Improvements in Nuclear Transfer Procedures will Increase Commercial Utilization of Animal Cloning - Review -

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ABSTRACT : Cloning technology continues to capture widespread attention by the international news media and biomedical and agricultural industries. The future uses of this technology could potentially contribute to major advances in biomedical and agricultural sciences. Cloned transgenic dairy cattle possessing milk promoters directing transgenes will produce pharmaceutical proteins in their milk faster, more efficiently and less expensively than transgenic cattle created using microinjection techniques. Additionally, cloned transgenic fetuses and animals may become a source of cells, tissue and organs for xenotransplantation. Lastly, but maybe most importantly, enhanced production traits and disease resistance may be realized in animal agriculture by utilizing these new technologies. The recent advances in the cattle cloning technology are important but there are still major obstacles preventing widespread commercial use of this technology. The type of donor nucleus, recipient cytoplasm, and cloning procedures used will impact the potential number of clones produced and the uses of the technology. In addition, the new advances in cloning methodology have not improved the relatively low pregnancy rates or reduced the incidence of health problems observed in cloned offspring. These problems may require novel techniques to decipher their cause and new methods of preventing and/or diagnosing them in the preimplantation embryo. The commercial potential is enormous for cloning technology; however, little has been done to improve the efficiencies of the procedure. Improving procedural efficiencies is a critical developmental milestone especially for potential uses of cloning technology in animal agriculture. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 6 : 856-860*)

Key Words : Nuclear Transfer, Embryo, Bovine, Porcine, Oocyte

INTRODUCTION

Recent progress in cloning mammals is attributed to improvements in treatment of the donor nucleus and better understanding of the nuclear reprogramming ability of the unfertilized oocyte. Despite these advances, the procedures used in cloning (nuclear transfer) are still inefficient, thus limiting commercial applications of this technology. In cloned cattle and sheep, the limited viability of embryos and/or offspring is reportedly a result of problems during nuclear reprogramming of the donor nucleus. Pigs are even more problematic, since the advances made in cloning in other species apparently are not sufficient for producing offspring derived from reprogrammed differentiated cells in pigs. Clearly, new and innovative approaches to inducing and monitoring nuclear reprogramming of donor nuclei are needed.

COMMERCIAL OPPORTUNITIES

In the 1980s cattle genetics companies envisioned using nuclear transfer technology to multiply genetically superior cattle; however, today the field of biomedicine appears to be the first major commercial opportunity for cloning technology. Nuclear transfer technology can produce transgenic cattle faster and more efficiently than traditional microinjection techniques. Microinjection techniques were used to produce transgenic rabbits, pigs, goats and sheep able to secrete blood proteins in their milk. These products are in human clinical trials and are expected to gain regulatory approval and be marketed in the next several years. Therefore, microinjection procedures are useful but they are very inefficient. In cattle only one in 1000 embryos injected with the DNA construct results in a transgenic calf. Nuclear transfer presents many advantages over microinjection. One is the fact that fewer embryos need to be produced to obtain a transgenic offspring. Second, all offspring produced are transgenic thus eliminating the cost of carrying non-transgenic pregnancies to term. All of the offspring are transgenic because all of the cells used to produce the nuclear transfer embryos were selected for the gene of interest being present in the donor cells. Thirdly, the sex of the cloned offspring is known since the sex of the starting donor cell is predetermined. All female offspring are of interest in this case since they will produce a product earlier than having to wait for the next generation of female offspring when using a microinjection founder bull. Nuclear transfer offers the potential of having a herd of cloned animals producing the pharmaceutical protein in three years. An estimated two years of developmental research can be eliminated when a herd of cloned transgenic females are produced. This means clinical trials can be started two years earlier. This

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Table 1. Species and donor cell type used to produce cloned mammals

Species	Cell type used to produce a nuclear transfer offspring (clones)		
	Embryonic	Fetal	Adult
Mouse	Cheong et al., 1993	NO	Wakayama et al., 1998
Rabbit	Stice and Rabl, 1988	NO	NO
Cattle	Prather et al., 1987	Cibelli et al., 1998	Kato et al., 1998
Sheep	Willadsen, 1986	Campbell et al., 1996	Wilmut et al., 1997
Pig	Prather et al., 1989	NO	NO

shortcut is a great attraction to pharmaceutical companies.

Nuclear transfer technology may also be used to enhance cell and tissue therapies. Previously we transplanted neural tissue derived from cloned transgenic bovine fetuses into a rat model for Parkinsons disease. This significantly reduced the Parkinsonian symptoms in the treated rats (Zawada et al., 1998). Therefore, therapeutic transgenes may be added or endogenous genes knocked out through nuclear transfer to produce a consistent source of genetically engineered animal fetal cells to be tested in rats prior to clinical trials. Cloning and transgenic improvements will eventually impact these disease states through more consistent sources of cells (genetically identical) for cell therapies and new and better animal models.

Yet another biomedical opportunity is developing porcine nuclear transfer technologies for organ transplantation applications. The potential of using this technology in this field is large and some suggest it could become a $6 billion global market at maturity. The attraction is to use cloning to add or remove (knockout) genes; however, first the cloning procedures must be developed for pigs to make these valuable animals. Therefore, cloned pigs produced through novel techniques will have an impact on this field and the increased efficiencies will facilitate the necessary genetic modifications faster than conventional micro-injection techniques.

In animal agriculture, cloning still has the potential for broad-based economic benefits. This will fill the needs of animal production industries that desire reliable sources of high quality breeding stock. Development of cloning and transgenics for use in food producing animals will provide the opportunity to develop animals with traits that improve both the efficiency of production and the quality of products for consumers.

WIDESPREAD COMMERCIALIZATION REQUIRES IMPROVED NUCLEAR TRANSFER EFFICIENCIES

The following is a general schematic of the nuclear transfer procedure in mammals (figure 1),

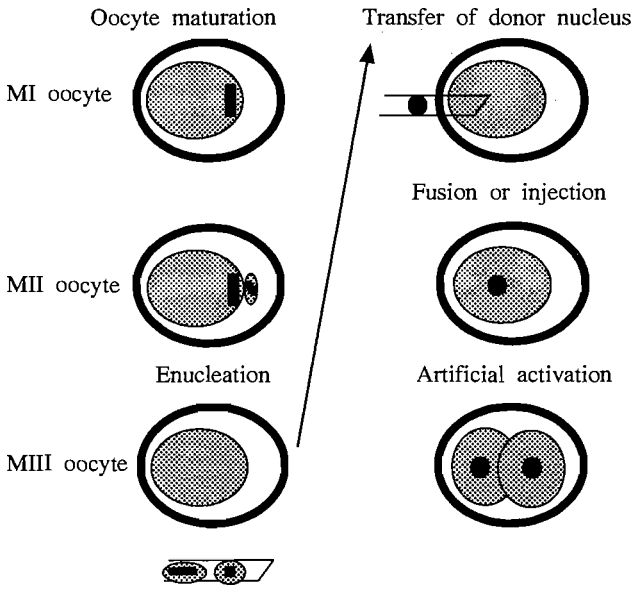

Figure 1. Traditional nuclear transfer procedures

developed first in the late 1980s and basically the same today. Some of the minor changes in the procedure, developed since then, will be described in following sections.

Over the last 11 years, the source of the donor nuclei has changed, but the basic nuclear transfer procedure has not changed dramatically. The first cloned rabbit (Stice and Robl, 1987) was produced using embryonic cells, whereas recently, the first cloned transgenic calves were produced using fetal cells (Cibelli et al., 1998; table 1). Although 11 years separate those experiments, both studies used unfertilized M II oocytes that were enucleated and then fused with the donor cell. The major difference other than donor cell type is the timing of fusion and activation appears to separate the use of embryonic verses differentiated donor cells. Bovine embryonic cell derived clones developed at a higher rate when the oocyte was activated first followed by introduction of the donor nucleus into the activated oocyte (Barnes et al., 1993; Stice et al., 1994). Thus, bovine fetal and adult cell cloning was successful because the donor nuclei were exposed to the reprogramming properties of the unfertilized oocyte for an extended period.

Table 2. The use of various cell types and procedures in the production of bovine nuclear transfer offspring

	1998 (Kato et al.)	1999 (Wells et al.)	1999 (Vignon et al.)
Cytoplast	MII oocyte	MII oocyte	MII oocyte
Donor nuclei	Quiescent cumulus and	Quiescent granulosa	Proliferating fetal and
Procedure	Fusion to non-activated	Fusion to non-activated	Fusion to activated
Outcome	Offspring	Offspring	Offspring

This was accomplished by reversing the fusion and activation steps in the cloning process, and resulted in the first cloned cattle fetuses from differentiated cell lines (Stice et al., 1996), and later in offspring from fetal cells (Cibelli et al., 1998; table 2). Thus, with differentiated cells, the basic nuclear transfer procedures and starting oocyte stage have not changed, but the donor nucleus is exposed to the enucleated M II oocyte cytoplast prior to activation.

Adult fibroblast and cumulus cell nuclei have now been shown to direct development to offspring in cattle (table 2). Again, a key difference appears to be an extended exposure to the reprogramming factors associated with the oocyte prior to initiating activation (Kato et al., 1998; Wells et al., 1999). The use of quiescent donor cells may have played a role in the success as well. Extended oocyte exposure also helped produce the first cloned adult mice (Wakayama et al., 1998). The results are less clear in sheep, since Dolly was produced using simultaneous activation and fusion (Wilmut et al., 1997). However, another laboratory working in cattle used non-quiescent bovine cell nuclei fused to activated oocytes and produced offspring (Vignon et al., 1999).

Dolly was produced using a quiescent nucleus, the authors theory being that a quiescent differentiated cell would facilitate nuclear reprogramming (Wilmut et al., 1997). However, more recent studies in the mouse using various cells that are naturally in a quiescent state when harvested (cumulus cells, sertoli cells and neural cells), produced very different results (Wakayama et al., 1998). The cumulus cells gave rise to offspring while the other quiescent cells did not. Arguably, the least quiescent of the three cell types is the cumulus cells since these are often mixed with granulosa cells which will propagate very well in culture (Wells et al., 1999). Therefore, the role of quiescence in the success of nuclear transfer is debatable and there is no conclusive evidence that quiescence is or is not mandatory for nuclear reprogramming.

Certain cell types like cumulus/granulosa cells and fibroblast cells do result in offspring when used in the nuclear transfer process (table 2). Based on the data from cattle and mice, it may be more important to have a functional cell type (either G0 or G1) capable of immediately directing development in the resulting nuclear transfer embryo than merely quiescence. This is the primary reason for using dividing or arrested fibroblast cells rather than non-dividing neural cells, truly quiescent donor nuclei. Successful cloning requires cell cycle synchrony between the donor nucleus and the recipient cytoplasm and/or the additional time that the donor nucleus is reprogrammed by the recipient oocyte.

Although cloned cattle were produced using either fetal or adult cells, the efficiencies are still low and losses of late term pregnancies and neonatal anomalies make the current procedures problematic. We have only limited knowledge of what causes these problems. Using fetal fibroblast cells as donor nuclei 13 cloned fetuses progressed into the third trimester of pregnancy (Hill et al., 1998). Of the 13 fetuses, eight live calves were born and six survived past one month of age. In the other pregnancies, four fetuses were recovered from three dead cows between seven days-and two months before parturition. The 13th fetus was aborted at eight months gestation but the surrogate cow survived. Placental edema was associated with the calves and fetuses with cardiopulmonary abnormalities. Hydrallantois and/or placental edema were observed in six cows resulting in only one of the six surviving calves. Six cows without hydrallantois or placental edema had five live calves and one aborted fetus. Therefore, five of the seven dead fetuses or calves were from pregnancies that showed obvious placental abnormalities.

Improvements in the nuclear transfer procedure that reduces or eliminates neonatal losses will improve the commercialization potential of cloning especially in animal agriculture. Alternatively, if embryos that produce late term abortions or neonatal losses could be diagnosed early then these problem pregnancies could be avoided. Ideally viable embryos would be diagnosed prior to transfer into recipient females. However, the best solution to this problem is a better basic understanding of the nuclear reprogramming process leading to normal offspring.

NUCLEAR REPROGRAMMING

Definitions for nuclear reprogramming vary greatly; for our purposes, the desired result in cloning is to modify an adult nucleus so it is capable of directing development from the one-cell embryo stage to offspring. This, of course, is the goal of cloning and

accomplished in various species using donor nuclei of differing states of differentiation (table 1). Other parameters, such as ability to produce a blastocyst stage embryo, are an indication of successful nuclear reprogramming. However, early cloning studies using embryonic cell lines indicated that embryo development to the blastocyst stage does not mean that offspring are forthcoming (Stice et al., 1996). Other parameters such as temporal and spatial development patterns were also used. When the first cloned rabbit was produced, we also reported morphological parameters for nuclear reprogramming (Stice and Robl, 1988). In this study, the time required to progress from the zygote to the blastocyst stage was similar for both fertilized embryos and nuclear transfer embryos. However, the donor cell that was not reprogrammed by the recipient cytoplast formed a blastoceole cavity earlier (72 hrs). Therefore, the oocyte cytoplasm at least partially reprogrammed the donor nucleus since it reverted to the same morphological and temporal pattern as the fertilized embryo. There are anecdotal and unpublished observations that spatial and temporal events between fertilized embryos and nuclear transfer embryos are not always the same, but these events have never been quantified. For example, bovine nuclear transfer embryos do not form a distinct compact morula stage but advance quickly from precompacted morula to the blastocyst stages (Stice, unpublished).

There are preliminary data suggesting that in fertilized sheep embryos, the time to development to blastocyst stage *in vitro* may be correlated with birth weight (Kuran et al., 1999). The most advanced staged embryos at day seven of development were more likely to have high lamb birth weights than lambs from earlier stage embryos. Although not quantified, others and we have observed that nuclear transfer embryos in both cattle and pigs often develop to blastocyst stage faster than fertilized embryos. They often form compact morula only briefly before developing a blastocyst cavity. This may indicate incomplete nuclear reprogramming as mentioned in a previous section (Stice and Robl, 1988). Fully reprogrammed nuclear transfer embryos capable of developing to normal offspring would have a developmental pattern similar to that of fertilized embryos.

Others have used ultrastructural and biochemical markers to observe nuclear reprogramming including nuclear lamina epitopes (Prather et al., 1989), nucleolar morphology (An et al., 1994), and protein synthesis (Yang et al., 1995). However, nuclear transfer embryo development within a group is highly variable. These previous techniques are not vital measurements; therefore, measurements in an embryo cannot be compared to the eventual development of each individual embryo.

Genomic imprinting is involved in nuclear reprogramming and may influence development in nuclear transfer embryos. Both the maternal and paternal chromatin compete for hyperacytelated histones (Adenot et al., 1997). Methylation and acetylation of histones are related and could potentially affect genomic imprinting and or nuclear reprogramming. Because the maternal chromosomes are normally removed before activation in the nuclear transfer procedure, genomic imprinting events may be affected more so in nuclear transfer embryos. Several research groups are currently investigating methylation and imprinting patterns in nuclear transfer embryos.

CLONING PIGS

Cloning pigs is technically difficult and has not yet been performed using either fetal or adult donor nuclei. The reasons for the difficulties in cloning pigs are not clear. In international peer reviewed journals, only one cloned pig derived from a four-cell stage embryo nucleus was produced (Prather et al., 1989; table 1). Several groups have produced blastocyst stage pig nuclear transfer embryos derived from differentiated cells, but no offspring have been produced (Stice et al., 1998 IETS annual meeting; Prather et al., 1999; Miyoshi and Sato, 1999). Nuclear transfer embryo developmental rates with *in vitro* and *in vivo* derived M II oocytes is poor. Because of the many uses for cloned pigs in both biomedicine and agriculture, many companies are pursuing this research (Geron Bio, Novartus and PPL Therapeutics). However, public and private comments by individuals within these companies (Ian Wilmut, Geron Bio and Alan Coleman at PPL Therapeutics) suggest that there has been little progress.

Progress has been made by increasing calcium and/or decreasing protein phosphorylation in other mammalian oocytes (mice, Szollosi et al., 1994; cattle, Susko-Parrish et al., 1994). Improvements in porcine oocyte activation have lagged behind other species particularly in parthenogenetic development of the activated oocyte. Recently, however, an improvement in development to the morula and blastocyst stage for parthenotes was obtain when thimerosal and dithiothreitol (DTT) were used in combination (43% to morula or blastocyst stage; Machaty et al., 1997). It is unclear, though, as to how this activation procedure will affect development of porcine nuclear transfer embryos.

CONCLUSION

The advances in using differentiated cells as donor cells in the nuclear transfer process have opened new opportunities to commercialize animal cloning. Now improvements in the nuclear transfer efficiencies and

the pregnancy outcomes will determine whether we can capitalize on these opportunities.

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