Advancements in somatic cell nuclear transfer and future perspectives

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Implications

- Reprogramming of somatic DNA by oocytes is inadequate and thus often causes an abnormal epigenetic status in embryos derived from somatic cell nuclear transfer (SCNT). Certain compounds are used to aid the reprogramming and increase the efficiency.
- High-throughput gene expression analysis can reveal responses to environments to which embryos are exposed in vitro and therefore provide clues to improve culture conditions. This information has been applied to improve the culture system.
- Somatic cell nuclear transfer can produce genetically engineered animals if somatic cells with mutations are used. Development of mega-nucleases permit genetic engineering of somatic cells at high efficiency and produce genetically engineered animals for various purposes through SCNT.

Key words: cloning, pig, somatic cell nuclear transfer

Introduction

Since the birth of the cloned sheep, Dolly, the technology of SCNT has been used to clone various species (reviewed by Niemann and Lucas-Hahn, 2012). Application of SCNT is not confined to producing clones but has a variety of applications (Figure 1). For example, SCNT can be used to preserve valuable genetic backgrounds that may face extinction. It can also be used to produce embryo-derived stem cells from a somatic cell by reprogramming somatic DNA. This can be a resource for regenerative medicine as patients with degenerative diseases could potentially generate stem cells to cure their diseases. In addition, SCNT can be used to produce genetically engineered animals by using donor cells that have been modified. Because there is no evidence of authentic embryonic stem cells in non-rodent species, the use of SCNT has been the only way to produce animals with specific genetic modifications. It has been over a decade since the first production of cloned pigs from adult cells using SCNT (Polejaeva et al., 2000), and SCNT is routinely used to produce cloned pigs for various uses. However, even with routine use, the efficiency of SCNT is still poor. Here we will summarize advancements in SCNT technology, especially in pigs, and discuss its future.

Reprogramming of Somatic Donor Nuclei

Normal development results from a carefully orchestrated sequential expression of genes. The regulation of gene expression is thought to be a result of different factors associated with the DNA. These associations modify the three-dimensional structure of the DNA, making individual genes more or less likely to undergo transcription. Since the sequence of bases in the DNA does not change, the factors that regulate gene expression are said to be epigenetic or above genetics. Examples of epigenetic regulation include DNA methylation and histone methylation (Zhao et al., 2010). During fertilization, the oocyte regulates the epigenetic status of paternal- and maternal-derived DNA to properly initiate development. This is a very important step, and interference in this process can lead to embryo lethality. Oocytes typically only actively reprogram paternal DNA and not maternal DNA, as they have different epigenetic statuses. During SCNT, the oocyte reprograms the DNA of a donor nucleus that contains both maternal and paternal contributions by changing its status from a somatic state to an embryonic state. This remarkable change is due to the unique combination of DNA-associating factors present in the oocyte. Since the production of clones is possible, it is known that this ability can successfully carry out the change; however, the process is not very efficient. Only about 1% of SCNT embryos routinely fully develop into an animal. This is because the reprogramming factors in the oocyte are not designed to handle the epigenetic status of the somatic cells. Therefore, this reprogramming of donor DNA by the oocytes often results in an incomplete transition, thus causing abnormal development after SCNT (reviewed by Whitworth and Prather, 2010). Multiple studies show that after reprogramming by the oocytes, SCNT embryos have different gene expression patterns when compared with in vivo-derived or in vitro-fertilized (IVF) embryos. Although we can observe the abnormality in SCNT-derived embryos, we still do not know the main cause for this developmental failure. We simply do not know the detailed mechanism(s) of nuclear reprogramming during SCNT. But based on observations, one of the main suggested defects of SCNT-derived embryos is the abnormal epigenetic status due to inadequate remodeling of the donor nucleus.

Studies show that SCNT-derived embryos and animals have aberrant patterns of DNA methylation, an epigenetic tag on the chromosome which can affect gene expression. This special tag is important for normal development as abnormal DNA methylation is lethal and stalls embryo development (Reik et al., 2001). Dynamic changes in DNA methylation are observed in SCNT embryos as in IVF embryos, but they show greater levels of DNA methylation on the euchromatin in cloned morula stage embryos when compared with IVF- and in vivo-derived embryos (Kang et al., 2001). In addition, later stage SCNT-derived embryos typically show a lack of lineage-specific DNA methylation patterns. This is specifically
seen when embryos develop into the morphologically distinguishable state known as the blastocyst stage, where two different types of cells are present: inner cell mass (ICM) and trophectoderm (TE). The DNA of ICM cells is highly methylated when compared with TE cells; however, there is a lack of these lineage-specific DNA methylation patterns in SCNT-derived embryos (Deshmukh et al., 2011). Aberrant patterns of DNA methylation are also seen in extraembryonic tissues from SCNT-derived embryos. Placenta derived from dead cloned pigs had abnormal expression of imprinted genes and level of methylation compared with live clones or control pigs (Wei et al., 2010). This suggests that the developmental defects in SCNT-derived embryos result in abnormal DNA methylation patterns observed at post-implantation stages. An abnormal pattern of gene expression was also reported in young clones, and the level of DNA methylation in the clones was greater than the controls (Park et al., 2011). Based on these findings, we can say abnormal patterns of DNA methylation are observed in the pre-implantation and post-implantation embryo, as well as in the neonate. As mentioned above, abnormal epigenetic marks, such as DNA methylation, could be the main reason for low efficiency after SCNT as DNA methylation can regulate gene expression. Although these epigenetic defects are detected in clones, their progeny, interestingly, do not typically show any sign of abnormalities (reviewed in Prather et al., 2004). This indicates that proper epigenetic remodeling occurs during the development of germ cells (sperm or eggs) in the clones, and the sperm or eggs that they produce are normal.

These aberrant patterns of DNA methylation are not only found in SCNT-derived embryos and clones, but also in in vitro-produced embryos (Bonk et al., 2008). One possible reason for the abnormality is due to the in vitro culture conditions. In vitro culture conditions are known to affect the epigenetic status of the embryos, and thus result in abnormal downstream development. Therefore, in addition to a better understanding of the epigenetic remodeling that must occur at the time of nuclear transfer, improvements in the embryo culture system are also needed to increase the efficiency of SCNT.
Methods to Improve Efficiency of SCNT by Assisting Nuclear Reprogramming

Aberrant DNA methylation patterns in SCNT embryos and clones suggest inadequate reprogramming of the donor DNA by the oocyte. This is not surprising as oocytes are designed to reprogram sperm-derived DNA, not donor cell DNA. To assist the reprogramming process, different approaches have been made. Because previous studies suggested that DNA methylation was commonly misregulated in SCNT embryos, a compound that can reduce the level of DNA methylation has been used; 5-aza-2’-deoxycytidine (5-aza-dC) is a chemical that can reduce the level of genomic methylation by inhibiting DNA methyltransferases. However, the beneficial effect in pigs is not consistent (Ning et al., 2012).

Another approach to change epigenetic marks in donor cells or SCNT embryos is with histone deacetylase (HDAC) inhibitors. Inhibition of HDAC can increase the global level of acetylation in SCNT-derived embryos, leading to more active gene expression during development. Different HDAC inhibitors have been shown to improve the development of SCNT embryos in many species. When SCNT embryos are treated with 6-(1,3-dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide (Scriptaid) for 14 to 16 h, the efficiency of SCNT embryo development was increased. Moreover, inbred miniature pigs could be cloned that had failed to be cloned without the compound (Zhao et al., 2009). Also, treatment of SCNT-derived embryos with Scriptaid could correct some misregulated genes in SCNT-derived embryos when compared with in vivo and IVF embryos (Whitworth et al., 2011). This demonstrates that certain chemicals can assist the oocyte in reprogramming the somatic DNA. Currently, many groups are focused on evaluating different HDAC inhibitors to identify an optimum inhibitor for the SCNT process.

Treatment of SCNT embryos with HDAC inhibitors clearly increases the efficiency of SCNT embryo production; however, because we still do not fully understand the mechanism of the SCNT process finding the specific mechanism(s) of exactly how the HDAC inhibitors help is still under investigation. More research on how the oocyte reprograms donor DNA during SCNT may guide us to discover a more efficient way to produce SCNT embryos.

Improvements in Culture

Improvements in culture in vitro have contributed to the increase in efficiency of SCNT. Until the early 1990s, pig embryos were considered difficult to culture in vitro. Since then, there have been significant improvements in the culture system; yet pig embryo culture is still considered suboptimal. Traditionally, embryo metabolism studies lead to designing culture systems for embryos. Most conventional culture media are designed based on these studies; however, recent development in high-throughput gene expression analysis have allowed us to understand which genes are misregulated in embryos under in vitro conditions, thus permitting improvements in the culture system. We have focused on analyzing gene expression patterns among embryos from various origins to better understand how embryos are stressed under in vitro conditions (Whitworth et al., 2005; Bauer et al., 2010). This approach could lead us to identify the factors that are demanded by the embryos under in vitro conditions. For example, from our RNA sequence study between in vivo- and in vitro-derived embryos, we have identified that arginine transporters are highly up-regulated in in vitro-derived embryos. This suggests that embryos need more arginine, thus up-regulating the level of arginine transporters. Based on this finding, we have added arginine into our current culture media and have improved the development of the embryos in these culture conditions.

When compared with IVF-derived embryos, SCNT-derived embryos are generally considered fragile. Therefore, conventional wisdom was to transfer SCNT embryos into the surrogate as early as possible. Pigs have a large litter size compared with other species; thus, it may not be an issue. However, there can be some disadvantages of transferring embryos at an early stage. For example, the quality of SCNT embryos cannot be measured before transfer. Because different sources of cells and oocytes are typically used for SCNT, there is variation in development. Transferring embryos at this early of a stage may result in transferring poor quality embryos that do not lead to a pregnancy. In addition, transferring too many embryos could lead to reduction in productivity in pigs (Berthelot et al., 2007). By analyzing the gene expression profile, we noticed that a specific receptor, granulocyte macrophage colony-stimulating factor (GM-CSF),
was highly up-regulated in in vitro-derived embryos compared with their in vivo counter parts. By introducing a specific cytokine, GM-CSF, responsible for stimulating the receptor, development of IVF- and SCNT-derived embryos in vitro could be improved. Addition of this cytokine produced more piglets than the early-stage transferred embryos (Lee et al., 2013). This is a good example of how molecular analysis can be applied to improve the embryo culture system.

Understanding the metabolism of embryos could lead us to develop a better culture medium for embryos. From various gene expression profiles and comparative studies with mice, it has been suggested that embryos have a similar metabolism to cancer cells (Krisher and Prather, 2012; Redel et al., 2012). Similar studies utilizing high-throughput analysis would lead us to better understand embryo metabolism and therefore enable us to design a culture system that better suits embryo development. Improvements in embryo culture will increase the availability of high quality embryos and thus increase the application of SCNT. Recent advances in molecular analysis suggest a bright future in the technology.

**Utilization of SCNT for Production of Genetically Engineered Pigs**

Pigs are genetically very similar to humans, which in many cases, makes them an excellent model for biomedical studies (reviewed by Whyte and Prather, 2011). For example, transgenic pigs could be a resource for replacement human organs. There is a high demand for human organs, but the supply is always insufficient. To use pig organs for replacement of poor human organs, certain genes responsible for immunological rejection in the pig need to be altered. When organs from such modified pigs were transplanted into primates, the hyperacute rejection usually observed was eliminated (Kuwaki et al., 2005). This demonstrates that genetically engineered pig organs may be able to overcome the immunorejection hurdles observed with xenotransplantation. Another example of the application of transgenic pig as human models is the study of human diseases. Transgenic rodent models are generally used to study human diseases due to their convenience; however, rodent models cannot accurately represent all human diseases because of the differences in physiology and life span. For instance, symptoms of cystic fibrosis disease in humans are not shown in rodent models that have the causative mutation; however, the symptoms are present in porcine models that have the causative mutation (Rogers et al., 2008).

The value of genetically engineered pigs has been acknowledged in various fields, although application of these pigs is limited by the low efficiency of producing the founders. Specifically, conventional genetic engineering of somatic cells is considered challenging and inefficient, and targeting mutations in somatic cells (i.e., gene targeting) is extremely difficult. Recent development in mega-nucleases such as zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN) demonstrated that gene targeting can be efficient. These mega-nucleases have the ability to recognize and bind to specific sequences on the chromosome and induce a double strand break (DSB) at a specific location. This DSB can be lethal if unrepairred; therefore, repair mechanisms are activated to repair it. Two mechanisms, non-homologous end-joining (NHEJ) and homologous recombination, are triggered after the DSB, and the NHEJ pathway can generate mutations at a specific location by introducing base pair insertions or deletions. Similar changes in the coding sequence of a gene can render the gene non-functional. These mega-nucleases were used in multiple species and could generate mutations in cultured cells (Gaj et al., 2013). In rodents, targeted mutations were also reported when mRNA of these mega-nucleases were introduced into early embryos. However, until now, this has only been reported in rodents and has not been demonstrated in other species. These mega-nucleases have been used to create mutations in cultured cells, and then these cells were used for SCNT to produce pigs. Some of the mega-nucleases were very effective and thus generated biallelic modification. Biallelic modification represents a significant improvement in genetic engineering as conventional gene targeting generally only creates monoallelic modifications. Both ZFN (Hauschild et al., 2011) and TALEN (Carlson et al., 2012) were used to produce transgenic pigs that could be used for xenotransplantation and for cardiovascular disease studies, respectively. As mentioned above, these mega-nucleases induce random mutations at specific genomic sites due to an inaccurate repair process after the DSB. We have reported utilizing mega-nucleases with donor DNA to introduce specific mutations rather than relying on random mutations caused by the mega-nucleases (Kwon et al., 2013). These cells were used to produce pigs that have the potential to be used for xenotransplantation.
A scheme of the generation of induced pluripotent stem (iPS) cells. Isolate and culture host cells.

1. Introduce the ES specific genes (iPS factors) into the cells by using retrovirus vector. Red cells indicate the cells expressing the exogenous genes.
2. Harvest and culture the cells according to the method for ES cell culture using feeder cells (gray).
3. A subset of the cells generates ES-like colonies, that is, iPS cells.
4. A small subset of the transfected cells become iPS cells and generate ES-like colonies.

Since these mega-nucleases are very effective in producing genetically engineered pigs, we expect to see more reports of modified pigs for application to various fields. We have generated multiple lines of engineered pigs using the mega-nucleases, and the entire production of each line took less than 6 months. This represents a great advancement in genetic engineering in pigs, and considering the effectiveness, more complicated genetic engineering is now possible in pigs. In addition, these mega-nucleases can generate genetic mutations without any trace of foreign DNA (i.e., no DNA footprint). Mega-nucleases have the potential to revolutionize the future of genetic engineering in domestic animals.

**SCNT for Therapeutic Cloning**

Somatic cell nuclear transfer has ability to reprogram cells into an undifferentiated state. This unique oocyte ability permits SCNT to be used to generate stem cells from somatic cells. Once somatic cells are dedifferentiated and returned to the embryonic state, the ICM of the embryo can be isolated and potentially produce cells that are like embryonic stem cells. In theory, by generating stem cells from the somatic cells of a patient, the stem cells could be a source to cure degenerative diseases. Since the stem cells are derived from the cells of the patient, there should be no immunological rejection once introduced into patients. Therefore, this therapeutic cloning was considered to be an ideal cure for degenerative diseases such as Parkinson’s or Alzheimer’s disease. This technology has been demonstrated in multiple species, including pigs (Munsie et al., 2000; Kim et al., 2010). Only recently, a study showed that stem cells could be generated from SCNT-derived human embryos (Tachibana et al., 2013). Besides the ethical issue of the technology, this suggests that SCNT could be used to produce embryonic stem cells that have potential application in regenerative medicine.

SCNT was the main technology in fully reprogramming somatic cells until the invention of induced pluripotent (iPS) cells (Takahashi and Yamanaka, 2006). iPS cells are somatic cells reprogrammed into stem cells by introducing specific factors: Oct4, Sox2, Klf4, and c-myc. When the technology was first introduced, many believed that it would replace the need for SCNT as generation of iPS cells is ethically less controversial. However, there are some safety concerns with iPS cell technology as viral vectors are often used to deliver the reprogramming factors (Mayshar et al., 2010). Understanding both SCNT and iPS cell technologies would guide us to better utilize both technologies.

**Future Perspectives**

Advancements in pig SCNT will facilitate various achievements currently beyond our imagination. Recent modifications have increased the efficiency of SCNT to make it commonly applied; some of the cell lines we use for SCNT yield up to 80% efficiency in early pregnancies. Since pigs can be a useful biomedical model, the NIH recognizes them as an important model for human diseases and has established a National Swine Resource and Research Center (http://nsrrc.missouri.edu/) at the University of Missouri. The center is founded to serve as a genetic resource for the biomedical community. The recent developments described above will assist us in producing genetic modifications for a variety of purposes, and we expect to see more applications of pigs to study human diseases.

Until now, most of the genetic modifications in pigs have been focused on biomedical fields because genetically engineered pigs are banned from entering the food chain, and because more funding is available in biomedical fields. Future studies will focus on generating transgenic pigs with precise modifications to minimize any potential side effects. Thus, we may see transgenic pigs with increased productivity for agriculture purposes. One example is disruption of myostatin. In cattle, a naturally occurring mutation of the gene results in double muscle formation, thus increasing the production of meat. Application to pigs may result in animals that grow faster and produce more meat.

As show in this review, there are many advancements occurring in SCNT technology. However, there are still unknown factors about SCNT embryos, and the detailed mechanism of reprogramming by the oocyte is not well characterized. Research clarifying the process of SCNT would provide us with more pathways to utilize SCNT for various purposes. It is hard to predict how this technology will evolve, but we can only expect that the technology will become more applied in various fields.

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