O-7

Epigenetic Reprogramming in Cloned Embryos

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Introduction

During early development, a dramatic reduction in methylation levels occurs in mouse (Monk *et al.*, 1987). 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, Sanford *et al.*, 1984). This genome-wide demethylation process may be a prerequisite for the formation of pluripotent stem cells that are important for the later development (Reik & Surani 1997). 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 takes place during gametogenesis and is necessary for resetting of genomic imprinting (Solter 1988). The dynamic epigenetic reprogramming events appear to be basic and are probably conserved in eutherian mammals (see below).

The cloning of mammals by nuclear transfer also 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 assume an expression program typical of a zygotic genome. Epigenetic reprogramming processes after somatic cell nuclear transfer include remodeling of chromatin structure, global changes in DNA methylation, adjustment of telomere length and X chromosome inactivation. Extensive studies on epigenetic reprogramming of the donor genome have been done in mammalian species (Eggan *et al.*, 2000, Eggan *et al.*, 2001, Humpherys *et al.*, 2001, Kang *et al.*, 2001a, Kang *et al.*, 2001b, Kang *et al.*, 2001c, Shiels *et al.*, 1999, Tian *et al.*, 2000). In this review, I will focus on aspects of epigenetic gene regulation and DNA methylation that are pertinent to our understanding of the reprogramming process.

Various Phenotypes of Nuclear Clones

A serious impediment to the practical use of the nuclear transfer (NT) procedure is the low survival rate of cloned animals; only, or less than, few % of reconstituted NT embryos survive to adulthood, 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 disease phenotypes have been reported including circulatory distress, placental edema, hydrallantois, and respiratory problems (Hill *et al.*, 1999, Hill *et al.*, 2000). Even the surviving offsprings show increased placental (Hill *et al.*, 1999, Ono *et al.*, 2001, Wakayama *et al.*, 1998) and birth weights (Eggan *et al.*, 2001), often referred to as 'large offspring syndrome', and those with seemingly healthy appearance may suffer from immune dysfunction or kidney/brain malformation which contribute to their death at later stages (Lanza *et al.*, 2000, McCreath *et al.*, 2000).

The large offspring syndrome (LOS) is a typical phenotype observed in cloned neonates of mammalian species such as cow, mouse and sheep (Cibelli *et al.*, 1998, Wakayama *et al.*, 1998, Wells *et al.*, 1997, Young *et al.*, 1998), but the factors responsible for LOS remains elusive. This phenomenon is not unique to cloned neonates. A variety of *in vitro* embryo culture procedures have been associated with LOS (Eggan *et al.*, 2001, Sinclair *et al.*, 2000, Young *et al.*, 1998). Epigenetic alteration of the imprinted Igf2r region and the resultant abnormal expression of the corresponding gene have been observed in sheep LOS fetuses derived by in vitro culture procedure (Young *et al.*, 2001).

Epigenetic Studies with Cloned Preimplantation Embryos

1. Demethylation process in early embryos derived by fertilization

Early work with mouse embryos by Monk *et al.* (Monk *et al.*, 1987) provided an overall view of the extent of global methylation at various stages of preimplantation development. It showed that extensive demethylation of the genome takes place in the early embryos between the 8-cell and blastocyst stages. The biological significance of this early embryonic demethylation is unknown, but this process appears to be essential to provide a mechanism for removing differences in gamete-specific methylation patterns and to reformat the genome prior to the initiation of the normal program of embryonic development (Jost & Saluz 1993).

The phenomenon of a genome-wide demethylation appears not limited to mouse. Our laboratory observed similar process in preimplantation bovine and pig embryos. In bovine, the *Bov-B* LINE sequences exhibited a gradual demethylation pattern during early development, like the *LINE-1* sequences in mice (Kang *et al.*, 2001b). In pig, the *PRE-1* SINE sequences also showed a demethylation pattern in preimplantation embryos (Kang *et al.*, 2001c). In addition to these repeated sequences, other genomic repeats that have been examined till now all showed undermethylation status at the blastocyst stage as if a global demethylation process has taken place in these species during cleavage stage. Single-copy sequences behave similarly to genomic

repeated sequences. The promoter sequences of bovine tissue-specific genes clearly showed demethylation events during preimplantation development (unpublished data). In pig preimplantation embryos produced *in vitro*, the *PRE-1* SINE sequences were shown to be gradually demethylated in accordance with the developmental stage. Therefore, it is highly likely that the genome-wide demethylation process is a cross-species phenomenon in mammals, which gives weight to the importance of this process in early mammalian development.

2. Poor epigenetic reprogramming in early embryos derived by nuclear transfer

The cloned donor genome, like the embryonic genome, should ride on a wave of demethylation during early cleavage stage and thus peel off its own differentiation tags encrusting the genome. The first study on epigenetic reprogramming was performed with the subject of X inactivation in mouse NT embryos/fetuses by Eggan *et al.* (Eggan *et al.*, 2000). They used an ES cell with X-linked reporter transgene (X^{GFP}) the expression of which is regulated by X inactivation, and monitored the expression in either blastocyst-stage or mid-gestation NT embryos. As in normal embryos where selective inactivation of paternal X occurs in extraembryonic trophectoderm, non-random and random X inactivation was observed in trophectoderm and epiblast regions, respectively, of NT embryos. The results indicate that the epigenetic marks that distinguish activated X and inactivated X in somatic cells can be removed and reestablished on either X in epiblast cells during the cloning process, which provided evidence for the occurrence of epigenetic reprogramming in NT embryos.

In spite of these encouraging results with mouse NT embryos, the reprogramming process does not appear to operate efficiently in other epigenetic aspects, as observed from the analyses of bovine preimplantation NT embryos for methylation status (Kang *et al.*, 2001a, Kang *et al.*, 2001b, Kang *et al.*, 2001c). Our laboratory found using bisulfite-sequencing technology that various genomic repeated sequences (satellite Ⅰ, satellite Ⅱ, 18S *rDNA* and *art-2* SINE sequences) showed aberrant methylation status in NT blastocysts the patterns of which closely resembled donor cells in the overall genomic methylation status but were quite different from normal blastocysts produced *in vitro* or *in vivo*. Demethylation 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 (Kang *et al.*, 2001b). These observations of abnormal methylation status in the genome of NT embryos were enough to raise serious skepticism about the future practical applications of cloning to agriculture and other biomedical areas.

Then, what happens in viable offsprings that are still born from the unlikely NT embryos having highly methylated repeated genomic regions? Is mammalian development actually rather tolerant to epigenetic aberrations of the donor genome? Are NT embryos all epigenetically incompetent to support full-term development of themselves? Some clues on these questions were derived by individual analyses of bovine NT embryos for methylation (Kang *et al.*, 2001b). Unexpected results were obtained from these analyses; the degree of methylation status of the

satellite sequences was greatly varied in individual NT embryos and, among the different NT blastocysts, only 25-30% was relatively undermethylated, although their methylation levels were still higher than the mean methylation value of control embryos. The stage-matched control embryos derived in vitro also exhibited varied methylation status but the proportion (around or less than 10%) showing abnormality was much lower than NT blastocysts. Methylation states of donor cell populations (starved cells vs. growing-phase cells) were also characterized but they exhibited no methylation variations within populations and also between cell populations. Although the possibility that individual variation of methylation shown in NT embryos depends upon innate methylation difference among different donor cells cannot be excluded, but considering the nature of centromeric satellite I DNA such as high copy number and the relatively stable epigenetic status, it is more likely that methylation variation in individual NT embryos is determined largely by different abilities of the reconstituted embryos to modify the epigenetic status of donor genome.

3. Epigenetic reprogramming in pig NT embryos

The results of methylation study with pig NT embryos (Kang *et al.*, 2001c) appears to well tune with the results obtained from the studies with normal mouse embryos, but were different from the results seen in NT bovine embryos. Both the satellite and the *PRE-1* SINE sequences exhibited a gradual demethylation pattern in a similar fashion to endogenous demethylation in cleavage-stage pig NT embryos. These species-specific different patterns of demethylation suggest that, when moved into recipient oocyte, the initial hypermethylation should be confronted with either of two contrasting fates of epigenetic modifications, being maintained by a maintenance methylation activity through successive cycles of replication, or being modified by demethylation. This may be determined by competition between unknown oocyte-specific factors and donor cell-specific factors transferred into the enucleated ooplasm that can block the ability of oocyte-specific factors to reprogram the nucleus. The former of maintenance methylation was observed in the studies with cloned bovine embryos where hypermethylation was observed to be maintained up to the blastocyst (Kang, Kang *et al.*, 2001b). The latter case of demethylation was shown in this cloned pig embryo. Likewise, the *PRE-1* SINE sequences in cloned pig embryo were gradually demethylated whereas the *art-2* SINE sequences were observed remained largely methylated in cloned bovine embryos.

It is uncertain why such a discrepancy in capability of demethylating donor genome arises between the two different mammalian species. It could be interpreted as that demethylation takes place much more efficiently in pig clones than in bovine ones. However, this simple explanation appears to conflict with the observed cloning efficiencies seen empirically in these two mammalian species since the production of cloned offsprings in cattle is now a routine work but, in pig, many cloning attempts have met with little successes until recently (Onishi *et al.*, 2000, Polejaeva *et al.*, 2000). Therefore, these discordant observations suggest that efficient demethylation of donor genome alone cannot warrant the successful development of cloned

embryos, and that there exist other important elements to be considered for successful cloning (Cross 2001, Prather 2000, Young *et al.*, 2001).

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 entails misregulation of gene expression at multiple loci, and the accumulated action of many abnormally expressed genes in cloned fetuses can disrupt normal full-term development of clones. The reason and the curative means for the epigenetic anomaly observed inthe cloned embryos are currently completely unknown. The fact we truly know is that the nuclear transfer is an incomplete technology to support efficient production of cloned animals. Till the advent of an advanced cloning technology, there is no way but studies the molecular events that control the reprogramming mechanisms in preimplantation embryos that affect epigenetic modifications and genomic function of cloned embryos.

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