iPSC Reprogramming Methods and Applications

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Abstract:

Induced pluripotent stem cells (iPSCs) are a recent development in the field of bioengineering and its applications are quickly gaining speed. iPSCs are pluripotent, meaning they can differentiate into various types of specialized cells. Their ability to reprogram and differentiate is what allows for a plethora of applications. IPSCs are obtained through reprogramming cells through transcription factors; they are first reprogrammed through the Yamanaka Factors and then specific differentiation factors are used to further induce specialization. They have many advantages which conquer ethical and sustainability issues that arise with use of animal models. These applications include disease modeling, regenerative medicine and drug discovery. However, despite their advantages, there are still shortcomings to iPSCs: reprogramming efficiency, the risk of carcinogenesis, epigenetic factors, and much more play a role in differentiation and application. As time progresses, it is likely we will see the use of this technology increase, as it provides an innovative route which offers solutions to regenerative medicine, disease modeling and other medical issues.

Introduction

Within this article, we will go over a brief introduction to induced pluripotent stem cells (iPSCs), including what they are and providing examples of how they are reprogrammed. Next, we dive into the therapeutic applications of iPSCs in disease modeling, regenerative medicine, and drug discovery. Finally, we discuss the challenges and liabilities of using iPSCs in the medical field.

Induced Pluripotent Stem Cells

An iPSC is the product of reprogramming other cells such as fibroblasts [1]. To reprogram cells, the Yamanaka Factors are used. The Yamanaka Factors consist of 4 genes; octamer binding transcription factor 4 (OCT4), SRY-related high mobility group box protein 2 (SOX2), Kruppel like factor 4 (KLF4), and cellular myelocytomatosis viral oncogene (c-MYC) [2]. C-Myc influences histone acetylation which loosens the chromatic structure of chromosomes, stimulating DNA transcription [3] whilst Klf4 maintains the self-renewal capacity in iPS cells [4]; both of these genes regulate proliferation and differentiation. Oct3/4 and Sox2 both regulate the gene transcription for cell development and function as molecular rheostats, controlling the self-renewal and pluripotency of iPSCs [5].

Methods of Reprogramming

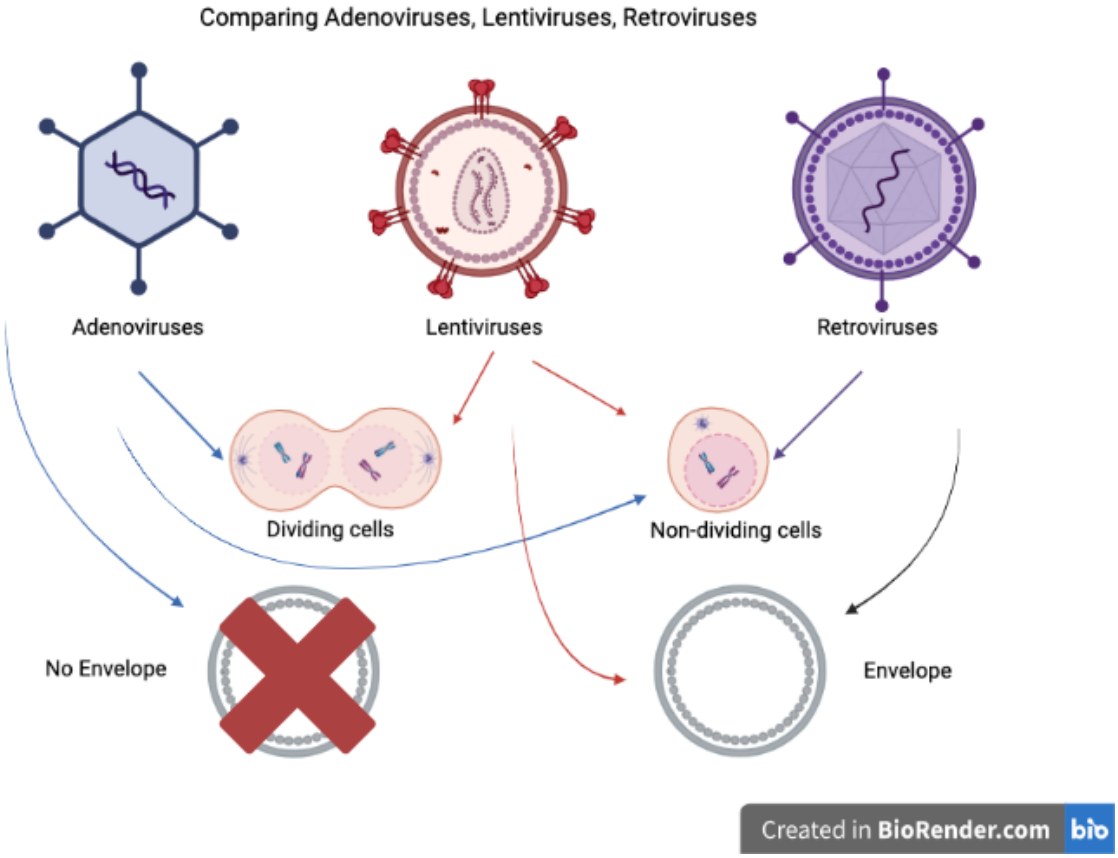

Figure 1. Comparison between Adenoviruses, Lentiviruses, and Retroviruses - Adenoviruses, Lentiviruses, and Retroviruses share many features but also have distinctions. The figure above compares their structure - whether they have an envelope or not - and their ability to infect dividing and non-dividing cells.

To differentiate iPSCs,both retroviral and non-retroviral methods are used within the field. Retroviral methods include the use of lentiviruses and retroviruses. Replication-defective retroviral vectors are the most common choice during iPSC studies as this class of retroviruses don't contain the coding regions required for the genes necessary for virion replication [6]. However, a major drawback of using retrovirus-mediated gene delivery is that cells must be dividing for transduction to occur[6]. To overcome this barrier, lentiviruses may be used as an alternative. Lentiviruses are a subclass of retroviruses which are capable of infecting both dividing and non-dividing cells [6]. Similar to other classes of retroviruses, their genome is reverse-transcribed when they enter the cell, producing DNA which is also passed on to the progeny of the cell when it divides [6]. Retroviruses carry the target DNA which is then inserted into the genome of the host cell, however, they also pose potential health risks since the DNA of the virus remains in the genome which can lead to the unwanted transcription of certain genes

[6]. For example, c-Myc is a cancer promoting gene, thus it is essential that it is suppressed after cell reprogramming [7].

The nonretroviral methods of iPSC production include excisions, adenoviral methods, and protein transduction (a non-DNA method). Excisions, also known as transient transfections, allows for a brief integration of transgenes into the cell's genome; once reprogramming has been achieved, the transgenes are removed from the cell [8]. One example of a transient transfection is the piggyBac enzyme system which can excise itself completely, without leaving any of its DNA in the iPSC genome. PiggyBac restores the donor site to its pretransposon state, making it useful for reversible transgenesis, a valuable feature which could be used to generate iPSCs without permanent alterations to genomic sequence [9]. Adenoviral methods utilize adenoviruses which hijack their host's cells and replicate their genome; however, unlike retroviruses, adenoviruses do not incorporate their genome into the host DNA [8]. Thus, these genes never have to be excised and the expression of the transgenes can be done directly through the virus' genome [8]. A disadvantage of the adenoviral method of reprogramming is that it has an extremely low reprogramming efficiency [8]. Protein transduction is a non-DNA method of reprogramming which utilizes fusion proteins [8]. These proteins fuse each of the transgenes into a polypeptide sequence which can penetrate into the cell, allowing the genes to enter the cellular membrane [8]. This version of reprogramming doesn't require the use of DNA intermediates, eliminating the risk of tumorgenesis and disturbed gene expression [8].

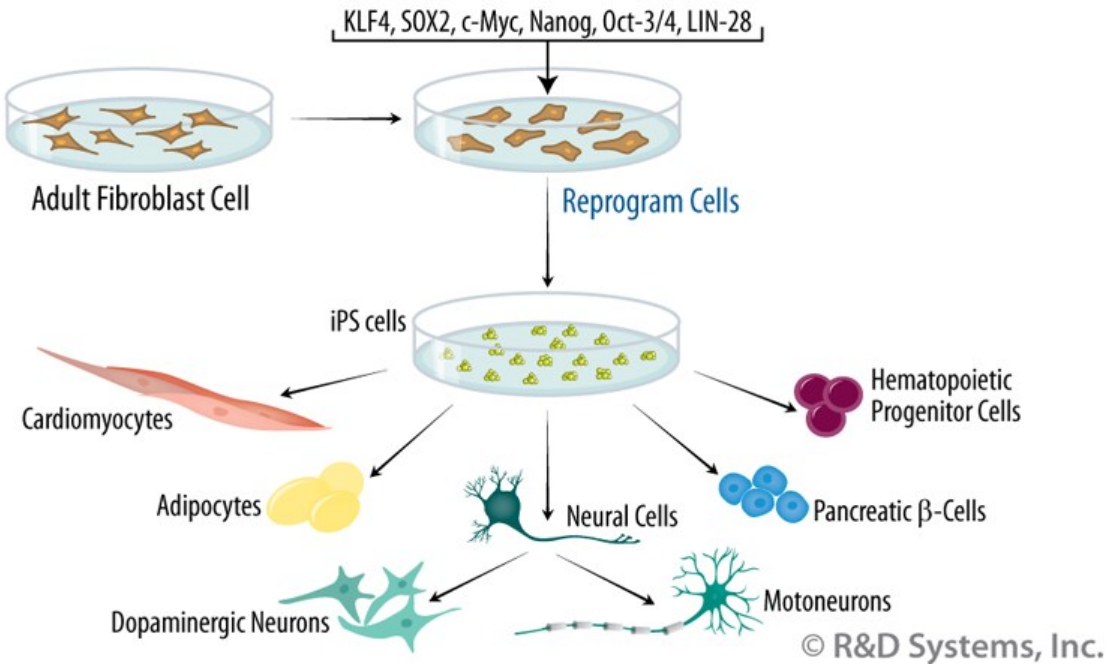

Figure 2. *(adapted from R&D Systems)* - For most cell types, four factors (c-Myc, Oct-3/4, SOX2, and KLF4) are used, although a combination of alternate factors (Oct-3/4, SOX2, Nanog, and LIN-28) has also been used successfully. Expression of these exogenous factors triggers a gradual process of silencing markers of the

differentiated phenotype and inducing markers of the pluripotent state in some cells. As pluripotent cells, iPS cells theoretically have the ability to generate all cell types found in the body [29].

Cardiomyocyte and Osteogenic Differentiation

Cardiomyocytes are contractile and excitable heart cells that contract rhythmically without rest [10]. Their differentiation is characterized by an initial decrease in the expression of undifferentiated pluripotent markers such as Oct4 and Nanog Homeobox (Nanog) [11]. Cardiac-related transcription factors include NK2 Homeobox 5 (NKX2-5), Myocyte Enhancer Factor 2C (MEF-2c), and Gata Binding Protein 4 (GATA-4); to differentiate iPSCs into Cardiomyocytes the OCT4, SOX2, NANOG, and Cell Lineage Abnormal 28 (LIN28) transcription factors are used (Figure 2) [11]. These iPSCs allow researchers to model cardiovascular diseases, study heart development, and accelerate predictive drug toxicology tests [30].

The inhibition of Wnt signaling is essential for cardiac induction of iPSC and Glycogen synthase 3 inhibition enhances cardiomyocyte differentiation [12]. Glycogen Synthase Kinase-3 (GSK3) inhibition followed by inducible expression of B-catenin shRNA (short hairpin RNA) or chemical inhibitors of Wnt signaling allows for the production of functional cardiomyocytes [12]. B-catenin is a key effector responsible for the triggering transcription within the Wnt signaling pathway which plays a crucial role in maintaining cellular homeostasis. B-catenin knockdown at the appropriate differentiation stage during monolayer-directed differentiation leads to enhanced Cardiomyocyte differentiation [12].

Osteogenic progenitor cells are stem cells, precursors to more specialized bone cells, which are located in the bone that play important roles in bone repair and growth[13] The Notch signaling pathway plays a large role in the differentiation of iPSCs into osteogenic progenitor cells [14]. In a study conducted, knockdown of the Notch signaling pathway via y-secretase inhibition enhanced induced pluripotent stem cell differentiation and commitment to osteogenic fate [14]. Notch inhibition has a stimulatory effect on Secreted Protein Acidic and Rich in Cysteine (SPARC) gene expression [14]. SPARC is a protein associated with the production of type 1 collagen [14]. The protein binds to collagen and hydroxyapatite crystals and release calcium mions which is essential for the mineralization of the collagen matrix in bones [14]. Osteogenic progenitor cells have the potential to help researchers find new breakthroughs in skeletal diseases through disease modeling.

APPLICATIONS OF iPSCs

iPSC disease modeling

iPSCs offer as an alternative to embryonic and animal models used to model diseases. Use of embryonic tissues and animal models is often the subject of ethical debates (destruction of human embryos, pain and suffering of animals, etc). Now, with iPSCs, we are able to access an unlimited supply of clinically relevant phenotypic cells with easy accessibility and scalability, allowing for an increased understanding in the etiology and progression of a diverse array of diseases [15].

The main challenge with iPSC disease modeling is gaining access to tissue with the disease phenotype of interest. Cells which are used within *in vitro* models must reflect the cell genotype and phenotype as if *in vivo*. To overcome this, there are two methods which have been developed: the procurement of tissue from a diseased individual or gene editing approaches to generate cells with the disease genotype of interest [16].

The former method can be done by obtaining source material (such as skin, blood, or urine) from a patient and filtering to isolate primary cells which can then be reprogrammed [16]. The disease phenotype will be exhibited within these primary cells, and therefore it will also be incorporated into cells that are differentiated from the collected primary cells [16].

Gene-editing approaches such as the use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas9) technology, which allows scientists to develop cells with specific disease genotypes. These cells are used to investigate disease mechanisms and experiment with treatments [16]. CRISPR-Cas9 technology utilizes 3 steps: designing an RNA guide, delivery, and screening/analysis of clones and their frequency to become enabled and proliferate with the desired edit [16]. REPROCELL is a corporation which uses CRISPR-SNIPER technology. SNIPER (Specification of Newly Integrated Position and Exclusion of Random-integration) uses a combination of long, high-stringency donor DNA with a digital PCR-based quantification method to determine bacterial colonies with highest likelihood to have the desired genome edits [16]. This allows for fewer clones needing to be selected for sequence verification, thus the success rate for isolating homoallelic and heteroallelic clones with the correct genomic change is greatly increased [16].

Cancer is an example of a disease that can be modeled by iPSCs. Cancer cells share a number of characteristics with induced pluripotent stem cells including indefinite capacity and expression of oncogenic markers such as c-Myc [17]. The generation of iPSCs from human cancer cells could present us with an opportunity to develop in vitro models of carcinogenesis [8].

iPSCs also offer as a source for studying pathogenesis[18]. The availability of these cells from individual patients allows for experiments on the therapy of diseases such as amyotrophic lateral sclerosis (ALS) or spinal muscular atrophy (SMA) [19, 20]. The development of inherited

diseases is associated with unique cell types which are hard to obtain with biopsy, thus with iPSCs, we are provided with a limitless resource.

iPSC in regenerative medicine

In addition to disease modeling, iPSCs research has the potential to foster new findings in organ development. To demonstrate their application, we will look at a research study conducted by He, R., Li, H., Wang, L. *et al* where researchers delve into how hiPSCs expand our knowledge and treatment towards Duchenne muscular dystrophy (DMD). DMD is characterized by progressive muscle weakness and hypertrophy. In a mouse model of DMD(*mdx* mice) the absence of dystrophin impairs polarity and subsequent asymmetric cell division of satellite cells which results in reduced myogenic potential and loss of muscle regenerative capacity. In this study, myogenic differentiation was performed on hiPSCs with the supplementation of basic fibroblast growth factor, forskolin, 6-bromoindirubin-3′-oxime as well as horse serum. These hiPSCs-derived myogenic progenitors were grafted into *mdx* mice via both intramuscular and intravenous injection. The myogenic progenitors placed in the mice then contributed to human-derived myofiber regeneration in host muscles, restored dystrophin expression, ameliorated pathological lesions, and seeded the satellite cell compartment in dystrophic muscles. Indeed, the authors observed that hiPSC derived myogenic progenitors successfully contributed to significant muscle regeneration and restored dystrophin expression in mdx mice [21].

The delivery of iPSC into internal organs can be done through intravenous injection of cells (with an expectation that they will navigate to the damaged site) or through local administration via catheter placement. For local delivery, doctors use both injectable and implantable biomaterial scaffolds—three-dimensional porous, or permeable biomaterials which are intended to allow transport of body liquids and gases— topromote cell interaction [22]. With their ability to transform into various types of cells, iPSCs can rapidly progress research in degenerative diseases.

iPSC in drug discovery

Selection of patients with different disease severity for reprogramming can allow the isolation of iPSCs with differing disease phenotypes

Viral transgene-containing iPSC models are capable of replicating human disease using available *in vitro* assays for both neurological and cardiac disorders and used to assess if candidate drugs can reverse diseased phenotypes. For example, In a study conducted by Itzhaki *et al,* iPSCs were differentiated into cardiomyocytes which have a K+ mutation associated with long QT syndrome – a disorder which is associated with cardiac arrhythmias.

The authors in this study were able to screen various pharmacological agents and test which ones could correct the defect [23].

iPSCs are a major approach to personalized medicine as we could use diseased cells from patients (through a skin biopsy) to create disease models [24]. iPSCs would be generated for each patient and redifferentiated into cell types most affected by the disease of interest. Then, after conducting studies on disease mechanisms, drug screens would be set up to discover lead compounds to correct the disease phenotype. Once potential lead compounds are identified, they will then be tested on individual patient-derived disease cells – this process aids in selecting a more reliable drug lead [25]. With this method, the cost of drug discovery would be significantly reduced.

Challenges with iPSCs

One of the main challenges with the use of iPSCs is to identify the best reprogramming method and cell source to allow for reproducible drug screening practices. The gene expression ratio within iPSCs is extremely important as various levels of certain differentiation factors can alter which cell progenitor may be derived. The timing of various events (the order they occur in) may also affect the cell's success [8]. This hinders screening due to less availability.

Integrating-virus methods are effective for iPSC generation, however, they also provide a liability. iPSCs made in this manner may have more altered gene expression profiles than transgene-free iPSCs [26]. This is because the DNA of viruses remains within the cell genome [27], potentially leading to the unwanted transcription of certain genes even after differentiation.

Another challenge is that cells derived from iPSCs exhibit immature characteristics which are similar to functional characteristics of embryonic cells [15]. It remains to be seen if they can be induced to a more mature state, which is nessecary for modeling adult diseases. For example, many neurodegenerative disorders, such as Alzheimer's, take decades to manifest so would iPSCs produce a reliable model to represent this disease?

Epigenetics also plays a factor in considering iPSC differentiation. Incubating cells over prolonged periods of time and exposing cells to high concentrations of differentiation factors can affect the pathology within the cell culture environment. This may lead to epigenetic reprogramming to erase important features which would allow the correct manifestation of disease essential for drug screening [28].

Conclusion

Induced pluripotent stem cells have immense potential within the medical field. Their applications in drug discovery, regenerative medicine, and disease modeling have facilitated

thousands of studies and led to new discoveries. For the future, scientists are looking towards the substitution of transgenes with small molecules that promote the iPSC generation, a method that would offer a safe, clinically appropriate way of creating these cells. Still, it remains to be seen if small molecules will be able to replace genetic methods of iPSC generation or are just useful as supplementary aids to the process [8]. With increased use of iPSCs, the medical field has been able to improve treatment options and create novel therapeutics. With some more advances to refine iPSC differentiation and use, we will overcome their shortcomings create one of the large impacts on the future of personalized medicine.

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