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Factors Involving Reprogramming in Cloned Embryos

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ABSTRACT

Although nuclear transfer (NT) techniques are used to clone animals, its efficiency is very low. Moreover, nuclear transfer has resulted in offspring with severe developmental problems, probably due to incomplete nuclear reprogramming. Nuclear reprogramming is characterized by functional modification of the transferred nucleus to allow it to direct normal embryo development with the potential to grow to term. Although the nature of the reprogramming factor(s) in mammals is not clear, various nuclear as well as cytoplasmic components are involved in the processes. In this article we review recent data on factors involved in the nuclear reprogramming of cloned embryos.

(Key words : Nuclear transfer, Reprogramming, Epigenetic factors, Cloned animal)

Ⅰ. INTRODUCTION

While the nuclear transfer (NT) procedure is a powerful tool for cloning animals, its efficiency, in terms of the numbers of live offspring born, is low because of the high rate of fetal loss after transfer (Wilmut et al., 1997; Cibelli et al., 1998; Daniels et al., 2000). This fetal loss is due to a high frequency of embryonic, fetal, and neonatal abnormalities (Young et al., 1998; Niemann et al., 2002). The major cause of these abnormalities is likely to be the incomplete reprogramming of the donor cell nucleus, which result in chromosomal abnormalities that lead to aberrant or absent expression of developmentally important genes (Jaenisch, 1997; Mizuki et al., 2001; Young et al., 2001).

Nuclear reprogramming is characterized by functional modification(s) of the transferred nucleus to allow it to direct normal embryo development with the potential to grow to term. The nature of the reprogramming factor(s) in mammals, however, is not clear. Reprogramming of a nucleus constitutes structural remodeling, which may be associated with fundamental changes in genomic activity. Nuclear envelope breakdown, premature chromosome condensation (PCC), and nuclear swelling are well-known morphological changes in reconstructed embryos following NT (Campbell et al., 1996). While nuclear remodeling processes following NT are evident, the interactive roles played by cytoplasmic and nuclear components in these processes, are not

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yet well understood. In this review we will concentrate on the recent data on epigenetic factors affecting nuclear reprogramming in reconstructed oocytes following nuclear transfer. Elucidating the molecular events which lead to the reprogramming of a donor genome will contribute to our understanding, and hopefully correcting, the developmental defects of cloned embryos.

Ⅱ. NUCLEAR REMODELING/REPROGRAMMING

The remodeling of the nucleus following somatic cell nuclear transfer is characterized by nuclear envelope breakdown and PCC (Campbell et al., 1996). The PCC is induced in interphase nuclei, which is fused with an M-phase cell, as a result of maturation-promoting factor (MPF) (Collas et al., 1992; Tani et al., 2001). Premature chromosome condensation could be beneficial because it provides an opportunity for the chromatin to be directly exposed to the cytoplasmic factors needed for efficient reprogramming. In a protocol where the oocyte is activated simultaneously with the transfer of a nucleus, both in mouse (Wakayama and Yanagimachi, 2001) and in bovine (Vignon et al., 2002), no characteristic PCC was observed during the first hours of development. Since this protocol also induces a high rate of blastocyst development (Renard et al., 2002), chromatin condensation may not be necessary for bovine embryo development.

Recently, Sullivan et al.(2003) identified several nuclear defects in NT embryos and increased resistance of DNA to DNAse I. These abnormalities may result from incomplete expression of differentiated cell-specific genes. Remodeling of nuclei *in vitro* and transplantation of condensed chromatin into oocytes alleviates these defects. Interestingly, however, transfer of M-phase cells into oocytes results in pronuclei resembling NT pronuclei with respect to lamin A/C and TBP expression and TBP anchoring. Thus, chromosome condensation following nuclear transfer may alleviate incomplete reprogramming (Sullivan et al., 2003).

The observation that somatic cells are reprogrammed when fused with enucleated MII oocytes, but not with oocytes activated 3~6 h previously (Tani et al., 2001; Shin et al., 2002), indicates that MPF is a candidate reprogramming factor. The mitogen-activated protein kinase (MAPK) cascade, such as Mos-MEK (MAPK kinase)-ERK1/2 (extra cellular signal-regulated protein kinase)-p90RSK1, is a principal regulator of transition from the MII phase to pronuclear formation after fertilization or parthenogenetic activation (Reimann and Jackson, 2002). Tani et al. (2003) examined whether the process of nuclear reprogramming is regulated by MPF and MAPK activity. Reprogramming of bovine somatic cell nuclei is not directly regulated by MPF or MAPK activity in oocytes. Recently, Gonda et al. (2003) demonstrated that a single protein (FRGY2) from Xenopus oocytes triggers reversible disassembly of somatic cell nuclei. However, it is not clear whether the FRGY2 protein can convert somatic cell nuclei to totipotent cells. Further molecular studies of the differences between oocytes with and without the potential to reprogram somatic cells are necessary to identify unknown reprogramming factor(s).

Ⅲ. CENTROSOME INHERITANCE AND MICROTUBULE ASSEMBLY

Microtubule configuration following nuclear transfer has been demonstrated in the blastomere nuclei of rabbits and cattle (Collas et al., 1992; Pinto-Correia et al., 1993; Navara et al., 1994). In rabbits, following transfer into enucleated oocytes, donor blastomere nuclei undergo nuclear breakdown with subsequent formation of a "transient spindle" (Col-

las et al., 1992). Navara et al. (1994) observed one or two microtubule asters in enucleated bovine oocytes following nuclear transfer, suggesting nuclear transfer introduces foreign centrosomes. Recently, we described nuclear and microtubule dynamics following somatic cell NT in cattle (Shin et al., 2002).

Animal centrosomes play important roles in regulating cell division, and are composed of a pair of centrioles and protein-rich pericentriolar material containing g-tubulin (Joshi et al., 1992). The centrosome is disassembled during gametogenesis in both males and females, and reassembled soon after fertilization, aprocess that requires components from both the sperm and egg (Schatten, 1994; Manandhar et al., 1998). The mechanism of centrosome reconstruction from the maternal and paternal components during fertilization is yet to be fully elucidated. The current NT procedure may be used to introduce the centrosome, since it is located close to the nucleus (Navara et al., 1994). A microtubule aster containing g-tubulin was observed close to a transferred nucleus that later divided, completing the first mitosis step following NT (Shin et al., 2002).

Ⅳ. MITOCHONDRIA

Cloned animals produced by current nuclear transfer procedures may not be genetically identical to the donor cell. In NT embryos, nucleus genes originate from donor cells, while the mitochondrial genes originate from recipient oocytes, even if a small amount of donor cytoplasm is added to the recipient's cytoplasm. If the donor mitochondrial genome is maintained throughout development, the clones are heteroplasmic. During normal fertilization, the sperm's mitochondria are destroyed and the oocyte-derived mitochondria are assumed to be transmitted to the offspring (Smith and Alcivar, 1993). The fate of foreign mitochondrial DNA following NT is controversial. The fate of mitochondria derived from a donor cell has been demonstrated using a molecular probe (MitoTracker Green FM fluorochrome, Do et al., 2001). The labeled mitochondria dispersed into the cytoplasm and became distributed among blastomeres and could still be identified up to the 8- to 16-cell stage. However, donor mitochondria were not detected in embryos at the 16-cell or subsequent developmental stages. In the control group, mitochondria could be identified up to 7 days in arrested one-cell embryos following nuclear transfer. Sheep cloned by somatic cell nuclear transfer inherited their mitochondria entirely from the oocyte and not from the donor cell (Evans et al., 1999). After nuclear transfer, the donor mitochondrial DNA (mtDNAs) gradually degenerated and/or disappeared by approximately the blastocyst stage (Takeda et al., 1999; Meirelles et al., 2001).

However, thepotential for mtDNA heteroplasmy was observed in NT embryos and offspring produced by both embryonic and somatic cell transfers (Steinborn et al., 1998a,b; Hiendleder et al., 1999; Takeda et al., 1999; Steinborn et al., 2000; Meirelles et al., 2001). Minute amounts of donor-derived mtDNA were detected in somatic cell-derived NT calves (Steinborn et al., 2000), and larger amounts of donor-derived mtDNA were detected in blastomere-derived NT calves (Steinborn et al., 1998b; Hiendleder et al., 1999). The proportion of donor-derived mtDNA in NT calves seems to be dependent upon the quantity of donor cell cytoplasm present post-reconstruction. Reduction or elimination of donor mtDNAs is then observed later in development in both NT embryos and offspring (Steinborn et al., 1998b; Evans et al., 1999; Takeda et al., 1999; Meirelles et al., 2001). On the other hand, Steinborn et al. (1998a) reported that mtDNA originating from donor blastomeres

was detected in cloned cattle using the allele-specific TaqMan PCR. Furthermore, cattle derived by the transfer of blastomere nuclei showed ambiguous contributions from both the donor cell and the oocyte (Hiendleder et al., 1999).

The cause of the disparity in mitochondria inheritance during nuclear transfer is not clear. Mitochondria transferred from donor cells may be broken down by cytoplasmic events, whereas mitochondrial DNA remains in the cytoplasm and is transported to endogenous mitochondria. It is also possible that the molecular probe for mitochondria disappears at the mitochondrial maturation stage during which mitochondria show extensive morphological transformations (Do et al., 2002). Further studies are required to determine the mechanism of mitochondrial destruction and the mode of transmission of mtDNA in both somatic cell NT and normal fertilization.

Ⅴ. GENE EXPRESSION

In mammals, the early developmental stages of preimplantation embryos rely on maternal RNA and protein, until the embryonic genome becomes activated (EGA) and controls the regulation of development. In bovine, EGA occurs at the 8- to 16-cell stage (Memili and First, 1998). Therefore, EGA is very important for a large number of genes that require activation before the embryo can undergo further development. After NT, the nuclei of differentiated somatic cells must undergo reprogramming to re-establish an embryonic gene expression profile for development following the EGA stage. Recent studies of cloned embryos during preimplantation development have revealed striking defects, indicating that cloned embryos do not faithfully recapitulate many of the essential early events of normal development. The successful reprogramming of a somatic cell following nuclear transfer would result in an embryo with the same profile of gene transcription asthat of embryos produced by IVF. Cloned embryos exhibit defects in the expression of key regulatory genes such as Oct4, a POU domain, class 5, transcription factor 1 (Botquin et al., 1998). These embryos display dramatic alterations in culture-medium preferences, with a shift toward somatic cell characteristics, indicating a lack of nuclear reprogramming affects genes influencing basic physiology and metabolism. Aberrant gene expression is frequently observed in NT embryos, which probably results in abnormal development and contributes to early loss cloned fetuses (Daniels et al., 2000; Rideout et al., 2001; Boiani et al., 2002). Daniels'group also found that transcription of some implantation-related genes, such as IL-6, FGF4, and FGFr2, is delayed and/or aberrant in some cloned embryos. More recently, Wrenzyciki et al.(2001) observed that the mRNA expression of some development-related genes is different in embryos created by nuclear transfer (NT) than in embryos created by *in vitro* fertilization (IVF). Recently Han et al.(2003) compared the transcription patterns of the IGF2-IGF2R axis in single IVF and NT embryos by RT-PCR. While the IGF2R mRNA transcript levels did not differ ($P > 0.05$), significantly greater expression of IGF2 mRNA was detected in the NT embryos relative to the IVF group ($P<0.05$). The IGF2 gene is a classic example of an imprinted gene as it appears to be biallelically transcribed in embryos up to the morula stage, but by the blastocyst stage the maternal IGF2 allele is silenced (Mizuki et al., 2001). Thus, it appears that the NT embryos suffer a loss of imprinting and therefore over express IGF2 and similarly imprinted genes.

Ⅵ. DNA METHYLATION

After fertilization, the parental genomes are first

abruptly, and then gradually demethylated in early cleavage-stage embryos, which is a prerequisite for normal embryonic development in mammals. In cloned bovine embryos, however, the epigenetic reprogramming mechanism, represented by the methylation/demethylation process, is likely disrupted (Kang et al, 2001a; Bourc'his et al., 2001; Kang et al., 2001b). Various repetitive genomic sequences, such as satellite I, satellite II, 18S rDNA, LINE and art-2 SINE sequences showed aberrant methylation status in cloned bovine embryos (Kang et al., 2001a). The methylation patterns of cloned embryos were quite different from those of normal embryos produced *in vitro* or *in vivo*, but closely resembled the overall genomic methylation status of donor cells. In contrast to the repetitive sequences, normal reprogrammed methylation patterns were observed on GC-rich sequences of single-copy genes, such as bovine epidermal cytokeratin gene, beta-lacto globulin gene, and galanin gene (Kang et al., 2002 Kang et al., 2003). Immunostainning results also showed differences in the dynamics of chromosome methylation between cloned embryos and embryos from normal reproduction at the preimplantation stages (Bourc'his et al., 2001). The centromeric heterochromatin in cloned embryos was heavily methylated in contrast to the low methylation in IVF-derived embryos, although the euchromatic methylation patterns were similar in cloned and normal embryos. These observations of an abnormal methylation status in the genome of cloned embryos are sufficient to explain the problems of the current cloning technology, and account for the low efficiency which remains an impediment to widespread agricultural and biomedical applications.

The phenomenon of differential demethylation appears not only among different genomic sequences, but also between the different regions of the cloned blastocyst. Aberrant allocations of inner cell mass (ICM) cells and trophectoderm (TE) cells were observed in cloned bovine blastocysts (Koo et al., 2002), showing a greater proportion of ICM in cloned embryos as compared with normal embryos produced both *in vitro* and *in vivo*. It was postulated that the methylation differences in cloned embryos might be derived from epigenetic differences between the ICM and TE genomes. In cloned embryos the satellite sequences were methylated to some extent in TE cells but not in ICM cells, although the cytokeratin gene sequence was equally demethylated in both TE and ICM regions (Kang et al., 2002). Even though a similar methylation difference between ICM and TE cells was not detected in IVF-derived embryos, this observation alone cannot exclude the possibility of differential demethylation in normal embryos. Since the satellite sequences maintain low methylation status in IVF-derived embryos throughout preimplantation stage, this makes it difficult to detect potential differences in their epigenetic status. Additional information on genomic sequences that remain methylated until the blastocyst stage would be helpful for elucidating methylation differences between ICM and TE regions. However, such sequences appear rare in bovine because the genomic sequences examined in the previous studies all showed hypomethylation at the blastocyst stage (Kang et al., 2001a,b). The results with bovine IVF-derived embryos is consistent with a previous observation that a low level of methylation of L1 sequences was detected uniformly across both cell lineages of the blastocyst in the mouse (Howlett et al., 1991). On the other hand, an early methylation study described a pattern of differential methylation in rabbit blastocysts on day 6, where higher levels of methylation were found in ICM cells, as compared to TE cells (Manes et al., 1981), which is opposite to the results from cloned bovine embryos. The molecular mechanisms of regional differences in the methylation patterns of cloned blastocysts remains to be elucidated.

Ⅶ. CONCLUSION

Poor epigenetic reprogramming may result in aberrant gene expression in early cleavage embryos. The accumulated consequences of several abnormally expressed genes in cloned embryos or fetuses can be detrimental to normal full-term development. The characterization of additional parameters that affect the developmental potential of embryos will assist the current nuclear transfer technology to identify its problems and to resolve them. The discovery of aberrant epigenetic patterns in early cloned embryosdemonstrates that parts of the somatic cell genome are, not susceptible to reprogramming forces. The nature of the donor cell genome may limit the complete reprogramming of its epigenetic marks, and can be one, if not the cause of chronic developmental defects inherent to clones.

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