Nuclear reprogramming by somatic cell nuclear transfer – the cattle story

XC Tian[1], SL Smith[1], SQ Zhang[1,2], C Kubota[3], C Curchoe[1] F Xue[4], L Yang[1], F Du[1], L-Y Sung[1] and X Yang[1]

[1]Department of Animal Science/Center for Regenerative Biology, [4]Department of Molecular and Cellular Biology, University of Connecticut, Storrs, CT, USA; [2]College of Animal Science, South China Agricultural University, Guangzhou 510642, People's Republic of China; [3]Department of Veterinary Medicine, Kagoshima University, Kagoshima City, Kagoshima, Japan

Somatic cell nuclear transfer (cloning) returns a differentiated cell to a totipotent status; a process termed nuclear reprogramming. Nuclear transfer has potential applications in agriculture and biomedicine, but is limited by low efficiency. To understand the deficiencies of nuclear reprogramming, our research has focused on both candidate genes (imprinted and X-linked genes) and global gene expression patterns in cloned bovine embryos/offspring as compared to those generated by conventional reproduction. We found aberrant expression patterns of *H19* and *Igf2r* as well as X-linked genes in term cloned calves. The expression profiles of cloned blastocysts, however, closely resembled those of the naturally fertilized embryos but were considerably different from those of their nuclear donor cells. Our findings suggest that cloned embryos have undergone significant nuclear reprogramming by the blastocyst stage. However, it is possible that during re-differentiation in later development gene expression aberrancies occur. Additionally, small initial nuclear reprogramming errors may be manifested during subsequent development.

Introduction

A long-held dogma in developmental biology was that mammalian somatic cell differentiation was considered irreversible. Fig. 1 shows the landscape model of cell differentiation (Waddington 1940; Keeton & Gould 1984). It likens the process of mammalian cell differentiation as a ball rolling down a hill with many valleys. When the ball is on top of the hill, it can roll down through any valleys below; this represents the process of a totipotent cell that can differentiate into any tissue of the body. However, as the ball rolls passed an intersection, the available valleys for the ball to roll down become limited. When the ball reaches the bottom of the hill, it cannot move to another valley or back to the top of the hill. This model was used to illustrate a totipotent cell choosing among different developmental paths; when the cell's fate is partially determined its differentiation potential becomes limited. Once the cell is terminally differentiated, it can no longer trans-differentiate into another cell type or become totipotent again.

Corresponding author E-mail: Xiuchun.tian@uconn.edu

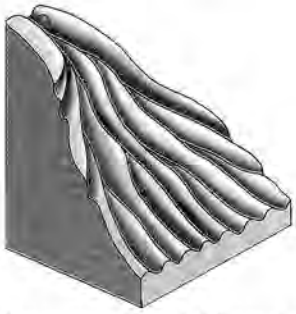

Fig. 1. The landscape model of mammalian cell differentiation (modified from Keeton & Gould 1984).

The success of cloning a whole animal using differentiated somatic cells, however, challenged this theory. During cloning, a differentiated somatic cell is injected into the oocyte's cytoplasm and a cloned embryo is created. The cloned embryo contains totipotent cells that can differentiate into any tissue type and result in a cloned animal (Fig. 2). The process of returning a differentiated somatic nucleus to a totipotent status is termed nuclear reprogramming. Currently, this process can only be accomplished by somatic cell nuclear transfer. During nuclear reprogramming, genes inactivated due to cell differentiation are subjected to re-activation, allowing the re-constructed cloned embryos to support development and generation of all tissue types in the cloned individual.

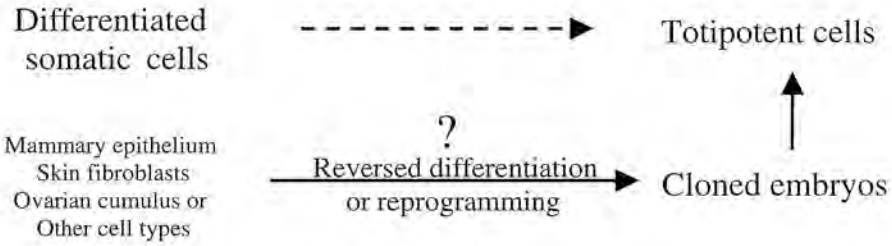

Fig. 2. Schematic drawing of the nuclear reprogramming process. Differentiated somatic cells used in nuclear transfer such as epithelial, fibroblast, cumulus or other cells can be made into cloned embryos. It has been shown that cloned embryos contain totipotent cells because embryonic stem cells can be readily generated from these embryos (Wang et al. *2005; Brambrink* et al. *2006; Wakayama* et al. *2006). Indirectly, the differentiated cells are reprogrammed to become totipotent cells by a yet unknown reversed differentiation process.*

Somatic cloning, however, is challenged with many problems, such as low efficiency, abnormally high rates of fetal death and abortions, premature birth is frequently observed in cloned pregnancies, neonatal death, and placental abnormalities to name a few (Hill *et al.* 1999; Kubota *et al.* 2000; Heyman *et al.* 2002; Xue *et al.* 2002). These observations suggest that nuclear transfer derived fetuses do not develop similarly to *in vivo* or *in vitro* fertilized embryos. These abnormalities are believed to result from incomplete re-activation of genes involved in embryonic development in the donor cells.

Epigenetics

The inactivation of genes during cell differentiation is believed to involve epigenetic modifications of chromatin. Epigenetics is defined as nuclear inheritance that is not based on differences in DNA sequence (Holliday 1987). It is best exemplified by different tissues comprised of cells expressing different proteins while having the same genetic makeup in an individual. Epigenetics is believed to involve differential DNA methylation, histone acetylation, chromatin configuration as well as other mechanisms. These epigenetic signals are stably transmitted during cell division but are reset in each generation in the gonads during fetal development (Goto & Monk 1998; Latham 1999). Therefore, epigenetic signals are not inheritable from one generation to the next but stably maintained within the generation.

To study gene re-activation and reprogramming by nuclear transfer, and to identify genes not expressed properly after cloning, we have employed two complementary approaches: 1) the candidate-gene approach to study individual gene expression; and 2) the gene-panning approach to study global gene expression.

Candidate gene approach

Two main epigenetic modifications of gene expression have been relatively well characterized: genetic imprinting and X chromosome inactivation. Both have been active areas of study in cloned animals because somatic cloning bypasses the natural process of parental specific erasure and re-establishment of epigenetic signals (occurs in the gonads). Cloning using somatic cells skips the gonads and epigenetic signal modifications. Genes that are subjected to epigenetic regulations are thus good candidates to study nuclear reprogramming. Additionally, clones of the same donor provide unique experimental materials in that they are genetically identical yet epigenetically different (Eggan *et al.* 2000; Xue *et al.* 2002). A thorough understanding of reprogramming of epigenetically regulated genes in cloned animals will also improve the young and promising technology by revealing the ideal conditions for complete reprogramming of the somatic nucleus.

Genetic imprinted genes

Genomic imprinting is an epigenetic phenomenon in which only one allele of a specific gene is expressed depending on its parental origin, mono-allelic expression (Latham 1999; Ferguson-Smith & Surani 2001). To date, more than 50 imprinted genes have been identified in the mouse and/or human (Dean *et al.* 2003) and many of them are involved in regulation of fetal growth. These genes are epigenetically modified in the gonads during natural reproduction. This is caused by differential "marks", in forms of differential methylation, established on the DNA of sperm and oocytes during gametogenesis. In nuclear transfer, however, both sets of chromosomes are derived from the same donor cell. It is therefore important to study how imprinted genes are regulated in cloned embryos/animals. Furthermore, many of the defects in large offspring syndrome (LOS) are similar to experimentally created imprinting disruptions (bi-allelic expression of imprinted genes) in mice and naturally occurring imprinting diseases in humans. Because most imprinted genes regulate fetal growth and many are essential for normal development, it is likely that some defects, especially LOS, and a portion of embryonic deaths, are caused by imprinting disruptions. We have chosen to study the *H19* and *IGF2R* genes in the cloned animals.

Imprinting status of the *H19* gene in clones

The *H19* gene encodes for an un-translated RNA molecule (Brannan *et al.* 1990) and is one of the best-studied imprinted genes in both the mouse and human. It is expressed from the maternal allele in both species with the paternal allele silent or nearly silent (Rachmilewitz *et al.* 1992; Bartolomei & Tilghman 1997). *H19* is expressed abundantly in the human placenta and in several embryonic tissues (Goshen *et al.* 1993). We identified a single nucleotide polymorphism (SNP) in exon 5 of bovine *H19*, and found that cattle produced by conventional breeding expressed the maternal allele of *H19* (Fig. 3a). In organs of three out of four deceased cloned calves, bi-allelic expression of *H19* was observed; supporting our hypothesis that imprinting disruption is present in cloned animals that suffered from developmental abnormalities at birth (Fig. 3b).

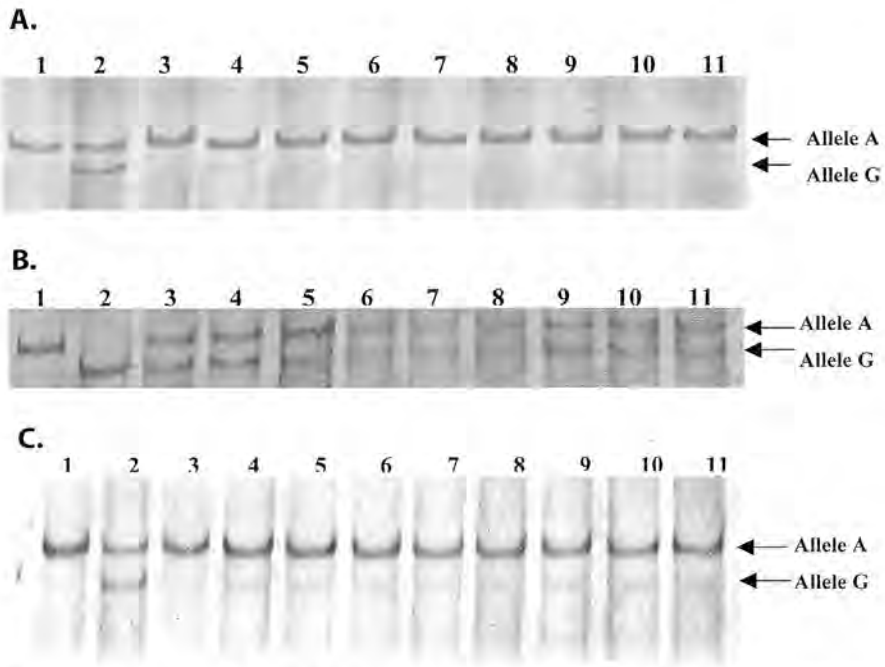

Fig. 3. SSCP images of the allele-specific expression pattern of the *H19* gene in cattle produced by natural reproduction and SCNT. a). Allelic expression of a beef calf: Lanes 1 and 2: Genotypes of a dam and her calf. The calf had two bands indicating the animal was heterozygous for the SNP while the dam only had one band (Allele A) indicating she was homozygous and the calf inherited the A allele from the maternal origin. Lanes 3-9: Expression pattern of *H19* in the calf's liver, kidney, heart, brain, lung, placenta, thymus, bladder, spleen. All organs were either predominantly or exclusively expressing the A allele, which was of maternal origin, indicating the *H19* is imprinted and maternally expressed. b). A representative SSCP image of the allele-specific expression pattern of the bovine *H19* gene in a deceased cloned calf showing bi-allelic expression of *H19*. Lanes 1 and 2: Genotypes of control animals homozygous for the *H19* SNP; Lane 3: genotype of the cloned animal, showing that she was heterozygous for the SNP; Lane 4: genotype of the donor cells; Lane 5: allelic expression of the donor cells, showing bi-allelic expression; Lanes 6-11: brain, heart, liver, lung, spleen and kidney of the cloned animal. c). An SSCP image of the allele-specific expression of *H19* in tissues of a cloned cow's offspring produced by artificial insemination. Lanes 1 and 2: genotypes of the clone's dam (the cloned cow) and her calf by natural reproduction; Lanes 3-11: allelic expression of the liver, kidney, heart, brain, lung, placenta, thymus, bladder, spleen of the clone's calf.

Interestingly, examination of the expression of *H19* in the offspring of a cloned animal produced by artificial insemination showed that the imprinting pattern in this animal was indistinguishable from those of control animals (Fig. 3c), suggesting that either imprinting disruptions in cloned animals are corrected through natural reproduction or that they are not present in healthy cloned animals capable of undergoing natural reproduction.

Insulin-like growth factor 2 receptor (*IGF2R*)

IGF2R, also called cation-independent mannose-6-phosphate receptor, was among the first imprinted genes discovered (Barlow *et al.* 1991). Species variations have been found for the imprinted status of this gene. It has been shown to be maternally expressed in the mouse (Willison 1991; Wutz & Barlow 1998) , sheep (Young *et al.* 2001; Young *et al.* 2003) , cattle (Killian *et al.* 2001b) and pig (Killian *et al.* 2001a) , but not in humans (Riesewijk *et al.* 1996; Wutz *et al.* 1998).

We analyzed allelic expression of *IGF2R* in placentas and organs of ten bovine clones derived from a 13-year-old cow. We found that the maternal *IGF2R* expression pattern of the donor cells was retained in the organs. In contrast, we found random preferential expression of either allele of *IGF2R* in the clones' placentas. Methylation analysis of the putative bovine imprinting control elements of *IGF2R* is underway. Our findings may indicate that independent epigenetic marks may exist for imprinting of *IGF2R*, and that nuclear reprogramming can erase those recognized by the placentas, but not by tissues from the epiblast.

Levels of expression of imprinted genes

By using real time reverse-transcription polymerase chain reaction (RT-PCR), we quantified the expression of the bovine *IGF2*, *IGF2R* and *H19* genes in eight major organs (brain, bladder, heart, kidney, liver, lung, spleen and thymus) of somatic cell cloned calves that died shortly after birth, in three tissues (skin, muscle and liver) of healthy clones that survived to adulthood, and in corresponding tissues of control animals from natural reproduction (Yang *et al.* 2005). We found that deceased bovine cloned calves exhibited abnormal expression of all three genes studied in various organs. Large variations in the expression levels of imprinted genes were also seen among these clones, which were produced from the same genetic donor. In surviving adult clones, however, the expression of these imprinted genes was largely normal, except for the expression of the *IGF2* gene in muscle, which was highly variable. Our data suggest that nuclear transfer can cause disruptions of expression of imprinted genes in bovine clones, possibly due to incomplete reprogramming of donor cell nuclei, and these abnormalities may contribute to the high neonatal mortality in cloned animals; clones that survived to adulthood, however, are not only physically healthy but also relatively normal at the molecular level (Yang *et al.* 2005).

X-linked genes

In mammals, males have one while females have two copies of the X chromosomes. This creates a situation in which there is unequal gene dosage between males and females. During evolution, this was solved by a process termed X chromosome inactivation (XCI) (Heard *et al.* 1997; Lyon 1999), the random transcriptional silencing of one of the two X chromosomes in somatic cells of females during early development. XCI occurs by the process of epigenetic modification, the inactivated X chromosome has hyper-methylated DNA and hypo-acetylated histones. Proper XCI is essential to embryonic development. Inactivation of both X chromosomes in mouse embryos leads to embryonic lethality, and having more than one active X chromosome is deleterious to extra-embryonic development and also causes early embryonic death in mice (Wang *et al.* 2001).

X chromosome inactivation occurs at the late blastocyst-stage in mice. As described in Fig. 4a, before fertilization, the egg carries an active X chromosome that is of maternal origin (Xam), while the sperm carries an inactive X of paternal origin (Xip). At the formation of the female zygote, both X chromosomes become active (XamXap). This state of activation persists through early blastocyst-stage. During late blastocyst-stage, the expression of the X-inactivation specific transcript (*Xist*) gene from one of the two X chromosomes, in a random fashion in the inner cell mass, leads to its inactivation (Xi). In extra-embryonic tissues, however, the paternal X chromosome is preferentially inactivated in the mouse, resulting in imprinted XCI. Once established, the inactive state of a particular X chromosome is epigenetically inherited throughout all subsequent cell divisions (Goto & Monk 1998).

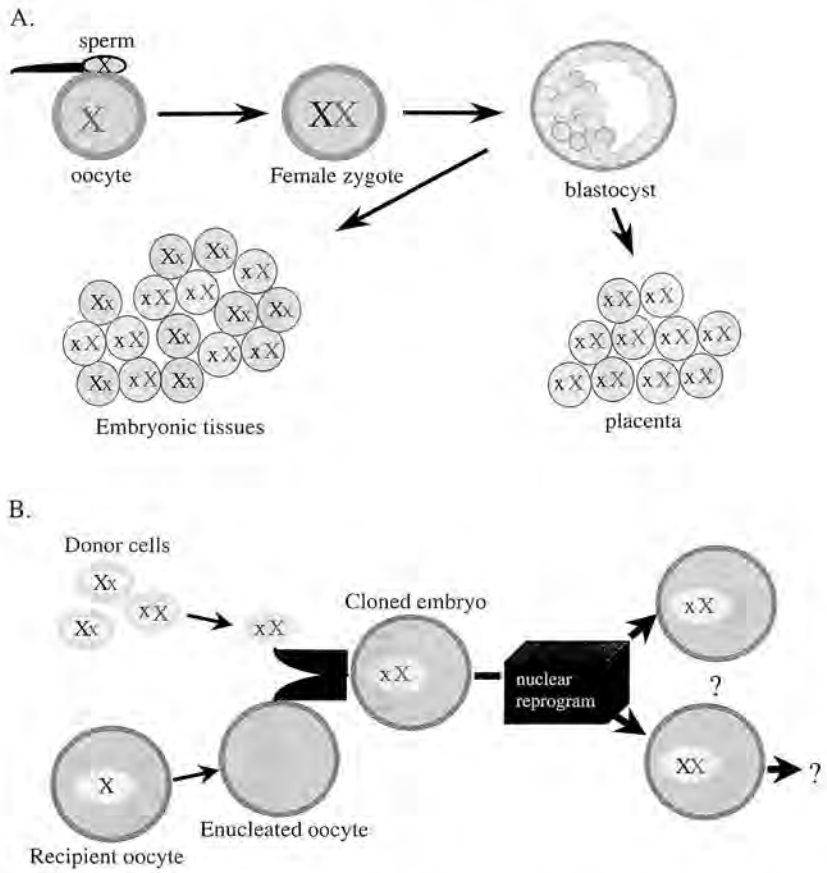

Fig. 4. XCI in early fertilized embryos (a). During natural fertilization, the sperm carries an inactive x (blue, lowercase) while the egg carries an active X (red, uppercase). Both X are active after formation of the female zygote (XX both uppercase). At the time of blastocyst formation, the inner cell mass randomly inactivates one X, either of paternal or maternal origin, resulting in random XCI. In the trophectoderm, which will become the placenta, the paternal X chromosome (blue) is preferentially inactivated, resulting imprinted XCI. b). Question of reprogramming of X chromosome inactivation in cloned embryos. During nuclear transfer, a somatic cell with a pre-existing active and inactive X chromosomes is transferred into an enucleated oocyte. After nuclear reprogramming, which is still largely a black box, it is unclear whether the inactivated X will become re-activated to result in two active X as in the naturally fertilized zygote, or the same pattern of XCI in the somatic donor cell will be maintained in the cloned animal.

In nuclear transfer, the cloned zygotes receive one active (Xa) and one inactive (Xi) X chromosome from the donor cell (Fig. 4b). This state of X inactivation is different from that in naturally fertilized female zygotes, in which both X are active. During nuclear reprogramming, it is unclear whether the inactivated X is re-activated or the pattern of XCI in the donor cell is maintained in the cloned animals. The first study of XCI reprogramming in cloned animals was conducted by Eggan *et al.* (2000) who reported that epigenetic marks on the somatic X chromosomes in mice were completely erased and then appropriately reestablished by the nuclear reprogramming process, leading to normal random XCI in the cloned embryos. The question remains whether or not this is universal across all species. We conducted an extensive study on XCI in cattle and established patterns of XCI in female cattle from natural reproduction (Xue *et al.* 2002). By following the allele specific expression of the X-linked house-keeping gene monoamine oxidase type A (*MAOA*), we found that, as in the mouse, XCI in cattle somatic cells is also random and is paternally imprinted in the placenta (Fig. 5). In cloned calves that died shortly after birth, however, expression of both alleles of the *MAOA* gene was observed, suggesting aberrant XCI. We also conducted a series of studies to examine the normalcy of XCI reprogramming in 9 full-term calves cloned from different cell types using 10 X-linked genes sampled from various available organs. We examined allele specific expression of *MAOA*, and the expression of 9 additional X-linked genes, in major organs or in skin and blood of these 9 full-term cloned XX calves. Surprisingly, we found aberrant expression patterns in 9 out of 10 X-linked genes in all deceased clones. Inactivation of both alleles of several X-linked genes was observed in organs of all 5 deceased clones. Interestingly, the transducin (beta) like 1 (*TBL1*) a gene known to escape XCI in humans and mice (Bassi *et al.* 1999; Carrel *et al.* 1999) was expressed in all organs of these clones.

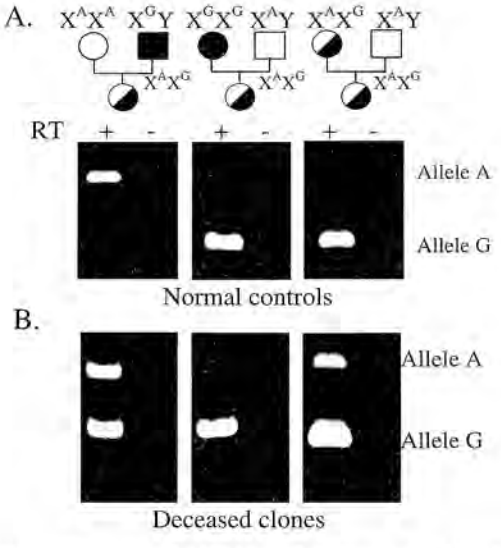

Fig. 5. Allele-specific expression of *MAOA* and expression of *XIST* in bovine placenta. a) three pedigrees showing the inheritance of *MAOA* gene during natural reproduction. circle = female, square = male, X^A=X chromosome carrying the A allele (clear), X^G=X chromosome carrying the G allele (filled), half-filled circle=heterozygous female. The three informative daughters are heterozygous for the *MAOA* gene, and the banding patterns of *MAOA* in their placentas demonstrate mono-allelic expression of the maternal allele of this gene. RT was added (+) or omitted (-) as a control for DNA contamination in RT-PCR. b) RTPCR-RFLP of *MAOA* showing expression of both alleles A and G in the placentas of deceased clones CA, E2 and I.

Consistent with observations of aberrant XCI in internal organs, we also found random XCI in the placenta of all deceased clones examined (Xue *et al.* 2002). Placental abnormalities have been reported in both live and deceased cloned calves (Hill *et al.* 1999; Hill *et al.* 2000).

The aberrant XCI in bovine clones may have resulted from incomplete erasure of the epigenetic marks on the X chromosomes of the somatic donor cells during nuclear reprogramming, which in turn may lead to only partial reactivation of the Xi or silencing of both X chromosomes prior to XCI. Upon differentiation, those epigenetic marks already present on the X chromosomes of the cloned embryo may interfere with the ones further imposed during XCI in clone development, ultimately leading to the observed aberrant expression patterns of X-linked genes.

The interesting finding that *TBL1*, a gene that escapes XCI, was properly expressed in all clones may indicate that regions of the X chromosomes not subjected to XCI, thus not epigenetically modified, are less affected by events involved in nuclear reprogramming. These data are consistent with the abnormal levels of DNA methylation found in cloned embryos and fetuses discussed earlier.

The global gene expression approach

Candidate gene expression studies can only analyze a handful of genes at a time; therefore, the study of global gene expression at early embryonic stages is a powerful approach to study nuclear reprogramming. We used a 7,872 cattle cDNA microarray to compare gene expression profiles between cloned and control blastocyst-stage embryos (Smith *et al.* 2005). This microarray was primarily derived from the bovine placental and spleen cDNA libraries, and was able to detect the expression of approximately 3,500 genes in the early embryos. In conjunction with linear amplification, individual NT embryos were compared to 1) their donor fibroblast cells; and 2) to control embryos created *in vivo* by artificial insemination (AI) and *in vitro* fertilization (IVF). Genes that differed by ≥ two fold (ANOVA $P < 0.05$ with False Discovery Rate (FDR) correction) in relative expression levels were considered differentially expressed.

Surprisingly, the NT embryos' gene expression profiles were drastically different from those of the donor cells (Fig. 6a). A total of 1,546 genes were differentially expressed, representing 29% of the total genes analyzed (n = 5,356). Among these, 751 were up-regulated and 795 were down-regulated in the donor cells versus the NT embryos. Gene Ontology (GO) analysis revealed that over-represented categories among genes up-regulated in the NT embryos were: "carrier activity," "mitochondrial inner membrane," "primary active transporter activity," "RNA splicing" and "ion transporter activity."

Because early embryos such as those generated from NT are expected to contain totipotent cells, we analyzed the expression of 94 genes on the microarray known to be highly enriched in human and mouse ES cells (Ivanova *et al.* 2002; Ramalho-Santos *et al.* 2002; Sato *et al.* 2003; Abeyta *et al.* 2004). Genes previously characterized as ES cell-specific: *ODC1*, *PECAM1* and *CCNE1* (Kelly *et al.* 2000) and an additional 20 genes had significantly higher expression in the NT embryos compared to the differentiated donor cells, suggesting that the enucleated bovine oocytes reprogrammed the differentiated fibroblast nuclei to totipotency. Additionally, the dissimilarity of gene expression profiles between the donor cells and NT embryos indicates that significant nuclear reprogramming of the donor cell nuclei is evident at the blastocyst stage after cloning.

Another surprising observation was that the gene expression profiles of the NT and AI embryos were more similar than those of the IVF and AI embryos (Fig. 6b). The correlation coefficient between the NT embryos and AI embryos was 0.808, but was only 0.714 between the IVF and AI embryos. One of the most interesting findings was that substantially less variation

was found among individual NT embryos, with the correlation coefficient being 0.838, as opposed to 0.733 observed among IVF embryos and 0.812 among the AI embryos. This exhibited lower variability among the NT embryos indicates that by the blastocyst stage NT embryos of the same donor animal behaved similarly at the molecular level, while AI and IVF embryos are more variable due to different genetic backgrounds.

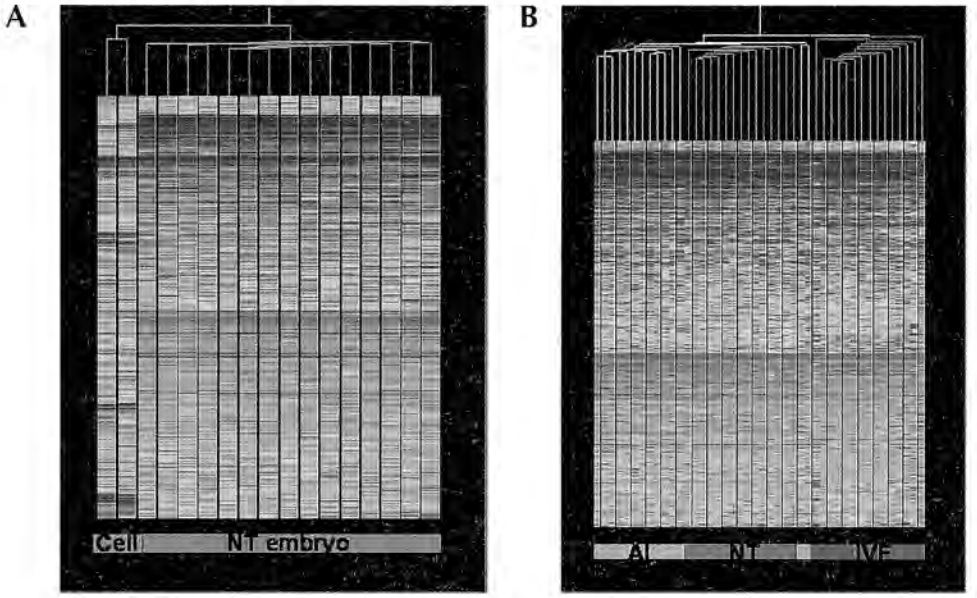

Fig. 6. Hierarchical cluster of global gene expression profiles. a) Cluster of donor cells (pink) and NT embryos (blue) reveals a difference of 84.2% in gene expression over 5,356 genes. b) Cluster of AI (yellow), NT (blue) and IVF (red) embryos illustrates more similarity between AI and NT embryos, with IVF embryos clustering as the outlier (n = 5174 genes). Color indicates the normalized expression values (sample: standard reference). Red equals high expression in the sample compared to the standard reference; yellow, equal expression; and green, low expression.

Even though the expression profiles between the NT embryos and the *in vivo* produced AI embryos displayed the most similarity, 50 out of 5,174 analyzed genes were identified as differentially expressed. Among these differentially expressed genes, eight were differentially expressed in the NT embryos when compared to both the AI and IVF embryos; this could be the result of a specific effect of nuclear reprogramming or it is possible that these genes were expressed from regions of the donor DNA still yet incompletely reprogrammed. Similarly, 17 genes were differentially expressed in the AI embryos versus both NT and IVF embryos; these genes may be important for the high developmental potential of AI embryos. Lastly, 25 genes were differentially expressed only between the AI and NT embryos. Some of these uniquely expressed genes (*COL4A1, DUSP6, FOLR1, MEIS2, MITF* and *TFAP2A*) are involved in development and down-regulated in the NT embryos. Thus, these genes could be potential candidates for perpetuating the abnormal development and mortality observed in NT fetuses.

Although many of abnormalities in cloned animals suggest imprinting disruptions (Mann *et al.* 2003; Ogawa *et al.* 2003), the expression of the 20 out of the 21 imprinted genes on the microarray were similarly expressed in the AI, IVF and NT embryos. Only *CD81*, a gene imprinted in the mouse placenta (Lewis *et al.* 2004), was differentially expressed between the NT and AI embryos. The observation indicates that the other 20 imprinted genes examined

were either properly reprogrammed in the NT embryos or that imprinting has not been established in bovine embryos at this stage. The former possibility is more likely because eleven of the imprinted genes studied—*CD81, COPG2, DCN, GNAS, GRB10, IGF2R, MEST, PEG3, PLAGL1, SDHD* and *SGCE*—were significantly differentially expressed between the donor cells and the NT embryos. Interestingly, the differentially expressed imprinted genes were not located on the same bovine chromosomes, suggesting that there was no clustering effect of imprinting reprogramming.

Aberrant expression of X-linked genes has been previously reported in bovine NT embryos and the tissues of deceased clones (Wrenzycki *et al.* 2002; Xue *et al.* 2002). Interestingly, no X-linked genes (n = 123 analyzed genes) were identified as differentially expressed between the NT and AI embryos. Previously in the bovine, it has been determined that by day 14-15, XCI is completely established *in vivo* (De La Fuente *et al.* 1999). Therefore, it is possible that XCI was not complete in the blastocyst-stage embryos examined here.

During normal bovine preimplantation embryonic development, dramatic methylation reprogramming takes place. These precise events could be difficult to recapitulate after nuclear transfer and indeed bovine NT embryos have been shown to be abnormally hypermethylated (Bourc'his *et al.* 2001; Kang *et al.* 2001; Dean *et al.* 2003) . We therefore sought to study genes that are involved in methylation regulation: *ATF7IP, DMAP1, DNMT2, DNMT3A, DNMT3B, FOS, MBD4, MIZF* and *p66alpha*. These genes were not differentially expressed among the three embryo types. This was consistent with our observation of similar expression of imprinted and X-linked genes between NT and normal AI embryos, and further indicates that the methylation regulation involved in nuclear reprogramming is not deficient in the NT embryos. Additional support for this observation came from the finding that both the *de novo* methyltransferases, *DNMT3A* and *DNMT3B,* were very highly and consistently expressed in the AI, NT and IVF embryos. This level of expression was not seen in the donor cells, signifying that these *de novo* methyltransferases were properly reprogrammed in the NT embryos at the blastocyst stage.

Chromatin remodeling and modification is crucial for mammalian development and efficient nuclear reprogramming (Li 2002). Twenty-six genes associated with chromatin modification and epigenetic regulation were examined: *ARID1A, ASF1A, BAT8, BAZ1B, CHD4, CHRAC1, CPA4, CTCF, CUGBP1, HDAC1, 2, 3, 7A, L3MBTL, MLL3, MSL3L1, MYST1, 4, RBM14, RPS6KA5, SET07, SIRT5, SMARCA5, SMARCC1, SMARCD3* and *TRIM28*. No differential expression was seen among the AI, IVF and NT embryos. However in the NT embryos and donor cell comparison, *ASF1A, BAZ1B, HDAC1, MLL3, RPS6KA5* and *TRIM28* were up-regulated in the NT embryos and *HDAC7A* and *SMARCD3* were up-regulated in the donor cells. This indicates that proper reprogramming of genes important for chromatin modification took place.

Conclusions

In summary, our data on global gene expression documented that the NT embryos' gene expression profiles were vastly different from those of their donor cells and look a great deal like those of AI embryos, suggesting that reprogramming of the differentiated somatic cell by the oocyte cytoplasm is effective and relatively complete by the blastocyst stage. This conclusion is supported by three lines of evidence: 1) blastocyst development rates of NT embryos are similar to those of IVF embryos, suggesting NT embryos are as competent as embryos fertilized by natural gametes in regards of preimplantation development; 2) embryonic stem cell (ESC) lines can be derived from NT embryos (ntESC) with high efficiency, suggesting NT embryos are reprogrammed and contain totipotent cells; 3) ntESC have similar global gene expression patterns as ESC's derived from fertilized embryos (Brambrink *et al.* 2006; Wakayama *et al.*

2006). Combined with our results obtained by the candidate gene approach in cloned fetuses and neonates, we hypothesize that the commonly observed low developmental efficiency of NT embryos is potentially caused by abnormal gene reprogramming during post-implantation fetal/placental development (i.e., gene re-differentiation). This hypothesis is supported by at least 2 lines of evidence: 1) the majority of the failure in NT embryo development occurs after implantation and/or around placentation (Heyman *et al.* 2002); 2) the small number of differentially expressed genes found in our microarray study were mainly involved in tissue differentiation/development, but not in pre-implantation development. Further research is required to determine if the aberrant expression of these genes at the blastocyst stage are magnified downstream in development.

Acknowledgments

This work is supported by funds from NIH and USDA to XCT and XY. The microarray study was conducted in collaboration with Drs. Harris A. Lewin and Robin E. Everts of the University of Illinois, Urbana-Champaign.

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