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Epigenetic Reprogramming of Adult Mammalian Cells into Induced Pluripotent Stem Cells (iPSCs) - An Emerging Paradigm

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ABSTRACT

The field of stem-cell biology has been catapulted forward by the startling development of reprogramming technology. The ability to restore pluripotency to somatic cells through the ectopic co-expression of reprogramming factors has created powerful new opportunities for modelling human diseases and offers hope for personalized regenerative cell therapies. Worldwide increases in life expectancy have been paralleled by a greater prevalence of chronic and age-associated disorders, particularly of the cardiovascular, neural and metabolic systems. Patient-specific induced pluripotent stem (iPS) cells are an emerging paradigm that may address this. Reprogrammed somatic cells from patients are already applied in disease modelling, drug testing and drug discovery, thus enabling researchers to undertake studies for treating diseases 'in a dish', which was previously inconceivable. Although there are currently several strategies to deliver reprogramming factors to induce iPSCs. In this study we have focus is on utilize plasmid vector to reprogramm because of convenience, reasonable efficiency and zero genes fingerprints and xeno free production of iPSCs. This virus-free technique reduces the safety concern for iPScell generation and application, and provides a source of cells for the investigation of the mechanisms underlying reprogramming and pluripotency.

Keywords: induced pluripotent stem cells, Organs-on-Chips, *OSKML*, Markers of pluripotency.

During the last few decades, the use of induced pluripotent stem cells (iPSCs) has gained attention among the researchers all around the world. It is a potentially important resource for many applications, ranging from basic research to drug discovery. PSCs can be an enormous cell source for *in vitro* model systems of development (Imamura *et al*., 2012). The iPSCs from patient's somatic cells could be a useful source for drug discovery and cell transplantation therapies. Reprogramming of somatic cells (SCs) into PSCs has been reported by introducing a combination of several transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) (Takahashi and Yamanaka, 2006; Okita *et al*., 2010; Stadtfeld and Hochedlinger, 2010). iPSC has generated a renewed interest in stem cell research. It promises to overcome several key issues, including the ethical concerns of using human embryonic stem cells (hESCs) and the difficulty in obtaining large numbers of adult stem cells (Anastasia *et al*., 2010).

In 1962, generation of tadpoles from unfertilized eggs that had received a nucleus from the intestinal cells of adult frogs revolutionized the whole world (Gurdon, 1962). Almost, three decades later, Ian Wilmut *et al*., (1997) reported the birth of Dolly, the first mammal generated by somatic cloning of mammary epithelial cells. These successes during the cloning of somatic cells demonstrated that even differentiated cells contain all of the genetic information that is required for the development of entire organism and an oocyte contains factors that can reprogram somatic cell nuclei. Moreover, ESCs are also reported to possess factors that can reprogram somatic cells (Tada *et al*., 2001). The second stream in this aura was the discovery of ''master'' transcriptional factors. In 1987, Antennapedia a Drosophila transcriptional factor was reported to induce the formation of legs instead of antennae when ectopically expressed (Schneuwly *et al*., 1987). These results led to the concept of a ''master regulator,'' a transcription factor that determine and induce the fate of a given lineage. Many researchers began to search for single master regulators for various lineages. The attempts failed, with a few exceptions (Yamanaka and Blau, 2010). In 2006, Takahashi and Yamanaka reported that stem cells with properties similar to ESCs could be generated from mouse fibroblasts by simultaneously introducing four genes and designated those cells as iPSCs. A similar approach is applicable for human fibroblasts and by introducing a handful of factors; human iPSCs can be generated (Takahashi *et al*., 2007; Yu *et al*., 2007). Generation of mouse ESCs was followed by the establishment of culture conditions to enable the long-term maintenance of pluripotency (Smith *et al*., 1988).

Now a days, convergence approach for direct deterministic turning of somatic cells into iPSCs using viral vectors such as retroviruses and lentivirus as a vehicle for genes insertion is in vogue. These vehicles (viruses) pose number of problems including copy number variations (CNVs), *de novo* generation of genetic mosaicism, protein-coding point mutations and overexpression of few specific genes (Hussein *et al*., 2011; Gore *et al*., 2011). It is observed that long-term studies

Table 1: Methods in vogue for reprogramming of somatic cells to iPSCs

Name of Method	Experimental design*	Vector type	Factors	Efficiency %	Advantages	Disadvantages	References
Retroviral integrating method	EF/AF,NSC, SC/LC KRC / AMC,BC/ AC into iPS	Retroviral	OSKM and VPA,	0.01–0.02%	- Reasonably efficient	-Genomic integration, -Incomplete pro-viral silencing and slow kinetics	Takahashi and Yamanaka, (2006), Okita *et al*., (2007) Park *et al*., (2008)
Lentiviral plus cre-lox mediated transgene excision method	Fibroblasts and KRC	Lentiviral	OSKM miR302 miR367 and VPA	0.1-2%	-Reasonably efficient and transduces dividing and non-dividing cells	-Genomic integration and incomplete proviral silencing	Chang *et al*., (2009) Soldner *et al*., (2009) Sommer *et al*., (2009) Somers *et al*., (2010)
Adenovirus Mediated non-integrating method	Fibroblasts and LC	Adenoviral	OSKM	0.001–0.0001%	-No genomic integration	-Low efficiency	Stadtfeld *et al*., (2008) Zhou and Freed, (2009)
Sendai virus mediated DNA free method	NF/DF	Sendai Viral	OSKM	0.1- 1%	-No genomic integration	-Sequence-sensitive RNA replicase, and -difficulty in purging cells	Fusaki *et al*., (2009) Seki *et al*., (2010)
Protein DNA free method	Fibroblasts	Protein	OS	0.001%-0.006%	-No genomic integration, -direct delivery of transcription factors	-Low efficiency, -short half-life, and -requirement of bulk amount of protein	Kim *et al*., (2009)

Contd.

mRNA Transfection method	Fibroblasts	Modified mRNA	OSKML and VPA	0.1- 4.4%	-No genomic integration, -faster reprogramming kinetics, high efficiency	-Requirement for multiple rounds of transfection	Warren *et al*., (2012)
miRNA infection/ transfection method	ASC/DF	miRNA	miR-200c miR-302s miR-369s	0.1%	-More efficient then lentiviral or retroviral methods - no risk of integration	-Lower efficiency than other commonly used methods	Miyoshi *et al*., (2011)
PiggyBac mediated method	MEF	PiggyBac	OKS	0.02–0.05%	-virus-independent iPS cell production -option of xeno-free iPS cells	-Lower efficiency than other commonly used methods	Woltjen *et al*., (2009) Tsukiyama *et al*., (2014)
Minicircle VectorsBased method	Fibroblasts / hASC	Minicircle vectors	Lin28, GFP, Nanog, OS	0.005%	-Xeno free production of human iPSC	-Lower efficiency	Jia *et al*., (2010)
Episomal Plasmids mediated method	Skin fibroblasts	EPISOMAL PLASMIDS	OSKM Nanog, and SV40 Large T antigen and Lin28.	0.0003–0.0006%	-zero-footprint iPSCs	-Very low efficiency	Yu *et al*., (2009)
Chemical Method	human fibroblasts	Chemo	OSKM	-	Xeno free production of iPSC	Very low efficiency	Anastasia *et al*., (2010)

***** EF/AF-embryonic / adult fibroblasts, NSC-Neuronal stem cells, SC/LC - Stomach cells / Liver cells, KRC/AMC - Keratinocytes/ Amniotic cells, BC/AC - Blood cells / Adipose cells, NF/DF - Neonatal fibroblasts/ Dermal fibroblasts ASC - Adipose stromal cells**,** MEF - Mouse embryonic fibroblasts, hASC - human adipose stem cells, OSKML - Oct4, Sox2, Klf4, Myc and Lin28)**,** miR - microRNA**,** VPA - Valproic acid, iPSCs - induced pluripotent stem cells.

must focus on functional characterization of reprogramming-associated mutations to aid to the creation of clinical safety standards. Further rigorous work on mutation rates, distributions during *in vitro* culturing and reprogramming of hiPSCs and hESCs is the dire need of the hour. It will be essentially helpful in the establishment of clinical safety standards for genomic integrity. The current study was planned with an objective to know about the various methods, their comparative efficacy to derive iPSCs and to outline the functional assessments of pluripotency. Moreover, how the iPSCs genes influence the role of each cell type in disease modeling, therapeutics and regenerative medicine as well as prediction for the evolution of the art of reprogramming of somatic cells has also been studied.

TECHNIQUES USED FOR REPROGRAMMING OF SOMATIC CELLS TO iPSCs

iPSC reprogramming got its existence through the over-expression "Yamanaka factors (transcription factors)". A retroviral delivery system in mouse and human fibroblasts was successfully adopted during the iPSC reprogramming (Takahashi and Yamanaka, 2006; Takahashi *et al*., 2007). The biggest disadvantage of iPSC reprogramming method from a translational perspective is that the reprogramming vectors are integrated into an infected cell's genome. Since Yamanaka's breakthrough discovery, many different methods are in vogue to develop iPSCs (Table 1). Although various viral, nonviral DNAs and miRNA are currently involved as delivery of reprogramming factors. The major lacunae faced in the most of the reprograming techniques are low efficiency, genetic finger printing, genome integration and mutations. The plasmid based approach of reprogramming is the most preferred among all these techniques because of it efficiency and its ability to excise the genome without leaving a footprint i.e. iPSCs genome without any genetic alteration (Yusa *et al*., 2009; Tsukiyama *et al*., 2014; Table 1).

MARKERS OF PLURIPOTENCY AND THEIR APPLICATIONS

Ectopic expression of the transcription factors Oct4, Sox2, c-Myc, and Klf4 in fibroblasts generates iPSCs (Takahashi and Yamanaka, 2006; Yu *et al*., 2007; Park *et al*., 2008; Yu *et al*., 2009; Okita *et al*., 2010; Stadtfeld and Hochedlinger, 2010; Warren *et al*., 2012). Pluripotency markers have been recently, validated for the Yamanaka's factors which can induce the reprogramming of cochlear cells, fibroblasts and somatic cells into iPSCs and express ESC like markers (Table 2). However, it exhibits pluripotency during in-vitro and in-vivo conditions. Recently a breakthrough has been achieved by generating fully pluripotent iPSCs from mammalian cochleae with defined exogenous genes (Du and Lou, 2014).

Table 2: Markers of pluripotency

Name of iPSC markers	Experiment Design	Techniques used for validation	References
Flt1+ , Flt4+	mouse embryonic stem cells and Cardiovascular progenitor cells (CPCs) vs normal cells	Microarray, FACS, Immuno-histochemistry, qRT-PCR	Nsair *et al*., (2012)
OCT4, NANOG and SOX2	Fibroblasts vs normal cells	FACS, IHC, qRT-PCR, Wetern blot,	Chang and Daley, (2008) Yu *et al*., (2009) Jia *et al*., (2010)
OSKM, SSEA-1, Thy1+, X-GFP	Viral and fibroblasts vs normal cells	FACS, qRT-PCR	Stadtfeld *et al*., (2008)

APPROACHES TO DISEASE MODELING WITH iPSCs

In recent decades, nanotechnology has attracted major interests in the field of drug delivery systems and therapies against broader range of diseases, such as cancer, and neurodegenerative diseases. Therapies involving stem cells are considered to have an outstanding potential in the treatment for numbers of maladies. Consequently, the combination of nano-medicines and iPSCs could actually be the potent arms for remedies in transplantation medicine and personalized medicine (Jang *et al*., 2014). iPSCs, currently have been used to model Parkinson's disease (PD) in human. Fibroblasts from patients carrying pathogenic mutations that lead to PD have been reprogrammed into iPSCs, which can subsequently be differentiated into important cell types (Beevers *et al*., 2013). The emerging "Organs-on-Chips" technology in combination with iPSCs offers unprecedented opportunities to develop human *in-vitro* models (Table 3). Moreover, this combined approach enabled to investigate the fundamental mechanisms involved in disease development, screening of drug toxicity, drug target discovery, testing of healthy and diseased organ tissues (van de Stolpe and den Toonder, 2013). In contrast, validation and optimization through these systems will likely be a long process. Organs that have been simulated by microfluidic devices include the heart, the lung, kidney, artery, bone, cartilage and skin. iPSCs can further be used in the artificial organ synthesis on bio-surface and generate desired tissues or organs on electronic circuit and substrate such as Artery-on-a-chip, nephron-on-a-chip and Kidney-on-a-chip.

All these theories of iPSCs application in the therapeutic approaches may open the new avenue for the animal biotechnology to develop the iPSCs based therapeutic regimens for the treatment of animal diseases.

Table 3: Approaches to Disease Modeling with iPSCs.

Name of disease	Molecular defects	Cell types differentiate from iPSC	References
Amyotrophic Lateral Sclerosis	mutations in the SOD1 gene	Motor neurons and glial cells	Chestkov *et al*., (2014)
Parkinson's disease	Mutation in the LRRK2, PARK2, PARK7, PINK1, GBA, UCHL1 and SNCA gene	Differentiation of DA Neurons from Pluripotent Stem Cells	Sundber *et al*., (2013)
Huntington's disease	72 CAG repeats in the huntingtin gene	Human fibroblast into iPSC-Derived NSC	Mattis *et al.,* (2012)
Sickle-cell anaemia	Homozygous HbS mutation	Haematopoietic cells	Kim, (2014); Sebastiano *et al*., (2011)
α1-Antitrypsin deficiency (A1ATD)	Homozygous mutation in the α1-antitrypsin gene	Hepatocyte-like cells (fetal)	Yusa *et al*., (2011)
Type 1 diabetes	Multifactorial; unknown	β-Cell-like cells (express somatostatin, glucagon and insulin; glucose-responsive)	Thatava *et al*., (2012)

iPSC DERIVATION, DIFFERENTIATION AND APPLICATIONS

Nowadays research on iPSCs application is emphasizing in human medicine and later this may bring the breakthrough in the animal biotechnology for treating numerous breakthroughs. As iPSCs derived from any patient, can self-renew and differentiate into many cell types. They offer a renewable tissue resource for disease modeling, developing novel strategies for drug discovery or drug 'rescue' and studying interactions between the gene of interest and drug, (Bellin *et al*., 2012; van de Stolpe and den Toonder, 2013).

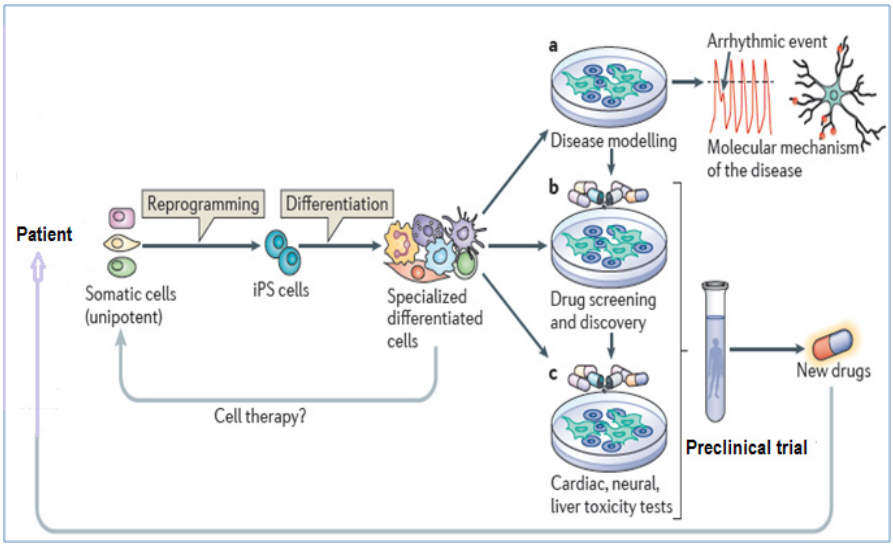

Figure 1: Reprogramming of adult stem cells (unipotent) into induced pluripotent stem cells (Modified from Bellin *et al*., 2012).

CONCLUSION

The possibility of reprogramming adult somatic cells into iPSCs has created a renewed interest in the arena of stem cell research. It promises to overcome several key issues, including the ethical concerns of using human embryonic stem cells and the difficulty of obtaining large numbers of adult stem cells (Izpisua Belmonte *et al*., 2009). The traditional approaches involved in the transformation of somatic cells to pluripotency revealed very low efficiency of reprogramming, genetic aberrations, manipulations and mutations which can cause several abnormalities at the molecular basis of the life (Stadtfeld *et al*., 2008; Fusaki *et al*., 2009; Kim *et al*., 2009; Zhou and Freed, 2009; Hussein *et al*., 2011; Gore *et al*., 2011). Recent scenarios of inventions are focusing at iPSCs reprogramming without genetic incorporations, zero genetic aberrations and zero foot print based approaches. To achieve successful gene therapy, development of proper gene delivery systems could be one of the most important factors. Several non-viral and viral gene transfer methods have been developed. Even though the viral agents have a high transferring efficiency but they are difficult to handle due to their toxic side effects. To overcome the safety problems of the viral counterpart, several non-viral *in-vitro* and *in-vivo* gene delivery systems have been developed. Out of these, the most promising and latest system include plasmid mediated gene insertion. Shunning of possible immunogenicity, toxicity, and the feasibility of repeated administration are some of the merits of non-viral gene delivery systems over viral gene delivery (Woltjen *et al*., 2009; Steffy B Manjila *et al*., 2013; Tsukiyama *et al*.,

2014). Plasmid mediated and transposon-based reversible genetic modification is a novel paradigm for iPS induction.

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