to its implication in the large offspring syndrome (LOS), is the imprinted gene insulin-like growth factor 2 receptor (IGF2R) (Lazzari et al., 2002). This gene has shown altered expression values in embryos produced *in vitro* and a marked misregulation in cloned embryos (Han et al., 2003a; Yang et al., 2005).

Several genes have been reported to be abnormally expressed in bovine cloned embryos including IL6, FGF4, FGFr2, FGF4, DNMT1, Mash2, HSP70, interferon tau, histone deacetylases and DNMT3A (Daniels et al., 2000; Niemann et al., 2002; Beyhan et al., 2007a). Oligonucleotide microarray analysis and Real Time PCR showed that developmentally crucial genes such as Desmocollin 3 (DSC3), a transmembrane

glycoprotein involved in cell adhesion, and the high mobility group nucleosomal binding domain 3 (HMGN3) were significantly downregulated in cloned bovine embryos compared to *in vitro* produced embryos (Rodriguez-Osorio, article in press). The same study found a significant downregulation in the signal transducer and activator of transcription 3 (STAT3) in cloned bovine blastocysts, contrary to a report of upregulation of this gene in cloned blastocyst (Zhou et al., 2007). The importance of these genes during morula and blastocyst formation could make them good candidates in understanding the poor developmental rates of cloned embryos. The lack of consistency in the pattern of gene expression of some genes in different SCNT studies makes it difficult to pinpoint the genes that are consistently misregulated after cloning. A recent study reported abnormal expression of DNMTs, interferon tau (INFT) and major histocompatibily 1 complex class 1(MHC1 1) transcripts in the majority of cloned bovine embryos. This study reports a downregulation of DNMT3B in the majority of cloned embryos on day 7 (Giraldo et al., 2008). Conversely, a significant upregulation in DNMT3A and DNMT3B transcripts was indentified in cloned bovine embryos compared to their *in vitro* produced counterparts (Rodriguez-Osorio, article in press). The role of DNMT3A and DNMT3B in *de novo* methylation could link these enzymes with the, already discussed, high methylation levels of cloned embryos. The lack of consistency in the pattern of gene misregulation in cloned embryos in different studies has lead several authors to suggest that nuclear reprogramming after somatic cell nuclear transfer is stochastic in nature. According to this hypothesis, the number and the role of misregulated genes determine the fate of each cloned embryo.

2.7 Alternatives for Improving Nuclear Reprogramming

Improving the efficiency of SCNT is directly related to knowledge about molecular reprogramming which is important for embryo formation and development after nuclear transfer. Ooplasmic factors contributing to nuclear reprogramming are being sought in hope of improving the outcome of SCNT and providing a better understanding of mammalian embryogenesis (Sutovsky and Prather, 2004). A DNA demethylation agent, 5-aza-29-deoxycytidine (5-aza-dC a derivative of the nucleoside cytidine), has lowered DNA methylation and induced overexpression of imprinted genes in mouse embryonic fibroblast cells by lowering DNA methylation levels (Eilertsen et al., 2007). Treatment of donor cells with 5-azacytidine prior to nuclear transfer, may remove epigenetic marks and improve the ability of somatic cells to be fully reprogrammed by the recipient karyoplast (Enright et al., 2003). Unfortunately, 5-aza-dC reduced blastocyst formation of cloned embryos (Tian et al., 2003).

A histone-deacetylase inhibitor, trichostatin A (TSA) enhances the pool of acetylated histones and induces overexpression of imprinted genes in embryonic stem cells (Yoshida et al., 1990; Wakayama, 2007). Demethylation of H3K9 tri- and dimethylation might be crucial for further development of cloned embryos. Whether this histone H3K9 demethylation is correlated with active DNA demethylation needs to be investigated further (Wang et al., 2007). Treatment of cloned embryos with TSA could affect the histone acetylation reprogramming.

No reports are yet available on how treating donor cells with these agents would affect the development of cloned embryos to term. Reducing methylation by knocking-

down DNMT1 gene expression using small interfering RNA (siRNA) technology has been applied to a bovine donor cell line with an approximately 30–60% decrease in global DNA methylation. Demethylated cells were used subsequently for SCNT, which doubled blastocyst rates suggesting that demethylation prior to NT may be beneficial for NT-induced reprogramming (Eilertsen et al., 2007).

Decondensation of sperm chromatin in eggs is achieved by the replacement of sperm-specific histone variants with egg-type histones by the egg protein nucleoplasmin. Nucleoplasmin can also decondense chromatin in undifferentiated mouse cells without overt histone exchanges but with specific epigenetic modifications that are relevant to open chromatin structure. These modifications included nucleus-wide multiple histone H3 phosphorylation, acetylation of Lysine 14 in histone H3, and release of heterochromatin proteins HP1beta and TIF1beta from the nuclei. At the functional level, nucleoplasmin pretreatment of mouse nuclei facilitated activation of four oocyte-specific genes (Tamada et al., 2006). Nucleoplasmin injected into bovine oocytes after nuclear transfer resulted in apparent differences in the rates of blastocyst development and pregnancy initiation. Over 200 genes were upregulated in nucleoplasmin treated cloned embryos, several of which were previously shown to be downregulated in cloned embryos when compared to bovine IVF embryos (Betthauser et al., 2006). These data suggest that addition of chromatin remodeling factors, such as nucleoplasmin, to the oocyte may improve development of NT embryos by facilitating reprogramming of the somatic nucleus.

Nuclear and cytoplasmic extracts that can transform one cell type into another have been used as reprogramming factors. The procedure involves the permeabilization

of a somatic cell (the "donor" cell) in a nuclear and cytoplasmic extract derived from another somatic "target" cell (Hakelien et al., 2006a). The reprogramming ability of these extracts has been evidenced by nuclear uptake and assembly of transcription factors, induction of activity of a chromatin remodeling complex, changes in chromatin composition, and expression of new genes (Hakelien et al., 2006b). These systems are likely to constitute a powerful tool to examine the process of nuclear reprogramming. In addition, cell-free extracts create possibilities for circumventing human SCNT (which raises ethical, moral and legal issues) by producing replacement cells for therapeutic applications (Collas, 2003). The recently reported use of four transcription factors (Oct4, Sox2, Klf4, and c-Myc) (Yu et al., 2007) to produce pluripotent stem cells raises the question of whether nuclear transfer is still necessary for the achievement of stem cells for therapeutic purposes (Cibelli, 2007a). Nevertheless, the therapeutic use of such induced pluripotent stem cells is not known yet.

A recent hypothesis suggests that failure in the oocyte reprogramming machinery to target the paternal genome of the somatic nucleus originates an unbalanced nuclear reprogramming between parental chromosomes. These authors suggest that exogenous expression in donor somatic cells of a sperm chromatin remodeling proteins, particularly the BRomo Domain Testis-specific protein (BRDT), could induce a male-like chromatin organization of the somatic genome (Loi et al., 2008). The real advantages of such a method remain to be observed since both the paternal and the maternal genomes, present in the somatic nucleus, need to undergo reprogramming after nuclear transfer.

In addition to the multiple proteins that participate in chromatin remodeling and DNA methylation, oocytes contain microRNAs (miRNAS) that regulate the expression of

genes by inhibiting translation (Bartel, 2004). Several specific miRNas have been isolated from *Xenopus* (Watanabe et al., 2005), Drosophyla (Nakahara et al., 2005), and mouse oocytes (Tang et al., 2007). The function of miRNAs during early development is not known yet, but their importance in early embryo development is supported by the fact that mouse oocytes lacking miRNAs fail to cleave (Tang et al., 2007). Although the exact role of miRNAs in nuclear reprogramming has not been explored, it has been proposed that some developmental failures of cloned embryos might be a consequence of miRNA alteration during nuclear transfer. Enucleation did not seem to remove substantial amounts of oocyte miRNAs, while nuclear transfer significantly increased the oocyte miRNA profile. Some miRNAs that play a role in somatic cells may be capable of regulating the same or different mRNAs with distinct roles in embryogenesis following their introduction to the oocyte by nuclear transfer (Amanai et al., 2006). Further studies should focus on the role of somatic miRNA in early embryonic development.

CHAPTER 3

TRANSCRIPTIONAL REPROGRAMMING OF GENE EXPRESSION IN BOVINE SOMATIC CELL CHROMATIN TRANSFER EMBRYOS

3.1 Abstract

Successful reprogramming of a somatic genome to produce a healthy clone by SCNT is a rare event and the mechanisms involved in this process are poorly defined. When serial or successive rounds of cloning are performed, blastocyst and full term rates decline even further with the increasing rounds of cloning. Identifying the "cumulative errors" could reveal the epigenetic reprogramming blocks in animal cloning. Bovine clones from up to four generations of successive cloning were produced by chromatin transfer. Using Affymetrix bovine microarrays we determined that the transcriptomes of blastocysts derived from the first and the fourth rounds of cloning (CT1 and CT4, respectively) have undergone an extensive reprogramming and were more similar to blastocysts derived from in vitro fertilization (IVF) than to the donor cells used for the first and the fourth rounds of chromatin transfer (DC1 and DC4 respectively). However a

set of transcripts in the cloned embryos showed a misregulated pattern compared to IVF embryos. Among the genes consistently upregulated in both CT groups compared to the IVF embryos were genes involved in regulation of cytoskeleton and cell shape. Among the genes consistently upregulated in IVF embryos compared to both CT groups were genes involved in chromatin remodeling and stress coping. The present study provides a unique data set for identifying epigenetic errors in somatic cell chromatin transfer and understanding cell plasticity. Identifying the "cumulative errors" could reveal the epigenetic reprogramming blocks in animal cloning shedding light on the reprogramming process.

3.2 Introduction

The process of early embryonic development is determined by activation of the embryonic genome, which for bovine embryos begins as a “minor genome activation” at the 1-cell stage (Memili and First, 2000) ascending to a “major genome activation” during the 8-cell to 16-cell stage (Whitworth et al., 2004). In the absence of proper genome activation, the developing embryo will die because it can no longer support its essential developmental functions (Latham and Schultz, 2001; Han et al., 2003b). In the case of embryos produced by somatic cell nuclear transfer (SCNT) the somatic nucleus has to be completely reprogrammed in order to restart and continue the developmental process. It is believed that, guided by the ooplasm, the somatic nucleus aborts its own program of somatic gene expression and re-establishes a particular program of embryonic gene expression necessary for normal embryo development (Han et al., 2003b).

Embryos produced by SCNT have lower developmental rates than their *in vitro* and *in vivo* produced counterparts (Vajta and Gjerris, 2006). They also have a greater incidence of apoptosis and consequently a lower number of cells (Prather, 2007). Cloned embryos have overall greater rates of embryo and fetal mortality, stillbirths and perinatal deaths, which bring down the overall efficiency of cloning and may be caused, at least partially, by incomplete epigenetic reprogramming of the somatic nuclei (Ng and Gurdon, 2005; Vajta and Gjerris, 2006). Somatic cell chromatin transfer (SCCT) attempts to facilitate the reprogramming process by exposing the somatic cells, prior to the transfer, to a mitotic cell extract, which is thought to induce chromosome condensation and promote removal and solubilization of nuclear factors, enhancing nuclear remodeling (Sullivan et al., 2004). Compared to nuclear transfer, SCCT shows greater survival of cloned calves up to at least 1 month and could be a useful tool in understanding the mechanisms of reprogramming. Remarkably, a recent study did not detect any significant differences in the global gene expression profiles of SCCT and SCNT embryos (Zhou et al., 2007).

Embryos derived from nuclear transfer have an abnormal pattern of chromatin methylation, in some cases resembling that of somatic cells (Bourc'his et al., 2001; Dean et al., 2001; Kang et al., 2001). This aberrant DNA methylation pattern has been inversely correlated with the developmental potential of the cloned embryos (Santos et al., 2003). Treatment of donor cells, before the nuclear transfer, with DNA demethylation agents may remove epigenetic marks improving the ability of the somatic cells to be fully reprogrammed by the recipient karyoplast (Enright et al., 2003). Global alteration of gene expression has been another finding in embryos produced by cloning. The abnormal

expression of genes playing important roles in early embryonic development, implantation and fetal development is of particular interest. Conversely, other studies have reported a significant reprogramming for SCNT embryos by the blastocyst stage and similar transcriptome profiles to those of embryos produced in vitro or in vivo, suggesting that defects in gene expression for SCNT embryos may occur later during redifferentiation and organogenesis (Smith et al., 2005; Somers et al., 2006).

Among the abnormally expressed genes reported in bovine cloned embryos are IL6, FGF4, and FGFr2 (Daniels et al., 2000); FGF4, DNMT1, Mash2, HSP70, and interferon tau (Niemann et al., 2002); Acrogranin, Cdx2, and ERR2 (Hall et al., 2005b). Cytokeratin 19, Cytokeratin 8, Vimentin, Hsp27, Nidogen2 and MHC-I (Pfister-Genskow et al., 2005); HDAC-1, 2, and 3, DNMT3A, and OCT4 (Beyhan et al., 2007a). Lower levels of transcripts involved in the retinoic acid signaling pathway (RARB, CRAB1, HLA-A, THBS2, and SERPINB5) were reported for cloned bovine embryos (Beyhan et al., 2007b). There have been conflicting results when it comes to the expression of particular genes in SCNT and IVF embryos. Such is the case of the developmentally important POU5F1 gene, which has been reported as misregulated in cloned embryos compared to IVF derived blastocysts in some studies (Boiani et al., 2002; Beyhan et al., 2007a), while being detected at similar concentration in others (Daniels et al., 2000; Smith et al., 2007).

SCNT is often used for the production of human proteins in the milk of transgenic animals. For the achievement of some specific transgenic phenotypes, multiple genetic modifications should be completed through sequential modifications in primary cells

prior to nuclear transfer. Since transfection and selection of transgenic cells requires nearly the entire lifespan of a cell, only one genetic modification can be completed in each cell lifespan (Wang and Zhou, 2003). Therefore, consecutive rounds of cloning (also referred to “repeated cloning”, “serial cloning”, “recloning” or “nuclear recycling”) are performed in order to facilitate the regeneration and rejuvenation of the cells (Kasinathan et al., 2001a). However, it has been hypothesized that epigenetic errors could accumulate in the embryos as a result of the serial cloning and decrease even more cloning efficiency (Kasinathan et al., 2001a). Serial cloning in mice up to four and six generations showed a general drop in cloning efficiency after the first generation. However no signs of premature ageing, or telomeres shortening were observed in the animals (Wakayama et al., 2000). A greatly reduced in vitro and in vivo developmental capacity was reported for bovine embryos derived after several rounds of serial cloning (Peura et al., 2001; Kubota et al., 2004). Normally appearing fetuses were recovered from a fifth generation of serial cloning and four genetic modifications (Kuroiwa et al., 2004).

The objective of the present study was to identify the "cumulative errors" on global gene expression, caused by serial rounds of SCCT, by comparing the transcriptome profile of IVF derived blastocysts to that of SCCT derived blastocysts from the first and fourth rounds of cloning (CT1 and CT4) using oligonucleotide microarray analysis (Affymetrix Bovine GeneChips). Donor cells used for first and fourth rounds of cloning (DC1 and DC4) were also the target of the study as we compared the global gene expression of the SCCT embryos with their respective donor cells. Additionally, we analyzed the expression patterns of a panel of selected genes, in fetal fibroblasts obtained from fetuses from zero to fifth rounds of chromatin transfer. Our results show that a

substantial reprogramming has taken place in the cloned embryos from both generations of chromatin transfer. However, there was a set of differential expressed genes in both groups of cloned embryos compared to their IVF counterparts. The number and functions of these genes could suggest cumulative misregulations probably caused by the successive rounds of cloning.

3.3 Methods

3.3.1 In Vitro Fertilization (IVF)

Bovine oocytes were aspirated from 2-8 mm follicles of abattoir-obtained ovaries from Holstein cows and matured in Tissue Culture Medium (TCM-199, Gibco/ Invitrogen, Grand Island, NY) supplemented with 0.2 mM pyruvate, 0.5 µg/ml FSH (Sioux Biochemicals, Sioux City, IA), 5 µg/ml LH (Sioux Biochemicals, Sioux City, IA), 10% FCS (Gibco/Invitrogen, Grand Island, NY), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco/Invitrogen, Grand Island, NY) in 5% CO_2 in air at 38.5ºC. For fertilization, matured oocytes were transferred to fertilization medium and were fertilized using thawed sperm from a Holstein bull separated by Percoll density gradient and further incubated for 24 hours. Presumptive zygotes were transferred to Gardner's culture medium 1 (G1) for 3 days, followed by 3-4 days culture in Gardner's culture medium 2 (G2). Blastocysts were evaluated and graded according the International Embryo Transfer Society (IETS) guidelines (Stringfellow and Seidel, 1998). Grade 1 blastocysts were selected, pooled in groups of 3 blastocysts per tube, frozen (with addition of lyses

buffer from RNeasy MicroKit (Qiagen Valencia, CA) in liquid nitrogen and stored in -80ºC until RNA isolation.

3.3.2 Chromatin Transfer

In vitro-matured oocytes were enucleated at 20 hours post maturation (hpm). Bovine fetal fibroblasts after one and four rounds of cloning were trypsinized and washed in Ca/Mg Hank's Balanced Salt Solution (HBSS) and permeabilized by incubation of 50,000 - 100,000 cells in 31.25 units Streptolysin O (SLO-Sigma, St. Louis, MO) in 100 µl for 30 minutes in a 37°C H_2O bath. Permeabilized fibroblasts were washed, pelleted and incubated in 40 µl of mitotic extract prepared from MDBK cells containing an ATP-generating system (1 mM ATP, 10 mM creatine phosphate and 25 µg/ml creatine kinase) for 30 min at 38°C. At the end of incubation, the reaction mix was diluted with 500 µl of cell culture media (Alpha MEM with 10% FBS), pelleted and resuspended in TL Hepes. These cells were fused to enucleated oocytes, activated 26 h after maturation with 5 µM calcium ionophore for 4 min followed by 10 µg/ml of cycloheximide and 2.5µg/ml of cytochalasin D for 5 h. After activation, embryos were washed, and cultured in SOF medium for the first 4 days with 8 mg/ml BSA and the last three days with 10% fetal calf serum at 38.5°C and 5% CO_2 in air. Grade 1 blastocysts were pooled (3 per tube) and frozen, with addition of lysis buffer. Embryos were stored in -80ºC until RNA isolation.

3.3.3 Fourth Generation of SCCT Embryos

For subsequent rounds of cloning, CT derived bovine blastocysts from the first generation were transferred into hormonally synchronized cows. At seventy-days,

pregnancies were interrupted, and fetuses recovered. Fetal fibroblast cultures were established and used for the next chromatin transfer process. The same procedure was done 3 times to provide a fourth generation of clones. Grade 1 blastocysts from the fourth generation were pooled (3 per tube) and frozen, with addition of lysis buffer. Embryos were stored in -80ºC until RNA isolation.

3.3.4 Establishment of Fetal Fibroblast Cell Lines

Seventy-day old male bovine fetuses were recovered and transported to the laboratory in Dulbecco's PBS (DPBS) with 16 ml/ml of antibiotic-antimycotic (Gibco, Grand Island, NY), 4 ml/ml tylosin tartrate (Sigma, St. Louis, MO), and 8 ml/ml fungizone (Gibco). Fetuses were rinsed in DPBS, the head and internal organs were removed, and remaining tissues were finely chopped into pieces with a scalpel blade. The fibroblasts were separated from the tissue pieces using 0.08% trypsin and 0.02% EDTA in PBS (trypsin-EDTA). The cells were seeded onto 100-mm tissue culture plates (Corning, VWR, Chicago, IL) in a minimal essential medium (a-MEM; Gibco) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT), 0.15 g/ml glutamine (Sigma), 0.003% b-mercaptoethanol (Gibco), and antibiotic-antimycotic (Gibco). On the same day of cloning (day 3 of seeding), the cells were harvested using DPBS with trypsin-EDTA solution and were counted. One million cells were frozen in MEM with 10% FCS, dimethyl sulfoxide (Sigma), and lysis buffer.

3.3.5 RNA Isolation

Total RNA was isolated from IVF blastocysts, SCCT blastocysts, and donor cells using the RNeasy MicroKit (Qiagen Valencia, CA) according to the manufacturer's specifications. Briefly, embryos and cells frozen at -80˚C in lysis buffer were transferred to silica-gel membrane spin columns and washed with RW1 wash buffer and 80% ethanol. Final RNA elution was conducted using 14 µl of RNAse free water provided in the kit. Concentration and purity of isolated RNA were determined using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Integrity and quality were analyzed using a Bioanalyzer 2100 RNA 6000 Picochip kit (Agilent Technologies, Palo Alto, CA).

3.3.6 Microarray

Microarray hybridizations were performed in triplicate for each of the experimental groups using Affymetrix Bovine DNA Chips as described by the manufacturer (Affymetrix Santa Clara, CA). Briefly, complementary DNA (cDNA) synthesis was performed from 10 ng total RNA using the Two-Cycle cDNA Synthesis Kit (Affymetrix Santa Clara, CA). The MEGAscript® T7 Kit (Ambion, Inc.) was used for the first in vitro transcription (IVT). GeneChip IVT Labeling Kit was used for the second IVT and labelling of RNA. Complementary RNA (cRNA) was fragmented and 10 µg of fragmented cRNA were hybridized to the Genechips in a Hybridization Oven, set to 45°C and rotations of 60 rpm for 16 hours. The chips were then washed and stained with streptavidin/phycoerythrin (SAPE) antibody solution using an Affymetrix FS-450 fluidics station. GeneChips were scanned using the Affymetrix GeneChip scanner 3300.

3.3.7 Microarray Data Processing

Images were processed with the Affymetrix GeneChip® Operating Software (GCOS) and expression quantified with MAS 5.0, which also provides information on signal, detection and calculated the detection p-value. Signal information is a numeric value indicating transcript abundance for a particular probe set. Detection information indicates whether the transcript is detected (P, present), undetected (A, absent), or if it is at the limit of detection (M, marginal). Detection p-value indicates the significance of the detection call for a probe set. Only probe sets that were called Present in at least one of the five groups were included in the analysis. A total of 5,599 probe sets were excluded from the analysis as they were called Absent in all groups. The data set for further analysis included 18,396 probe sets.

3.3.8. Hybridization Quality Check

Metrics like noise, background, Scale factor, and the ratio of intensities of 3′ probes to 5′ probes for Actin and GAPDH genes were analyzed for chip quality control. Spiked in controls (*B. subtilis* genes *lys, phe, thr*, and *dap*) were added to the total RNA at known concentrations at the beginning of the experiment. Their intensity values were used to monitor the linear amplification and labeling process. The performance of the hybridization control genes (*E. coli* genes *BioB, BioC* and *BioD* and P1 Bacteriophage *cre*) was also used for determining the quality of each chip.

3.3.9 Microarray Data Analysis

For data visualization, the raw GeneChip signals were uploaded into GeneTraffic UNO (Iobion Informatics LLC), which generated scatter plots of pairwise hybridization comparisons and Heat maps from all hybridizations using hierarchical clustering. Power Atlas, a web-based resource from the University of Alabama at Birmingham, was used to estimating the power of the hybridization given the sample size (Page et al., 2006). HDBStat was used for statistical analysis (Trivedi et al., 2005). Data were quantile-quantile normalized and examined for outliers using Person's correlation. Quality control statistics included a deleted residuals approach (Chen, 2004; Persson et al., 2005; Trivedi et al., 2005). False discovery rates (FDR) for the genes were calculated using t-test (Benjamini et al., 2001). Fold changes were calculated based upon the unadjusted data means in pairwise comparisons. Probe sets in each pairwise comparison with a $p<0.01$, and FDR of <20%, and a Fold Change (FC) in excess of 2.0 were considered to be significant and examined further. For multiple comparisons, One-way analysis of variance (ANOVA) from PROC GLM in SAS 9.1 (SAS Institute inc. Carey, NC) was performed on the complete data set. The Least Significant Difference (LSD) test was used to detect significant differences between groups.

3.3.10 Gene Ontology Annotation

The probe sets corresponding to differentially expressed genes were uploaded into the Affymetrix Netaffx Analysis Center (Bovine GeneChip annotation from November 6 2007) to retrieve updated information regarding gene symbol, gene title, Biological Process (BP), Molecular function (MF), and Cellular Component (CC) (Liu et al.,

2003a). To complement the annotation from Netaffx, we used the GOAnna tool (reference) from AgBase, a Mississippi State University curated, web-accessible resource for functional analysis of agricultural plant and animal gene products (available at http://agbase.msstate.edu/GOAnna.html). For data visualization, all the GO terms associated to each gene were uploaded into GOSlimViewer (available at http://agbase.msstate.edu/GoSlimViewer.html) another AgBase tool that provides a high level summary of the GO categories found in the dataset allowing a better visualization of the data.

3.3.11 Data Modeling

Ingenuity Pathway Analysis 5.0 from Ingenuity Systems was used for data modeling and the analysis of networks related to the generated data sets. Genes upregulated in IVF embryos compared to CT embryos and donor cells (figure 7) and genes downregulated in IVF embryos compared to CT embryos and donor cells (figure 8) were uploaded in the Ingenuity Pathway Analysis 5.0. Since Ingenuity Pathway Analysis database is based on human, mouse, and rat genes, some of the bovine names were not recognized by the software, mostly because of different gene symbols. For those genes, we manually identified the human orthologous symbol.

3.3.12 Real time RT-PCR Gene Expression Analysis

DNA microarray derived gene expression results for genes DNMT3A, DNMT3B, IGF2R, PLAC8, PGR, BIT1, HMGN3, HSPA1A, NGDN, FBXO9, and GNAI2 were

confirmed by Real time PCR using GAPDH as the reference gene. Complementary DNA was generated with the First-Strand cDNA Synthesis system for RT-PCR using SuperScript III Platinum® Two-Step qRT-PCR Kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The samples were incubated for 10 min at 25°C, 50 min at 42°C and at 85°C for 5 min. Then 2U of *E. coli* Rnase H was added to each tube and incubated at 37°C for 20 min. The cDNA was used for quantitative real-time PCR amplification with SYBR Green I chemistry (Roche Applied Sciences, Indianapolis, IN). Real-time quantitative PCR was performed using the LightCyclerTM instrument (Roche Applied Sciences, Indianapolis, IN). The real time PCR reactions were carried out in a total volume of 10 µl according to the manufacturer's manuals for DNA Master SYBR Green I mix (Roche Applied Sciences, IN). The primer concentrations were adjusted to 0.5 µM for each gene. Primers were designed using Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA). Primer sequences used for real time PCR are shown in Table 1. The cycling parameters were 30 seconds at 95°C for denaturation, 50 cycles of 2 seconds at 95°C, 10 seconds at 55°C for amplification (quantification was performed at this step), and 12 seconds at 72°C for extension. The specificity of all individual amplification reactions was confirmed by melting curve analysis. Real-time expression values were calculated through the relative standard curve method, using 10-fold serial dilutions for both the target and the endogenous reference genes by measuring the cycle number at which exponential amplification occurred in a dilution series of samples. Values were normalized to the relative amounts of the control mRNA, which were obtained from a similar standard

curve. In real time PCR reactions, the same initial amounts of target molecules were used, and the Cp values of control mRNA were constant in all samples.

3.3.13 Real time RT-PCR Gene Expression Analysis from Fetal Donor Cells

Donor cell lines included in the study were fibroblasts from non-cloned fetuses (DC0), and fetal fibroblasts from first, second, fourth, and fifth rounds of cloning (DC1, DC2, DC4, and DC5). RNA isolation from donor cells and subsequent cDNA synthesis were performed according to the above mentioned protocols. Relative mRNA abundance was determined for paladin (PALLD), nuclear transcription factor Y alpha (NFYA), glycine amidinotransferase (GATM) and Taspase 1 (C20orf13). Quantitative assessment of RNA amplification was detected by SYBR® GreenER™ qPCR SuperMixes for iCycler (Invitrogen Life Technologies, Carlsbad, CA, 11761-100). Real-time PCR reactions were performed using the iCycler iQ Real-Time PCR instrument (BIO-RAD). The cycling parameters were 50°C for 2 min, 95°C for 8 min 30 s for denaturation, 40 cycles of 15 s at 95°C and 30 s at 60°C and 30 s at 72°C for amplification and extension respectively. The melting curve was performed starting at 55°C with a 0.5°C increase for 10 s in 80 cycles. Expression values were calculated using the relative standard curve method. Standard curves were generated using 10-fold serial dilutions for both GAPDH and 18S ribosomal RNA. Standard curves were also generated for all target genes by measuring the cycle number at which exponential amplification occurred.

3.3.14 Statistical analysis of Real Time PCR results

Results from different groups were analyzed by one-way analysis of variance (ANOVA) by SAS 9.1 (SAS Institute inc. Carey, NC). Differences at $p<0.001$ were considered significant. An additional analysis was performed using Relative expression software tool (REST©, 384-beta version May 2005) to compare all samples of each group. The mathematical model used in the REST software is based on the PCR efficiencies (E) and the crossing point deviation between the samples (CP) (Pfaffl, 2001; Pfaffl et al., 2002; Misirlioglu et al., 2006).

3.4 Results

3.4.1 Isolation of Total RNA

The amount of total RNA isolated from pools of 3 embryos was 12.2 ng (between 3.2 and 4.5 ng per blastocyst). RNA integrity ranged from 1.8 to 1.96, based on the ratio between the 28S and 18S ribosomal RNA bands from the Bioanalyzer gel-like image (Figure 3.1).

3.4.2 Transcriptome analyses

The Affymetrix GeneChip® Bovine Genome Array contains 24,129 probe sets representing over 23,998 bovine transcripts, including assemblies from approximately 19,000 UniGene Clusters. In order to assess the influence of the two cycles of linear amplification, on the representation of original transcripts, we compared microarray

experiments from one-cycle and two-cycle amplifications using total RNA from DC1. The results showed that amplification of messages using 1 *vs.* 2 cycles were highly consistent with a correlation coefficient of 0.93 (data not shown). These data confirm the manufacturer's results using 1 and 2 cycles of linear amplification.

Microarray experiments were performed in three biological replicates for all blastocysts (CT1, CT4 and IVF) and donor cells (DC1 and DC4). Images were processed with GCOS and data extracted using MAS 5.0. However, one of the CT1 blastocyst chips did not pass the quality control analysis (Persson et al., 2005) and was excluded from the study. The analyses for CT1 are based on the remaining two chips in this group, which showed an appropriate p-value distribution. The GCOS software expression data report showed that 56% of the probe sets were called 'present' for all donor cell chips. This number was lower for all blastocyst chips with 44%, 41%, and 47% for IVF, CT1, and CT4 respectively, suggesting that a lower number of transcripts were present in the blastocysts. Hierarchical clustering classified all donor cells chips in one single group indicating small differences in their gene expression profiles. All embryos were classified in 2 distinctive clusters with IVF blastocysts in one group and all cloned blastocysts in other group (Figure 3.2).

Pairwise comparisons among all five groups, included only transcripts with a p-value <0.01, a False Discovery Rate (FDR) of 20%, and a Fold Change >2.0. The numbers of differentially expressed transcripts in all the pairwise comparisons are presented in Table 3.1. In general, the number of probe sets that were differentially expressed between all 3 groups of blastocysts was significantly lower compared to the number of differentially expressed transcripts between donor cells and embryos (P<0.01).

This numeric difference alone may indicate a substantial chromatin reprogramming for blastocysts obtained from first and fourth rounds of cloning. However there were significantly less differentially expressed transcripts between cloned embryos and donor cells than between IVF blastocysts and donor cells ($P<0.01$). Out of 83 differentially expressed transcripts between both cell lines, 79 corresponded to absent or marginal signals, leaving only 4 differentially expressed transcripts. Chemokine binding protein 2 (CCBP2) and myocilin, trabecular meshwork inducible glucocorticoid response (MYOC) were upregulated in DC1 compared to DC4. Similar to hemicentin (LOC528634) and similar to dolichyl pyrophosphate phosphatase 1 (LOC504908) were the genes upregulated in DC4 compared to DC1.

Because the bovine genome has not been fully annotated, the annotation information available from NetAffx Analysis Center (Affymetrix) classifies probe sets as: 1) fully annotated bovine genes; 2) transcripts similar to specific genes, but not confirmed; 3) hypothetical proteins based on sequence similarity; 4) cDNA clones; and 5) transcripts with strong, moderate or weak similarity to genes from other species. Table 3.2 presents a breakdown of the differentially expressed transcripts according to these categories. Only transcripts corresponding to annotated bovine genes were included in further analyses.

Multiple comparisons through one-way analysis of variance (ANOVA) using a Bonferronni correction and Least Significant Differences (Gurdon et al.) showed a set of 109 genes that were differentially expressed in the cloned embryos and donor cells compared to their IVF counterparts. Out of 109 genes, 67 were upregulated in IVF

embryos compared to CT embryos and donor cells (top 30 in Table 3.3). Forty two genes were upregulated in CT embryos (top 30 in Table 3.4).

3.4.3 Functional classification of genes

The Gene Ontology (GO) information for each probe set recovered from NetAffx Analysis Center (Bovine GeneChip November 2007 annotation) was still incomplete for several probe sets, which lacked annotation for at least one of the three ontologies Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). The annotation was complemented with information retrieved using the GOAnna tool part of the AgBase resource at Mississippi State University. All the GO terms associated to each gene were uploaded into the AgBase tool GOSlimViewer in order to obtain a high level summary of the GO categories and create graphs for a better visualization of the data, determining which classes of gene products are over-represented or under-represented on each of the three ontologies for cloned embryos compared to IVF embryos. GOSlimViewer results are summarized in Figures 3.3 through 3.5.

3.4.4 Gene expression analysis by Real Time PCR:

In order to confirm the accuracy of microarray data, the following 11 genes were selected based on their relevance during embryonic development: DNMT3A, DNMT3B, IGF2R, PLAC8, PGR, BIT1, HMGN3, HSPA1A, NGDN, FBXO9, and GNAI2. The expression patterns of the selected genes, obtained by Real time PCR, were consistent with the results from the DNA microarray analysis (Figures 3.6 trough 3.10).

The analysis of gene expression in the cell lines showed that both housekeeping genes, GAPDH and 18S ribosomal RNA, had a similar pattern of expression. The internal standard 18S ribosomal RNA values were 1.5 times greater in all groups than those of GAPDH. After normalization based on both housekeeping genes, there were no differences among the groups for NFYA and Taspase 1 genes. Both G1 and G2 cell lines had significantly greater concentration of PALLD transcript compared to G0, G4 and, and G5. For GATM, the transcript levels of G5 were significantly lower than in all of the other groups (Figure 3.11).

3.4.5 Data modeling

The pathways originated using Ingenuity Pathway Analysis show the most important pathways in which the differentially expressed genes participate. The top networks formed by the genes upregulated in IVF embryos compared to both CT groups included cellular growth and proliferation, embryonic development, cellular assembly and organization, cellular death and response to stress (Figure 3.12). On the other hand the networks obtained from the transcripts more abundant in the cloned blastocysts compared to IVF embryos were cellular morphology cellular development, cell signaling, and metabolism (Figure 3.13).

3.5 Discussion

It has been reported that in vitro culture conditions alter gene expression and may lead to developmental aberrations in IVF derived cattle, commonly referred to as the large offspring syndrome (Wrenzycki et al., 1999; McEvoy, 2003; Wrenzycki et al., 2004). In the case of embryos produced by SCNT, besides the alterations due to in vitro culture conditions, gene expression defects may be caused by improper silencing and activation of specific genes, altered chromatin remodeling, and epigenetic alterations (Lazzari et al., 2002). But identifying key genes responsible for the general developmental failure in cloned embryos is not an easy task, since the alterations may be caused by a variety of factors including donor cell type, cell cycle stage, nuclear transfer protocol, source of the oocytes, embryo culture system, embryo transfer procedure, recipient cows management, and operators' skills (Oback and Wells, 2007a). Consequently, there is a big variety of alterations that are not always shared by all cloned embryos. Still, the common thread uniting many of the SCNT failures can be traced to epigenetic alterations, specifically failures in chromatin remodeling and DNA and histone methylation (Vignon et al., 2002; Santos et al., 2003; Suteevun et al., 2006).

Microarray analysis has been used to explore the transcriptome profile of cloned embryos relative to that of the donor cells and IVF embryos as a control. However, the appropriate microarray platform is crucial in order to detect changes in particular genes. Smith and colleagues reported similar transcriptome profiles for cloned blastocysts and blastocysts produced by artificial insemination (Smith et al., 2005). However, the cDNA microarray used by Smith and colleagues consisted of placenta and spleen cDNA

libraries, lacking embryonic genes, which therefore were not analyzed. The results from the present study show an extensive reprogramming in cloned embryos by the blastocyst stage. However, the data point to a group of differentially expressed transcripts between IVF and cloned blastocysts.

Serial cloning is often performed for the production of transgenic animals. Although apparently healthy animals can be obtained after serial cloning, the efficiency of cloning decreases from generation to generation despite comparable blastocyst and early pregnancy rates. This increase in pregnancy losses and perinatal deaths could be caused by gene expression defects accumulated throughout the serial cloning procedures, which could be detected in blastocysts, although no phenotypic alterations are observed at this stage. Furthermore, it has been proposed that the extended culture, associated with transfection and selection procedures, may induce changes in the donor cells (Zakhartchenko et al., 2001). To our knowledge this is the first study to focus on the influence of serial cloning on global transcriptome profile of embryos and donor cells. Only a small proportion of the data set generated by the present study corresponded to fully annotated bovine genes (Table 2). The rest of the probe sets were excluded from further analyses due to lack of annotations. Progress in the annotation of the bovine genome will greatly facilitate global gene expression studies in the bovine species.

In the present study, multiple comparisons revealed four distinctive patterns of differential gene expression among all embryos and donor cells. The first pattern corresponded to genes that had similar expression in IVF embryos and CT embryos, but had a very different pattern of expression in both donor cell lines. It could be assumed that these genes completely switched from the "donor cell gene expression mode" to the

"embryo gene expression mode". A big proportion of genes in the present study followed this pattern, including some imprinted and embryonic specific genes such as the Oct-4 protein coding gene (POU5F1), which has been reported as differentially expressed for cloned embryos in previous studies (Boiani et al., 2002; Beyhan et al., 2007a). Placenta specific 8 (PLAC8) also showed this pattern of expression (Figure 5.6). It is possible that some genes, due to their methylation pattern in the somatic cells or to their location in the chromosome, are more likely to be reprogrammed by the oocyte factors.

The second pattern corresponded to genes with a similar pattern of expression for CT embryos and donor cells, and a very different expression pattern in IVF embryos. These were genes with apparently incomplete reprogramming, still showing a somatic cell pattern of expression. The heat shock 70 kD protein 1 (HSPA1A), involved in cell protection from stress and apoptosis was significantly higher in IVF embryos when compared to CT embryos and donor cells (Figure 5.7 A). Important embryonic genes showed this pattern of expression. Desmocollin 3 (DSC3) a trans-membrane glycoprotein, involved in cell adhesion that belongs to the cadherin family, was present in IVF embryos but was absent in CT embryos and donor cells. The signal transducer and activator of transcription 3 (STAT3), was significantly upregulated in IVF embryos when compared to both groups of cloned embryos and donor cells. A similar pattern was observed for high mobility group nucleosomal binding domain 3 (HMGN3) a gene involved in chromatin remodeling, a vital process during embryonic genome activation (Figure 5.7 B). The importance of both genes during morula and blastocyst formation could make them good candidates in understanding the lower developmental rates of cloned embryos.

The third group of genes corresponded to those with a similar pattern of expression in IVF embryos and donor cells, but with a marked differential expression in all cloned embryos. Genes over expressed after chromatin transfer could point to a compensation mechanism. Genes with this kind of expression pattern included prostaglandin-endoperoxide synthase (PTGS2) and the transcription factor GATA-2. Both genes had a greater microarray signal in all CT embryos, but low expression in IVF and donor cells. The imprinted gene glycine amidinotransferase (GATM), showed significantly greater values in the cloned embryos compared to IVF embryos and donor cells. Two interesting genes in this group were DNMT3a and DNMT3a transcripts, which are responsible for *de novo* methylation. The mRNA abundance was significantly greater in CT-1 and CT-4 embryos compared to IVF blastocysts (Figure 5.8 A and B). This pattern of transcription is consistent with the hypermethylation often reported in cloned blastocysts, which could indicate that *de novo* DNA methylation occurs on a major scale in cloned embryos. These results do not agree with previous findings, in which DNMT3A was downregulated in NT embryos compared to IVF embryos (Beyhan et al., 2007a). Zhou et al., reported similar levels of DNMT3B for embryos produced *in vivo*, *in vitro*, and by different nuclear transfer methods, including chromatin transfer (Zhou et al., 2007). These contrasting results confirm that alterations greatly vary and are not shared by all cloned embryos.

A fourth pattern corresponded to genes that had an increasing or a decreasing pattern of expression form IVF embryos through donor cells showing an intermediate pattern of expression in CT embryos. It could be assumed that these genes have been partially reprogrammed. The imprinted gene insulin-like growth factor 2 receptor

(IGF2R), one of the most studied genes in the large offspring syndrome, showed similar expression values in IVF and CT1 embryos, but significantly higher signals in CT4 embryos, and very high signals in both donor cells (Figure 6 F). These higher mRNA levels in the fourth generation of cloning could indicate a cumulative misregulation of this gene. The Bcl-2 inhibitor of transcription (BIT1) showed the greatest values in IVF embryos, intermediate values in CT embryos and the lowest values in donor cells (Figure 6 G). The nuclear transcription factor Y, alpha (NFYA), showed a similar expression pattern in both IVF and CT1 embryos; although it was significantly lower in CT4 embryos and donor cells. Neuroguidin (NGDN), an eukaryotic translation initiation factor with important functions in embryonic development was another gene with a decreasing pattern of expression (Figure 6 H). Genes with and increasing pattern of expression included F-box protein 9 (FBXO9), and guanine nucleotide binding protein alpha inhibiting activity polypeptide 2 (GNAI2) represented in Figure 6 I and Figure 6 J, respectively.

Based on the difference in gene expression for RARB, CRAB1, THBS, SERPINB5, and HLA-A, Beyhan et al. suggest a possible role for the retinoic acid signaling pathway in the failures observed in cloned bovine embryos (Beyhan et al., 2007b). However, the bovine GeneChip does not contain a Retinoic Acid Receptor Beta (RARB) probe set. It only contains a probe set that corresponds to a bovine EST with similarity to the rat RARB (Bt.21044.2.A1_at). In the present data, CRAB1 and THBS2 were slightly higher in IVF embryos, although without statistical significance. They also found differential gene expression among several genes in both donor cells (CDKN1C, COPG2, DCN, GATM, MEST, NDN, NNAT, PON3, and SGCE). In the current study

GATM was significantly downregulated in donor cells from the fifth successive generation of chromatin transfer (Figure 3.9).

At the blastocyst stage there was an extensive reprogramming of cloned embryos leading to very similar transcriptomes in IVF and CT blastocysts. However, there were around 200 differentially expressed genes in both CT embryos compared to IVF. For some genes, the differences were significantly greater in CT4 when compared to CT1, suggesting a possible cumulative misregulation caused by serial cloning. Genes involved in transcription, cellular proliferation, embryonic development, cellular death, and response to stress are over represented in IVF embryos; many of these genes are present in the nucleus, which was the cell component overrepresented in IVF embryos. Genes involved in cell morphology, cell development, and metabolism were over expressed in donor cells and in cloned embryos when compared to IVF, suggesting that they were not properly silenced in the donor nucleus. The upregulation of genes involved in metabolism should be further explored as it could be linked to the large size of cloned animals.

3.6 Conclusions

The present study provides a unique data set for identifying the epigenetic errors in somatic cloning and may facilitate a better understanding of the reprogramming process in SCCT. Future studies should involve all of the successive generations of cloned embryos and their respective donor cells to identify cumulative misregulated genes. Gene expression studies from fetal, newborn, and placental tissues could identify genes that are responsible for abnormalities, abortions, stillborns and low birth rate.

As gene expression profile can only show one step in cell phenotype and function control, namely transcriptome regulation, proteomic analysis could complement this study by providing a more complete picture of the regulation of embryonic development. With the advances in bovine genome annotation, more of the differentially expressed transcripts could be analyzed further providing more information for the currently unidentified transcripts, which, in the present study represented around 23% of the dataset. Gene Ontology information for a proportion of the differentially expressed genes is still incomplete. Thus, for some of the genes the cellular component is known, but the biological process and/or its molecular function is not documented. It is interesting that the majority of genes upregulated in CT blastocysts participate in metabolism processes, while the percentage of metabolism genes in IVF blastocyst was lower compared to signaling pathway genes.

Our next set of studies will include structural and functional genomics analysis of genes involved in chromatin remodeling and DNA methylation in bovine. These studies would provide valuable information regarding the conservation of these proteins in

mammals, their role in early embryo development, and molecular reprogramming. Some of these proteins have not been completely annotate and the available information comes from ESTs or cDNAs. Annotation of genes involved in DNA methylation and chromatin remodeling in cattle would complement the present study and contribute to our knowledge of key genes in embryonic development.

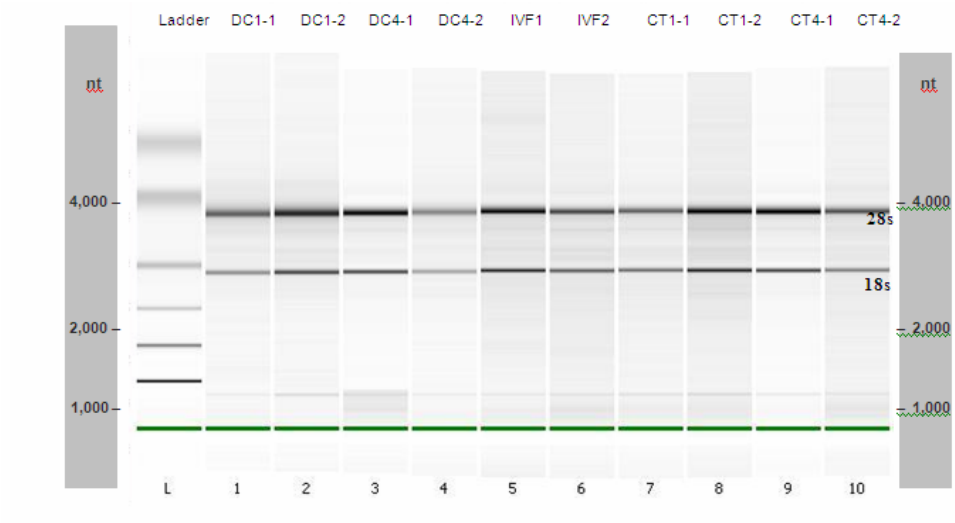

Figure 3. 1 Agilent Bioanalyzer gel-like image of total RNA.

Note: The image shows a total RNA gel like-image produced by the Bioanalyzer. (Ten out of the 15 samples used in the microarray experiment are shown since no more than 11 samples can be run at one time). Lane L: Size markers. Lanes 1 and 2: total RNA from 10^6 donor cells used for the first round of SCCT. Lanes 3 and 4: total RNA from 10^6 donor cell used for the fourth round of cloning. Lanes 5 and 6: total RNA from a pool of 3 In Vitro Produced embryos. Lanes 7 and 8: total RNA from a pool of 3 embryos produced by the first round of chromatin transfer. Lanes 9 and 10: total RNA from a pool of 3 embryos produced by the fourth round of chromatin transfer. The 28S and 18S distinctive ribosomal RNA bands are observed for all samples.

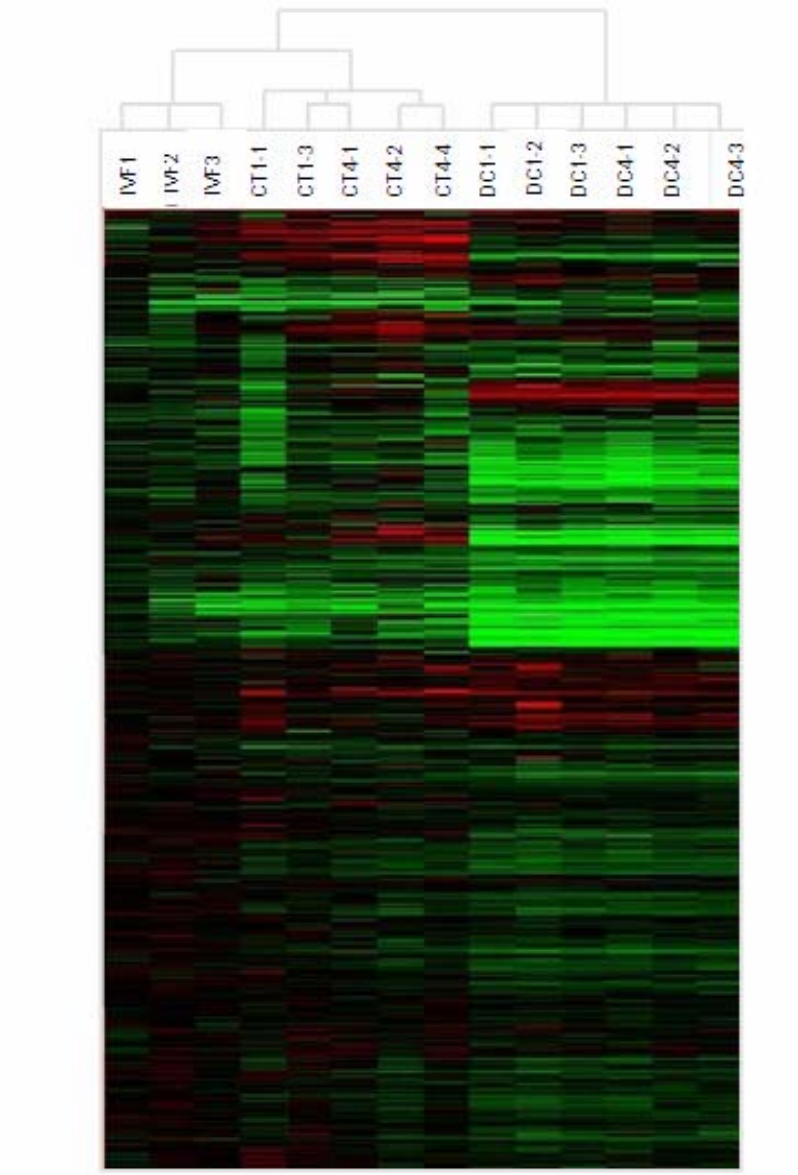

Figure 3.2 Hierarchical clustering of microarray hybridizations.

Note: Cluster analysis of hybridizations and genes performed using GeneTraffic UNO (Iobion Informatics LLC). All donor cells were clustered in one group, while all the embryos were clustered in a second group. The embryos clearly separate into two groups: a group containing the IVF embryos and a group containing the chromatin transfer embryos.

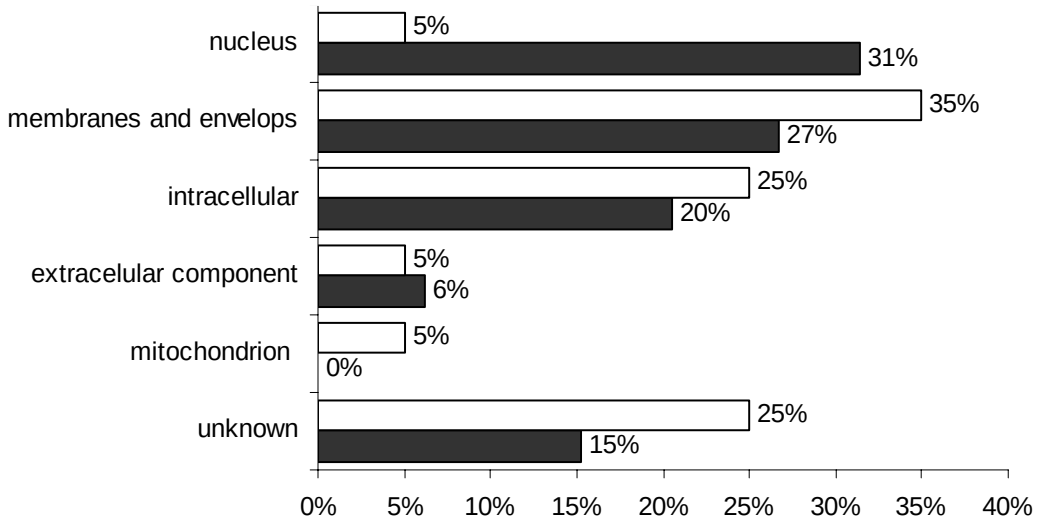

Figure 3.3 GoSlimViewer graph of Cellular Component over-represented terms in IVF and CT embryos.

Note: Sub-cellular locations of gene products found at high levels in both IVF blastocysts (solid bars) and both groups of CT blastocysts (open bars). The proportion of genes present in the nucleus was higher in IVF embryos (31%) compared to CT embryos (5%). There were more membrane and intracellular genes in CT embryos compared to IVF embryos.

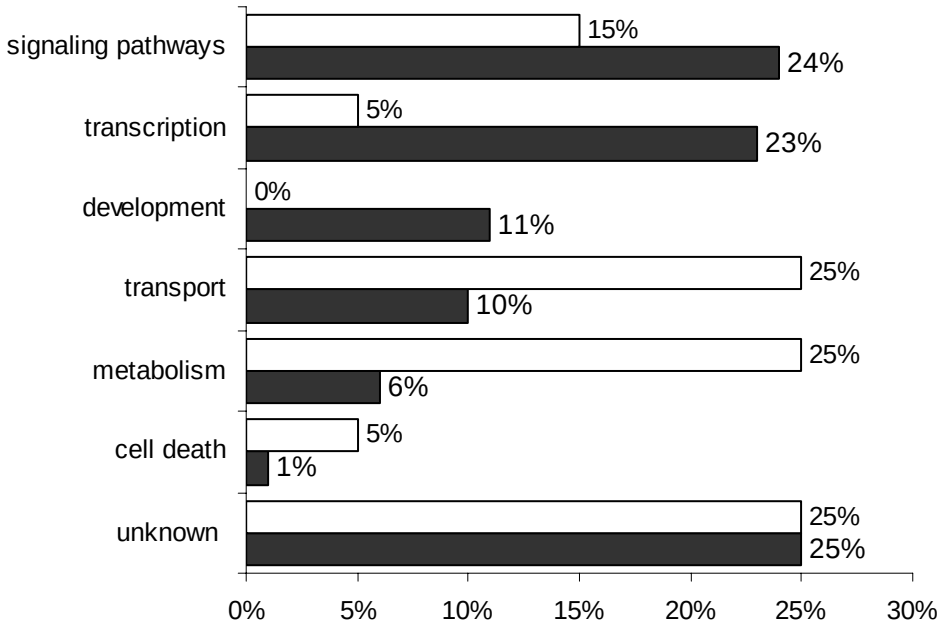

Figure 3.4 GoSlimViewer graph of Biological Process over-represented terms in IVF and CT embryos.

Note: Biological processes of gene products found at high levels in both IVF blastocysts (solid bars) and CT blastocysts (open bars). No genes involved in development were upregulated in CT blastocysts compared to IVF blastocysts, for which 11% of the genes were involved in development. Conversely a greater proportion of metabolism genes were overrepresented in CT embryos compared to IVF embryos.

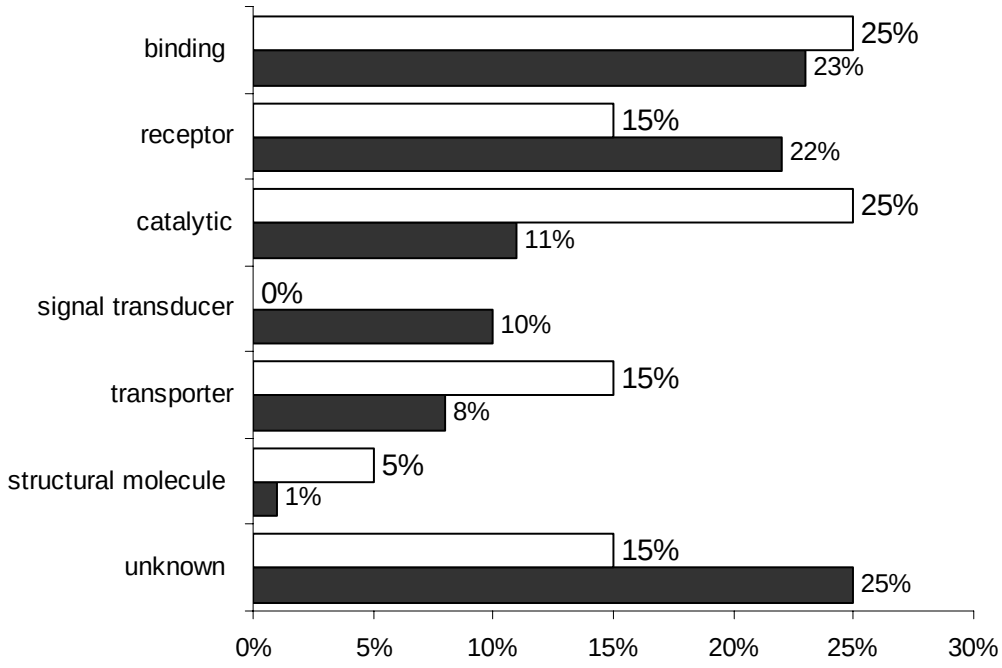

Figure 3.5 GoSlimViewer graph of Molecular Function over-represented terms in IVF and CT embryos

Note: Molecular functions of gene products found at high levels in IVF blastocysts (solid bars) and CT blastocysts (open bars). Genes with receptor function were higher in IVF blastocysts, while genes with catalytic, signal transduction and transporter functions were overrepresented in CT blastocysts.

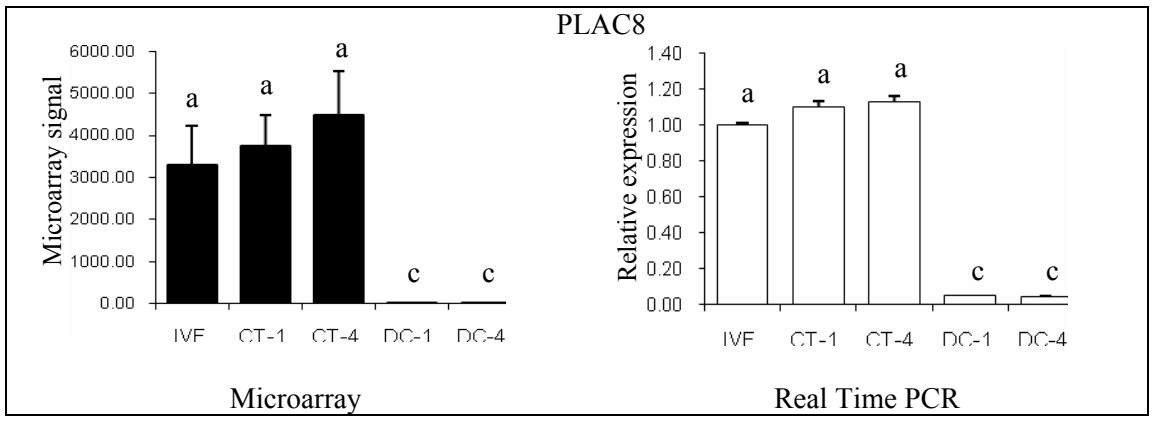

Figure 3.6 Real Time PCR Validation of PLAC8 transcript abundance.

Note: Gene expression patterns from microarray analysis (solid bars) and relative quantification through Real time PCR (open bars). Microarray units indicate signal intensity values. Real time PCR units indicate relative expression to the internal standard GAPDH. Different letters on top of each bar indicate significant differences in expression ($P < 0.01$).

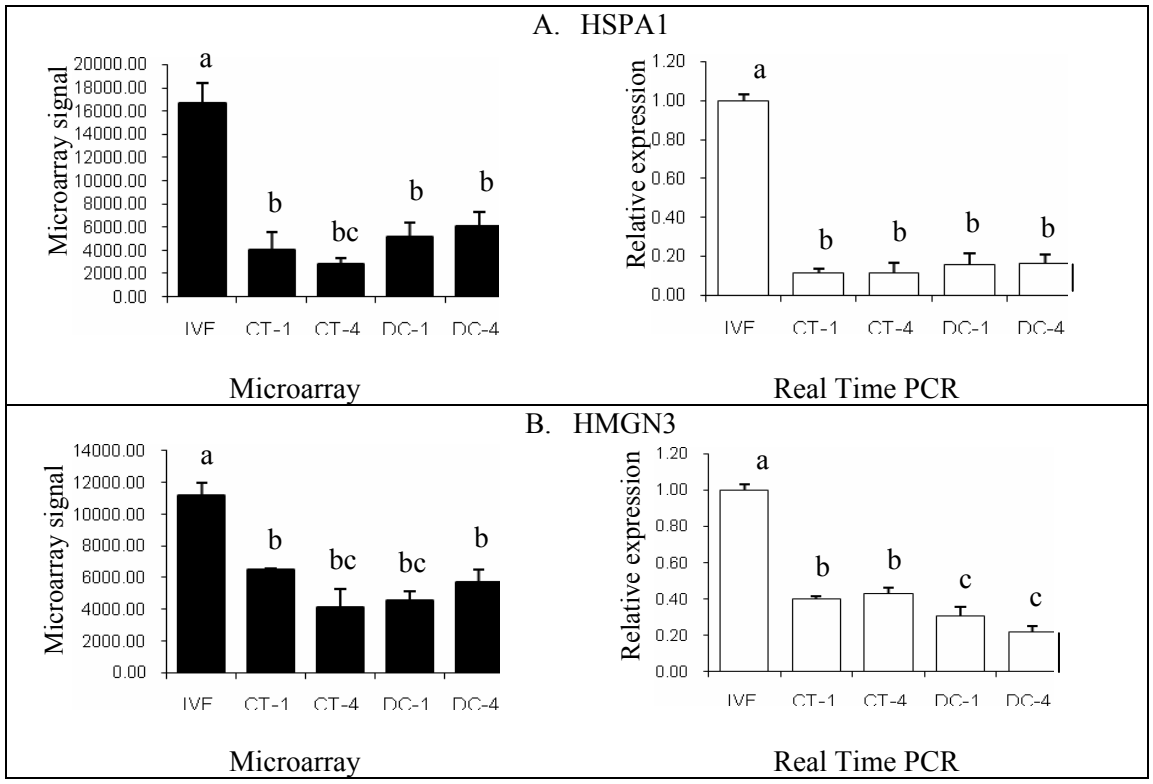

Figure 3.7 Real Time PCR Validation of HSPA1 and HMGN3 transcript abundance.

Note: Gene expression patterns from microarray analysis (solid bars) and relative quantification through Real time PCR (open bars). Microarray units indicate signal intensity values. Real time PCR units indicate relative expression to the internal standard GAPDH. Different letters on top of each bar indicate significant differences in expression ($P < 0.01$).

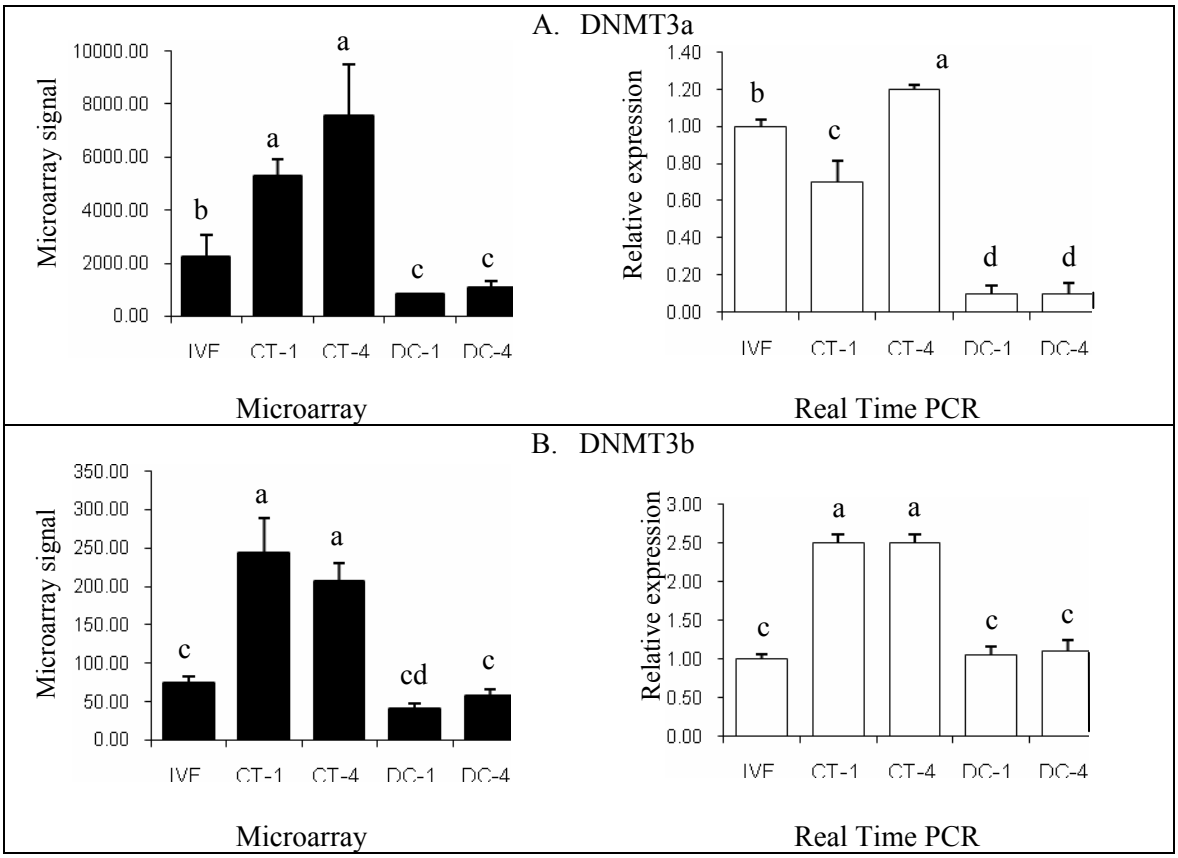

Figure 3.8 Real Time PCR Validation of DNMT3a and DNMT3b transcript abundance.

Note: Gene expression patterns from microarray analysis (solid bars) and relative quantification through Real time PCR (open bars). Microarray units indicate signal intensity values. Real time PCR units indicate relative expression to the internal standard GAPDH. Different letters on top of each bar indicate significant differences in expression
($P < 0.01$).

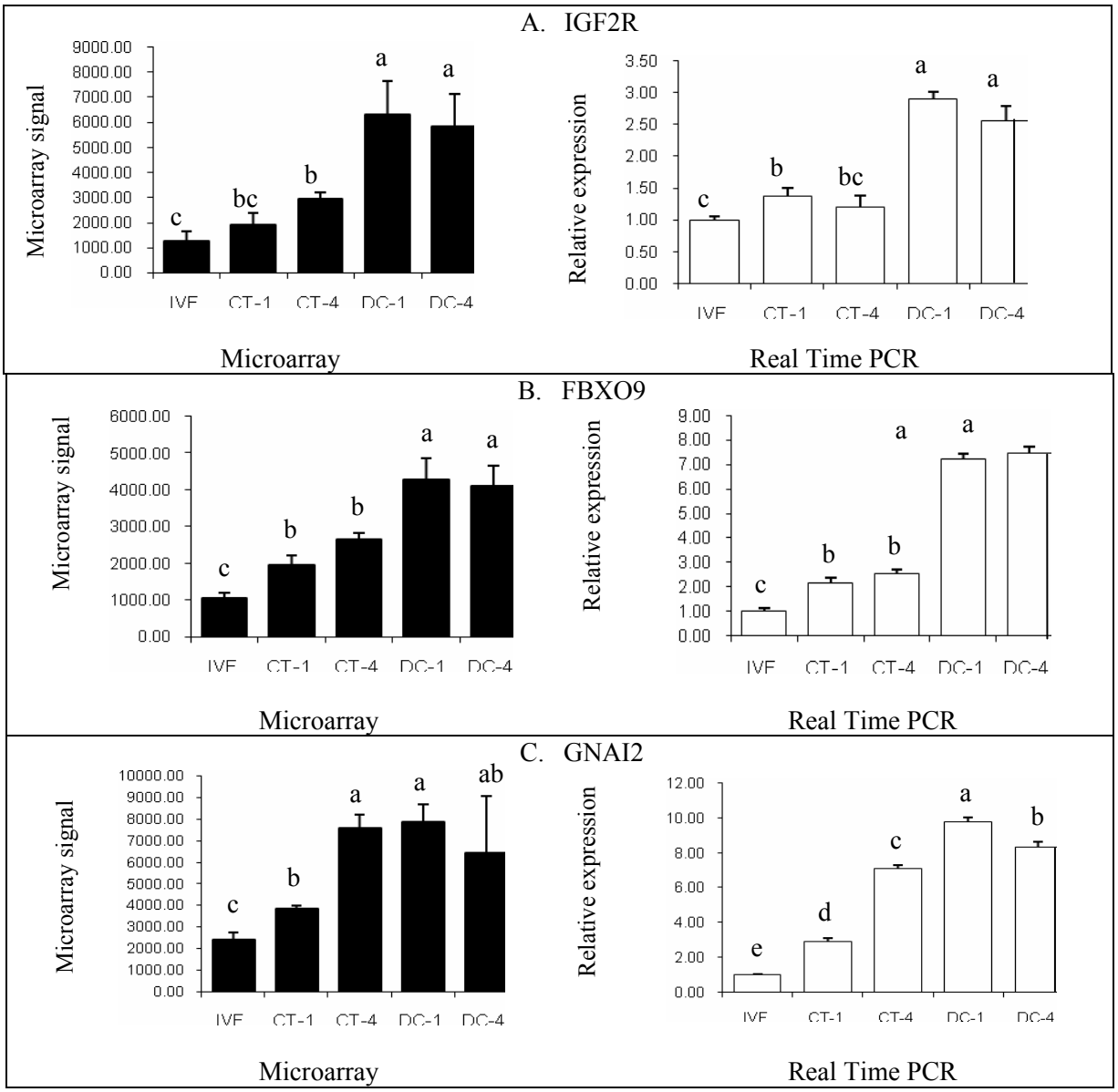

Figure 3.9 Real Time PCR Validation of IGF2R, FBXO9, and GNAI2 transcript abundance.

Note: Gene expression patterns from microarray analysis (solid bars) and relative quantification through Real time PCR (open bars). Microarray units indicate signal intensity values. Real time PCR units indicate relative expression to GAPDH. Different letters on top of each bar indicate significant differences in expression ($P < 0.01$).

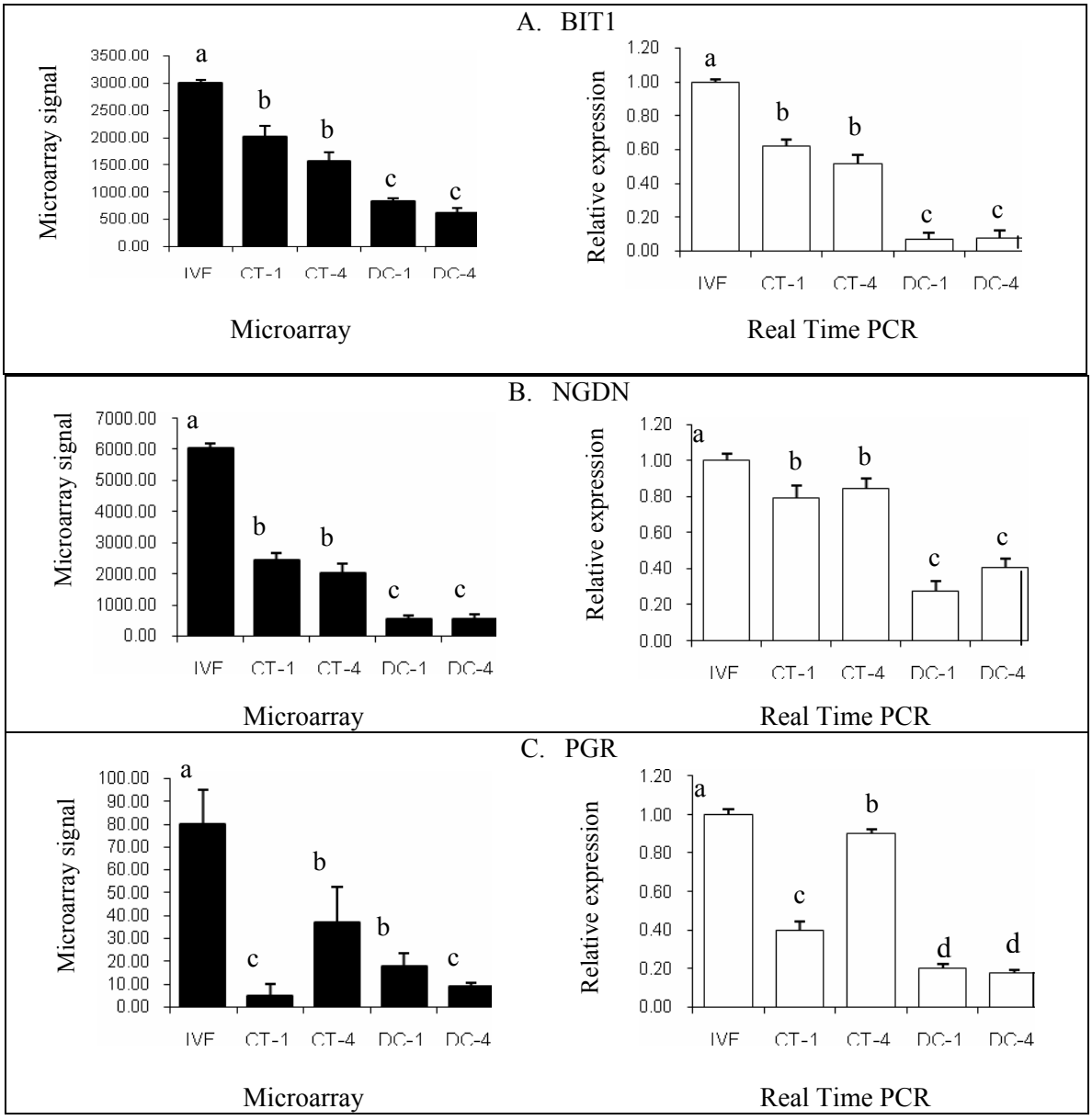

Figure 3.10 Real Time PCR Validation of BIT1, NGDN, and PGR transcript abundance.

Note: Gene expression patterns from microarray analysis (solid bars) and relative quantification through Real time PCR (open bars). Microarray units indicate signal intensity values. Real time PCR units indicate relative expression to GAPDH. Different letters on top of each bar indicate significant differences in expression ($P < 0.01$).

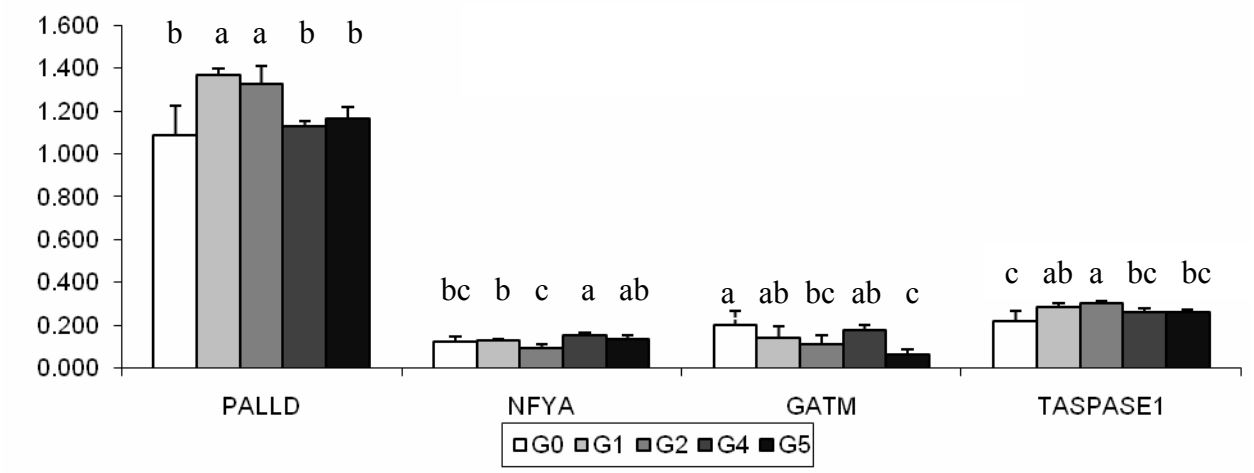

Figure 3.11 Real Time PCR Validation of PALLD, NFYA, GATM and Taspase1 in cells lines obtained from serial cloning.

Note: Cell lines derived from 0 rounds of cloning (G0) first round of cloning (G1), second round of cloning (G2), fourth round of cloning (G4), and fifth round of cloning (G5). Units indicate relative expression to the internal standards GAPDH and 18S rRNA. Different letters indicate significant differences in expression between different donor cell lines ($P<0.01$).

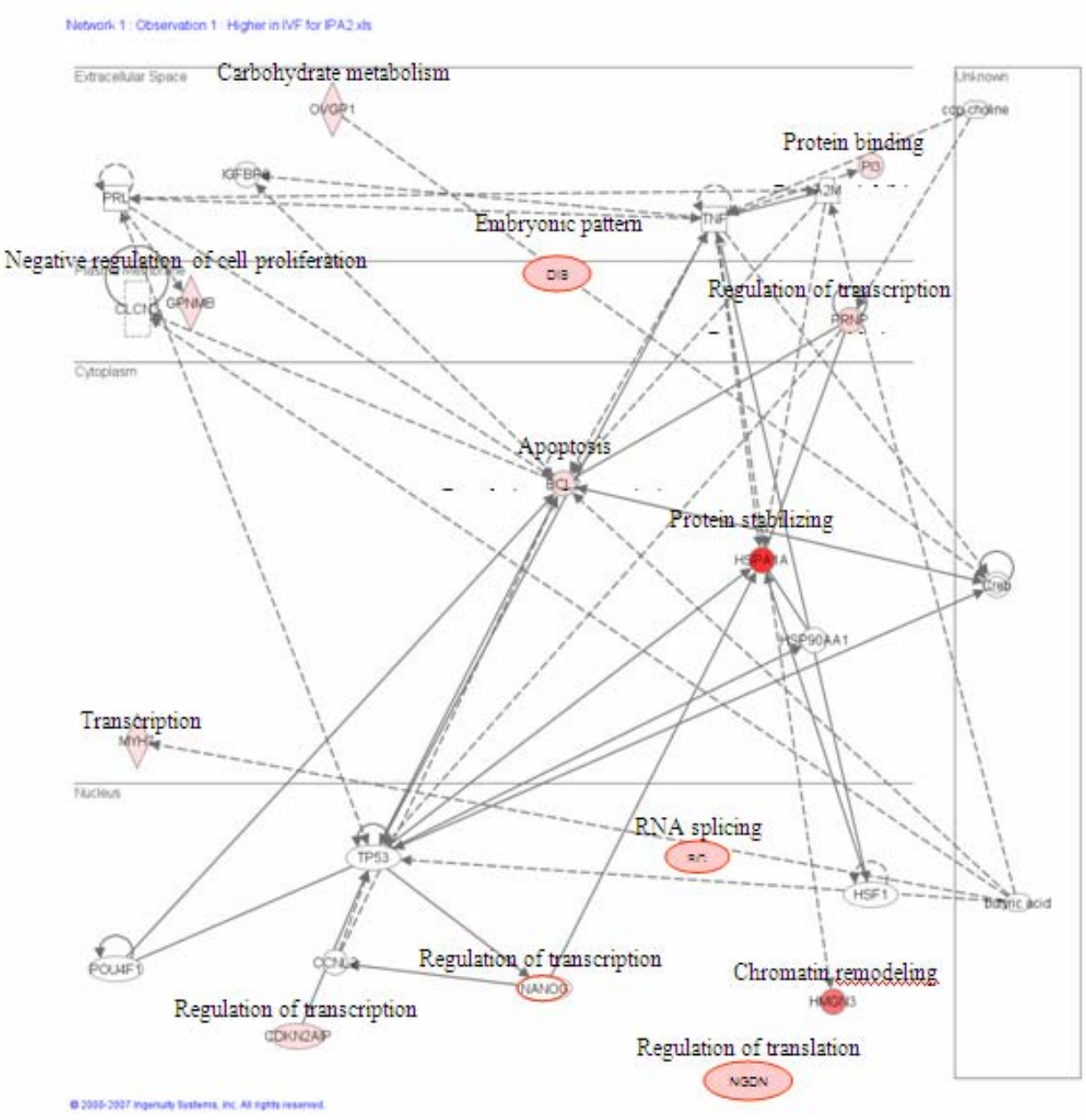

Figure 3.12 Network of genes with high expression levels in IVF embryos.

Note: Data modeling of genes with high expression in IVF embryos compared to cloned embryos. The top networks in the pathway include cellular growth and proliferation, embryonic development, cellular assembly and organization, cellular death and response to stress and cancer.

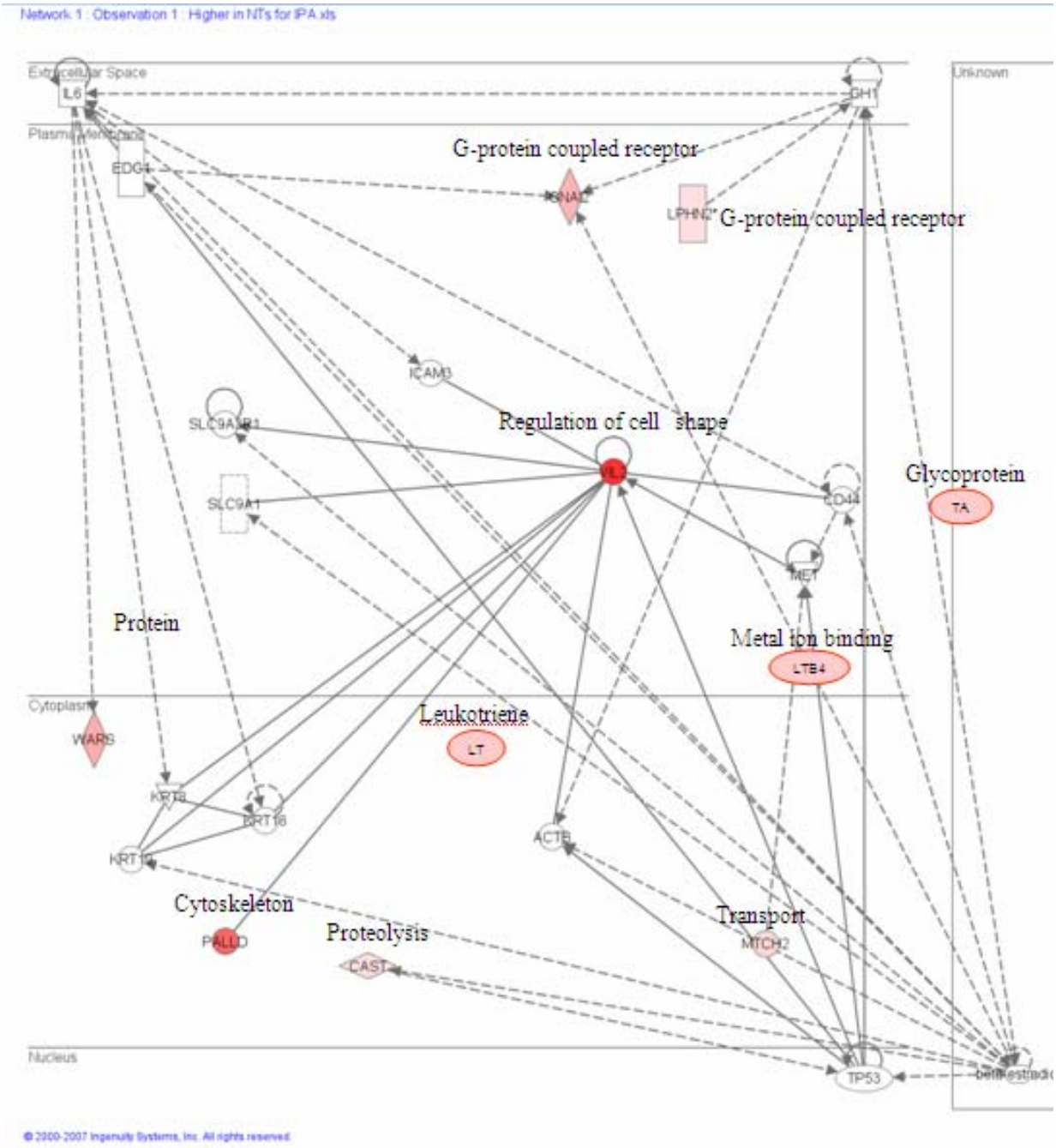

Figure 3.13 Network of genes with high expression levels in NT embryos.

Note: Data modeling of genes with high expression in CT embryos compared to IVF embryos. The top networks in the pathway include cellular morphology, cellular development, cell signalling and metabolism.

Table 3.1 Number of differentially expressed transcripts in microarray pairwise comparisons.

	Comparison Group 1 *vs.* Group 2	Differentially expressed transcripts	Higher in the first group	Higher in the second group
1	IVF embryos *vs.* CT-1 embryos	270 [a]	123	147
2	IVF embryos *vs.* CT-4 embryos	411 [a]	193	218
3	IVF embryos *vs.* DC-1 cells	3360 [c]	1548	1812
4	IVF embryos *vs.* DC-4 cells	3428 [c]	1593	1835
5	CT-1 embryos *vs.* CT-4 embryos	193 [a]	91	101
6	CT-1 embryos *vs.* DC-1 cells	2459 [b]	1238	1221
7	CT-1 embryos *vs.* DC-4 cells	2588 [b]	1379	1209
8	CT-4 embryos *vs.* DC-1 cells	2036 [b]	1151	885
9	CT-4 embryos *vs.* DC-4 cells	2276 [b]	1287	989
10	DC-1 cells *vs.* DC-4 cells	83 [d]	34	49

Comparisons were performed between a pair of groups IVF embryos, CT1 embryos, CT4 embryos, DC1 cells, and DC4 cells. (p-value <0.01 and fold change <2.0).
Different subscripts indicate statistically significant differences in the number of differentially expressed transcripts.

Table 3.2 Classification of differentially expressed probe sets in pairwise comparisons.

	Comparisons									
Probe set category	IVF *vs.* CT1	IVF *vs.* CT4	IVF *vs.* DC1	IVF *vs.* DC4	CT1 *vs.* CT4	CT1 *vs.* DC1	CT1 *vs.* DC4	CT4 *vs.* DC1	CT4 *vs.* DC4	DC1 *vs.* DC4
Genes	63	104	747	763	44	574	563	421	461	23
"Similar to…	106	180	1564	1597	81	1071	1132	898	995	34
Hypothetical proteins	4	10	90	102	0	69	80	65	76	6
cDNA clones	0	1	24	28	0	19	16	17	16	0
Transcripts with strong similarity to a known gene	1	3	26	23	0	17	17	19	21	0
Transcripts with moderate similarity to a known gene	2	0	24	27	2	13	16	12	14	2
Transcripts with weak similarity to a known gene	1	0	13	15	1	10	17	8	10	0
Unknown transcripts	93	113	872	873	64	686	747	596	683	18
Total	**270**	**411**	**3360**	**3428**	**192**	**2459**	**2588**	**2036**	**2276**	**83**

The probe set categories correspond to NetAffx Bovine GeneChip (Annotation, November 2007).

Table 3.3 Top 25 upregulated genes in IVF blastocysts compared to CT blastocysts.

Probe set ID	Gene Title	Gene ID	P value	FC IVF/CT1	FC IVF/CT4
Bt.28010.1.S1_at	Peptidase inhibitor 3, skin-derived (SKALP)	PI3	0.000000	5.58	9.12
Bt.21013.1.S1_at	Polo-like kinase 3 (Drosophila)	PLK3	0.000001	3.99	9.09
Bt.28223.1.S1_at	20-beta-hydroxysteroid dehydrogenase-like	MGC127133	0.000009	2.13	1.71
Bt.9525.1.A1_at	Zinc finger protein 183	ZNF183	0.000057	2.13	3.62
Bt.2892.1.S1_at	Fatty acid binding protein 7, brain	FABP7	0.00014	1.22	6.35
Bt.4430.1.S2_at	ATPase, H+ transporting, lysosomal V0 subunit a1	ATP6V0A1	0.00014	1.89	1.93
Bt.5154.1.S1_at	Heat shock 70kDa protein 1A	HSPA1A	0.0002	4.14	7.17
Bt.15787.1.S1_at	Bcl-2 inhibitor of transcription	BIT1	0.0002	1.49	2.01
Bt.13544.2.S1_a_at	Zinc finger protein 410	ZNF410	0.0003	2.01	1.90
Bt.2005.1.S1_at	LSM1 homolog, U6 small nuclear RNA associated	LSM1	0.0003	1.86	1.57
Bt.16291.1.A1_at	Testis expressed 12	TEX12	0.0004	3.40	3.64
Bt.27854.1.S1_at	Nuclear factor, interleukin 3 regulated	NFIL3	0.0004	1.72	3.08
Bt.13928.2.S1_a_at	Sodium channel modifier 1	SCNM1	0.0004	2.02	4.05
Bt.15334.2.A1_at	Signal transducer and activator of transcription 3	STAT3	0.0005	4.71	16.06
Bt.12506.1.S1_at	Serpin peptidase inhibitor, E member 2	SERPINE2	0.0005	1.52	1.42
Bt.20204.1.S1_at	Sjogren's syndrome/scleroderma autoantigen	SSSCA1	0.0005	1.61	2.93
Bt.20199.1.A1_at	DEAD (Asp-Glu-Ala-Asp) polypeptide 56	DDX56	0.0006	1.45	1.79
Bt.3359.1.S1_at	General transcription factor IIF, polypeptide	GTF2F1	0.0009	1.53	1.98
Bt.2958.1.A1_at	Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	UBE2A	0.001	2.03	3.08
Bt.3002.1.S1_at	BUB3 budding uninhibited by benzimidazoles 3	BUB3	0.001	1.27	1.62
Bt.6087.1.S1_at	Transmembrane 4 superfamily member 1	TM4SF1	0.001	2.29	6.62
Bt.4737.1.S2_s_at	Prion protein	PRNP	0.001	2.20	2.99
Bt.1854.1.S1_at	Intraflagellar transport 20 homolog (Chlamydomonas)	IFT20	0.001	1.65	2.30
Bt.5340.1.S1_s_at	Nucleoside-diphosphate kinase NBR-A	NBR-A	0.002	1.42	1.76
Bt.8.1.S1_at	Keratin 10 (epidermolytic hyperkeratosis)	KRT10	0.002	1.98	3.39

Genes were analyzed by one-way ANOVA and sorted by P-value. FC: Fold Change

Table 3.4 Top 25 upregulated genes in CT blastocysts and donor cells compared to IVF blastocysts.

Probe set ID	Gene Title	Gene ID	P value	FC CT1/IVF	FC CT4/IVF
Bt.8933.1.S1_at	Adaptor-related protein complex 3, sigma 2	AP3S2	0.0001	1.61	2.21
Bt.27382.1.A1_s_at	X-ray repair complementing defective repair in Chinese hamster cells 1	XRCC1	0.0001	2.60	2.54
Bt.22224.1.S1_at	insulin receptor substrate 4	IRS4	0.0002	2.31	2.10
Bt.3220.1.S1_at	Crystallin, lambda 1	CRYL1	0.0003	1.94	1.82
Bt.7805.2.S1_a_at	Nuclear casein kinase and cyclin-dependent kinase substrate 1	NUCKS1	0.0003	3.14	3.47
Bt.29540.1.S1_at	Arginine/serine-rich coiled-coil 1	RSRC1	0.0004	1.57	3.09
Bt.19690.1.A1_at	Paraoxonase 1	PON1	0.0005	1.53	3.74
Bt.20444.1.S1_at	thyroid hormone receptor associated protein 5	THRAP	0.0006	1.60	1.20
Bt.16122.1.S1_at	Sorbitol dehydrogenase	SORD	0.0008	2.59	3.40
Bt.5737.1.S1_at	vacuolar protein sorting 26 homolog A	VPS26	0.0008	2.33	3.38
Bt.4292.1.S1_at	ARP3 actin-related protein 3 homolog (yeast)	ACTR3	0.0008	1.69	1.69
Bt.18230.1.S1_a_at	Nuclear autoantigenic sperm protein	NASP	0.0011	1.95	2.75
Bt.9107.1.S1_a_at	phosphatidylinositol binding clathrin assembly protein	PIBCAP	0.0014	1.83	2.93
Bt.663.1.S1_at	Palladin, cytoskeletal associated protein	PALLD	0.0019	2.88	3.08
Bt.1743.2.S1_a_at	Phenylalanyl-tRNA synthetase 2, mitochondrial	FARS2	0.0021	1.64	2.98
Bt.13205.1.A1_at	Mitochondrial ribosomal protein S35	MRPS35	0.0023	1.44	2.29
Bt.25100.1.A1_at	Cortactin	CTTN	0.0026	1.26	1.58
Bt.783.1.S1_at	Aldehyde oxidase 1	AOX1	0.0029	1.99	3.67
Bt.23608.1.S1_s_at	Keratin 8	KRT8	0.0030	3.99	4.59
Bt.27284.1.S1_at	Eukaryotic translation initiation factor 4H isoform 2	WBSCR1	0.0038	2.21	1.55
Bt.10898.1.S1_at	Tumor differentially expressed 2-like	TDE2L	0.0041	1.98	5.08
Bt.28745.1.S1_at	Coagulation factor II receptor-like 1	F2RL1	0.0045	2.25	1.85
Bt.5267.1.S1_at	Annexin A6	ANXA6	0.0046	1.92	3.79
Bt.355.1.S1_at	Caldesmon 1	CALD1	0.0047	1.71	1.79
Bt.20084.2.S1_at	Casein kinase 1, epsilon	CSNK1E	0.0053	3.90	2.64

Genes were analyzed by one-way ANOVA and sorted by P-value. FC: Fold Change

Table 3.5 Primers used for Real time PCR validation

Genes	Primer sequences and positions (5' - 3')		Fragment size (bp)	Accession Number
GAPDH_F	TGCTGGTGCTGAGTATGTGGT	(333-354)	295	XM_865742
GAPDH_R	AGTCTTCTGGGTGGCAGTGAT	(627-648)		
DNMT3A_F	CTGGCTCTTTGAGAATGTGGTG	(2372-2394)	236	XM_867643
DNMT3A_R	TCACTTTGCTGAACTTGGCTATT	(2607-2630)		
DNMT3B_F	GGGAAGGAGTTTGGAATAGGAG	(698-720)	417	NM_181813
DNMT3B_R	CTCTGGTTGCTTGTTGTTAGGTT	(1114-1137)		
IGF2R_F	AACCAGGTGATTTAGAAAGTGCC	(1939-1962)	397	NM_174352
IGF2R_R	CGCTTCTCGTTATTGTAGGGTG	(2335-2357)		
PLAC8_F	TGTTTCACAGCCAGGTTACAGC	(168-190)	200	NM_001025325
PLAC8_R	GGGTCCGATACATTGTCCTCAT	(367-389)		
PGR_F	TAAATGACCAGCAAGCAGAAACT	(562-585)	394	XM_613908
PGR_R	GGTAATTGTGCAGCAATAACCTC	(955-978)		
BIT1_F	CGGAGCCAGAGGAAGAATGA	(75-95)	445	NM_001034519.1
BIT1_R	TGCTTGTAGGCAGAAACAGCA	(519-530)		
HMGN3_F	GTTCCAGCCCGTTGCTTTAC	(22-42)	355	NM_001034504.1
HMGN3_R	GACCATTCATTCTCCCTCGTTAG	(376-399)		
HSPA1A_F	CACGATGTTGATCCTGTGGG	(86-106)	380	NM_174550.1
HSPA1A_R	CACCTTAGGCTTGTCTCCGTC	(465-487)		
NGDN_F	GTGAGAATGACCCACTCCGTT	(403-424)	397	NM_001046459
NGDN_R	TCCCGCTTGCTGACACTTAA	(799-819)		
FBXO9_F	GCAGACGGCAGGAGTAGACAC	(231-252)	445	NM_001034412.1
FBXO9_R	ACAAGTTGCATAGCCCTACGAT	(675-697)		
GNAI2_F	TCCAGACAACTGCCAACATCA	(1978-1999)	215	XM_589440.3
GNAI2_R	CAAACCAGGTGAACAATTCCATA	(2192-2215)		
PALLD_F	AGGTTGACCTACGAGGAAAGGA	(2071-2092)	292	XM_869983.2
PALLD_R	ATGTGAACGTCGCAGGCATA	(2362-2382)		
NFYA_F	CGGGCTAAATTAGAAGCAGAAG	(998-1020)	311	NM_001014956.1
NFYA_R	AGGGCAGAATGTGATCGTCAG	(1308-1329)		
GATM_F	ATTGGCTGCTCAGGGAAAGT	(824-844)	262	NM_001045878.1
GATM_R	ACATGGTCGGTCAGGGTTG	(1085-1104)		
TASPASE1_F	CAAGACTCATATTTCCAGACTCCC	(1145-1169)	264	NM_001034577.1
TASPASE1-R	CCAAGCACTAACTACAGCAGCAC	(1408-1431)		

CHAPTER 4

COMPARATIVE FUNCTIONAL GENOMICS OF MAMMALIAN DNA METHYLTRANSFERASES

4.1 Abstract

As an essential element of the epigenome, mammalian DNA methylation is catalyzed by the DNA methyltransferases (DNMTs). These enzymes play a crucial role in regulation of gene expression in disease and development.

Three mammalian DNMT's (DNMT1, DNMT3A and DNMT3B), together with the accessory protein DNMT3L, are responsible for the acquisition of specific DNA methylation patterns during gametogenesis, embryogenesis and somatic tissue development. There is no consensus on DNA methylation activity of DNMT2, however, this enzyme has recently been shown to catalyze methylation of $tRNA^{Asp}$. The present study focuses on structural and functional genomics analysis of cattle DNMT's comparing them to the humans and mice proteins. Our previous studies have shown greater mRNA abundance of DNMT3a and DNMT3b in cloned embryos. Therefore, we also wanted to establish the patter of expression of these enzymes during embryogenesis

to improve our understanding of epigenetic regulation in early mammalian development. Our findings showed a high degree of structural and functional conservation among the human, mouse, and bovine DNMT's. In addition, our results showed similar patterns of transcript abundance for all of the proteins at different stages of early embryo development. A predicted protein sequence for bovine DNMT3L is available (XP_869990.2), i.e., its RNA or protein has not been confirmed yet. Remarkably, all of the DNMTs with an important role in DNA methylation (DNMT1, DNMT3A, DNMT3B, and DNMT3L) show greater degree of structural similarity between human and bovine than that between human and mouse. These results have important implications for the selection of an appropriate model for study of DNA methylation during early development in humans.

4.2 Introduction

Regulation of gene expression without any actual modification of DNA sequence, epigenetics, is a topic that has garnered increasing attention in the post-genomic era. Epigenetic regulation causes differential expression of genes depending on the type of tissue and stage of development. DNA methylation is a well-studied epigenetic process with a variety of key roles in gene repression (Nan et al., 1998; Beaujean et al., 2004), control of cellular differentiation (Shin et al., 2002; Ehrlich, 2003a), gene regulation during embryonic development (Okano et al., 1999; Shiels et al., 1999; Reik et al., 2001), X chromosome inactivation, and genomic imprinting (Shin et al., 2002; Chow and

Brown, 2003). Other important roles of DNA methylation include silencing of endogenous retroviruses, suppression of homologous recombination, and protection from the mutagenic effects of the abundant transposable elements in mammalian genomes (Yoder et al., 1997; Bestor, 2000). DNA methylation results from the activity of a family of enzymes called DNA methyltransferases (DNMT's) that catalyze the addition of a methyl group to the cytosine residues at CpG dinucleotides (Nan et al., 1998). Four different DNA methyltransferases have been identified and their structure as well as functions have been extensively reviewed (Kumar et al., 1994; Bestor, 2000; Hermann et al., 2003). These DNA methyltransferases are widely conserved among different species. Mammalian DNMT's contain at least three structural regions: the N-terminal regulatory domain, which is responsible for the localization of DNMT's in the nucleus, the C-terminal catalytic domain, which is responsible for the methyltransferase activity, and the central linker, consisting of repeated GK dipeptides (Araujo et al., 2001). The regulatory N-terminal domain contains a proliferating cell nuclear antigen-binding domain (PBD), a nuclear localization signal (NLS), a cysteine-rich zinc finger DNA-binding motif (ATRX), a polybromo homology domain (PHD), and a PWWP tetrapeptide chromatin-binding domain (Bestor, 2000). The C-terminal DNMT's catalytic domain contains ten different characteristic sequence motifs, six of which are evolutionally conserved: I, IV, VI, VIII, IX, and X (Turek-Plewa and Jagodzinski, 2005).

The first identified DNA methyltransferase, DNMT1, plays a key role in maintenance of DNA methylation by restoring the methylation pattern on newly synthesized hemi-methylated DNA strands during replication (Bestor et al., 1992; Pradhan et al., 1999). An interesting DNMT1 isoform lacking 118 amino acids from the

N-terminal domain (DNMT1o) is exclusively active in oocytes and preimplantation embryos and is later replaced by the regular somatic DNMT1 (Bestor, 2000). DNMT2, the smallest mammalian DNMT, contains only the methyltransferase motifs of the C-terminal domain, and, although it is highly conserved, its biological function has been enigmatic (Yoder and Bestor, 1998; Dong et al., 2001). Some studies show that DNMT2 acts as a DNA methyltransferase (Kunert et al., 2003) while other studies have detected little DNA methylation activity. Recent research has demonstrated that DNMT2 methylates $tRNA^{Asp}$ in the cytoplasm (Goll et al., 2006; Rai et al., 2007). DNMT3a and DNMT3b are similar proteins that have been identified as *de novo* DNA methyltransferases acting upon hemi-methylated and unmethylated DNA with equal efficiency during early embryonic development and gametogenesis (Okano et al., 1998; Okano et al., 1999). The DNA cytosine-like 5-methyltransferase (DNMT3L) protein lacks the most important C-terminal methyltransferase motifs, but possesses an active nuclear localization signal sequence (NLS) and the ATRX zinc finger motif, (identical to the ones in DNMT3A and DNMT3B enzymes) that enable nucleus translocation and DNA binding. DNMT3L has a PHD-like motif that activates Histone deacetylase 1 (HDAC1) (Deplus et al., 2002; Turek-Plewa and Jagodzinski, 2005) and has recently been shown to also recognize histone H3 tails that are unmethylated at lysine 4 and induce *de novo* DNA methylation by recruitment or activation of DNMT3A (Ooi et al., 2007). Thus, DNMT3L has a dual role in de novo DNA methylation, interacting with unmethylated lysine 4 of histone H3 through its PHD-like domain while interacting and activating DNMT3A through its carboxy-terminal domain (Jia et al., 2007).

Modulation of DNA methylation early embryogenesis is dynamic and developmentally regulated. Genomewide DNA demethylation, with the exception of methylation marks at imprinted genes, occurs during the first embryonic stages (Oswald et al., 2000; Reik et al., 2001). The paternal genome is significantly and actively demethylated within hours of fertilization, before the onset of DNA replication, whereas the maternal genome is demethylated after several cleavage divisions (Mayer et al., 2000). This demethylation is followed by *de novo* DNA methylation that establishes a new embryonic methylation pattern. The DNA of blastocysts is thus relatively undermethylated. The exact biological function of this dynamic reprogramming of DNA methylation in early development is unknown. Several studies support the hypothesis that DNA methylation is crucial for the establishment of gene expression during embryonic development (Eden and Cedar, 1994; Jones et al., 1998). However, recent data suggest that DNA methylation may only affect genes that are already silenced by other mechanisms in the embryo, indicating that DNA methylation could be a consequence rather than a cause of gene silencing during development (Nan et al., 1998; Walsh and Bestor, 1999; Bestor, 2000; Szyf, 2005a).

The objectives of the present study were to determine the structural and functional conservation among DNA methyltransferases of human, mouse, and bovine as means of better understanding the role of these enzymes in epigenetic regulation during early mammalian embryonic development. Additionally we intended to improve the gene annotation of bovine DNMT's, for which annotation was incomplete. Our study confirms a high degree of conservation in the protein sequences and functional domains among the studied species. Although mouse is routinely used as a model for mammalian embryonic

development, our results showed that bovine and human DNMTs all have much higher similarities than the mouse and human DNMTs. This difference is especially striking for DNMT3L that has recently been shown to have dual roles in de novo methylation of DNA. These results have important implications for the selection of appropriate models to study mammalian DNA methylation during embryogenesis.

4.3 Materials and Methods

The nucleotide and protein reference sequences for mouse, human, and bovine DNMT's (DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L) were obtained from NCBI (see Table 4.1).

4.3.1 Structural analyses of DNMTs

Pairwise visual sequence comparisons were performed using dot matrix alignment of the protein reference sequences. The comparisons were generated for each DNMT for mouse *vs.* human, mouse *vs.* cow, and human *vs.* cow using the on-line dottup tool from the European Molecular Biology Open Software Suite EMBOSS using a word size of 10 (http://emboss.bioinformatics.nl/cgi-bin/emboss/dottup). A solid diagonal line indicates sequence similarity. A break in the line with a shift indicates an insertion or a deletion in one of the sequences. A gap indicates low similarity.

Pairwise sequence similarity was computed for the mouse, human, and bovine sequences using the GAP program (Huang X, 1991) with Blosum62 as the scoring matrix

and the following alignment parameters: match 11, mismatch -4, gap-open penalty 10, and gap-extension penalty 2. The program is available from Michigan Tech University (http://genome.cs.mtu.edu/align/align.html). Multiple sequence alignments for mouse, human and bovine DNMTs were done using both ClustalW (Chenna et al., 2003) and T-Coffee (Notredame et al., 2000) available on-line from the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw) using the Gonnet scoring matrix, a gap-open penalty of 10, and gap-extension penalty of 0.5. High level views of multiple sequence alignments and conserved domains were generated using in-house software that is part of the MSAVIS package (Lindeman et al., 2007).

Phylogenetic trees for the DNMTs were generated using the sequences listed in Table 4.3 with chicken used as the out-group for drawing the trees for all proteins except for DNMT3L, where opossum was used as the out-group. Multiple sequence alignments were generated using T-Coffee with the parameters listed above. Phylogenentic trees were generated using both neighbor joining and maximum likelihood methods implemented in the Phylip program (Felsenstein, 2005). Phylip programs used to generate the trees were PROTDIST, NEIGHBOR, PROMLK, SEQBOOT, and CONSENSE. The distance matrix for neighbor joining was computed using the JTT model (Jones et al., 1992). The molecular clock assumption was tested using a procedure described by Tuimala in 2006 (Tuimala, 2006). Because the assumption of a molecular clock was rejected with p=0.05 for DNMT1 and DNMT3b, but not for DNMT2 and DNMT3a, we used PROML (no molecular clock) for all phylogenetic analyses. The ML program PROML was run with the iterative search option (s) and with the global search (g) option for subtree pruning and regrafting to improve the quality of the tree. The

consensus trees were inferred from 250 bootstrap replications for the ML and NJ methods. Trees generated by the two methods were similar and ML trees are shown in the results since this method is generally considered to be more accurate and the branches in the ML trees had higher bootstrap values than those in the NJ trees. Trees were drawn using the online Interactive Tree of Life (iTOL) tool (Letunic and Bork, 2007).

4.3.2 Annotation of genes involved in DNA methylation

Annotations of DNMT2, DNMT3b and DNMT3L were performed using the Apollo software, an interactive tool that enables gene annotators to inspect computationally obtained gene predictions, and edit them by evaluating all the data supporting each annotation (Lewis et al., 2002). Apollo was successfully used to annotate the *Drosophila melanogaster* genome (Drysdale, 2003), and was the tool recommended by the Bovine Genome Sequencing Consortium for manual annotation of bovine genes. Each gene was uploaded to Apollo by specifying its region on the chromosome, (scaffold), by using the BLAST tool at BovineGenome.org to identify the official gene model (GLEAN) and its location. Apollo software was used to confirm the protein sequence accuracy by translating the DNA and identifying untranslated region (UTR), translation start, exons, introns, and translation stop. Previous protein information from NCBI or Ensembl, was compared to the GLEAN sequence and errors in the proteins were analyzed in detail. Annotations were submitted to the Bovine Genome Annotation Submission Database at BovineGenome.org.

4.3.3 Functional Analyses of DNMTs

Protein sequences were analyzed using the conserved domain database (CDD) at the NCBI website (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The program imports multiple sequence alignments from SMART (Simple Modular Architecture Research Tool), Pfam (Pfam-A seed alignments from the Protein families database of alignments and HMMs), and COGs (Clusters of Orthologous Groups of proteins). Which are used to compare the amino acid sequences in the query protein to sequences with known domains (Marchler-Bauer et al., 2005).

4.3.4 Determination of DNMT transcript abundance

4.3.4.1 In vitro maturation, fertilization and culture of embryos

Oocytes were collected from 2-8 mm follicles of bovine ovaries obtained from a local slaughterhouse. Only oocytes containing several layers of cumulus cells and homogenous cytoplasm were selected. Oocytes were washed three times in TL-HEPES before transferring into maturation media. The maturation medium used was Tissue Culture Medium (TCM) 199 (Gibco/Invitrogen) supplemented with 0.2 mM pyruvate, 0.5 µg/ml follicle-stimulating hormone (FSH; Sioux Biochemicals, Sioux City, IA, USA), 5 µg/ml luteinizing hormone (LH; Sioux Biochemicals), 10% fetal calf serum (FCS, Gibco/Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco/Invitrogen). Ten oocytes in each 50 µl maturation drop were covered with mineral oil and incubated for 24h at 39C° in a humidified incubator with 5% CO_2 (Misirlioglu et al., 2006). After 24 hours, mature oocytes were washed twice with TL-HEPES. Mature

oocytes were randomly selected for either RNA isolation or fertilization. Pools of 100 oocytes were frozen at -80°C on RLT lysis buffer (Qiagen Valencia, CA) until RNA isolation.

For fertilization, groups of 10 oocytes washed with TL-HEPES were transferred into 44 µl drops of fertilization medium (glucose-free TALP supplemented with 0.2 mM pyruvate, 6 mg/ml fatty acid-free BSA, 100 U/ml penicillin and 100 mg/ml streptomycin). Percoll gradient was used for separation of live spermatozoa in frozen-thawed semen (Misirlioglu et al., 2006). Briefly, sperm was thawed at 36°C for 1 min, and then carefully layered on top of the Percoll gradient system. Sperm was diluted in L-HEPES to 5.0 x10^7 cells/ml and 2 µl of diluted sperm were added to the 44 µl fertilization drops, which produced a final sperm concentration of 2.0 x10^6 cell/ml. Fertilization was completed by adding 2 µl of 5 µg/ml heparin, and 2 µl PHE solution (20 mM penicillamine, 10mM hypotaurine, 1 mM epinephrine) and co-culture of oocytes and sperm for 18h in the incubator (Leibfried and Bavister, 1982).

After 18 hours, cumulus cells were removed from oocytes by vortexing in a 1.5 ml Eppendorf tube for 3 min. Presumptive zygotes were washed three times by TL-HEPES and transferred into 50 µl culture drops of SOF under mineral oil (25 zygotes per drop). At 48 hours post in (hpi), cleavage rate (proportion of zygotes that reached the 2-cell stage) was recorded and 2-cell embryos were randomly selected for RNA isolation or further development. Pools of 100 2-cell stage embryos were frozen at -80°C on RLT lysis buffer (Qiagen Valencia, CA) until RNA isolation. Embryos selected for further development were kept under the same cultured conditions.

At 96 hpi, the proportion of embryos reaching the 8- cell stage was recorded and 8-cell stage embryos were randomly selected for RNA isolation or further development. Pools of 100 8-cell embryos were frozen at -80°C on RLT lysis buffer (Qiagen Valencia, CA) until RNA isolation. Embryos selected for further development were kept under the same cultured conditions. Five µl of FCS was added into each culture drop.

4.3.4.2 Isolation of RNA

Total RNA was isolated from pools of 100 oocytes, 100 2-cell embryos, 100 8-cell embryos and 10 blastocysts using an RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Quality of total RNA was estimated using the Bioanalyzer 2100 RNA 6000 picochip kit (Agilent, Palo Alto, CA, USA). RNA quantity and purity was determined using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA from all groups was normalized to 4 ng and used for cDNA synthesis using SuperScript III Platinum Two Step qRT-PCR kit according to the manufacturer's protocol. Cycling temperatures and times were 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min.

4.3.4.3 Real time PCR

Primers were designed using Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA). All primers were designed to span exon – intron boundaries to differentiate genomic DNA amplification (Table 3). Complementary DNA was generated using the SuperScript III Platinum® Two-Step qRT-PCR Kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The samples

were incubated for 10 min at 25°C, 50 min at 42°C and at 85°C for 5 min. Then 2U of E. coli Rnase H was added to each tube and incubated at 37°C for 20 min. Real-time quantitative PCR was performed to assess transcripts of DNMT1, DNMT3a, and DNMT3b relative to the housekeeping gene GAPDH. Quantitative assessment of RNA amplification was detected by SYBR® GreenER™ qPCR SuperMixes for iCycler (Invitrogen Life Technologies, Carlsbad, CA, 11761-100). Five µl cDNA were used for quantitative Real-time PCR reactions according to the iCycler iQ Real-Time PCR instrument (BIO-RAD). The primer concentration was adjusted to 10 µM. The cycling parameters were 50°C for 2 min, 95°C for 8 min 30 s for denaturation, 40 cycles of 15 s at 95°C and 30 s at 60°C and 30 s at 72°C for amplification and extension respectively. The melting curve was performed starting at 55°C with 0.5°C increase for 10 s in 80 cycles. Expression values were calculated using the relative standard curve method. Standard curves were generated using 10-fold serial dilutions for GAPDH and all target genes by measuring the cycle number at which exponential amplification occurred. Results from different groups were analyzed by one-way analysis of variance (ANOVA) by SAS 9.1 (SAS Institute inc. Carey, NC). Relative expression software tool (Whitworth et al., 2004) was used to compare all samples of each group. The mathematical model used in the is REST software is based on the PCR efficiencies and the crossing point deviation between samples (Pfaffl et al., 2002).

Complementary mRNA abundance data for bovine DNMT's in oocytes and 8 cell embryos was obtained from a bovine microarray experiment conducted earlier by our laboratory (Misirlioglu et al., 2006). Expression data for DNMT's from mouse was collected from two separate studies (Ratnam et al., 2002; Vassena et al., 2005). Human

DNMT's expression data was obtained from Huntriss et al., 2004 (Huntriss et al., 2004) and the patterns of expression were compared to those of bovine DNMT's in order to establish the dynamics of expression of the different enzymes in oocytes and embryogenesis.

4.4 Results

4.4.1 Structural analyses of DNMTs

Human DNMT2 (also known as TRDMT1) has 3 isoforms. In this study human isoform "a" and the corresponding isoform for the other two species was used for alignments. DNMT3a has 2 isoforms in mouse, 3 in humans and 3 in bovines. We used isoforms corresponding to human isoform a, for the other two species (mouse isoform 1, and bovine isoform 2). The enzyme DNMT3b has 4 isoforms in humans and mouse and 4 isoforms have also been reported for cattle (Golding and Westhusin, 2003) although they are not yet available in the databases. The bovine isoform AY244710 corresponds to human isoform 1. For the present study isoform 1 was used for all species. Complete information regarding the chromosome, gene, accession numbers, protein lengths, and isoforms used is summarized in Table 4.1.

All DNMT's showed a high degree of structural conservation at the protein level. The protein pairwise sequence alignments for all DNMT's from the three species (mouse *vs.* human, mouse *vs.* cow and cow *vs.* human) produced similarity scores however, human and bovine proteins produced higher similarity scores. These results are summarized in Table 4.2. Dottup graphs of the pairwise alignments are presented in

figures 4.1 through 4.15. Solid diagonal lines indicate similar sequences between both species. Gaps indicate low similarities. As in the pairwise alignments, the human and cow comparison produced higher similarities. The relatively low similarity of human and mouse DNMT3L when compared to human and bovine is particularly striking.

Figures 4.16 through 4.20 show the phylogenetic trees generated for DNMTs from eight mammals, opossum, and chicken. Chicken was used as the outgroup for drawing all trees except DNMT3L where opossum was used as the outgroup. Rat and mouse are shown to be more distantly related to human than cow for all enzymes with the exception of DNMT2. The extraordinary degree of conservation of DNMT3A results in a very shallow tree indicating that this enzyme is essential for survival and/or development. Recent results have shown that the C-terminal domains of both DNMT3A and DNMT3L interact forming a dimmer. The complexed C-terminal domains of DNMT3a and Dnmt3L further dimerize through a DNMT3a–DNMT3a interaction, forming a tetrameric complex with two active sites. Both interfaces (DNMT3a–Dnmt3L and DNMT3a–DNMT3a) are essential for the de novo methylation activity of DNMT3A (Jia et al., 2007). Both sequence alignment and phylogenetic results indicate that bovine and human DNMT3L show much greater similarity than mouse and human DNMT3L.

4.4.2 Annotation of genes involved in methylation

Manual annotation of DNMT3b showed that the currently available protein sequence at the time lacked the C-terminal amino acids due to a missing exon and an incorrect end codon. The corrected sequence was uploaded to the Bovine Genome

Annotation submission site. The corrected sequence has been uploaded to NCBI with the accession number NP_861529.2. Annotation of DNMT2 and DNMT3L showed no errors in the predicted sequences.

4.4.3 Functional Analyses of DNMTs

All the known mammalian DNMTases have a common structure consisting of a catalytic C-terminal Cytosine-C5 specific DNA methylase domain. This domain is found in both prokaryotes and eukaryotes and six of the 10 conserved motifs from prokaryotes are also conserved in vertebrates. In addition, with the exception of DNMT2, all enzymes contain a large N-terminal domain that has been identified as having regulatory functions. Figures 4.21 through 4.25 provide a high level view of the multiple sequence alignment of the DNMT enzymes with conserved domains from eight mammalian species (7 placental mammals and one marsupial), for which genome sequencing is complete.

4.4.4 Determination of DNMT transcript abundance

Bioanalyzer assessment showed RNA degradation for 2-cell and 8-cell embryos consistent with the physiological maternal mRNA degradation occurring at these stages. At the blastocyst stage, total RNA integrity was high as the 28S:18S ribosomal RNA band ratio was >1.9.

No differences in the levels of DNMT1 mRNA were found among MII, 2-cell, and 8-cell groups. However, the mRNA level of DNMT1 in the blastocyst group was significantly lower compared to the other groups. The transcript levels of DNMT3a were similar between the MII and 8-cell groups, but they were significantly higher in the 2-cell

and blastocyst groups compared to the oocytes. The transcript levels of DNMT3b were similar among all the four groups.

4.5 Discussion

The predominant isoform of DNMT1 in somatic cells has 1619, 1616, and 1611 amino acids in mouse, human, and bovine species respectively. A shorter isoform of Dnmt1, called Dnmt1o, is found specifically in growing oocytes and during early preimplantation development (Ratnam et al., 2002). Dnmt1o lacks the N-terminal 114 amino acid residues, since its translation initiation lies on exon 4 instead of exon 1. Dnmt1o displays an increased in vivo stability against degradation and stable ooplasmic stores of Dnmt1o are available in the oocytes and early embryos.

Parwise comparisons obtained for all of the DNMT proteins for mouse, bovine, and human species showed a higher sequence similarity between human and bovine than between mouse and the other two species. The almost complete conservation of DNMT3A among the three organisms is particularly noteworthy. Multiple sequence alignments produced using both T-Coffee and ClustalW gave similar alignments. However, the T-Coffee alignments had fewer gaps and maintained the structure of conserved domains.

T-Coffee alignments were used as input for Phylip to build the phylogenetic trees. Golding and Westhusin reported a high level of sequence conservation for DNMT2 among species (Golding and Westhusin, 2003). In the present study, conservation of DNMT2 sequences among the studied species was comparable to that of DNMT1.

However, the phylogenetic tree of DNMT2 showed a different branching compared to that observed for the other DNA methyltransferases, with mouse and rat proteins being closer to the proteins of primates. The function of DNMT2 in DNA methylation has not been resolved.

It is sometimes difficult to evaluate structural conservation across a wide range of mammals including the newly sequenced genomes because of the large number of predicted protein isoforms. This is particularly true for DNMT1, for which there are 11 predicted isoforms in the chimpanzee, all of which with strong sequence similarities to human DNMT1, but with deletions or insertions when compared to human isoform a. Thus for all sequences, we have used the isoform that most closely aligns with the human isoform listed in Table 1 in our analyses. For some predicted proteins, we have used manual annotation to correct apparent missed exons and missed translation start sites. For example, in the predicted DNMT1 protein for rhesus monkey, there is a large deletion with respect to the chimp and human proteins. The first part of this deletion aligns with the rhesus genome within the predicted gene on Chromosome 19. A second part of the deletion matches an unassembled contig. Likewise, some of the predicted proteins, such as DNMT1 in dog, use a different translation start site than the reference sequences in other species, although the more typical translation start site is present. In these cases, we have re-annotated the protein to use the canonical translation start site. Supplemental Data 1 describes all manual re-annotation and provides both the original and revised protein sequences.

As expected, all of the DNMTs have high levels of conservation of the conserved motifs in the C-terminal domain. DNMT1 has an N-terminal domain that binds

a DMAP-1 transcriptional co-repressor (Rountree et al., 2000), a CXXC zinc finger domain containing eight conserved cysteine residues that bind to zinc, and two Bromo Adjacent Homology (BAH) domains. The BAH domain is thought to mediate protein-protein interaction and to play a role in transcriptional silencing and remodeling of chromatin (Callebaut et al., 1999). Both DNMT3a and DNMT3b contain a PWWP domain that is essential for DNMT binding to chromatin (Turek-Plewa and Jagodzinski, 2005) and that is almost completely conserved in all species shown.

According to Carlson et al (Carlson et al., 1992), DNMT1 is expressed approximately 50,000 fold higher in oocytes as compared to somatic cells. The splice variant DNMT1o lacks the first 114 N-terminal amino acids and is expressed in mouse oocytes. Another study shows that mouse Dnmt1o is expressed in oocytes and zygotes and is later replaced by the complete variant (Ratnam et al., 2002). The Affymetrix Bovine GeneChips do not include particular probe sets for DNMT1o, therefore it was not possible to differentiate this variant in our previous microarray experiment, which showed a 6-fold decrease in DNMT1 transcript abundance in 8-cell embryos compared to that in MII oocytes (Misirlioglu et al., 2006). However, the sole analysis of mRNA levels for DNMT1 in pre-implantation embryos may underestimate protein levels, which are known to be very high in both mature oocytes and embryos (Bestor, 2000). In the present study, we did not find any significant difference in the levels of DNMT1 transcripts in the MII oocytes, 2-, and 8-cell embryos. However, the levels of DNMT1 transcripts were more than 2.5 times lower at the blastocyst stage as compared to the other developmental stages tested.

Examination of Dnmt2 mRNA in mouse determined a very low transcript abundance during earlier embryonic development increasing significantly between the 8-cell and morulae/blastocyst stages (Vassena et al., 2005). This pattern of expression contrasted with that of rhesus monkey and human. In rhesus monkey DNMT2 mRNA was detected in similar levels from the GV oocyte stage to the morulae stage with a slight decrease at the blastocyst stage (Vassena et al., 2005). In human, DNMT2 mRNA was variably detected in MII oocytes and blastocysts (Huntriss et al., 2004). Golding and Westhusin reported the presence of DNMT2 mRNA in all bovine tissues, being particularly abundant in adult testis and ovaries. They also detected bovine DNMT2 mRNA at all embryonic stages from the two-cell through blastocyst stage (Golding and Westhusin, 2003). In our microarray study, DNMT2 transcripts were 10 fold higher in bovine oocytes compared to 8-cell embryos (Misirlioglu et al., 2006). In other study we found similar levels of DNMT2 transcripts in bovine fibroblasts and blastocysts (data not published). These different patterns of DNMT2 mRNA abundance during early embryonic stages could suggest species specific differences in this enzyme function, which has been heavily debated in recent years. The available data suggest that DNMT2 has a weak methyltransferase activity on unmodified DNA and RNA. By contrast, the enzyme activity on a tRNA ^{Asp} template seems comparatively strong (Goll et al., 2006), which might indicate DNMT2 participation in complex nucleic acid modification pathways.

The previously reported pattern of DNMT3b expression in mouse oocytes and embryos was reciprocal to that of DNMT3a with low abundance in oocytes and early embryos, and then a sharp increase in abundance at the blastocyst stage (Vassena et al.,

2005). In human, DNMT3B was detected continuously from the MII stage oocyte to the blastocyst stage (Huntriss et al., 2004). In bovine, we reported a similar pattern of expression for DNMT3a and DNMT3b with an average 3-fold increase in mRNA abundance in the 8-cell embryos compared to the oocytes (Misirlioglu et al., 2006). In contrast, the present study did not find significant differences in DNMT3b abundance in the developmental stages studied and only significantly lower DNMT3a transcripts in MII oocytes compared to 2-cell embryos and blastocysts. Relative mRNA expression values are summarized in figure 4.26.

Although DNMT3a and DNMT3b have high structural and functional similarities, it has been proposed that they have distinct genomic targets and functions (Okano et al., 1999). In humans, mutations in the DNMT3b gene cause the Immunodeficiency, Centromeric instability and Facial anomalies syndrome known as ICF. Patients with ICF have hypomethylated DNA and abnormalities localized mostly to the juxtacentromeric heterochromatin of chromosomes 1 and 16 (Ehrlich, 2003b). From this syndrome it is clear that *de novo* DNA methylation through DNMT3b has an important role in late immune function and facial phenotype. The role of DNMT3a and DNMT3b is paramount during embryonic development, yet again at this level functional differences between both enzyme are evident since DNMT3a deficient mice develop to term and appear normal at birth, while DNMT3b deficient mice die *in utero* (Ueda et al., 2006).

One of the functional differences between DNMT3a and DNMT3b could be their interaction with the enzyme-like protein DNMT3L, which lacks the catalytic domain common to the DNA methyltransferases. It has been reported that DNMT3L stimulates de novo methylation by interacting with DNMT3a to (Chedin et al., 2002). Therefore,

DNMT3L acts as a stimulatory factor for DNA methylation by DNMT3a. This is particularly true for de novo methylation of imprinted genes in mammalian germ cells (Jia et al., 2007). Homozygous Dnmt3L mutant male and female mice are viable, but infertile (Bourc'his et al., 2001; Hata et al., 2002; Hata et al., 2006). Furthermore, Dnmt3L deficient oocytes showed aberrant methylation of the imprinted genes Small Nuclear Ribonucleoprotein Polypeptide N (Snrpn), paternally expressed 3 (Peg3) and insulin-like growth factor 2 receptor (Igf2r) (Lucifero et al., 2007). The level of interactions between the different DNMT's has not been fully established yet since depletion of either DNMT3L or DNMT1o in growing oocytes results in an increase in DNMT3B, suggesting a potential compensation mechanism by this enzyme, since an interaction between DNMT3L and DNMT3A is crucial for de novo methylation.

The high degree of structural and functional conservation among the different DNMT's, not only within mammals but in all eukaryotes, highlights the importance of DNA methylation patterns in the regulation of gene expression, particularly at the onset of development during gametogenesis and embryogenesis. Understanding the complex interactions between these enzymes and their roles could shed light on the role of epigenetics in human reproduction and disease. Because of the importance of these enzymes, it is essential that we identify appropriate models for study of DNA methylation during early embryonic development.

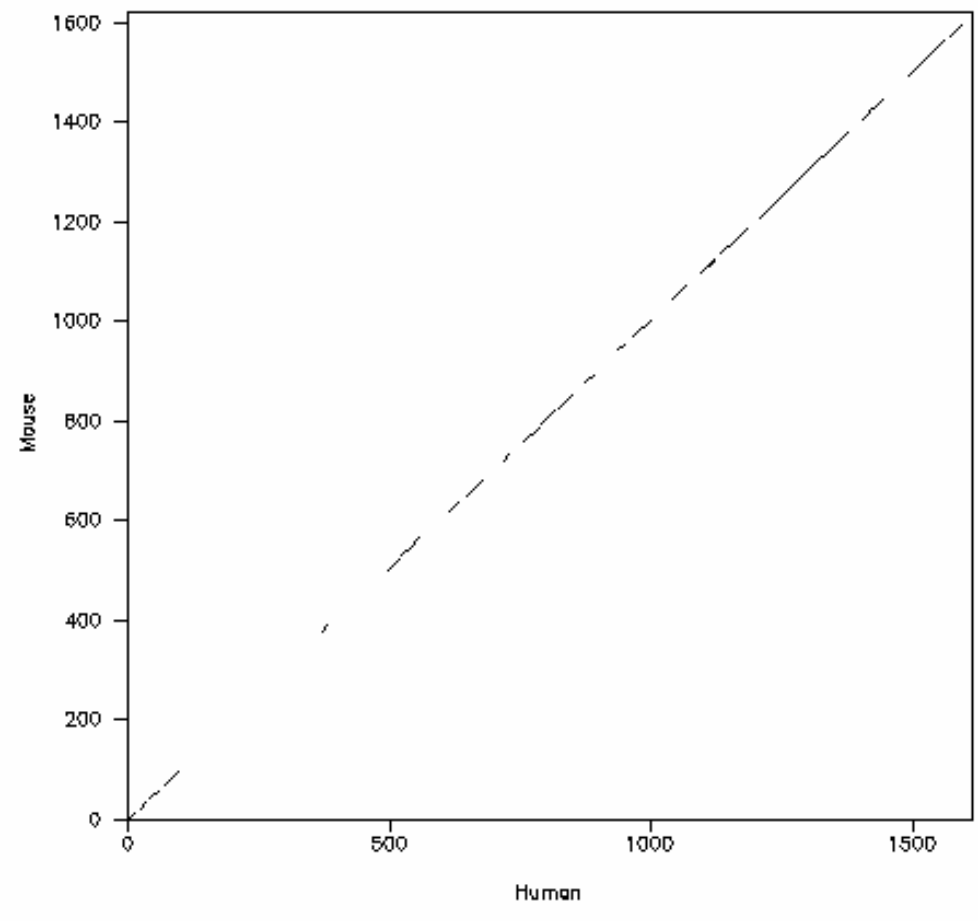

Figure 4.1 Dottup analysis of mouse and human DNMT1 sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion.

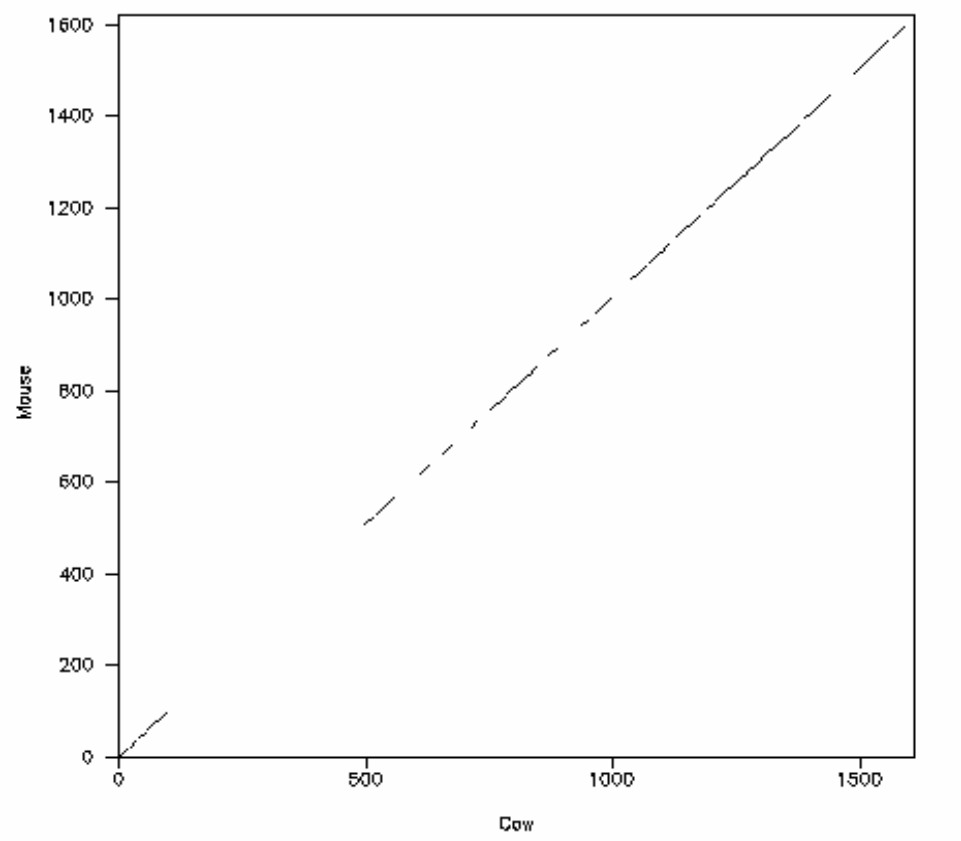

Figure 4.2 Dottup analysis of mouse and cow DNMT1 sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion. There is low homology in the initial part of the sequence.

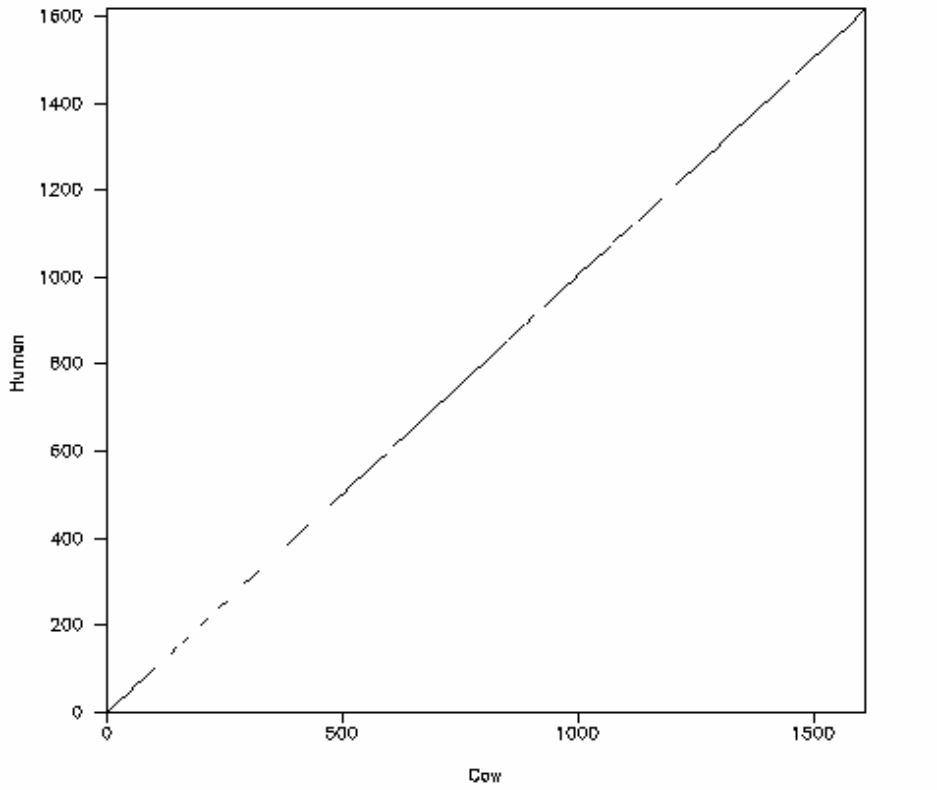

Figure 4.3 Dottup analysis of human and cow DNMT1 sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion.

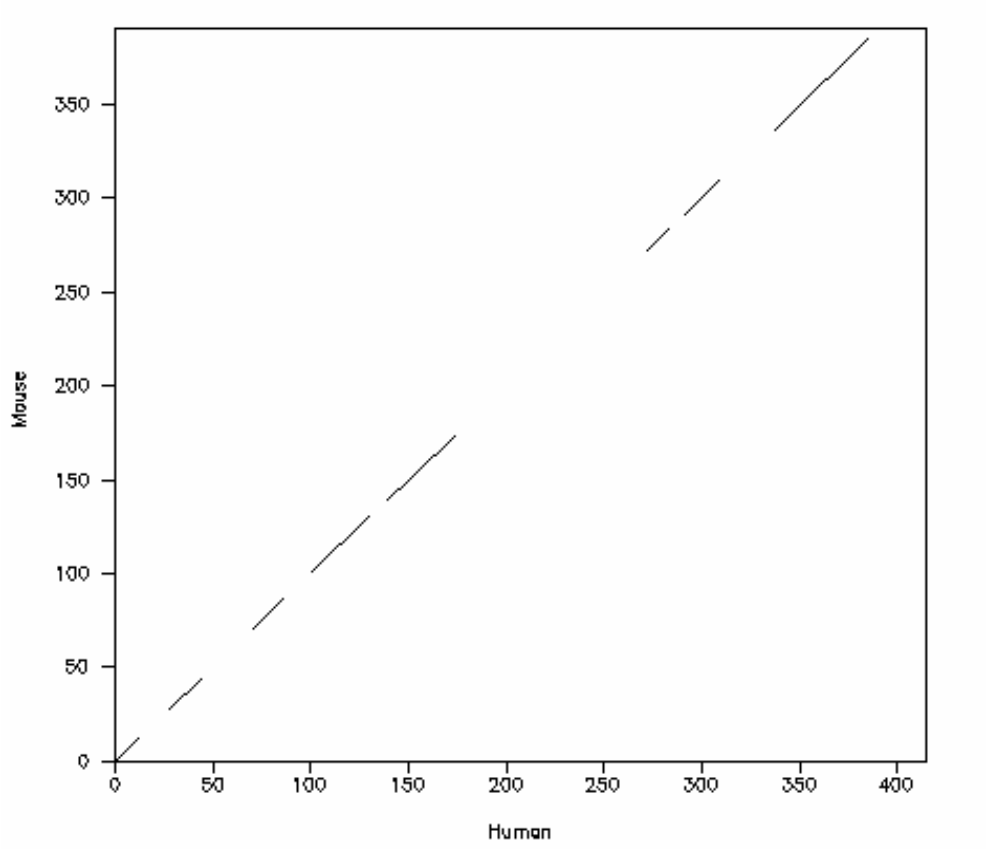

Figure 4.4 Dottup analysis of mouse and human DNMT2 sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion.

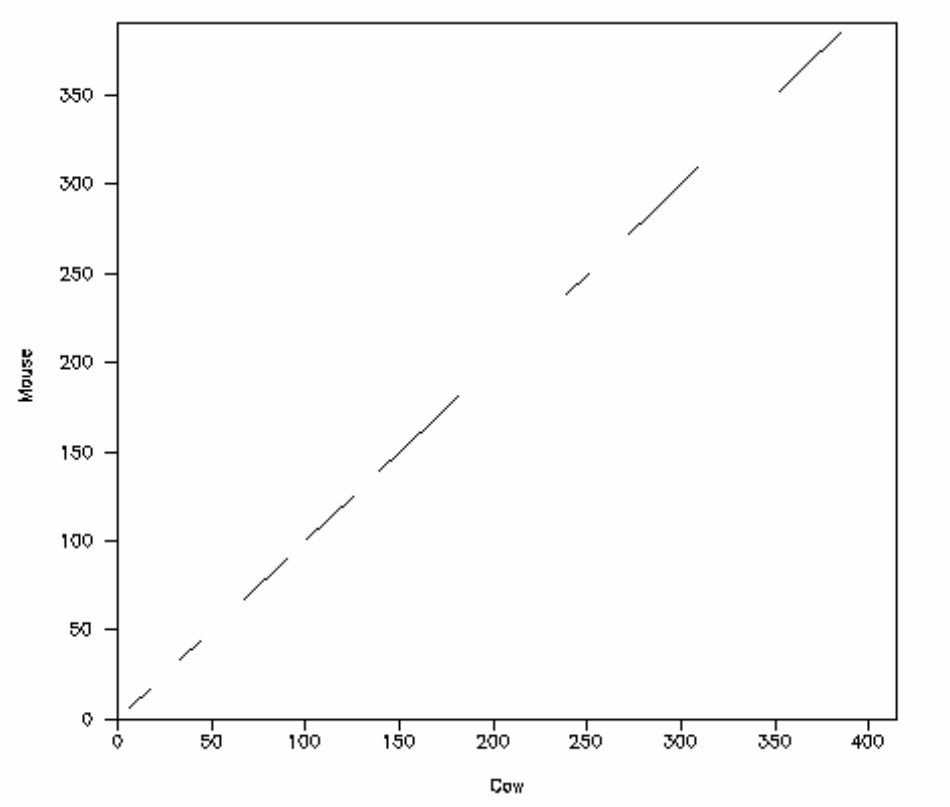

Figure 4.5 Dottup analysis of mouse and cow DNMT2 sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion.

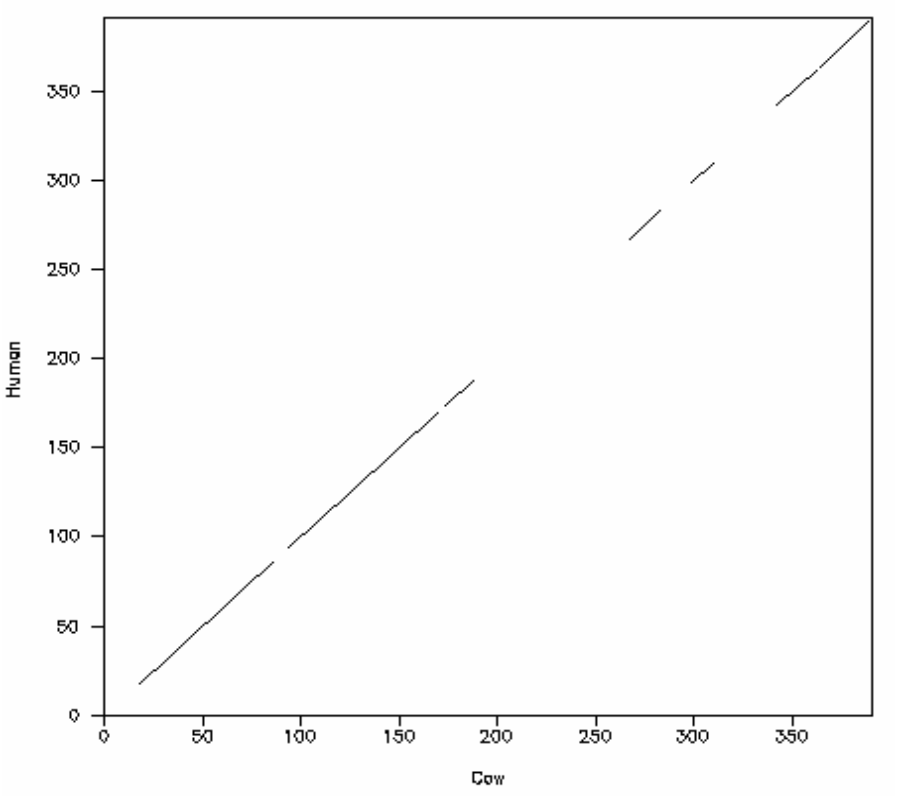

Figure 4.6 Dottup analysis of human and cow DNMT2 sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion.

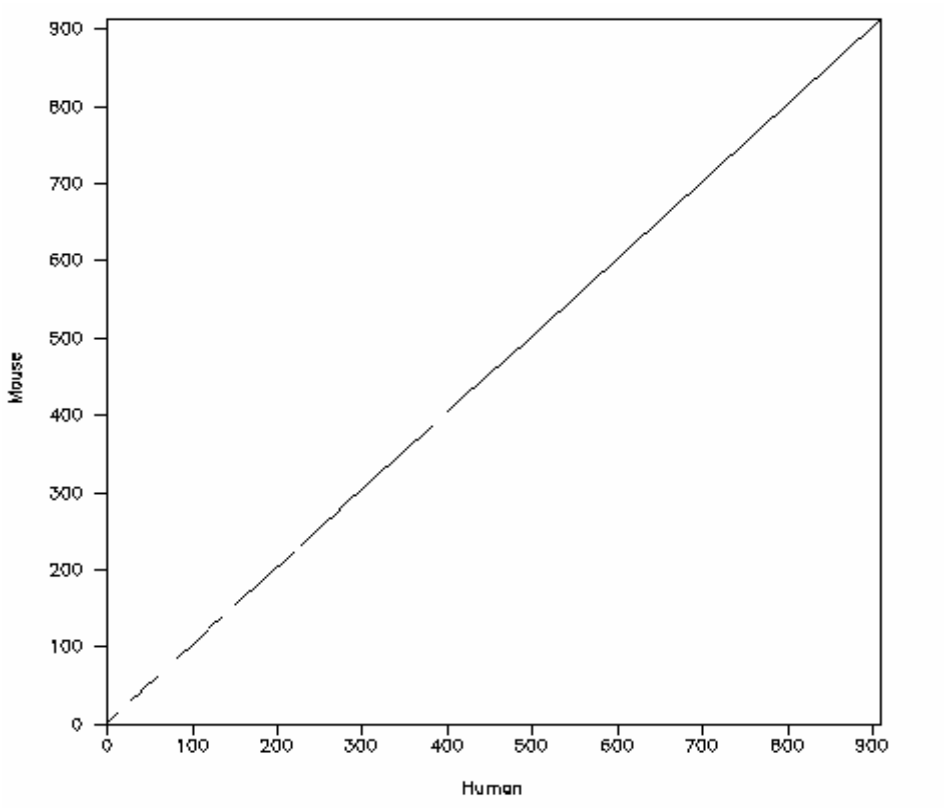

Figure 4.7 Dottup analysis of mouse and human DNMT3a sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion. There is a high sequence similarity

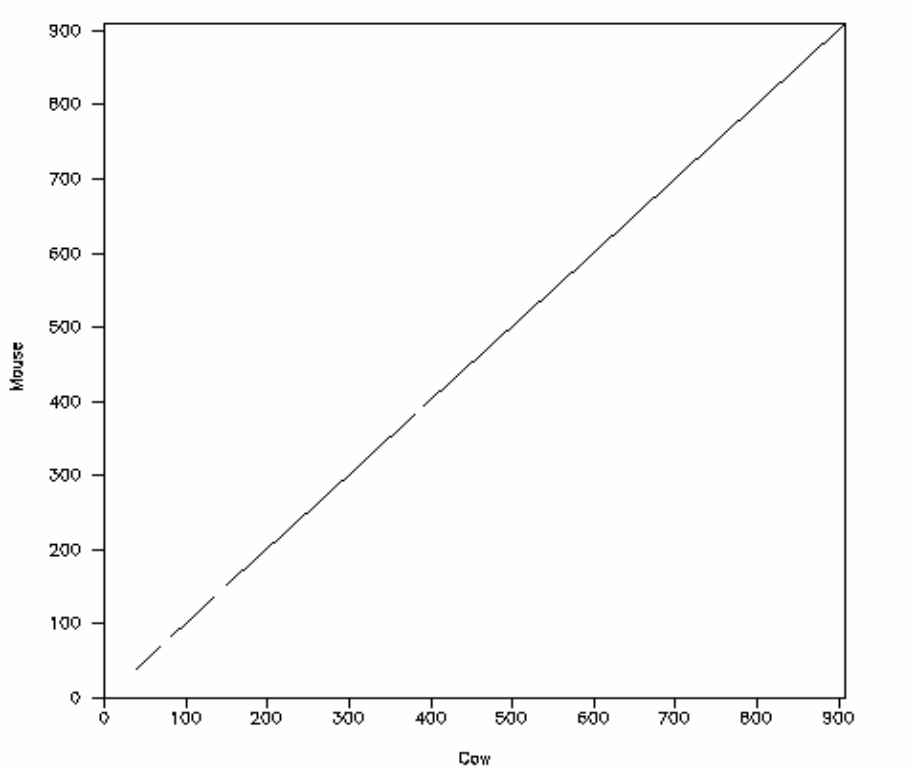

Figure 4.8 Dottup analysis of mouse and cow DNMT3a sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion. There is a high sequence similarity

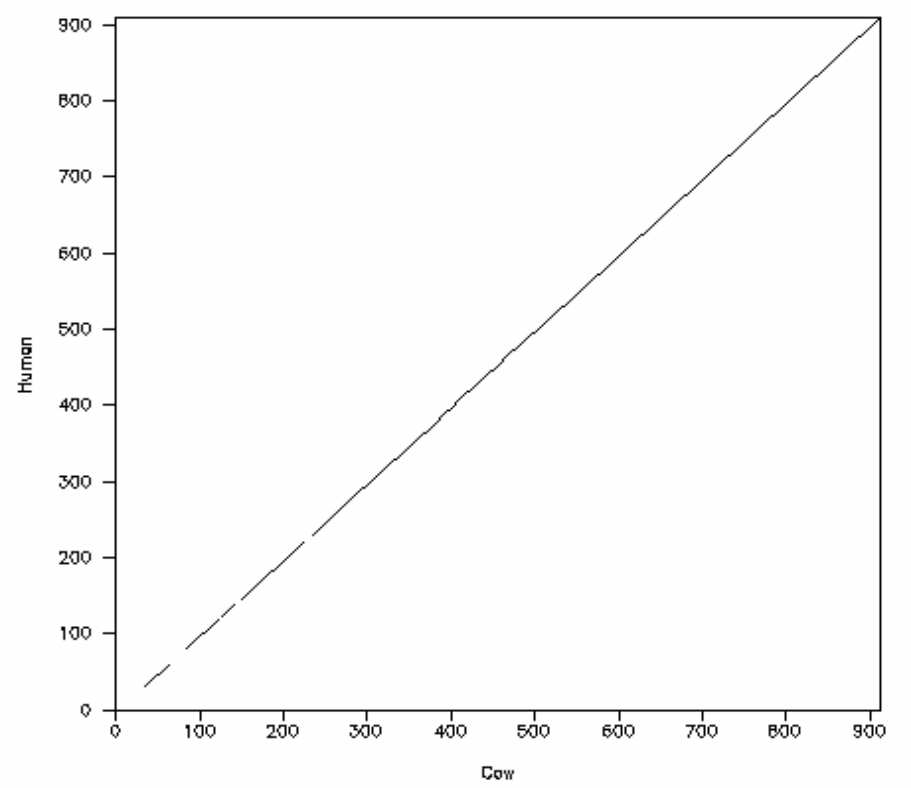

Figure 4.9 Dottup analysis of human and cow DNMT3a sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion. There is a high sequence similarity

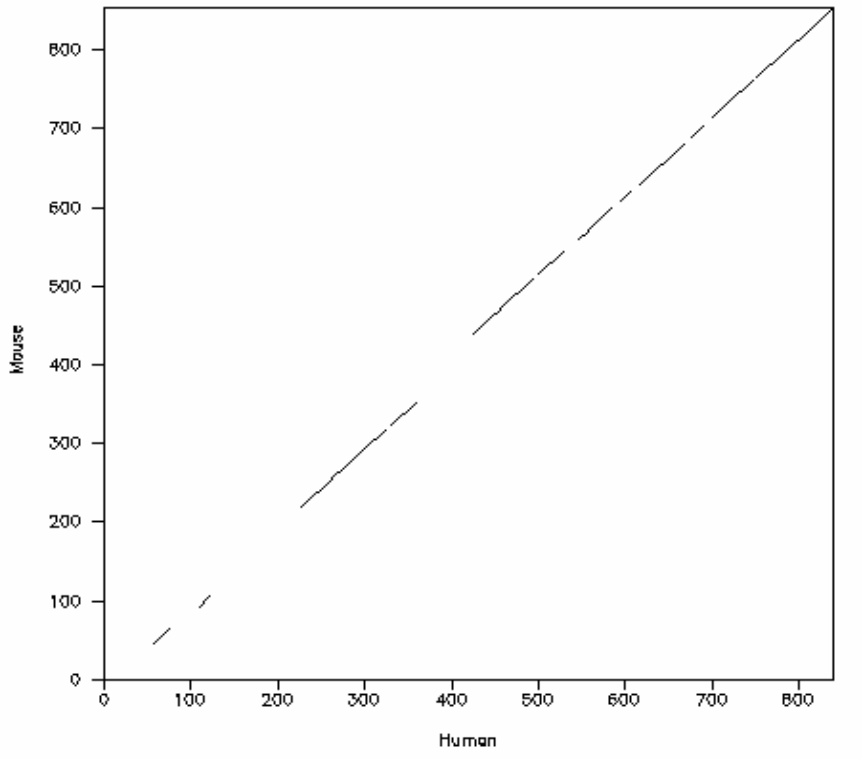

Figure 4.10 Dottup analysis of mouse and human DNMT3b sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion.

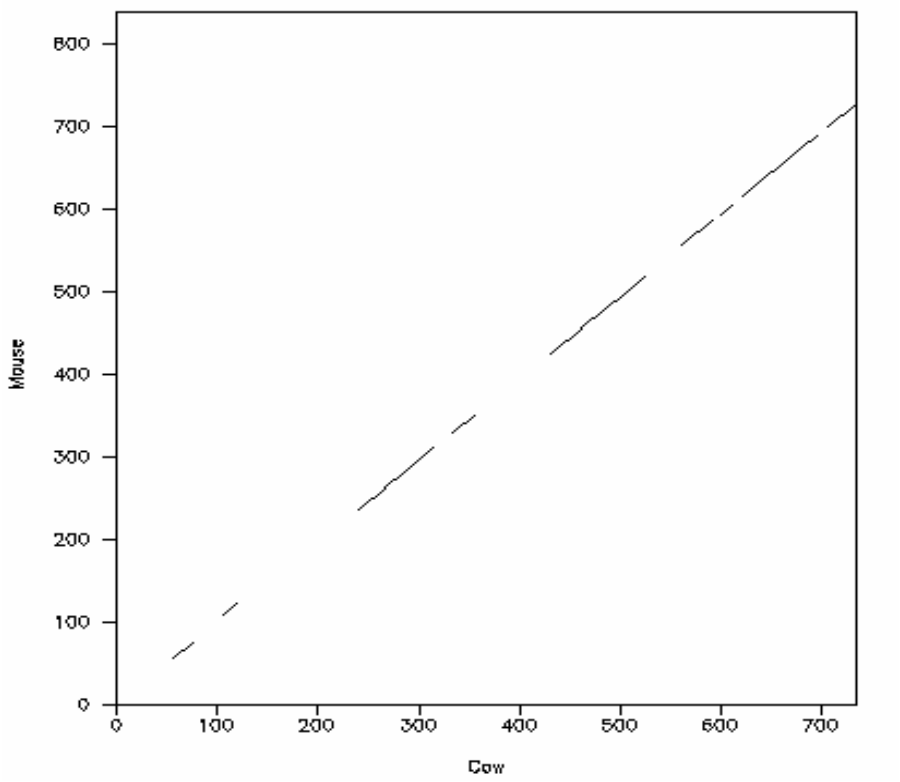

Figure 4.11 Dottup analysis of mouse and cow DNMT3b sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion. A deletion of the last part of the cow sequence is observed.

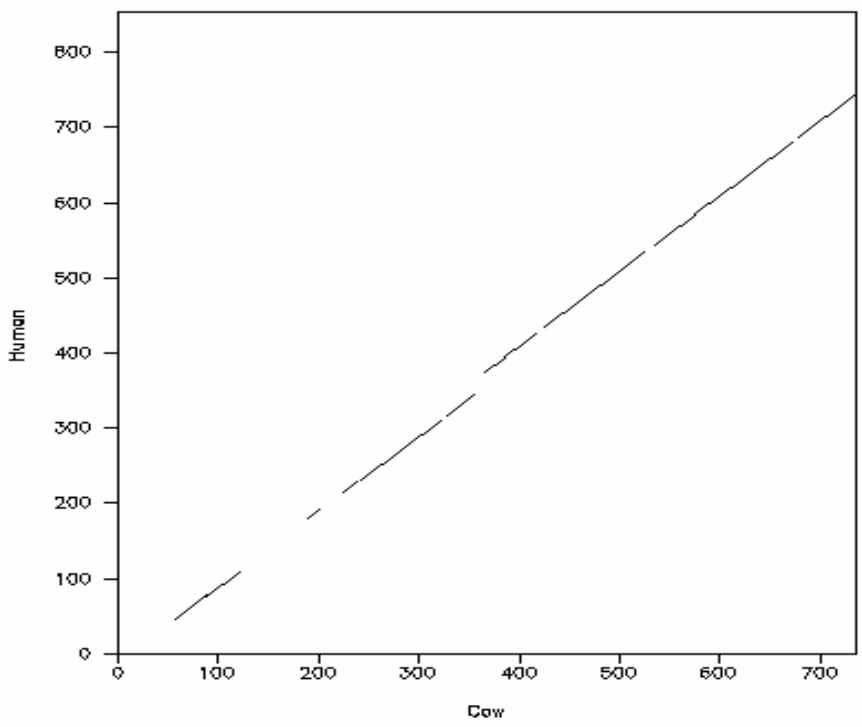

Figure 4.12 Dottup analysis of human and cow DNMT3b sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion. A deletion of the last part of the cow sequence is observed.

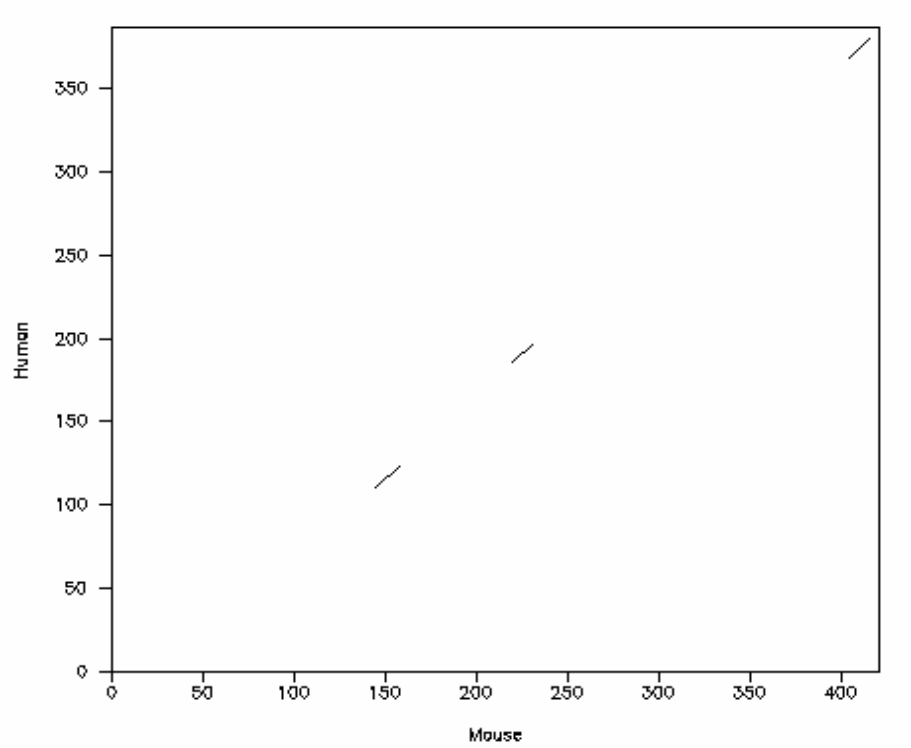

Figure 4.13 Dottup analysis of mouse and human DNMT3L sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion. There is a low sequence similarity for this protein.

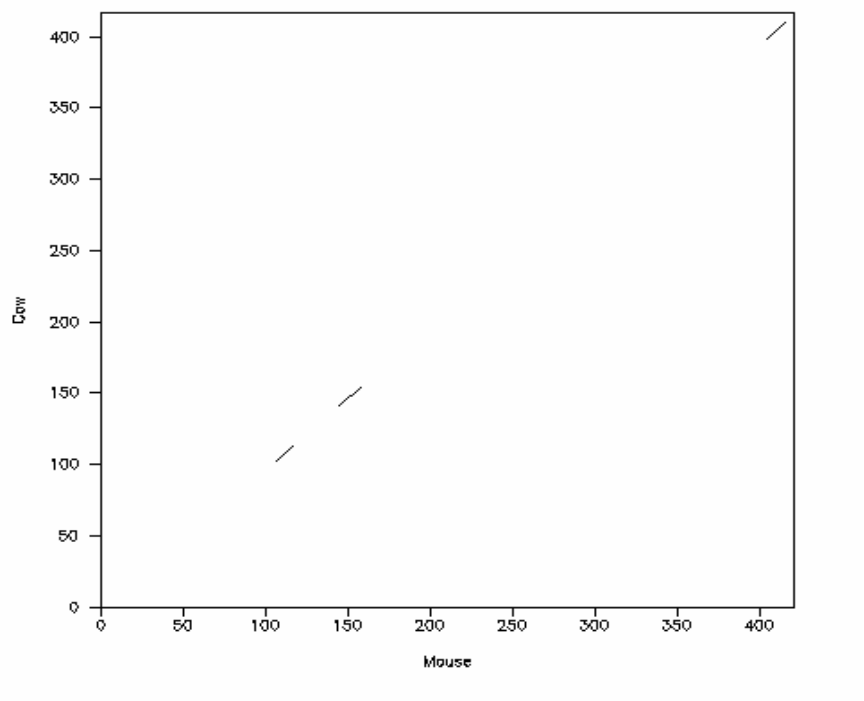

Figure 4.14 Dottup analysis of mouse and cow DNMT3L sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion. There is a low sequence similarity for this protein.

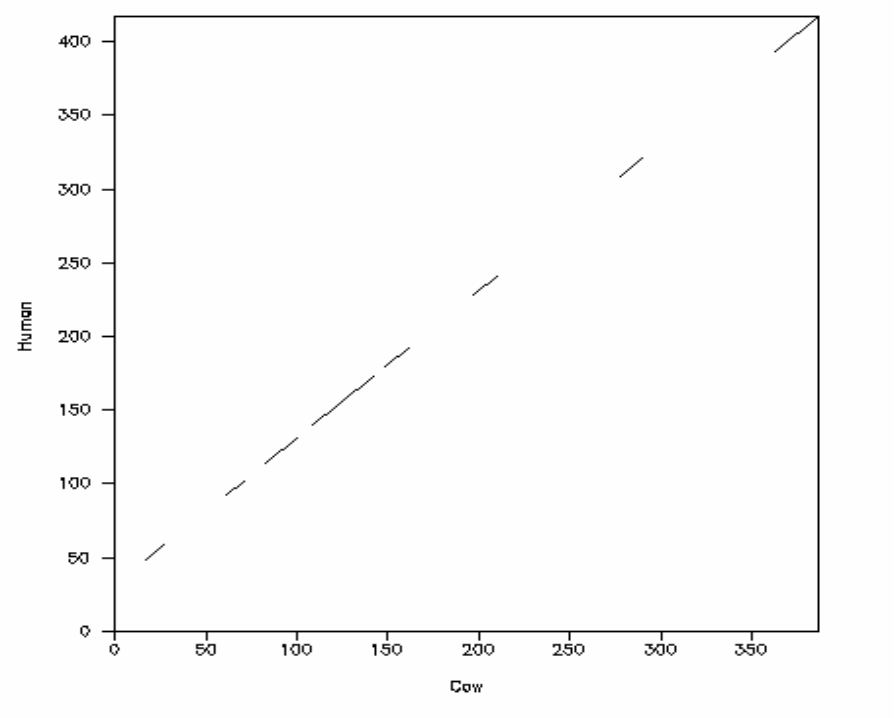

Figure 4.15 Dottup analysis of human and cow DNMT3L sequence similarities.

Note: Black dots represent identical amino acids. Blank spaces represent different amino acids. A shift in the line represents an insertion or a deletion. There is a low sequence similarity for this protein.

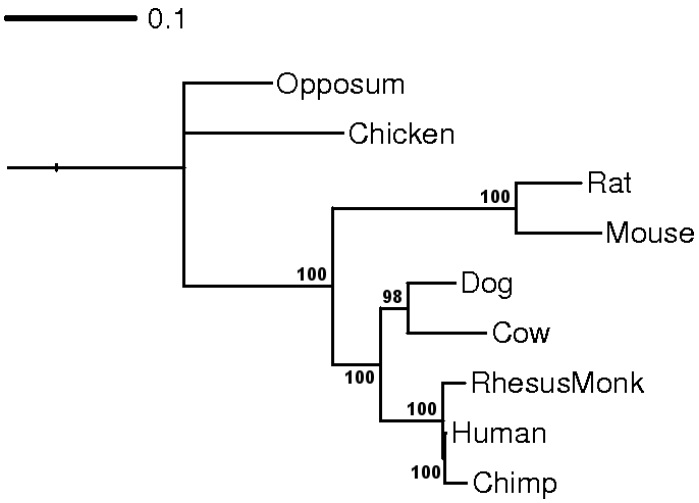

Figure 4.16 Phylogenetic tree of DNMT1.

Note: Chicken was the outgroup for generating the trees. Bootstrap values out of 250 replicates are shown for branches involving mammals.

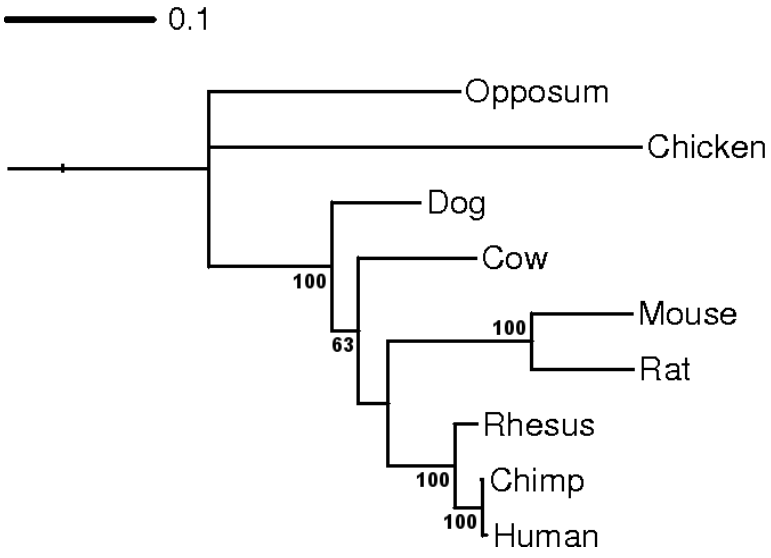

Figure 4.17 Phylogenetic tree of DNMT2.

Note: Chicken was the outgroup for generating the trees. Bootstrap values out of 250 replicates are shown for branches involving mammals.

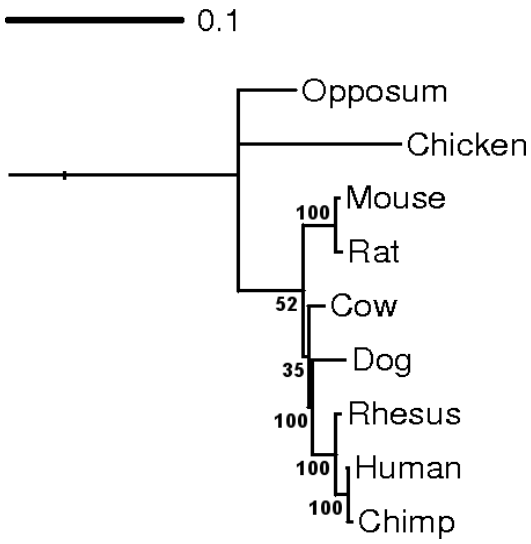

Figure 4.18 Phylogenetic tree of DNMT3a.

Note: Chicken was the outgroup for generating the trees. Bootstrap values out of 250 replicates are shown for branches involving mammals.

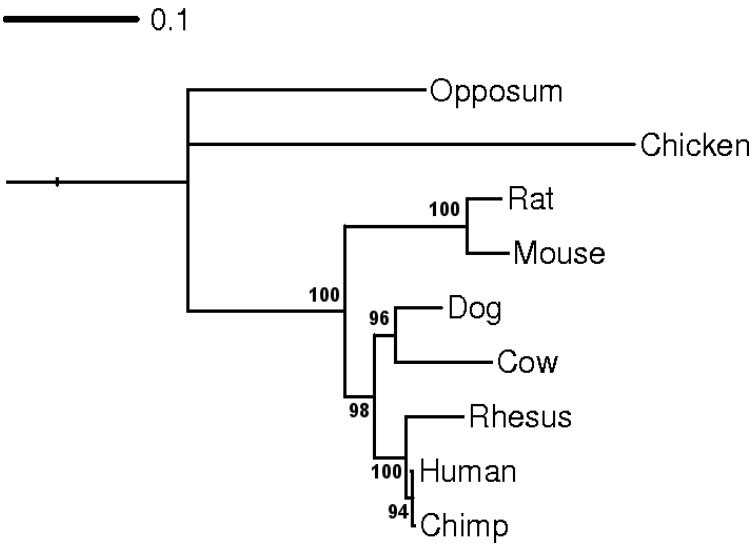

Figure 4.19 Phylogenetic tree of DNMT3b.

Note: Chicken was the outgroup for generating the trees. Bootstrap values out of 250 replicates are shown for branches involving mammals.

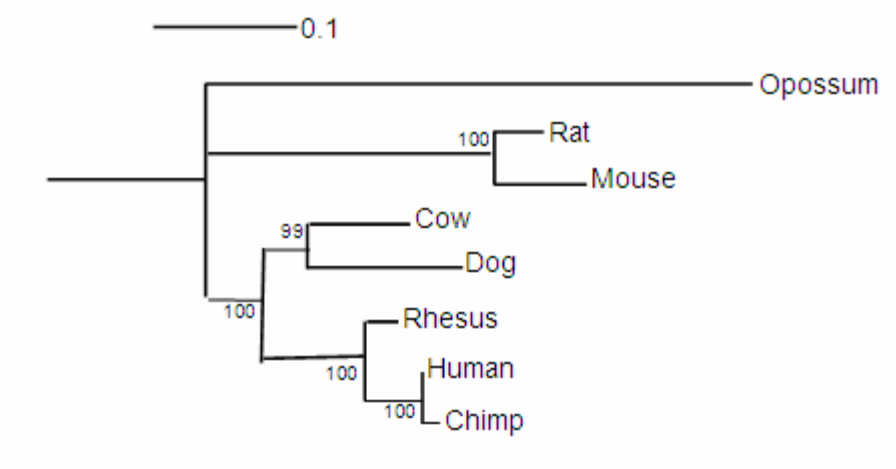

Figure 4.20 Phylogenetic tree of DNMT3L.

Note: Opossum was the outgroup for generating the trees. Bootstrap values out of 250 replicates are shown for branches involving mammals.

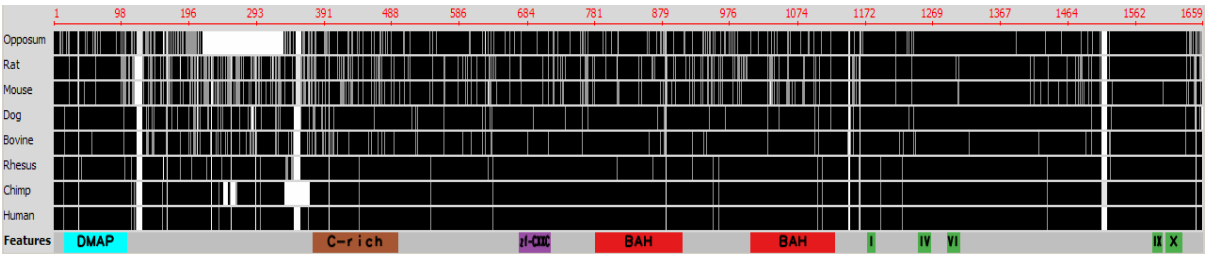

Figure 4.21 Multiple sequence alignment and functional domains of DNMT1.

Note: The species compared are opossum, rat, mouse, dog, bovine, macaque, chimpanzee, and human, using Different color tags at the bottom represent conserved domains. Black represents identical amino acids for that column in all the species. Gray represent amino acids that differ. White indicates a gap.

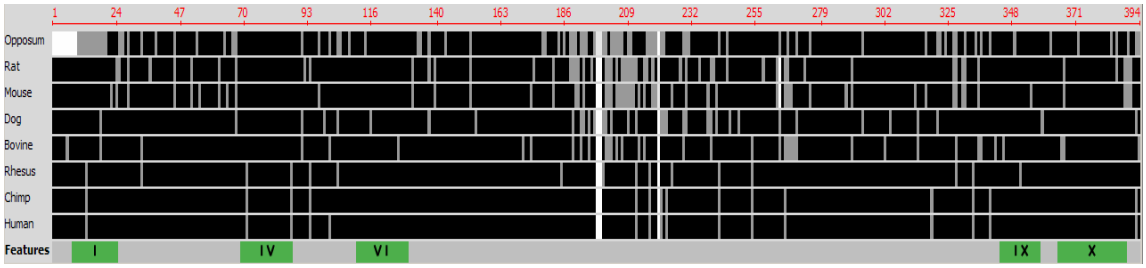

Figure 4.22 Multiple sequence alignment and functional domains of DNMT2

Note: The species compared are opossum, rat, mouse, dog, bovine, macaque, chimpanzee, and human, using Different color tags at the bottom represent conserved domains. Black represents identical amino acids for that column in all the species. Gray represent amino acids that differ. White indicates a gap.

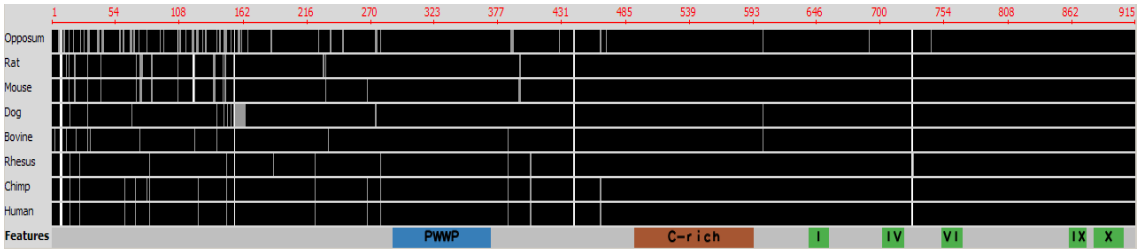

Figure 4.23 Multiple sequence alignment and functional domains of DNMT3a.

Note: The species compared are opossum, rat, mouse, dog, bovine, macaque, chimpanzee, and human, using Different color tags at the bottom represent conserved domains. Black represents identical amino acids for that column in all the species. Gray represent amino acids that differ. White indicates a gap.

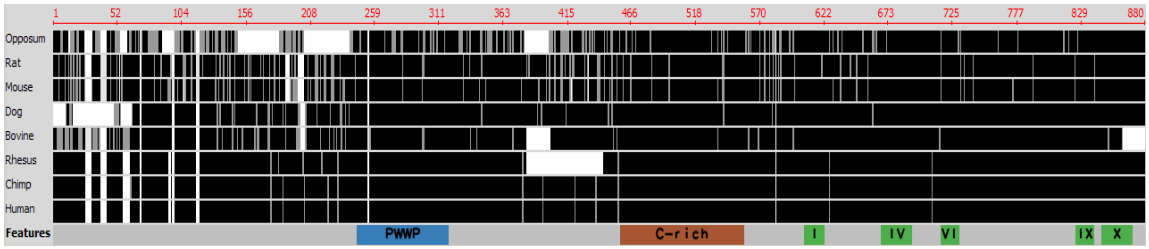

Figure 4.24 Multiple sequence alignment and functional domains of DNMT3b.

Note: The species compared are opossum, rat, mouse, dog, bovine, macaque, chimpanzee, and human, using Different color tags at the bottom represent conserved domains. Black represents identical amino acids for that column in all the species. Gray represent amino acids that differ. White indicates a gap.

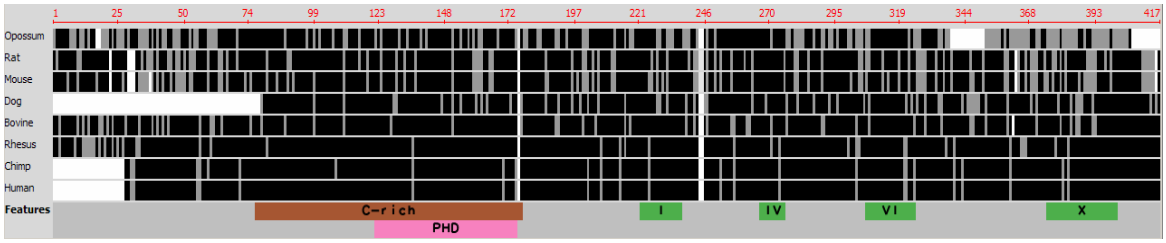

Figure 4.25 Multiple sequence alignment and functional domains of DNMT3L.

Note: The species compared are opossum, rat, mouse, dog, bovine, macaque, chimpanzee, and human, using Different color tags at the bottom represent conserved domains. Black represents identical amino acids for that column in all the species. Gray represent amino acids that differ. White indicates a gap.

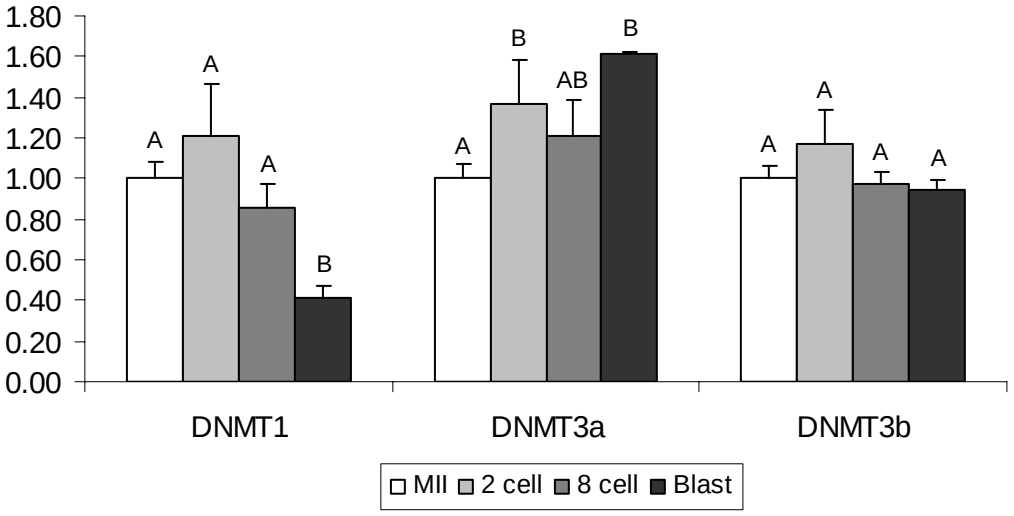

Figure 4.26 Real Time PCR for analysis of DNMT1, DNMT3a, and DNMT3b mRNA abundance in bovine oocytes and embryos.

Note: Bars represent relative expression values of 2-cell embryos, 8-cell embryos, and blastocysts to the expression in MII oocytes. Different letters represent statistically significant differences ($P<0.05$).

Table 4.1 Isoforms of the different DNMT's from mouse, human and cow included in the study

Enzyme	Species	Chrom	Gene ID	mRNA	Protein	aa
DNMT1	Mouse	9 5.0 cM	13433	NM_010066.3	NP_034196.3	1619
	Human	19p13.2	1786	NM_001379.1	NP_001370.1	1616
	Cow	7q15	281119	NM_182651.1	NP_872592.1	1611
DNMT2	Mouse	2 A1	13434	NM_010067.2	NP_034197.2	415
	Human	10p15.1	1787	NM_004412.3	NP_004403.1	391
	Cow	13	353353	NM_181812.1	NP_861528.1	391
DNMT3a	Mouse	12 A2-A3	13435	NM_007872.4	NP_031898.1	908
	Human	2p23	1788	NM_022552.3	NP_072046.2	912
	Cow	11	359716	XM_867643.2	XP_872736.1	909
DNMT3b	Mouse	2 A2-A3	13436	NM_001003961.1	NP_001003961.1	859
	Human	20q11.2	1789	NM_006892.3	NP_008823.1	853
	Cow	13	31074162	AY244710	AAP20552.1	826
DNMT3L	Mouse	10 C1	54427	NM_001081695.1	NP_001075164.1	421
	Human	21q22.3	29947	NM_013369.2	NP_037501.2	387
	Cow	1	613785	XM_864897.2	XP_869990.2	417

Table 4.2 Percent identity scores for DNMT methyltransferases in human, mouse and cow.

	DNMT1	DNMT2	DNMT3a	DNMT3b	DNMT3L
Human *vs.* Mouse	77%	75%	95%	81%	57%
Mouse *vs.* Cow	75%	76%	97%	75%	60%
Human *vs.* Cow	88%	85%	96%	84%	72%

Percent identity scores were obtained from Pairwise Sequence Alignments as the number of identities in the alignment divided by the number of residues compared (gap positions are excluded).

Table 4.3 DNA methyltransferase sequences used for phylogenetic trees.

DNMT	Taxon	Database	Accession	Status
DNMT1	Gallus gallus	NCBI	NP_996835.1	Refseq
	Monodelphis domestica	NCBI	NP_001028141.1	Refseq
	Bos taurus	NCBI	NP_872592.1	Refseq
	Homo sapiens	NCBI	NP_001370.1	Refseq
	Pan troglodytes	UCSD	chr19.11.012.a	Predicted Build 2, v1[2]
	Macaca mulatta	NCBI	XP_001104704.1	Predicted[1,2]
	Mus musculus	NCBI	NP_034196.3	Refseq
	Rattus norvegicus	NCBI	NP_445806.1	Refseq
	Canis familiaris	UCSD	chr20.54.038.a	Predicted v 2.0 May 2005[2]
DNMT2	Gallus gallus	NCBI	NP_001020002.1	Curated
	Monodelphis domestica	NCBI	XP_001377353.1	Predicted
	Bos taurus	NCBI	NP_861528.1	Refseq
	Homo sapiens	NCBI	NP_004403.1	Refseq
	Pan troglodytes	NCBI	XP_001151907.1	Predicted
	Macaca mulatto	NCBI	hmm23493[1]	Predicted ab initio Build 1.1
	Mus musculus	NCBI	NP_034197.2	Refseq
	Rattus norvegicus	NCBI	NP_001026813.1	Refseq
	Canis familaris	NCBI	XP_848593.1	Predicted

Chicken was the outgroup for analysis except for DNMT3L, for which it was opossum.

Table 4.3 Continued

DNMT	Taxon	Database	Accession	Status
DNMT3a	Gallus gallus	NCBI	NP_001020003.1	Refseq
	Monodelphis domestica	NCBI	XP_001380132.1	Predicted
	Bos taurus	NCBI	XP_872736.1	Predicted
	Homo sapiens	NCBI	NP_072046.2	Refseq
	Pan troglodytes	NCBI	XP_001148246.1	Predicted
	Macaca mulatto	NCBI	XP_001083234.1	Predicted
	Mus musculus	NCBI	NP_031898.1	Refseq
	Rattus norvegicus	NCBI	NP_001003958.1	Refseq
	Canis familaris	NCBI	XP_540110.2	Predicted
DNMT3b	Gallus gallus	NCBI	NP_001019999.1	Refseq
	Monodelphis domestica	NCBI	XP_001362485.1	Predicted
	Bos taurus	NCBI	AAP20552.1	From mRNA
	Homo sapiens	NCBI	NP_008823.1	Refseq
	Pan troglodytes	NCBI	XP_514580.2	Predicted
	Macaca mulatta	NCBI	XP_001107249.1	Predicted
	Mus musculus	NCBI	NP_001003961.1	Refseq
	Rattus norvegicus	NCBI	NP_001003959.1	Refseq
	Canis familaris	NCBI	hmm47423	Predicted ab initio Build 2.1
DNMT3L	Gallus gallus	NCBI	NA	NA
	Monodelphis domestica	NCBI	XP_001377724.1|	Predicted
	Bos taurus	NCBI	XP_869990.2	Predicted
	Homo sapiens	NCBI	NP_037501.2	Refseq
	Pan troglodytes	NCBI	XP_525483.2	Predicted
	Macaca mulatta	NCBI	XP_001118368.1	Predicted
	Mus musculus	NCBI	NP_001075164.1	Refseq
	Rattus norvegicus	NCBI	NP_001003964.1	Refseq
	Canis familaris	NCBI	XP_849972.1	Predicted ab initio Build 2.1

Table 4.4 Primer used for gene expression analysis by Real Time PCR

Genes	Primer sequences and positions (5' - 3')		Fragment size (bp)	Accession Number
DNMT1_F	AATGGGCAGATGTTCCATGC	(2356-2376)	298	NM_182651.1
DNMT1_R	CCTCCGTCGGCTGAGTTTT	(2653-2672)		
DNMT3A_F	CTGGCTCTTTGAGAATGTGGTG	(2372-2394)	236	XM_867643
DNMT3A_R	TCACTTTGCTGAACTTGGCTATT	(2607-2630)		
DNMT3B_F	GGGAAGGAGTTTGGAATAGGAG	(698-720)	417	NM_181813
DNMT3B_R	CTCTGGTTGCTTGTTGTTAGGTT	(1114-1137)		
GAPDH_F	TGCTGGTGCTGAGTATGTGGT	(333-354)	295	XM_865742
GAPDH_R	AGTCTTCTGGGTGGCAGTGAT	(627-648)		

CHAPTER 5

CONCLUSIONS

Nuclear reprogramming after NT has been extensively reviewed over the past few years (Rideout et al., 2001; Jaenisch, 2002; Mann and Bartolomei, 2002; Cezar, 2003; Han et al., 2003b; Jouneau and Renard, 2003; Latham, 2005; Rodolfa and Eggan, 2006; Eilertsen et al., 2007). These reviews and several studies have indicated that SCNT extensively alters the gene expression of differentiated somatic cells to more closely resemble that of embryonic nuclei. However, a combination of *in vitro* culture conditions, aggressive manipulation and insufficient reprogramming, compromises the developmental potential of SCNT embryos. Cloned embryos present different degrees of aberrations in chromatin structure and DNA methylation, which cause inadequate repression of developmental genes or the expression of unnecessary somatic genes. The outcomes of SCNT are very variable, ranging from early embryonic death, lack of implantation, abortions, perinatal deaths, up to the few cloned animals that have reached adulthood with no evident pathologies. These variable outcomes could be the manifestation of different degrees of nuclear reprogramming and have lead some authors to suggest that nuclear reprogramming is a haphazard and stochastic phenomenon

(Somers et al., 2006; Eilertsen et al., 2007; Niemann et al., 2008). Conversely a recent study has suggested that the small mRNA differences in a selected panel of genes indicate a uniform rather than random course of reprogramming (Cavaleri et al., 2008). The variability in the phenotypes observed in clones derived from the same donor cell line, makes it harder to establish a unique theory for nuclear reprogramming.

Microarray analysis has been used to explore the transcriptome profile of cloned embryos relative to that of donor cells and IVF embryos. However, the appropriate microarray platform is crucial in order to detect changes in particular genes. Microarray developed from cDNA libraries should have a representation of embryonic genes to be sensitive to changes in the expression of genes that are expressed exclusively during early embryonic development. The Affymetrix GeneChip Bovine Genome Array is built from cDNA libraries and predicted sequences to represent all tissues including early embryos (see Appendix A). The lack of annotation for the bovine genome is still a limitation even when an appropriate microarray platform is used. The fact the bovine genome annotation is still an ongoing process generates inconsistency in annotations over time, which is one of the biggest pitfalls in microarray data analysis (Noth and Benecke, 2005; Perez-Iratxeta and Andrade, 2005; Stalteri and Harrison, 2006).

Inconsistencies in microarray annotation should be carefully considered during and after microarray data analysis. To minimize the impact of annotation inconsistencies on Affymetrix microarray studies, the probe set identifiers and the annotation version should be recorded along with gene titles and gene symbols. It could be stated that a microarray data set can only be considered completely analyzed if the annotation for the whole genes set is complete. Another constrain of microarray technology is the

variability of results due to several factors including amplification and hybridization efficiency, starting RNA quality, and expertise of the person performing the experiments. Affymetrix microarrays have several amplification and hybridization controls that help identify any failure during the process (see Appendix B).

To minimize variations due to RNA we started with similar amounts of total RNA with comparable quality for all groups. A complete report from, each microarray hybridization, was used to exclude samples that did not pass the quality control (see Appendix C). All procedures were performed by the same person to prevent the inclusion of more extraneous variation.

The present studies corroborate the extensive transcriptional reprogramming that has been reported for cloned embryos using global gene expression analyses. Seven days after nuclear transfer, the transcriptome profile of the cloned blastocysts has changed so drastically that it no longer resembles that of the donor cells. The cloned embryos global gene expression closely resembles that of blastocyst produced by fertilization. Based on this finding alone, it could be suggested that nuclear reprogramming is complete. However, the alterations in a small set of genes involved in DNA methylation and chromatin remodeling, could cause epigenetic alterations downstream.

Nuclear reprogramming seems to be influenced by the genomic background of donor somatic cells and their reprogramming potential. Increasing the ability of chromatin to be reprogrammed by the oocyte has been attempted with Somatic Cell Chromatin Transfer (Sullivan et al., 2004). When somatic cells are exposed to cellular extracts from mitotic cells prior to transfer, their chromosomes are condensed and somatic nuclear factors are removed. Although the overall cloning efficiency does not

seem to be affected by SCCT, a trend toward enhanced survival of cloned calves after one month postpartum has been observed. However, a global gene expression study that included both NT and CT embryos found no difference in their gene expression pattern by the blastocyst stage (Zhou et al., 2007). These results could suggest that the condensation of the somatic chromatin prior to nuclear transfer has no effects on transcriptional reprogramming.

Studies on the effect of serial cloning on nuclear reprogramming have produced conflicting results. Some authors have suggested that consecutive rounds of cloning produce cell rejuvenation (Hill et al., 2000; Hill et al., 2001; Liu et al., 2001; Kuroiwa et al., 2004) and increase the reprogramming potential of somatic cells (Cho et al., 2007). Conversely, other reports suggest that epigenetic errors could accumulate as a result of serial cloning and prolonged *in vitro* culture decreasing cloning efficiency (Wakayama et al., 2000; Peura et al., 2001; Kubota et al., 2004). The present studies show that serial cloning does not significantly affect transcriptional reprogramming of cloned blastocysts. The global transcriptome profile of blastocysts from four consecutive rounds of cloning did not significantly differ from the one obtained from blastocysts after only one round of cloning. However, for a set of genes, misregulation was more marked in the blastocysts obtained from four rounds of cloning (see Appendixes D and E).

When the transcript abundance of four selected genes was analyzed in fetal fibroblasts isolated from 70-day old fetuses, obtained from successive rounds of cloning, no conclusive results could be obtained. These findings could suggest that misregulations in the expression of some genes could be completely reprogrammed in clones that survive beyond the early stages of development. However, alterations in the transcription

of other genes could persist throughout fetal development and only become manifest after birth. In fact, genes that were altered in cloned blastocyst, were also misregulated in the organs of clones that died shortly after birth (Li et al., 2005).

Multiple studies have pointed to alterations in DNA methylation in cloned embryos, particularly a genome-wide hypermethylation (Kang et al., 2001; Han et al., 2003b; Beaujean et al., 2004). Global demethylation soon after fertilization appears to be a prerequisite for successful reprogramming later in embryonic development, and possibly for successful SCNT outcomes. The results of the present study indicate that both *de novo* DNA methyltransferases are being transcribed at a significantly higher rate in the cloned embryos. Surprisingly alterations in DNA methylation do not seem to be life threatening for the cloned embryos, although extensive aberrations may be fatal. Epigenetic alterations can result in different phenotypic manifestations in each embryo, which would account for the variable outcomes of SCNT. The traditional view has maintained that DNA methylation is the primary epigenetic mark responsible for repressive chromatin structure. According to this theory, DNA methylation attracts methylated cytosine binding proteins, which in turn recruit repressor complexes and histone deacetylases to further silence chromatin. An alternative model suggests that it is chromatin structure which determines the DNA methylation or demethylation (Szyf, 2005a). The precise sequence of events leading to gene silencing would determine the right approach for improving reprogramming after SCNT.

Species-specific differences in DNA methylation reprogramming have been suggested, although the overall process appears to be conserved among the clones of different species. Aberrations in genome-wide reprogramming have been reported for

mouse, rat, pig, and bovine NT embryos (Bourc'his et al., 2001; Dean et al., 2001) . We analyzed the structural and functional conservation of DNA methyltransferases during preimplantation embryo development in mouse, human and cow by multiple sequence alignment, phylogenetic analysis, and study of conserved domains. We observed a closer homology between human and cow proteins with the exception of DNMT3b. The available DNMT3b sequence (AAP20552.1) lacked several amino acids in the C-terminal domain, which were present in all other mammalian species analyzed. After annotation and correction, cow and human DNMT3b sequences showed a higher degree of homology. These findings have corroborated the closer phylogenetic relationship between human and bovine *de novo* methylation genes, which could suggest that the bovine model would be more appropriate for studying DNA methylation during embryogenesis. The corrected sequence was submitted to the Bovine Genome Annotation Submission site (see Appendix F). Additionally, we analyzed the changes in mRNA abundance of DNMT1, DNMT3a and DNMT3b in oocytes and preimplantation embryos to determine the moment in which these transcripts are higher in the embryos. As expected, DNMT1 was low throughout early embryo development. Consistent with our microarray analyses, DNMT3a transcript abundance was higher in IVF derived blastocysts compared to DNMT3b, which could indicate an earlier role of DNMT3a in *de novo* DNA methylation during early embryogenesis.

Chromatin associated proteins play a key role in nuclear remodeling. In the present studies HMGN3 was significantly higher in IVF derived blastocysts compared to blastocysts produced by CT. The levels of HMGN3 transcript in CT embryos resembled the ones detected in somatic cells. Although the exact function of HMGN3 during early

embryonic development has not been determined, its role in facilitating chromatin modifications and enhancing transcription, replication, and DNA repair is critical for early embryo development (West et al., 2001).

Reprogramming of DNA methylation and histone modifications to ensure a pattern of gene expression compatible with embryonic development is essential for successful cloning. Identification of the specific factors present in the ooplasm, which are necessary for epigenetic reprogramming, will provide a better understanding of the underlying mechanisms and would improve cloning efficiency. Although several questions regarding the low efficiency of SCNT still remain unanswered, the central role of nuclear reprogramming on the outcome of cloning is evident. Increasing the efficiency of SCNT would have a great impact on biomedical sciences and agriculture, particularly therapeutic cloning, and the production of animals with desired qualities. Understanding the reprogramming process of SCNT derived embryos would be instrumental to increase the success rate of cloning. Several strategies have been used to determine the extent of nuclear reprogramming in cloned embryos.

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APPENDIX A

BOVINE GENECHIP SPECIFICATIONS

GeneChip Bovine Genome Array Specifications	
GeneChip probe sets	24,072
Bovine transcripts	approximately 23,000
UniGene clusters	approximately 19,000
Array format	100
Feature size	11 μm
Oligonucleotide probe length	25-mer
Probe pairs/sequence	11
Hybridization controls:	bioB, bioC, bioD, from E. coli cre from P1 B. subtilis
Poly-A controls:	dap, lys, phe, thr, trp from B. subtilis
Housekeeping/Control genes:	actin, GAPDH, eflα, 5.8S rRNA, 12S rRNA, 18S rRNA, cyclophilin B, glutathione S-transferase, lactophorin, translation initiation factor eIF-4E
Detection sensitivity	1:100,0001[1]

[1] As measured by detection in comparative analysis between a complex target containing spiked control transcriptions and a complex target with no spikes

The design of the array is based on content from Bovine UniGene Build 57 (March 24, 2004) and GenBank® mRNAs.

The gene annotation used for the present dissertation was the November 2007 update.

APPENDIX B

MICROARRAY INTERNAL CONTROLS

Origin of control	Control Gene Name	Type of controls
B. subtilis	*lys* *phe* *thr* *dap*	Poly-A-tailed sense RNAs used as controls for the labeling and hybridization process. They are also used to estimate assay sensitivity.
E. coli	*bioB* *bioC* *bioD*	Antisense biotinylated cRNAs used as hybridization controls.
P1 Bacteriophage	*cre*	
synthetic	*B2 Oligo*	Grid alignment.

APPENDIX C

HYBRIDIZATION PERFORMANCE OF MICROARRAYS

Sample	Backgr	Scale factor	Noise	Present	Average Signal (P)	Average Signal (A)	Actin-5'	Actin-3'	Actin 3'/5'ratio	Gapd-5'	Gapd-3'	GAPDH ratio 3'/5'
IVF1	47.3	3.443	1.63	48.30%	1579.0	25.1	44.6	583.8	13.1	1152.9	6351.9	5.5
IVF2	70.0	4.994	2.40	41.50%	1896.3	58.9	112.7	491.9	4.4	1560.5	10485.9	6.7
IVF3	57.0	5.966	2.00	42.70%	1957.0	51.1	37.5	295.1	7.9	1328.3	20239.5	15.2
CT1-1	60.8	7.606	2.13	39.00%	2076.5	70.2	24.7	233.4	9.4	2723.5	17147.8	6.3
CT1-2*	54.2	45.634	1.92	9.50%	5806.5	417.8	1359.9	5460.2	4.0	82.8	7979.5	96.4
CT1-3	67.5	5.059	2.33	43.50%	1776.8	48.0	69.7	347.3	5.0	1103.2	17142.8	15.5
CT1-4*	54.7	41.442	1.96	18.60%	3801.2	264.7	483.4	85.3	0.2	430.1	10201.7	23.7
CT4-1	55.8	4.877	2.01	43.60%	1849.0	38.6	7.7	349.6	45.4	1748.0	18766.2	10.7
CT4-2*	56.0	36.20	1.94	23.30%	3557.6	178.6	330.4	878.1	2.7	208.7	9698	46.5
CT4-3	59.8	3.31	2.09	49.00%	1515.8	30.6	50.9	266.1	5.2	1009.5	12757.2	12.6
CT4-4	55.1	3.44	1.93	48.10%	1595.4	37.7	44.3	316.3	7.1	5432.1	21479.1	4.0
DC1-1	47.4	2.337	1.68	55.30%	1282.8	28.5	57.7	584.3	10.1	2108.0	22469.9	10.7
DC1-2	38.7	2.246	1.39	59.10%	1196.8	22.2	63.6	395.6	6.2	9686.6	20649.8	2.1
DC1-3	54.2	1.665	1.94	56.70%	1240.0	28.9	26.7	678.1	25.4	8504.9	7019.6	0.8
DC4-1	46.5	2.350	1.59	55.40%	1287.1	27.1	35.8	540.8	15.1	1826.4	23085.8	12.6
DC4-2	66.1	1.549	2.31	54.30%	1294.1	30.7	44.2	514.3	11.6	20518.6	18656.5	0.9
DC4-3	71.4	1.526	2.50	55.30%	1266.8	27.7	37.8	500.5	13.2	6592.7	18854.6	2.9

*Samples excluded from the microarray analysis

APPENDIX D

GENES WITH PUTATIVE CUMULATIVE DOWNREGULATION IN BLASTOCYSTS OBTAINED AFTER SERIAL ROUNDS OF CHROMATIN TRANFER

Probe Set ID	Gene Title	Gene Symbol	IVF	NT1	NT4	Fold change IVF/NT1	Fold change IVF/NT4
Bt.5154.1.S1_at	heat shock 70 kD protein 1 /// heat shock 70 kD protein 2	HSPA1A	16655.53	4021.00	2975.26	4.14	5.60
Bt.9759.1.S1_a_at	neuroguidin, EIF4E binding protein	NGDN	11691.84	5346.60	3041.70	2.19	3.84
Bt.5039.1.S1_at	high mobility group nucleosomal binding domain 3	HMGN3	11195.32	6522.85	4078.53	1.72	2.74
Bt.9759.2.S1_at	neuroguidin, EIF4E binding protein	NGDN	5999.87	2431.02	1665.35	2.47	3.60
Bt.4737.1.S2_s_at	prion protein	PRNP	3552.73	1614.40	1425.30	2.20	2.49
Bt.1854.1.S1_at	intraflagellar transport protein 20	IFT20	3526.47	2139.25	1380.10	1.65	2.56
Bt.27874.1.S1_s_at	phosphatidylserine receptor	PTDSR	3476.73	1517.25	980.58	2.29	3.55
Bt.15787.1.S1_at	Bcl-2 inhibitor of transcription	BIT1	2989.58	2007.15	1415.27	1.49	2.11
Bt.20204.1.S1_at	Sjogren's syndrome/scleroderma autoantigen 1	SSSCA1	1695.08	1056.05	579.62	1.61	2.92
Bt.4595.1.S1_at	TSR2, 20S rRNA accumulation, homolog (S. cerevisiae)	TSR2	1567.39	755.35	568.11	2.08	2.76
Bt.12250.1.S1_at	chromosome 14 open reading frame 10	C14orf10	1525.13	981.80	567.59	1.55	2.69
Bt.27095.1.S1_at	collaborates/cooperates with ARF (alternate reading frame) protein	CARF	1390.25	907.40	668.85	1.53	2.08
Bt.13928.2.S1_a_at	sodium channel modifier 1	SCNM1	786.05	390.50	249.35	2.01	3.15
Bt.6620.1.S1_at	myosin, heavy polypeptide 7, cardiac muscle, beta	MYH7	673.53	219.15	135.85	3.07	4.96
Bt.19972.1.S1_at	proton-dependent gastrointestinal peptide transporter	PEPT1	567.85	189.46	170.27	3.00	3.34
Bt.28010.1.S1_at	protease inhibitor 3, skin-derived (SKALP)	PI3	510.98	91.50	56.05	5.58	9.12
Bt.5126.1.S1_at	hypertension-related calcium-regulated gene	COMMD5	449.40	335.50	176.24	1.34	2.55
Bt.22523.1.S1_at	dispatched homolog 1 (Drosophila)	DISP1	402.17	174.75	155.13	2.30	2.59
Bt.5828.1.S1_at	SERTA domain containing 1	SERTAD1	357.71	287.95	157.44	1.24	2.27
Bt.333.1.S1_at	transition protein 1 (during histone to protamine replacement)	TNP1	233.38	155.00	98.93	1.51	2.36
Bt.14098.1.S1_at	microtubule-associated protein, RP/EB family, member 2	MAPRE2	199.89	183.45	69.82	1.09	2.86
Bt.4158.1.A1_at	oviduct specific glycoprotein	OVGP1	196.48	168.70	78.09	1.16	2.52
Bt.22856.1.S1_at	neurofilament, medium polypeptide	NEF3	188.69	126.35	46.89	1.49	4.02

Bt.9807.1.S1_at	glycoprotein (transmembrane) nmb	GPNMB	154.95	52.30	24.03	2.96	6.45
Bt.23151.1.S1_at	fucosyltransferase 10 (alpha (1,3) fucosyltransferase)	FUT10	154.43	114.10	55.12	1.35	2.80
Bt.7239.1.S1_at	solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	SLC6A3	149.32	48.30	21.24	3.09	7.03
Bt.12739.2.S1_a_at	membrane-associated ring finger (C3HC4) 2	C3HC4	110.18	51.40	23.87	2.14	4.62
Bt.6556.1.S1_at	regakine-1 protein	LOC504773	89.66	25.75	39.07	3.48	2.29
Bt.12080.2.S1_at	Bernardinelli-Seip congenital lipodystrophy 2	BSCL2	88.59	38.70	13.83	2.29	6.41
Bt.13036.1.S1_at	progesterone receptor	PGR	79.73	4.69	36.57	17.02	2.18
Bt.2157.1.S1_a_at	RPGR-interacting protein 1	RPGRIP1	77.03	58.90	6.56	1.31	11.75
Bt.28409.2.S1_at	DNA replication factor	CDT1	71.69	55.20	12.73	1.30	5.63
Bt.3771.1.A1_at	Nucleolar protein family A, member 1	NOLA1	69.73	21.50	21.26	3.24	3.28
Bt.27752.1.S1_at	tensin 4	TNS4	69.66	43.05	8.73	1.62	7.98
Bt.13024.2.S1_at	purinergic receptor P2Y G-protein coupled, 2	P2RY2	67.08	46.15	22.11	1.45	3.03
Bt.28017.1.S1_at	vacuolar H+-ATPase	LOC407191	65.07	34.20	17.47	1.90	3.73
Bt.512.1.S1_at	nucleotide phosphodiesterase, 3'-5'-cyclic	PDE1A	60.70	15.59	15.85	3.89	3.83
Bt.12928.1.S1_at	Interleukin 13	IL13	58.85	37.70	9.25	1.56	6.36
Bt.29129.1.S1_at	anterior gradient 2 homologue	agr2	45.07	39.00	21.29	1.16	2.12

APPENDIX E

GENES WITH PUTATIVE CUMULATIVE UPREGULATION IN BLASTOCYSTS OBTAINED AFTER SERIAL ROUNDS OF CHROMATIN TRANFER

Probe Set ID	Gene Title	Gene Symbol	IVF	NT1	NT4	Fold change NT1/IVF	Fold change NT4/IVF
Bt.4475.1.S1_at	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase)	NDUFS2	6724.02	13373.15	14960.42	1.99	2.22
Bt.3583.1.S1_at	villin 2	VIL2	6698.24	13698.40	17209.52	2.05	2.57
Bt.663.1.S1_at	palladin, cytoskeletal associated protein	PALLD	5038.25	14502.45	19368.34	2.88	3.84
Bt.9068.1.S1_at	non-muscle myosin heavy chain	LOC404108	3,972.71	6,504.05	8,152.57	1.64	2.05
Bt.2841.1.S1_at	tryptophanyl-tRNA synthetase	WARS	2,665.06	4,276.85	5,569.16	1.60	2.09
Bt.4311.1.S1_at	guanidine nucleotide binding protein, (G protein), alpha inhibiting activity polypeptide 2	GNAI2	2,389.08	3,859.15	7,740.86	1.62	3.24
Bt.962.1.S1_at	golgi autoantigen, golgin subfamily a, 7	GOLGA7	1,689.70	2,728.90	4,288.07	1.62	2.54
Bt.760.1.S1_at	zinc finger protein 313	Znf313	1,523.55	2,140.45	3,126.63	1.40	2.05
Bt.803.1.A1_at	chromatin modifying protein 1B	CHMP1B	1,315.99	2,093.75	3,934.13	1.59	2.99
Bt.4503.1.S1_at	mitochondrial carrier homolog 2	Mtch2	1,279.84	3,359.75	4,555.63	2.63	3.56
Bt.23603.3.S1_at	F-box protein 9	FBXO9	1,058.76	1,948.25	2,813.78	1.84	2.66
Bt.7169.1.S1_at	methylmalonyl Coenzyme A mutase	MUT	898.23	1,622.10	1,943.02	1.81	2.16
Bt.14010.1.S1_at	leukotriene B4 12-hydroxydehydrogenase	LTB4DH	841.63	5688.55	11345.50	6.76	13.48
Bt.8933.1.S1_at	adaptor-related protein complex 3, sigma 2 subunit	AP3S2	667.54	1,071.50	1,425.67	1.61	2.14
Bt.12261.1.A1_at	taspase 1	C20orf13	435.56	1,113.20	1,293.73	2.56	2.97
Bt.4738.1.S1_at	calpastatin	CAST	329.41	504.45	890.74	1.53	2.70
Bt.26764.1.A1_at	Lectomedin 2	LEC2	307.46	1,085.70	1,567.79	3.53	5.10
Bt.1388.1.S1_at	Abl-philin 2 isoform 2	ZDHHC16	286.19	630.40	948.26	2.20	3.31
Bt.20236.1.S1_at	thrombospondin repeat containing 1	ADAMTSL4	211.65	322.35	522.48	1.52	2.47
Bt.5330.1.S1_at	lysosomal-associated membrane protein 1	LAMP1	194.91	174.90	1,195.48	0.90	6.13
Bt.8870.3.S1_at	CGI-119 protein	CGI-119	128.16	218.35	403.76	1.70	3.15
Bt.23209.1.S1_a_at	lectomedin 2	LEC2	83.06	279.90	418.46	3.37	5.04
Bt.318.1.S1_at	adrenergic, beta 3, receptor	ADRB3	26.17	52.65	89.07	2.01	3.40

Bt.4057.1.S1_at	myosin, heavy polypeptide 10, non-muscle	MYH10	21.07	38.95	83.71	1.85	3.97
Bt.4560.1.S1_s_at	trophoblast Kunitz domain protein 1	TKDP1	21.03	43.25	88.08	2.06	4.19
Bt.22858.1.S1_at	uroplakin IIIB	UPK3B	16.02	16.70	100.71	1.04	6.29
Bt.12304.1.S1_at	interferon-stimulated protein, 15 kDa	ISG15	15.51	67.95	66.46	4.38	4.29
Bt.26830.2.S1_a_at	5,10-methylenetetrahydrofolate reductase (NADPH)	MTHFR	12.02	57.85	79.11	4.81	6.58
Bt.5101.1.S1_at	prion protein interacting protein	PRNPIP	8.97	32.00	74.73	3.57	8.33
Bt.17862.1.A1_at	Guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide 1	GNAS1	8.03	42.00	44.87	5.23	5.59
Bt.2301.1.S1_at	Zinc finger protein 325 (gonadotropin inducible transcription repressor-3)	ZNF325	3.81	22.10	121.65	5.80	31.93
Bt.17862.1.A1_at	Guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide 1	GNAS1	8.03	42.00	44.87	5.23	5.59
Bt.12304.1.S1_at	interferon-stimulated protein, 15 kDa	ISG15	15.51	67.95	66.46	4.38	4.29
Bt.12261.1.A1_at	taspase 1	C20orf13	435.56	1113.20	1293.73	2.56	2.97
Bt.3583.1.S1_at	villin 2	VIL2	6698.24	13698.40	17209.52	2.05	2.57

APPENDIX F

GENES ANNOTATED AND SUBMITTED TO THE BOVINE

GENOME SEQUENCING CONSORTIUM 2007

Gene symbol	Glean	Landmark	Start	End	Strand	Gene ID	Predited mRNA	Predicted Protein
ANP32A	GLEAN_11459	Chr10.20:24030..29969	21549	29170	-			
AQP8	GLEAN_04832	Chr25.32:1581000..1655000	1420670	1588474	-	450206	XM_583253.3	XP_583253.3
DNMT3L	GLEAN_15626	Chr1.170:55000..70000	56219	70607	-	613785	XM_864897	XP_869990
DNMT2	GLEAN_20779	Chr13.27:1109000..1145000	1110166	1163352	-	353353	NM_181812.1	NP_861528.1
DNMT3B	GLEAN_08037	Chr13.65:734000..758000	393306	756255	+	353354	NM_181813	NP_861529.1
BCL2L	GLEAN_10559	Chr13.67:376000..428000	377692	426902	-	282152	NM_001077486.2	NP_001070954.1
BMP6	GLEAN_00235	Chr23.57:1110000..1162213	1113316	1161213	-	617566	XM_869844.2	XP_874937.2
BMP8B	GLEAN_02029	Chr14.89:150000..180000	159588	175685	+			
EED	GLEAN_06204	Chr29.14:470000..504000	471825	502151	-	404183	NM_001040494.1	NP_001035584.1
EGF	GLEAN_06456 + GLEAN_06457	Chr6.13:889000..995000	916453	993377	-	530315	XM_001253862	XP_001253863.1
GATA3	GLEAN_15109	Chr13.15:381000..402000	382644	400398	-	505169	NM_001076804.1	NP_001070272.1
GATA6	GLEAN_00216	Chr24.45:782000..813000	782580	811772	+			
HMGN3A	GLEAN_08006	Chr9.20:2024000..2060000	2024869	2059808	-	515652	NM_001034504.1	NP_001029676.1
IFITM3	GLEAN_22223	Chr29.68:19000..21500	20015	20932	+	282255	NM_181867.1	NP_863657.1
MBD3	GLEAN_09279	Chr7.60:7000..13000	4124	11374	+	616090	XM_868057	
MECP2	GLEAN_23979	ChrX.59:114000..163000	114897	161821	+	539629	XM_588477.3	XP_588477.3
NOTCH3	GLEAN_12973	Chr7.14:120000..165000	121620	160252	-		XR_028762.1	
PLAC1	GLEAN_25707	ChrUn.104:462000..465000	463224	463763	-	767997	NM_001077057.1	NP_001070525.1
PLAC8	GLEAN_20429	Chr6.101:1137000..1208000	1138588	1148466	+	509228	NM_001025325.1	NP_001020496.1
SMARCA1	GLEAN_09261	ChrX.27:647000..703000	643271	714992	+	535439	XR_028639.1	
STAT1	GLEAN_20786	Chr2.93:762000..968000	763383	900710	-	510814	XM_001253473.1	XP_001253474.1
STAT4	GLEAN_17376	Chr19.41:140000..198000	165609	196381	-	282086		
SMARCAL1	GLEAN_20241	Chr2.124:232000..285000	233017	345663	-	338072	NM_176666.1	NP_788839.1