Reprogramming of Cloned Embryos During Early Embryogenesis

초기 발생에 있어서 복제수정란의 리프로그래밍

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1. Summary

Animal clones derived from somatic cells have been successfully produced in a variety of mammalian species such as sheep, cattle, mice, goats, pigs, cat and rabbits. However, there are still many unsolved problems in the present cloning technology. Somatic cell nuclear transfer has shown several developmental aberrancies including high rate of abortion in early gestation and increased perinatal death. These developmental failures of cloned embryos may arise from abnormal reprogramming of donor genome and/or incomplete cloning procedure. We have found that overall genomic methylation status of cloned bovine embryos is quite different from that of normal embryos in various genomic regions, suggesting that the developmental failures of cloned embryos may be due to incomplete reprogramming of donor genomic DNA. Many of the advances in understanding the molecular events for reprogramming of donor genome will more clarify the developmental defects of cloned embryos.

2. Introduction

Although successful production of animal clones from somatic cells have been achieved in various species, till now its efficiency is very low in that only less than 1% of the reconstructed embryos with nuclei of somatic cells give rise to live-born animals. Moreover, somatic cell nuclear transfer has raised severe developmental failures including high rate of abortion during early gestation and increased perinatal death (Hill et al., 1999; Hill et al., 2000; Schnieke et al., 1997; Wilmut et al., 1997). However, unclear is whether the developmental failures of cloned embryos are due to the incomplete nuclear reprogramming or to the cloning procedure itself. Nuclear transfer involves a series of complex procedures including culture of donor cells, in vitro maturation of oocytes, enucleation, cell or nucleus injection, fusion, activation, in vitro culture of reconstructed embryos and embryo transfer. If any part of these steps is not properly done, cloned embryos or animals may not be produced. Although many research groups worldwide are technically sufficient to develop cloned animals, we still have limited information about nuclear reprogramming in cloned embryos. It is no doubt that a differentiated somatic cell nucleus transferred into the cytoplasm of

an enucleated oocyte should become reprogrammed to support normal embryonic development of cloned embryos. Telomere restoration of donor chromatin may be one factor affecting developmental failures of cloned embryos. In bovine the shortened telomeres of donor somatic cells were restored to normal or longer length after nuclear transfer (Lanza et al., 2000; Tian et al., 2000), whereas the clone sheep inherited shortened telomeres from her donor cell (Shiels et al., 1999). Cloning or non-physiological culture environments may result in inappropriate epigenetic modification of imprinted genes during early embryogenesis when many allele-specific imprints are established or maintained (Young & Fairburn, 2000). Manipulation or non-physiological embryo culture environments could lead to defective fetal reprogramming in livestock (Young et al., 2001). Some metabolic enzyme genes were normally expressed in cloned bovine embryos (Winger et al., 2000), demonstrating that expression of these genes may be properly reprogrammed even following somatic cell nuclear transfer. On the contrary, some genes essential for early embryonic development were abnormally expressed in cloned bovine embryos, suggesting that the aberrant transcription patterns detected in cloned embryos could lead to a reduction in embryo viability (Daniels et al., 2000). Recently, we have demonstrated that aberrant methylation patterns are detected in various genomic regions of cloned bovine embryos and methylation level of donor cells is maintained throughout preimplantation development (Kang et al., 2001b), although epigenetic modification takes place differentially in donor genome of cloned bovine embryos (Kang et al., 2002). These results provide an indirect evidence that the developmental failures of cloned embryos may be due to inefficient epigenetic reprogramming of donor genomic DNA. This paper will discuss reprogramming of the cloned embryos in two aspects of early embryonic development and DNA methylation.

3. Various Abnormalities of Cloned Animals

A serious impediment to the practical use of somatic cell nuclear transfer technology is the low survival of cloned embryos to live-born animals; only a few percent of reconstructed oocytes develop to term, and of those, many die shortly after birth (Cibelli et al., 1998; Wakayama et al., 1998; Wells et al., 1997; Wilmut et al., 1997; Young et al., 1998). Various abnormalities include circulatory distress, placental edema, hydrallantois, and chronic pulmonary hypertension (Hill et al., 1999; Hill et al., 2000). Even the surviving offsprings show large placenta (Hill et al., 1999; Ono et al., 2001; Wakayama et al., 1998) and increased birth weights (Eggan et al., 2001), referred to as 'large offspring syndrome' (LOS), and those with seemingly healthy appearance suffer from immune dysfunction or kidney/brain malformation which contribute to the death later (Lanza et al., 2000; McCreath et al., 2000). The LOS is a typical phenotype observed in cloned neonates of mammalian species such as cattle (Cibelli et al., 1998; Wells et al., 1997), mouse (Wakayama et al., 1998) and sheep (Young et al., 1998), but the factors responsible for LOS still remain elusive. This phenomenon is not unique to cloned neonates. It has been reported that the LOS is attributed to a variety of in vitro culture procedures before embryo transfer (Eggan et al., 2001; Sinclair et al., 2000; Young et al., 1998). Epigenetic alteration of the imprinted Igf2r region and abnormal expression of the corresponding gene have been detected in sheep LOS fetuses derived from in vitro-produced embryos (Young et al., 2001).

4. Epigenetic Reprogramming of Donor Genome in Early Cloned Embryos

Epigenetic modification such as DNA methylation has been considered to be one of candidates regulating nuclear reprogramming. In mammals DNA methylation plays an important role in the regulation of gene expression and is essential for normal embryonic development. The most dramatic changes in the methylation level occur during gametogenesis and early development (Monk et al., 1987; Sanford et al., 1987). During early embryonic development, the overall methylation level sharply decreases and reaches a low point at the blastocyst stage (Monk et al., 1987; Razin & Shemer, 1995). The process of epigenetic reprogramming in early embryos erases gamete-specific methylation patterns inherited from the parents (Howlett & Reik, 1991; Monk et al., 1987; Oswald et al., 2000). This genome-wide demethylation process may be a prerequisite for the formation of pluripotent stem cells that are important for the later development. During post-implantation development, a wave of de novo methylation takes place; most of the genomic DNA is methylated at defined developmental timepoints, whereas tissue-specific genes undergo demethylation in their tissues of expression (Kafri et al., 1992; Razin & Kafri, 1994). Another demethylation-remethylation cycle of epigenetic reprogramming occurs during gametogenesis and is necessary for resetting of genomic imprinting (Solter, 1988). Therefore, dynamic epigenetic changes appear to be essential and probably are unique during embryonic development in mammals.

Successful cloning of animals by nuclear transfer probably requires epigenetic reprogramming of the differentiated state of the donor cell to a totipotent, embryonic ground state (Gurdon & Colman, 1999). It means that the donor cell must cease its own program of gene expression and restore a particular program of the embryonic expression necessary for normal development. Epigenetic reprogramming processes after somatic cell nuclear transfer include remodeling of chromatin structure, global changes in DNA methylation, expression of imprinted genes, restoration of telomere length, X chromosome inactivation and other events during early embryonic development. Recently, some interesting results on epigenetic reprogramming of the donor genome have been taken out in cloned embryos (Kang et al., 2001a; Kang et al., 2001b; Kang et al., 2001c) and animals (Eggan et al., 2000; Eggan et al., 2001; Humpherys et al., 2001; Ohgane et al., 2001; Shiels et al., 1999; Tian et al., 2000).

It has been known that a dramatic demethylation of the maternal and paternal genomes occurs in the early embryos, predominantly from 8-cell to blastocyst stages (Monk et al., 1987). Although the biological significance of early embryonic demethylation remains unclear, this demethylation process appears to be essential to remove differences in gamete-specific methylation patterns and then to reformat the genome prior to initiation of the normal development. A genome-wide demethylation process generally appears to be unique during early embryogenesis in mammals. In the IVF-derived bovine embryos, the *Bov-B* LINE sequences exhibited a gradual demethylation pattern at the preimplantation stages (Kang et al., 2001b), like the L1 repeats in the mouse (Howlett & Reik, 1991). The *PRE-1* SINE sequences also showed a demethylation pattern in early pig embryos (Kang et al., 2001c). In addition to these repeated sequences, other genomic repeats were also substantially undermethylated at the blastocyst stage as if a global demethyla-

tion process had taken place during cleavage stage. Our experiments also demonstrate that single-copy sequences behave similarly to genomic repeated sequences. The promoter sequences of bovine tissue-specific genes clearly represented demethylation events in IVF-derived bovine embryos. Based on these findings, we strongly suggest that the genome-wide demethylation process is a typical phenomenon in various mammals, which plays an important role for normal development of early embryos.

It has been suggested that in cloned bovine embryos the epigenetic reprogramming mechanism, represented by the methylation and demethylation process, probably is inappropriately operated (Kang et al., 2001a; Kang et al., 2001b; Bourchis et al., 2001). We found that various genomic repeated sequences (satellite I, satellite II, 18S rDNA and *art-2* SINE sequences) showed aberrant methylation status in cloned embryos. The methylation patterns of cloned embryos closely resembled those of donor cells in the overall genomic methylation status, but were quite different from those of normal embryos produced in vitro or in vivo. A gradual demethylation process of the *Bov-B* LINE sequence was detected in normal embryos, but not in cloned embryos where the donor-type methylation was simply maintained during preimplantation development. Bourchis et al. (2001) also reported differences in the dynamics of chromosome methylation between cloned and normal embryos at the preimplantation stages. They demonstrated that the centromeric heterochromatin in cloned embryos was heavily methylated in contrast to the low methylation in IVF-derived embryos, although the euchromatic methylation pattern was similar in cloned and normal embryos. These observations of abnormal methylation status in the genome of cloned embryos are enough to raise serious skepticism about the practical applications of cloning technology to agriculture and biomedical areas.

What can viable offsprings really develop from the unlikely cloned embryos showing heavy methylation states? Is the cloned embryo actually rather tolerant to epigenetic reprogramming for normal development? Some clues on these questions could be answered by analyses of individual cloned embryos for the methylation state. Unexpectedly, the methylation level of bovine satellite sequences was greatly varied in individual cloned embryos (Kang et al., 2001b). Among individual cloned blastocysts, only 26% (7/27) were relatively undermethylated, although their methylation levels were still higher than the mean methylation value of normal control embryos, whereas 88% (23/26) of IVF-derived embryos showed substantially demethylated states. At present, it is hard to make a correlation of these diverse methylation states with subsequent developmental potential of cloned embryos. However, it is likely that the undermethylated cloned embryos probably have developmental competence because their methylation states are more close to those of normal embryos. In general, two cell types, serum-starved and growing-phase cells, have been used for cloning as donor nuclei. When overall methylation states of these different cell populations were compared, there was no variation in the methylation state between two cell types. The possibility that individual variation of methylation level shown in cloned embryos may depend upon innate methylation difference among individual donor cells cannot be excluded. However, considering the nature of centromeric satellite I DNA such as high copy number and relatively stable epigenetic status, it is more likely that methylation variations observed in individual cloned embryos are determined largely by different abilities of the recipient oocytes or reconstituted embryos to modify the epigenetic status of donor genome. It is

plausible that the extent of oocyte cytoplasmic factors may influence reprogramming of a differentiated somatic cell nucleus to totipotency when transferred into the enucleated oocyte.

5. Conclusion

In the aspect of DNA methylation status, the observations of the high-frequency, various-phenotype and cross-species similarities in abnormalities inherent to cloned animals lead us to speculate that these developmental problems come into being from faulty epigenetic reprogramming process that should be necessarily accomplished in cloned donor genome during preimplantation development. Poor epigenetic reprogramming in early cleavage embryos may entail aberrant expression of the genes at multiple loci, and then accumulated actions of many abnormally expressed genes in cloned embryos or fetuses can disrupt normal full-term development. The precise understanding for the epigenetic anomaly observed in cloned embryos remains largely unclear. Thus, somatic cell nuclear transfer is still an incomplete technology to support efficient production of cloned animals. To improve efficiency of the present cloning system, more extensive studies should be performed on the reprogramming mechanisms of donor genome during early embryo- genesis.

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