—Mini Review—

Animal Cloning: Reprogramming the Donor Genome

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Introduction

Nuclear transfer (NT) involves transferring the nucleus from a diploid cell to an unfertilized egg from which the maternal nucleus has been removed. The NT technique involves several steps. The nucleus itself can be injected or the intact cell can be transferred into the oocyte. In the latter case, the oocyte and donor cell are normally fused and the reconstructed embryo activated by an electrical pulse. The reconstructed embryos are then cultured and those that appear to be developing normally are implanted into foster mothers. The NT technique was first used to clone sheep [1, 2] and cattle [3] by using cells taken directly from early embryos. In 1995, Campbell et al. [4] produced live lambs from embryo derived cells from a ‘differentiated’ cell line that had been cultured for several weeks. In 1996, Wilmut et al. [5] created Dolly, the first animal cloned from a cell taken from an adult animal. Since then, although somatic cloned animals have been produced in several species [6–28], success rates remain low in all species, with published data showing that only 1% to 5% of reconstructed embryos result in live births (see Roslin Institute web site [www.roslin.ac.uk/public/cloning.html]). Many cloned offspring die late in pregnancy or soon after birth [8, 29, 30], often from respiratory or cardiovascular dysfunction [31–33]. Abnormal placental development [34–41] is common and this is probably the major cause of fetal loss earlier in pregnancy [42]. Many of the cloned cattle and sheep that are born are much larger [29, 30, 43, 44] than normal and apparently normal clones may carry unrecognized abnormalities [45].

Differences Between Embryonic and Somatic Clones

Oocyte cytoplasmic factor(s) can reprogram either embryonic or somatic cell genome during cloning. It is clear from cloning experiments that an embryonic/somatic nucleus can be reprogrammed in the enucleated unfertilized egg and can form the entire body of an animal. When an embryonic cell (blastomere) is used as a donor nucleus source, either activated or unactivated ooplasm receiving a donor nucleus can develop to term as a result of nuclear transfer [46, 47]. But when a somatic nucleus is transferred into activated ooplasm, the reconstructed egg is arrested during several cell divisions, although unactivated ooplasm receiving a somatic nucleus can develop to term [29, 48]. It is suggested that an oocyte plasmic factor(s), which is essential for reprogramming the somatic genome, but not for an embryonic genome, exists in an MII oocyte, and the factor(s) or its activity dissipates several hours after oocyte activation.

The conception rate of somatic NT embryos is not much lower than that of embryonic NT embryos, but a greater number of fetal losses and abortions occur during the gestation of somatic clones than of embryonic clones [44]. Neonatal death and large offspring are also common in somatic clone calves, therefore reprogramming of the genome in somatic NT embryos is insufficient for fetal and placental development or neonatal maturation during gestation. Somatic NT embryos can develop to the blastocyst stage and the developed blastocysts can be successfully implanted. But incomplete or inappropriate reprogramming leads to dysregulation of gene expression and phenotypes [32, 36, 49–55]. Even if it is hypothesized that ‘difficulty’ in reprogramming the somatic nucleus is closely related to totipotency that dissipates with the cell differentiation process, no clear
proof has been established.

Epigenetic Modification of DNA in Cloned Embryo

Epigenetic modification of DNA by methylation in mammals occurs predominantly at CpG dinucleotides and is involved in a number of key genome functions. These include roles in imprinting, X chromosome inactivation, genome stability, silencing of endogenous retrovirus, inactivation of genes related to cancers, and embryonic development [56]. Normal embryo development is dependent on the methylation state of the DNA contributed by the sperm and egg and on the appropriate reconfiguration of the chromatin structure after fertilization.

Genome-wide reprogramming of DNA methylation occurs in two steps: the first during germline development, when DNA methylation imprints are erased [57, 58], and the second during fertilization and preimplantation development [59–61]. Fertilization of sperm and oocyte achieve paternal genome introduction into the oocyte, where the oocyte cytoplasm quickly replaces protamines in sperm chromatin with oocyte histones, and active demethylation of paternal DNA (not of maternal DNA, asymmetric methylation) occurs before the onset of the first DNA replication [62]. Active demethylation is displayed in most mammalian species (mouse, rat, pig, and cattle) [63], with the exception of sheep [33, 64] and rabbits [65]. Passive demethylation of the maternal genome is observed in preimplantation embryos [60, 61]. Genome-wide de novo methylation (remethylation) occurs at the blastocyst stage in mouse (at 8- to 16-cell stage in cattle), preferentially in the inner cell mass [61, 63]. In marked contrast to the mouse, male pronuclear demethylation does not occur in sheep and rabbit embryos [64, 65].

In somatic cloning, however, the donor cell genome is compelled to bypass gametogenesis. Somatic cells have a very different chromatin structure from that of sperm and ‘reprogramming’ of the transferred nuclei must occur in the reconstructed embryos in order to achieve totipotency. Nevertheless the extent to which epigenetic reprogramming is conserved in cloned embryo development is unknown, and inefficient reprogramming of DNA methylation patterns may be partly responsible for the low birth rates and developmental abnormalities that often result from nuclear transfer [66]. As well as investigating the conservation of methylation reprogramming events in early mammalian development, Dean et al. [63] analyzed demethylation events in cloned bovine embryos. Interestingly, the initial demethylation event appears to be conserved in cloned 1 cell embryos, with some loss of methylation in the somatic donor nucleus occurring shortly after fusion with the enucleated oocyte. Further demethylation is observed at the 2 cell stage, but spurious de novo methylation is found in many 4–8 cell stage embryos. The observations of Dean et al. [63] show that methylation reprogramming in somatic nuclei is partially conserved in cloned embryos, with different extents of inappropriate methylation in individual embryos. But more detailed information on which sequences are aberrantly methylated in cloned embryos is required to understand the observed phenotypes and low success rates of cloning [67]. Another study reports a change in nuclear methylation in early stage somatic NT embryos. With the 5-mC antibody, Bourc’his et al. [68] reported that bovine cloned embryos fail to reproduce distinguishable paternal-chromosome methylation after fusion and to maintain their somatic pattern during subsequent stages. They were able to observe the methylation status of euchromatic and heterochromatic sequences in IVF and cloned bovine preimplantation embryos [68]. Compared to their normal counterparts, cloned bovine blastocysts show increased levels of methylation in centromeric heterochromatin, but comparable levels in euchromatin. In agreement with Dean et al. [69], this study found inefficient passive demethylation during the initial embryonic cell divisions. In contrast however, Bourchis et al. [68] could find no evidence for the active demethylation of somatic nuclei after fusion. This seems to conflict with the two previous reports; the former report shows that active demethylation occurs in somatic NT embryos, but not in the latter report, although the same anti-5-mC antibody is used in both studies. In fact, differences may result from the immunostaining procedure, intensity comparison and/or microscopic resolution [70, 71]. Passive demethylation occurs during the cleavage stage in normal embryos, leading to global hypomethylation until implantation. After passive demethylation, de novo DNA methylation occurs in normal blastocysts [61]. Bisulphite mutagenesis of bovine embryos shows that heterochromatic Bov-B LINE (long interspersed repeat sequence) and unique sequences in epidermal cytokeratin and beta-lactoglobulin are normally demethylated and remain that way until the blastocyst stage [72, 73]. In bovine cloned blastocysts, the Bov-B LINE and satellite I sequence—a major sequence
consisting of centrometric heterochromatin—are abnormally methylated. In addition, individual variation in the DNA methylation pattern is observed among different cloned blastocysts [72]. The same method demonstrates that differential demethylation events take place between tissue-specific gene (cytokeratin and beta-lactoglobulin) sequences and the satellite I DNA sequences, and also inner cell mass and trophoectoderm in cloned blastocysts [73]. Surprisingly, cloned porcine embryos do not appear to show highly aberrant methylation patterns, with cloned blastocysts retaining similar methylation levels to their normal counterparts in centromeric satellite and PRE-1 SINE sequences [74]. Overall, these studies present a picture of partial, but incomplete methylation reprogramming in cloned embryos. The details of the reprogramming failure differ among the three studies. The reasons for this are not entirely clear, but may lie in the methods—donor cell type, evidence of serum starvation, the oocyte source, and embryo culture conditions [75]—used in the different studies [67].

Santos et al. [76] examined histon H3 lysin 9 (H3-K9) methylation and acetylation, because links between DNA methylation and H3-K9 methylation have been established in other organisms [77–83]. They found that the majority of cloned embryos exhibit H3-K9 hypermethylation and hyperacetylation associated with DNA hypermethylation, although H3-K9 methylation and acetylation are reprogrammed in parallel with DNA methylation in normal bovine embryos [76].

**Can Cloning Efficiency Be Improved?**

Aberrant epigenetic profiles in NT embryos and cloned fetuses/offspring [84, 85] suggest that most somatic nuclei fail to be reprogrammed [70, 86]. It is clear that gene expression patterns and phenotypes can be disrupted in both somatic and extra-embryonic tissues of cloned animals [36, 87–92]. At present, to improve the success rate of normal offspring production, the only way to assess the ‘quality’ of embryos is to view them under a microscope and it is clear that the majority of embryos classified as ‘normal’ do not develop properly after implantation. If only ‘healthy’ embryos are selected from a number of junk embryos based on gene expression profiles etc., it may be a great tool to improve cloned animal production. A substantial effort is being made to use technological advances in genomics to screen the expression patterns of genes to identify differences between the development of cloned embryos, fetus, and offspring and those produced by *in vivo* or *in vitro* fertilization [39, 55, 93, 94]. Several studies with mouse clones show abnormal expression of Oct4 in the blastocyst [89], and imprinted genes and other non-imprinted genes in later stages [32, 36, 49–55]. Boiani et al. [95] reported that aggregated mouse cloned embryos express Oct4 normally and have higher rates of fetal and postnatal development. They propose that the regulation of gene expression and, possibly, reprogramming of nuclei in clones has a non-cell-autonomous component that can be complemented by clone-clone embryo aggregation [95].

Another possibility is to identify systematic ways of improving reprogramming in cloned embryos. Sullivan et al. [96] have developed a novel system for remodeling mammalian somatic nuclei *in vitro* prior to cloning. The system involves permeabilization of the donor cells and treatment in a mitotic cell extract to condense somatic nuclei. The condensed chromosomes are transferred into enucleated oocytes prior to activation. Cloned calves have been produced by this new method [96]. Santos et al. [76] reported that the precise epigenotype in cloned embryos depends on the donor cell type, and the proportion of embryos with normal epigenotypes correlates with the proportion of embryos developing to the blastocyst stage. This suggests that it may be possible through characterization of a donor population and the assessment of NT embryos at the blastocyst stage to begin to test manipulations of donor cells that in turn could improve cloning efficiency [76]. As a manipulation of donor cell epigenotype, pre-treatment of donor cells with trichostain A (TSA, a histone deacetylase inhibitor) or 5-aza-2’-deoxycytidine (5-aza-dC, a DNA methyltransferase inhibitor) was examined [97]. Adult fibroblast cells treated with TSA have elevated histone H3 acetylation compared with untreated controls. Cells treated with 5-aza-dC have decreased methylation. Development to blastocysts for embryos cloned from donor cells after 5-aza-dC treatment is lower than in untreated controls, whereas TSA treatment of donor cells increased blastocyst development compared to controls. The results indicate that partial erasure of preexisting epigenetic marks of donor cells improves subsequent *in vitro* development of cloned embryos [97].

**Outlook for Applicability of Animal Cloning**

Some of the more utilitarian opportunities provided by animal cloning are the ability to expand populations of
valuable, high-quality or endangered animals [19, 98]. The other opportunities provided by clone technology combined with gene engineering are production of transgenic animals producing valuable recombinant proteins [31, 99] and animals for xenotransplantation [100–103]. Whether it is feasible to produce large numbers of clones, all of which express the same desirable phenotype (performance) as the nuclear donor, remains to be determined [104]. Cloning results to date reveal that, while many cloned offspring appear outwardly normal and healthy [105, 106], in many cases cloned animals suffer from epigenetic abnormalities [45], which may affect the degree to which desirable characteristics are obtained. Also, epigenetic abnormalities may affect the risk of endogenous retroviruses [107], carcinogenesis, and the instability of genomes or transgenes in cloned animals. It has been seen that cloning failures are ‘epigenetic’ in the mouse. The obese phenotype frequently observed in cloned mice is not transmitted to offspring generated by mating male clone and female clone mice [108], indicating that failures associated with cloning are epigenetic and not inheritable. This is, in fact, ‘good news’ to use progenies from clones for genetic applications.

Some questions persist: Why is cloning inefficient? How can cloning efficiency be improved? What is genome reprogramming? These are all important questions that must be addressed with NT technology, approaching valuable applications and developmental biology.

References

20) Zou, X., Chen, Y., Wang, Y., Luo, J., Zhang, Q., Zhang,


44) Heyman, Y., Chavatte-Palmer, P., LeBourhis, D., Camous,


97) Enright, B.P., Kubota, C., Yang, X. and Tian, X.C. (2003): Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin a or 5-aza-


