Generation of transgenic livestock by somatic cell nuclear transfer

REVISIÓN

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ABSTRACT

The ability to produce transgenic animals through the introduction of exogenous DNA has existed for many years. However, past methods available to generate transgenic animals, such as pronuclear microinjection or the use of embryonic stem cells, have either been inefficient or not available in all animals. More recently, somatic cell nuclear transfer has provided a method to create transgenic animals that overcomes many limitations of other methods. This review summarizes the major advantages and disadvantages of currently available techniques to produce genetically modified livestock with special emphasis on the benefits of using somatic cell nuclear transfer (SCNT) to create genetically modified livestock for agriculture and biomedical applications. Potential applications of transgenesis by SCNT in farm animals are discussed.

Keywords: nuclear transfer, transgenic, livestock, stem cells, somatic cells

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RESUMEN

Generación de ganado transgénico mediante transferencia nuclear de células somáticas. La posibilidad de producir animales transgénicos mediante la introducción de ADN exógeno existe desde hace muchos años. Sin embargo, los métodos disponibles hasta el presente para generar animales transgénicos, tales como la microinyección pronuclear o el uso de células madre embrionarias, han sido ineficientes o no han estado disponibles para todos los animales. La transferencia nuclear de células somáticas, recientemente introducida, representa un procedimiento alternativo para crear animales transgénicos, la que supera muchas de las limitaciones de otros métodos. En esta revisión se presenta en forma resumida las principales ventajas y desventajas de las técnicas disponibles para producir ganado genéticamente modificado con especial énfasis en los beneficios del uso de la transferencia nuclear de células somáticas para crear ganado transgénico. Se discuten también las aplicaciones potenciales de los animales transgénicos generados mediante transferencia nuclear de células somáticas en agricultura y biomedicina.

Palabras claves: transferencia nuclear, animales transgénicos, ganado, células madres, células somáticas

*I*ntroduction

Genetic modification of the mammalian genome has its origins in the pioneering work of Gordon and coworkers [1, 2]. They demonstrated that exogenous DNA injected into the pronuclei of one-cell embryos can be integrated, expressed and transmitted to the progeny of transgenic mice. For the first time the term "transgenic" was introduced to denote animals bearing this induced genetic modification. Although there have been major advances in the field of animal transgenics since its inception [1], the full potential of this technology has not been realized in part due to the limitations of commonly used transgenic technologies. More recently, the success of somatic cell nuclear transfer (SCNT) has provided a new and faster way to produce transgenic animals while circumventing many of the limitations of other transgenic techniques. Recent improvements in reproductive technologies such as artificial insemination, multiple ovulation and embryo transfer, and oocyte and embryo culture, in combination with the ability to insert DNA into livestock [3, 4], will provide limitless possibilities for both agriculture (e.g., inserting genes affecting milk and beef production) and biomedicine (e.g., producing pharmaceuticals in milk for human use). This review will focus on summarizing the recent past and present state of transgenics, and how somatic cell nuclear transfer will affect the future potential of transgenic technology in livestock.

*M*ethods to generate transgenic animals

There are multiple ways to produce a transgenic animal. Briefly discussed below are the strengths and weaknesses of the most common ways currently available to make transgenic animals. The success, or lack thereof, of each method in creating transgenic livestock is also summarized.

Pronuclear microinjection

Pronuclear injection is a straightforward procedure, which involves the placement of DNA containing the gene of interest into a pronucleus of a zygote followed by transfer of the zygotes to a surrogate mother. Eventually, the gene of interest randomly integrates in the embryonic genome. Microinjection was the first technique used to generate transgenic mice [1], and then it was extended to other animals, including livestock [3, 4]. The success of pronuclear injection with respect to transgene integration ranges from around 3% for mice, rats and rabbits to only 1% for cattle, pigs and sheep [5]. In addition to the poor efficiency of pronuclear microinjection, this method usually re-

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sults in a high percentage of mosaics in which not all cells of the animal contain the transgene. The time and cost of screening for germline transmission in mosaic animals can be prohibitive to generating more transgenic animals through breeding. Using pronuclear injection to create transgenic animals can also lead to high variability in transgene expression between animals due to mosaicism, variable efficiency in transgene integration and chromosomal position effects that occur during the random integration of the transgene [6]. Testing multiple lines of animals for proper transgene expression is necessary in a pronuclear microinjection approach to creating transgenic animals. Microinjection is also limited in that it only allows for the random addition of exogenous DNA rather than targeting to specific sites. DNA targeting is necessary in generating gene knockouts, for instance to model human diseases. The success of pronuclear microinjection is evident in the generation of transgenic pigs [3], goats [7], sheep [8] and cattle [4]; but its limitations have hindered the progress of transgenesis in livestock.

Embryonic Stem cells

Embryonic stem (ES) cells are used extensively as a way to create transgenic mice. These cells are isolated from the inner cell mass of a blastocyst, and when kept in the right culture conditions, they have the potential to divide endlessly [9]. This immortal-like characteristic allows for easy propagation and eventual DNA manipulation through the insertion of transgenes. When transgenic ES cells are isolated and inserted into a growing mouse embryo, they multiply and contribute to the resulting fetus giving rise to almost any tissue type. These chimeric animals are then tested for germline transmission and used to create fully transgenic animals through breeding strategies. One big advantage of using ES cells over microinjection is the ability to select for transgene integration through the use of selectable markers. This ability ensures the creation of transgenic offspring even if they are chimeras. The use of ES cells also allows for the targeted alteration of DNA by homologous recombination leading to the creation of gene knockouts [10]. The ability to easily create gene knockouts has lead to the mouse being considered one of the best models for genetic studies.

Due to the success of mouse ES cells, many attempts have been made to isolate similar cells in farm animals with limited success [11-13]. Genetic modification of livestock ES-like cells is not easy, due to the culture system required to maintain them as undifferentiated ES cells and their inability to expand clonally *in vitro* [14]. These intrinsic features that limit genetic manipulation of ES-like cells were circumvented by means of nuclear transfer (NT) technique. It was demonstrated that genetically modified fetal fibroblasts can be de-differentiated through nuclear transfer, and transgenic ES-like cells can be derived from those NT embryos. When these ES-like cells were reintroduced into preimplantation embryos they contributed to tissues of the resulting transgenic calves [14]. Unfortunately, even if livestock ES cells were comparable to mouse ES cells, the generation time and maintenance cost of multiple mosaic animals would be prohibitive to testing for germline transmission.

Sperm-mediated transgenesis

The ability of sperm cells to carry exogenous DNA into the oocyte during fertilization was first reported by Brackett and coworkers in 1971 [15]. However, the concept of sperm mediated transgenesis rested for 18 years until Lavitrano and coworkers [16] reported the use of spermatozoa as DNA carriers to produce transgenic mice. This work was met with skepticism by the scientific community being rapidly challenged by an unsuccessful attempt to replicate the experiment [17]. Since then, transgene delivery by sperm cells has been used to produce transgenic animals in a wide variety of species, including cattle [18], pigs [19, 20], rabbits [21], frogs [22] and zebrafish [23]. Sperm-mediated transgenesis is probably the most straightforward approach envisioned to date to produce transgenic animals. In the original protocol spermatozoa are incubated with the DNA containing the gene of interest followed by *in vivo* or *in vitro* insemination. DNA binds to the sperm's plasma membrane through specific DNA-binding proteins. Part of it (15-20% of the total sperm-cell bound DNA) is internalized by a mechanism mediated by CD4 molecules [24] and carried into the oocyte upon fertilization.

The most appealing characteristics of using sperm as vectors to produce transgenic animals are its simplicity (no embryo manipulation is required) and the possibility of performing mass production of genetically modified animals through *in vivo* or *in vitro* insemination of many oocytes. On the other hand, this technique also has limitations. Like pronuclear microinjection, no targeted modifications by homologous recombination can be achieved with this method due to the random integration of transgenes. Despite this drawback, the effectiveness of sperm-mediated gene transfer was recently demonstrated by Lavitrano and coworkers. They recently reported successful generation of transgenic pigs carrying a human gene (human decay accelerating factor, hDAF) [20]. In this study, approximately 80% of generated pigs had integrated the construct containing an hDAF minigene; from this 80%, approximately 53% expressed the foreign protein at different levels. There is no doubt that this success represents an important step toward production of humanized pig organs and tissues for human transplantation. If these findings are readily reproducible, sperm-mediated gene transfer may displace in short time the popular pronuclear microinjection method.

Viral vectors

Although viral vectors have been extensively used to transfer genes to somatic cells for therapeutic purposes (gene therapy), its application to produce germline transgenic animals, i.e., capable of transmission of transgene to subsequent generations, has been much more restricted. Both retrovirus [25-27] and replication-defective adenovirus [28] have been used as vectors to introduce exogenous DNA into animals. Researchers have taken advantage of natural infective mechanisms of retroviruses to develop methods to deliver transgenes into the target genome. During a retroviral infection the genetic material is released into the cells as RNA, which is subsequently reverse-transcribed to DNA and integrated into the

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host genome by retroviral integrases [29-31]. The only way the retroviral pre-integration complexes containing the transgene can reach and subsequently integrate in the host's chromatin is after nuclear membrane breakdown during mitosis [32, 33]. Therefore, retroviral integration is restricted to dividing cells. Initial attempts to produce transgenics by retroviral infection of early embryos invariably resulted in genetic mosaics caused by multiple insertion sites of the transgene [Reviewed in 34]. The short window of opportunity for the viral preintegration complexes to reach the embryonic chromatin during M-phase explains the delayed viral insertion, resulting in cell lineages with different insertion sites or no insertion at all. A significant advancement of this technique has been achieved recently by exposing metaphase II (MII) oocytes to transgene-containing retrovirus [26, 27]. Arrested oocytes are particularly appropriate because they have undergone nuclear envelope breakdown and remain at MII for a longer period of time compared with the M-phase of somatic cells. This maximizes the probability of preintegration complexes gaining access to the oocyte chromatin. Reverse-transcribed gene transfer has been used to produce transgenic cattle with high efficiency (4 out 4 born animals were transgenic) [26]; unfortunately, transgene expression in these animals was not pursued. More recently transgenic rhesus monkeys [27] and pigs [35] were reported. Although this approach seems to be highly efficient to produce transgenic animals, expression of the transgenes introduced in this way remains to be demonstrated.

Adenovirus represents an alternative to retrovirus as vectors to insert recombinant DNA into the mammalian germ line. There are some clear advantages of using adenovirus for transgenic purposes: it can infect a wide range of cell types, it can accommodate large pieces of exogenous DNA (>20 Kb), and high viral titers can be produced. Despite these comparative advantages of adenoviruses, there have been controversial reports on the effectiveness of adenoviral gene transfer into gametes or embryos. In one study germ line transgenic mice were produced by incubation of zone-free zygotes with replication-defective adenoviruses [28], however, in other studies the use of adenovirus proved to be very inefficient [36, 37], mainly due to low integration frequencies and high toxicity. Adenoviral gene transfer could be a useful alternative tool for germ line gene insertion, if investigators can find an appropriate dose of infective particles that renders maximal integration frequencies with acceptable toxicity along with improvements in viral construct design.

Somatic Cell Nuclear Transfer

Due to the absence of proven ES cells and the recent advances in nuclear transfer (NT), current emphasis for creating transgenic animals has been placed on somatic cell nuclear transfer (SCNT). Nuclear transfer is a technique that can be used to create a genetically identical copy, or a clone, of an animal. Nuclear transfer commonly involves the transfer or placement of a donor nucleus into the cytoplasm of an enucleated MII oocyte (Figure 1). Donor cells can originate from a wide variety of cell types ranging from embryonic blastomeres all the way up to adult cells. Although initial work in NT focused on using embryonic blastomeres as a donor source [38], the process was hampered by the limited number of cells available in an early embryo. More recently fetal or adult cells have been used successfully to clone all major livestock species, including sheep [39,40] cattle [41, 42], goats [43], and swine [44]. The ability to use cells that can be cultured increases the number of cells available to clone, thereby facilitating the ability to make transgenic animals. Transgenes can be introduced into cultured cells that can be used as donor cells for SCNT (Figure 1).

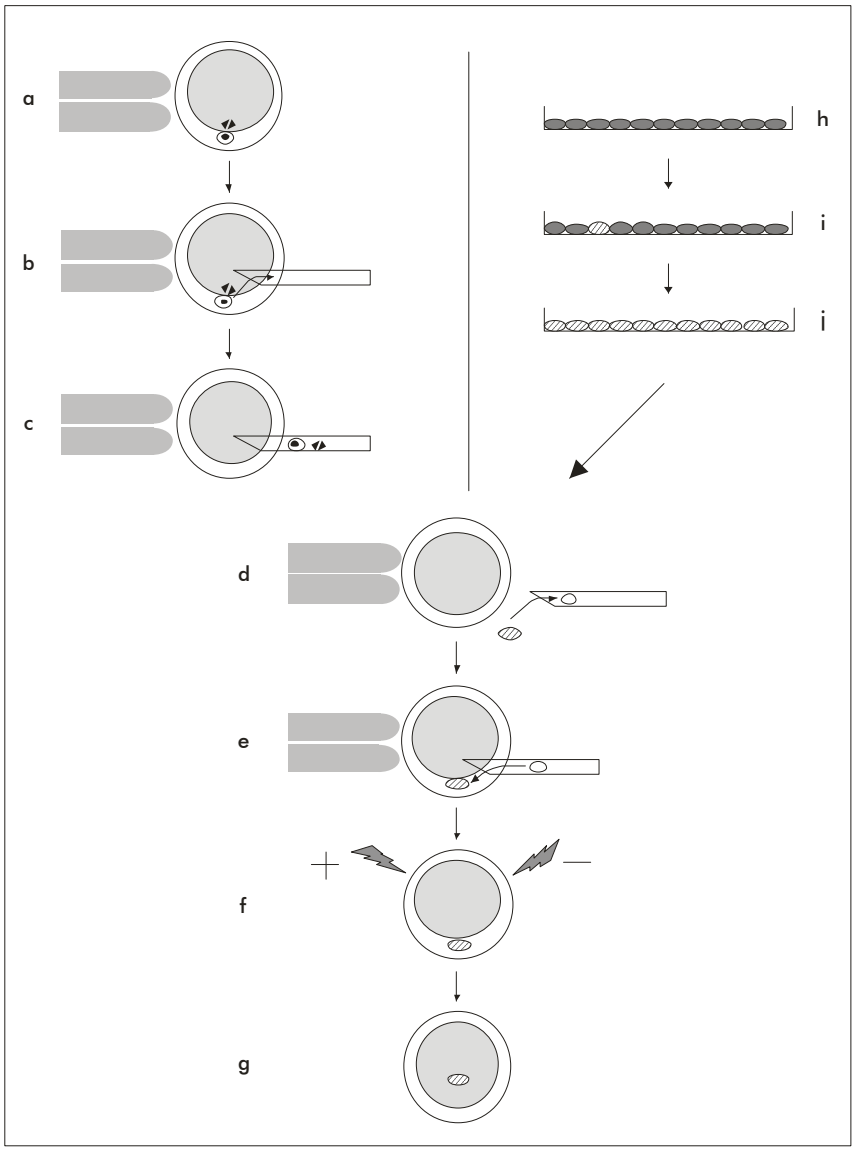

Figure 1. Steps involved in somatic cell nuclear transfer with transgenic cells. A mature oocyte is enucleated by a glass pipette (a-c) and a transgenic somatic cell (striped cell) is transferred under the zona pelucida of the oocyte (d-e). An electrical pulse is then given to fuse the two cell membranes (f), transferring the transgenic donor nucleus and cytoplasm into the oocyte (g). The reconstructed embryo is either immediately transferred to synchronized females or cultured *in vitro* before embryo transfer. Somatic cells (h) are transfected with a transgene (i) and appropriate selection is used to isolate a clonal population of transgenic somatic cells (j).

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Advantages in using SCNT for transgenesis

Somatic cell nuclear transfer has facilitated the ability to make transgenic animals by circumventing most of the shortcomings of other transgenic techniques [41]. One of the first advantages over microinjection is that the sex of the animal can be predetermined by choosing the donor material (i.e., male or female tissue). For example, the ability to select the sex of the animal would increase efficiency and facilitate the manipulation of milk production through transgenesis [45]. Second, the use of cell culture to propagate donor cells can lead to large numbers of transgenic cells that can be frozen and stored for long periods of time. In conjunction with SCNT these transgenic donor cells can eventually give rise to numerous cloned transgenic animals. Transgene structure and expression can be tested by molecular techniques, such as PCR, Southern blot analysis, fluorescence *in situ* hybridization and Western blot analysis, before initiating NT and transferring the embryo to a recipient cow with a lengthy gestation time of 9 months. The proper use of SCNT also ensures that 100% of animals produced are transgenic and that every cell of a cloned animal will have the transgene, thereby saving time and cost associated with recipient animals. The ability to use a clonal population of transgenic cells guarantees the same transgene insertion site for each clone, thus decreasing animal to animal variation in transgene expression levels. Further, transgenes can be added to a specific genetic background. For example, a female that is above average in milk protein production may be used as the genetic background (donor somatic cells) in which the transgene is placed. Lastly, SCNT allows for not only the addition of DNA at random sites but also targeted insertion of DNA by homologous recombination, which is vital in modulating specific gene expression as well as creating gene knockouts. Gene targeted pigs [46, 47] and sheep [48, 49] have been produced using SCNT techniques.

SCNT can be improved

Although somatic cell nuclear transfer has led to various accomplishments and offers many advantages over current transgenic techniques available, improvements are warranted to increase future success. First and foremost is the need to increase overall efficiency. The success rate for somatic cell nuclear transfer averages 1-3% in most animals [50]. The majority of embryos are lost during pregnancy with a 60% higher fetal loss between gestational days 35-60 when compared to embryos created through *in vitro* fertilization (IVF) [51]. In cloned cattle, there is higher perinatal loss than that observed in the general population. These losses are not due to any one anomaly, but rather to complications that can include increased birth weight (referred to as "large offspring syndrome"), pulmonary abnormalities, respiratory problems, and metabolic deficiencies [reviewed in 52]. It should be noted that these complications were first observed in blastomere-derived NT offspring and reported by Willadsen and coworkers in 1991 [53]. Placental abnormalities are also common in bovine NT offspring clones as first observed in bovine pluripotent cell NT pregnancies [54]. Term NT placentae often have large but few placentomes, edema and hydroallantois [55]. Most SCNT calves survive early postnatal development and seem to be quite normal and fertile [56].

Cloning in the pig has had to cope with some limitations associated with *in vitro* embryo technologies in this species, posing additional challenges for researchers. The lack of reliable *in vitro* culture systems for pig oocytes and embryos has favored the use of *in vivo* mature oocytes as recipient cytoplasts [44, 57-60] and surgical transfer of early-stage cloned embryos to the oviduct of surrogate mothers [44, 59, 60] to avoid the deleterious effects of extended exposure to *in vitro* conditions. However, cloned piglets generated from in vitro matured oocytes [46, 47, 61-65] and embryos cultured for short periods of time *in vitro* before transfer [57] have been reported. In most laboratories seeking term development of cloned pigs, high numbers (60-150) of NT embryos are transferred by surgical methods into the oviduct of recipient animals. Despite efforts to minimize the negative influences of *in vitro* manipulation on the embryo's developmental potential, overall efficiency in pig cloning remains very low. A major component of the low efficiency reported is the poor viability of NT embryos. An additional potential source of embryonic loss in pigs is the presence of insufficient number of viable embryos in the uterus around day 11-12 to trigger antiluteolitic mechanisms. In the pig, pregnancy maintenance depends on the presence of at least 4-5 embryos during the critical window of maternal recognition of pregnancy [66]. Therefore, if less than 4-5 cloned embryos survive by day 11-12 of gestation, pregnancy will not be established. Hormonal supplementation or co-transfer of partenotes with NT porcine embryos has been proposed as strategies to rescue pregnancies with limited numbers of viable embryos [60, 67].

Our knowledge of developmental abnormalities associated with cloned pigs is scarce, mainly due to the limited number of documented cloned pigs and the lack of systematic studies in this area. Reports of near normal birth weights of cloned piglets [63-65] and normal placentae [64] suggest that the large offspring syndrome, a common finding in ruminant clones, is not associated with pig cloning.

Most, if not all, developmental abnormalities in cloned animals are believed to originate in deficient or inadequate resetting of the developmental clock present in the differentiated nucleus used as donor of the genetic material. The mechanisms and factors that affect the progression of this process, known as nuclear reprogramming, have not been completely elucidated, but the identification of these factors and their subsequent manipulation would increase cloning efficiency. In the meantime, improvements in cloning efficiency may be expected by careful selection of the starting biological materials (i. e., recipient oocytes and donor cells) used to produce the cloned embryos [68].

Nuclear Reprogramming

Current research to decrease the number of pregnancy losses and thus increase the efficiency of NT has recently focused on understanding nuclear reprogramming. Nuclear reprogramming can be loosely defined as a set of epigenetic changes (those not involving a change in DNA sequence) required for a nucleus to

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change developmental fates. During the NT process the oocyte changes the fate of the donor nucleus from its original status (e.g., skin, granulose, etc.) to that of a zygotic nucleus. Improper nuclear reprogramming of the donor nucleus in the oocyte is thought to be the major reason of failure in the cloning process. In cattle, several studies have shown that methylation [69-71] and gene expression [72-74] are abnormal in NT embryos when compared to *in vivo*-and *in vitro*-generated counterparts. There seems to be some species-specific differences in epigenetic reprogramming of the cloned donor genome. Using bisulfite-sequencing technology, Kang and coworkers [75] have found that the patterns of genomic demethylation in repetitive sequences of the cloned donor genome were similar between cloned and IVF pig embryos. These findings contrast with the maintenance of hypermethylation of satellite sequences observed in bovine cloned embryos up to blastocyt stage [76]. Recently, Enright and coworkers showed that histone acetylation levels in cells changed with respect to the stage of cell, cell type and numbers of cell passages, suggesting that histone acetylation could be a factor in improper nuclear reprogramming in NT [77]. Epigenetic abnormalities caused by the NT process, however, are not passed on to the offspring of cloned animals, as shown in mice [78].

A recent idea that may increase efficiencies in nuclear reprogramming during the NT process is the exposure of donor cells to remodeling factors through *in vitro* systems before NT is initiated [79]. The addition of *Xenopus laevis* egg extracts was shown to successfully inhibit transcription, which has been hypothesized to facilitate nuclear reprogramming [79]. In the same line, Sullivan and coworkers [80] have recently introduced the concepts of *in vitro* nuclear remodeling and chromatin transfer. In this novel system, permeabilized donor cells are exposed to a mitotic cell extract in vitro, followed by transfer of condensed chromosomes into enucleated oocytes prior to activation. There is evidence that this treatment initiates remodeling of mammalian somatic nuclei in vitro prior to cloning procedures [80]. Although chromatin transplantation was successfully used to generate live cloned calves [80], the superiority of this new cloning procedure over the classic nuclear transfer technique has not yet been demonstrated.

A complementary approach to improve the efficiency of producing viable cloned offspring is through careful selection of recipient oocytes and donor cells that will produce cloned embryos with functionally reprogrammed nuclei.

Selection of recipient oocytes

Metaphase II arrested oocytes are considered the cytoplast of choice for nuclear transfer procedures [81]. High level of maturation/meiosis/mitosis promoting factor (MPF) present in MII oocytes has been associated with successful nuclear reprogramming. Since MPF activity is maximal at both metaphase I (MI) and MII [82],, in theory, MI oocytes might be suitable as cytoplast recipients as well. However, the rate of blastocyst formation by embryos reconstituted with somatic cells and MI oocytes was significantly lower than that of embryos reconstituted with MII oocytes [83]. This result suggests that MII oocytes rather than MI oocytes are more appropriate recipients for production of differentiated cell-derived cloned embryos, and that presence of MPF is not sufficient for maximum developmental ability of reconstructed embryos.

Both *in vivo* –and *in vitro*– matured oocytes have been used as recipients for production of cloned animals from differentiated cells. In vitro-matured oocytes have been commonly used for production of cloned calves [41, 84-86]. In pigs the lack of a robust in vitro maturation system has favored the use of in vivo-matured oocytes as recipients [44, 57-60]. The use of in vitro matured oocytes from sow [46, 47, 62] or prepuberal gilt [61, 63, 64] ovaries has been limited to a few laboratories. Improvements in in vitro maturation systems for livestock oocytes will provide an abundant and stable supply of recipient oocytes from slaughter animals. Regardless the origin of the oocytes, a high variability in quality has been commonly reported, suggesting that methods to identify and select superior oocytes for use in cloning are needed.

Another consideration is the source of oocytes, i.e., derived from prepuberal animals versus adult animals. It is accepted that the oocytes from prepuberal animals have reduced developmental competence compared with that of oocytes from adult animals, as indicated by the decreased blastocyst formation after in vitro fertilization [62, 87-89]. Furthermore, oocytes derived from sows and then matured in vitro were better able to support the development to the blastocyst stage of cloned embryos than were similarly matured prepuberal gilt oocytes [62]. However, the ability to support development into offspring of cloned embryos has not been directly compared for oocytes derived from prepubertal and adult animals in either cattle or pigs.

Based on the asynchronous meiotic progression observed in porcine oocytes during in vitro maturation, we have described an approach to identify a subpopulation of oocytes with enhanced developmental capacity [90]. Rapidly matured oocytes, i.e., oocytes that reach MII stage by 24 h of culture, had significant higher developmental ability to blastocyst stage compared with 42-h matured oocytes [90]. Use of the rapidly matured oocyte subpopulation in cloning would represent a novel way to improve developmental rates for cloned offspring.

Selection of donor cells

Various cell types, such as embryonic cells, fibroblasts, mammary gland cells, cumulus cells, oviductal cells, leukocytes, granulosa cells, germ cells, and liver cells have been used as donors for production of cloned animals [91]. However, it is still unclear if which cell type is the most successful for nuclear transfer into oocytes. It may be difficult to show significant differences in the rate for development into cloned animals among different cell types because of the overall low cloning efficiency. On the other hand, the ability to support development of cloned embryos differs among donor cell lines [92] even if they are derived from the same tissue or organ [86]. The differences among cell lines may be due to epigenetic effects, because even within a pri-

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mary cell culture, the generation of cell lines from that culture shows that some lines are more suitable than others as donors for cloning [92]. Modifications that occur during primary cell culture may result in genomes that are either more or less capable of being reprogrammed. The profiling of gene expression patterns with DNA microarrays will allow direct comparison of thousands of genes simultaneously among cell lines allowing for efficient selection of those cells lines that are best suited, based on specific markers, for use as nuclear donors.

Some studies have reported improved postimplantational survival of mouse clones originated from embryonic stem (ES) cells [93-95] what might indicate that nuclei from undifferentiated cells could be more amenable to correct reprogramming. Our laboratory is currently using adult mesenchymal stem cells collected from pig bone marrow as nuclear donors to produce cloned embryos [96]. If these cells require less extensive nuclear reprogramming compared with terminally differentiated cells normally used as karyoplasts (e.g. fibroblast), then improved developmental rates would be expected. Use of these or other adult stem cells as nuclear donor may represent an avenue to improve the overall efficiency of cloning.

Another variable affecting cloning outcome that has received much attention is the stage of the donor cell cycle at the moment of nuclear transfer outcome. Quiescent donor cells arrested in G0/G1 phases of the cycle have been commonly used to produce cloned animals [40-44, 58, 62, 84, 85, 97, 98]. However, the specific methods used to arrest the cells in G0/G1 phases significantly affected fetal survival to term and neonatal survival [85]. Serum starvation and growth arrest induced by contact inhibition are two methods used to synchronize cells in the G0/G1 cell cycle stage. Cloned animals from cells arrested at different stages of the cell cycle by specific cell cycle inhibitors have been reported [61, 99-101]. The addition of roscovitine, a cyclin dependent kinase 2 inhibitor, to donor cells successfully synchronized donor cell cycle and increased survivability of cloned calves and thus may increase the nuclear reprogramming capacity of the donor cells [85]. Studies such as this give evidence that increasing NT efficiency is possible and will only improve in the future as we expand our knowledge of the basic mechanisms that govern nuclear reprogramming.

Cell lifespan

Another difficulty in using SCNT to create transgenic animals is that unlike ES cells somatic cells have a finite lifespan. Bovine fetal fibroblast cells, which are commonly used to make transgenic cattle, have 30-50 population doublings (PDs) before senescence [102]. Although Cibelli and coworkers were able to create transgenic calves from a clonally derived transgenic cell line with a capacity for 30 PDs [41], Clarke and coworkers have estimated that gene targeting requires around 45 PDs [103]. Recent evidence has shown that the doubling capacity can vary widely between cell lines and that genetics may play a major role in determining this capacity, illustrating the importance of picking the right cell line to work with [104]. Even though there has been some success using late passage cells for NT [105, 106] the extended cell culture necessary for clonal propagation of a transgenic cell most likely leads to senescence. For example, out of the 25 gene targeted colonies identified by Denning and coworkers, 23 senesced before they could be expanded for NT [107].

Development of strategies to increase the lifespan of cultured cells would expand the window of opportunity for gene targeting. For example, addition of L-carnosine in the culture medium extended the lifespan of human fetal foreskin or lung cells by 10 PDs [108]. Agents like the superoxide dismutase mimetic MnTMPyP [109] or culture under reduced O_2 tension (2%) may contribute to postpone senescence in cultured cells by reducing oxidative damage to DNA [110]. A completely different approach involved the generation of pig fibroblasts expressing UP1 [111], a shortened derivative of heterogeneous nuclear riboprotein A1, known to protect and elongate telomeres in mammalian cells through direct interaction [112, 113]. Although UP1-expressing cells showed an extended lifespan, this was associated with high percentage of cells (60-70%) bearing abnormal karyotype [111]. Another approach recently suggested to overcome the problem of senescence of somatic cells is the introduction of TERT [114]. TERT is the catalytic component of telomerase and has been shown to immortalize cell lines when it is expressed [115]. Even cell lines with active TERT that have been passaged numerous times show no sign of phenotypic or chromosomal abnormalities that are hallmarks of a transformed cell line [116, 117]. However, when these transformed cells were used as nuclear donors, they were unable to support development to term of cloned sheep embryos, suggesting that TERT-immortalized cells cannot be completely reprogrammed [118]. A reversible system has been reported, making it possible to express TERT until genetic modifications are completed and then silencing it before NT [119]. The future use of such a system would facilitate transgenic work in primary cell cultures and improve the chances of deri ving SCNT offspring from these modified donor cells.

Combining transgenics and SCNT

Although SCNT efficiencies can be improved, it is currently considered one of the most promising methods to produce transgenic livestock [120]. The number of studies reporting successful generation of transgenic farm animals by SCNT is rising and commercial companies are adopting this approach to produce their transgenic founders. The success of SCNT in creating transgenic livestock has provided a means for generating gene-targeted animals. McCreath and coworkers produced the first gene-targeted mammal other than mouse when they inserted a transgene into the ovine a1 procollagen locus [48]. The first transgenic cattle produced through SCNT were transgenic for a fusion α-galactosidase-neomycin gene and demonstrated that cattle could be produced by using transgenic culture cells for SCNT [41]. Gene targeting success has also been achieved in pigs [46, 47, 107]. It is conceivable that the number of gene targeting studies will multiply as the amount of genetic sequence information increases.

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Models for human diseases

The success of SCNT has provided a means by which the generation of mammalian animal models other than mice is now available. The mouse is the usual animal model of choice due to factors like short generation time, low maintenance and cost, and ease of availabi lity. Although livestock, like pigs and cattle, do not have these attributes that one might consider as a prerequisite for an animal model, they do have similarities to humans that may make them great animal models for human diseases. For instance, factors such as lifespan, size, and possibly genomic organization [121, 122] are all more similar between cattle or pigs and humans than between mice and humans. Although no one would dispute the impact that the mouse has had as a model on understanding human disease, there are diseases like cystic fibrosis in which the mouse does not display all of the human phenotypes of the disease [123]. Due to similarities between the lung in sheep and humans, sheep have been suggested as a possible model for cystic fibrosis [123]. Similarities between humans and livestock may help advance our understanding of certain diseases and thus successfully alleviate them.

Xenotransplantation

The gap between the demand for and the availability of human organs for transplantation is growing; a tendency that is not likely to reverse in the near future. The number of patients on waiting lists for organs has been growing every year, reflecting both an increased demand for organs for transplantation and a pronounced shortage of donors. In the United States during 2002 only one-third of patients on waiting lists actually received an organ on time [124]. During the same period, more than 6,000 patients were reported to have died before a suitable organ was available [124]. Pigs have long been considered as an alternative source of organs for xenotransplantation (i.e., transplantation of organs/tissues between different species, e.g., from animals to humans) to satisfy this increasing demand. Pigs seem to meet most of the requirements for an ideal animal donor: they are anatomically and physiologically similar to humans with organs of appropriate size, they are prolific, and they can be maintained under specific pathogen-free conditions. However, there are barriers to pig-to-human xenotransplantation, one of the preeminent challenges being to avoid the hyperacute immunological rejection of the grafted tissue. When organs or tissues are transplanted between discordant species like pig to human, the host's immune system initiates a fast reaction known as hyperacute rejection (HAR) [125]. In this reaction, naturally occurring human xenoantibodies [126, 127] react with Gal α1,3 Gal residues present on the surface of cells of the foreign tissue, followed by complement mediated vascular damage that completely shuts down the xenograft [128]. In most animals, including pigs, α1,3 galactosyl transferase catalyzes the addition of galactose residues to glycoproteins and glycolipids [129] targeted to the cell plasma membrane. Unlike pigs, in humans and Old World primates the α1,3 galactosyl transferase gene is inactive, therefore there is neither the enzyme nor the Gal α1,3 Gal epitope [130].

Since HAR is initiated by antibody recognition of Gal α1,3 Gal followed by complement mediated tissue damage, there are two different strategies to avoid or reduced HAR: knocking down the Gal α1,3 Gal from pig cells and/or inactivation of the complement pathway. It is conceivable that through genetic modification it would be possible to generate "humanized" organs that would not be rejected. To this end, a combination of cell-based transgenesis and nuclear transfer has enabled the production of pigs in which one [46, 47, 131] or both alleles [132] for α1,3 galactosyl transferase were knocked down. Pigs bearing transgenes encoding major components of the complement regulatory pathway have been produced by pronuclear microinjection [133, 134] or sperm-mediated transgenesis [20]. Expression of functional complement regulatory proteins by transgenic pig organs has led to extended survival of xenotransplanted primates [135-138]. However, the complexity of the immunological rejection process will probably require combinations of genetic modifications in the same animal in order to obtain organs or tissues that would be accepted by the host. In this context cloning has opened the possibility of adding genetic modifications to an already transgenic background [131]: cells from a transgenic animal can be collected, genetically manipulated *in vitro* and used in nuclear transfer to produce transgenic clones.

Agriculture applications

The development of SCNT along with remarkable progress in gene mapping and genome sequencing endeavors in livestock will open a new set of possibilities for introduction of precise genetic modifications for agricultural applications. The host of possibilities includes progress in areas like milk production, growth rate, carcass composition, reproductive performance, and disease resistance. We are now witnessing how some of these potential opportunities are being put into practice. One recent study used SCNT to create calves transgenic for two casein genes involved in milk protein production [45]. When the resulting calves were induced to lactate, the levels of β-and κ-casein protein in milk were altered, suggesting that the transgene did influence milk content. Another example of a practical application of SCNT combined with cell-based trangenesis is the production of 4 cloned calves carrying a genetic modification that would render them resistant to bovine spongiform encephalopathy (BSE) [139].

Production of pharmaceuticals

Possibilities in biomedical applications include the production of important therapeutic proteins in milk such as α1 antitrypsin for cystic fibrosis, blood clotting factors like antithrombin III, factor IX and fibrinogen for bleeding disorders, and human serum albumin, which could be useful for treatment of burns [140]. Although such possibilities originated when transgenic work was started using pronuclear microinjection, these ideas are now being realized through SCNT [120].

Conclusions

Somatic cell nuclear transfer has provided a new way to create transgenic animals without the drawbacks of

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past transgenic techniques. Although the combination of transgenic technology and SCNT is still in its infancy, the possibilities it provides are almost limitless. Further research focused on improving our understanding of nuclear reprogramming will ultimately increase the efficiency of NT and facilitate the creation of transgenic SCNT animals. Improvements in DNA transfection, colony selection and extending cell lifespan will also increase success in creating SCNT transgenic livestock.. The future possibilities of using artificial chromosomes as vectors in donor cells [141] and increasing the number of oocytes available for NT through ES cell differentiation [142] could also boost transgenesis in farm animals. All of these improvements will facilitate the realization of goals for transgenic livestock in agriculture and biomedicine.

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