SZENT ISTVÁN UNIVERSITY

Animal Husbandry Science PhD School

GENERATION OF MOUSE INDUCED PLURIPOTENT STEM (iPS) CELLS BY *SLEEPING BEAUTY* (SB) TRANSPOSON

Suchitra Muenthaisong

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The PhD program

Name:	**Animal Husbandry Science PhD School**
Discipline:	**Animal Husbandry Science**
Leader of the shool:	**Professor Dr. Mézes Miklós D.Sc**. Head of Department, Szent István University, Faculty of Agricultural and Environment Science, Department of Nutrition
Supervisor:	**Professor Dr. Dinnyés András D.Sc.** Head of Molecular Animal Biotechnology Laboratory, Szent István University, Faculty of Agricultural and Environment Science, Institute for Basic Animal Sciences

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Approval of the PhD School leader Approval of the Supervisor

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ABBREVIATIONS

ADA-SCID: Adenosine Deaminase-Severe Combined Immunodeficiency

ALS: Amyotryophic Lateral Sclerosis

AP: Alkaline Phosphatase

AV: Adenovirus

AZA: Azacytidine

bFGF: Basic Fibroblast Growth Factor

BMD: Becker Muscular Dystrophy

BSA: Bovine Serum Albumin

C57BL/6: C57 Black 6

CHIR99021: Glycogen synthase kinase-3 inhibitor

c-Myc: Cellular Myelocytomatosis Oncogene

CO_2: Carbondioxide

CPP: Cell-Penetrating Peptide

d.p.c: Days Post Coitum

DA: Dopaminergic

DAPI: 4',6'-diamidino-2-phenylindole

Dlk1-Dio3: Delta-like homolog 1 gene and the type III iodothyronine deiodinase gene

DMEM: Dulbecco's Modified Eagle's Medium

DNA: Deoxyribonucleic Acid

DNMT: DNA Methyltransferase

DS: Down Syndrome

DMD: Duchenne Muscular Dystrophy

EBs : Embryoid Bodies

EBNA1: Epstein-Barr Nuclear Antigen 1

EDTA: Ethylenediaminetetraacetic Acid

EF1α: Elongation Factor-1 Alpha

EP: Episomal Plasmid

Esrrb: Estrogen-Related Receptor Beta

Essrrg: Estrogen-Related Receptor Gamma

ES: Embryonic Stem

ET: Embryo Transfer

F1: Filial-1

FBS: Fetal Bovine Serum

FM: Fibroblasts Medium

Fbx15: F-box only Protein 15

HDAC: Hintone Deacetylase

IR: Inverted Repeat

GD: Gaucher Disease

GFP: Green Fluorescence Protein

GLT : Germline Transmission

GSK-3: Glycogen Synthase Kinase

HD: Hanging Drop

HD: Huntington Disease

HEMA: Hydroxyethyl Methacrylate

HFFs: Human Fetal Fibroblasts

ICM: Inner Cell Mass

ICR: Imprinting Control Region

iPS: Induced Pluripotent Stem

IRES: Internal Ribosomal Entry Site

JDM: Juvenile-onset type I diabetes mellitus

KK20: Kinase Calcium 20 serum-free medium

Klf-4: Krüppel-like Factor-4

KSOM: Keck School of Medicine

L: Lin28

LIF: Leukemia Inhibiting Factor

LV: Lentivirus

M: Minicycle

MAPKK: Mitogen Activated Protein Kinase

MEFs: Mouse Embryonic Fibroblasts

miPS: Mouse Induced Pluripotent Stem

MMLV: Moloney Murine Leukemia Virus

N: Nanog

NEAA: Non-Essential Amino Acid

NPCs: Neuron Progenitor Cells

O_2: Oxygen

Oct-4: Octamer-binding transcription factor-4

oriP/EBNA1: Replication origin/Encoded-Barr Nuclear Antigen-1

OSKM: Oct4-Sox2-Klf4-cMyc

OSLN: Oct4-Sox2-Lin28-Nanog

PM: Plasmid

PB: *piggybac* Transposon

PD: Parkinson Disease

poly-2-HEMA: Poly-2-Hydroxyethyl Methacrylate

PT: Protein

RA: Retinoic Acid

RNA: Ribonucleic Acid

RT-PCR: Reverse Transcription Polychain Reaction

RV: Retrovirus

SB: *Sleeping Beauty* Transposon

SBDS: Shwachman-Bodian-Diamond Syndrome

SCNT: Somatic Cell Nuclear Transfer

SMA: Spinal Muscular Atrophy

Sox-2: Sex-determining region-Y

SSEA-1: Stage-Specific Embryonic Antigen-1

SV: Sendai virus

SV40LT: Simian Virusa-40 Large-T Antigen

T: SV40-large T-antigen

T2A: *Thosea asigna* virus

Wnt3A: Wingless-type MMTV integration site family, member 3A

VPA: Valproic Acid

β-ME: β-mercaptoethanol

CHAPTER 1. INTRODUCTION

Embryonic stem (ES) cells have an unlimited expansion potential and are able to produce many differentiated and functional cell types. Directed differentiation of ES cells can provide a valuable source of specialized cells for regenerative cell therapy of damaged tissues. However the generation of human ES cell lines requires the sacrifice of human embryos. The generation of patient-specific ES cells requires also access to high quality human oocytes. Thus, the generation of human ES cells, raises serious ethical issues.

Recent advances of induced pluripotency in mouse and human fibroblast cells have resulted in the generation of a new type of stem cell, called induced Pluripotent Stem (iPS) cells (Takahashi and Yamanaka 2006; Okita *et al*. 2007; Wernig *et al*. 2007; Kaji *et al*. 2009; Woltjen *et al*. 2009). The iPS cells have been produced from adult differentiated cells in mouse and human by introducing a few key pluripotency genes. These cells have shown characteristics surprisingly similar to ES cells and like ES cells, are also able to differentiate into many somatic tissues. To date, the best demonstration of their ES-like differentiation potential has been made through the generation of whole mice by tetraploid complementation (Zhao *et al*. 2009). The iPS cells might be a replacement for ES cells, as they overcome the ethical and legal limitations of embryo and cloning research. The long-term goal of iPS technology is to generate patient-specific donor cells for transplantation, which can be expanded and differentiated to multiple cell types, and also be genetically modified for gene therapy purposes.

Despite the success with retro- and lentivirus based iPS cell generation, there are reports on increased prevalence of tumor formation in mice generated from such cells (Okita *et al*. 2007; Nakagawa *et al*. 2008). The scientific aim of this study is to improve techniques for the generation of iPS cells, in order to find the safest and most efficient way to de-differentiate adult mouse cells into the pluripotent state. In this study, I used a non-viral, transposon-based gene delivery method, the *Sleeping Beauty* (SB) expression system (Ivics *et al*. 1997). The advantage of this system over viral methods is that the transposon integrates randomly at the genome level and does not show a pronounced bias for integration into genes. I were able to generate iPS lines from three different genetic backgrounds by using this technique. These lines were found to be pluripotent and differentiated into multiple lineages both *in vitro* and *in vivo*. Therefore, the generation of pluripotent stem cells from differentiated somatic cells has

various therapeutic implications, particularly in the aim of disease modeling, pharmaceutical screening, and xeno-transplantation therapies.

This scientific study is aimed to generate novel information on the generation and maintenance of iPS cells from mouse fibroblasts on their differentiation towards cardiac lineage. I systematically investigated the effect of the origin of the genetic background in order to be clarified this novel technology prior to clinical progress. The iPS cells were analyzed and compared comprehensively with existing ES cell lines. In addition, practical approaches can be the basis for the researcher to improve the understanding on the reprogramming of the cells.

CHAPTER 2. LITERATURE REVIEWS

2.1 INTRODUCTION

Pluripotent cells have a potential to give rise into any of all three germ layers: endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system). Pluripotent cells, however, cannot make an extra-embryonic tissue such as amnion, chorion, and placenta. Pluripotency by *in vivo*, is observed in early embryos while *in vitro*, pluripotency can be maintained in ES cells state which derived from inner cell mass (ICM) of blastocyst stage embryos (Figure 1a). These ES cells were first found from mouse embryos (Evans and Kaufman 1981; Martin 1981) which they can be unlimited proliferate and have the potential to develop into many different cell types.

The first breakthrough was when Takahashi and Yamanaka discovered the direct reprogramming of somatic cells to pluripotency state from mouse embryonic fibroblasts (MEF) to iPS cells (Figure 1b) by retroviral transduction of a select group of transcription factor (Takahashi and Yamanaka 2006). The discovery of these iPS cells gave a new insight in order to develop the stem cell research. This technology offers a significant technical simplicity and enables generate specific stem cell lines with reduce ethical concerns.

Recently, many new methods of generating iPS cells have advanced rapidly with less or no exogenous genetic modifications. Many research generated iPS cells with nearly identical functionally to ES cells by optimized iPS technology. However, the establishment of iPS lines is still low efficiency, remain to be characterized and many mechanism still unknown. Several approaches must be considered in order to reproduce the iPS cells including of many factors for example, reprogramming factors, delivery system, the donor cell type, culture condition method and identification and characterization.

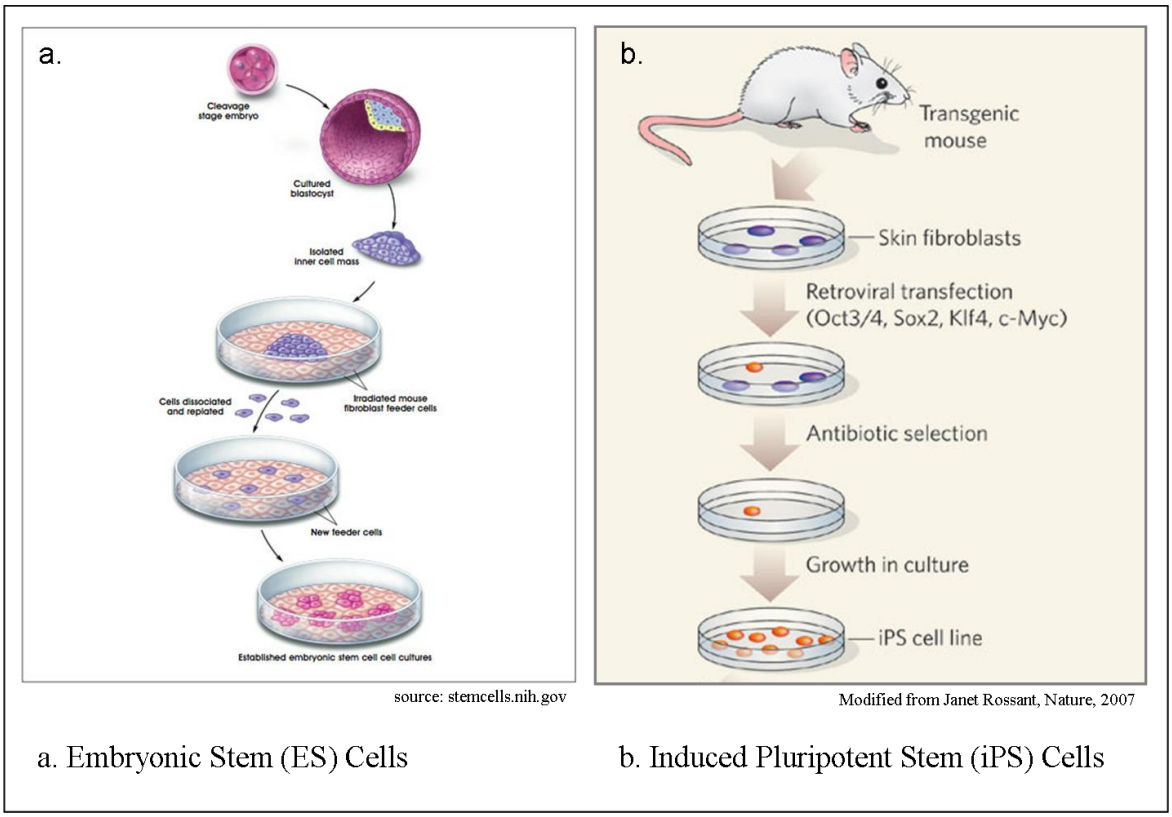


Figure 1. Generation of pluripotent cells from ES and iPS cells.

2.2 ESTABLISHMENT FACTORS OF IPS CELLS

Mouse induced pluripotent stem cells (miPS cells) were first generated by introducing four transcription factor of Pou5f1 (Oct-4), Sex-determining region Y-Box2 (Sox-2), Krüpple-like factor 4 (Klf-4) and cellular-myelocytomatosis oncogene (c-Myc) via retroviral delivery coupled to reactivation of a neomycin-resistant reporter gene knocked into the Fbx15 locus (Takahashi and Yamanaka 2006). Initially, direct reprogramming was performed with 24 different factors which were implicated to have a potential role in the induction of pluripotency. These 24 factors was ultimately selected to four transcription factors, Oct-4, Sox-2, Klf-4 and c-Myc, that were sufficient to establishment and maintenance of the pluripotent state. Gene expression and epigenetic profiling demonstrating the iPS cells are similar, although not identical to ES cells. The iPS cloned is pluripotent and having the ability to differentiate into all three germ layers, endoderm, mesoderm and ectoderm (Takahashi and Yamanaka 2006). This reprogramming factor set has been shown to work in several of mouse cell types (Table 1, modified from Patel and Yang 2010; Okita and Yamanaka 2011).

2.1.1 Reprogramming factors

a. Oct-4

Oct-4 (Octamer-binding transcription factor-4, also known as Oct3/4 and Pou5f1) is a transcription factor known to play a key role in the maintenance and self-renewal of pluripotent cells. Oct-4 was first described as a protein present in unfertilized oocytes, ES cells and primodial germ cells (Scholer *et al*. 1989) which is an important factor for the development of the inner cell mass (ICM) both *in vivo* and *in vitro* (Nichols *et al*. 1998). Oct-4 is specially expressed in the pluripotent cells, such as ES cells. As such, it is frequently used as a marker for undifferentiated cells. The expression of Oct-4 by less than 2-fold increase in expression causes differentiation into primitive endoderm and mesoderm whereas repression of Oct-4 induces loss of pluripotency and de-differentiation to trophectoderm (Niwa *et al*. 2000). Oct-4 has been shown to have a various number of target genes, many of which possess regulatory elements for Sox-2 and Nanog (Boyer *et al*. 2005). In mouse and human stem cells, only Oct-4 transgene can generate the iPS cells although the reprogramming efficiency is dramatically low (Kim *et al*. 2009b).

b. Sox-2

Sox-2 (Sex-determining region Y-box 2), *Sox-2*, is a transcription factor involved in the self-renewal of ES cells and is one of the key factor required in iPS cells. It has also an important factor which plays a role of maintenance pluripotent state of ES cells. In addition, Sox-2 is also expressed in the extra-embryonic ectoderm, trophoblast stem cells and the neural stem cells (Avilion *et al*. 2003). Furthermore, forced expression of Oct-4 can compensate for loss of Sox-2 in ES cells which may concluded that the primary role of Sox-2 in iPS cells is controlling Oct-4 expression (Masui *et al*. 2007). In mouse and human iPS cells, Sox-2 is dispensable for the reprogramming of neural stem cells, melanocytes and melanoma cells (Maherali and Hochedlinger 2009). Sox-2 is functionally redundant in part with other Sox family proteins, including Sox-4, Sox-11 and Sox-15 (Maruyama *et al*. 2005; Masui *et al*. 2007). In direct reprogramming, Sox-2 can be replaced by Sox-1, Sox-3 and, to a lesser extent, Sox-15 or Sox-18 (Nakagawa *et al*. 2008). However, iPS cells cannot be generated with the normal strategy in the absence of Sox transgenes. Recently, it has been shown that the transduction of Nanog gene could cover for the Sox-2 functional (Ichida *et al*. 2009). Therefore, exogenous Sox-2 is probably not essential for reprogramming. However, there is no many evidence of Sox dispensation in reprogramming and many approaches are different

method in order to improve only reprogramming efficiency.

c. Klf-4

Klf-4 (Krüpple-like factor-4, Klf-4) is a transcription factor expressed in a variety of tissues, including the epithelium of the intestine, particularly the epithelium of the intestine, kidney and skin (Zhao *et al*. 2004). Klf-4 was first reported as one of the downstream targets of LIF/Stat3 signaling in mouse ES cells (Li *et al*. 2005). Depending on the target gene and interaction partner, Klf-4 can both activate and repress transcription (Rowland and Peeper 2006). It has been reported that constitutive expression of Klf-4 suppresses cell proliferation by blocking G1-S cell cycle. By functional, Klf-4 can be both as an oncoprotein and tumor suppressor (Zhao *et al*. 2004). In reprogramming process, it is not fully understood the exact role of Klf-4 and it also can be replaced with other Klf family members (Klf-2 and Klf-5) (Nakagawa *et al*. 2008) or the unrelated factors Nanog and Lin28 (Yu *et al*. 2007). Recently, it has been demonstrated that Klf-4 can be substituted with estrogen-related receptor beta (Esrrb) or gamma (Esrrg) in order to generate the mouse iPS cells derived from MEFs (Feng *et al*. 2009).

d. c-Myc

C-Myc, Cellular-Myelocytomatosis Oncogen, is a helix-loop-helix transcription factor that has been linked to several cellular functions including cell proliferation, cell-cycle regulation, growth, metabolism and differentiation (Schmidt 1999). C-Myc is a key regulator of cytostasis and apoptosis through repression of the cyclin-dependent kinase (CDK) inhibitor p21Cip1 (Seoane *et al*. 2002). C-Myc reportedly functions during both self-renewal and diffierentiation of stem cells and progenitor cells by interactions between stem cells and the local microenvironment (Masui *et al*. 2007). This transcription factor highly expressed in the majority of rapidly proliferating cells and it is generally low or absent during quiescence (Murphy *et al*. 2005). As a proto-oncogene, c-Myc greatly enhances reprogramming efficiency, although it is dispensable for generate the iPS cells in mouse and human (Huangfu *et al*. 2008; Kim *et al*. 2008; Nakagawa *et al*. 2008; Wernig *et al*. 2008b; Sridharan *et al*. 2009). To reprogram somatic cell to ES-like cell state, C-Myc also can be replace by other family members such as N-Myc and L-Myc (Nakagawa *et al*. 2008). C-Myc is expressed in most of the mouse and human cell types whereas the N-Myc and L-Myc are limited expressed. Nonetheless, the role of c-Myc is remaining unclear in direct reprogramming.

Variation on the four-factor cocktail have been used to successfully reprogram cells and with improved selection methods, these reprogrammed cells can contribute to germlines in chimeric mice (Maherali *et al*. 2007; Okita *et al*. 2007; Wernig *et al*. 2007). Furthermore, iPS cells derived from MEFs can produce cloned live pups by tetraploid complementation which is one of the strictest hurdles of pluripotency (Boland *et al*. 2009; Kang *et al*. 2009; Zhao *et al*. 2009). It has also been reported that a partially different set of reprogramming factors, Oct-4, Sox-2, Nanog and Lin28, is sufficient to establish human fibroblasts to an ES-like cell state (Takahashi *et al*. 2007b; Yu *et al*. 2007). These data suggested that the differentiation potentials of iPS cells are already equivalent to those ES cells. However, it has been showed that Sox-2 could be replaced with Sox-1, Sox-3, Sox-7, Sox-17, or Sox-18. It has also been demonstrated that Klf-4 was able to be substituted by Klf2 or Klf5. Furthermore, Myc family genes such as N-Myc and L-Myc mimic c-Myc function during direct reprogramming. However, Oct families such as Oct1 and Oct6 could not be replace for Oct-4 (Nakagawa *et al*. 2008).

Table 1. Summary of iPS cells derived from different cell type, reprogramming factors, and method in different species.

Species	Cell types	Factors[a]	Method	Efficiency (%)	Reference
Mouse	Embryonic and adult fibroblasts	OSKM	RV	0.02	Takahashi and Yamanaka 2006
	Embryonic and tail tip fibroblasts	OSKM	RV	0.1	Wernig *et al*. 2007
	Embryonic fibroblasts	OSKM	LV	0.01	Okita *et al*. 2007
	Embryonic fibroblasts	OSKM	PM	0.01	Okita *et al*. 2008
	Fibroblasts	OSKM	RV	ND	Nakagawa *et al*. 2008
	Multiple somatic tissues	OSKM	LV	0.1	Wernig *et al*. 2008a, b
	B lymphocytes	OSKM	LV	> 0.5	Hanna *et al*. 2008
	Hepatocytes and fibroblasts	OSKM	AV	0.1	Stadtfeld *et al*. 2008a
	Neural stem cells	OK, OM	RV	0.02-0.05	Kim *et al*. 2008
	Neural stem cells	O	RV	0.014	Kim *et al*. 2009b
	Fibroblasts	OSKM	PB	ND	Kaji *et al*. 2009
	Mature B and T cells	OSKM	LV	0.02	Eminli *et al*. 2009
	Myeloid progenitors	OSKM	LV	2	Eminli *et al*. 2009
	Hematopoietic stem cells	OSKM	LV	13	Eminli *et al*. 2009
Mouse	Dermal papilla	OKM	RV	1.4	Tsai *et al*. 2010
	Dermal papilla	OK	RV	0.02	Tsai *et al*. 2010

Species	Cell types	Factors[a]	Method	Efficiency (%)	Reference
Human	Fibroblasts	OSKM	RV	0.02	Takahashi *et al*. 2007b
	Fibroblasts	OSNL	LV	0.02	Yu *et al*. 2007
	Keratinocytes	OSKM	RV	ND	Aasen *et al*. 2008
	Fibroblasts	OSK	RV	0.002	Nakagawa *et al*. 2008
	Fibroblasts	OSKM	AV	< 0.001	Zhou and Freed 2009
	Fibroblasts	OSKM	SV	> 0.1	Fusaki *et al*. 2009
	Fibroblasts	OSKM NLT	EP	< 0.001	Yu *et al*. 2009
	Embryonic fibroblasts	OSKM	PB	< 0.01	Kaji *et al*. 2009; Woltjen *et al*. 2009
	Adipose stem cells	OSNL	M	< 0.001	Jia *et al*. 2010
	Keratinocytes and foreskin fibroblasts	OSKM	RNA	< 0.01	Warren *et al*. 2010
	Cord blood stem cells	OSKM		ND	Eminli *et al*. 2009
	Cord blood endothelial cells	OSLN	RV	0.01-0.03	Haase *et al*. 2009
	Fibroblasts	OSKM	PT	< 0.001	Kim *et al*. 2009
	Adipose stem cells	OSKM	RV	0.5	Sugii *et al*. 2010
Human	Peripheral blood cells	OSKM	SV	< 0.01	Seki *et al*. 2012
Rat	Fibroblasts	OSKM	RV	0.05	Liao *et al*. 2009
	Liver progenitor cells	OSK	RV	ND	Li *et al*. 2009
	Neural progenitor cells	OSK	RV	0.01	Chang *et al*. 2010

Species	Cell types	Factors[a]	Method	Efficiency (%)	Reference
	Embryonic fibroblasts	OSK	LV	ND	Hamanaka *et al*. 2011
Pig	Embryonic fibroblasts	OSKM	RV	ND	Esteban *et al*. 2009
	Fetal fibroblasts	OSKM	LV	0.1	Ezashi *et al*. 2009
	Primary ear fibroblasts	OSKM	LV	ND	Wu, Z. *et al*. 2009
	Primary ear fibroblasts	OSKM	PM	ND	Montserrat *et al*. 2011
Rhesus monkey	Fibroblasts	OSKM	RV	ND	Liu, H. *et al*. 2008
Marmoset	Skin fibroblasts	OSKM	RV	0.1	Wu, Y. *et al*. 2010
Rabbit	Adult fibroblasts	OSKMN	LV	0.25-0.5	Honda *et al*. 2010
Buffalo	Fetal fibroblasts	OSKM	RV	< 0.01	Deng *et al*. 2012

[a]O, *Oct-4*; S, *Sox-2*; K, *Klf-4*; M, c-Myc; N, Nanog; L, Lin28; T, SV40-large T-antigen; RV, Retrovirus; LV, Lentivirus; AV, Adenovirus; PB, *piggyBac* transposon; M, Minicycle; PM, Plasmid; SV, Sendai virus; EP, Episomal plasmid; PT, Protein

2.2.2 Delivery system

Although direct reprogramming is being widely studied because it presents a possibility of generating reprogrammed somatic cells just by introducing a set of gene. However, the efficiency of reprogramming is still low including the majority problems of using retroviruses may result in insertional mutagenesis within a genome. Therefore, to overcome the viral effect, various techniques are being explored to perform direct reprogramming in order to establish the integration-free iPS cell cloned and improve the reprogramming efficiency. Based on delivery system of the reprogramming factor, the method can be divided into three groups: integration, excisable and non-integration delivery system.

2.2.2.1 Integrative delivery systems

a. Retroviral delivery system

First generations of mouse and human iPS cells were produced via retroviral system (Takahashi and Yamanaka 2006; Takahashi *et al*. 2007a) and constitutive lentiviral transduction (Blelloch *et al*. 2007; Yu *et al*. 2007). The first attempts to deliver transcription factors into mouse or human fibroblasts originally used moloney murine leukaemia virus (MMLV)-based retroviral vectors. This system is known to undergo silencing in the ES cell state (Takahashi *et al*. 2007a; Wernig *et al*. 2007; Aasen *et al*. 2008). These self-silencing vectors have cloning capacities which allow to deliver genes into the genome of dividing cells and they are usually silenced in ES cells. Silencing is important to iPS cells because it has potentially up-regulated the endogenous pluripotency gene network and down-regulated the expression of the transgenes. This silencing can effected to be fully reprogrammed of iPS cells (Hotta and Ellis 2008). To reprogram cells with retroviruses, silencing occurs gradually during the course of iPS cell induction, resulting in a lowered efficiency of conversion compared to non-silencing viral methods (Stadtfeld *et al*. 2008b). The retroviruses-derived iPS cells are often maintained viral gene expression (Dimos *et al*. 2008; Park *et al*. 2008) thus limiting their utility.

Consequences by using retrovirus-based induction are: 1) random insertion of the viruses within a host genome, 2) requirement of proliferating cells for transduction and infectivity, 3) presence of a low titer number, 4) occurrence of insertion mutagenesis and 5) low transfection efficiency. Moreover, it is also difficult to store and control the quality of retroviruses. The

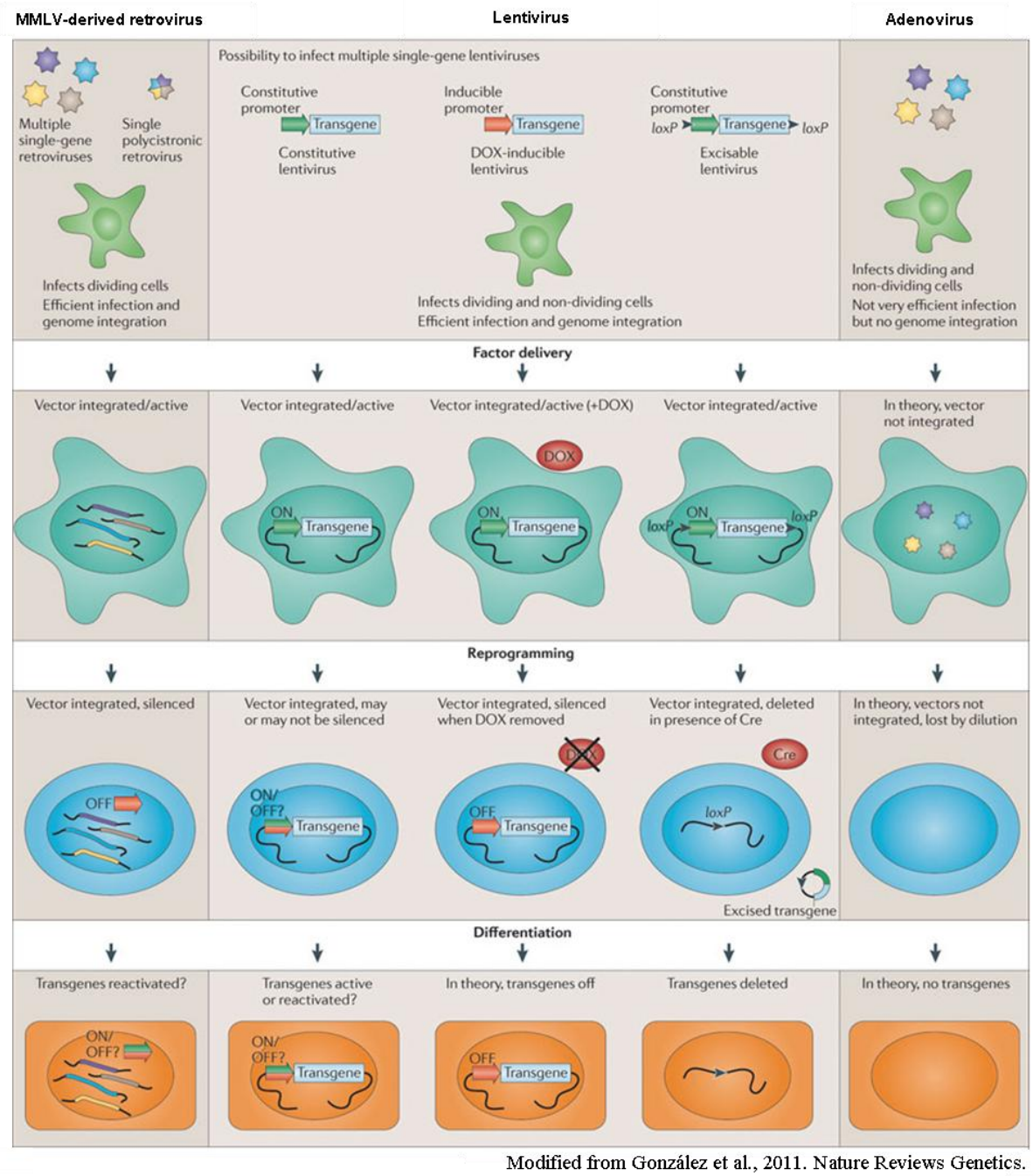
major disadvantage is that occurrence of tumours. It has been reported that viral transgene reactivation in iPS-derived chimeric mice has been implicated in tumorigenesis (Nakagawa *et al*. 2008). The integration site analysis of iPS cells yielded no common target or a pathway which indicates the genomic integration is not necessary for reprogramming (Aoi *et al*. 2008; Varas *et al*. 2009). The result of this derivation has confirmed in mouse iPS cells using transient delivery method (Okita *et al*. 2008; Stadtfeld *et al*. 2008c). Later, it had been used inducible lentiviruses to generate the iPS cells (Brambrink *et al*. 2008; Hockemeyer *et al*. 2008; Maherali and Hochedlinger 2008; Stadtfeld *et al*. 2008b).

b. Lentiviral delivery system

Lentiviruses have been used as a gene transfer vector since the discovery, they can infect both proliferating and non-proliferating cells. Lentiviruses can be used to integrate into the host chromosome without the viral gene expression in the target cells (Naldini *et al*. 1996). For non-dividing cell types, lentiviruses permit the transduction with high expression levels. However, the major disadvantage is that it is derived from immunodeficiency viruses which posses a safety concern, difficult to storage and quality control. Using lentivirus vectors also limit the size of insert. Generally, lentiviruses are derived from HIV which exhibit slightly higher cloning capacities and usually have higher infection efficiency than MMLV-based retroviruses. Tet-inducible reprogramming lentiviruses vectors have been successfully used in somatic cells to express different sets of reprogramming factors (Blelloch *et al*. 2007; Yu *et al*. 2007). It has been found the efficiency of reprogramming using lentiviral vectors is less effectively repressed compared to MMLV-base retroviral vectors in pluripotent stem cells but the main advantage that they present is their availability as inducible system.

Lentiviral vectors are efficient and reproducible, however, iPS cells line which generated using these vector carry randomly distributed viral transgene insertion (Varas *et al*. 2009). They could disrupt the expression of tumour suppressor genes if there are inserted nearby the open reading frames or disturb later the expression of oncogenes. Moreover, it complicates comparative analysis as they unavoidably generate heterogenous iPS cells line. In case of properly silenced, eventually it leads to tumours as viral transgenes can reactivated during differentiation or during the life time of transplanted or iPS cell-derived animal (Okita *et al*. 2007). Although iPS cells made with constitutive lentiviruses have been used (Blelloch *et al*. 2007; Yu *et al*. 2007), it is still unclear how differentiation proceeds during transgene

expression and also integrate into the host genome (Brambrink *et al*. 2008; Stadtfeld *et al*. 2008b), therefore integrative-viral delivery systems do not represents a safety method for therapeutic applications (Figure 2).



Modified from González et al., 2011. Nature Reviews Genetics.

Figure 2. A summary diagram of viral delivery methods.

2.2.2.2 Excisable delivery systems

In order to reprogram fibroblasts, the main problems of retrovirus-based system are oncogenicity and mutagenesis. Chimeric mice derived from iPS cells as well as their offsprings developed tumors, probably because the reactivation of the proviral c-Myc oncogene (Okita *et al*. 2007). Even thought to reprogram with three factors; Oct-4, Sox-2, Klf-4, iPS-derived animal did not develop tumors (Nakagawa *et al*. 2008), ectopic expression of any one of these genes may have deleterious consequences. Furthermore, retroviral integration itself may cause insertional mutagenesis and also may change the expression pattern of genes (Nair 2008). Therefore, non-viral vector for gene transfer are promising tools for genetic studies and therapies because of their high productivity and high safety. One of the approaches to overcome this limitation is utilization of transposons (Ivics and Izsvak 2006).

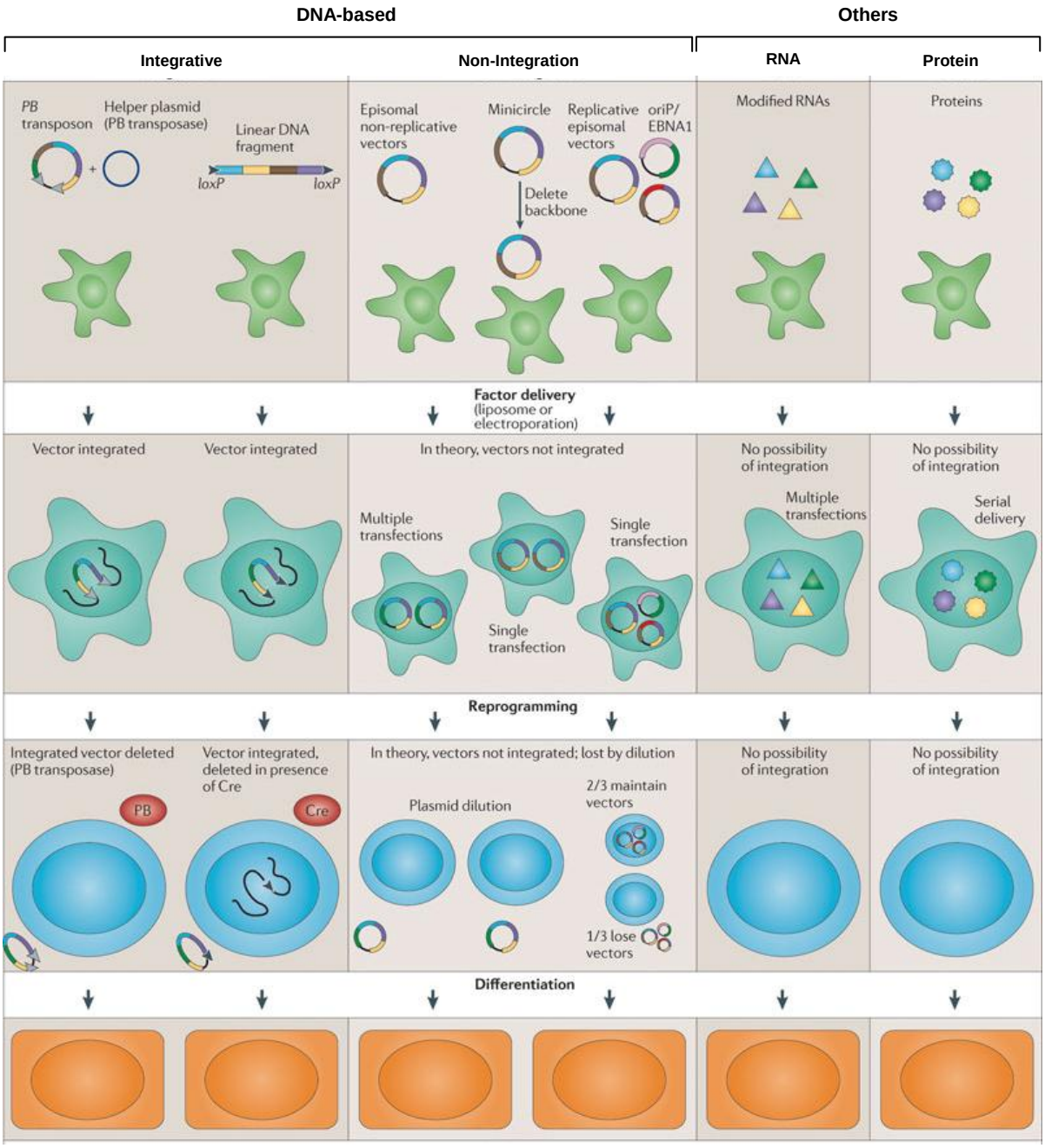
Transposon (or transposable element) is a DNA sequences that can change its relative position within the genome of a single cell. The mechanism of transposition can be either "copy-and-paste" or "cut-and-paste". Transposition can create phenotypically significant mutations and alter the cell's genome size (Mc 1950). Transposon are only one of several types of mobile genetic elements which they are assigned into 2 categories based on their mechanism of transposition: 1) retrotransposon that undergo transposition via an RNA intermediate and can be described as "copy-and-paste" mechanism of transposition, and 2) DNA transposon that move directly as DNA and can be described as "cut-and-paste" (Wicker *et al*. 2007). The transposon system consists of two components: a DNA element flanked by two terminal inverted repeats (IRs) and a transposase that catalyzes the transposon's mobilization by either "copy-and-paste" or "cut-and-paste" mechanism. The transposase first bind to the IRs, then excise the DNA segment flanked by the IRs from the genome and finally reintegrates the segment into a new location (Ivics *et al*. 1997).

a. *piggyBac* (PB) transposon delivery system

Unlike viral vectors, this system does not require special storage or quality control conditions. It does not need to be prepared in high titers and does not have a limited lifetime. Its transfection efficiency is increased and commercial products for gene delivery are available. Another advantage of this system over viral vectors is the non-occurrence of viral infections. This system was first used to reprogram fibroblasts to iPS cells by using *piggyBac* transposon (Woltjen *et al*. 2009; Yusa *et al*. 2009). The PB transposition is host-factor independent and

has recently been demonstrated to be functional in various human and mouse cell lines (Ding *et al*. 2005; Wu *et al*. 2006; Cadinanos and Bradley 2007; Wang *et al*. 2008). The PB element was originally discovered from cabbage looper moth *Trichoplusia ni* cell line TN-368 as a repetitive element (Fraser *et al*. 1996). The PB transposon/transposase system requires only the inverted terminal repeats flanking a transgene and transient expression of the transposase enzyme to catalyze insertion or excision events (Fraser *et al*. 1996). Subsequently, it was isolated (Cary *et al*. 1989) and found to efficiently transpose in many different species (Lobo *et al*. 1999; Thibault *et al*. 2004; Ding *et al*. 2005). One important feature of the PB transposition is that it does not leave any footprints behind and it nearly always excises itself precisely when a transposition takes place (Elick *et al*. 1996; Fraser *et al*. 1996; Thibault *et al*. 2004). Furthermore, the PB transposon system has a very large cargo capacity. Up to 10 kb DNA fragments can be transposed without losing transposition efficiency (Ding *et al*. 2005).

Taking advantage of PB transposon system, they have recently demonstrated the generation of factor-free mouse iPS cells (Woltjen *et al*. 2009; Yusa *et al*. 2009), which were linked by 2A-self cleaving peptides. The approximately 20-amino acid-long 2A peptides from foot-and-mouth disease virus (F2A) and *Thosea asigna* virus (T2A) work as self-cleaving signals and enable expression of several gene products from a single transcript facilitating multi-gene delivery to target cells (Szymczak *et al*. 2004). By using PB system, they have induce mouse and human pluripotent stem cells from fibroblasts and subsequently they could remove transposon from their primay iPS cells by re-expressing transposase which PB excised without a footprint or any genetic alteration (Woltjen *et al*. 2009; Yusa *et al*. 2009). Among integrative methodologies, this approach is the only one that confirms a precise deletion of the transgenes, although alterations are sometimes observed in the integration site, which therefore need to verify the sequence (Park *et al*. 2008). Additional, the low error rate of this process allows for a seamless excision, but requires characterization of integration sites in iPS cells before and after transposon removal. It also remains unclear if transposase expression can induce non-specific alterations in iPS cells (Stadtfeld and Hochedlinger 2009).



Modified from González *et al*., 2011. Nature Reviews Genetics.

Figure 3. A summary diagram of non-viral delivery methods.

b. *Sleeping Beauty* (SB) transposon delivery system

Since Barbara McClintock (1950) discovered the DNA transposition in maize, many different transposable elements have been described and developed further as a tool for genetic analysis in a variety of organisms. SB was the first DNA transposon system discovered to be functional in mammalian cells (Mc 1950). SB transposon represents a type of mobile genetic element that belongs to the *Tcl/mariner* class of transposons. This SB transpose by DNA element movement. SB was assembled by combining fragments of silent and defective *Tc1/mariner* elements from salmonid fish, and probably resembles an ancestral transposon that had become inactivated during evolution (Ivics *et al*. 1997). One limitation of transposons of the mariner family, including SB, is that transposition efficiency decreases in the presence of an excess of transposase, a phenomenon termed "overproduction inhibition". Hence, the transposon-to-transposase ratio needs to be optimized (review in VandenDriessche *et al*. 2009).

The purpose of using SB transposon system can be considered as a vector delivery vehicles. This SB vector combines the advantages of viral vectors which directed integration of a single copy of a gene and with the advantage of non-viral vector as the absence of protein factors that can elicit adverse reactions (Liu *et al*. 2004). In contrast to viral vectors, SB transposon can also be maintained and propagated as plasmid DNA. This advantage also includes its reduced immunogenicity (Yant *et al*. 2000), improved safety/toxicity profiles (Ivics *et al*. 2007; Moldt *et al*. 2007; Walisko *et al*. 2008; VandenDriessche *et al*. 2009), and relaxed limitation on the size of expression cassettes (Zayed *et al*. 2004). The SB transposon system consists of two functional components: i) a transposon, specific part of DNA that made up of a gene interest flanked by terminal inverted repeats (IR), and ii) the transposase, a protein that mobilizes the transposon (Figure 4). During transposition occur, the SB transposase recognizes the ends of the IRs and catalyzes the excision from the delivered plasmid DNA, subsequently it then inserts the transposon into another DNA site. The transposase can be provided in *cis* or in *trans* in the form of a second plasmid (Review in Izsvak *et al*. 2010). The transposition-integration process occurs at TA dinucleotides by SB transposase cleaves the transposon-donor site at the flanking TA-dinucleotide basepairs. Note that the following integration, the original TA-dinucleotide basepair target sequence is duplicated on the both flanks of the transposon.

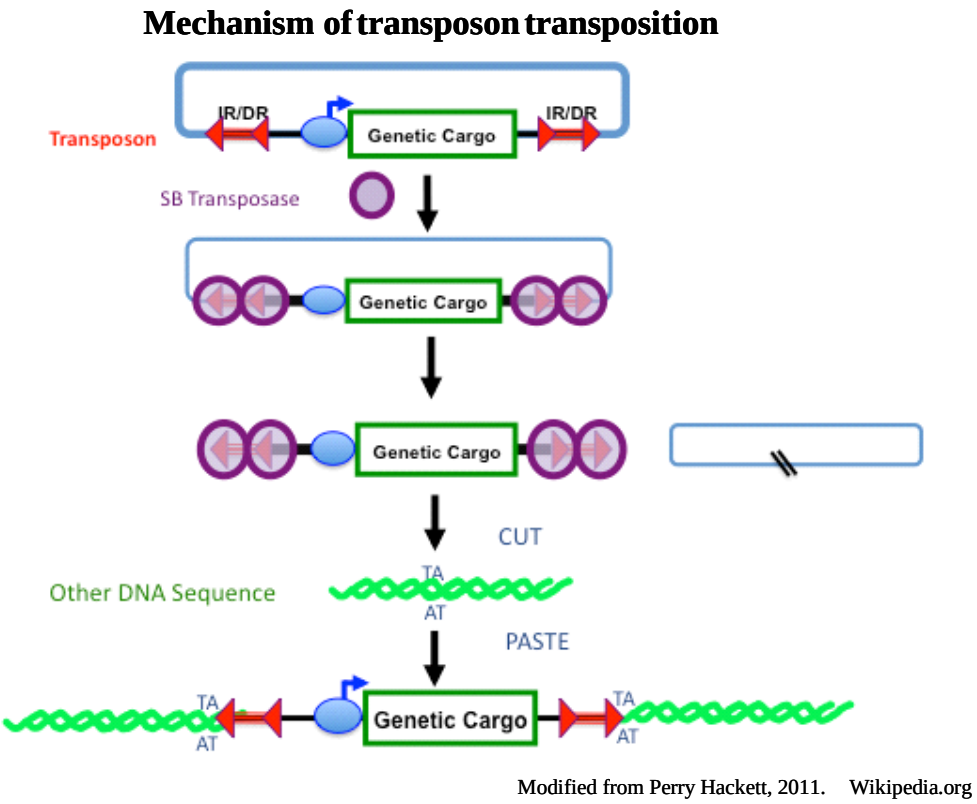


Figure 4. Mechanism of SB-mediated transposition.

Compared the SB transposon with retroviral vector (Schroder *et al*. 2002; Bushman 2003; Narezkina *et al*. 2004), or *piggyBac* transposon vector (Wilson *et al*. 2007), SB has very weak enhancer/promoter activity that could transcriptionally trans-activate neighboring gene sequences (Walisko *et al*. 2008). Recently, the hyperactive version of SB transposase designated SB100X, has been developed and characterized which approaches stable gene transfer efficiencies of integrating viral vectors (Mates *et al*. 2009). In contrast to retroviral (Narezkina *et al*. 2004), lentiviral (Schroder *et al*. 2002), recombinant adeno-associated viral vectors (Nakai *et al*. 2003), or PB transposon (Wilson *et al*. 2007), integration of SB transposon into the genome does not exhibit such biased integration pattern and has a random integration profile (Vigdal *et al*. 2002; Yant *et al*. 2005). These intrinsic advantages of the SB transposon system make it preferable choice as a stable gene delivery vector for both somatic or germ line transgenesis.

c. Plasmid delivery system

Many studies have the aim to avoid using viral vectors when considering iPS cells technology in therapeutic settings. Plasmid delivery system is an alterative non-viral vector transfection method in which to generated integration-free iPS cells (Kaji *et al*. 2009). Using of polycistronic vector, is a crucial improvement that allows the expression of several cDNAs from the same promoter. Kaji and colleagues successfully reprogrammed mouse fibroblasts to iPS cells by using a linearized 2A-peptide-based polycistronic vector flanked by *loxP* sites. They have been showed that single insertion of a single-copy polycistronic OSKM expression cassette were sufficient to achieve direct reprogramming. Once, reprogramming was achieved, the reprogramming cassette was then deleted with *Cre* recombinase in the between of *loxP* sites by transiently expression (Kaji *et al*. 2009). Additional, they succeed establishing reprogrammed human cell lines from embryonic fibroblasts by using the single-vector combined with PB transposon (Wang *et al*. 2008; Woltjen *et al*. 2009). This approach has a main advantage in order to delete the reprogramming factors and avoids the reactivation or constitutive expression of the reprogramming factors in iPS cells. These may improve the differentiation and reduce their oncogenic potential. Furthermore, this plasmid vectors have no tendency for chromosomal integration and gene expression from these vectors is transient (Figure 3).

However, efficiency of transduction is very much lower compared to viral system and it requires a large number of donor cells to obtain for certain cell types as substantially fewer donor cells receive the full set of reprogramming factors. Furthermore, it indicates that many clones obtaining transgene-deleted iPS cells are not necessarily straightforward as they represent reprogramming intermediates because many of colonies with the deletion start to differentiate (Kaji *et al*. 2009). The polycistronic vectors which allow reprogramming of somatic cells through a single insertion have been also used with integrative MMLV-derived retroviral vector (Rodriguez-Piza *et al*. 2010) and lentiviral vectors (Carey *et al*. 2009; Sommer *et al*. 2010). It showed that substantially reducing the number of genomic insertions compared with single factor expression viruses. By using *Cre-LoxP* system, such vectors represent a simply way to induce transgene-free iPS cells from various donor sources with higher transduction efficiencies than plasmid DNA. It has been shown these vectors eliminate the oncogenic risk related to transgene reactivation and have a positive effect on the differentiation potential of the resulting iPS cells (Sommer *et al*. 2010).

2.2.2.3 Non- integrative delivery systems

To avoid the permanent genetic modification resulting from integration of classical retroviruses or lentiviruses vectors, non-integrative approaches address a major limitation of iPS cells. The different approaches that are currently available can be subdivided into three categories: excisable, integration-defective viral and DNA-free delivery systems. Usually, the generation of the stable iPS cells requires several weeks to complete although the kinetics of reprogramming vary between different starting cell types and species.

a. Non-integration adenoviral delivery system

Adenoviral vectors were the first method to generate integration-free iPS cells. This method allows for transient and high-level expression of exogenous genes without integrating into the host genomes included avoids harmful effects of insertional mutagenesis and genetic alterations (Figure 3). However, the efficiency of using this system in mouse is approximately between 0.0001% and 0.0018% which is lower than retroviruses delivery system. It has been shown that iPS could generated from adult mouse hepatocytes and mouse embryonic fibroblast (Okita *et al*. 2008; Stadtfeld *et al*. 2008c). They were used adenoviral vector in order to generate transgene-free iPS cells contained 4 factor of reprogramming genes: Oct-4, Sox-2, Klf-4 and c-Myc without evidence of gene integration and also expressed endogenous pluripotency genes, produced teratomas and contributed to adult chimeras. Importantly, these reports proved that the principle of transient expression of the four classical reprogramming genes is sufficient to induced pluritpotent state in somatic cells as they showed DNA demethylation similar to that of reprogrammed cells.

b. Transient episomal delivery system

To improve further the reprogramming approaches, integration-defective viruses represent an alternative method based on direct delivery of non-replicating (Okita *et al*. 2008; Gonzalez *et al*. 2009; Jia *et al*. 2010) or replicating episomal vectors (Yu *et al*. 2009). These approaches are relatively easy to set up with standard laboratory and molecular biology experiences which avoid the time-consuming and labor-intensive production of viral particles. Especially they have been found that the iPS cell lines were free of plasmid integration even the efficiency of reprogramming are low by using serial transfection of two plasmid or single

plasmid expression OSKM set as polycistronic (Okita *et al*. 2008; Gonzalez *et al*. 2009). Because of a few cells received the appropriate amount of plasmid over the full reprogramming period that resulting in a low efficiency and no sign of plasmid integration. Furthermore, premature dilution of the vectors in actively proliferating cells or the active silencing of sequences contained in the backbone of these vector in mammalian cells leading to down-regulation of reprogramming factors (Pollack *et al*. 1980).

Yu and colleagues have been used oriP/Epstein-Barr nuclear antigen-1-based episomal vectors (oriP/EBNA1) to solve the problem of episome dilution through cell division (Yu *et al*. 2009). These vectors are maintained as stable extra-chromosome replications through the cell division and the cells were kept under selection conditions. It requires only *cis*-acting oriP elements, a trans-acting EBNA1 gene and a positive selection gene (Yates *et al*. 1984; Yates *et al*. 1985). They have been used three oriP/EBNA1 vectors expressing respectively, *OCT4–SOX2–NANOG– KLF4*, *OCT4–SOX2–SV40LT–KLF4 and MYC–LIN28*, by co-transfection to transduce the vector into donor cells (Figure 3). By removing, they successfully generated iPS colonies from HFFs in the absence of drug selection (Yu *et al*. 2009). However, the reprogramming efficiency by using oriP/EBNA1 vectors is extremely low as 3 of 6 colonies per million cells were transfected. The reason of these results can be explained with the facts that: such large plasmids (more than 12 kb) which their gradual loss efficiency through the cell division in the absence of drug selection, or may active silencing through DNA methylation in which resulting in low expression levels of reprogramming factors. As one of the reprogramming factors they used in this system is SV40LT antigen, potent viral oncoprotein, which is able to inactivate both p53 and the retinoblastoma pathways and can be caused tumorigenic in the generation of iPS lines. However, it still needs to be properly addressed for this aspect.

Minicircle vectors represent one of alternative method that is free of bacterial DNA and can be decreased the size of the reprogramming episomes. Minicircle vectors are supercoiled DNA molecules which lack a bacterial origin of replication and antibiotic resistance gene because their backbone is removed by PhiC31-mediated intramolecular recombination before purification (Chen *et al*. 2003; Chen *et al*. 2005). This method allows with high level expression of reprogramming factors as non-integrating and non-replicating episomes (Jia *et al*. 2010). This vector shows higher transfection efficiencies (their size is usually reduced by at least by 3 kb, the average size of the backbones usually found in episomal vectors) and

longer ectopic expression of the transgenes due to lower activation of exogenous DNA-silencing mechanisms compared to plasmids. Jia and colleagues constructed plasmid (P2PhiC31-LGNSO) by cloning a 2A-peptide-based polycistronic cassette comprise with Oct-4, Sox-2, Lin28 and Nanog (OSLN), plus a GFP reporter gene and reprogrammed human adipose stem cells in 14-16 days with an average efficiency of ~0.005%. None of these iPS cell lines carried integration of these minicircle vectors analyzed by southern blot (Jia *et al*. 2010).

c. Proteins delivery system

To address the safety issues arose and avoid the introduction of exogenous genetic material into the target cells genome, protein delivery system is another way in which iPS cells can be generated with potentially reduced risks of unexpected genetic modification by the exogenous sequences in the donor cells (Zhou and Freed 2009). Previous studies have demonstrated that proteins can be directly delivered into cells *in vitro* and *in vivo* by fused them with a short peptide mediating their transduction, such as HIV trans-activation of transcription (Tat) and poly-arginine (Wadia and Dowdy 2002; Michiue *et al*. 2005; Inoue *et al*. 2006). In addition, many process of solubilisations and refolding techniques include expression in *E. coli* to bioactive proteins have been developed to allow facile and large-scale production of therapeutic proteins (Lafevre-Bernt *et al*. 2008). Using this approach to generate the recombinant proteins, Zhou and colleagues fused the recombinant OSKM proteins with a poly-arginine transduction domain at the C-terminus of four reprogramming factors. These proteins were subsequently expressed in inclusion bodies of *E. Coli* which then were solubilised, refolded and further purified. To test the cell permeability and stability of the proteins, MEFs were serial transduced with *Oct-4*-GFP reporter by recombinant proteins and examined cell morphology and protein presence by immunocytochemistry. They have been found that GFP positive colonies obtained if the histone deacetylate (HDAC) inhibitor valproic acid (VPA) was also added to the culture media (Zhou and Freed 2009).

Recently, they have also been generated iPS cells from human fibroblasts by fused each of OSKM factors with a Myc tag and a tract composed of 9 arginines, known as CPP (Kim *et al*. 2009). They could be generated stable HEK293 cell lines which express each of the four human OSKM reprogramming factors and applied the extracts of these cells to human neonatal fibroblasts for 8 hours per week in total of 6 weeks. In particular, the whole protein

extracts used in this study limited the concentrations of factors delivered into the donor cells in the absence of any chemical treatment. Compared with protein-miPS-derived cells (Zhou and Freed 2009), they were not generated when only recombinant proteins were used, it has also combined with the small molecule VPA. However, the generation of protein-hiPS-derived cells is still very slow kinetics, inefficient of reprogramming and requires further optimization. Moreover, the recombinant proteins used in this approach are usually difficult to reproducibly purify in the required amount of proteins and also make them difficult to use routinely in the laboratory although such protein-iPS derived cells can long-term self-renew and are pluripotent both *in vitro* and *in vivo* (Kim *et al*. 2009a; Zhou and Freed 2009).

d. RNAs delivery system

A more efficient and safer way of producing integration-free iPS cells may be the introduction of modified RNA molecules encoding for the reprogramming factors into somatic cells (Figure 3), which has been validated recently (Warren *et al*. 2010). This system requires modification of *in vitro* transcribed RNAs in order to escape the endogenous antiviral cell defence response to ssRNA. Phosphatase treatment, incorporation of modified ribonucleoside bases substituting 5 methylcytidine for cytidine and pseudo-uridine for uridine, combined with the addition of a recombinant version of B18R protein in the medium, allowed for high, dose-dependent levels of protein expression with high cell viability. This approach showed that multiple human cell types can be reprogram to pluripotent state with higher efficiency compared with other non-integrative system (2% of neonatal fibroblasts being converted into iPS cells in 17 days). By delivering synthetic RNAs encoding OSKM and Lin28, reprogramming was achieved by serial transfection of different donor populations using a cationic vehicle (Warren *et al*. 2010). This method developed a system that achieves the efficient conversion of different human somatic cells into iPS cells using direct delivery of sysnthetic mRNAs modified to overcome innate antiviral response. Moreover, this approach allows protein stoichiometry to be exquisitely regulated within cultures while avoiding the stochastic variation of expression typical of integrating vectors, as well as the uncontrollable effects of viral silencing. Although this system is extremely appealing for its simplicity and efficiency, the high gene dosages of the reprogramming factors resulting from direct mRNA delivery may represent an oncogeneic risk owing to higher expression levels of Myc affecting genomic stability (Warren *et al*. 2010).

Some of the non-integrative approaches are difficult to apply owing to poor infection or transfection efficiencies, poor cell survival, long reprogramming kinetics or other limitations depending on the starting population (Table 2). These methodologies consider underline one of the major drawbacks as they are usually inefficient and poorly reproducible which is the principle reason why no conclusion has yet been reached in the community regarding a method of choice (Review in Gonzalez *et al*. 2011). Although the kinetics of reprogramming varies between different starting cell types and species, the generation of stable iPS cells usually requires several weeks to complete.

Table 2. Advantages and disadvantages of method of delivery system

METHOD	ADVANTAGES	DISADVANTAGE
Moloney-based retrovirus	Silenced in pluripotent cells	Genomic integration (Many viral integrations, fewer integration with polycistronic system)
	Self-silencing eliminates need for timed factor withdrawal	Risk of insertional mutagenesis
	Very efficient and stable	Limited to dividing cells
		Expression often maintained in iPS cells
		Increased tumor incidence in chimeric mice due to transgene reactivation
Lentivirus	Constitutive, inducible	Genomic integration
	Transduction of both dividing and nondividing cells	Risk of insertional mutagenesis
	Very efficient and stable	Lack of silencing in pluripotent state
	Temporal control over factor expression	Possibility of leaky expression
	Integrate-Deficient	Lower expression levels than integrated form
	Low frequency of genomic integration	Integration provides selective advantage and necessitates clone screening
		Possible long terminal repeat integration close to oncogene
Adenovirus	No genomic integration	Repeated infection required for certain cell types
	Transgene-free and vector free	Delayed kinetics of reprogramming
		Slow and inefficient
Transposon	Transgene-free and vector-free	Genomic integration
	Average efficiency	Negative selection strongly advised
Plasmid DNA	Transgene-free and vector-free	Genomic integration
	Average efficiency	Negative selection strongly advised

METHOD	ADVANTAGES	DISADVANTAGE
Transient transfection	No genomic integration	Multiple rounds of transfection are required
	Transgene-free and vector-free	Slow and inefficient
	Low frequency of genomic integration	Lower levels of expression than when integrated
	Technically simple procedure	Delayed kinetics of reprogramming
		Integration provides selective advantage and necessitates clone screening
RNA	Transgene-free and vector-free	Multiple transfections required
	No genomic integration	
	No need to screen numerous colonies	
	All integration-free as efficient as retrovirus	
Protein transduction	Direct delivery of transcription factors avoids complications of nucleic-acid-based delivery	Slow and inefficient
		Some proteins difficult to purify and short half-life
		Multiple applications required

Modified from Maherali and Hochedlinger, 2008; González *et al*., 2011.

2.2.3 Donor cell types

An important consideration for iPS technology is determining an appropriate starting somatic cell type. A majority of iPS studies uses fibroblast cells, most likely due to their ease of derivation and extensive use in SCNT and fusion-based reprogramming studies (Wakayama and Yanagimachi 1999; Tada *et al*. 2001). Various other starting somatic cell populations have been used for iPS induction in mouse including adult stomach and liver cells (Aoi *et al*. 2008), embryonic or newborn neural progenitor cells (Eminli *et al*. 2008; Kim *et al*. 2008), pancreatic β cells (Stadtfeld *et al*. 2008a), mature B lymphocytes (Hanna *et al*. 2008) and bone marrow hematopoietic cells (Kunisato *et al*. 2010). An important observation from these studies is that the somatic cell type chosen had a significant influence on the efficiency of iPS generation and the level of reprogramming. Similarly, for human somatic cells, fibroblasts (Takahashi *et al*. 2007b; Yu *et al*. 2007), keratinocytes (Aasen *et al*. 2008), and blood cells

(Giorgetti *et al*. 2009; Haase *et al*. 2009; Loh *et al*. 2009), neural progenitor cells (Kim *et al*. 2008) and adipose cells (Sun *et al*. 2009) have been reported to yield successful iPS populations.

Although these iPS cell lines have been shown to express pluripotency genes and support the differentiation into cell types of all three germ layers, the molecular and functional differences were detected among iPS-derived cells from different cell types. In mice, for example, iPS cells produced from stomach and liver cells showed different propensities to form tumors. To identified persistent donor cell-specific gene expression patterns from different cell types, suggesting an influence of the somatic cell of origin on the molecular properties of resultant iPS cells (Ghosh *et al*. 2010). To resolve the question whether iPS cells produced from different cell types are molecularly and functionally equivalent is crucial for using these cells in modelling disease and therapeutic settings. The identification of somatic cells influence on the differentiation capacities of resultant iPS cells into desired cell lineages, need to be further study (Maehr *et al*. 2009). Different tissues show variable susceptibility to reprogramming. This may reflect an epigenetic memory retained by the iPS cells from the parental donor somatic cell type. Keratinocytes reprogram more readily than fibroblasts (Maherali *et al*. 2008), and iPS cells from stomach or liver cells harbour fewer integrated proviruses than fibroblasts, indicating that they require lower levels of the reprogramming factors to achieve pluripotency (Aoi *et al*. 2008). When iPS cells from adult tail-tip fibroblasts differentiated into neurospheres, they retain more teratoma-forming cells than iPS cells from embryonic fibroblasts that indicated again the heterogeneity based on the tissue of origin (Miura *et al*. 2009). Moreover, cells can exist in intermediate states of reprogramming with continuous passage or treatment with chromatin-modifying agent (Mikkelsen *et al*. 2008).

Although the iPS cells are highly similar to ES cells, but not all iPS cells generated from various tissues may harbor functional and molecular different. Mikkelsen *et al*. performed a detailed analysis on several partially reprogrammed clonal cell lines (e.g. MCV8, MCV6, BIV1). MCV8 cells express SSEA-1 pluripotency marker, however, critical endogenous genes, *Oct-4*, *Sox-2* and Nanog, remain inactive (Mikkelsen *et al*. 2008). Analysis of the methylation status of *Oct-4* and Nanog genes, that promoter are methylated in somatic cells and demethylated in iPS and ES cells, indicates that these promoters remain largely methylated in partially reprogrammed cells. Failure to demethylate pluripotency genes is associated with intermediate or partial states of reprogramming (Wernig *et al*. 2008b; Chin *et*

al. 2009). Knock down of the maintenance methyltransferase DNMT1 or treatment with the demethylating agent 5-aza-cytidine (AZA) can convert intermediate states to full pluripotency. AZA is a methyltransferase inhibitor shown to impede the maintenance of CpG methylation at promoters of essential pluripotency genes, such as *Oct-4* (Mikkelsen *et al*. 2008). The choice of cell type is therefore one of an important aspect to consider before starting any experiment. It will usually depend on cell availability and will affect later the requirement of ectopic factors, the efficiency and kinetics of reprogramming, and the quality of the resulting iPS cells.

2.2.4 Culture condition methods

After decide on the combination of reprogramming factors that are suited to a specific cell types. Later, a key aspect of a successfully reprogram is the culture conditions. The culture conditions which supportive cells and medium compositions are all parameters that have been shown to modulate reprogramming efficiencies. Regarding to maintain the pluripotency state, iPS cells also require the same culture conditions as those of ES cells. Both mouse and human iPS cells can be maintained in the pluripotent stage when supported by feeder cells or addition of LIF (mouse) and bFGF (human) into the medium for self-renewal (Thomson *et al*. 1998; Maherali *et al*. 2007; Okita *et al*. 2007; Takahashi *et al*. 2007a; Wernig *et al*. 2007; Yu *et al*. 2007; Ying *et al*. 2008).

There will be an important toward creating defined and xeno-free cultures in efforts to generate iPS cells which will be more suitable for clinical used. It has been reported to improve the reprogramming of mouse fibroblast by use of knockout serum replacement instead of fetal bovine serum (Blelloch *et al*. 2007). However, it is unsuitable to use a knockout serum replacement because variability of serum batches and may not elicit reproducible effects to reprogramming of the cells. Therefore, it is important to screen the serum batch-to-batch for supportive capacity (review in (Maherali and Hochedlinger 2008). The ES cells conditions rely on fibroblast-derived factors which support their growth and maintain their pluripotency. However, mouse ES cells can be derived and cultured on gelatin-coated dish without any feeders and addition of growth factos (Ying *et al*. 2008) and similar with mouse iPS cells which can also be derived under feeder-free culture conditions (Stadtfeld *et al*. 2008b; Wernig *et al*. 2008a).

Wnt signaling also supports the self-renewal of ES cells. Adding medium conditioned by cells expressing WNT3a with Wnt3a or CHIR99021 also promotes the generation of iPS cells in the absence of Myc (Marson *et al*. 2008; Li *et al*. 2009). In the combination of glycogen synthase kinase (GSK3) and mitogen activated protein kinase (MAPKK also known as MEK) inhibitors not only support self-renewal of iPS cells in the serum- and feeders-free but it is also improve the reprogramming efficiency (Silva *et al*. 2008). Additional, optimal seeding-cell density need to create for achieves the suitable culture conditions. As low cells density may senesce and can be less amenable to reprogramming. Whereas high cells seeding can quickly become over-confluent and hindering the outgrowth of the new colonies which posing the risk of cell layer lifting, particularly in prolonged culture during reprogramming process (review in (Maherali and Hochedlinger 2008). Oxygen tension is also important for stem cell maintenance and differentiation. It has been reported that iPS cell induction preformed under hypoxic conditions (5% O_2) showed the enhancement of reprogramming efficiency of mouse and human cells up to fourfold. Furthermore, the reprogramming efficiency increases to 200-fold in mouse cells when combined with VPA (Yoshida *et al*. 2009). Furthermore, serum-free medium (KK20) allows iPS cells to be obtained at an earlier time point by testing different culture conditions (Okada *et al*. 2010). Modifications of culture conditions, may also increase the efficiency and rate at which somatic cells are reprogrammed (Cox and Rizzino 2010). Therefore, it has a various questions that need to be considered before initiate the reprogramming process such of which reprogramming factor, which delivery method and which cell type should be used.

2.2.5 Identification and characterization

To identify whether iPS cells are equivalent to ES cells, is a complex and unresolved question. As genetic or epigenetic abnormalities may influences iPS cells during differentiation and/or transplantation. Several approaches have been made in order to achieve the effective and therapeutically compatible methods to identify and obtain reprogrammed cells. The biological assays are a criteria used to compare ES cells and iPS cells, for example, the development potency testing including molecular assays that are used to compare epigenetic status and gene expression. In the first report to generate mouse iPS cells, it was obtained via selection for ESC specific gene, Fbx15, which is nonessential. It was used a selection system whereby only cells that reactivated ES cell-specific genes could survive

during the reprogramming (Takahashi and Yamanaka 2006). Even the cells showed pluripotency in term of teratoma formation, iPS cells were still not fully reprogrammed as they could not generate chimera mouse. Also, they have different the gene expression profiles and DNA methylation status compared to ES cells. Later, it has been found that the selection for ES cell-specific genes are Oct-4 and Nanog which permitted the iPS cells were much more similar to ES cells (Maherali *et al*. 2007; Okita *et al*. 2007; Wernig *et al*. 2007).

In the level of cellular biological level, morphological criteria are the one of classical assays to identify reprogrammed cells equivalent to ES cells. Generally, mouse ES cells colonies can be indicated by their appearance as large nucleolus, scant cytoplasm, shiny, tight packed and round shape with clear borders, while human ES cells colonies show a cobblestone shape, tightly-packed colonies with pronounced individual cell borders and prominent nucleoli. The stepwise morphological changes have been depicted during reprogramming both in mouse (Stadtfeld *et al*. 2008b) and human (Maherali *et al*. 2008). In case of human fibroblast reprogramming, it was noted that morphologically is similar to ES cells but non-iPS cell colonies also arise during the time course of reprogramming. These colonies are distinguished from other iPS cells as they are display granular and loose shape in appearance (Takahashi *et al*. 2007b; Lowry *et al*. 2008). Furthermore, the cellular biological level assays include also growth rate properties were equal to ES cells. They expressed stem cells markers specific to ES cells, and expressed gene which specific to undifferentiated ES cells.

The iPS and ES cells have been functionally compared for pluripotency at molecular level which shown a high degree of similarity between iPS cells and ES cells. These studies included profiling of genome-wide expression pattern, global gene expression, modifications of histone tails, X-chromosome inactivation and global differences of DNA methylation (Maherali *et al*. 2007; Okita *et al*. 2007; Wernig *et al*. 2007; Mikkelsen *et al*. 2008). However, some studies using global expression analyses concluded that even iPS cells are a unique pluripotent cells but they are still retain a consistent gene expression signature distinguishable from ES cells (Chin *et al*. 2009). The re-analysis of a large collection of gene expression and histone modification profiles led to the conclusion that small variations between iPS cells and ES cells in global gene expression and chromatin structure may constitute experimental "noise" (review in Hanna *et al*. 2008) and do not reflect a consistent signature differences iPS cells from ES cells (Guenther *et al*. 2010; Newman and Cooper 2010).

Assays for developmental potency are considered to be a crucial for concluding the pluripotent state which is capable fully differentiate into differentiated tissues in a similar term to ES cells. Particularly in mice, chimera formation and germline contribution are used to determine the developmental potential of iPS cells. Recently, it has been shown that iPS cells can indeed contain the same developmental potential as ES cells by produce iPS mice by tetraploid complementation (Boland *et al*. 2009; Kang *et al*. 2009; Zhao *et al*. 2009). Recently, it has shown that repression of maternally imprinted gene cluster, Dlk1-Dio3, may distinguish the functional level between mouse iPS cells from ES cells (Stedtfeld *et al*., 2010a). These experiments suggest that reprogramming is complete in some cases of mouse iPS cells, for example, the ES cells or iPS cells that show normal expression at this locus are able to contribute the entirely animal in the tetraploid complementation assay. While those ES or iPS cells that do not show normal expression of these genes are incapable for the tetraploid complementation assay (Liu, L. *et al*. 2010). Although, these non-coding RNAs show interesting 'landmark' of reprogramming, the conclusions need to be address on this issue as it have just begun the explored. Thus, it will be an important to minimize requirement and methodology criteria that define the safety of reprogramming cells for disease research and clinical use.

2.3 APPLICATION

Towards clinical application of iPS cells, including the potential of iPS cells to generate all lineage of the embryo and to contribute germline chimera, the iPS cells have a differentiation ability comparable to ES cells. Furthermore, iPS cells have already been differentiated into various differentiated cell lineages such as neurons, cardiomyocytes and hematopoietic cells (Hanna *et al*. 2007; Dimos *et al*. 2008; Wernig *et al*. 2008b). At present in the iPS cells technology, it is still difficult to address the suitable reprogramming methods that fulfill for all purpose. Especially, in order to consider in the mechanisms of reprogramming and to generate clinically relevant iPS cells. Currently, many researches have been shown the differences between iPS and ES cells which is further support the evidence that the reprogramming process requires a vast variety of molecular changes. Furthermore, it is too difficult to describe that the cells can either be only partial reprogramming or fully reprogramming without any effect of epigenetic memory.

Recently, it has been generated iPS cells and differentiated into motor neuron from patients with amyotryophic lateral sclerosis (ALS) (Dimos *et al*. 2008). This study demonstrated that patient specific iPS cells posses properties of ES cells and can be fully reprogrammed. Furthermore, the iPS cells have also been generated for drug testing and disease. The iPS cells were generated from several of genetic diseases from patient with adenosine deaminase deficiency-related severe combined immunodeficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS), Gaucher disease (GD) type III, Duchenne (DMD) and Becker muscular dystrophy (BMD), Parkinson disease (PD), Huntington disease (HD), juvenile-onset, type 1 diabetes mellitus (JDM), Down syndrome (DS)/trisomy 21 and the carrier state of Lesch-Nyhan syndrome (Park *et al*. 2008). These patient specific-derived iPS lines can be used to compare with normal cell lines in order to understand at molecular mechanism level of disease conditions. Furthermore, iPS technology can also be applies for drug screening in treatment approach.

Several studies have now been successfully generated human iPS cells *in vitro* for disease models which they mainly focus on genetically inherited disease of neurological and cardiovascular disease (Dimos *et al*. 2008; Park *et al*. 2008; Ebert *et al*. 2009; Soldner *et al*. 2009; Hu and Zhang 2010; Moretti *et al*. 2010; Swistowski *et al*. 2010). The primary human fibroblasts were successfully reprogrammed from sporadic PD patients (Park *et al*. 2008;

Soldner *et al*. 2009) and differentiated into dopaminergic (DA) neurons (Chambers *et al*. 2009; Soldner *et al*. 2009). They have been reported that functional DA neurons could be efficient reprogrammed from human iPS cells with validated scalable method (Swistowski *et al*. 2010). Subsequent iPS studies have also outlined approach to produce functional and relative pure populations of motor neurons from patient with Spinal Muscular Atrophy (SMA) (Ebert *et al*. 2009) and ALS (Dimos *et al*. 2008). Recently, Hu and Chang were established functional spinal motor neurons by chemically defined protocol. (Hu and Zhang 2010)

In addition, cardiomyocytes derived from iPS cells also provide the new possibilities to study of cardiac disease. It has been reported that iPS-derived cardiomyocytes from fibroblasts of patient with Timothy syndrome could be reprogrammed (Yazawa *et al*. 2011). Several studies have also been improved the reprogramming efficiency with small molecules which make a new attractive approach for further research (Ruau *et al*. 2008; Ebert *et al*. 2009; Ichida *et al*. 2009). However, the generations of somatic cells into iPS cells of patient and differentiated cell lineages are not the same which means the epigenetic memory from different cell types may effect the reprogramming and accuracy in order to apply for disease model. Although these advances remain the challenging to address the therapeutic effect and clinical safety relevant, the iPS technology still offers significant potential to overcome the obstacles in the field of therapeutic medicine, disease models and patient-specific drug screening.

CHAPTER 3. MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), unless otherwise specified. Cells were cultured at 37°C in humidified atmosphere containing 5% CO_2. Medium was changed daily on mouse ES and iPS cell cultures and every two days during differentiation.

3.1 PLASMID CONSTRUCTION

The SB transposon-based expression vector (Figure 5) was constructed as follows. First, an IRES/eGFP cassette was cut at BsrGI/EcoRI sites from the pEF-GFP construct (Matsuda and Cepko 2004) (Addgene plasmid 11154) and inserted into the EcoRI/EcoRV sites in the SB transposon vector pT2BH (Ivics *et al*. 1997). Second, an EF1α promoter which was also obtained from the pEF-GFP construct was cut at PacI/EcoRI sites and inserted into the pT2BH-IRES/eGFP construct at the EagI/EcoRI sites. Third, the OSKM fragment from the FUW-OSKM constructs (Carey *et al*. 2009) (Addgene plasmid 20328) was cut at EcoRI sites, and then inserted into the pT2BH-IRES/eGFP construct at the EcoRI site, resulting in pT2BH-EF1α-OSKM-IRES/eGFP (SB-OSKM).

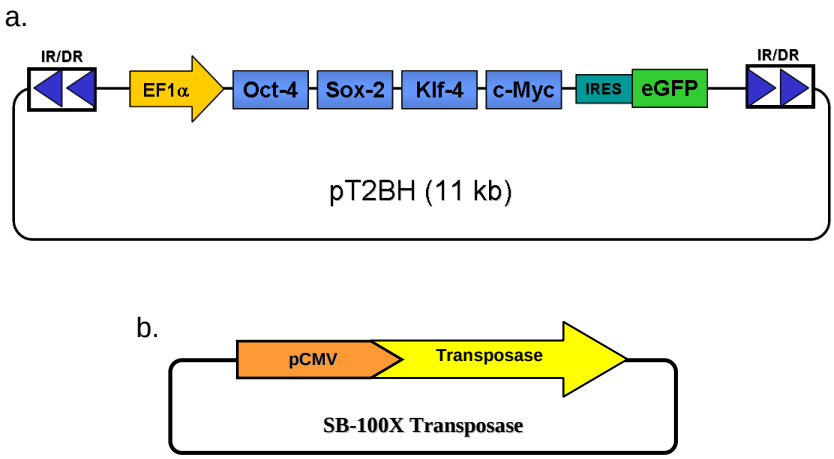


Figure 5. A *Sleeping Beauty* transposon-mediated gene construct used for reprogramming; expression transposon construct (a) and transposase constract (b)

3.2 CELL CULTURE

Primary mouse embryonic fibroblasts (MEFs) were prepared from 13.5 d.p.c mouse fetuses derived from three different genetic backgrounds: C57Bl6 inbred, C57Bl6xDBA/2J F1 hybrid, and ICR outbred using standard protocols (Robertson 1987). MEFs were cultured in FM medium (Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone) and 50 U/mL penicillin, 50 µg/mL streptomycin). ES cells and iPS cells were cultured in ES medium (DMEM supplemented with 15% (v/v) FBS (Sera Laboratories International, West Sussex, RH17 5PB, UK), 1,000 U/mL mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon International), 0.1 mM nonessential amino acids (NEAA, Gibco BRL, Life Technologies), 0.1 mM β-mercaptoethanol (β-ME, Gibco) and 50 U/mL penicillin, 50 µg/mL streptomycin). Mouse ES cells and iPS cells were cultured on mitomycin C-treated MEFs in serum-based ES medium or on 0.1% gelatin-coated dishes. All the cells were passaged with 0.25% trypsin, 0.1% EDTA and cultured at 37ºC in a CO_2 incubator.

3.3 REPROGRAMMING OF MEFS USING SB VECTORS

MEFs were seeded onto 6-well plates at a density 5×10^5 cells/well one day prior to transfection in FM medium without antibiotics. The next day (day 0), 4 µg of pT2BH- EF1α-OSKM-IRES/eGFP expression vector and 0.4 µg of transposase (SB100X (Mates *et al.* 2009)) were co-transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. On day 2, transfected MEFs were trypsinized with 0.25% trypsin, 0.1% EDTA and re-plated onto 10 cm^3 tissue culture dishes containing mitomycin-C treated MEFs at a split ratio of 1:10 in ES medium. The medium was replaced every other day. On day 14, colonies were either analyzed for alkaline phosphatase (as stated by Kobolak *et al.* 2010) and counted, or picked and further expanded for other characterization analyses (Figure 6).

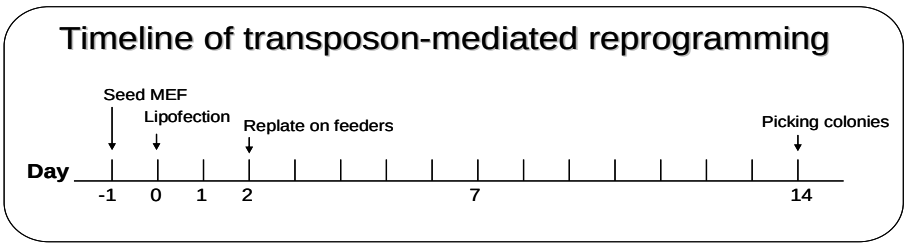


Figure 6. Timeline of SB transposon-mediated reprogramming

3.4 EB FORMATION

To form embryoid body (EB), the ES and three different genetic backgrounds of iPS cells were cultured on feeder cells for at least one cell passage. EBs were made by use of the hanging drop method (Rungarunlert *et al*. 2009). Briefly, on the starting day of differentiation, ES and iPS cells were trypsinized with 0.25% trypsin, 0.1% EDTA. EBs were formed in hanging drops (Figure 7) by placing 800 cells in 20 µl of differentiation medium (ES medium without LIF) on the lid of Petri-dishes, the dish bottom was filled with PBS to prevent drying the cell droplets. On day 2, EBs were collected and placed into 10 mg/ml poly 2-hydroxyethyl methacrylate (poly 2-HEMA) treated-bacterial dishes and maintained in differentiation medium for 2 days.

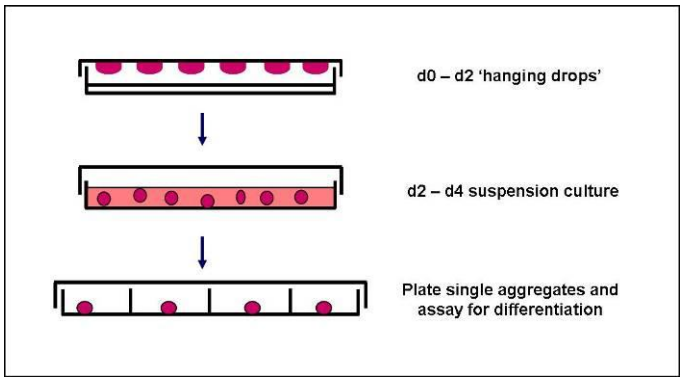


Figure 7. Hanging drop method

3.5 *IN VITRO* CARDIAC DIFFERENTIATION ASSAY

By spontaneously differentiation, individual EB was placed into a well of 24-well plate containing 0.1% gelatin coated cover-slips on day4 after hanging drop (Figure 7). The differentiation medium was changed every second day. The EBs were cultured for a further 14 days and observed for beating cells daily under phase-contrast microscope.

3.6 *IN VITRO* NEURONAL DIFFERENTIATION

Mouse pluripotent cells were induced to differentiate into neuronal lineage previously described with some modifications (Bibel *et al*., 2007). Mouse ES and iPS cells were disaggregated with 0.25% Trypsin into single cells, then seeded at density 3×10^5 cells/ml of differentiation medium onto a bacteriological Petri Greiner dish pre-coated with Poly 2-

hydroxyethyl methacrylate (Poly-HEMA) to prevent EBs attachment. First step (Day0-8), iPS cells were allowed to aggregated as EB formation for 4 days. Then EBs were cultured in differentiated medium supplemented with 5 µM all-trans retinoic acid for additional 4 days. Second step (Day8-14), eight days old EBs were dissociated and plated on Poly-L-ornithine and laminin (Roche, CA) coated dishes at a density of 2×10^5 cells/cm^2 in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) containing 3 mg/ml D-(+)-Glucose, 3 mg/ml AlbuMaxI, 50 U penicillin/ml, 50 µg streptomycin/ml, 1% N2 supplement and 10 ng/ml recombinant human FGF basic (bFGF) for 2 days neuronal precursor culture before changing medium into DMEM/F12 : Neurobasal medium (1:1)**,** 1 mM glutamax**,** 3 mg/ml AlbuMaxI**,** 50 U penicillin/ml, 50 µg streptomycin/ml, 0.5% N_2 supplement and 1% B27 supplement**.** The medium was renewed every second days until day 14.

3.7 *IN VIVO* DIFFERENTIATION

Chimera production was used to examine the potential of iPS cell to differentiate *in vivo*. The standard method of stem cells preparation for injection has already been described previously (Nagy *et al*. 2003). Chimeras were produced by injection of 6-8 iPS cells into the perivitelline space of 8-cell stage embryos by using a laser system (Hamilton Thorne, Inc., XY Clone). Host embryos were obtained from ICR [(in case of F1 and C57Bl/6 iPS cells) and C57BL/6xDBA/2J (in case of ICR iPS)] mice and were collected at the 8-cell stage. Manipulated chimeric embryos were cultured in KSOM medium until the blastocyst stage and transferred into uterine horns (6-10 blastocysts in each horn of the uterus) of 2.5day pseudo-pregnant recipients. Pregnancy was allowed to progress to term, followed by spontaneous parturition. Phenotypically coat color chimeras were naturally mated with ICR mice for testing germline transmission.

3.8 RT-PCR ANALYSIS

Total RNA was collected using the RNeasy Mini Kit (Qiagen) from ES and iPS cells. One µg of total RNA was reverse-transcribed using an oligo (dT) primer by SuperScript III Reverse Transcriptase (Invitrogen), and subjected to PCR using primers listed in Table 3. Standard PCR conditions were 94 ºC for 30 s, 55–62 ºC for 30 s, 72 ºC for 10 s; for 30–35 cycles. RT-PCR was performed using Gene Amp® PCR System 9700 (AB Applied System).

Table 3. RT-PCR primer sequences

Primer sequence	**Sequence 5'-> 3'**
Oct-4	
Forward	GAGGAGTCCCAGGACATGAA
Reverse	AGATGGTGGTCTGGCTGAAC
Sox-2	
Forward	ACCAGCTCGCAGACCTACAT
Reverse	GTGGGAGGAAGAGGTAACCA
Klf-4	
Forward	GTGCCCCAAGATTAAGCAAG
Reverse	CGGGACTCAGTGTAGGGGTA
c-Myc	
Forward	TCCTGTACCTCGTCCGATTC
Reverse	GGTTTGCCTCTTCTCCACAG

3.9 IMMUNOFLUORESCENCE STAINING

For immunofluorescence staining of the cells, cells were plated onto 0.1% gelatin-coated coverslips and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Cells were permeabilized using 0.05% Triton-X100 for 10 minutes at room temperature, followed by 1% bovine serum albumin (BSA) for 1 hour at room temperature. Cells were washed with PBS and incubated with primary antibody overnight at 4°C. Primary antibodies used for this study include: Oct-4 antibody (C-10, 1:100, Santa Cruz), Nanog antibody (1:20, R&D), SSEA-1 (480, 1:100, Abcam), cardiac Troponin T (1:200, Abcam), Desmin (1:200, Abcam), Nestin (Rat-401, dilution: 1:200, DSHB) and β-III Tubulin (Tuj1, dilution 1:2,000; Covance, PRB-435P). Following 3 washes with PBS, cells were labeled with Alexa Flour®594-conjugated secondary antibodies (Invitrogen) for 1 hour at room temperature. Cells were then washed 3 times with PBS and covered with DAPI mounting medium (VectaShield, Vector Laboratories). The cells were analyzed and imaged by using a Zeiss AxioImager Z1 microscope (Carl Zeiss MicroImaging GmbH, Germany).

CHAPTER 4. RESULTS

4.1 GENERATION OF IPS CELLS USING THE SB TRANSPOSON SYSTEM

4.1.1 Transfection of *Sleeping Beauty* transposon-mediated construct into MEFs

In order to deliver the reprogramming cassette to the fibroblast, I constructed SB transposon-based reprogramming vectors (Figure 5). The reprogramming factors (c-Myc, Klf-4, Oct-4 and Sox-2) were cloned into the pT2BH *Sleeping Beauty* transposon plasmid under the transcriptional control of the EF1α constitutively active promoter. In order to minimize the number of integration sites, I used a polycistronic expression cassette where the four factors were separated by 2A peptides (Carey *et al*. 2009). The construct were linked with green fluorescent protein (GFP) using an IRES sequence, which allowed us to monitor the transgene expression. As a negative control for iPS generation, SB-GFP were used the same transposon construct, which expressed only GFP (Figure 8).

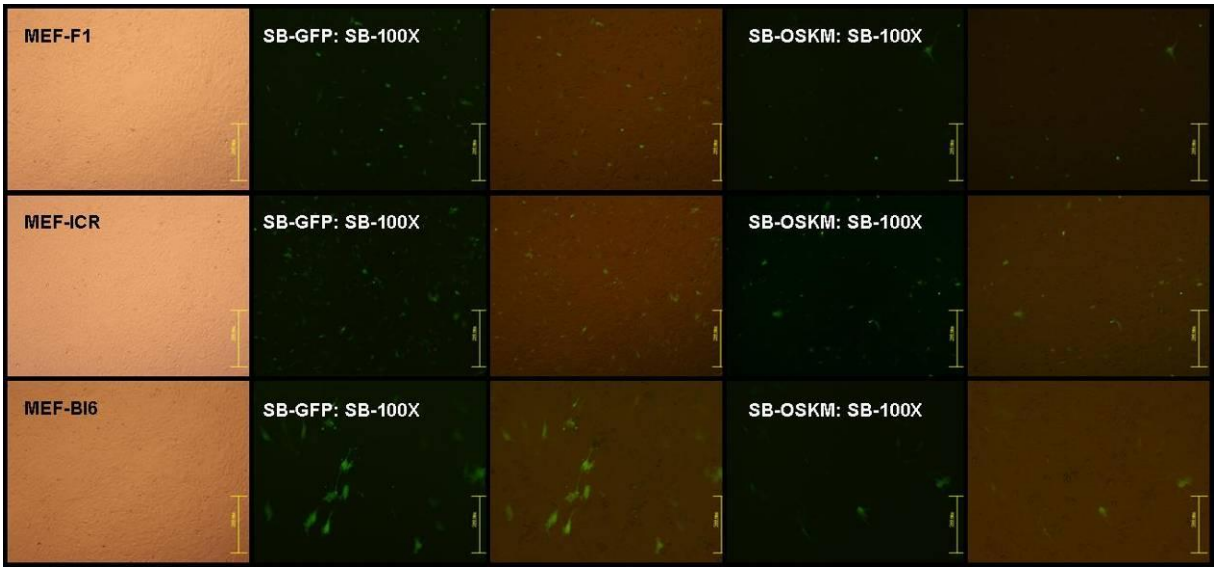


Figure 8. GFP expression resulting after co-transfection with SB-GFP (control) and SB-OSKM with SB-100X transposase.

4.1.2 Generation of iPS cell colonies

I next investigated whether SB transpons carrying 4 factors could induce reprogramming in mouse fibroblasts from three different genetic backgrounds. In order to evaluate the effect of the genetic background on the generation of iPS cells, mouse embryonic fibroblasts (MEFs) were used from three genetic backgrounds, an outbred (ICR), an inbred (C57BL/6) and an F1 hybrid (C57BL/6 x DBA/2J). The work flow is illustrated in Figure 6. The MEFs were transfected with the transposon construct containing the polycistronic reprogramming cassette in the presence of SB100X hyperactive transposase (Mates *et al*. 2009) by using Lipofectamine 2000. Iused co-transfection with 4 µg of SB transposon-expression vector and 0.4 µg of SB-100X transposase vector in 6-well plate. Two day post transduction, MEFs were replated onto a mitomycin-C treated feeder-layer at a split ratio 1:10 and cultured using serum-based ES medium.

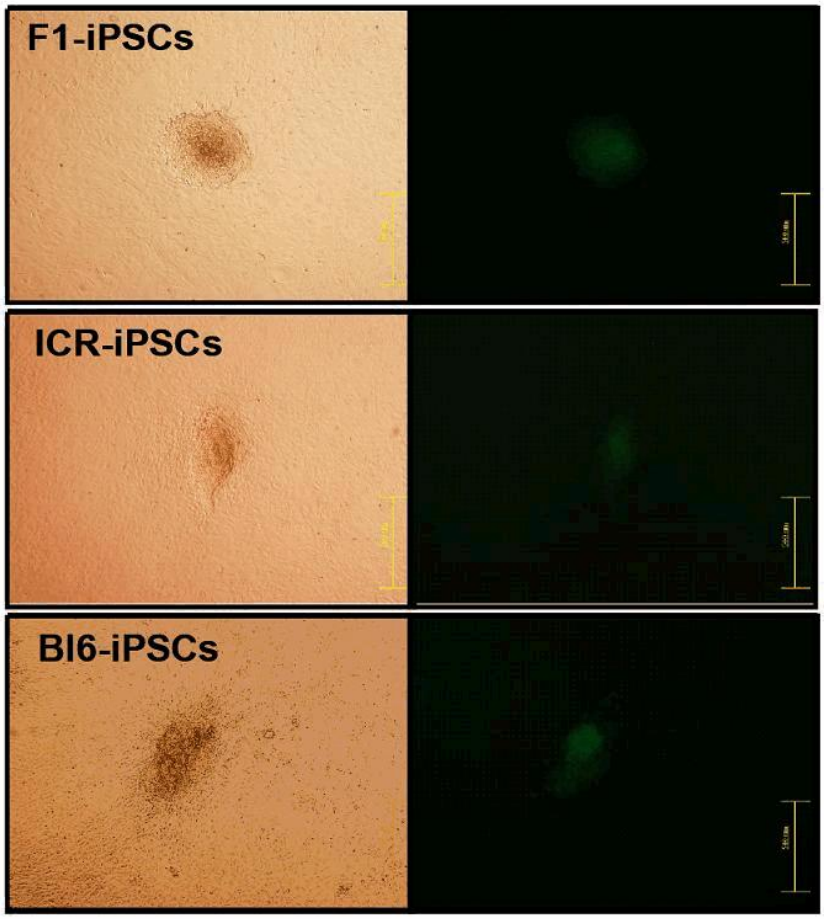


Figure 9. Morphology of iPS cell colonies generated from three different genetic backgrounds at day 14 after transfection.

Under this condition, GFP expressing iPS-like colonies appeared 10-12 days after transfection. At day 14, colonies were sufficiently large to be picked (Figure 9). On day 14, I picked colonies which could be cultured and passaged repeatedly, resulting in the establishment of several stable cell lines. I did not observe a significant difference in the colony appearance, nor in the reprogramming efficiency among the three different genetic backgrounds. All ES cell-like colonies were positive for GFP expression (Figure 9) as well as AP staining (Figure 10), indicating successful reprogramming of these colonies.

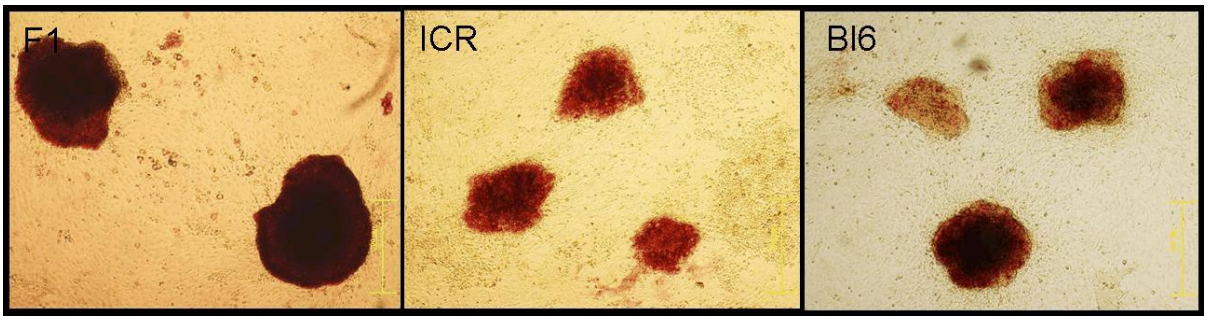


Figure 10. Alkaline phosphatase staining of day14 colonies from three different genetic backgrounds

4.2 CHARACTERIZATION OF IPS CELLS

This study characterized 6 lines in depth from each genetic background for pluripotent characteristics. To study the undifferentiated state of iPS cells lines, each iPS cell line first were scored for morphology, growth rate, GFP expression and alkaline phosphatase activity (Table 4). The colonies were picked from the cultured and established as primary iPS cell lines, which were further characterized and observed that are indistinguishable from ES cells.

Table 4. Characteristics of iPS cell lines generated from three different genetic backgrounds.

ES/iPS lines	Morphology	Growth rate	GFP expression	AP staining	*In vitro* differentiation		*In vivo* differentiation
					EBs	cardiac cells (Beating rate)	Chimera test
F1 ES	+++	+++	-	+++	+++	11/23 (48%)	
F1 iPS D3	++	+++	+	+++	+++	23/24 (96%)	+
F1 iPS F7	+	++	+	++	+	0	
F1 iPS D11	++	++	+++	+++	+++	3/21 (14%)	
F1 iPS C1	+++	++	+	+++	++	15/24 (63%)	+ germ line
F1 iPS D10	+	++	++	+++	+++	0	
F1 iPS E8	+	++	+++	++	+	0	
ICR ES	+	+	-	++	+	1/24 (4%)	
ICR iPS B7	++	+++	+++	+++	+	10/24 (42%)	
ICR iPS F8	++	+	++	+++	+++	17/24 (71%)	
ICR iPS A1	++	++	+++	++	+	14/24 (58%)	
ICR iPS G11	+++	++	+	+	++	19/24 (79%)	
ICR iPS H6	+++	++	+	++	+++	23/24 (96%)	+
ICR iPS H10	+++	+	++	+++	+	20/24 (83%)	
Bl6 ES	++	++	-	++	++	9/24 (38%)	
Bl6 iPS A7	+	+++	++	+	++	1/16 (6%)	
Bl6 iPS B3	++	++	+++	++	++	0	
Bl6 iPS A2	++	++	++	+++	++	6/24 (25%)	
Bl6 iPS A6	++	++	+	+++	+++	23/24 (96%)	
Bl6 iPS A4	+++	++	++	++	++	20/24 (83%)	-
Bl6 iPS B5	++	++	+	++	++	11/24 (46%)	

4.2.1 Morphology of iPS cells

To investigate whether these iPS cells are pluripotent, the morphology and alkaline phosphatase expression are the first observe of one line from each genetic background compared to ES cells. The iPS cells formed typical ES-like colonies and exhibited positive staining for alkaline phosphatase (Figure 11). I were able to expand these cells long-term (over 20 passages) and the proliferating cell colonies remained morphologically undifferentiated. These lines could be cryopreserved and recovered with high efficiency using standard techniques. I found that the GFP expression differed among the different iPS cell lines and in between the genetic backgrounds. The iPS derived from F1 background showed lower GFP expressed compared to ICR and Bl6 background, respectively. In some lines, a subset of the cells did not express GFP, indicating silencing of the pluripotency cassette may occur. The differences in the GFP expression might be due to differences in the copy number, number of integration sites or in silencing of the promoter in each clone.

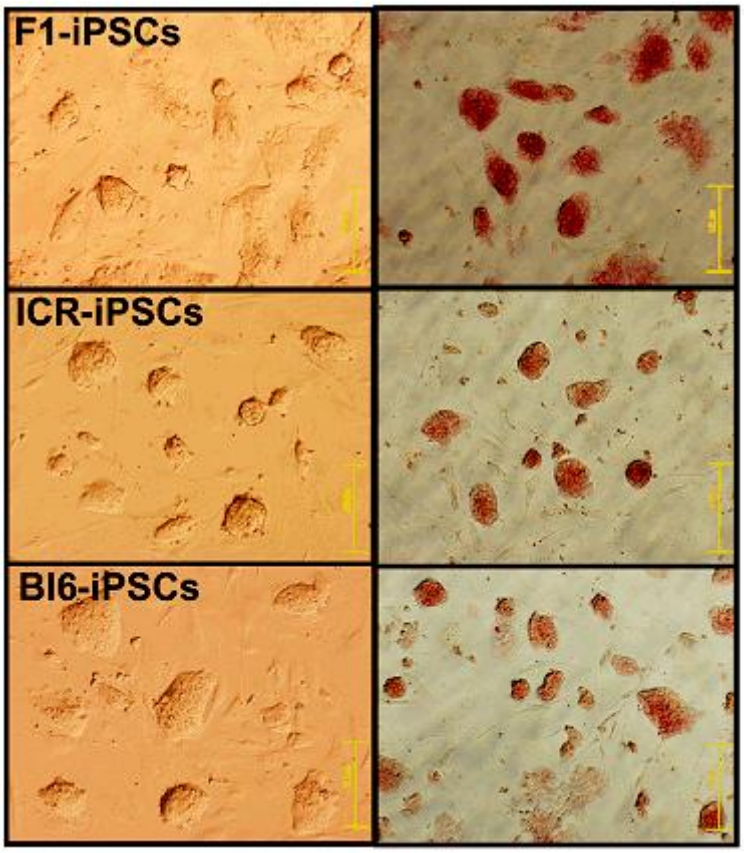


Figure 11. Morphology (left column) and alkaline phophatase staining (right column) of iPS cells generated from three different genetic backgrounds.

4.2.2 Immunofluorescence staining of ES cells marker

I also analyzed the iPS lines for endogenously expressed pluripotency markers, such as, *Oct-4*, Nanog and SSEA-1. The iPS lines showed positive nuclear staining for *Oct-4*, Nanog and the positive plasma membrane marker, SSEA1, similar to that observed in ES cells (Figure 12).

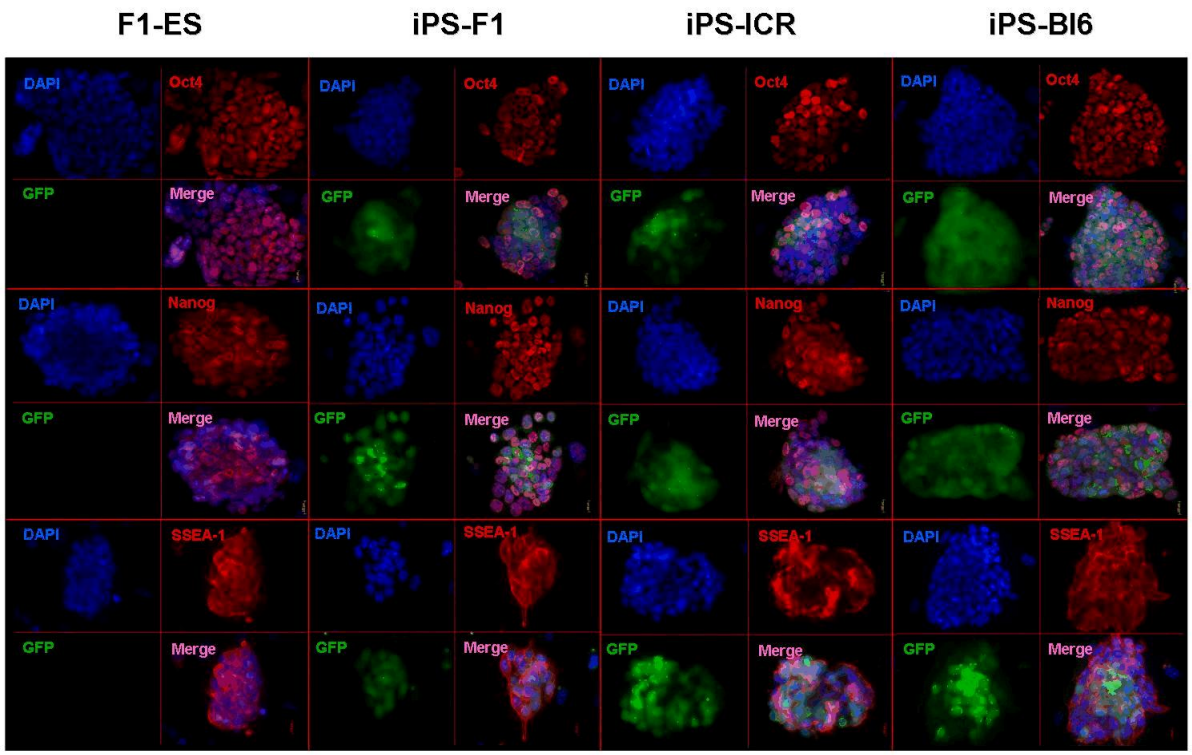


Figure 12. Immunofluorescense analysis of ES cell markers; *Oct-4*, Nanog and SSEA-1.

4.2.3 RT-PCR analysis

To further evaluate the expression of pluripotency markers, I performed RT-PCR analysis to identify if the exogenous expression of pluripotency genes (OSKM) could induce endogenous gene expression (Figure 13). I used primers for the endogenous sequences. I also analyzed the expression of additional pluripotency genes, such as Nanog, Rex1, Dax1, FoxD3, Fbxo15 and Eras. All the 18 examined iPS clones were found to express these endogenous pluripotency markers at similar levels to ES cells. Therefore, the derived iPS lines displayed typical characteristics of a pluripotent stem cell.

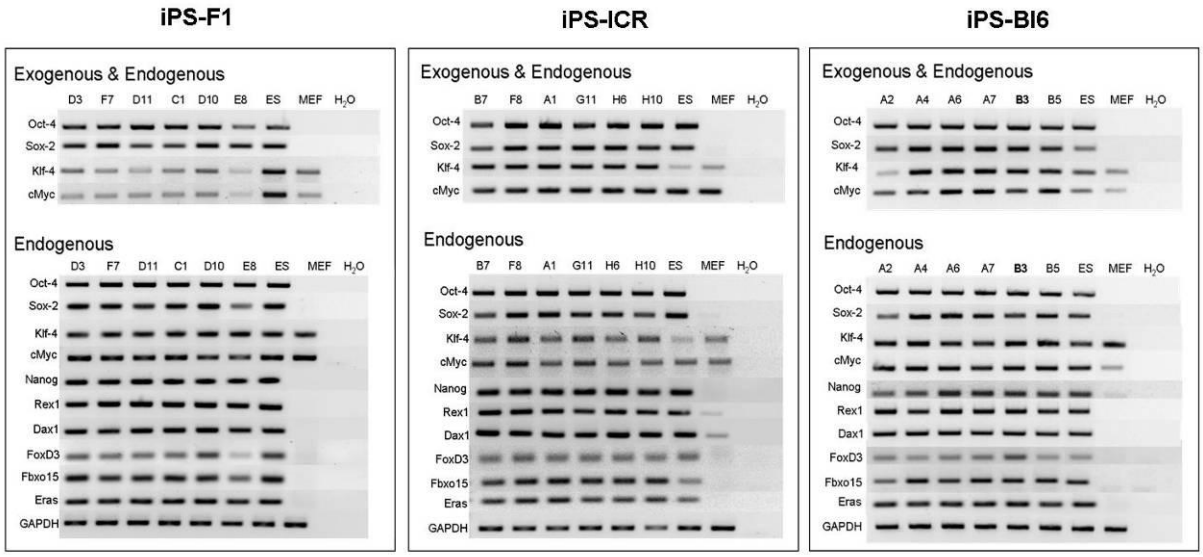


Figure 13. RT-PCR analysis of exogenous and endogenous pluripotency genes derived from three genetic backgrounds.

4.3 DIFFERENTIATION OF IPS CELLS

4.3.1 *In vitro* cardiac differentiation

Next, I tested the *in vitro* differentiation potential of these cell lines. The classical method to induce ES cell differentiation is to allow ES cells to grow in suspension after LIF withdrawal from the culture medium and form aggregates known as EB. I determined that all of 6 lines from each background formed EB. However, I observed some differences in the morphology of the EBs (see scores in Table 4, Figure 14). I did not find a significant relationship between the ES-like characteristics (ES-morphology or intensity of AP staining) and the capacity to form EB when comparing the iPS lines. In addition, the activity of the reprogramming cassette (GFP expression) did not appear to influence the morphology of the differentiated EBs *in vitro*.

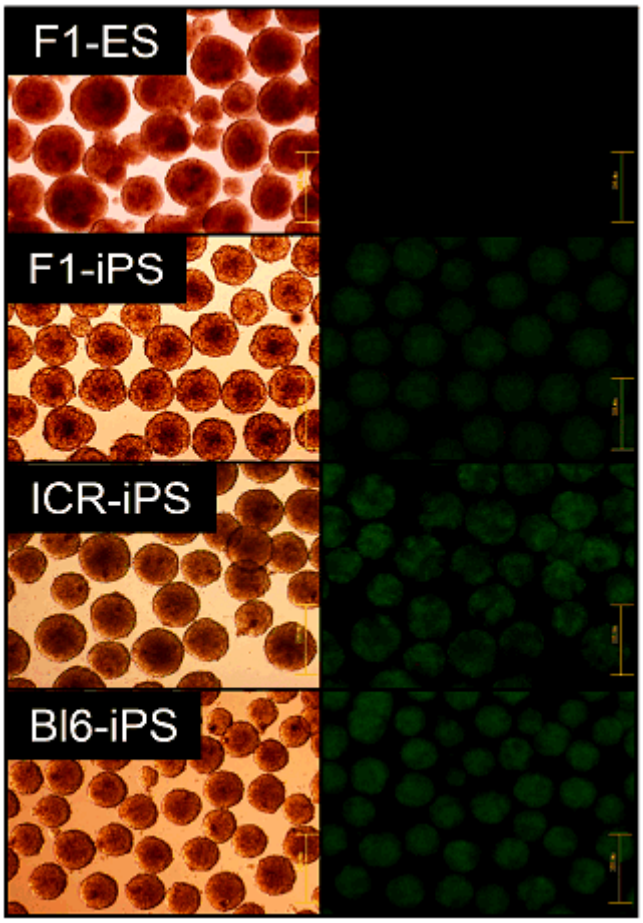


Figure 14. Embryoid body formation of ES and iPS cells by hanging drop method. Scale bar = 500 μm.

Thereafter, I differentiated these lines towards the cardiac lineage and scored their differentiation potential. For the cardiac differentiation, I first differentiated these cells into EBs using the hanging drop method. Two days later, I transferred the EBs in suspension culture. On day 4 of differentiation, I transferred the EBs onto gelatin-coated cover slips (one EB/cover slip) and grew them for a further 10-17 days. From day 7 onwards, I observed spontaneously beating areas in the culture. During days 7-14, I identified and counted the number of beating EBs (Table 4 contains the beating rates of each line at day14). I found large differences existed in the cardiac differentiation potential of the different iPS clones. The iPS lines derived from the ICR background performed the best in this assay. The beating rate in 4 out of 6 examined clones was over 70% and the lowest was 42%. The iPS lines derived from the C57BL/6 genetic background had the lowest differentiation rate, where this value was below 40% in 3 of the 6 lines, and only 2 lines performed over 70%.

In this study, I obtained higher percentage of beating cells from the iPS cells than from the parental ES cells (Figure 15). I found a negative correlation existed between the level of GFP expression and the cardiac differentiation capacity of the cells within the genetic backgrounds. For example, in the F1 hybrid background, the iPS line containing the highest level of GFP expression (F1-D11) had the lowest beating rate (14%). In addition, two iPS lines derived from the ICR background, which had the highest GFP expression, also had the lowest beating rate (ICR-B7-42%, ICR-A1-58%). This observation, however, did not apply to iPS cells derived from the C57BL/6 background. These differences in the differentiation capacity might be an effect of the different genetic backgrounds.

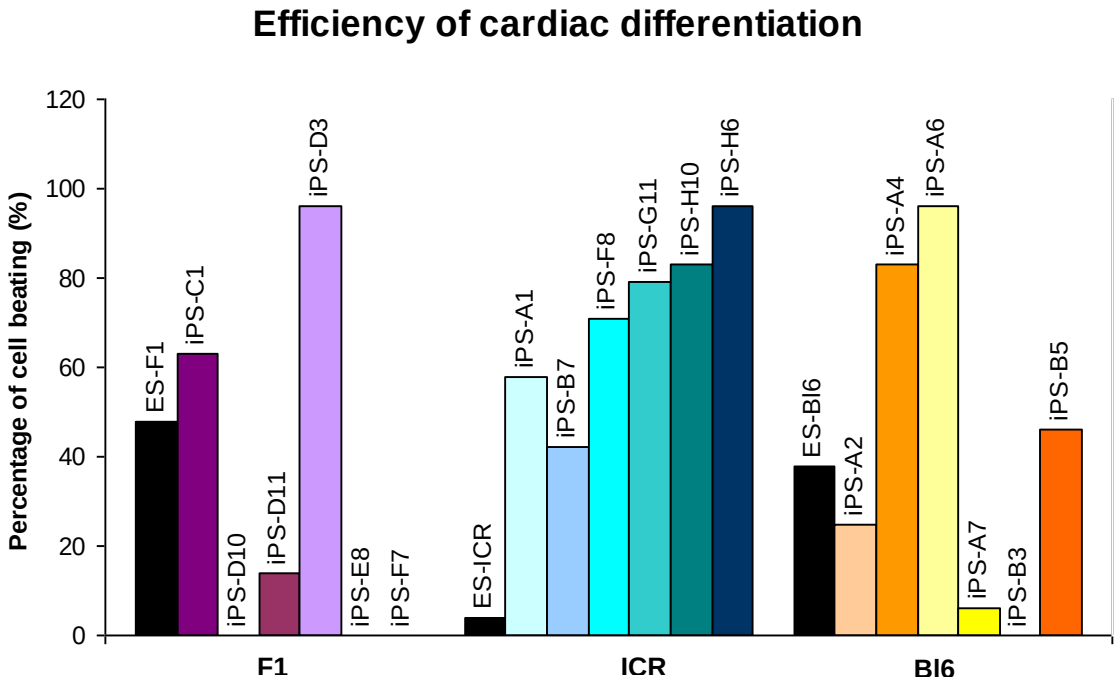


Figure 15. Cardiac differentiation efficiency of EBs after 21days of differentiation.

I also analyzed the differentiated EBs for cardiac differentiation markers, desmin and cardiac TroponinT. The cells within the beating areas stained positive for these two cardiac markers. Figure16 represents typical expression observed from each genetic background.

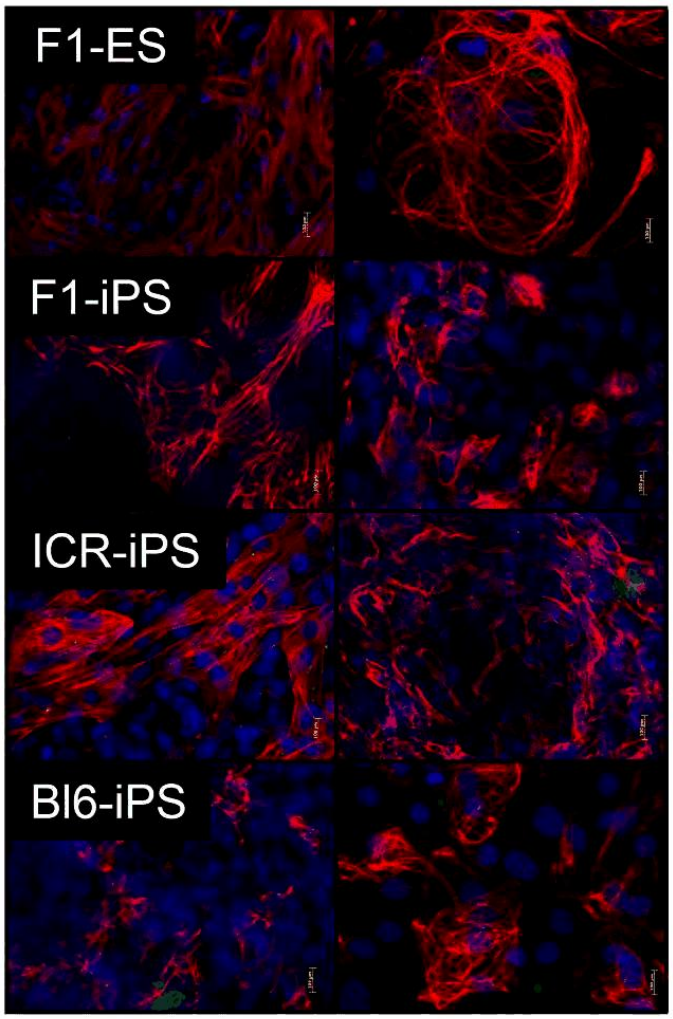


Figure 16. Immunofluorescense analysis of cardiac markers, left column – cardiac TroponinT (red color), right column – desmin (red color).

4.3.2 *In vitro* neuronal differentiation

To investigate the capability of mouse ES and iPS cells in order to differentiate into neuronal lineage, these ES and iPS cells were induced through EB formation and supplementation of RA (Rungarunlert *et al*. 2011). I found that the EBs at day8 from both ES and iPS cells show the spherical structure with various sizes. Two days after plating the cells onto culture dishes (day10 of differentiation), the cells exhibited a neuron-like appearance with neurite processes organized in network.

After performing the neuronal differentiation procedure, ES and iPS cells subsequently furthered analyzed the differentiated EBs for neuronal markers, nestin and Tuj-1 (Figure17).

Within differentiation culture period, ES and iPS cells were able to differentiate into cells expressing nestin, a specific antibody against the intermediate filament protein of NPCs. Furthermore, ES and iPS cells were also showed a few amount of post-mitotic neuronal marker Tuj-1. Interestingly, neuronal lineage-derived iPS cell show with approximately 2-3 times higher in number when compared to ES cells. These results demonstrated that mouse ES and iPS cells have ability to generate NPCs and differentiate further into neurons through EB formation in culture.

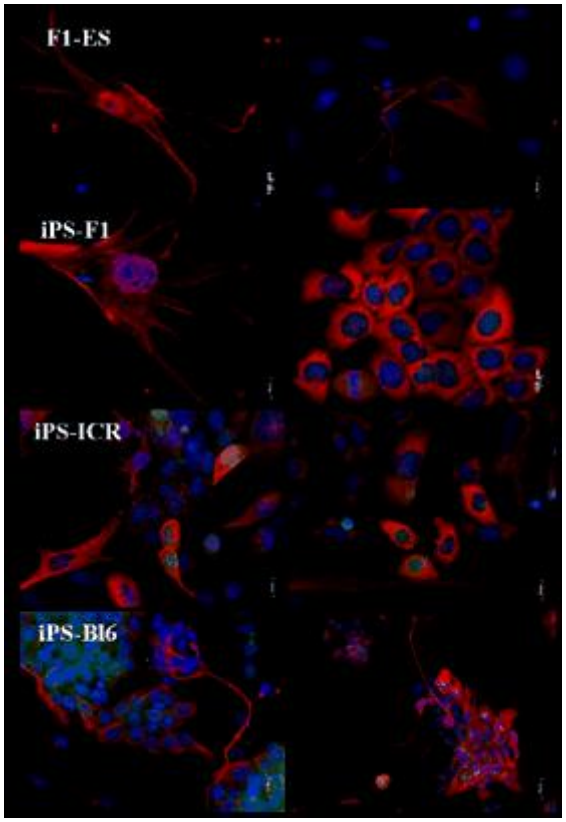


Figure 17. Immunofluorescense analysis of neuronal markers, left column – nestin (red colour), right column – Tuj-1 (red colour).

4.3.3 *In vivo* differentiation

One defining feature of authentic pluripotent stem cells is their capacity to incorporate into developing embryos and transfer through the germ line. In order to evaluate the chimera formation potential of our cell lines, I picked the iPS lines (based on their ability to differentiate) from each genetic background and injected individual cells into host blastocysts.

The iPS cells from F1 hybrid and C57BL/6 backgrounds were injected into ICR blastocysts, whereas, iPS cells derived from the ICR background were injected into C57BL/6 blastocysts. I obtained chimeric mice from the iPS lines derived from F1 hybrid and ICR mice (Figure 18). The Bl6-A4 line derived from C57BL/6 mice did not form chimeras. The chimeras from the F1 hybrid and ICR backgrounds were then mated to identify whether the cells could contribute to the germ-line. Two females mated with the chimeras derived from the F1 hybrid background produced offspring with black color, indicating germ-line transmission (Table 4).

Table 4. Summary of embryo transfer and chimera production for *in vivo* differentiation

Cell line	8-cell stage injection	Host embryo	ET*	Live offspring (ET%)	Live Chi. (%)	GLT**
iPS: F1-C1	179	CD1	9	6 (3%)	2 (33%)	1 (50%)
iPS: ICR-H6	184	B6D2	9	9 (10%)	1 (13%)	0
iPS:Bl6-A4	157	CD1	8	17 (6%)	0	0

*ET, Embryo Transfer; GLT, Germline Transmission

The *in vivo* differentiation assay revealed that the F1 hybrid iPS cells had the best differentiation potential, even though the ICR lines performed the best *in vitro.* The cell lines with the least *in vitro* and *in vivo* differentiation potential were derived from the C57BL/6 background.

Figure 18. *In vivo* differentiation of iPS cells

CHAPTER 5. NEW SCIENTIFIC RESULTS

1. I have generated for the first time of mouse iPS cells by non-viral, *Sleeping Beauty* transposon-mediated gene delivery, with four transcription factor (OSKM).

2. For the first time, the capabilities of SB transposon-derived mouse iPS cells to be fully reprogrammed have been proven by both *in vitro* and *in vivo*.

3. For the first time, a novel comparative *in vitro* study has been performed with iPS cells generated from mouse fibroblasts from three different genetic backgrounds: ICR (outbred), C57BL/6 (inbred) and F1 hybrid (C57BL/6 x DBA/2J).

CHAPTER 6. DISCUSSION AND SUGGESTION

Since the first successfully generated iPS from fibroblasts was reprogrammed by retroviral delivery by four transcription factors (OSKM), a substantial number of alternative approaches have been developed to induce pluripotency in many kind of somatic cells. To properly assess the improvement that each of the methods provides and to give a more precise picture of their real contribution to reprogramming, it will be crucial to test them using commonly accepted standards. In addition to the use of oncogenes in reprogramming cocktails and the issue of viral integration, reprogramming itself may have an effect on a cell's genome, especially given that the process takes many weeks and is rather inefficient. Although reprogramming by using retroviral delivery is efficient and widely used, iPS cells-derived from retroviral vectors have insertional mutations and may cause the tumors.

In this study, I have shown that the *Sleeping Beauty* transposon system, containing the polycistronic reprogramming cassette is able to reprogram MEFs to the pluripotent state in three different genetic backgrounds, including an inbred (C57BL/6), an outbred (ICR) and an F1 hybrid (C57BL/6 x DBA/2J) strain. To achieve this, I utilized the SB-transposon system to deliver the reprogramming cassettes, which were linked by self-cleaving peptide. This peptide has approximately 20-amino acid long 2A peptides from foot-and-mouth disease virus (F2A) and *Thosea asigna* virus (T2A). They work as self-cleaving signals and enable expression of several gene products from a single transcript (Szymczak *et al*. 2004), which facilitated multi-gene delivery to target cells. I also used a polycistronic expression cassette where the four reprogramming factors were separated by 2A peptides in order to generate the iPS cells and minimize the number of integration sites (Carey *et al*. 2009).

The *Sleeping Beauty* transposon system has similar advantages to other transposon-based systems such as *piggyBac* (Kaji *et al*. 2009; Woltjen *et al*. 2009). This gene delivery method is simple compared to viral systems. The SB transposon system has a very large cargo capacity. Our reprogramming construct has size around 11 kb. Unlike most other DNA transposons, *piggyBac* has a capacity up to 10 kb (Ding *et al*. 2005). I were able to reprogram the cells by simple transfection, avoiding the preparation of the viral stocks in a biohazard facility. However, it is also have a disadvantage by this transient expression method which has a very low reprogramming efficiency (Okita *et al*. 2008; Stadtfeld *et al*. 2008c). By using the

hyperactive form of the SB transposase, the frequency of the genomic integration was relatively high. It may also be possible to remove the integrated transgene following insertion, by using a mutant version of the transposase, which is able to remove the transgene from the genome, but not able to re-integrate (Ivics unpublished). Therefore, it may be possible in the future to generate therapeutically safe, reprogramming factor-free iPS cells. These experiments are currently in progress in our laboratory.

The genetic backgrounds of mice have crucial differences which implicate their use for studying different diseases (Erickson 1996). Success in the generation of mouse ES cells highly depends on the mouse strain used. The most commonly used strain for ES cell generation is the 129/SV strain (Stevens 1973; Threadgill *et al*. 1997; Auerbach *et al*. 2000). The generation of ES cell lines from other inbred C57BL/6 or outbred strains (e.g. ICR) appears to be more difficult to propagate *in vitro* (Suzuki *et al*. 1999; Cheng *et al*. 2004; Tanimoto *et al*. 2008), including ES cells derived from inbred C57BL/6 strains are less efficiency for chimera formation and less frequently contribute to the germline transmission than ES cell lines from the 129 strains (Brook and Gardner 1997; Auerbach *et al*. 2000). However, C57BL6 strains mouse breed well, highly characterized and preferably study in immunological and behavioral research (Waterston *et al*. 2002; Keskintepe *et al*. 2007). Resulting in C57BL/6 strain is become to use in the research study. This strain is the most commonly used background of genetically modified mouse strains and is currently the only inbred strain whose genome has been fully sequenced (Waterston *et al*. 2002).

Several studies have been reported strain-dependent differences mostly in inbred mouse strains for cardiovascular function (Blizard and Welty 1971; Bendall *et al*. 2002; Hoit *et al*. 2002; Stull *et al*. 2006). This was particularly noticeable from lines derived from the outbred strain, where it is known that the generation of pluripotent stem cells is more difficult than in hybrid or inbred strains. The difficulties of outbred strain may be there is some genetic variation and each individual is genetically unique (Festing 2009). Interestingly in this study, the majority of the examined iPS lines from each background had a better differentiation potential compared to the parental ES cells. In addition, the iPS line derived from ICR outbred also performed the best *in vitro* cardiac differentiation. However, the iPS cells derived from ICR background could not develop *in vivo* differentiaton. Many types of pluripotent stem cells are needed for detailed analysis of genetic diseases. Outbred lines are important for modeling human diseases, such as diabetes or neuronal diseases (Sullivan *et al*. 2007). Therefore, the

generation of pluripotent stem cells from outbred strains might lead to improvement of these disease models. Here, I showed that iPS technology is suitable for reprogramming cells from different genetic backgrounds, even from backgrounds (e.g. ICR), where it is difficult to generate pluripotent ES cell lines. In the iPS-ICR background, I also observed a higher efficiency of differentiation than the parental ES cell line.

Additionally, these methods could be readily applied to other cell types such as keratinocytes, which has higher reprogramming efficiency than fibroblasts to generate iPS cells (Aasen *et al*. 2008; Stadtfeld *et al*. 2010a). To examine the development potential of the iPS cells derived from 3 different genetic backgrounds, all iPS cell lines showed that the endogenous pluripotency genes were switched on following reprogramming which was detected by immunostaining (*Oct-4*, Nanog, SSEA-1) and RT-PCR (endogenous and endogenous-exogenous). From all three backgrounds, the cells were able to differentiate *in vitro* into cardiac and neuronal lineages by using embryoid bodies or monolayer chemically defined stepwise differentiation. Recent studies showed that cell extracted from different somatic tissues can be re-programmed by different efficiency, e.g. liver cells require lower level of the reprogramming factors to achieve pluripotency (Aoi *et al*. 2008). Furthermore, iPS cells derived from different sources have different differentiation potential, e.g. differentiated into neurospheres, generated from adult tail-tip fibroblasts derived iPS cells retain more teratoma-forming cells than iPS cells from embryonic fibroblasts (Miura *et al*. 2009).

These results showed that iPS cells to aggregate and differentiate in hanging drop and in suspension culture. Embryoid bodies recapitulate many aspects of cell differentiation during early mammalian embryogenesis and the cells can be terminally differentiated into various cell types belonging to the three germ layers (Keller 1995). The lack of structural organization and positional information within EBs during differentiation of the cells result in heterogeneity both within and between EBs. Interestingly, high yield of cardiac and neuronal population can be generated from iPS cells compared with ES cells. The differentiated cells also showed the positive expression of differentiation marker for cardiac (cardiac Troponin T and desmin) and neuron (nestin and Tuj-1). Most importantly, two lines from F1 and ICR backgrounds, the cells formed chimeras after blastocyst injection and one line from the F1 background transmitted to the germ line, this confirming this line to be an authentic pluripotent stem cell line. A potential limitation of the studies is that ES cells were compared with iPS cells of different genetic backgrounds which are know to affect functional

(Takahashi and Yamanaka 2006; Okita *et al*. 2007) and gene expression pattern (Brambrink *et al*. 2006; Soldner *et al*. 2009) of the cells

Some researchers advised that perhaps the combination of single-cell analysis and cell tracking with high-resolution time-lapse imaging might be the only way to truly understand the reprogramming events (Chan *et al*. 2009; Smith *et al*. 2010). In addition, the reprogramming approach still needs to be improving for a robust and efficient. For example, small molecules were used to improve the efficiency although they must be treated with caution as some of them can be tumorigenic (review in (Feng *et al*. 2009; Stadtfeld *et al*. 2010b)). The delivery method, reprogramming factors and cell types of tissues is also require, regardless of the presence of genomic modifications, however, many approaches have been reported with inefficiently reprogramming rate.

iPS technology opened up new possibilities in regenerative medicine. By reprogramming somatic cells to pluripotent stage and then differentiate them to specific lineages, the iPS technology allows patient specific stem cell therapy, without immunological side effects. Despite many optimistic predictions, the generation of safe and efficient cells for therapy is more difficult than expected. For generation therapeutically safe iPS cells one of the most crucial issue is the choice of gene-delivery system. To avoid the danger of malignant transformation, non-integrating (plasmid transfection, chemical inducers) or removable (transposone, excisable lentiviral) techniques are applicable, instead of the most commonly used retroviral system. Nevertheless, another very important issue for the generation of good quality iPS cells is the source of the cells to be reprogrammed.

In summary, the study presented here shows for the first time that the *Sleeping Beauty* transposon system is suitable for reprogramming differentiated cells into pluripotent cells. It remains to be tested, however, if iPS cell clones can be removed the transgene after complete reprogramming, even they could give rise to germline chimeras. This system provides a new non-viral methodology for the generation of therapeutically safe pluripotent stem cells. The iPS cells generated in our system were able to differentiate both *in vitro* and *in vivo* even without the excision of the pluripotency cassette. Our results also show, that the iPS technology provides a new tool for the generation of pluripotent stem cells from genetic backgrounds where ES cell generation has been difficult.

CHAPTER 7. SUMMARY (EN)

Induced pluripotent stem (iPS) cell technology involves reprogramming somatic cells to a pluripotent state. The original technology used to produce these cells requires viral gene transduction and results in the permanent integration of exogenous genes into the genome. This can lead to the development of abnormalities in the derived iPS cells. Here, I reported that non-viral transfection of *Sleeping Beauty* (SB) transposon containing the coding sequences c-Myc, Klf-4, *Oct-4* (Pou5f1) and Sox-2 linked with 2A peptides, can reprogram mouse fibroblasts. I have established reprogrammed mouse cell lines from three different genetic backgrounds: (1) ICR (outbred), (2) C57BL/6 (inbred) and (3) F1 hybrid (C57BL/6 x DBA/2J), with parallel robust expression of all exogenous (c-Myc, Klf-4, *Oct-4* and Sox-2) and endogenous (e.g. Nanog) pluripotency genes. The iPS cell lines exhibited typical characteristics typical for undifferentiated embryonic stem (ES) cell lines: ES cell-like morphology, alkaline phosphatase positivity and gene expression pattern (shown by quantitative real-time PCR, and immunofluorescence of ES cell markers - e.g. *Oct-4*, SSEA1, Nanog). Furthermore, cells were able to form embryoid bodies (EBs), to beat rhythmically, and express cardiac markers (assayed by immunofluorescence, e.g. cardiac Troponin T, desmin). The *in vitro* differentiation potential was found to be the highest in the ICR-derived iPS lines (ICR-iPS). Interestingly, the ICR-iPS lines had even higher differentiation potential than the ICR-ES cell lines: the rate of EBs forming rhythmically beating cardiomyocytes was 4% in ICR-ES and 79% in ICR-iPS cells, respectively. *In vivo*, the ICR and F1 hybrid iPS cells formed chimeras and one of the iPS cells from the F1 hybrid background transmitted to the germline. Our results suggest that iPS technology may be useful for generating pluripotent stem cells from genetic backgrounds of which good quality ES cell generation is difficult. These studies provide new insights into viral-free iPS technology and may contribute towards defining future cell-based therapies, drug-screening methods and production of transgenic animals using genetically modified iPS cells.

CHAPTER 7. SUMMARY (HU)

Az indukált pluripotens őssejt (iPS) technológia. a szomatikus sejtek pluripotens sejtekké történő átprogramozását jelenti. Az eredeti technológia általában virális génekkel történő transzdukció útján állítja elő ezen sejteket, mely az idegen gének tartós. Beépülését eredményezi a genomba, ezért. Az így előállított iPS sejteknél a későbbiekben fejlődési rendellenesség jelentkezhet. Jelen tanulmányomban bemutatom, hogy a nem virális eredetű *Sleeping Beauty* (SB) transzpozon transzfekciója, amely tartalmazza a c-myc, KLF-4, Oct3 / 4 (Pou5f1) és a Sox-2 kapcsolt 2A peptideket. Kódoló szekvenciákat, lehetővé teszi az egér fibroblasztsejtek újraprogramozását. Három különböző genetikai háttérrel rendelkező, átprogramozott egér sejtvonalat hoztunk létre: (1) ICR (outbred), (2) C57BL/6 (inbred) és (3) F1 hibrid (C57BL/6 x DBA/2J), a melyek párhuzamosan expresszálják az összes exogén (c-myc, KLF-4, Oct3 / 4 és a Sox-2) és endogén (pl. Nanog) pluripotencia géneket. Az iPS sejtvonalak jellemző tulajdonságaikban megegyeznek a differenciálatlan embrionális őssejtvonalakkal (ES): az ES-sejt szerű morfológia, alkalikus foszfatáz pozitivitás és génexpressziós mintázat (kvantitatív real-time PCR, és immunfluoreszcens ES sejt markerek - pl *Oct-4*, SSEA1, Nanog). Továbbá, a sejtek képesek voltak embrioid testeket (EBS) létrehozni, ritmikusan verni, és szív specifikus markereket expresszálni (például szív eredetű troponin T, dezmin). Az *in vitro* differenciálódási potenciált a legmagasabbnak az ICR-eredetű iPS vonalak (ICR-IPS) esetében találtam. Érdekes módon az ICR-IPS vonalak nagyobb differenciációs potenciált mutattak, mint az ICR-ES sejtvonalak: az EBs-t alkotó ritmikusan összehúzódó szívizomsejtek aránya 4% volt az ICR-ES és 79%-ICR-IPS sejtek esetében. Eredményeim arra utalnak, hogy az IPS technológia hasznos lehet pluripotens őssejtek létrehozására, azokban az esetekben, ahol a jó minőségű ES sejtek előállítása akadályba ütközik. A tanulmány betekintést nyújt a vírus mentes IPS technológiába és a jövőben hozzájárulhat meghatározó sejt-alapú terápiák kidolgozásához, gyógyszer-szűrési módszerekhez és transzgenikus állatok előállításához genetikailag módosított iPS sejtek felhasználásával.

APPENDIX

Mediums and Solutions

PBS

KCl.....20 mg

KH_2PO_4 .....20 mg

$MgCl_2(6XH_2O)$.....21,24 mg

NaCl.....800 mg

Na_2HPO_4 .....115 mg

$CaCl_2(2xH_2O)$.....13,25 mg

MQ water.....100 ml

Autoclave and storage at room temperature for up to 3 month.

FM medium

DMEM.....78 ml

FBS.....20 ml

Penicillin/Streptomycin.....1 ml

Filter sterile and storage at 4°C for up to 2 week.

10% DMSO (Freezing medium)

DMEM.....7 ml

FBS.....2 ml

DMSO.....1 ml

Filter sterile and storage at 4°C for up to 1 week.

ES medium

DMEM.....78 ml

FBS.....20 ml

LIF (1,000 U/ µl) ……10 µl

NEAA (100X).....1 ml

β-ME (50mM),.....200 µl

Penicillin/Streptomycin.....1 ml

Filter sterile and storage at 4°C for up to 1 week.

EM medium

DMEM.....78 ml

FBS.....20 ml

NEAA (100X).....1 ml

β-ME (50mM),.....200 µl

Penicillin/Streptomycin.....1 ml

Filter sterile and storage at 4°C for up to 1 week.

Neuronal differentiation (D0-8)

DMEM/F12.....98 ml

Glucose.....0.3 g

AlbuMaxI.....0.3 g

N_2 supplement.....1 ml

bFGF (stock 100 µg/ml).....10 µl

Penicillin/Streptomycin.....1 ml

Filter sterile and storage at 4°C for up to 1 week.

Neuronal differentiation (D8-14)

DMEM/F12.....48.5 ml

Neurobasal medium.....48.5 ml

GlutaMax.....1 ml

N_2 supplement.....0.5 ml

B27 .....1 ml

Penicillin/Streptomycin.....1 ml

Filter sterile and storage at 4°C for up to 1 week.

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