Factors Affecting the Efficiency of Animal Cloning by Somatic Cell Nuclear Transfer

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ABSTRACT

Since the birth of Dolly using fully differentiated somatic cells as a nuclear donor, viable clones were generated successfully in many mammalian species. These achievements in animal cloning demonstrate developmental potential of terminally differentiated somatic cells. At the same time, the somatic cell nuclear transfer (SCNT) technique provides the opportunities to study basic and applied biosciences. However, the efficiency generating viable offsprings by SCNT remains extremely low. There are several explanations why cloned embryos cannot fully develop into viable animals and what factors affect developmental potency of reconstructed embryos by the SCNT technique. The most critical and persuasive explanation for inefficiency in SCNT cloning is incomplete genomic reprogramming, such as DNA methylation and histone modification. Numerous studies on genomic reprogramming demonstrated that incorrect DNA methylation and aberrant epigenetic reprogramming are considerably correlated with abnormal development of SCNT cloned embryos even though its mechanism is not fully understood. The SCNT technique is useful in cloning farm animals because pluripotent stem cells are not established in farm animal species. Therapeutic cloning combined with genetic manipulation will help to control various human diseases. Also, the SCNT technique provides a chance to overcome excessive demand for the organs by production of transgenic animals as xenotransplantation resources. Here, we describe the factors affecting the efficiency of generating cloned farm animals by the SCNT technique and discuss future directions of animal cloning by SCNT to improve the cloning efficiency.

(Key words : animal, cloning, somatic cell nuclear transfer, DNA methylation, epigenetic reprogramming)

INTRODUCTION

Animal cloning by the somatic cell nuclear transfer (SCNT) technique provided the opportunities to study many aspects of both basic sciences and applied sciences such as transgenic animal production, xenotransplantation, cell therapy, pharmacology and conservation of endangered animal. Initially, the SCNT technique was proposed to study cell differentiation by Spemann (1938), which was successfully done in amphibians by Briggs and King (1952). Thereafter, developmental potency of fully differentiated epithelial cells was demonstrated by production of adult frog using epithelial cells of developing tadpole intestine (Gurdon, 1962). A decade ago, a sheep was produced from cultured mammalian epithelial cells as nuclear donor cells (Wilmut *et al.*, 1997). Since the production of the cloned sheep, Dolly, the SCNT technique was employed to clone many animal species during the last 10 years (Zhou *et al.*, 2003; Loi *et al.*, 2001; Betthauser *et al.*, 2000; Lanza *et al.*, 2000; Polejaeva *et al.*, 2000; Wakayama *et al.*, 1999; Wakayama *et al.*, 1998; Wilmut *et al.*, 1997). Successful production of viable offsprings using the SCNT technique indicates that most cell types have developmental potency to generate viable clones at certain conditions.

Although the SCNT technique is an attractive and beneficial tool for both basic and applied sciences, the overall efficiency of generating viable cloned animals using the technique remains extremely low. The overall cloning efficiency of SCNT reconstructed embryos to delivery of viable progeny is less than 5 %

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(Campbell *et al.* 2005). Several factors such as methods of enucleation, embryo handling and selection, type of the donor cells, activation and culture conditions may affect the cloning efficiency. Recent studies suggest that the low efficiency of generating viable offspring is due to incomplete nuclear reprogramming of donor nucleus, which includes DNA methylation, histone acetylation and chromatin remodeling (Farin *et al.*, 2006; Humpherys *et al.* 2001). To increase the cloning efficiency, the loss of embryo during the *in vitro* manipulation period and the gestational period should be minimized. Loss of embryo before transferring to the recipient may be associated with the sub-optimal culture conditions and manipulation process, and gestational loss with placental abnormalities (Ka *et al.*, 2007; Hill *et al.*, 2000, 1999). Since SCNT cloning is consisted of sequential processes of *in vitro* manipulation and embryo development in the recipient uterus, species-specific adjustment for the manipulation processes is needed for the successful SCNT cloning (Hinrichs *et al.*, 2007; Ribas *et al.*, 2006). In this review, we will discuss general factors affecting the efficiency of generating viable cloned offsprings to provide further insights about the SCNT technique.

IN VITRO MANIPULATION OF OOCYTES, DONOR CELLS AND CLONED EMBRYOS

1. Selection of Nuclear Donor Cells and Cell Cycle Synchronization

Cloning efficiency depends on considerably the donor cell type. Generation of embryonic stem (ES) cells by SCNT using neural stem cells or ES cells is more efficient than that using mature neural cells in mice (Eggan *et al.*, 2004; Li *et al.*, 2004). It is known that mature lymphocytes, such as B and T cells, are inefficient as nuclear donors, but natural killer T cells are more efficient than mature lymphocytes (Hochedlinger and Jaenisch, 2002). In mice, viable SCNT cloned offsprings have been produced using cumulus cells, tail tip cells, Sertoli cells, fetal cells, ES cells, NKT cells, primordial germ cells, hematopoietic stem cells, granulocytes, fetal neuronal cells and neuronal stem cells as donor cells (Wakayama, 2007). The use of ES cells as donor cells are more beneficial in nuclear transfer (Wakayama *et al.*, 1999), but ES cells are not established yet in most farm animal species. Nevertheless, it is not clear whether differentiation status is truly related to developmental potency of cloned embryos (Oback and Wells, 2007).

Cell cycle of recipient oocytes and donor cells is also important for the success of nuclear transfer. Oocyte quality as a nuclear recipient is related to the maturation status. Studies on influence of cell cycle of recipient suggest that metaphase Ⅱ (M-Ⅱ) stage-arrested oocytes are desirable (Campbell *et al.*, 2005). Synchronization of donor cell cycle is also critical for improvement of cloning efficiency (Wells *et al.*, 2003). Serum starvation is commonly used technique to arrest donor cell cycle, which adversely effects chromosome integrity (Kues *et al.*, 2000). Roscovitine and nocodazol are used for synchronization of cell cycle, but they also have toxic side effects (Amano *et al.*, 2001; Tanaka and Kanagawa, 1997). A simple mitotic shake-off method and contact inhibition may be used to select cells in G0/G1 (Boquest *et al.*, 1999).

2. Preparation and Selection of Oocytes as Nuclear Recipients

Since the quality of recipient can influence the cloning efficiency, both oocytes derived from *in vitro* and *in vivo* have been used to obtain good quality of nuclear recipients. The *in vitro* oocytes are obtained from the slaughterhouse that is the most useful place to obtain oocytes of cattle and pigs. Oocytes are harvested by aspiration of antral follicles after recovery of ovaries followed by *in vitro* maturation (IVM) process. However, oocytes for a recipient in SCNT should be selected again after IVM because of unequivalent quality of oocytes. Oocytes can be obtained from uterine flushing or hysterectomy after ovulation. Superovulation regimens are used in the *in vivo* approach to increase the harvest of large number of oocytes. However, ovarian stimulation regimens often result in abnormal development of cloned embryos, including reduced embryo development, retarded fetal growth and aberrant DNA methylation (Shi and Haaf, 2002; Van der Auwera and D'Hooghe 2001). In cloning of endangered animals, oocytes of closely related species is replaced with own oocytes because it is difficult to obtain optimal recipient (Lanza *et al.*, 2000). It is subsequently demonstrated that oocytes obtained closely related species are possible alternatives as nuclear recipients (Loi *et al.*, 2001; Lanza *et al.*, 2000).

3. Enucleation in the Recipient Oocytes

Enucleation is a process to remove genetic materials of recipients and is undertaken at MⅡ stage of recipient oocytes. At MⅡ stage DNA is present as highly condensed chromosomes around a metaphase spindle or plate. Using the first polar body (PB1) as a marker, small volume of oocyte cytoplasm is removed using micropipette because DNA is invisible under the microscope due to lipid. However, because all maternal DNA

is not located close to PB1, incomplete enucleation may occur after enucleation (Li *et al.*, 2004). In addition, the removal of cumulus cells prior to enucleation can disrupt the connection of MⅡ spindle and PB1 (Campbell *et al.*, 2005), and too much aspiration of the cytoplasm adversely affects development of reconstructed embryos and cloning efficiency. Many studies demonstrated that incomplete enucleation and removal of excessive amount of cytoplasm showed limited developmental potency in some animal systems (McGrath and Solter, 1983; Prather *et al.*, 1989). It is suggested that enucleated oocyte lost large proportion of the cytoplasm could not support nuclear epigenetic reprogramming of donor cells (Campbell *et al.*, 2005). Furthermore, during enucleation Hoechst 33342 (bisbenzimide) DNA staining and ultraviolet light exposure are commonly used, but the exposure of oocyte to staining materials and UV light cause damages to genomic and mitochondrial DNA of oocytes (Forsberg *et al.*, 2002; Loi *et al.*, 2001). Telophase Ⅱ (TⅡ) enucleation is an alternative method to remove maternal genome including second PB and surrounding the cytoplasm after activation of oocytes without cytotoxic materials and loss of large portion of cytoplasm. TⅡ enucleation method is required for activiation of oocytes prior to enucleation, which results in decreased MPF activity (Fulka and Moor, 1993). In addition, there are some other enucleation methods using chemicals, such as oposide, cycloheximide, ethanol, demecolcine and sucrose (Ibanez *et al.*, 2003; Wang *et al.*, 2001; Elsheikh *et al.*, 1998; Fulka and Moor, 1993).

4. Reconstruction and Activation of Oocytes with the Somatic Cell Nucleus

Electrofusion is preferentially used to introduce the donor nucleus into the enucleated oocyte in most farm animals, while piezo-driven microinjection is routinely used in mice because mouse eggs become prematurely activated through the electropulse (Wakayama *et al.*, 1998).

Mammalian oocytes are ovulated at the MⅡ-arrested status. Oocytes obtain a full competency to fertilize during the maturation period (Miyazaki *et al.*, 1993). Fertilization triggers escape from meiotic arrest of oocytes and a cellular and molecular cascade to activate oocytes is initiated. Accordingly, an artificial stimulation by physical or chemical stimuli must be conducted to activate oocytes to induce various cellular events such as increase of Ca^{2+} and completion of meiosis (Saunders *et al.*, 2002). It has been suggested that decrease of both maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activity is critical for the reconstructed embryos to form a pronucleus and start the developmental process (Liu and Yang, 1999). This activation step is one of the key factors to improve development of SCNT embryos in rodent and horse (Hinrichs *et al.*, 2007; Zhou *et al.*, 2003).

Several methods are used to activate reconstructed oocytes; electrical pulse, which causes transmembrane Ca^{2+} influx through the formation of transient pores in the plasma membranes (Zimmermann and Vienken, 1982), and Ca^{2+} ionophore which also induces Ca^{2+} influx to activate oocytes (Steinhardt and Epel, 1974). Electrical stimulation alone or combination with dithiothreitol, 6-dimethylaminoprutine (6-DMAP) or cyclohexamide is commonly used for activation of oocytes (Im *et al.*, 2004; Polejaeva *et al.*, 2000). But, chemical treatments after multiple low pulse stimuli result in improved developmental potentials but early apoptosis than electrical stimulation alone (Im *et al.*, 2004). In addition, it has been shown that the treatment of 6-DMAP or cyclohexamide after chemical stimulation by inonomycin or calcium inophore has no beneficial effect on the developmental potency of SCNT embryos during the culture period or results in increased chromosomal abnormalities (Bhak *et al.*, 2006). It also has been reported that inomycin induces species-specific calcium response in some animal systems (Machaty *et al.* 1998; Jones *et al.*, 1995).

Inhibition of protein synthesis by puromycin and cyclohexamide treatment induces oocyte activation, possibly by preventing the production of cyclin B, a component of MEF (Tanaka and Kanagawa, 1997; Nussbaum and Prather, 1995; Presicce and Yang, 1994). However, inhibition of protein synthesis by treatment with puromycin or cycloheximide can induce oocyte activation in mouse and human, but not in pig oocytes (Nussbaum and Prather, 1995). Another factor to consider for oocyte activation is the age of oocytes, because young oocytes are not easy to activate than aged oocytes due to higher levels of cytostatic factors in young oocytes (Tanaka and Kanagawa, 1997; Yang *et al.*, 1994). Therefore, the procedure for oocyte activation should be optimized with regard to species, age of oocytes, activation condition and post-treatment of chemical to improve the developmental potential of SCNT embryos.

5. Culture of Reconstructed Embryos

The majority of reconstructed SCNT embryos are cultured to the blastocyst stage *in vitro* before transferred to surrogates except for rodents and pigs (Polejaeva *et al.*, 2000; Wakayama *et al.*, 1998). In rodents, reconstructed SCNT embryos are

should be measured for the survival of reconstructed embryos *in vitro* and after embryo transfer to surrogates. Reconstructed embryos have been cultured in media supplemented with fetal bovine serum and with feeder layers of oviductal cells or uterine cells (Menezo and Herubel, 2002; Thompson, 2000), but defined culture media for individual species are preferred nowadays; SOF for cattle and sheep (Matsuyama *et al.*, 1993; Walker *et al.*, 1992), NCSU23 for pigs (Machaty *et al.* 1998), CZB (Chatot *et al.*, 1989) and KSOM (Erbach *et al.*, 1994) or G1/G2 media (Barnes *et al.*, 1995) for mice. PZM-3, a culture medium for pig embryo, improved the development of *in vitro* produced porcine SCNT embryos (Im *et al.*, 2004). Sequential culture with different media such as G2.1/G2.2 (Swain *et al.*, 2001) and NCSU37-Pry-Lac/NCSU37-Glu (Medvedev *et al.*, 2004) 67 are also used to support the development of the reconstructed SCNT embryo. It has been reported that supplementations of BSA, FBS, platelet activating factor, IGF-1, GST and β-mercaptoethanol could enhance the developmental rate to the blastocyst stage and quality of reconstructed porcine embryo by SCNT (Vajta *et al.*, 2007). However, it still needs that culture media be adjusted for individual species because embryos produced *in vitro* showed aberrant gene expression compared to those produced *in vivo* (Li *et al.*, 2004; Doherty *et al.*, 2000).

EPIGENETIC REPROGRAMMING AND DEVELOPMENTAL ABNORMALITIES

1. Epigenetic Reprogramming of the Nucleus in Reconstructed Embryos

DNA methylation is an addition of methyl group to the 5' position in the symmetrical CpG nucleotide of promoter region of genes and is a major feature of epigenetic reprogramming (Bestor, 2000). DNA methylation regulates epigenetic reprogramming and participates in a variety of cell function, including embryo development, tissue specificity, differentiation, imprinting, X-chromosome inactivation, chromatin remodeling, chromatin structure stability, carcinogenesis, and aging (Bird, 2002). Genomic imprinting and X chromosome inactivation are well-known examples which are mediated by DNA methylation (Heard, 2004; Young *et al.*, 2001).

Imprinted genes inherited from paternal genome or maternal genome is known to play a key role in fetal growth and placental development (Dean *et al.*, 2001; Walker *et al.*, 1998). Some imprinted genes such as *H19*, *XIST*, *IGF-I*, *IGF-II* and *IGF2R* are not properly regulated in embryos derived by SCNT (Dindot *et al.*, 2004; Ravelich *et al.*, 2004a; Doherty *et al.*, 2000). Accordingly, defects in imprinted genes influence fetal and placental development of the SCNT embryos. Surprisingly, genome-wide analysis revealed that nearly 50% of cloned bovine and ovine blastocysts showed defects or aberrant patterns of methylation and histone acetylation status (Han *et al.*, 2003; Santos *et al.*, 2002). Furthermore, approximately 4% of a panel of 10,000 murine genes showed unexpected expression patterns in the placenta carrying embryo produced by NT, possibly due to the aberrant regulation of genes, including imprinted genes (Humpherys *et al.*, 2001).

DNA methylation is maintained by DNA methyltransferases (DNMTs), and among them, DNMT1, DNMT3a and DNMT3b are believed to play critical roles in DNA methylation (Rhee *et al.*, 2000; Okano *et al.*, 1999; Jaenish, 1997). DNMT1 is responsible for the maintenance of methylation because DNMT1 preferentially adds methyl groups on new CpG sites when their partners on the parental strand have methyl groups (Bestor, 1992). DNMT3a and DNMT3b are responsible for de novo methylation that occurs after implantation (Okano *et al.*, 1999). It has been shown that mutant mice devoid of DNA methyltransfeases are lethal during early embryonic development (DNMT1 and DNMT3b) or shortly after birth (DNMT3a) (Bird, 2002; Bestor, 2000).

DNA methylation status changes dynamically during the early mammalian development. Demethylation of paternal genome occurs after protamine-histone exchange in the male pronucleus by an active mechanism, which is a loss of methylation in the absence of DNA replication (Bird, 2002; Santos *et al.*, 2002), while demethylation of maternal genome is demethylated by a passive DNA replication mechanism (Santos *et al.*, 2002; Reik *et al.*, 2001). DNA demethylase(s) involved in a loss of DNA methylation has not been found in oocyte so far.

During embryo development, methylation levels decline at the morula state, but remain in some repetitive elements and imprinted genes (Reik *et al.*, 2001; Sanford *et al.*, 1987). At the blastocyst stage inner cell mass is hypermethylated, while trophectoderm is hypomethylated (Santos *et al.*, 2002). The significance of methylation status during development of SCNT embryos has also been investigated. In SCNT cloned mice, several imprinted genes showed abnormal methylation and expression patterns (Mann *et al.*, 2003). The methylation patterns in some satellite DNA sequences of SCNT bovine embryos were iden-

tical to those of somatic donor cells with high methylation levels in trophectoderm than inner cell mass (Han *et al.*, 2003; Kang *et al.*, 2002, 2001a). In addition, abnormal methylation patterns of de novo methylation and hypo- and hypermethylation were also found during development of bovine SCNT embryos (Dean *et al.*, 2001; Bourc'his *et al.*, 2001). On the other hand, analysis of the satellite DNA sequences of porcine embryos showed that methylation levels were not significantly different between IVF embryos and SCNT cloned embryos (Kang *et al.*, 2001a; 2001b). These findings suggest that methylation and demethylation patterns of genome of SCNT embryos may not be uniform in the chromosomal regions, and that methylation levels are differentially regulated in the normal or SCNT-cloned embryos in a species-specific manner.

2. Developmental Abnormalities in the Maternal Uterus

The efficiency of SCNT cloning is influenced by several factors, but largely depends on the success of reprogramming process. When incomplete reprogramming occurs, reconstructed SCNT embryos undergo abnormal development. There are many reports on abnormalities of SCNT cloned embryos during development in the maternal uterus. Large offspring syndrome (LOS) is a typical abnormality of cloned animals but not limited to SCNT cloned animals (Walker *et al.*, 1998; Sinclair *et al.*, 1997). In sheep, LOS is caused by aberrant methylation of IGF2R (Young *et al.*, 2001). Enlarged placentas are observed during development of SCNT cloned embryos in mice and cow (Wakayama *et al.*, 1999; Cibelli *et al.*, 1998). Prolonged gestation is also common to SCNT clones (Cibelli *et al.*, 1998).

Evidence shows that the abnormalities of fetuses or placentas derived from SCNT cloning are caused by aberrant expressions of imprinted or non-imprinted genes during pregnancy (Dean *et al.*, 2001). The *XIST* gene was expressed biallelically in chorion, while the *IGF2R* gene was paternally expressed during bovine SCNT embryo development (Dindot *et al.*, 2004). Aberrant expression of IGF-I and IGFBP1-3 is also observed in the placenta carrying SCNT cloned embryos (Ravelich *et al.*, 2004a), which results in poor viability of fetuses and placental hyperplasia. Furthermore, placental lactogen expression increases while placental leptin expression decreases in amniotic fluid of bovine SCNT fetus (Ravelich *et al.*, 2004b). Abnormal expression of placental lactogen and leptin may induce incomplete attachment between cotyledon and caruncle during pregnancy of bovine SCNT cloned fetuses (Ravelich *et al.*, 2004b). Real time RT-PCR analysis show that expression of transforming growth factors beta (TGFβs) is elevated, while expression of their receptors, TGFβRI and TGFβRⅡ, decreases in the bovine placentas carrying SCNT cloned fetuses (Ravelich *et al.*, 2006).

It has been attempted to understand the uterine function and the uterine responsiveness interacting with the transferred SCNT cloned embryos during implantation and placentation in pigs (Kim *et al.*, 2008; Ka *et al.*, 2007). Expression of retinol binding protein and fibroblast growth factor 7 in the uterine endometrium carrying SCNT embryos at term was decreases, but osteopontin protein increases compared to that in the uterine endometrium carrying normal embryos (Kim *et al.*, 2008). These results show that the uterine secretory activity is impaired in the endometrium of gilts carrying SCNT cloned embryos compared to that in the endometrium of gilts carrying normal embryos at term, and that expression of the uterine secretory molecules is also affected by the normality of the developing SCNT cloned fetus (Kim *et al.*, 2008). Further, the transcriptional profiling analysis show that genes in the uterus with SCNT cloned embryos is regulated differently compared to those in the uterus with normal embryos on day 30 of pregnancy, indicating that the maternal responsiveness to the SCNT embryos is impaired (Ka *et al.*, 2007). Therefore, SCNT cloned embryos cannot interact appropriately with the maternal uterine endometrium to induce uterine responses for implantation and/or maintenance of pregnancy. Fetal/placental defects of the developing SCNT cloned embryos cause the inappropriate signaling to the maternal uterus to induce altered gene expression, resulting in inappropriate interactions between the uterus and the developing embryos for normal pregnancy. The uterus with aberrantly altered gene expression, in turn, is not fully supportive to the developing NT cloned embryos, resulting in decreased fetal size or even embryonic loss (Kim *et al.*, 2008).

PERSPECTIVES

The SCNT technique is an invaluable tool to propagate animals with economic traits of high performance in farm animals. Furthermore, this technique can be applied to generate disease model animals to study pathology of specific diseases or to develop pharmacological agents for treatments. Cloning animal by SCNT can also be applied to generate bioreactor animals to produce bioactive materials, and many animal species including goat, cow, sheep, pig and chicken have been successfully generated to produce bioactive agents such as coagulation fac-

tors and anti-inflammatory proteins in mammary gland, urinary bladder or egg (Vajta and Gjerris, 2006). Animal cloning by SCNT in association with transgenesis has been tried to provide organs for xenotransplantation. Currently, the pig is considered as the most suitable animal for the xenotransplantaion because of the similar organ size compared to that of humans, short reproductive cycle, large litter size and relatively short growth period (Pinkert, 1994). Production of transgenic pigs with immunosuppressive genes or without immunoactive genes such as the alpha 1,3-galactosyltraserase gene has been tried and some promising results have been obtained (Lai *et al.*, 2002). Therapeutic cloning in human is an alternative approach to control serious diseases such as diabetes, autoimmune disease, Parkinson's and Alzheimer disease. Generation of human SCNT-ES cells would help customized therapy for the patients suffering from incurable diseases by traditional treatments.

Improvement of cloning efficiency is essential to availability of the SCNT technique. To increase early embryonic development of SCNT embryos many chemicals and cytokines have been applied. Trichostatin A (TSA), an inhibitor of histone deacetylase, increased rate of blastocyst formation during *in vitro* culture of bovine SCNT embryos and production of viable offsprings in mouse SCNT (Kishikami *et al.*, 2006). Treatment of bone morphogenetic protein 15 and growth and differentiation factor 9, which are secreted by oocytes, also enhance developmental potential of SCNT embryos during the *in vitro* culture period (Hussein *et al.*, 2006). However, the most important feature to improve overall cloning efficiency for generating viable offspring is to enhance genetic reprogramming of donor cells in the recipient oocyte and during embryo development. Recently, it has been reported that introduction of *Oct-4*, *Sox2*, *c-Myc* and *Klf4* can induce mouse embryonic and adult fibroblast cells to pluripotent stem cells under the embryonic stem cell culture condition (Takahashi and Yamanaka, 2006). Introduction of *OCT-4*, *SOX2*, *NANOG* and *LIN28* can dedifferentiate human fibroblast cells to pluripotent stem cells (Park *et al.*, 2008), and oocytes extracts or direct OCT-4 activation are able to reprogram somatic donor cells (Byrne *et al.*, 2003; Hansis *et al.*, 2004). These findings suggest that nuclear factors such as *OCT-4* and *SOX2* are involved in regulation of nuclear reprogramming event. Therefore, employment of oocyte extracts or nuclear factors that are associated with genetic reprogramming along with chemicals and cytokines could be treated to the nuclear donor cells to help increase of cloning efficiency. The establishment of optimal SCNT procedure and understanding the mechanisms of epigenetic reprogramming would greatly increase the efficiency of animal cloning by the SCNT technique which can be widely used in reproductive cloning, therapeutic cloning and production of transgenic animals.

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