Can Somatic Cell Nuclear Transfer Produce Human Pluripotent Stem Cells for Regenerative Medicine?

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Abstract

In the last half a century, researchers and scientists discovered the application of somatic cell nuclear transfer (SCNT) to clone mammalian embryos to produce a line of pluripotent stem cells for medical and laboratory use. This is a breakthrough technology that is applied to stem cell research, regenerative medicine, and cloning. Somatic cells are non-germ cells that are differentiated but provide the nuclei that are transferred to enucleated oocytes. The replacement of the nuclei results in a developing embryo that contains the genetic information of the donated nucleus, which can either be transplanted into a surrogate mother to produce a genetically similar offspring or grow in-vitro to extract embryonic stem cells (ESC). This process has made it possible for the cloning of numerous mammalian species, such as pigs, cattle, mice, and, recently, primates. Although success has been evident in mammals, human derivation of pluripotent embryonic stem cells has been difficult to obtain. The difficulty stems from the premature activation of the oocyte and the improper reprogramming of the donated nucleus. This paper focuses on the development of human nuclear transfer embryonic stem cells (ntESC) and its application in regenerative medicine. Studies done on primates provide information on the barriers of this procedure on humans and the proper modifications on regular SCNT protocol. The use of deacetylase inhibitor TSA, phosphate inhibitor caffeine, and HVJ-E for proper membrane fusion are only some of the recent methods found for proper nuclear reprogramming and embryonic development. Breakthroughs in the methylation of DNA and histones in mice provided insight to a barrier in human embryo development. As a result, derivation of embryonic stem cells was successful and tested for pluripotency. Insulin beta cells and cardiomyocytes have been produced using this modified SCNT protocol and hold great potential for the future of science. The use of nuclear transfer embryonic stem cells is important in the development of stem cells that can differentiate into specialized cells, such as neurons, that can potentially be used to cure disease, like Parkinson's. Even more so, these cells will retain the genome of the patient and reduce immune incompatibility. The paper goes on to discuss the ethical issues that impede researchers from advancing in this area.

Introduction

Somatic cell nuclear transfer (SCNT) is a laboratory technique in which the somatic cell nucleus is transferred into an enucleated oocyte. The egg is developed into a blastocyst, an early embryonic stage where the opening of a cavity in the morula between the inner cell mass and the enveloping layer is filled with fluid. This procedure was first theorized in 1938 by Hans Spemann, a German embryologist, in his book Embryonic Development and Induction. He proposed an experiment to replace the nucleus of an unfertilized egg with a differentiated embryo nucleus. He pondered on whether such a transplant of a differentiated nucleus can give rise to an organism (Wellner, 2010). This was a start to the idea of cloning that was initially attempted in 1952. Scientists Robert Briggs and Thomas King transplanted nuclei from an advanced blastula cell into enucleated eggs of Rana pipiens, also known as leopard frogs. They saw a normal development of embryo resulting in the first cloned organism. Their study provided a basis for the SCNT protocol. As well, they determined that success is attributed to the transfer of a nucleus from the same species of frog. Transplanting the nucleus of a bullfrog led to an arrest at the blastula stage and inevitably died (Briggs et. al, 1952). Alluding to the core principle in SCNT mechanics of donor nucleus and oocyte recipient compatibility.

In 1996, the first cloned mammal using a fully differentiated adult cell was executed by Campbell et al. (1996) in Scotland. Dolly, the cloned sheep born in 1997, was a breakthrough in determining the nuances of SCNT. They figured out that the development of embryos reconstructed by nuclear transfer is highly dependent on the interaction of the donor nucleus and recipient cytoplasm (Campbell et al., 1996). Their findings aided in further successes of cloned organisms and nuclear transfer embryonic stem cells (ntESC) derivation. Such findings include the effects of cytoplasmic kinase activity and maturation promoting factor (MPF) on chromosomal damage. The expression of cytokeratin and lamin A/C, markers associated with differentiation, was also reported indicating the development of ESC. Research in this area after this incident paved the road to develop human ntESC and the extensive studies done on primates contributed to a modified SCNT approach.

The important application of this process is to produce a culture of embryonic stem cells that are created from the inner cell mass of the blastocyst. Stem cells are undifferentiated cells that can either produce cells that continue as stem cells or are differentiated into specialized cells. The implications of this procedure of generating personalized embryonic stem cells is useful for disease mechanism, and development of therapies (Tachibana et al, 2013), i.e. regenerative medicine or replacing damaged cells in patients, like cardiomyocytes to replace damaged heart tissue or insulin-producing beta cells for diabetic patients, by eliminating the prospect of immune incompatibility (Lanza et al, 1999). However, therapeutic cloning refers to the derivation of nuclear transfer embryonic stem cells without uterine transplantation that has been successful in mice and cattle, derived from ntESC lines in cloned blastocysts (Yang et. al, 2007). However, human ntESC has been notoriously difficult due to its failure to progress past the eight-cell stage because of inactivation of critical embryonic genes. Due to ethical and legal restrictions, further research in this area is moving at a slower pace. Therefore the question arises, can SCNT be used to produce human pluripotent stem cells for regenerative medicine?

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Methods

The information gathered in this paper was collected from several sources such as Touro College Online Library and NCBI. The searches for "Somatic cell nuclear transfer", "Cloning", and "Therapeutic Cloning" led to the majority of articles used obtained from PubMed and Nature. The articles discuss the experimental studies done at various times and an analysis of these studies provided material for this discussion. Some review articles were necessary in the development of this analysis.

Discussion: Nuclear Reprogramming

For successful embryonic development the proper nuclear and cytoplast-mediated reprogramming is necessary. Campbell and colleagues (1993) specified morphological events that occur in nuclear transfer at the merging of a nucleated blastomere or karyoplast into the cytoplast of an enucleated metaphase II arrested oocyte (MII). Oocytes in MII state have a higher success rate in nuclear reprogramming in the cytoplasm due to certain factors. In mammals, the fusion of the two specimens is induced by an electric pulse to promote fusion and activation of the enucleated egg. Studies in mice, pigs, and cattle display that certain morphological events occur at the point of fusion, including nuclear envelop breakdown (NEBD) followed by premature chromosome condensation (PCC), dispersal of nucleoli, reformation of the nuclear envelope, and nuclear swelling (Campbell et. al, 1993). The induction of NEBD and PCC are important for the reprogramming of gene expression and increased embryo development. These events vary depending on both the species and nuclei at different cycle stages. In rabbits, blastocyst development was found to be greater when the blastomeres were in G1 or early S phase than in late S phase or G2 (Collas et. al, 1992). A possible explanation for such observations was found in the cell cycle regulation with an emphasis on cellular DNA replication by maturation promoting factor (MPF).

A study showed the necessary implication of MPF activity at G1/S and G2 stages. MPF is a complex of two proteins, cyclins and p34cdc2. P34cdc2 is a protein kinase that is regulated by phosphorylation changes and interaction with cyclins. P34cdc2 does not change during the cell division cycle while cyclin does. P34cdc2 kinase triggers the entry of the cell into the M phase that produces NEBD, chromatin condensation, and other morphological changes in the cell. Therefore, MPF primary function is to promote nuclear breakdown, spindle formation, and chromatin condensation. Mammalian oocytes at the MII phase contain high levels of MPF. Upon fertilization or activation, MPF levels decline resulting in the decondensation of chromatin and formation of pronuclei. Therefore, NEBD and PCC occurrence in the donor nucleus suggests a correlation between MPF with NEBD and PCC activity. NEBD and PCC probably remove the nuclear membrane to allow ooplasmic remodeling factors access to the donor cell chromatin for the re-replication of previously replicated DNA and the synthesis of DNA in the donor nucleus at any cell stage. As well as, contributes to the reformation of the nuclear membrane. A problem arises when MPF activity is low at the time of fusion (Campbell et al., 1993).

When MPF is low at the time of fusion, from premature activation of the enucleated egg, the nuclear membrane is maintained. G1 donor nuclei will replicate their DNA, however, G2 nuclei will not rereplicate and a low frequency of development arises. However, interestingly enough, this suggests that, at slow MPF decline at the time of fusion, in any other phase other than G1, the DNA will rereplicate resulting in aneuploidy. This correlates with the results of the study done by Collas et. al (1992) regarding the development of rabbit blastocyst. In rabbits, at G1, early S, and late S phases, metaphase plates, and spindles were detected but abnormalities, like incomplete spindle formation and incomplete chromosome condensation, was present in late S phases (Campbell et. al, 1993).

A study was done by Mitalipov et. al (2007) built on the aspect of MPF regulation in primates and nuclear remodeling through lamin A/C staining. Lamins, an intermediate filament superfamily of proteins, are part of the nuclear lamina found on the inner layer of the nuclear envelope. Lamin types A and C are expressed in many differentiated somatic cells. Lamins A/C are essential in size, shape, and strength determination of the nuclear envelop as well as maintaining lamina structure. (Hutchison and Worman, 2004). These proteins tend to depolymerize during late prophase and become undetectable in the MI or MII oocytes. At fertilization, cytoplasmic lamins are gathered into the forming pronucleus (Prather et al., 1989). Lamin A/C has been used to assess the extent of nuclear remodeling following SCNT because of the changes in the nuclear lamina, as shown in this 1study, in monkeys.

MPF levels, as mentioned before, result from premature oocyte activation and a failure to induce nuclear remodeling. In studies with mice and cattle, the fusion of a donor cell with an oocyte was done through electroporation. Electroporation in a calcium ion fusion medium increases the calcium levels that trigger a rapid decline of histone H1 kinase and even MPF activity. Using electrofusion with calcium ion or magnesium ion free buffers or performing all manipulations free of these ions can minimize premature activation of the oocyte (Mitalipov, 2007). An improvement in the in-vitro development of pig (Boquest et al., 2002) and primate blastocysts (Mitalipov et al., 2007), which was shown from lamin A/C profile under these modifications was similar to those detected in sperm-fertilized controls. To supplement electroporation as a fusing agent in human SCNT, HVJ-E virus, hemagglutinating virus of Japan-envelope, a non-infectious vesicle used as an agent for cell fusion, showed a high rate of fusion. However, embryonic development past the compact morula failed, even though ionomycin and 6-Dimethylaminopyridine (DMAP) were used to activate the cell at the appropriate time.

Therefore, implementing electroporation, which is not necessary for cell fusion but rather for timely cell activation, increased blastocyst development. It is necessary as an activation stimulus in conjunction with ionomycin and DMAP (Tachibana et. al, 2013).

Extended exposure of the chromatin to the non-activated MII cytoplast enhances embryonic development. An MII oocyte has high levels of MPF but enucleation, which also removes cytoplasm from the oocyte, reduces MPF levels (Lee & Campbell, 2006). The disassembly of the nuclear lamina with the chromosome condensation is attributed to the phosphorylation of MPF. To prolong the presence of MPF, use of caffeine, a protein phosphatase inhibitor, or MG-132, a proteasome inhibitor, increased nuclear remodeling in the donor nucleus. MG-132 prevents spontaneous oocyte activation, as seen by the weak/partial lamin A/C signal in C/C1 (figure 1). More exposure to MG-132 reduces the lamin A/C signal and increases the chromatin condensation, as seen by DAPI blue stain (Mitalipov et. al, 2007). However, proteasome activity impacts development events, in contrast to caffeine that has high cleavage rates and regular blastocyst formation. However, caffeine does not affect the frequency of blastocyst formation (Lee & Campbell, 2006; Mitalipov et. al, 2007). Embryos usually do not develop past day-12. These conclusions suggest there is more than merely nuclear remodeling that affects the success of SCNT.
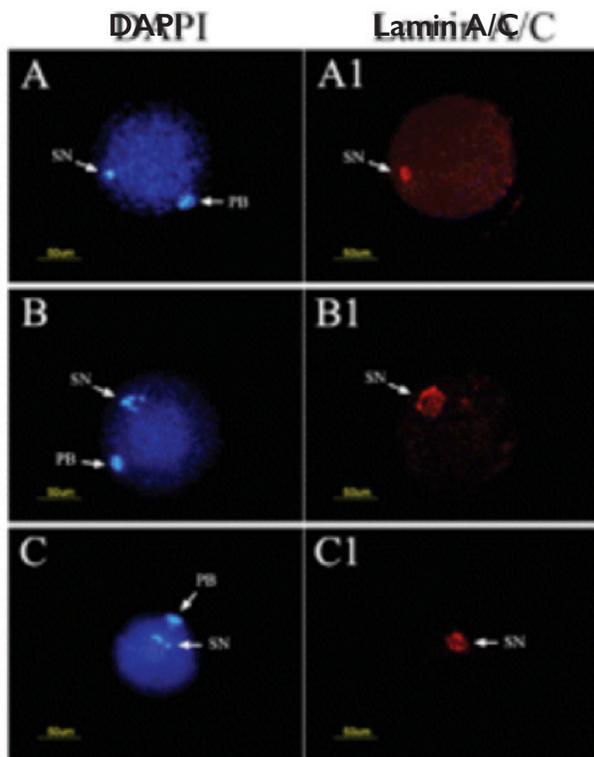

Figure 1: Lamin A/C signaling and DAPI staining for exposure of MG-132 (Mitalipov et al, 2007)

Premature activation of the MII oocyte is a likely occurrence, especially in aged or overstimulated oocytes. Therapeutic cloning or ESC derivation from discarded, aged, or failure-to-fertilize oocytes are unlikely to succeed (Mitalipov et al., 2007). Ovarian stimulation can also affect the quality of the oocyte, thus, tampering with the success of the procedure. A study was conducted to compare three groups of oocyte donation cycles, <10 oocytes per cycle, 11-15 oocytes per cycle, and >16 oocytes per cycle. Survival of the egg after spindle removal until embryonic cleavage was similar amongst the three groups. However, more >16 oocyte/cycle groups arrested after the eighth cell stage. Additionally, the quality of the blastocysts correlated inversely with the number of oocytes collected per cycle. ntESC derived from these oocytes were mainly from the <10 oocyte/cycle group and none from the >16 oocyte/cycle group (Tachibana et al., 2013). This shows that both the donated nucleus and recipient oocyte contribute to the success of ntESC derivation and development.

Another challenge to SCNT embryonic development is the consequence of microtubule and centrosomal protein depletion. This is a result of enucleation of the oocyte that extracts the spindle with defective reformation of the spindle by the donor nucleus. However, when the somatic cell nucleus is transferred to an oocyte containing its own chromosomes it results in a functional polyploid blastocyst that can develop into ESC. This can mean that certain reprogramming factors, associated with the spindle apparatus, are present in the MII oocyte (Tachibana et al., 2013) and are removed upon enucleation. Modification in the SCNT protocol of spindle removal is necessary to successfully execute nuclear reprogramming and blastocyst formation. The standard procedure of enucleation and spindle extraction is by adding bisbenzimide staining and UV exposure in mammals, such was executed in pigs (Polejaeva et al., 2000). However, if meiosis-specific factors are retained during spindle removal then only spontaneous activation of the cell causes its decline. By using the methods above to protect against premature activation, SCNT embryo development increases and spindle-like structures are formed. This method increased ESC derivation in primates that displayed similarities to human IVF-derived blastocysts than the manipulated spindle transfer embryos (Tachibana et al., 2013).

Epigenetic Regulation

Nuclear reprogramming is a crucial step for the success of embryonic development. However, it is not limited to the first steps of activation. Reprogramming gene expression in the inner cell mass (ICM) is a necessary component for the continuation of embryonic development and especially for derivation of pluripotent ntESCs. Normal development requires epigenetic modifications, which includes DNA methylation and histone modification. The ICM and trophectoderm are distinguished in the blastocyst with a predominant increase of DNA and histone

methylation in the ICM, affecting genomic imprinting and X chromosome activation in females (Yang et al., 2007).

In normal embryonic development, methylation occurs preferentially in the ICM, a process in which a methyl group is added to the 5th carbon of the cytosine ring next to a guanine base, through DNA methyltransferases (DNMT). In female mice, for example, demethylation occurs due to DNMT during subsequent cleavages. In male mice, it is demethylated in the pronucleus after fertilization. Chromatin configuration establishes the 'histone code,' a cellular memory responsible for maintaining the identity of differentiated cells. Histones are proteins that contribute to the make-up of chromatin. They have an exposed N-terminus end, which is either modified through methylation, acetylation, ubiquitinylation, or phosphorylation. For example, the acetylation of histone 3 Lys9 (H3K9) induces an open chromatin configuration giving transcription factors access while the methylation or demethylation of H3K9 inactivates it (Yang et al., 2007).

Trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, has shown a significant impact on embryonic development in mammals and primates (Sawai et al., 2012; Mitalipov et al., 2007). A study was conducted on bovine animals using 5nM and 50nM of TSA. The group that was treated with 50nM of TSA showed significant blastocyst development. TSA increased histone H4K8 and histone H3K9K14 acetylation, which activates gene tran-
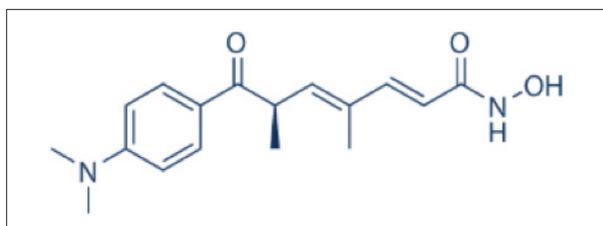

Figure 3: TSA structure. Selleckchem.com

scripts. However, FGF4 in bovine, required for ICM formation and trophectoderm differentiation, was still considerably low and did not change through the treatment of TSA. It is mainly affected by DNA methylation signifying that DNA methylation is an important factor for nuclear reprogramming (Sawai et al., 2012). Therefore, it is proper to conclude that TSA is necessary, as Mitalipov's study on primates and Tachibana's study on human ntESC both come to the same conclusion, yet DNA methylation is another component to embryonic development.

DNA and histone methylation is pertinent to improve the ntESC derivation. The first developmental defects of SCNT appears at the time of zygotic genome activation (ZGA) which occurs at the 4 to 8 cell stage in humans and bovine and at the 2-cell stage in mice. Difficulties in ZGA are due to epigenetic barriers existing in the genome. Certain genomic domains resistant to ZGA in SCNT embryos, known as reprogramming resistant regions (RRRs), have been identified. Enrichment of mouse RRRs with histone H3, lysine 9 methylation (H3K9me3)

in the donor genome results in a barrier to transcriptional reprogramming. They must be removed by specific demethylase or by ridding it of H3K9 methyltransferases. Kdm4d, H3K9me3-specific histone demethylase, greatly reduces H3K9me3 levels. The injection of Kdm4d mRNA increased the blastocyst rate up to 88.6% (Matoba et al., 2014).

In the study done by Chung et al. (2015), it was found that H3K9me3 also serves as a barrier in human SCNT. Using the same oocytes that failed to develop into blastocysts, they demonstrated that Kdm4a overexpression significantly improved embryonic development. Compared to the IVF derived embryos, of 707 genomic regions in SCNT embryos, 308 were termed as RRRs with the same enrichment of H3K9me3 as seen in mice. This study also showed that the injection of Kdm4a wild-type, not Kdm4d catalytic mutant used in the original study on mice, had a greater blastocyst rate, 90.3%, in SCNT embryos. To test the efficiency in this demethylase on humans, Kdm4a mRNA was injected at different stages of SCNT embryonic develop-
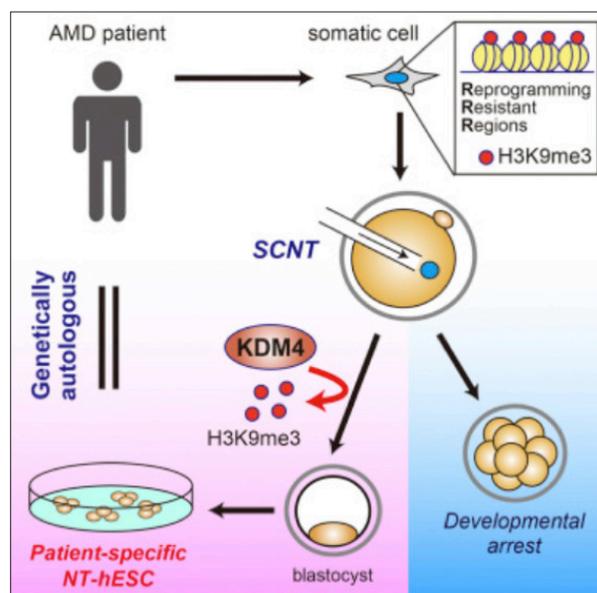

Figure 4 (Chung et al., 2015)

ment. Injecting it upon activation, after fusion by HVJ-E, showed no effect prior to ZGA completion. However, the effect was noticeable at the end when 8 blastocysts were developed, with no development of the control group. In a conventional ESC derivation medium, seven attached to the MEF feeder cell and four stable ntESC lines were derived. As shown in Figure 5, immunostaining revealed that NANOG, OCT4, SOX2, SSEA-4, and TRA1-60, transcription factors that maintain ESC differentiation, were all expressed similar to human ESC line derived by IVF. Likewise, these ntESC's expressed pluripotency marker genes that were indistinguishable from IVF derived ESC. Additionally, these ntESCs underwent immunostaining of embryoid bodies (EB), aggregates of ESC, for two weeks in vitro giving rise to the

differentiation into all three germ layers. Proving its differentiation ability. Karyotyping, the process of chromosome pairing, demonstrated normal chromosomal number and the same sex chromosome of the donor nucleus, and Short Tandem Repeat (STR) analysis showed all 16 repeat markers on the genome is perfectly matched to the donor somatic cell. A sequence analysis of mitochondrial DNA (mtDNA) demonstrated that the single-nucleotide polymorphism of ntESCs matched the mtDNA of the donor oocyte and not the donor nucleus. A necessary analysis that demonstrates the idea that mtDNA is transmitted by the fertilized oocyte and not from the DNA in the cell's nucleus (Chung et al., 2015).

Although blastocyst development in SCNT embryos was improved, the success of humans, merely 27%, compared to the success of mice, 90%, suggests that all modifications to SCNT protocol must be utilized, especially proper oocyte quality. That is a major factor for ntESC derivation. Human oocyte varies tremendously even from a single ovulation. Therefore, even some blastocysts formed through IVF were not supported (Chung et al., 2015).

Fetal vs. Adult Somatic Cell

In order for SCNT to be regarded as having practical medical significance there must be a way to generate ntESC from adult somatic cells. Most experiments use various fetal fibroblasts as somatic nuclei donors. However, patients that are in need of this procedure are mainly aged. Therefore, a recent study done by Chung et al. (2014) determined whether dermal fibroblasts from 35 and 75- year-old males can undergo successful SCNT procedure. The debate on adult vs. fetal cells is regarding the age-related changes, such as shortened telomeres and oxidative DNA damage that can potentially obstruct nuclear reprogramming. Even though fetal fibroblasts were used in numerous researches, there were no clear claims of age impedance on SCNT. Using the non-modified, standard SCNT protocol, no aneuploidy was produced in the resultant human ESC. Therefore, the modified SCNT protocol established by Mitalipov et al. (2007) and Tachibana et al. (2013) on fetal fibroblasts was used, with two groups forming. One group was activated 2 hr after implantation and the other group was activated after 30 min. However, only two blastocysts hatched from the 2 hr group, one from each age subtype, leading to the generation of ESC lines. The difference in time duration can be explained by the possibility for the necessary extra time needed for reprogramming that is dependent on the exposure of the donor nucleus to the recipient oocyte cytoplast. Both ntESC lines displayed the same sixteen STR markers located on the human autosomal and allosomal loci as the donor somatic cell and differed from the oocyte donor. The mtDNA was verified being inherited by the oocyte donor in both ntESC lines and expression of transcription factors OCT-4, SSEA-4, TRA-1-60, and TRA-1-81 was present. Pluripotency was confirmed through spontaneous differentiation into the three

germ layers by immunostaining of embryoid bodies. Further confirmation of pluripotency was through teratoma formation assays that demonstrated outgrowth of tissue from the germ layers. Despite the low success rate, this study portrayed the efficiency of the modified SCNT protocol and greatly implies the possibility of using SCNT to generate patient-specific ESC for medical use (Chung et al., 2014).

Application of Therapeutic Cloning on Regenerative Medicine

Therapeutic cloning holds great promise in regenerative medicine and gene therapy. It has numerous research and clinical applications, such as producing a vector for gene delivery, creating animal models of human diseases, and cell replacement therapy in regenerative medicine. It can permanently cure Parkinson's disease or diabetes mellitus. Barberi et al. (2003) conducted a study on mice using SCNT with nuclei from cumulus and tail-tip cells. Two ntESC lines were differentiated into motor, GABAminergic, serotonergic, and dopaminergic neurons, that displayed synapse formation and normal electrophysiological properties in vitro. Mice with Parkinson-like lesion were injected with dopaminergic neurons into the cortical striatum induced by 6-hydroxydopamine. An 80% survival rate 8 weeks post-transplantation was observed from ntESC contrary to 40% for stem-cell derived neurons (Kfoury, 2007).

Furthermore, SCNT can allow for organogenesis and patient-specific rescue of genetic mutations. In cell replacement therapy, therapeutic cloning has the potential to create various types of tissues such as osteoblasts to counteract osteoporosis. Deshpande et al. (2006) transferred motor neurons derived from ESC to rats with a severed spinal cord showing SCNT application for spinal cord regeneration. A potential cure for paralysis in humans. Therapeutic cloning eliminates the issue of immune rejection and organ shortage by engineering tissues and organs. Patient-specific cardiomyocytes, blood vessels, and skin can treat infarctus, atherosclerosis, and severe burns (Kfoury, 2007). With the use of the modified SCNT protocols, diploid pluripotent stem cells that were derived from a patient with type 1 diabetes were produced (Yamada et al, 2014). Such advancements in medicine hold hope for cures of various diseases.

iPSC vs. ntESC

IPSCs, induced pluripotent stem cells, is a laboratory technique that reprograms differentiated cells back to pluripotency using specific transcriptional factors. The medical application of human iPSCs has opened the door for rapid stem cell therapy. A set of essential transcription factors, called reprogramming factors, trigger the destruction of the existing state of somatic cells by changing their epigenetic status, leading to alterations in their gene expression. The changes in the gene expression induce secondary epigenetic changes, including DNA methylation and

alterations of the nuclear structure. However, there are road-blocks to reprogramming that need to be removed because cellular identity is stabilized. However, transcription factors such as Oct4, Sox2, Klf4, and c-Myc are sufficient to destabilize the existing order in the original cells and re-construct a new order. Therefore, transcription factors associated with development may undertake important actions during the reprogramming process. This produces stem cells that are similar to embryonic stem cells but has a few disadvantages when compared to ntE-SCs (Takahashi & Yamanaka, 2013).

ESC is considered the ground state which is believed to have a greater chance of successful differentiation than iPSC. During reprogramming, iPSCs-specific methylation and transcriptional abnormalities in imprinted regions and X chromosomes were observed with such abnormalities less frequent in ntESCs (Nazor et al.,2012). In some instances, a number of highly pro-liferative colonies appear that are not pluripotent and can form into tumors. Furthermore, iPSCs do not efficiently silence the expression pattern from where they are derived from and fail to induce some ESC specific genes (Bilic & Belmonte, 2011).

A major advantage of SCNT-based ntESCs is the fact that it contains mtDNA originating from the oocyte. Irrespective of the nuclear donor cell mtDNA, ntESCs have the potential to produce functional cells and tissues for cell therapies. Thus, SCNT offers a strategy for correcting mtDNA mutations and retaining the metabolic function of pluripotent cells from patients with inherited mtDNA diseases.

MtDNA that is specific to the oocyte recipient of cloned embryos was first determined in the study done by Evans et al. (1999), with co-researcher Dr. John Loike. They showed nuclear transfer-derived sheep were homoplasmic. The random partitioning of mtDNAs does not occur. This may be due to the failure of the donor mitochondria to enter the ooplasm following electrofusion. It can be hypothesized that an active mechanism operates to destroy the donor mitochondria in the recipient ooplasm, similarly to what is thought to happen to sperm-derived mitochondria in fertilized ova in human reproduction. These results have implications for future attempts to correct maternally inherited mitochondrial genetic disorders by nuclear transfer involving a somatic or germline cell from a woman containing a pathogenic mtDNA mutation but normal nuclear DNA and a recipient enucleated oocyte.

Ethical and Legal Issues
Research in this field has been held back due to controversial ethical issues. Legal constraints and lack of funding results in the impediment of therapeutic cloning (Lo & Parham, 2009). A major objection to SCNT is the belief that creating embryos for research, with the intention of mutilating these embryos, violates human respect and integrity. Sandel (2004) argued in his article on embryo ethics, "Although every oak tree was once an acorn, it does not follow that acorns are oak trees, or that I should treat the loss of an acorn . . . as the same kind of loss as the death of an oak tree." This is an opinion based question whether an embryo varies in essence from a developed human being. Evaluation whether the use of embryos for medical research justifies its destruction. Meanwhile, legislative actions, especially in Europe, hinders SCNT advancement that contributes to its slow progression.

Conclusion
Therapeutic cloning is feasible through modified SCNT protocol. Research in mammals and primates aided in the success of human ntESC derivation by establishing the correlation between oocyte and donor nucleus quality, nuclear reprogramming, and proper gene expression. All steps led to the derivation of pluripotent ESC that were differentiated into various specialized cells, such as cardiomyocytes and insulin beta cells. The developed embryo harboring mtDNA from the recipient oocyte holds an advantage over iPSC cells by constituting metabolically functional cells from mitochondrial diseased cells. More research is needed to expand and apply this technique in medical practice; however, ethical and legislative actions repress the advancement in this area.

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