Epigenetic Reprogramming and Cloning

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ABSTRACT : Zygote genome should entail a complex process of epigenetic reprogramming including a global DNA demethylation to reach a totipotency or pluripotency during early mammalian development. In this study, we have analyzed methylation patterns in cloned bovine embryos to monitor the epigenetic reprogramming process of donor genomic DNA. Aberrant DNA methylation patterns were observed in various genomic regions of cloned embryos except single-copy gene sequences. The overall genomic methylation status of cloned embryos was quite different from that of normal embryos produced *in vitro* or *in vivo*. Abnormal methylation profiles were also specifically represented in trophectoderm cells of cloned embryos, which probably result in widespread gene dysregulation in extraembryonic region or placental dysfunction familiar to cloned animals. Our findings suggest that developmental failures of cloned embryos are due to incomplete epigenetic reprogramming of donor genomic DNA. Understanding the epigenetic reprogramming processes of donor genome will clearly define the faulty development of cloned embryos.

   Key words : Somatic cell nuclear transfer, Reprogramming, DNA methylation, Embryo, Bovine.

요 약 : 포유동물의 초기 발생과정 중 접합체가 전능성이나 다능성을 가지기 위해서는 전반적인 DNA 메틸화를 포함하는 후성 유전학적 리프로그래밍의 복잡한 과정을 거쳐야만 한다. 본 연구팀에서는 공여핵의 후성 유전학적 리프로그래밍 과정을 조사하기 위하여 소 복제수정란에서 메틸화 양상을 분석하였다. 복제수정란의 비정상적인 메틸화 양상이 다양한 반복염기서열에서 관찰되었지만 single-copy 유전자들의 염기서열은 정상적인 메틸화 양상을 보여주었다. 전반적으로 복제수정란의 전반적인 메틸화 상태는 정상수정란과 완전히 다른 양상을 보여주었다. 또한 복제 배반포의 영양외배엽세포에서 특이적으로 높은 메틸화 수준은 현 복제동물에서 빈번히 나타나는 불완전한 태반형성에 작용할 수 있을 것이다. 결론적으로 복제수정란의 비정상적 발생은 공여핵의 불완전한 후성 유전학적 리프로그래밍에 기인할 수 있다는 사실을 제시하게 되었다. 이러한 공여핵의 후성 유전학적 과정의 이해는 복제수정란의 비정상적 발생을 보다 분명히 밝힐 수 있을 것이다.

INTRODUCTION

Somatic cell nuclear transfer is a powerful technology for multiplication of unique animal genotypes(Kato et al., 1998) and for preservation of endangered animals(Wells et al., 1998), and its application is further being expanded to the areas of transgenic(Schnieke et al., 1997), knock-in(McCreath et al., 2000)

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and knock-out livestock(Lai et al., 2002). Although successful production of clones from somatic cells has been achieved in various species such as sheep(Wilmut et al., 1997), cattle(Cibelli et al., 1998), mice(Wakayama et al., 1998), goats(Baguisi et al., 1999), pigs(Onishi et al., 2000; Polejaeva et al., 2000), cats(Shin et al., 2002), rabbits(Chesne et al., 2002) and mule(Woods et al., 2003), till now its efficiency is very low in that only less than 1% of the reconstructed embryos give rise to live-born animals. Moreover, the nuclear transfer has raised severe developmental problems including high rate of abortion during early gestation and increased perinatal death(Wilmut et al., 1997; Hill et al., 1999; Hill et al., 2000; Heyman et al., 2002). It is uncertain

whether these developmental failures of cloned embryos are due to the incomplete nuclear reprogramming or 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 the nuclear transfer procedures is improperly performed, the production of cloned embryos or animals can be tackled. Although cloned animals have been successfully generated from many research groups worldwide, we still have limited information about nuclear reprogramming of cloned embryos. It is no doubt that a differentiated cell nucleus transferred into the cytoplasm of an enucleated oocyte should become reprogrammed to restore normal embryonic development. Telomere restoration of donor chromatin may be one factor affecting developmental ability 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 cell donor(Shiels et al., 1999). It was suggested that different donor cell types might lead to different telomere lengths in the resultant cloned animals (Miyashita et al., 2002). 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 and 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), demonstratingthat 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(Daniels et al., 2000) and in cloned mouse blastocysts or ES cells(Boiani et al., 2002), suggesting that aberrant transcription patterns detected in cloned embryos may lead to abnormalities at various embryonic stages. Abnormal methylation patterns were detected in various genomic regions of cloned bovine embryos and methylation level of donor cells was maintained throughout preimplantation development(Kang et al., 2001a). These results provide indirect evidence that aberrant

epigenetic reprogramming of donor genomic DNA may result in developmental failures of cloned embryos. Here, epigenetic reprogramming, or DNA methylation state of cloned embryos during early development is described in details.

VARIOUS ABNORMALITIES OF CLONED ANIMALS

A serious impediment to the practical use of somatic cell nuclear transfer technique is a low efficiency in the viability of cloned embryos during embryonic development; only a few percent of reconstructed oocytes develop to term, and of those, many die shortly after birth(Wilmut et al., 1997; Cibelli et al., 1998; Wakayama et al., 1998; Young et al., 1998). Various abnormalities include circulatory distress, placenta edema, hydrallantois, and chronic pulmonary hypertension(Hill et al., 2000). Even the surviving offsprings show large placentae(Hill et al., 1999; Ono et al., 2001; Chavatte-Palmer et al., 2002) 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), mouse(Wakayama et al., 1998) and sheep (Young et al., 1998), but 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* embryo culture procedures(Young et al., 1998; Sinclair et al., 2000; Eggan et al., 2001). 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).

EPIGENETIC REPROGRAMMING IN EARLY 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 me-

thylation state occur during gametogenesis and early embryonic development(Monk et al., 1987; Sanford et al., 1987). During early embryonic development, the overall methylation level sharply decreases and reaches to 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(Monk et al., 1987; Howlett & Reik, 1991; Oswald et al., 2000). This genome-wide demethylation process may be crucial 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 and most of the genomic DNAs are methylated at defined developmental time points. Another demethylation/remethylation event occurs during gametogenesis and is necessary for resetting of genomic imprinting(Solter, 1988). Therefore, dynamic epigenetic changes of the genomic DNAs appear to be essential and unique during embryonic development in mammals.

Overall view of global methylation was suggested at various stages of preimplantation development(Monk et al., 1987). A dramatic demethylation of the maternal and paternal genomes occurs in the early embryos after fertilization, especially from 8-cell to blastocyst stages. Although the biological significance of early embryonic demethylation remains unclear, this deme-thylation 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. In general, a genome-wide demethylation process appears to be unique during early embryonic development in mammals. In 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). In addition to the repeated sequences, our experiments also demonstrated that single-copy sequences behaved similarly to genomic repeated sequences. The promoter sequences of bovine tissue-specific genes clearly represented demethylation events in IVF-derived bovine embryos(Kang et al., 2002). In IVF-derived pig embryos, the PRE-1 SINE sequences were gradually demethylated during preimplantation development (Kang et al., 2001a). Therefore, 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.

ABERRANT EPIGENETIC REPROGRAMMING IN CLONED EMBRYOS

Successful cloning of animals by nuclear transfer 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 nuclei 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 chan-ges in DNA methylation, expression of imprinted genes, res-toration 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(Bourc'his et al., 2001; Kang et al., 2001a; Kang et al., 2001b; Kang et al., 2001c; Kang et al., 2002) and cloned animals(Shiels et al., 1999; Eggan et al., 2000; Tian et al., 2000; Eggan et al., 2001; Humpherys et al., 2001; Ohgane et al., 2001; Young et al., 2001).

In cloned bovine embryos, the epigenetic reprogramming mechanism represented by the methylation/demethylation process probably is not working well(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 embryos(Kang et al., 2001b). The methylation patterns of cloned embryos were quite different from those of normal embryos produced *in vitro* or *in vivo*, but closely resembled those of donor cells in the overall genomic methylation status.In contrast to the repetitive sequen-ces, normal methylation reprogramming was observed on GC-rich sequences of single-copy genes such as bovine epidermal cytokeratin gene, beta-lactoglobulin gene and galanin gene, giving a cue for the potential of cloned embryo to superintend the epigenetic states of foreign genome, even after global demethylation(Kang et al., 2002; Kang et al., 2003). Immuno-stainning results also showed differences in the dynamics of chromosome methylation between cloned and normal embryos at the preimplantation stages(Bourc'his et al., 2001). The centro-

meric 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 explain a problem of the present cloning technology, the low efficiency of which is still an impediment to the agricultural and biomedical applications.

Then, 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? Are all cloned embryos epigenetically incompetent to support full-term 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. In contrast to the cloned embryos, most of IVF-derived embryos(88%, 23/26) 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. Two cell types, serum-starved and growing-phase cells, have been generally 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 them. 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. Considering the nature of centromeric satellite I DNA such as high copy number and relatively stable epigenetic status, however, 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. In addition, we have demonstrated that epigenetic modification can take place differentially in donor genome of

cloned bovine embryos(Kang et al., 2002). In contrast to the genomic repeats, the promoter sequences of tissue-specific genes such as bovine epidermal cytokeratin gene and beta-lactoglobulin gene were completely demethylated at the blastocyst stage of cloned embryos. Another sigle-copy gene, bovine galanin gene, in the cloned embryos showed a normal methylation process during preimplantion development(Kang et al., 2003). These results indicate the possibility that single-copy genes that are important for embryonic development can be selectively demethylated in cloned embryos, like tissue-specific genes. If it is true, mammalian development is rather tolerant to epigenetic reprogramming as previously suggested by Humpherys et al. (2001). Our findings could be answered for the question how viable offsprings are still born from the unlikely cloned embryos carrying abnormally methylated genome.

The phenomenon of differential demethylation appears not only among different genomic sequences, but also between the different regions of cloned embryos at the blastocyst stage. Aberrant allocations of inner cell mass(ICM) cells and trophectoderm(TE) cells were observed in cloned bovine blastocysts(Koo et al., 2002), showing a higher proportion of ICM in cloned embryos as compared with normal embryos produced in vitro and in vivo. It then was postulated whether the methylation differences in cloned embryos might be derived from epigenetic difference between ICM and TE genomes. We observed in cloned blastocysts that the satellite sequences remain methylated in trophectoderm cells but not ICM cells, and that the cytokeratin gene sequence was demethylated equally in both TE and ICM regions(Kang et al., 2002). However, it is unknown that the unequal methylation between ICM and TE cells observed in cloned blastocysts is a general phenomenon that appears also in normal bovine embryos derived by fertilization. Although 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 for the two distinct lineage cells to manifest the potential ability to modify their epigenetic status differentially. More information is needed on other genomic sequences that remain methylated till the blastocyst, which can be very helpful for elucidating methylation differen-

ces between ICM and TE regions. However, such sequences appear rare in bovine because the genomic sequences examined in the previous studies all showed hypomethylation status at the blastocyst stage(Kang et al., 2001b). The result with bovine IVF-derived blastocysts is consistent with a previous observation that a low level of methylation of L1 sequences was detected uniformly across both lineage cells of the blastocyst in the mouse(Howlett & Reik, 1991). On the other hand, an early methylation study described a pattern of differential methylation in 6-day rabbit blastocysts where high levels of methylation were found in ICM cells as compared to TE cells(Manes & Menzel, 1981), which is opposite to the results of cloned bovine embryos. At present, it is difficult to make a decision about generality of the phenomenon of differential methylation between two different regions of the blastocyst because of a paucity of related studies. Although whether methylation status is maintained differentially between ICM and TE regions of the normal blastocyst is obscure, it has been well recognized that, after implantation, methylation level increases in fetus proper (primitive ectoderm lineage) whereas, in extraembryonic tissues derived from trophectoderm or primitive endoderm lineages, methylation levels remain low throughout gastrulation as if *de novo* methylation has not been activated in these cells(Chapman et al., 1984; Rossant et al., 1986).

Whether or not the methylation difference between ICM and TE cells is a unique feature of cloned embryos remains to be elucidated yet, but it is clear that TE lineage cells of the blastocyst clones with normal ICM/TE ratios are aberrantly methylated in the satellite sequences(Kang et al., 2002). TE cells do not remain as a simple epithelium after implantation, but perform several active functions such as invasion into the deciduas by synthesis of various proteases(Strickland & Richards, 1992), formation of primary trophoblastic giant cells carrying polytene chromosomes(Varmuza et al., 1988) and expressions of the TE-specific genes such as c-fms(Regenstreif & Rossant, 1989) and Mash-2(Guillemot et al., 1994). These various processes programmed by the TE cells may be essential to establish and maintain the stable placentation and thereby to support the growth of embryo proper. The functional integrity of these all processes can be guaranteed by the proper methylation status in the corresponding cells. In this line of connection, it is likely that the trophectoderm cells of cloned blastocysts become

strayed out of the developmental orbit since the observation of abnormal DNA methylation of the satellite sequences leads us to easily infer methylation aberrancy in other genomic regions including single-copy genes with important roles in normal development of cloned embryos. Abnormal methylation of unique gene sequences necessarily gives rise to misregulation of gene expressions, and the cumulative action of many abnormally expressed genes may affect subsequent viability of cloned embryos after implantation. Intuitively, placental dysfunction should be the most likely phenotype that could be explained by the epigenetic anomaly of TE cells. Deficient placentation is most frequently observed in dead fetal clones of various mammalian species, and also has been recognized as a potential cause of early fetal loss(Hill et al., 2000) and neonatal mortality(Wakayama et al., 1998) in cloned animals. Although our results did not present direct evidence for this hypothesis and thus appear to be more or less suggestive, it seems natural to believe the correlation of abnormal methylation in TE cells of cloned blastocysts with the placental defects observed in cloned fetuses/ animals.

CONCLUSIONS

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 the accumulated actions of many abnormally expressed genes in cloned embryos or fetuses can be detrimental to normal full-term development. However, the precise understanding for the epigenetic anomaly in the cloned embryos remains mainly elusive. The characterization of more parameters that affect the development of cloned embryos helps the current nuclear transfer technology to find its problems and to address what should be done to resolve these problems. It is reasonable that early cloned embryos are one of the most valuable materials for epigenetic reprogramming studies. Therefore, studies on epigenetic reprogramming at the preimplantation stage contribute to understand the molecular basis for develop-

ment of cloned embryos. To improve efficiency of the present cloning system, molecular mechanisms underlying the reprogramming of donor genome during early embryonic development should be understood.

ACKNOWLEDGEMENTS

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