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Genetic Reprogramming after Somatic Cell cloning

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Abstract

Cloning by nuclear transfer in mammals using somatic cells has enormous potential applications. However, somatic cloning has been inefficient in all species in which NT is successful. High abortion and fetal death rates have been observed. These developmental defects have been attributed to incomplete nuclear reprogramming by the somatic cloning process. In this review, we will discuss studies conducted in our labs to understand the nuclear reprogramming process.

Introduction

Somatic cell cloning (cloning or nuclear transfer) is a technology in which the nucleus (DNA) of a somatic cell is transferred into an enucleated metaphase-II oocyte for the generation of an identical individual. The success of cloning an entire animal, Dolly, from a differentiated adult mammary epithelial cell (37) has created a revolution in science. It demonstrated that genes inactivated during tissue differentiation can be completely re-activated by a process called nuclear reprogramming: the conversion of a differentiated nucleus to a totipotent stage. Somatic cloning may be used to generate multiple copies of endangered animals, genetically elite farm animals and transgenic animals for pharmaceutical protein production or xeno-transplantation (2, 25, 32). With further optimization, it also holds enormous potential in biomedicine for therapeutic cloning and allo-transplantation (21). In addition to its practical applications, cloning is also

an essential tool for studying gene function (6, 25), genomic imprinting (33), genomic re-programming (8, 39), regulation of development, genetic diseases and gene therapy as well as many other areas (6, 23, 29, 43).

One of the challenges for cloning, however, is its low efficiency and high incidence of developmental abnormalities (12, 26, 36, 38, 44). Developmental defects including abnormalities in cloned fetuses and placentas, and high rates of pregnancy loss and neonatal death have been encountered by nearly every research team studying somatic cloning. It has been proposed that the low cloning efficiency may be largely attributed to the incomplete reprogramming of epigenetic signals (9, 15-17, 43).

In this review, I will discuss studies conducted in our laboratories for the understanding and improvement of nuclear reprogramming.

Cloning competence of various somatic cell types

Many adult cell types, including mammary epithelial cells, ovarian cumulus cells and fibroblast cells from skin and internal organs, have been utilized for nuclear transfer (19, 20, 30, 31, 37, 45, 47). A clear consensus, however, has not been reached as to the superior cell type to reprogram for nuclear transfer. This is due in part because different laboratories employ diverse procedures, and cell culture, nuclear transfer and the technical skill involved in micromanipulation all are critical. In order to make these comparisons, the procedures and techniques used as well as the skill of personnel must be identical for each donor animal and cell type. To compare the competence of different cell types for reprogramming by cloning, we avoided variations and compared the cloning competence of three cell types, ovarian cumulus, mammary epithelial and skin fibroblast cells, all from the same donor animal, a 13-year-old elite diary cow.

The ability of donor cells to be reprogrammed was assessed by the development of cloned embryos *in vitro* and by the development of clones to term following embryo transfer. As shown in Tables 1 and 2, although no differences were detected in the cleavage rates of three groups of embryos from different cell types, cumulus cells produced the highest rate of blastocyst development and resulted in 6 full-term cloned calves. Furthermore, four out of

six calves (Clones A, B, C and D, born between June and July of 1999) derived from cumulus cells survived after birth and are still healthy at two years of age (Table 2). In contrast, poorest *in vitro* development and no full-term survival was obtained with mammary epithelial cells. Skin fibroblast cells resulted in an intermediate rate of *in vitro* development and gave rise to 4 full-term cloned calves.

Table 1. Summary of *in vitro* development of cloned embryos from different cell types

Cell types	No. reconstructed embryos	Embryo development(%)	
		Cleavage	Blastocyst
Cumulus	92	65a	57a
Fibroblast	110	63a	34b
Epithelium	96	66a	23c

Numbers with different superscripts within columns are significantly different, $P < 0.05$.

Table 2. Summary of embryo transfer and calving of cloned embryos from different cell types

Cell type	No. embryo Transferred	No. of recipient		No. (%) calves born	Alive to adulthood	Animal ID
		Total	Pregnant*			
Cumulus	109	58	10	6 (5.5)**	4	A, B, B2, C, D, G
Fibroblast	57	29	8	4 (7.0)**	0	E1, E2, F, I
Epithelium	34	24	1	0	0	-

* Pregnancy determined by ultrasound examination at 60 days of gestation.

** A set of twins included.

Our results showed that donor cell type can significantly affect embryo

development *in vitro* as well as in vivo. Cumulus cells proved to be the most effective cell type for somatic cloning according to both the *in vitro* development test as well as full-term survival. These results suggest that DNA from cumulus cells is more effectively reprogrammed following nuclear transfer, which agrees with a recent report that cumulus cell-derived cloned mice do not have widespread dysregulation of imprinting (28).

Reprogramming of Telomere lengths

Telomeres are the DNA-protein structures located at the ends of eukaryotic chromosomes. Telomeric DNA (5-TTAGGG-3) is progressively lost with each round of cell division because the conventional DNA polymerase is unable to replicate lagging strand to the very 5 end (24). Since its shortening is well correlated with passage number, several teams of scientists proposed telomere as a mitotic clock for normal cell divisions (14). Telomerase, active in germ cells and cancer cells, is the enzyme that helps restore telomere length by synthesizing telomeric repeat DNA from its own RNA template, compensating telomere loss from conventional DNA replication (46).

Telomerase activity is highly regulated during embryo development. Studies in rat germ cells showed that the telomerase activity in oocytes from early antral and pre-ovulatory follicles was high, while its activity in ovulated oocytes became significantly lower (10). The same study also showed that after fertilization, telomerase activity was present in 4-cell-stage embryos (10). Telomerase activity was shown to be relatively high in blastocyst stage embryos of different species, including human and cattle (4, 40).

Our group examined telomerase activity in early stages of bovine IVF embryos (41). We demonstrated that telomerase activity increases after *in vitro* fertilization and then decreases gradually until the eight-cell stage. In addition, we found that from the eight-cell stage onward, telomerase activity increases progressively with advancing embryo stage and reaches its highest level at the blastocyst stage. Our results were consistent with those by Betts and King (4).

We also examined parthenogenetically activated (PA) and nuclear transferred (NT) bovine embryos. Telomerase activity in these embryos followed similar

transition patterns as those from IVF (42). In the PA system, telomerase activity was not significantly different among stages from oocyte to morula. However, we saw a trend involving an increase from the oocyte to zygote stage, a decrease at the eight-cell stage, and then an increase again at the morula stage. A significant increase was found at the blastocyst stage. In the NT system, telomerase activity decreased from the oocyte to the cleaved stage. From the cleaved stage onward, the activity increased gradually to the blastocyst stage, during which the highest telomerase activity was detected.

We demonstrated that these telomerase changes were due to increased enzymatic activity but not an increase in total protein. We have found a relative constant protein concentration (0.257 + 0.025 ug/ul) in the IVF embryo extracts among the different stages examined. It is worth noting that when the telomerase activity was expressed on a per-cell basis, a progressive decrease in activity was found from zygote stage to blastocyst stage in all three systems. The biological significance of this is not clearly understood. More studies are needed to examine the underlying mechanisms.

As we noticed, the telomerase activity of cloned blastocyst was lower than that in PA and IVF blastocysts. This may come from a loss of cytoplasm in the enucleation step, or possibly, from the variance between embryos. We also noticed that a significantly higher telomerase activity was found in the PA blastocysts than in those derived from either IVF or NT. The reason for this observation is not known, but the non-maternal genes from sperm or somatic cells may have actually suppressed the telomerase gene expression and hence, reduced the telomerase activity. In a study by Chian et al. (7) that bovine oocyte activation by sperm and PA induced different cytoplasmic responses for protein synthesis.

In all three systems, a decrease in telomerase activity was found in cleavage-stage embryos. This coincides with the maternal-zygotic transition model established by Barnes and First (3), who suggested that embryonic transcription of *in vitro*-cultured bovine embryos was initiated sometime between the four- to six-cell stage of development. It is reasonable to speculate that telomerase activity follows a pattern similar to that of the maternal-zygotic transition during dearly developmental stages in the IVF, PA and NT systems in the bovine. The maternal telomerase activity gradually decreases until approximately eight-cell-stage, during

which embryonic transcription starts. Once embryonic transcription initiates, new telomerase proteins are synthesized, and these could account for the increased activity detected during later stages.

More importantly, the activity found in NT blastocysts suggests successful reprogramming of telomerase in embryos after activation or cloning treatments. We have observed normal telomere lengths in the four cloned calves, as compared to the age-matched control calves from conventional reproduction (34). Our observations are consistent with those of Wakayama el. al. (35) and Betts et. al. (5). These results have important implications to the so called pre-mature aging of cloned animals, with regard to telomere length. The successful telomere restoration found in these cloned animals implied that cloned animals are not born pre-matured; rather, the telomere length has been restored. This may be attributed to the successful reprogramming of telomerase activity at early embryo stages.

We conclude here with the following: The telomerase activity is low in matured bovine oocytes. Telomerase activity is present throughout early development stages in bovine embryos derived from IVF, PA and NT. Telomerase activity follows similar patterns in these three systems, low in cleavage stages and high in blastocyst stages. The level of telomerase activity per cell, however, decreases during early embryonic development in the IVF, PA and NT systems.

Reprogramming of Epigenetic inheritance

Epigenetics is defined as nuclear inheritance which is not based on differences in DNA sequences (13). It is believed to involve differential DNA methylation and chromatin configuration and epigenetic signals are reset in each generation in the gonads (11, 13).

Somatic cloning bypasses the natural process of parental specific erasure and re-establishment of epigenetic signals (occurs in the gonads). Cloning thus provides an excellent model to investigate whether and how erasure and re-establishment of epigenetic marks (15, 16, 18). Clones of the same donor provide unique experimental materials in that they are genetically identical yet epigenetically different, at least so far as imprinted and X-linked genes are concerned (9, 43). This animal model provides insights into epigenetic regulation

that cannot be studied by models from natural reproduction, thus revealing unique features of epigenetics not possibly shown previously.

At the formation of the female zygote during natural fertilization, both X chromosomes are active. Expression of the X-inactive specific transcript (*Xist*) gene from one of the X chromosomes leads to its inactivation at the late blastocyst stage (11, 22). Once established, the inactive state of a particular X chromosome is epigenetically inherited throughout all subsequent cell divisions (11, 22).

In mammals, epigenetic marks are involved in dosage compensation by the process of X chromosome inactivation (XCI), which leads to the random transcriptional silencing of one of the two X chromosomes in female cells during early development, making females mosaic in the expression of X-linked genes9-11.

In somatic cloning through nuclear transfer, the cloned zygotes receive one active (Xa) and one inactive (Xi) X chromosome from the donor cells. Eggan *et al.* (9) 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 a universal observation for other species. We therefore conducted a series of studies to examine the normalcy of XCI reprogramming in 9 full-term cattle cloned from different cell types using 10 X-linked genes in various organs where samples were available.

In our study, we established for the first time the patterns of XCI in cattle from natural reproduction and from somatic cell cloning by nuclear transfer by studying allele-specific expression of the MaoA gene and expression patterns of 9 other X-linked genes. We found aberrant patterns of XCI in organs of all deceased clones, most of which were derived from fibroblast cells (Clones E1, E2, F, and I). Consistent with observations of aberrant XCI in internal organs, we also found random XCI in the placenta of all deceased clones examined. Placental abnormalities have been reported in both live and deceased cloned calves (12).

The aberrant XCI in bovine clones found in the present study may have resulted from incomplete erasure of the epigenetic marks in the X chromosomes

of the somatic donor cells during nuclear reprogramming, which in turn may lead to only partial reactivation of the Xi or to abnormal methylation patterns of the *Xist* gene in one or 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 X chromosomes not subjected to XCI, thus not epigenetically modified, were less affected by events involved in nuclear reprogramming. Additionally, the fact that living animals cloned from cumulus cells have normal XCI in skin and blood may suggest that the epigenetic marks that differentiate Xa from Xi can be properly erased and re-established. Analyses of XCI in the organs of these live clones will provide a clearer picture of whether improper reprogramming of epigenetic signals on the X chromosome is associated with the deaths and the low efficiency of somatic cell cloning.

Epigenetic instability and aberrant expression of imprinted genes have been reported in mice cloned from stem cells (15, 16). Epigenetic inheritance is a common feature between XCI and imprinting (11, 22, 33). The extent to which the epigenetic marks involved in imprinting and XCI overlap remains to be determined. Our recent research findings provide evidence that, similar to the instability of epigenetic signals in imprinted genes, the epigenetic signals involved in both random and imprinted XCI were not correctly erased and/or maintained during the process of somatic cell nuclear reprogramming in cattle. A thorough understanding of epigenetic reprogramming in cloned animals will improve the young and promising technology of cloning by revealing the ideal conditions for a complete reprogramming of the somatic nucleus.

Conditions tested to improve nuclear reprogramming

To improve the efficiency of nuclear transfer, various methods have been employed to modify the donor cells and nuclear transfer procedure. These include: a) synchrony of cell cycle stage of donor cells as well as synchrony

between donor cells and recipient oocytes (1, 4, 20, 45), b) using somatic cells from donors of various ages (27, our unpublished data), tissue origins (19, 30), passages (20) and culture conditions (20, 45); and c) transfer of cells without epigenetic marks (1, 18, 47). None of these attempts, however, significantly improved the success rate of nuclear transfer. These observations suggest that further studies on nuclear reprogramming and novel approaches for nuclear transfer are needed in order to understand the underlying mechanisms for reprogramming and significantly improve the ability of the differentiated somatic nuclei to be reprogrammed.

ACKNOWLEDGMENTS

The research projects reported here were supported by grants to XY and XCT from Connecticut Innovations Inc, UCONN Research Foundation, USDA and NIH.

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