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A highly efficient method for generation of therapeutic quality human pluripotent stem cells by using naive induced pluripotent stem cells nucleus for nuclear transfer

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Abstract

Even after several years since the discovery of human embryonic stem cells and induced pluripotent stem cells (iPSC), we are still unable to make any significant therapeutic benefits out of them such as cell therapy or generation of organs for transplantation. Recent success in somatic cell nuclear transfer (SCNT) made it possible to generate diploid embryonic stem cells, which opens up the way to make high-quality pluripotent stem cells. However, the process is highly inefficient and hence expensive compared to the generation of iPSC. Even with the latest SCNT technology, we are not sure whether one can make therapeutic quality pluripotent stem cell from any patient's somatic cells or by using oocytes from any donor. Combining iPSC technology with SCNT, that is, by using the nucleus of the candidate somatic cell which got reprogrammed to pluripotent state instead that of the unmodified nucleus of the candidate somatic cell, would boost the efficiency of the technique, and we would be able to generate therapeutic quality pluripotent stem cells. Induced pluripotent stem cell nuclear transfer (iPSCNT) combines the efficiency of iPSC generation with the speed and natural reprogramming environment of SCNT. The new technique may be called iPSCNT. This technique could prove to have very revolutionary benefits for humankind. This could be useful in generating organs for transplantation for patients and for reproductive cloning, especially for childless men and women who cannot have children by any other techniques. When combined with advanced gene editing techniques (such as CRISPR-Cas system) this technique might also prove useful to those who want to have healthy children but suffer from inherited diseases. The current code of ethics may be against reproductive cloning. However, this will change with time as it happened with most of the revolutionary scientific breakthroughs. After all, it is the right of every human to have healthy offspring and it is the question of reproductive freedom and existence.

Keywords

Somatic cell nuclear transfer, induced pluripotent stem cells, reproductive cloning, assisted reproductive technique

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Introduction

Although it is over a decade since human embryonic stem cells (h-ES) were made by Thompson in 1998 or induced pluripotent stem cells (iPSCs) by Yamanaka in 2007,[1–3] no one has ever succeeded in translating these into clinical practice for potential applications such as generation of transplant-able organs. One of the most remarkable advancement in this direction was by a Japanese group which made a primitive liver bud, although it failed to grow and survive into a useful larger organ.[3] However, it is possible to generate organs of one animal species in another one with the recent advancement in iPSC technology, transgenic technology, and embryo manipulation.[4–6] Pioneering work by another Japanese group, Kobayashi et al.,[6] reported in 2010

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generation of an entirely iPSC-derived rat pancreas inside the mouse. The techniques they used are difficult to apply in human beings to generate transplant-able organs due to several ethical and technical reasons in the immediate future. Although we cloned Dolly the sheep in 2003 through somatic cell nuclear transfer (SCNT), we were unsuccessful in generating human pluripotent stem cells (PSCs) through SCNT or cloning till 2013 when Mitalipov's group made a breakthrough. However, this technique is highly inefficient.[7–9]

SCNT

The history of induced pluripotency perhaps started in 1894 when Jacques Loeb occasionally observed formation of a large bleb in some of the early embryos when he attempted to induce parthenogenesis in sea urchin embryos using different salt concentrations.[10] He found some unique properties with this bleb. This bleb remained unchanged while the rest of the embryo developed. However, he observed that if a nucleus moved into the bleb, this part of the embryo started to develop. This part had the potential to develop independently even if it was severed from the original embryo. Loeb thus discovered that embryos could be created by moving the nucleus between cells. These laid the foundation for the modern nuclear transfer experiments which continued to evolve in complexity of techniques and perfection and across the spectrum of evolution from sea urchins to tadpoles[11,12] and from mice to sheep (Wilmut in 2003) and finally to primates (Hwang Woo-suk in 2005 and Mitalipov in 2013). Loeb's roads were furthered by Yves Delage (1895) and Hans Spemann (1936) independently.[11–14] Spemann's "delayed nucleation experiment" in the newt egg and his prophecies on "obtaining a normal development following transfer of a mature cell nucleus into an oocyte" made him the father of SCNT. On the other hand, Delage predicted that an embryonic nucleus would be found to be equivalent to a zygotic nucleus if it could be transferred into an enucleated egg and, at the same time, would have the properties of a "sex cell nucleus."[10] Thus, an SCNT involving the transfer of the nuclear material from an embryonic stem cell or iPSCs would result in a zygote that will be the donor of the nuclear genetic material. This is a very promising approach for therapeutic cloning, reproductive cloning for childless couple, or couple suffering from dominant inherited diseases.

iPSCs

In 2006, Yamanaka and colleagues[2] discovered that pluripotency can be induced in somatic cells by continued expression of the transcription factors Oct4, Sox2, Klf4, and Myc. iPSCs can be generated by different methods for expression of the Yamanaka's transcription factors. Use of integrating viral vectors such as lentivirus or retrovirus is efficient but has disadvantages of introduction of foreign genetic material in a less predictable location in the genome and induction of mutations which may have harmful effects.[15,16] The integrated viral genome undergoes epigenetic inactivation or silencing the genes. However, this process is not uniform and predictable and holds the theoretical risk of reactivation of the inserted genes. Adenoviral vectors (DNA virus) rarely integrate but are less efficient and have technical issues associated with production of the virus. Use of episomal vectors are efficient but have a small risk of persistence of the episome and subsequent integration to the genome. Use of transposon vectors such as PiggyBac vectors and Sleeping Beauty for carrying Yamanaka's factors is relatively safe to the host genome but is inefficient.[16,17] Minicircles (minimal vectors the genes of interest under a eukaryotic promoter) were used for reprogramming human fibroblasts but are very inefficient. Sendai virus is a good vector for generating iPSCs because it is an RNA virus which remains in the cytoplasm and does not integrate with the host genome but has high efficiency. However, this virus is more difficult to work with compared to lentivirus or retrovirus and is expensive. Transduction of the fully functional proteins (Yamanaka's factor proteins) is a great method in terms of genome safety, but this is extremely costly and inefficient. Generation of iPSCs by transfection of stable RNAs is efficient and genome safe but relatively expensive.[15,16]

iPSCs are of different types. The common h-ES and iPSC are similar to epiblast cells found in the embryo, which have a flatter morphology and are also pluripotent but cannot differentiate into extra-embryonic tissues. On the other hand, mouse ES and iPSC form more spherical colonies, require leukemia inhibitory factor (LIF) for growth and maintenance, and are more naive. The common h-ES/iPSCs do not dependent on LIF for growth and maintenance.[18,19] It is possible to convert common iPSCs/ES to the naive state cells which are LIF dependent. Other differences between human iPSCs/ES and naive iPSCs/ES include high levels of X chromosome inactivation in female ES/iPSC lines, predominant use of the proximal enhancer element to maintain OCT4 expression, increase in DNA methylation and prominent deposition of H3K27me3, and bivalent domain acquisition on lineage regulatory genes.[18,19]

Generation of pluripotent human stem cells: SCNT versus induction of pluripotence by Yamanaka's factors

Efforts to make h-ES from somatic cells as well as to make human clones were also largely unsuccessful.[7,8] Reprogramming adult cells into PSCs by SCNT is useful in studying disease mechanisms, generation of human organs, or therapeutic cloning.[8] However, SCNT has many problems such as low efficiency, huge costs on labor of experienced technicians, deficiency of mitochondrial inheritance, need of egg donors, and ethical issues associated with egg donation and "destruction" of human embryos.[8,9] On the other hand, iPSCs can be generated from almost any adult tissue, highly efficient, less

labor intensive, 100% mitochondrial inheritance, and after all there is no need of egg donors and associated ethical issues.[15,20] Some of the recent reports claim near 100% efficiency in generating human and murine iPSCs. Deterministic and synchronized iPSC reprogramming can be achieved by transduction of Yamanaka's factors together with depletion of Mbd3, a core member of the Mbd3/NuRD (nucleosome remodeling and deacetylation) repressor complex and reprogramming in naive pluripotency promoting conditions.[21] iPSCs, like embryonic stem cells, can contribute to all the three germ layers and contribute to germ cells producing a healthy chimeric animal when injected into a developing embryo.[17] Live healthy mice were produced from solely iPSCs through tetraploid complementation, although the process was relatively inefficient.[22] However, iPSCs suffer from incomplete reprogramming, clonal heterogeneity, incomplete or non-physiological erasure of epigenetic memory, and genetic variations. There are many reports, studies which projected downside of iPSCs such as increased abnormal epigenetic marks, epigenetic memory, mutations in coding regions, and copy number variations in a small proportion of cells.[43–45] Although there is no solid proof that the genome and phenome of PSCs generated by iPSCs are inferior compared to that generated by SCNT, it is still a possibility because generation of iPSCs involves forced expression of genes under conditions which are non-physiological or unnatural compared to the reprogramming mediated by the natural milieu of transcription factors and reprogramming environment inside an oocyte.

The epigenome of organisms become more stabilized as the longevity increases (vide-infra). However, gametogenesis, ovulation, fertilization, and subsequent events require epigenetic and chromosomal destabilization and remodeling to provide transcriptional activation or inactivation of key genes pertinent to different sub-stages post fertilization. This involves massive erasure and new imprinting of several genes.[23] Maternal and zygote specific factors (certain proteins, microRNAs, etc.) are evolved over millions of years of selection for this purpose. Therefore, it would be logical to use these natural factors and environment for generation of PSCs for therapeutic applications including cloning.

Yamanaka's reprogramming process involves forced unnatural expression of four transcription factors, Oct4, Sox2, Klf4, and Myc. This is a long process which takes over 20 days, and the efficiency is low because the process is stochastic (though not entirely).[24–27] On the other hand, in SCNT, reprogramming is more deterministic, quicker and results in about 24 h. The efficiency of SCNT is higher if we consider few facts: (a) SCNT needs two successful manual and individual manipulations—one of the donor cell and the other of the recipient cell (the egg cell). It is difficult to handle more than few tens of cells a day. (b) The SCNT process can cause physical damage to cells. On the other hand, the process of generating iPSCs can be performed on millions of cells simultaneously and effortlessly in less than an hour, and the process is much less damaging (DNA or messenger RNA (mRNA) transfection or viral transduction) to cells. However, only a small percentage of the transfected or transducted cells eventually become PSCs. The entire process of iPSCs generation can be mechanized and scaled up.[5,28]

iPSC nuclear transfer—highly efficient method for generation of therapeutic quality human PSCs

Here, I propose that we can compensate the deficiencies of these two approaches by combining both approaches to generate therapeutic quality PSCs which can be used in disease management, cosmetics, structural and functional improvements of organs, and therapeutic or reproductive cloning for individuals who are otherwise incapable to reproduce (even with the help of advanced assisted reproductive techniques (ARTs)). This can be achieved by transferring the nucleus of an iPSC instead of a somatic cell into the egg during the nuclear transfer procedure. The new technique may be called iPSC nuclear transfer (iPSCNT).

Before Mitalipov's success in 2013, numerous attempts by several groups all over the world failed to create pluripotent human stem cells through SCNT or attempts toward human cloning from an adult cell using the SCNT technique failed.[7] Noggle et al.[29] used an oocyte to reprogram a somatic cell to pluripotent state, but in order to achieve this, they had to retain the oocyte nucleus intact, which resulted in a triploid PSC line with very little clinical use. Some Harvard-based scientists suggested that it may not be even technically possible to use SCNT in humans the way it was used to clone Dolly the sheep. The Harvard team replaced human zygotic genome with that of a somatic cell, and they observed that the embryonic development got arrested before the morula stage. This development block according to them was associated with a failure to activate transcription in the transferred somatic genome. This block they observed only in human embryos but not in murine embryos. This group therefore concluded that "there may be a previously unappreciated barrier to successful human nuclear transfer."[30] Mitalipov and colleagues[7,8] overturned this view in 2013 and made a landmark. After years of extensive research, they identified premature exit from meiosis in human oocytes and suboptimal activation as key factors which are responsible for the failure of the earlier groups. They optimized SCNT approaches by tweaking several physical (osmotic pressure, pH, mechanical stress, etc.) and chemical parameters to circumvent these limitations, which allowed the derivation of human nuclear transfer embryonic stem cells. Use of caffeine made a big difference in their experiment in inducing somatic nuclear cell spindles in cytoplasts following oocyte enucleation and fusion.[8] However, it may be noted that the efficiency of the technique adopted by Mitalipov's group is still poor, and the group was not able to generate a clone from every human

subject specimen they tried or every oocytes worked across the donors.

It is possible that naive iPSCs[18,19] are more suitable for therapeutic applications/cloning because of their "more naive" and ground state compared to iPSCs, although so far this is not experimentally proven. These naive human iPSCs may eventually require tetraploid complementation[31] to form a viable embryo which may be used in therapeutic cloning or for generation of human organs. Considering these facts, it is logical to use the nucleus from reprogrammed naive iPSCs (generated by transfection of stable mRNAs of Yamanaka's factors) for SCNT for generating human PSCs for therapeutic applications or cloning in humans. This combined approach will increase the efficiency compared to the use of ES/iPSC combined with tetraploid complementation or the state of the art SCNT developed by Mitalipov et al. iPSCNT has the advantage that it do not require tetraploid complementation unlike iPSCs or naive iPSCs to generate a viable embryo which can be implanted in the uterus using the conventional protocols used in ARTs.[32,33]

Discussion

Evolution of epigenetic stability and reprogramming efficiency—a balance between longevity and cancer

Ability to regenerate and stability of an epigenetic state are highly correlated. When cells organize to form multicellular "societies" (organisms), the functional specialization of cells in an organism increases and the epigenomic stability increases, and this is evident in the evolutionary ladder from sea anemones to planarian to mouse to sheep to humans. Several strategies evolved during early evolution of multicellularity to prevent emergence of parasitic sub-populations within a multicellular organism to ensure the "common good"—the survival of the organism as a whole (or benefit of the majority of the cells and their genome). Cancer suppressor signaling pathways and epigenetic and chromosomal stability evolved as a result.[34–36] In higher organisms such as mammals, cancer is the result of mutations which provide proliferative and survival advantage to mutated cells at the cost of the whole organism. It may be noted that mutation alone may not result in cancer unless it is facilitated by epigenetic changes which improve the adaptation of the mutated cells according to the needs of the dynamic environment. Mutations accumulate as any organism gets older. This is especially true for longer living mammals such as humans compared to short lived organisms such as mice. Therefore, stronger epigenome stabilizing mechanisms are selected by evolution in humans. The epigenome stabilizing mechanisms make the reprogramming of a mature nucleus extremely difficult in a long living mammal like man compared to mouse. This could be the reason why it was extremely difficult to make viable human embryos through SCNT. As mentioned before, only in 2013, it was possible to derive human pluripotent cells by SCNT. However, Yamanaka's factors realize reprogramming but through forced and unnatural expression of four master transcription factors—Oct4, Sox2, Myc, and Klf4.

Evidence from literature supporting iPSCNT hypothesis

There is some evidence based on mice experiments that SCNT involving the transfer of the nuclear material from an embryonic stem cell or iPSC could be efficient. Zhou et al.[22] performed SCNT using mouse embryonic fibroblast (MEF) nucleus and the efficiency was 71%, while the efficiency using the nucleus of iPSC line IP14D-10 was 90%. I believe the efficiency would have been even less if they used true fibroblasts/adult fibroblasts instead of murine embryonic fibroblasts. The animals generated by Zhou et al.[22] had the same growth rate and lifespan as wild type mice. There is a report by Li-Ying Sung et al.[37] claiming that differentiated cells are more efficient than adult stem cells for cloning by SCNT. It may be noted that Briggs and King[11] used nucleus from early embryonic cells to perform SCNT. However, later, many scientists tried with nucleus from more mature cells but failed. In 1962, Gurdon[12] successfully cloned a frog using intact nuclei from the intestinal epithelial cells of a feeding tadpole, and for this work, he was awarded the Nobel Prize. These experiments are well documented and part of the history. The author has not come across yet another work in the same direction as reported by Li-Ying Sung. It is possible that the complex isolation and purification procedure for hematopoietic stem cells might have damaged these cells. The abundance and ease of isolation of granulocytes might have resulted in granulocytes which are healthier, resulting in higher success rate during SCNT.

The prospect of iPSCNT

One of the most revolutionary use of iPSCNT is that couples who have genetic diseases can hope to have healthy offspring. Genetic diseases can be corrected at ES/iPSC stage using a variety of evolving gene correction and editing strategies such as those that make use of zinc finger nucleases, Transcription Activator-Like Effector Nucleases (TALENS are artificial restriction enzymes generated by fusing a Transcription Activator-Like (TAL) effector DNA binding domain to a DNA cleavage domain), or Clustered Regularly Inter-spaced Short Palindromic Repeats (CRISPR)/Cas systems.[5,9,38] The right to have at least one healthy child is a fundamental right to every human, but this concept is controversial.[39] iPSCNT technique when combined with gene correction techniques, ARTs, and the use of surrogate uterus opens the way for those individuals who cannot have their own child to have a healthy child. This includes individuals suffering from inherited genetic diseases; infertility due to

anatomical, physiological, psychological, or social reasons (example nuns); same sex (gay or lesbian) couples; men or women who prefer to live alone; and so on.

Another major use would be therapeutic cloning or for harvest of organs, which is considered unethical.[4,5,39–41] However, it may be possible that in future, we would be able to generate patient-specific human organs completely in vitro or inside an artificial uterus (ectogenesis), although we have a long way to go.[5,42] iPSCNT would generate high-quality PSCs for this purpose. Less revolutionary and near future applications would involve iPSCNT for cell therapy in various diseases such as cell therapy in inherited blood and bone marrow disorders such as hemophilia, thalassemia, myelodysplastic syndromes, aplastic anemia, and re-population of bone marrow following radiotherapy or chemotherapy following malignant diseases. Gene-corrected hematopoietic stem cells derived from patients' own cells could potentially cure diseases like hemophilia or thalassemia.[5,38] Pluripotent cells derived through iPSCNT can be differentiated to mature cell types such as hepatocytes which may be used in hepatocyte transplantation in inherited liver diseases such as Crigler Najjar syndrome or fatal urea cycle disorders. Hepatocyte transplantation is also an alternative to liver transplantation or as a bridge to transplantation or physiological regeneration in acute liver failure, for example, following paracetamol (acetaminophen) overdose.[4,5]

Conclusion

To conclude SCNT involving the transfer of nucleus extracted from ES/naive iPSC (preferably generated by genome safe methods such as transfection of stabilized mRNA coding Yamanaka's factors) to an egg cell at appropriate stage of development would result in the generation of high-quality pluripotent stem cells useful for therapeutic applications. Alternatively the resultant embryo which upon implantation in uterus could grow into a normal fetus. This technique could prove to have very revolutionary benefits for humankind. iPSCNT could be useful for cell therapy in inherited diseases such as hemophilia, cell therapy in liver failure, therapeutic cloning, and reproductive cloning especially for childless men and women who cannot have children by any known technologies. This might prove useful to those who want to have healthy children but suffer from inherited diseases when combined with advanced gene editing techniques. The current code of ethics may be against reproductive cloning. However, this will change with time as happened in most of the revolutionary scientific breakthroughs. After all, it is the right of every human to have healthy offspring and it is the question of reproductive freedom and existence.[5,26]

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The author of this manuscript has no conflict of interests to declare.

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