MINIREVIEW

Nuclear Remodeling and Reprogramming in Transgenic Pig Production

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The manufacture of pigs with modifications to specific chromosomal regions requires that the modification first be made in somatic cells. The modified cells can then be used as donors for nuclear transfer (NT) in an attempt to clone that cell into a newborn animal. Unfortunately the procedures are inefficient and sometimes lead to animals that are abnormal. The cause of these abnormalities is likely established during the first cell cycle after the NT. Either the donor cell was abnormal or the oocyte cytoplasm was unable to adequately remodel the donor nucleus such that it was structured similar to the pronucleus of a zygote. A better understanding of chromatin remodeling and subsequent developmental gene expression will provide clues as to how procedures can be modified to generate fertile animals more efficiently. Exp Biol Med 229:1120–1126, 2004

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Mice have traditionally served as model mammals in studies of human physiology. Although they have been useful in providing a basic understanding of genetics and physiology, their use as a model for humans has many limitations. In some cases the mouse is simply not suited for the condition to be studied. Cystic fibrosis in humans is caused by a mutation in the gene for cystic fibrosis transmembrane conductance regulator. When this gene is similarly mutated in mice, it does not cause the same airway phenotype as in humans. In other cases the mouse is simply too small for researchers to take certain measurements (e.g., measuring blood flow in cardiovascular studies). Because of their size and differences in physiology, genetically modified pigs may provide a system to better model the human condition. In addition, genetic modification may help improve production agriculture (1).

Pronuclear DNA microinjection has long been the most reliable method to produce transgenic pigs (2). Despite the ease with which transgenic animals can be generated, this technique has limitations. The DNA integrates randomly and potentially in multiple copies. In addition, the random sites of integration limit the ability to control expression in the desired tissues or at the appropriate level. Moreover, the animal's endogenous genes cannot be specifically altered by this technique. Similarly, other methods of transgenic pig production (3, 4) also result in random integration of the transgene.

Successful nuclear transfer (NT) of cultured cells, first demonstrated in cattle (5), has provided an alternative for obtaining genetically modified pigs. McCreath *et al.* (6) first demonstrated in sheep the capability to selectively target specific genes in donor cells before NT. Although NT in pigs once lagged behind that in mice, cattle, and sheep, the first piglet from somatic cell NT (SCNT) was reported in 2000 (7). Since then, tremendous progress has been made. Successful production of pigs resulting from random genetic modification *in vitro* followed by NT (8–10), as well as those with a specific modification (i.e., gene ablation), has been reported by several groups in a short period (11–14). Despite its low efficiency, the production of cloned transgenic pigs has now transitioned from investigation to practical application. Indeed, many useful swine models that

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will benefit human health as well as production agriculture are expected to be established in the near future.

The developmental program that occurs during embryogenesis requires stage-specific gene expression. Because gene expression (RNA synthesis) is regulated by chromatin structure, the structure of the chromatin during the first cell cycle after NT is integral for correct gene expression. In this review the changes that occur to the structure of the nucleus as a result of NT will be referred to as nuclear remodeling, and the ensuing changes in gene expression will be referred to as nuclear reprogramming.

Nuclear Remodeling and Reprogramming

Swine embryos have served as an important model for many studies describing the nuclear remodeling that occurs after NT. The premise is that if the donor nucleus is to be successfully reprogrammed, it first must be remodeled to a state similar to that of a pronucleus.

At the chromosomal level, telomere length can be evaluated. Telomere length is maintained by telomerase, and the length of the telomere generally decreases as the cells divide. As the telomere shortens, the chromosome becomes unstable and degenerates and the cells senesce. Telomeres shorten through gestation as well as after birth and maturation of the individual. After cells are isolated for NT they are generally grown for a number of cell divisions before cloning. This additional culture may further erode the length of the telomere. Although the length of the sheep telomere may not be completely reset after NT (15), telomere lengths are reset in both cattle (16–18) and pigs (19) and appear to be a result of telomere elongation during embryogenesis (18).

At the level of transcription, apart from minor transcription of ribosomal genes, pronuclei are relatively transcriptionally inactive. At a species-specific cell stage the embryo begins producing significant amounts of mRNA. In the pig this transition occurs during the four-cell stage (20). This cell stage is correlated with a number of morphological changes to the nuclei. Thus, the initial discussion here will focus on morphological changes after NT.

The most obvious difference in morphology between a pronucleus and a nuclei from other cell types is the size of the pronucleus. In the fertilized egg the pronuclei are large, larger than the nuclei of most somatic cells. During cleavage the size of the nucleus diminishes as the size of the cell diminishes. When nuclei from 8- and 16-cell stage embryos are transferred to enucleated (and subsequently activated) meiotic metaphase II oocytes, the diameters of the nuclei swell from 13 or 14 μm to 27 μm (21). Other studies have clearly shown that this swelling is an important aspect of the remodeling process because it indirectly reflects an exchange of proteins that occurs when the donor nucleus is transferred into the cytoplasm of the oocyte.

One specific class of proteins that is known to change in composition during early cleavage is the nuclear lamins; consequently, these proteins can be used to illustrate this exchange. In mammals there are at least two families of these proteins, broadly categorized into nuclear lamins B and A/C. Lamins A and C are identical, except for an 82 amino acid tail on lamin A. The nuclear lamins are intermediate filament-type proteins that polymerize into a sphere surrounding the chromatin within the nuclear envelope and are thought to regulate the overall three-dimensional architecture of the nucleus. These proteins depolymerize during late prophase and are thus depolymerized in the oocyte. Upon fertilization these cytoplasmic lamins are recruited into the newly forming pronuclei. The composition of the nuclear lamins changes after the species-specific transition to embryonic control of transcription, and NT results in the normal species-specific nuclear lamin composition in the resulting pronucleus (22, 23).

Another morphological change that occurs is in the structure of the nucleoli. The nucleoli are the sites of rRNA synthesis. Studies using electron microscopy of sections through the nuclei have determined that during the pronuclear stage the nucleoli (also called nucleolus precursor bodies at this stage of development) have a tight compact appearance with few reticulations and a smooth surface. In contrast, a cell undergoing active rRNA synthesis at and beyond the four-cell stage in the pig has nucleoli with a reticulated or vacuolated appearance and a very rough or granular periphery. When nuclei with nucleoli that have an "active" nucleolar morphology are transferred to an enucleated oocyte and subsequently activated, the nucleolar morphology is reversed to be similar to the nucleoli in pronuclei (i.e., compact, agranular, and without reticulations or vacuoles) (24, 25). Such observations indirectly indicate that rRNA synthesis has ceased. Similar to the observations made for nuclear lamins, this modification is not complete if the oocyte is fertilized or preactivated before NT (26).

A third method for monitoring the degree of remodeling is to evaluate another component of the nucleus that indirectly indicates the activity of RNA synthesis: the small nuclear ribonuclear proteins (snRNPs). The snRNPs are responsible for the processing of pre-mRNA before it leaves the nucleus and enters the cytoplasm. The B and D core protein of the snRNP can be identified by a monoclonal antibody, Y12. This antibody does not localize to the nucleus between germinal vesicle breakdown and the late four-cell stage in the pig (27). The appearance of the Y12 epitope during the four-cell stage is sensitive to α-amanitin, an inhibitor of transcription preventing the neosynthesis of mRNA. When nuclei that have the Y12 epitope readily detectable are transferred to an enucleated oocyte and the oocyte is activated, the nuclei lose their reactivity to the Y12 epitope (27). This is an indirect indication that mRNA processing has ceased in response to the absence of mRNA and hence little mRNA synthesis. Thus, as was the situation with nuclear size, nuclear lamin composition, and nucleolar morphology, the transferred nucleus exhibits reactivity to the Y12 antibody that is similar to that of a normal pronucleus.

A final method of evaluating RNA synthesis is to look at the incorporation of ^{3}H-uridine in cells before and after NT. Little RNA synthesis is in the pronuclear-stage embryo, but considerable ^{3}H incorporation occurs beyond the 8-cell stage, which is reflective of significant RNA synthesis. Therefore, if a nucleus is really remodeled and reprogrammed after transfer to an oocyte, there should be no significant incorporation of ^{3}H-uridine into the pronuclei. Hyttel *et al.* (28) performed such experiments on *in vitro* matured oocytes and embryos and found that, although there was a significant decrease in ^{3}H-uridine incorporation after NT, some incorporation was still present. This observation suggested that complete remodeling and reprogramming often does not occur after NT.

An extension of the above experiments is to look at the expression of specific genes rather than nuclear morphology and classes of RNA. Winger *et al.* (29) evaluated messages for a number of key regulatory enzymes in NT-derived bovine embryos. They found that lactate dehydrogenase, citrate synthase, and phosphofructokinase were all correctly reprogrammed. Park *et al.* (30) found that only half of transferred nuclei were reprogrammed, as measured by expression of an integrated enhanced green fluorescent protein (EGFP). Other studies with transgenic ear-derived fibroblasts revealed that EGFP expression in NT embryos was often mosaic (31), suggesting that reprogramming in all blastomeres was not uniform. Because no other studies in the pig specifically address the issue of nuclear reprogramming, examples from another farm species, the cow, will be used. In an initial study, DeSousa *et al.* (32) showed by differential display that 95% of the transcripts in NT blastocysts were similar to the control *in vitro* produced embryos. However, that also means that 5% of the transcripts were different. Apparently, Daniels *et al.* (33, 34) have identified some of the transcripts representing this 5% population. They show that IL6, FGF4, and FGFr2 are not expressed correctly after NT. Similarly, NT-derived mouse embryos exhibited aberrant expression in about 4% of the transcriptome as determined via microarray analysis (35) and included such important regulators of development as Oct4 (36). However, it should be remembered that these measurements were taken in only the embryos that developed to the blastocyst stage. Thus, although much remodeling occurs normally after NT, clearly in some cases it is not complete.

Proteasomal Involvement in Remodeling

Some of the remodeling that occurs during the first cell cycle after NT may be mediated by the proteasomes (37). The 26S proteasome, a multisubunit holoenzyme, specifically degrades proteins that are modified postranslationally by covalent ligation of a multiubiquitin chain. Our study and other recent studies (38, 39) document the accumulation of proteasomes in the porcine zygotic male and female pronuclei (Fig. 1A), suggesting a possible role in pronuclear development after natural fertilization. It has been shown in the mouse that within 60 mins of NT and oocyte activation the somatic cell-type histones are replaced by oocyte-derived histones (40, 41). Then, again at the two- to four-cell stage, the oocyte-derived histones are replaced by embryonic-derived somatic histones. Because histones are known ubiquitin substrates (42) and ubiquitination targets proteins for proteolytic degradation via the proteasome, histone replacement may be mediated by proteasome-dependent proteolytic degradation.

Interestingly, the first success of cloning rats was by using MG132, a fully reversible, highly specific proteasomal inhibitor (43). The purpose of MG132 treatment was to block spontaneous resumption of meiosis during the recovery of the oocytes before NT, as the inhibition of proteasomal activity prevents metaphase-anaphase transition during meiotic cell cycle. Although cell-cycle progression can also be blocked by using protein kinase inhibitors, the inhibitors do not yield improved development. It is thus possible that the treatment with MG132 may have beneficial effects other than simple prevention of premature, spontaneous oocyte activation before SCNT (37). Our new data indicate that a continuous exposure to MG132 during the first 20–24 hrs after SCNT prevents donor cell nuclear remodeling (36; Fig. 1B–E′). In contrast, a brief pulse with MG132 in the first 2 hrs after SCNT may result in improved embryo development and blastocyst quality (44) and may lower the rates of embryo fragmentation and apoptosis after NT (45). We now have produced a pig as a result of such a transient MG132 treatment; therefore, these treatments are compatible with development.[2] It is plausible that transient exposure to MG132 could exert protective effects over reconstructed embryos by inducing overexpression of proteasomal subunits and cell-protective heat-shock proteins, as observed in cardiomyocytes (46). An alternative explanation is that an MG132 pulse extends the period during which donor cell nucleus is devoid of nuclear envelope. The nuclear envelope is an interphase structure not sustained by meiotic ooplasm during early stages of zygotic development before completion of oocyte meiosis. If this meiotic period is extended by a transient exposure to MG132, it may facilitate the access of ooplasmic remodeling factors to the donor cell chromatin. Interestingly, paternal pronuclear demethylation (see the next section on DNA methylation) is intricately linked with pronuclear formation (47). In summary, our data (as well as results from other laboratories) indicate that ubiquitin-controlled protein turnover and substrate-specific proteasomal degradation are required for proper remodeling of the donor cell nucleus after NT.

Although significant remodeling occurs normally after NT, clearly it is not complete as evidenced by the lack of

[2] Unpublished observations.

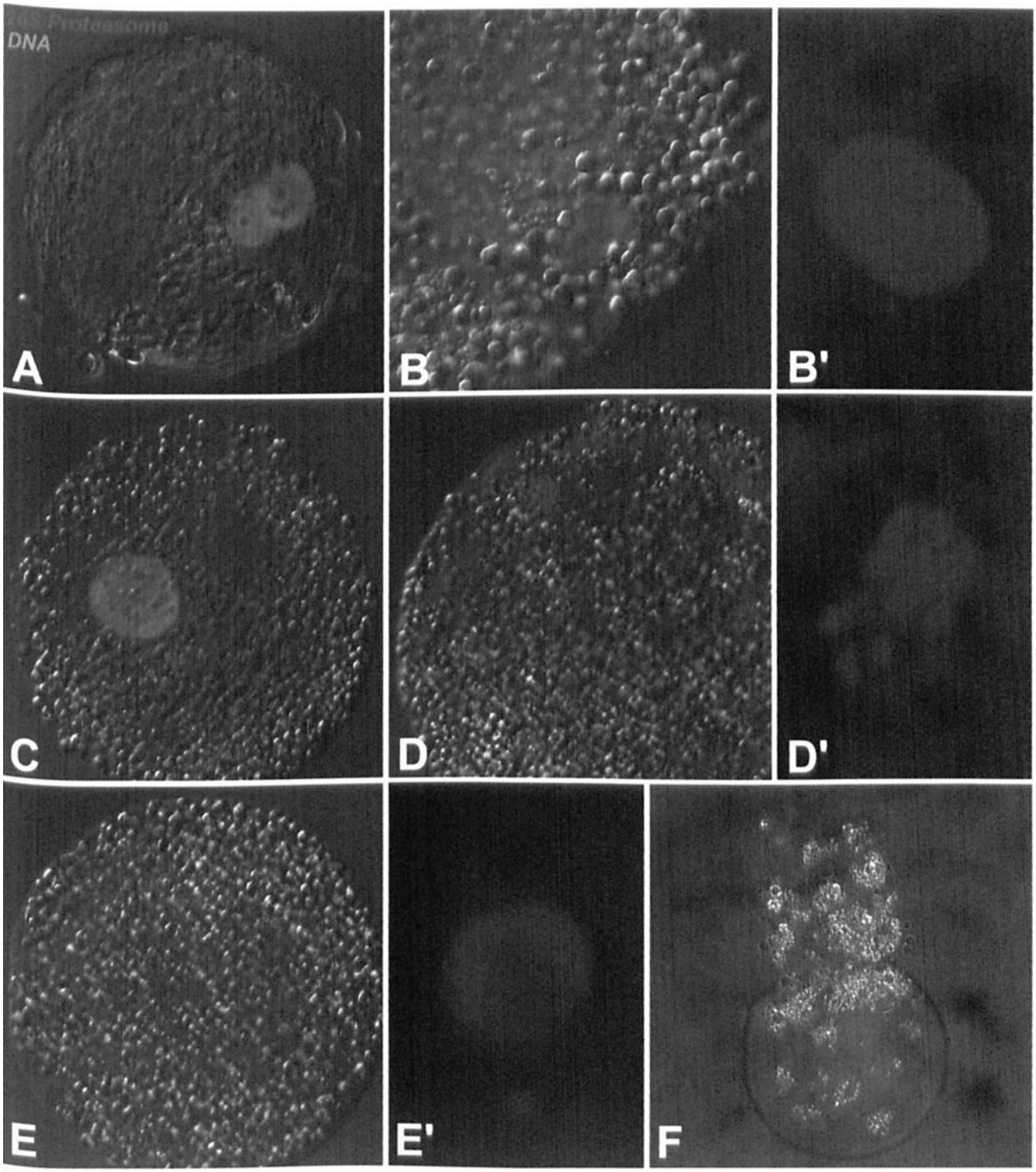

Figure 1. Accumulation of proteasomes (red) in the nuclear compartment of porcine zygotes. (A) Pronuclear zygote 20 hrs after fertilization *in vitro*. (B) A reconstructed zygote 1 hr after somatic cell nuclear transfer (SCNT). (B′) Detail of the intact donor cell nucleus before initiation of nuclear remodeling inside the zygote shown in Figure 1B. (C) Sequestration of proteasomes (red) within the remodeled pronucleus of an SCNT zygote at 20 hrs after nuclear transfer. (D) Abnormal remodeling of a donor cell nucleus, reminiscent of premature chromatin condensation, in an SCNT zygote cultured for 24 hrs in the presence of 10 μM MG132, a specific inhibitor of proteasomal protein degradation. (D′) Detail of condensed chromatin inside the zygote shown in Figure 1D; note the absence of proteasomes from donor cell chromatin. (E) An SCNT zygote cultured with 100 μM MG132 for first 24 hrs after nuclear transfer. (E′) A ring-shaped pattern of nuclear remodeling in the SCNT zygote shown in Figure 1E; proteasomes are not detectable within the nucleus. (F) A normal, Day 7, hatching blastocyst from a zygote pulsed with 10 μM MG132 for the first 2 hrs after SCNT. Proteasomes (red) were detected by using polyclonal antibody α/β (Biomol, Plymouth Meeting, PA) generated by immunization of rabbits with purified 26S proteasomes prepared from human erythrocytes. DNA (blue) was counterstained with DAPI (Molecular Probes, Eugene, OR). Color images were superimposed over a differential interference contrast micrograph of the corresponding focal plane acquired by Nikon Eclipse 800 microscope and CoolSnap HQ CCD camera with MetaMorph software. Primary magnification: A, C, E: ×400; B, D: ×600; B′, D′, E′: ×1500.

complete genomic reprogramming and normal development after embryo transfer. This leads us to a discussion of other factors that affect gene expression, namely, epigenetics and "large-offspring syndrome" (LOS).

Large-Offspring Syndrome

Any discussion of animals derived by NT requires a section about abnormal phenotypes in the offspring. Generally, these aberrant phenotypes are referred to as LOS and were first described in cattle derived from *in vitro* oocyte maturation, fertilization, and culture before embryo transfer. The most prevalent phenotype was a skewed distribution of birth weights (48, 49). These aberrant phenotypes tend to be species specific (e.g., contracted tendons in pigs). Our first transgenic pig created by oocyte transduction, *in vitro* fertilization, and culture to the blastocyst stage had contracted tendons (this was the first pig in the literature resulting from oocyte maturation, fertilization, and development to the blastocyst all *in vitro* before embryo transfer) (3). This female pig subsequently had 24 offspring, none of which had a contracted tendon.[3] When this female was cloned, only one of the four clones had a contracted tendon (31). A second specific example is that of large birth weight in cattle. Two cloned bulls were used on a herd of females. One of the clones had a large birth weight and the other had a normal birth weight. All of both bull's offspring were of a normal birth weight (50). A third example is that of obesity in cloned mice. Although the cloned animals have an obese phenotype, this phenotype is not transmitted to their offspring (51). Even when an animal with an abnormal phenotype is cloned, such abnormalities generally do not appear in the resulting offspring (52). Presumably, these phenotypes are not transmitted to the next generation because the DNA methylation pattern of the genome is reestablished during gametogenesis (53, 54) or altered during culture of the donor cells or embryo (52).

The role of DNA methylation in regulating gene expression is well described and can even affect phenotypic characteristics such as coat color (55, 56). Aberrant DNA methylation is also clearly implicated in some abnormalities of NT-derived embryos and offspring (57). One example in pigs is an enlarged tongue (macroglossia), which is consistent with Beckwith-Wiedemann syndrome and aberrant *IGF2* gene methylation and expression in humans (58). Active demethylation occurs in the paternal genome in the zygote followed by gene-specific passive (or dilution) demethylation during early cleavage and *de novo* methylation of the inner cell mass cells of the blastocyst (47). Cloned embryos fail to recapitulate the normal pattern of global demethylation and gene-specific methylation observed during normal embryogenesis (59, 60). Further research needs to be completed to better understand how the normal methylation pattern is established and how the normal pattern can be recapitulated during *in vitro* culture and after NT.

[3] Unpublished results.

Future Prospects

Although the efficiencies of genetic modification of somatic cells followed by NT are low, this technology is currently the only method to make specific genetic modifications (knockouts or knockins) to the germ line of pigs and other livestock. Compounded with the low efficiencies of producing animals, LOS can claim an NT-derived animal at most any stage of its life. The good news is that if such animals can reach puberty and produce offspring, the offspring do not have symptoms of LOS and can carry the genetic modification. To better understand the nuclear remodeling and reprogramming that occurs (or does not occur) after NT in mammals, a more thorough description is needed of the remodeling that occurs to male- and female-derived chromatin during normal fertilization. Because each species seems to have a specific subset of LOS, improved species-specific tools need to be created to study genomic DNA methylation and transcription. We hope that results gathered from such studies will result in new or refined procedures for producing cloned and transgenic animals.

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