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Cover Page Footnote
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Abstract

The foundational advancements of John Gurdon and Shinya Yamanaka have improved understanding of dedifferentiation of cells to a pluripotent state. The seminal discovery established a novel system to study disease pathogenesis, drug screening, and toxicity, as well as sprouted the new field of regenerative medicine. In this article, the methodology to obtain dedifferentiated cells, known as induced pluripotent stem (iPS) cells, subsequent validation, and application of which are reviewed. The experiments investigated here aim to demonstrate the capacity of iPS cells to replace the ethically-gray human embryonic cells by developing human livers and viable, healthy animals. It is concluded that the reported methods pave the way for a bright future of iPS cell application in both basic and applied sciences.

Introduction

Stem cells have immense potential for treatment of diseases including diabetes and heart disease through regenerative cell based therapies[1]. As a result, the scientific community has put forth tireless effort to better understand these cells and their unique capacity to differentiate into the plethora of cells observed in biological systems. The undertaking began with basic science experimentation where many researchers turned to the ovum to elucidate the beginning steps of cell differentiation. John Gurdon, winner of the Nobel Prize in Physiology or Medicine in 2012, was among the first to investigate what changes the somatic cell nucleus undergoes during embryonic differentiation[2]. After isolating recipient eggs and tagging donor embryo nuclei from *Xenopus laevis*, Gurdon exploited the previously established nuclear transplantation protocol to move the tagged nuclei into the donor eggs. Gurdon observed that some transplant frogs could grow to a normal size, morphology, and have normal reproductive function similar to non-transplant frogs. From his work, it was concluded that somatic cell nuclei had all genes necessary for normal developmental progression[3]. Strelchenko took the same principle of somatic cell nuclear transfer and developed a novel technique that fused a somatic cell with a human embryonic stem (hES) cell. By exploiting pre-existing factors in the cytoplast of hES cells, Strelchenko was able to culture cells harboring the nuclear genome of the somatic cell but had the differentiation capacity of hES cells[4]. Although amazing, both Gurdon and Strelchenko's work lack an understanding of the factors associated with reverting a somatic nuclei to one reminiscent of an embryonic nuclei.

The Generation of iPS Cells

In a ground-breaking endeavor worthy of sharing the Nobel Prize in Physiology or Medicine with Gurdon in 2012, Shinya Yamanaka of Kyoto University identified the elusive factors necessary to induce pluripotency[5,6]. To do this, Yamanaka developed a retrovirus containing green fluorescent protein (GFP) and four essential factors later coined the Yamanaka cocktail: Oct3/4, Sox2, Klf4, c-Myc. Adult human dermal fibroblasts (HDF) that had previously been supplemented with artificial mouse receptors for retrovirus were then transfected using the previously

developed retrovirus. Oct3/4 and Sox2 are known to encode for transcription factors associated with the developmentally critical homeodomain proteins as well as contribute to pluripotency and self-renewal[7]. Klf4 is a bona fide component of the pre-transcriptional machinery for Tert expression through interactions with ß-catenin[8]. C-Myc is traditionally known to be a transcription factor but has also been proven to be responsible for promoting formation of euchromatin in the human genome[9]. Six days after transfection, Yamanaka and colleagues collected cells via trypsinization and subsequently plated collected cells with SNL feeder cells[10].

The remaining majority of the protocol implemented by Yamanaka aimed to isolate and culture iPS cells. This started with first growing cells in a medium containing 10% fetal bovine serum (FBS). The medium was shortly thereafter replaced with a medium designed for primate ES cells supplemented with basic fibroblast growth factor (bFGF). After a full month of culturing, flat colonies resemblant of hES colonies were isolated via mechanic disaggregation and then transferred to fresh primate ES cell medium containing bFGF. At this stage, individual cells and cell colonies displayed morphologic characteristics similar to hES cells; these similarities include large nuclei with little cytoplasm and aggregates that formed in tightly packed flat colonies. In hES cell cultures, cells located in the center of aggregates tend to differentiate. At this stage of experimentation, the same phenomenon was observed in the hES-like cell aggregates. To sustain the suspected undifferentiated capacity of cells that resembled hES, cells were transferred to plates in mouse embryo fibroblast (MEF)-conditioned ES cell medium. These cells were then tested for pluripotency and were then on referred to as induced pluripotent stem (iPS) cells. To confirm that iPS cells were analogous to hES cells, Yamanaka compared iPS cell similarity to hES cells based on the following criteria: morphology, proliferation, feeder dependence, surface markers, gene expression, promoter activities, telomerase activities, in-vitro differentiation to three different germ layers, and teratoma formation. Yamanaka also observed that transgenic genes had been silenced by the time the cell reached an hES-like state[10].

Validation of iPSCs

Since the establishment of the Yamanaka cocktail, researchers hoped that iPS cells could replace the ethically controversial ES cells and set out to do so. Kristin Baldwin of the Dorris Neuroscience Center at Scripps Research Institute has recently generated iPS-derived mice to validate their candidacy to substitute for ES cells[11,12]. Baldwin used the procedure developed by Yamanaka but added a method to control expression of the Yamanaka cocktail through a tetO promoter. The tetO promoter is activated by a reverse tetracycline transactivator (rtTA) protein in the presence of tetracycline analogue doxycycline (dox). In this way, Baldwin could drive expression of the Yamanaka cocktail with the addition of dox and rtTA. Baldwin also exposed MEFs to a histone deacetylase inhibitor valproic acid (VPA) to enhance reprogramming efficiency and select against incompletely reprogrammed cells by restricting cellular division[11].

After developing several iPS cell lines, Baldwin strived to generate an iPS-derived adult mouse. To do so, Baldwin first conducted a tetraploid complementation assay. This was used to develop embryos with an albino phenotype from each of the iPS cell lines. This was accomplished by fusing the two cells of an embryo at the two cell stage to make one tetraploid cell. Normal development can take place up to blastocyst formation. At the blastocyst stage, the cell is anucleated and fused with a diploid iPS cell that will have an agouti phenotype[11,13]. The agouti blastocysts were then injected into separate albino female mice and pups were collected a day prior to estimated delivery via Caesarean section. During early stages of development, some pups from each cells line were either cannibalized, non-viable, or viable. All pups were observed to have normal morphologies. Of the non-viable pups, most presented with respiratory complications. Adult male iPS cell-derived mice later demonstrated successful reproductive capacity to produce viable offspring[11]. Although Baldwin's work provided an initial foundation demonstrating that iPS cells had the potential to replace ES cells, research was still far from developing satisfactory results to initiate clinical studies.

Using Baldwin and Yamanaka's concepts and techniques, Hideki Taniguchi of Yokohama City University in Japan set out to demonstrate iPS cells are bona fide substitutes of hES cells by creating a human liver from an iPS cell organ bud[14]. Taniguchi prepared hepatic endoderm cells from human iPS cells (iPSC-HEs). To mimic early organogenesis of the liver, human iPSC-HEs were grown in conjunction with a stromal cell population to activate the FGF and BMP pathways. The stromal cell population was composed of human umbilical vein endothelial cells (HUVECs)

and human mesenchymal stem cells (MSCs). After 48 hours of plating, the cell culture was observed to self-organize into three dimensional aggregates. These aggregates were proven to be iPS cell-derived liver buds (iPSC-LBs) via antibody staining. To see if the liver-bud cells more closely resembled human livers or human iPS cells derived from mature hepatocyte-like cells, Taniguchi implemented microarray analysis to assess the expression of 83 genes known to be upregulated during liver development. Microarray analysis showed that the liver-bud cells resembled human livers 22-40 weeks into gestation. Qualitative analysis with fluorescence-activated cell sorting (FACS) indicated that cells expressed proteins associated with adult livers; these proteins include the tight junction protein zona occludens 1 (ZO1), ALB, CK8/18, asialoglycoprotein receptor 1 (ASGR1), and collagen IV. Histologic examination revealed the presence of a hepatic cord-like structure - a defining characteristic of adult livers[15].

After developing viable organ buds, Hideki Taniguchi tested if the iPSC-LBs had the capacity to generate fully functional livers. Using the cranial window model, liver-buds were transplanted and monitored. Microarray analysis indicated that human iPSC-LBs were more similar to matured hepatocytes than iPSC-MHs. Considering that hepatocytes are the main cell of the liver tissue, this provided an encouraging indicator that an iPSC-LB derived liver had formed. To assess its functionality, mice with the human iPSC-LB transplant were injected with either ketoprofen or debrisoquine. Ketoprofen and debrisoquine are drugs known to be metabolized differently in humans than in mice. In serum and urine samples collected from mice transplanted with the human iPSC-LBs, metabolites unique to a human liver were found. Taniguchi then assessed the metabolome for metabolites of sugars, amino acids and nucleotides. Of the 222 metabolites assessed, the metabolome bore strong resemblance to a human adult liver rather than human iPS cells. In addition to its metabolic capability, the human iPSC-LB-derived liver displayed full vascularization[15]. This research validated iPS cells as candidates for replacing ES cells and paved the way for their application in regenerative medicine.

Current Status of iPSC Application

Masayo Takahashi of RIKEN laboratory in Japan has recently received government approval in July 2013 for a pilot study using iPS cell therapy in wet-type age-related macular degeneration (AMD)[16]. Although the cause of AMD is unknown, the disease has been well characterized. AMD patients present with an abnormal growth of new blood vessels in the tissue proximal to the eye. This abnormal growth causes damage to the retinal pigment epithelium (RPE) and sensory retina. Takahashi plans to collect a biopsy of skin cells from each patient to produce iPS cells unique to each patient. The iPS cells can then be differentiated to form a monolayer cell culture of RPE. After surgically removing damaged RPE and other undesired growths, the missing epithelium can be replaced by the monolayer of iPS cell-derived RPE cells. The initial treatment would then be followed up by a one year monitoring period and then a three year period of follow up examinations. The current challenges for the pilot study involve taking a biopsy and inhibiting the formation of tumors. Dr. Takahashi has thus far approved six patients for treatment[16]. The risks associated with Takahashi's pilot study may seem few, but there are many experimental hurdles to overcome prior to transplantation.

Current Issues and Future of iPSCs

The problems faced with iPS cell research stem from the recent evolution of iPS cells and the consequent ambiguities of a system not well characterized. For example, the mechanism by which the Yamanaka cocktail induces pluripotency is not well understood. An understanding of the signal transduction cascades involved may resolve other issues such as low iPS cell yield. In Yamanaka's study, 5×10^4 fibroblasts were used to yield roughly ten hES cell-like colonies. The undesirably low induction efficiency may be due to one or a combination of the following factors: low efficiency of HDF transduction, mechanistic difficulty teasing apart iPS cells from differentiated cells, or the transfection integration site may be site-specific at a loci[10]. Similar challenges beget similar complications as Baldwin had found with her experimentation that had low yields for iPS cell-derived mice. Baldwin reports that 0.3-13% of iPS cells were able to generate mice. Another problem emerged as roughly 20% of viable mice developed tumors resulting from c-Myc reactivation[11]. The high rate of tumor development could also be attributed to the approximate 20 retroviral integration sites per clone[10]. Due to the absence of reliable transplantation techniques for therapeutic application, Taniguchi also faced issues of low yield when transplanting iPSC-LBs[15]. Not only are iPS cells and iPS cell derivatives

challenging to generate, they are notidentical to hES cells. An ideal candidate to substitute hES cells would have high similarity in expression, morphology, and proliferation capacity. Some differences have been highlighted through microarray analyses. Researchers have concluded that iPS cells are like ES cells but are not ES cells10. Due to their dissimilarity and difficulty to generate, many researchers have turned to developing alternative techniques to induce pluripotency.

Dr. Andrew Wan of the Institute of Bioengineering and Nanotechnology in Singapore is one of many working on developing a non-viral vector method to induce pluripotency in somatic cells. Wan has developed a protein delivery system that requires the synthesis of a long hydrocarbon chain with hydrophilic functional groups incorporated at both ends of the molecule; this system is formerly known as a cationic bolammphiphile protein delivery system[18]. The delivered proteins are able to induce pluripotency at low toxicity but also low yield. Because this system requires the synthesis of an intricate cationic bolaamphiphile complex, the technique is challenging to some of the most experienced scientists[19]. Currently, the most reliable method to revert cells back to an ES-like state relies on the transfection of the Yamanaka cocktail.

Closing Remarks

IPS cell cultures can replace hES cells for understanding disease mechanisms, drug screening, and toxicology[10]. Using Taniguchi's method of developing human livers in mice models, in vivo drug screening can be performed to test for harmful effects or metabolites. Taniguchi's experiment also validates a discussion of using organ-bud transplantation as an alternative avenue for the generation of organs when treating organ failure[15]. Since iPS cells can be made from ordinary fibroblasts, such iPS cells are unique to the patient because they will harbor the same nuclear genome as the patient[10]. This allows for patient specific cultures that, in principle, should reduce or eliminate patient transplant rejection. Though iPS provides an optimistic future for basic and applied science research, further characterization is necessary.

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