INDUCED PLURIPOTENT STEM CELLS: WHERE ARE WE TODAY?

by

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Abstract

For many years, scientists have been trying to elucidate the molecular mechanisms that convey pluripotency and self-renewal to stem cells. With this valuable knowledge, they hoped to discover relevant information in the development, growth and regeneration of cells, tissues, and organisms that allow organisms to live as long as they do. These characteristics were once thought to be present only in embryonic stem cells (ESCs), and a few adult multipotent cells such as hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). Recent studies have tapped into the potential of our once thought to be terminally differentiated adult cells to produce self-renewable, pluripotentiating cells, now termed induced pluripotent stem cells (iPSCs). This critical review will discuss the advantages and limitations of the methods developed to generate and characterize iPSCs. Advantages and challenges of the use of iPSCs in applications such as research and therapeutics will also be discussed.

Keywords: stem cells, somatic cells, induced pluripotent stem cells, self-renewal, pluripotency, terminally differentiated, characterisation, reprogramming, disease modeling, toxicology, drug development, therapeutics.

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List of Abbreviations

Genes/proteins

A2B5	Anti-neuron cell surface antigen
AID	Activation-induced cytidine deaminase
ALK4/5	Activin-like kinase 4/5
AOF1/2	Amine oxidase family protein 1/2
AP	Alkaline Phosphatase
Apobec	Apolipoprotein-B mRNA editing enzyme catalytic polypeptide-like
BCtnn	Beta-catenin
BIM1	Binding to microtubules 1
BMP	Bone morphogenic protein
BMP4	Bone morphogenic protein 4
Ccnb1	Cyclin B1
CD13/CD14	Cluster of differentiation 13/14
CD133	Cluster of differentiation 133
CD44	Cluster of differentiation 44
CD56	Cluster of differentiation 56
Cdc2a/CDK1	Cyclin dependant kinase 1
Cdkn1a/p21	Cyclin dependant kinase inhibitor 1a
Cdkn2a/ARF or p16INK4	Cyclin dependant kinase inhibitor 2a
Cre	Cre recombinase
DAX1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, chromosome X, gene1
Dkk1	Dickkopf WNT Signaling Pathway Inhibitor 1

Dnmt	DNA methyl transferase
DNMT1	DNA methyltransferase 1
DNMT3B	DNA methyltransferase 3B
Dppa4	Developmental pluripotency associated factor 4
E-cadh (Cdh1)	E-cadherin
EpCam	Epithelial cell adhesion molecule
ERK	Extracellular signal-regulated kinase
Esrrβ	Estrogen related receptor beta
FAT/CD36	Fatty acid translocase CD36
Fbx15	F-box containing protein 15
Fgf4	Fibroblast growth factor 4
GABRB3	Gamma-aminobutyric acid receptor beta 3
GD2/GD3	Ganglioside Precursor Disialohematoside 2/3
GFAP	Glial fibrillic acidic protein
GFP	Green fluorescent protein
Gli1	Glioma-associated oncogene-similar transcription factor 1
GSK-3β	Glycogen synthase kinase-3 beta
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigen
hTERT	Human telomerase reverse transcriptase
ICAM1	Intercellular adhesion molecule 1
IP3K	Inositol triphosphate 3 kinase
Jhdm1a/1b	Jumanji/ARID domain containing histone demethylase 1a/1b

KDM	Lysine demethylase
Klf2/4/5	Krüpple-like factor 2, 4 and 5
Lats2	Large tumor suppressor kinase 2
LeftyA	Left-right determination factor A
Let7	Lethal 7
LIN28	Lineage protein 28
LMyc	Lung myelomonocytosis
LSD1	Lysine specific demethylase
MAPK	Mitogen activated protein kinase
MCEP	Methyl CpG-binding protein
Mdm2	Mouse double minute 2
MEK	MAP/ERK kinase
NANOG	Tŕr na nÓg
NMyc	Neuroblastoma myelomonocytosis
Nr5a2	Nuclear receptor subfamily 5, group a, member 2
Oct4	Octamer-binding transcription factor3/4
OKSM	Oct4/Klf4/Sox2/cMyc
PDK1	3’ phosphoinositide-dependant kinase 1
PE2	Prostaglandin E2
Pecam	Platelet endothelial cell adhesion molecule 1 (also known as CD31)
PFK1	Phosphofructokinase 1
PP1	Protein phosphatase 1
RB2 or Rbl2	Retinoblastoma 2
RCOR2	Rest compressor 2
REX1	Zinc finger protein 42 gene

ROCK	Rho-associated protein kinase
Sall4	Sal-like protein 4
Shh	Sonic hedgehog
Snai1/2	Small zinc finger protein 1 and 1
SNEL	Sox2/NANOG/Esrrβ/LIN28
SOX1/2/18	Sex-determining region Y-box 1, 2, and 18
Src	Sarcoma
SSEA	Stage Specific Embryonic Antigen
Stat3	Signal transducer and activator of transcription 3
Tat	Transactivator of transcription
TCF	Transcription factor 4
TDGF	Teratocarcinoma-derived growth factor
Tet1	Ten-eleven translocation 1
TGFβ	Transforming growth factor beta
TGFβRII	Transforming growth factor beta receptor II
TH2A/B	Testes-specific histone 2A/B
THO/TREX	Suppressors of the transcription defects of hpr1Δmutants by overexpression-Transcription and Export
Thoc2/5	THO complex subunit 2 and 5
Thy1	Thymocyte antigen 1
TUJ1	Beta III tubulin 1
Utf1	Undifferentiated embryonic cell transcription factor 1
vMyc	Avian myelocytomatosis viral oncogene homolog
Wnt	Wingless-related integration site
Zeb1/2	Zinc finger E-box homeobox protein 1 and 2
Zfp281	Zinc finger protein 281

γ-H2A.X	gamma histone 2AX

Others

5-aza-2	5-Aza-2'-deoxycytidine
5-aza-C	5-Azacytidine
5hmC	5-hydroxymethylcytidine
5mC	5-methylcytidine
8-Br-cAMP	8-Bromoadenine-3'-5' cAMP
A	Adenine
AFDC	Amniotic fluid derived cell
AHEK	Adult human epidermal keratinocytes
ALS	Amyotrophic lateral sclerosis
AMI-5	Arginine methyl transferase inhibitor 5
ARCA	Anti-reverse di-guanosine cap analog
ASC	Adipocyte stem cells
BSPP	Bisulphite padlock probes
C	Cytosine
C19MC	Chromosome 19 miRNA cluster
C2H2	Cysteine 2 Histidine 2
C57BL6	Common 57 black 6 mice
CAG promoter	Cytomegalovirus early enhancer/chicken embryo beta-actin promoter
cAMP	Cyclic adenosine monophosphate
Ccnb1	Cyclin B1
cDNA	Complementary DNA
Cdy1	Compound of designated yellow 1
CGH	Comparative genomic hybridization

CHARM	Comprehensive high-throughput array-base relative methylation
ChIP	Chromatin immunoprecipitation
CMP	Common myeloid progenitor
cMyc	Cellular myelocytomatosis
CpG	Cytosine Guanine islets
CPP	Cell penetrating peptide
DBS	Direct bisulphite sequencing
DNA	Deoxyribonucleic acid
DNMTi	DNA methyl transferase inhibitor
DOX	Doxycycline
dU3	3’ deleted upstream promotor region
DZNep	3-deazaneplanocin A
EB	Embryoid body
ESCAPE	Embryonic Stem Cell Atlas from Pluripotency Evidence
ESCC miRNA	Embryonic stem cell-specific cell cycle-regulating miRNA
ESCs	Embryonic stem cells
F2,6P	Fructose-2,6-biphosphate
FACS	Fluorescence activated cell sorting
FDA	Food and Drug Administration
FISH	Fluorescent in situ hybridization
FSK	Forskolin
G	Guanine
GMP	Granulocyte and Macrophage progenitor
GSK-3βi	Glycogen synthase kinase-3 beta inhibitor
H	Histone

H&E	Haematoxylin & Eosin
hASC	Human adipose stem cells
HDF	Human dermal fibroblasts
HDI/HDACi	Histone deacetylase inhibitors
hESC	Human embryonic stem cell
hHFCs	Human hair follicle cells
hiPSCs	Human induced pluripotent stem cells
HIV	Human immunodeficiency virus
HMG	High mobility group
HMT	Histone methyl transferase
HMTi	Histone methyl transferase inhibitor
HPV	Human papillomavirus
HSCs	Hematopoietic stem cells
HUVEC	Human umbilical vein endothelial cells
ICR	Imprinting control region
iPSCs	Induced pluripotent stem cells
ISCBI	International Stem Cell Banking Initiative
ISCF	International Stem Cell Forum
ISCI	International Stem Cell Initiative
ITR	Inverted Terminal Repeats
IVT	*In vitro* transcription
KP	Kenpaullone
L	Lysine
LAB	Lactic acid bacteria
LiCl	Lithium chloride

LoxP	Locus of X over P1
LTR	Long terminal repeat
LT-SCs	Long-term stem cells
me	Monomethylation
me2	Dimethylation
me3	Trimethylation
MeDIP	Methylated DNA immunoprecipitation
MeDIP-ChIP	Methylated DNA immunoprecipitation-Chromatin immunoprecipitation
MeDIP-seq	Methylated DNA immunoprecipitation-sequencing
MEF	Mouse embryonic fibroblast
MEKi	MAP/ERK kinase inhibitor
MEP	Myeloid and erythroid progenitor
MET	Mesenchymal-to-epithelial transition
M-FISH	Multiplex fluorescent in situ hybridization
miRNA	MicroRNA
MMLV	Maloney murine leukemia virus
MPSS	Massively parallel signature sequencing
mRNA	Messenger RNA
MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
NaB	Sodium Butyrate
NGS	Next generation sequencing
NHEK	Neonatal human epidermal keratinocytes
NLS	Nuclear localization signal
NOC	Non-obese diabetic

NOC/SCID	Non-obese diabetic severe combined immune-deficient
NPC	Neural progenitor cell
nt	Nucleotide
OriP/EBNA-1	Epstein-Barr nuclear antigen 1 origin of replication plasmid
PB	PiggyBac
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDK1i	3' phosphoinositide-dependant kinase 1 inhibitor
PGD	Pre-implantation genetic disorder
polyA	Polyadenylate
qRT-PCR	Quantitative RT-PCR
RNA	Ribonucleic acid
ROCKi	Rho-associated protein kinase inhibitor
RPE	Retinal pigment epithelium
rRNA	Ribosomal RNA
RRSB	reduced-representation bisulphite sequencing
RT-PCR	Reverse transcription PCR
rt-PCR	Real time PCR
SAGE	Serial analysis of gene expression
SAGE	Serial analysis of gene expression
SAHA	Suberanilohydroxamic acid
SCID	Severe combined immune-deficient
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SeV	Sendai Virus
SFFV	Spleen focus forming virus

SKY	Spectral karyotyping
SMA	Smooth muscle actin
SNP	Single nucleotide polymorphism
STR	Short tandem repeats
ST-SCs	Short-term stem cells
SV40LT	Simian Vacuolating Virus 40 Tag large T gene
T	Thymine
TF	Transcription factor
TGFβi	Transforming growth factor beta inhibitor
TLDA	Taqman low-density array
TRA	Keratan Sulfate Antigen
tRNA	Transfer RNA
TSA	Trichostatin A
TTF	Tail tip fibroblasts
TTNBP	4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid
U	Uracil
UDC	Urine derived progenitor cells
UTR	Untranslated region
VitC	Vitamin C
VPA	Valproic acid
Wndchrm OR WND-CHARM	Weighted neighbour distances using a compound hierarchy of algorithms representing morphology
Wpre	Woodchuck transcriptional regulatory element
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
βgeo	Beta geo

Introduction

The ultimate goal of many scientific studies is the discovery of molecular mechanisms that convey normal cellular functioning and pathological disease in order to introduce new and improved ways to diagnose, treat, and or prevent such occurrences. These studies use many different types of cells including different types of stem cells. The advent of induced pluripotent stem cells (iPSCs) only ten years ago has revolutionized the way that scientist view somatic cells and their apparently limited differentiation potential.

Since the discovery of iPSCs, there has been an explosion of studies done in order to understand the reprogramming process and refine the techniques to characterize and generate these types of cells. Many studies also show the use of these cells in basic science, disease modeling, drug development and screening, toxicology testing as well as therapeutics. With any new discovery comes much scrutiny, especially when the use of this technology is to be considered in the treatment of diseases in humans.

The purpose of this critical review is to shed light on the recent advances in the field of stem cell technology with regards to the use of reprogrammable adult human cells in the discovery of normal cellular functioning, pathological diagnosis, and therapeutics. A brief introduction on stem cells and iPSCs will be followed by a detailed look at techniques used for the characterization of these cells. A thorough analysis of the reprogramming techniques available as well as their advantages and limitations will be provided. Lastly, the applications of iPSCs as well as the limitations of their use will be discussed.

Chapter 1 – Stem Cells

1.1 Stem Cells: Overview

Stem cells have two main characteristics that distinguish them from other non-stem and germ cells. Firstly, they can self-renew for prolonged periods of time when maintained in the appropriate environment. Secondly, they have the potential to transform themselves into other specialized cell types [1-3], a process known as differentiation. Understanding the mechanisms that control and convey these properties is important for understanding the steps and processes that occur during development (embryogenesis). During embryogenesis, cells are actively proliferating and differentiating in order to produce all the cells, tissues and organs of the organism. In adults, stem cells allow the regeneration of cells and tissues, such as skin [4], blood [1], bone [5, 6], liver [7], neurons [8, 9], and many more, necessary for proper functioning and prolonged existence. Therefore understanding the mechanisms that regulate proliferation and differentiation in adults can provide insight on normal aging processes, as well as abnormal or diseased phenotypes. More importantly, this understanding can lead to the development and application of more appropriate and effective target-based treatments for diseases.

1.2 Pluripotency and Differentiation

From embryogenesis to adulthood, a plethora of different stem cells exist in the body at different times with varying potentials for self-renewal and differentiation. In humans, the totipotent stem cell has the most differentiation potential of all stem cells. They are the first few cells created immediately after fertilization of an oocyte. These cells can create all other cells necessary to

produce all three germ layers (endoderm, mesoderm and ectoderm) of the embryo, the reproductive cells, as well as all the necessary extraembryonic cells to support the embryo's development (cells of the trophoblast and the placenta)(Figure 1). Four to five days after fertilization of the human oocyte, the cells of the inner cell mass of blastocysts are now pluripotent. Pluripotent cells can still differentiate into all three germ layers of the embryo, and reproductive cells. However they have lost the potential to differentiate into the supporting cells that produce the trophoblast and the placenta [3, 10]. These pluripotent cells are known as embryonic stem cells (ESCs). Postnatally, somatic and reproductive stem cells exist. Reproductive or germ stem cells will produce gametes and somatic or adult stem cells mature into all other tissues. Most adult stem cells are multipotent, and can produce many lineages of a particular tissue type [10, 11](Figure 1). An example is the hematopoietic stem cell (HSC) that can produce multiple different blood cells from both lymphoid and myeloid lineages [1] (Figure 2), while others will be oligopotent, producing differentiated cells of only one lineage.

Long-term stem cells (LT-SCs) do not differentiate but will proliferate indefinitely to regenerate a specific tissue for the lifetime of the organism, like epidermal stem cells that regenerate skin [12]. Conversely, short-term stem cells (ST-SCs) will self-renew to regenerate a tissue only for a limited period of time and then will differentiate into more committed cells such as the progenitor. Although progenitors may still be multipotent, giving rise to different lineages, they have very limited proliferative activity. These cells very seldom self-renew, and if they do, it is limited to one or two cell cycles [13]. One such example is the common myeloid progenitor (CMP) that leads to the production of only two other multipotent progenitors: the Megakaryocyte and Erythroid Progenitor (MEP) and the Granulocyte and Macrophage Progenitor (GMP)(Figure 2). Each of these cells will then differentiate into distinct oligopotent precursors that can only

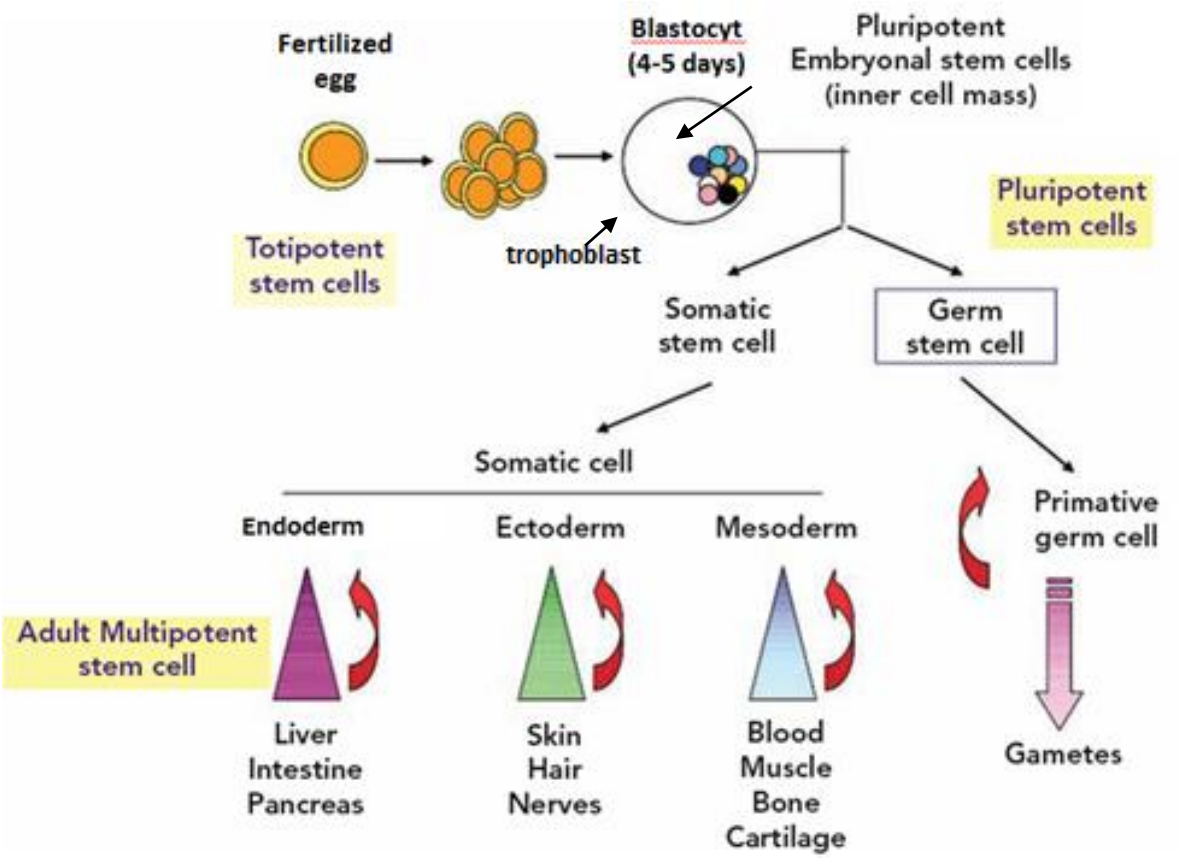


Figure 1- The Stem Cell Lineage

(Reprinted from Tyndall & Leblanc 2006 with permission)[11]

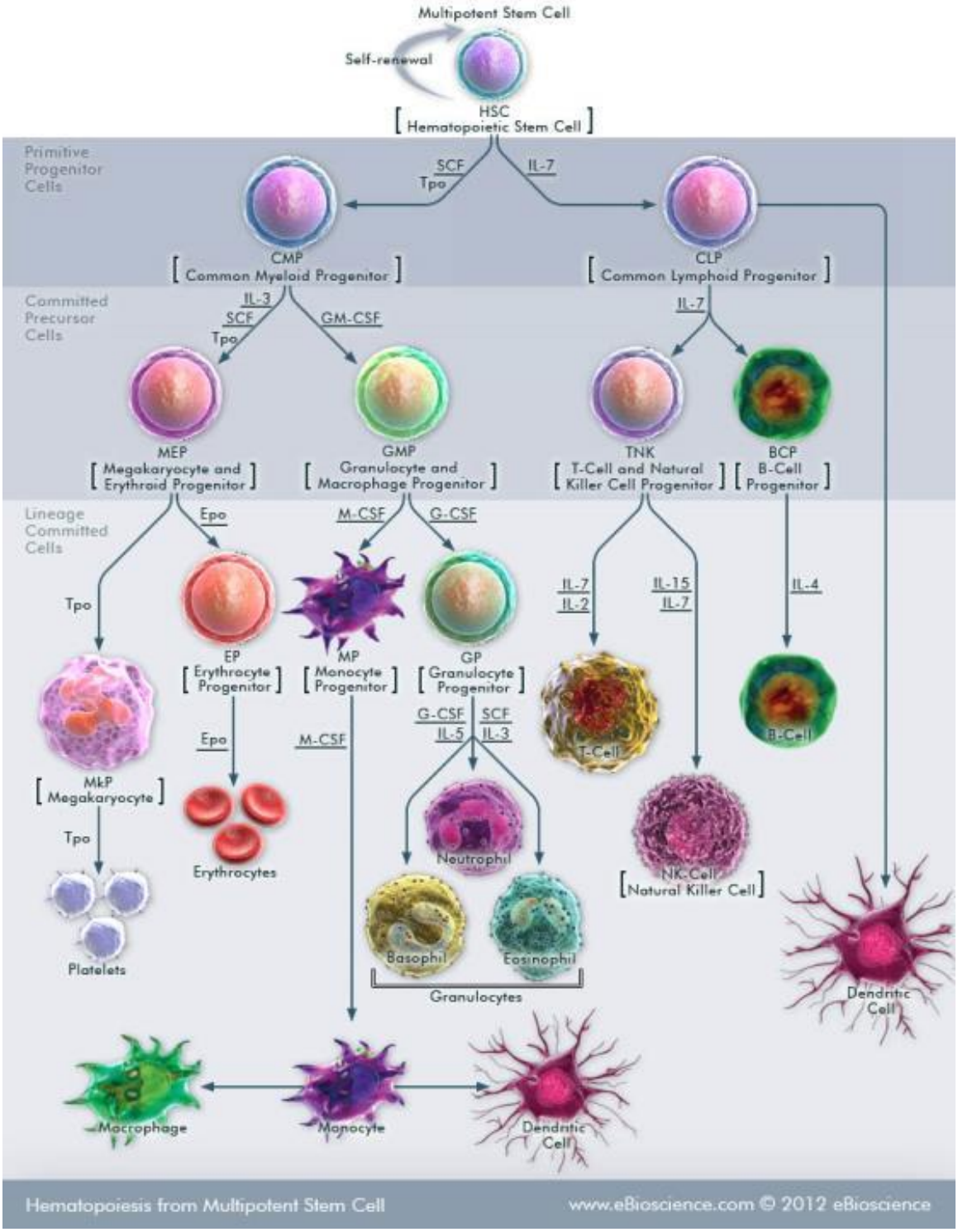


Figure 2 - Hematopoietic Stem Cell Lineage
(Reprinted from eBioscience.com 2012, courtesy of Affimatrix Inc. with permission)[14]

produce specialized cells from one lineage (Figure 2). For example, the MEP produces the lineage-committed megakaryocyte and the erythrocyte progenitor which in turn produce the terminally differentiated platelets and erythrocytes respectively (Figure 2). Fully differentiated cells have no self-renewal or differentiation potential. They have a limited life-span during which they will accomplish their own specialized functions until they die by natural cell death [15, 16], and/or are recycled like the erythrocyte [17]. The regeneration of terminally differentiated cells and tissue depends on the balance between the survival, proliferation, differentiation and death of the different types of adult stem and differentiated cells within each lineage. When this balance is shifted, it can lead to problematic phenotypes, making their study important in understanding normal and diseased states

1.3 Important Stem Cells Discoveries

The study of stem cells is a growing field. To date, many different types of stem cells have been identified and isolated for their use in scientific research and medicine. HSCs were first discovered in bone marrow by Till & McCullogh in 1961 during their investigation of the effects of radiation on the hematopoietic system[1]. Mesenchymal stem cells (MSCs), named by Caplan [18] were discovered and isolated from bone marrow by Friedenstein's group in 1970 [19]. This group as well as many others worked on the characterization of MSC to discover their ability to produce many different mesodermal tissues like bone, cartilage and muscle [18, 20-23]. Human embryonic stem cells (hESCs) were isolated from the blastocyst stage of embryos and studied by Thomson in 1998[3]. Although ESCs, HSCs, and MSCs have been fairly well studied and have important applications, their availability and isolation can be difficult. ESCs require the destruction of embryonic tissue, while MSC are mostly found in bone marrow [3]. Furthermore

MSC do not have a uniquely defined cell surface marker [24]. Other sources of stem cells include epidermal stem cells [4], intestinal stem cells [25, 26] as well as stem cells found in umbilical cord blood[27]. Recently, stem cells have been isolated from menstrual blood, endometrial tissue [28] lactating breast tissue and breastmilk [29]. These more recent findings are of scientific value since these sources are more readily available and collectable. This accessibility would allow for more thorough stem cell studies to be conducted.

1.4 Induced Pluripotent Stem Cells

Recent advances have led to the production of pluripotent stem cells from terminally differentiated adult somatic cells rather than the classical stem cells found in embryonic tissue or bone marrow. This achievement has been under development since the 1950's in some vertebrates and is still in the midst of being perfected. Attempts began in 1958 when John Gurdon produced viable frogs by the partial nuclear transfer of a haploid nucleus of a differentiated gut cell into the pronucleus of a female frog [30]. By doing so, he showed the scientific community that differentiated cell nuclei have an innate potential to behave like the nucleus of a germ cell. In 1997, Wilmut and his group continued this line of work and were successful in producing Dolly, the first cloned sheep, from the complete nuclear transfer of an adult mammary cell nucleus into an enucleated oocyte [31]. Again, their work showed that differentiated nuclei have the potential to be reprogrammed by the appropriate environment. In 2006, Takahashi and Yamanaka revolutionized the production of pluripotent stem cells by transforming whole cells by introducing exogenous factors [32]. The group used viral transfection technology in order to reprogram adult mouse tail-tip fibroblasts to their original embryonic stem cell state [32]. Not only were these cells germ line competent (as in Thompson and Wilmut's experiments)[3, 31], but they exhibited self-renewal capabilities for

extended periods of time when kept in the appropriate medium. The group showed that these cells, now termed induced pluripotent stem cells (iPSCs), can spontaneously differentiate into all three germ layers like ESCs, and can be directed to differentiate into different specialized cells (cardiomyocytes and dopaminergic neurons) upon exposure to different factors (bone morphogenic protein 4 (BMP4) and PA6 respectively)[32]. The group then repeated their experiments to reprogram the first human dermal fibroblasts (HDFs) into iPSCs [33]. Since then, scientists have been studying the process of reprogramming and redifferentiation of iPSCs in order to discover the most efficient, cost effective, and safest methods of producing the cells needed for research and medical applications.

The chapters that follow will review methods for iPSC characterization, and reprogramming. Applications and advantages of iPSC over other stem cells in research and clinical applications will also be discussed.

Chapter 2 – iPSC Characterization

To evaluate reprogramming efficiency, scientists must perform an in-depth molecular characterization of cells, during and after reprogramming. Since iPSCs are somatic cells that have been returned to an embryonic state, iPSCs are essentially tested for their resemblance to ESCs. The more the treated cells exhibit ESC-like features, the more fully reprogrammed they are considered to be. The International Stem Cell Banking Initiative (ISCBI) has published guidelines for banking and supply of ESC lines that are applicable to iPSC lines [34]. They also provide suggestions of minimal criteria for the classification (self-renewal, pluripotency, genetic stability) and advanced characterization for putative iPSC lines [35]. The bulk of this chapter will review the methods available to achieve minimal classification criteria and advanced characterization of putative iPSC lines. Advantages and limitations of methods will be discussed and other potential methods for characterization will be presented. A brief explanation of reprogramming efficiency calculations will end the chapter.

Since the reprogramming process is not immediate, identification and characterization of iPSCs consist of a series of different assays and tests performed throughout the entire reprogramming process. A summary of the timeline for different techniques, and their relative use is depicted in Figure 3 [36]. Characterization methods will be discussed in approximately the order shown in the timeline.

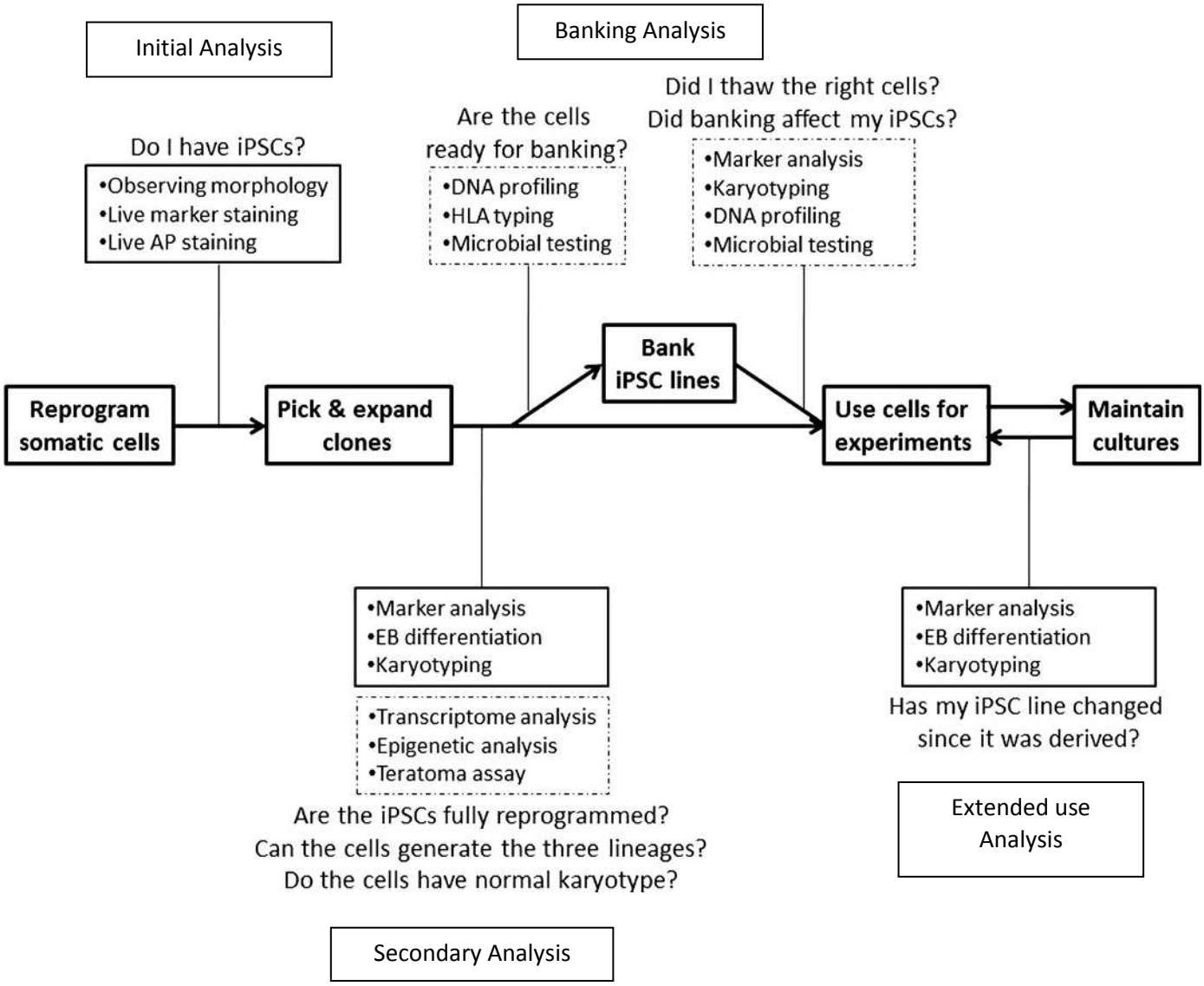


Figure 3 - Diagram of the iPSC Characterization Workflow Starting from the Reprogramming Process

Common characterization studies are found in solid boxes, while less common analyses are in the dotted boxes. (Reprinted from Asprer & Lakshmipathy 2015 with permission)[36].

2.1 Initial Analysis

In the early stages of reprogramming, shortly after they have been exposed to reprogramming factors, a small number of cells will begin the transformation process. The initial morphological changes that occur in these cells allow scientists to identify, choose, and finally reseed them for final expansion. Since these cells will be used in the lab afterward, it is important that the testing done does not affect their viability, growth or reprogramming. Prior to reseeding, scientists usually limit their testing to microscopic analysis of cell morphology, live immunostaining for cell-surface pluripotency markers, and possibly testing for enzymatic activity of alkaline phosphatase (AP), another pluripotency marker.

iPSCs can be identified from a feeder-cell layer by their compact organization, well-defined borders (Figure 4, Panel E), as well as their prominent nucleoli and limited cytoplasmic space (Figure 4, Panel F)[32, 37-39]. ISCBI provides governmentally-funded research labs with images of ideal colonies for each cell line, which can be quite helpful for scientists that are new to the field [35]. Also, the Human Stem Cell Manual Laboratory Guide contains microscopic images and descriptive analysis of the differences between differentiating and undifferentiated cells [40].

Live cell staining can also be used to identify stem cell-surface markers. This technique allows cells to be stained and visualized in culture with minimal effect on the cells themselves [41]. This consists of immunostaining with appropriate antibodies for pluripotency cell surface antigens followed by microscopic analysis. Minimal criteria proposed the ISCBI are positive staining for stage specific embryonic antigens 3 and 4 (SSEA3, SSEA4), keratin sulfate antigens 1-60 and 1-81 (TRA-1-60, TRA-1-81) [34-37, 42], as well as negative staining for SSEA1 (fibroblast cell surface antigen). In negative staining, the absence of cell-surface antigen would represent the

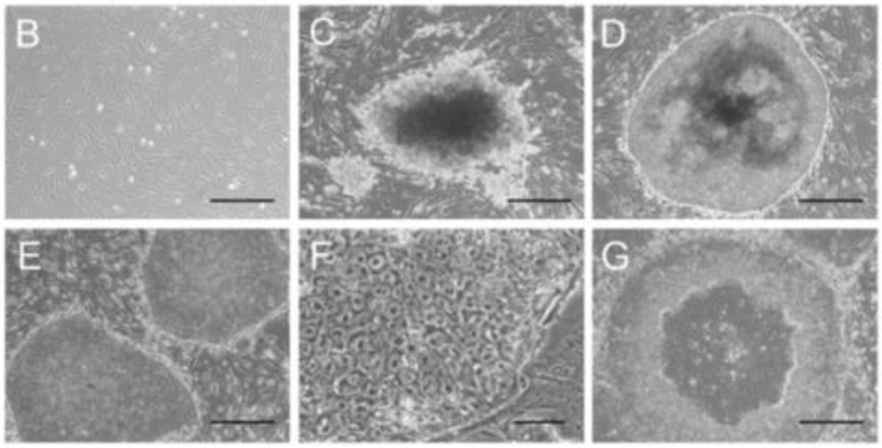


Figure 4 - Induction of iPS Cells from Adult HDFs
[A] Time schedule of iPS cell generation. [B] Morphology of HDF. [C] Typical image of non-ES cell-like colony.[D] Typical image of hES cell-like colony. [E] Morphology of established iPS cell line at passage number 6 (clone 201B7).[F] Image of iPS cells with high magnification. [G] Spontaneously differentiated cells in the center part of human iPS cell colonies. (Reprinted from Takahashi 2007 with permission)[37]

transition of somatic cell toward a reprogramming cell. Other markers that have been used for this negative staining are anti-neuron cell surface antigen (A2B5), cluster of differentiation 56, 13, and 44 (CD56, CD13, and CD44), ganglioside precursor disialohematoside 2 and 3 (GD2, GD3).[43-46]. An enzymatic assay for AP activity is also sometimes performed and recommended [35], consisting of treatment of the cells with a AP substrate prior to imaging [36](Figure 5, Panel A Live iPSCs). Theoretically, antibodies and substrates are non-toxic, and can be washed out prior to reseeding and expansion of the iPSCs, however introduction of chemicals into cells always carries the potential of influencing cellular mechanisms.

Reporter constructs can been used to identify early reprogramming cells. In the early experiments, scientists transfected cells with plasmids where green fluorescent protein (GFP) gene was inserted upstream of the promoter of pluripotency genes such as F-box containing protein 15 (Fbx15) [32], Tứr na nỞg (NANOG) [47, 48] or Octamer-binding transcription factor 4 (Oct4) [49]. When endogenous pluripotency genes begin to be expressed, as in reprogramming cells, the fluorescent reporter protein gene is transcribed, and then the mRNA of the fluorescent protein is translated. Fluorescence can be visualised under the microscope with the use of appropriate filters. The disadvantage of this technique is that reporter genes can integrate into the genome affecting the reprograming process itself or the integrity of DNA and the cell. Furthermore, NANOG and Oct4 genes are expressed only in late reprogramming [36] and this late expression may cause scientists to eliminate an early reprogramming cell populations by mistake [32, 50].

Since the introduction of stains and reporters into cells could affect reprogramming, efforts have been made to identify iPSCs based solely on their morphology using computational methods. A known automated image classifying program, weighted neighbour distances using a compound

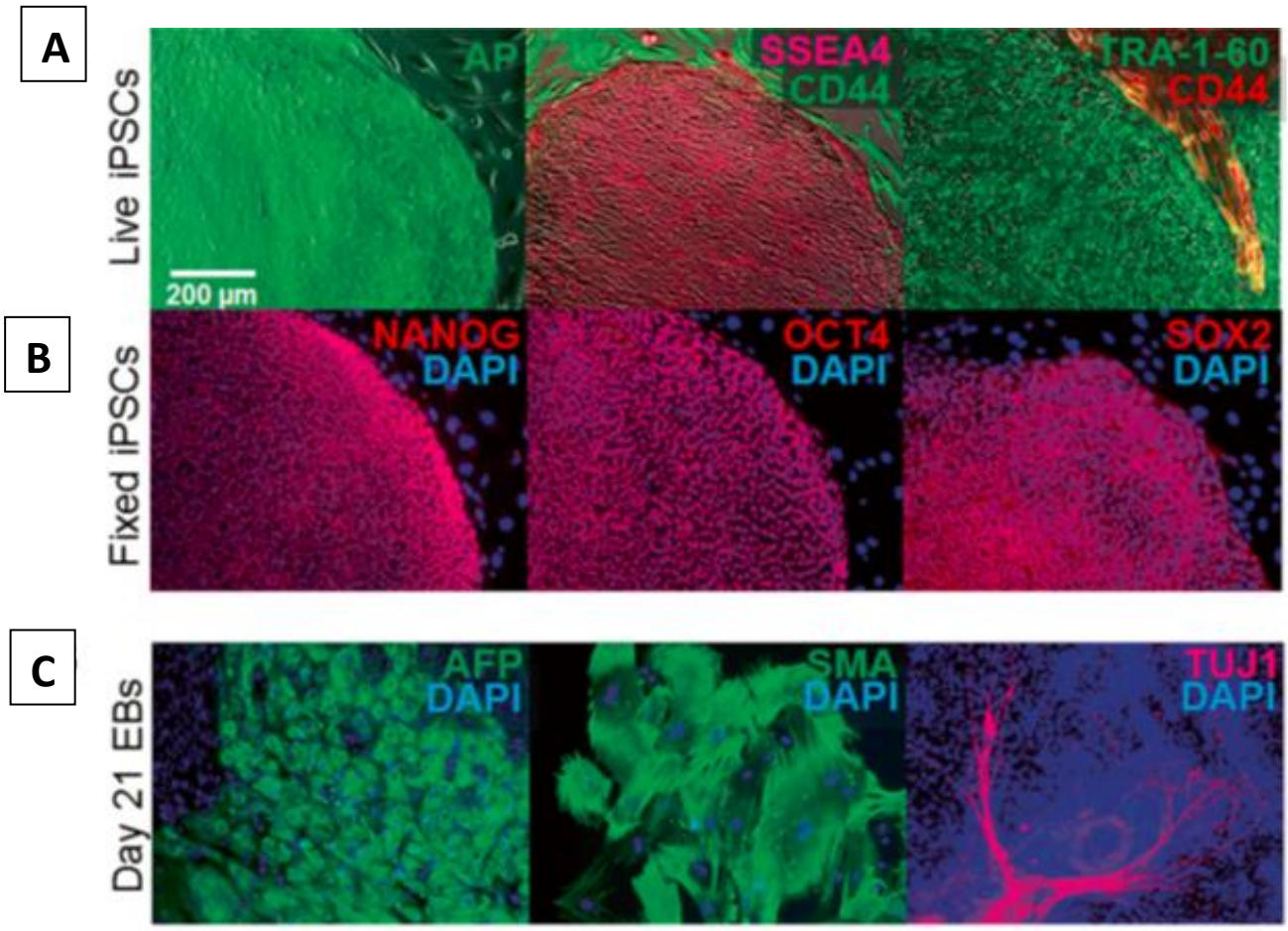


Figure 5 - Representative Characterization Panel for a Newly Derived iPSC Line
The minimum requirement for the characterization of a newly derived iPSC line is to show the expression of multiple self-renewal genes through cellular and molecular analyses, to demonstrate the ability to differentiate into the three germ lineages, and to confirm normal karyotype. [A] In this panel, a newly-derived feeder-dependent iPSC line is characterized using a variety of self-renewal markers. Live iPSCs are stained using a fluorescent substrate for AP or using antibodies against the positive surface markers SSEA4 and TRA-1-60 and the negative surface marker CD44. Images are shown as a merge of the phase contrast with the fluorescence channels. Fixed iPSCs are stained with antibodies against the intracellular self-renewal markers NANOG, OCT4, and SOX2, along with DAPI as a nuclear stain. [B] Additionally, iPSCs are allowed to form EBs and differentiate spontaneously for 21 days, then tested for the ability to generate the three germ lineages. The Day 21 EBs are stained with DAPI and antibodies against Alpha Fetoprotein (AFP) for endoderm, Smooth Muscle Actin (SMA) for mesoderm and Beta-IIITubulin (TUJ1) for ectoderm. [C] For the molecular analysis, the feeder dependent iPSCs are used to make feeder-free cultures and Day 7 EBs. Gene expression is analyzed using the TaqMan® hPSC Scorecard ™ Panel and is compared against a known set of reference samples through a data analysis software based on Bock *et al*.[51, 52]. (Reprinted from Asprer & Lakshmipathy 2015 with permission)[36]

hierarchy of algorithms representing morphology (wndchrm or WND-CHARM) is a potential avenue for iPSC identification [53, 54]. This machine-supervised algorithm automatically assesses over one thousand features from a multitude of images in order to create classes of images. These features can be grouped into four categories: 1) polynomial decompositions, 2) high contrast features, 3) pixel statistics, and 4) textures. Once the classes have been defined, the algorithm can automatically classify images based on the similarities to the known classes. Yamanaka's group created a model based on morphological similarities and differences of previously identified ESCs, iPSCs, and differentiated cells using this system [54]. Their study confirmed that WND-CHARM is effective in discriminating between hiPSCs and non-hiPSCs with almost 100% accuracy. Thus far, this is the only non-invasive method that has been successful in validating early iPSCs, and could be of significant value for scientists in the future.

2.2 Secondary Analysis

Once reprogramming cells have been identified, reseeded and expanded, they will be subjected to a more thorough characterization process before use in the lab, registration as iPSC line, and/or iPSC banking. Methods to establish self-renewal, pluripotency, and genetic stability will be presented followed by advanced characterization methods [35](Figure 3).

2.2.1 Self-renewal

Firstly, cells must be able to go through a minimum of 10 passages in a feeder-dependant manner without differentiating in order to be labelled as iPSCs [35]. Self-renewal can also be assessed by calculating the doubling time during extended proliferation [37, 55] and compared to the doubling time of ESCs of approximately thirty two hours [56].

2.2.2 Pluripotency

Pluripotency can be determined by using a variety of techniques that identify expression of cell-surface pluripotency markers, and genes, as well as differentiation into the three germ layers.

2.2.2.1 Marker Analysis

Minimal requirements for iPSC registration require positive results from different tests. Firstly, fixed immunostaining and western blotting of cell-surface markers SSEA3, SSEA4, TRA-1-60/TRA-1-81, AP, SSEA1^{-} , as well as intracellular pluripotency proteins Oct4, NANOG, Sex-determining region Y-box 2 (Sox2), zinc-finger protein 42 (Rex1), teratocarcinoma-derived growth factor (TDGF)[34] must be accomplished (Figure 5, Panel A Fixed iPSCs). Although these are the recommended markers from the ISCBI, groups have performed immunostaining and western blotting for other pluripotency markers identified by the ISCI such as gamma-aminobutyric acid receptor beta 3 (GABRB3), DNA methyltransferase 3B (DNMT3B), and human Telomerase Reverse transcriptase (hTERT)[37, 55] and negative staining for CD44. Fixed staining provides information about the location of the proteins, whereas western blotting allows quantification of protein levels within a cell. This process is accomplished by denaturing isolated proteins with sodium dodecyl sulfate prior to separation according to size by polyacrylamide gel electrophoresis (SDS-PAGE). This allows proteins to be identified by their size. Once transferred to a nitrocellulose paper, proteins can be stained with appropriate fluorescent antibodies and fluorescence can be quantified [57]. Furthermore, bands in the electrophoresed gel could potentially be excised and sequenced in order to verify the protein identify [58].

The downfall of this technique is that cell preparation requires fixing and permeabilization of cells with cytotoxic and carcinogenic agents [59]. Furthermore, western blotting requires lysis of the cells in order to isolate the proteins. Therefore, cells used for these procedures are useless for reseeding, expansion or further testing.

Flow cytometry or fluorescence activated cell sorting (FACS) are the most common and recommended methods for identification of cell-surface markers, cell-counting and sorting [34]. In flow cytometry, cells are placed in a liquid medium and incubated with fluorescently labelled antibodies for pluripotency or differentiation markers. They are then forced through a micronozzle under pressure. The nozzle vibrates axially to produce uniform droplets of liquid containing one cell. Prior to being expelled from the micronozzle, the cells pass through an argon ion laser beam that excites the fluorescent antibodies. The signal from a fluorescent antibody-labelled cell is captured by two detectors (front scatter and side scatter detector). Fluorescence signals can be analysed by a computer in order to quantify the number of positively stained cells in the solution. In FACS, cells go through flow cytometry process, however the droplets are negatively charged as they are released from the micronozzle. Cells exiting the nozzle are subjected to an electric field that can deflect cells based on their charge. The detection of an above threshold fluorescence read by the detectors, transmits a signal that will ensure the electric field is applied in a way to deflect the negatively charged droplets into the appropriate containers [60](Figure 6). Cells identified and isolated by FACS can be recultured, and used for research as long as they have not been contaminated.

Young-Tae Chang's group claim to have discovered a more appropriate staining technique using a fluorescent, non-toxic molecule compound of designated yellow 1 (Cdy1) instead of an antibody, that selectively stains pluripotent cells[61]. Cdy1 is a rosamine, lipophilic cation with

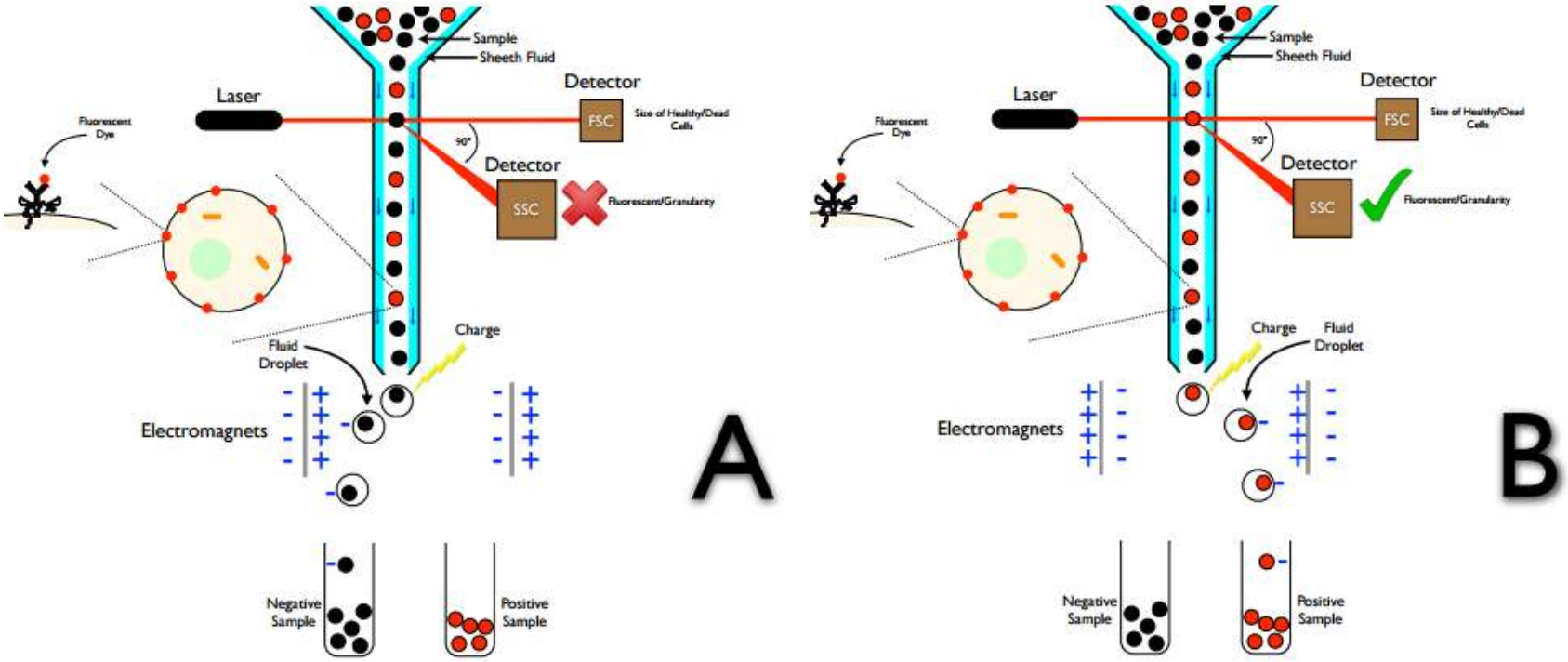


Figure 6 - Diagram Explaining Fluorescence-Activated Cell Sorting (FACS)
This figure is showing a representation of flow cytometry at the top of each diagram, but also shows how additional equipment can enable it to be used for FACS. [A] the sample containing cells is injected in the middle of a fluid sheeth and funneled to give a single line of ordered cells. The cells are passed through a laser beam that measures the forward scatter (FSC) and side scatter (SSC). Usually this flow cytometry result is sufficient for analytical methods, but for a preparatory cell sorting method (FACS) the cells passed through a nozzle that vibrates and results in cell droplets with approximately one cell per drop, this drop is then electrically charged to give a negative charge on it. The machine computer calculates (since the distance between the laser and the electromagnets is known) when the cell reaches the area between the electromagnets; it will switch them on to divert the cell path so it lands on the correct vial, and thus accumulate the sorted cells. In this case the beam detects no fluorescence and therefore sends the droplet of cell into the negative sample vial. In [B] the laser beam detects the correct fluorescence and therefore sends the charged cell droplet into the correct positive sample vial. (Reprinted from Sabban 2011 with permission)[62]

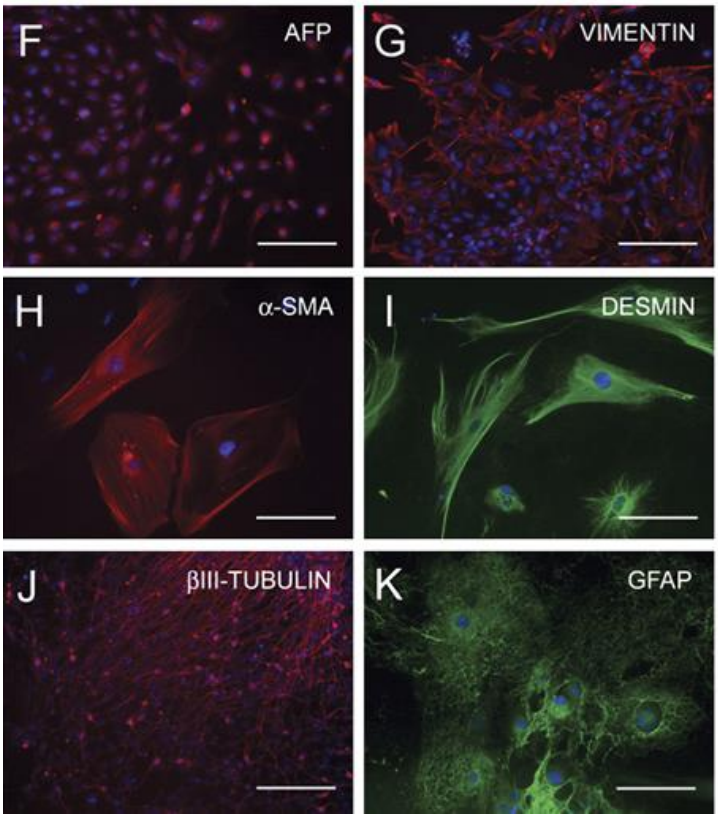
peripheral amines that preferentially stains the mitochondria of mESCs, hESCs and miPSCs as opposed to cancer cells, and fibroblasts. Interestingly, this stain was shown to target early reprogramming cells prior to the expression of Oct4-GFP construct, and ESC-like morphological changes [63]. This stain can be used with FACS in the identification and sorting of iPSCs [64]. The efficiency of this stain has yet to be verified in human induced pluripotent stem cells hiPSCs. Furthermore, since it has recently been shown to positively stain neural stem cells in the subventricular zone and the hippocampus [65], as well as drug-resistant cancer stem cells [61], its use would be limited to reprogramming of somatic cells that have shown negative staining for Cdy1, such as fibroblasts.

Some scientists verify pluripotency by measuring the expression level of mRNA of pluripotency-related genes by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)[55, 66], however quantification of mRNA levels of pluripotency genes mentioned above via quantitative-RT-PCR (qRT-PCR) is suggested as a minimal requirement for classification of iPSC lines [34]. In both cases, mRNA is isolated from cells and reverse transcribed into complementary DNA (cDNA) that will be amplified by PCR reaction. In RT-PCR, analysis is done at the end of the process by detecting the amplified DNA after separation by electrophoresis. These bands can further be quantified by densitometry or phosphorimaging [67]. In qRT-PCR, the quantification of mRNA levels is performed throughout the amplification process by the continuous measurement of fluorescence. These last two techniques allow scientist to quickly analyse the expression levels of a small number of genes associated with pluripotency at the same time. Their use is limited to providing information about gene expression levels only, as previous studies have shown that mRNA levels and protein levels do not necessarily change proportionally [68-71]. A group studying the effects of Nanog depletion on ESCs revealed that

changes in many nuclear protein levels were not consistent with corresponding mRNA level changes [72]. More recently, Wang's study on the transcription/export complex (THO/TREX) revealed that mRNA levels of Nanog, Sox2, krüpple-like factor 4 (Klf4) and estrogen-related receptor beta (Esrrβ) remained unchanged after knock out of THO complex subunits 2 or 5 (Thoc2 or Thoc5), while their corresponding protein levels were significantly decreased [73]. For these reasons, mRNA data should be supplemented by immunostaining and western blotting which provide information about localization and protein levels necessary for thorough analysis.

2.2.2.2 Differentiation

The second aspect to consider when evaluating the pluripotency of iPSCs is their ability to differentiate into the three cell lineages (ectoderm, mesoderm and endoderm). An *in vitro* method to verify pluripotency is the spontaneous formation of embryoid bodies (EBs) [32, 42, 55]. There are many different protocols for this assay [74], however the basics remain the same. Essentially iPSCs are removed from their feeder-layer, and grown on a medium deprived of pluripotency signals, without which they differentiate spontaneously [40]. Once they have formed masses, termed EBs, these are dissected, analysed for lineage-specific markers by immunostaining, FACS or quantification of lineage-specific mRNA levels by qRT-PCR [32]. Markers used include: beta III tubulin (TUJ1) [38, 55] and glial fibrillary acidic protein (GFAP) for neural ectoderm; vimentin, smooth muscle actin (SMA) and desmin for mesoderm; and alpha-fetoprotein (AFP) for endoderm, [55](Figure 5, Panel C and Figure 7, Panels F - K). EBs can also be dissected, stained via Haematoxylin & Eosin (H&E), and analysed for morphological features of different tissues like cartilage, bone (mesoderm) or structures like glands (endoderm), gut-like epithelium (endoderm) and neural rosettes (ectoderm) [32, 47, 55, 75, 76]. Minimal requirements for the iPSCs to be used in further studies are immunostaining for TuJ1, GFAP, and quantification by



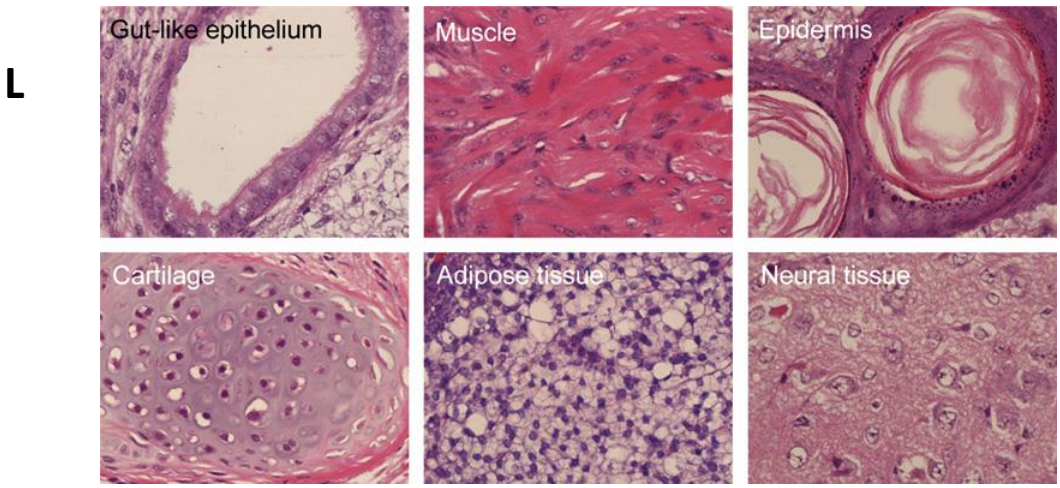


Figure 7 - Embryoid Body-Mediated and Teratoma Differentiation of Human iPS Cells **[**F–K] Immunocytochemistry of a-fetoprotein (AFP)([F], vimentin [G], alpha-smooth muscle actin (α-SMA) [H], desmin [I], betaIII-tubulin (βIII-TUBULIN)[J], and glial fibrillic acidic protein (GFAP) [K]. Bars = 200 mm [A and B] and 100 mm [C–K]. Nuclei were stained with Hoechst 33342 (blue). [L] Haematoxylin and eosin (H&E) staining of teratoma derived from iPS cells (clone 201B7). Cells were transplanted subcutaneously into four parts of a severe combined immune deficient (SCID) mouse. A tumor developed from one injection site. (Reprinted from Takahashi 2007 with permission)[37]

qRT-PCR for a choice of markers from all three cell lineages [34].

Teratoma formation is an *in vivo* method of verifying differentiation of iPSCs. iPSCs are injected into non-obese diabetic severe combined immunodeficient (NOC/SCID) mice. Lack of immunity in the mice allows rapid growth of teratomas that can be dissected and analysed for lineage-specific markers by the same methods as can be embryoid bodies (immunostaining or qRT-PCR of mRNA levels) [32, 37](Figure 7, Panel L). The histological analysis of teratomas is more difficult than that of the EBs since the differentiating iPSCs can mesh with surrounding tissue, making it difficult to distinguish host cells from injected cells.

Even though this technique is more laborious than EB analysis, confirming differentiation under physiological conditions is more biologically relevant than *in vitro* methods. The ISCBI strongly suggests that this procedure be a standard for characterizing iPSCs [34, 35], however only about half of the published ESC and iPSC cell lines have been validated with this assay [77].

A factor to consider when performing teratoma assay is the site of injection. Although iPSCs are usually injected subcutaneously into the dorsal flank of NOD/SCID mice [32, 47, 55, 66], they have also been injected into the liver, kidneys, testes, or leg muscle [78]. Studies have shown that ESC prefer to differentiate into one lineage over another depending on the site of injection [78-80]. If iPSCs had lineage-preferred differentiation depending on site of injection as ESCs, this could lead to false conclusion about pluripotency during their characterization. If teratoma formation is to be a gold standard for pluripotency determination of iPS cell lines, clear protocols with specific injection sites would need to be established.

Lastly, this assay is usually performed in mice regardless of iPSC origin. It remains unclear if this cross-species injection could also be influencing differentiation patterns since there is no ethical way of comparing results of hiPSC injection into humans.

The ultimate test for pluripotency is the establishment of germ-line competency. This requires that the cells be injected into blastocyst, implanted *in vivo*, and left to produce fertile offspring [48]. This procedure also allows scientists to verify the formation of complete chimeras that are fertile and can contribute to the germ line [48, 81, 82]. This is a significant feature that can set apart iPSC colonies, however it cannot be tested in primates such as humans due to ethical issues. Furthermore, since it involves the creation of life that may be immediately terminated after birth, and is not a necessity for characterization or banking, this technical approach is not common practice [34].

2.2.3 Genetic stability

Before cells are used for research, and/or clinical purposes, or banked, it is important to verify that testing or treatment has not altered their genetic stability (Figure 3). The last suggested minimal criterion for iPSCs is that they exhibit normal diploid karyotype. G-pattern karyotyping involves staining of the cells' chromosomes during mitosis (metaphase), with Giemsa's reagent. This technique allows scientists to observe and analyse the number, size, arrangement and structure of the chromosomes to detect any abnormalities such as aneuploidy, deletions, duplications, and/or translocations [83](Figure 8, Panel A). Although ISCBI's minimal suggested requirement is a diploid karyotype many groups verify and note other anomalies that are present in the lines.

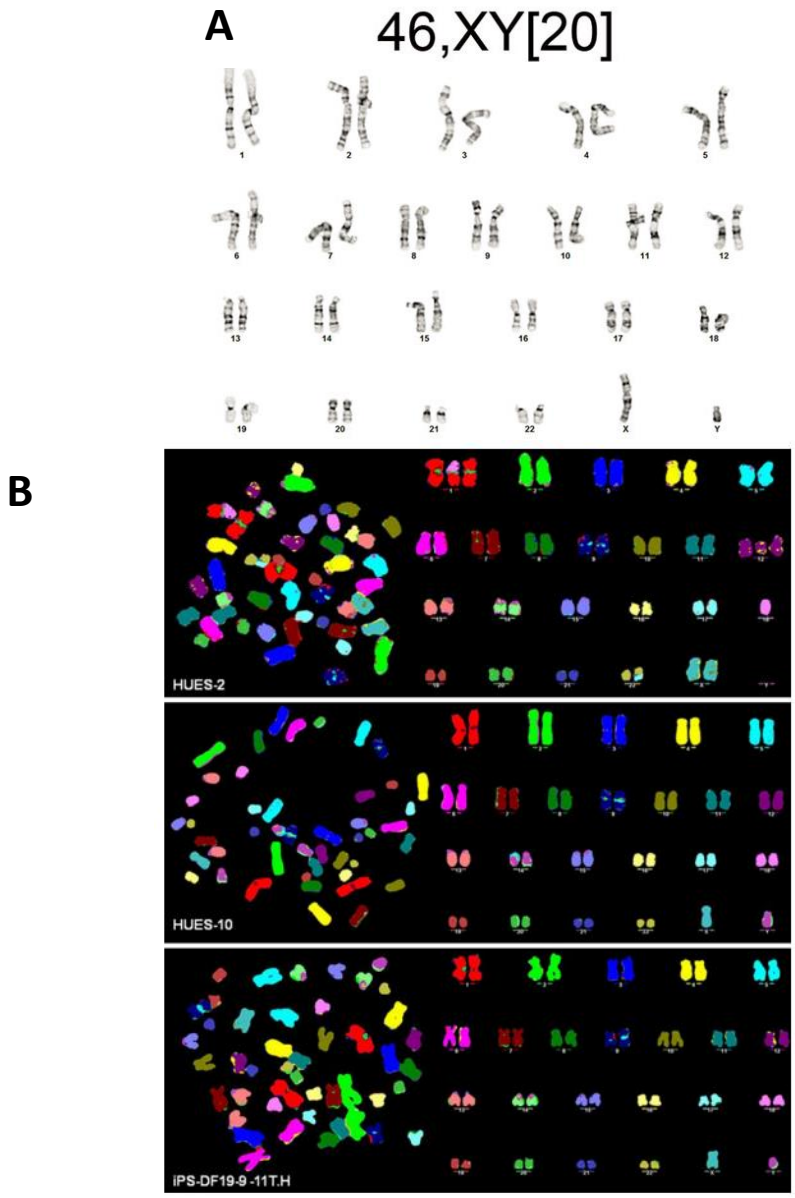


Figure 8 - Karyotyping
[A] iPSCs G-banding of a normal male karyotype in 20 cells analyzed. (From Asprer & Lakshmipathy 2015 with permission)[36]
[B] Twenty-four color karyotyping of hES cells (HUES-2 and HUES-10) and iPS-DF19-9-11T.H by M-FISH. The high standard and improved speed of the M-FISH analysis have together confirmed the newly identified optimal mitotic arrest and hypotonic conditions to provide a significant technical breakthrough for chromosomal analysis of hES and hiPS cells. While HUES-10 (passage 37) and iPS-DF19-9-11T.H (passage 29) presented a normal karyotype, M-FISH analysis on HUES-2 at passage 40 revealed, as well as chromosome 12 partial trisomy, a couple of structural abnormalities to include a translocation involving an extra copy of chromosome 1q and chromosome 18, and an unbalanced translocation involving chromosomes 17 and 22 (Reprinted from Moralli 2011 with permission)[84]

Spectral karyotyping (SKY) and multiplex fluorescent in situ hybridization (M-FISH) are adapted karyotyping techniques that may facilitate karyotyping interpretation (Figure 8, Panel B). In these techniques, the chromosomes are hybridized with differently coloured fluorescent probes and therefore appear in distinct colours as opposed to the black and white appearance of G-pattern results [84].

Comparative genomic hybridization (CGH) array or single nucleotide polymorphism (SNP) analysis are also methods that allow for identification of genetic abnormalities in cell lines [34, 35, 85-87]. Panels A and B of Figure 9 represent simplified models for the CGH and SNP analysis [88]. These methods were found to be more efficient at identifying genetic abnormalities in hESC and hiPSC lines that could not be detected using G-banding karyotyping techniques [89-91]. Notably, Dekel-Naftal demonstrated through M-FISH that iPSCs had increased aneuploidy rates correlated to their number of passages, and their CGH array showed a recurrent gain of chromosome q1 [91]. Laurent was able to detect deletions and duplications in genes analysed by SNP genotyping that had been missed by G- banding karyotyping technique [90]. These studies establish the importance of the techniques suggested by the ISCBI in the characterization of iPSCs, as well as the importance of repeated genomic monitoring of iPSC lines that will be used for research or in clinical applications.

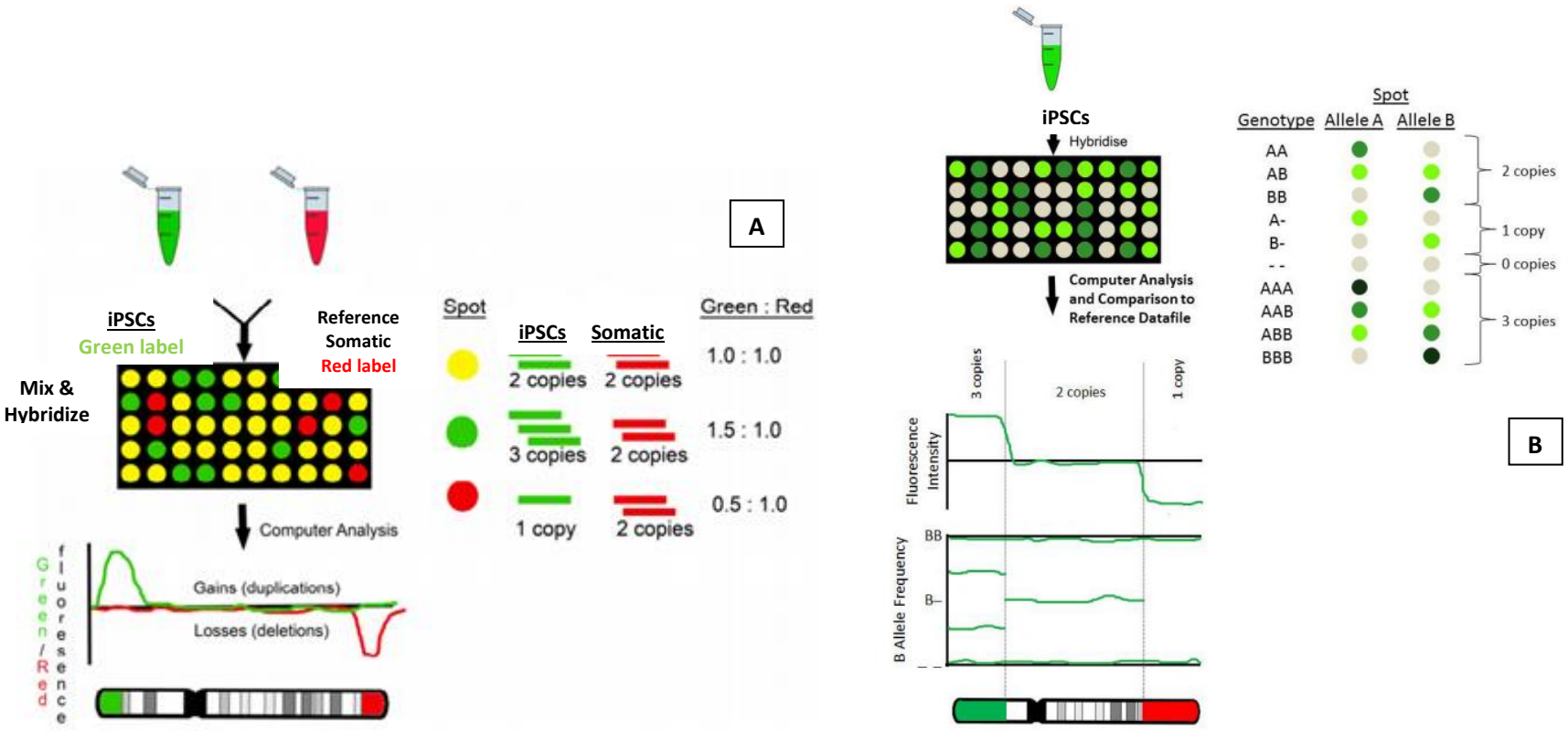


Figure 9 - CGH and SNP microarray

[A] In array CGH, iPSCs and somatic cells are differentially labeled using fluorescent dyes then mixed together and co-hybridized to a microarray slide bearing oligonucleotides. Each probe represents a specific locus in the genome. The slide is then scanned and the fluorescent of each dye for each probe is measured. Yellow dots indicate genomic segments with equal number for both cell types. Red and Green dots indicate the loss or gain of chromosomal material in the iPSC respectively. Analysis software can also plot the probes along the length of the chromosome depending on its location and also on, above or below a baseline according to its relative fluorescent dye intensity. [B] In SNP array, only iPSCs DNA would be labelled and hybridized. The intensity of each oligonucleotide on the array is compared to the intensity of the same oligonucleotide in a set of controls established with the patient's somatic cells and the probes can be plotted along the length of a chromosome depending on its location as well as on the B Allele Frequency plot depending on its presence or absence and fluorescent intensity. (Modified from Karampetsou et al 2014 with permission)[88]

2.4 Advanced Characterization

Unlike the initial and secondary analyses of iPSC colonies, which are based on the use of a few known markers, advanced characterization involves a broader scale of testing. This testing includes high throughput analysis and comparison of the transcriptome and the epigenome of multiple iPSC lines in order to gain insight on the internal cellular mechanisms controlling reprograming, and also to aid in the development of methods capable of properly identifying completely reprogrammed cells.

2.4.1 Transcriptome analysis

Transcriptome analysis involves the use of high-throughput techniques that allow generation of gene expression profiles for cell lines by analysing and quantifying mRNA levels of hundreds to thousands of genes at once. Focused array or q-RT-PCR panels allow the analysis tens to hundreds of genes [51, 92-94], while microarrays allow the quantification of thousands of predetermined pluripotency genes [66, 95-100]. Although the number of genes tested may vary, these three techniques are quite technically similar. They involve the creation of cDNA via RT-PCR from isolated mRNA, tagging of the cDNA with Biotin or fluorescent dyes, then hybridization of the labelled cDNA with a microarray containing a series of known probes. Excitation of the dyes by "laser" allows for the capture and measurement of fluorescence and analysis of up-regulation or down-regulation of specific genes. The amount of fluorescence is proportional to the amount of dye, hence cDNA, that has hybridized to the microarray probes [101](Figure 10). ISCBI suggests the quantification of gene expression using Taqman low-density array cards (TLDA) [35]. These methods are convenient when scientists want to focus on specific genes, however

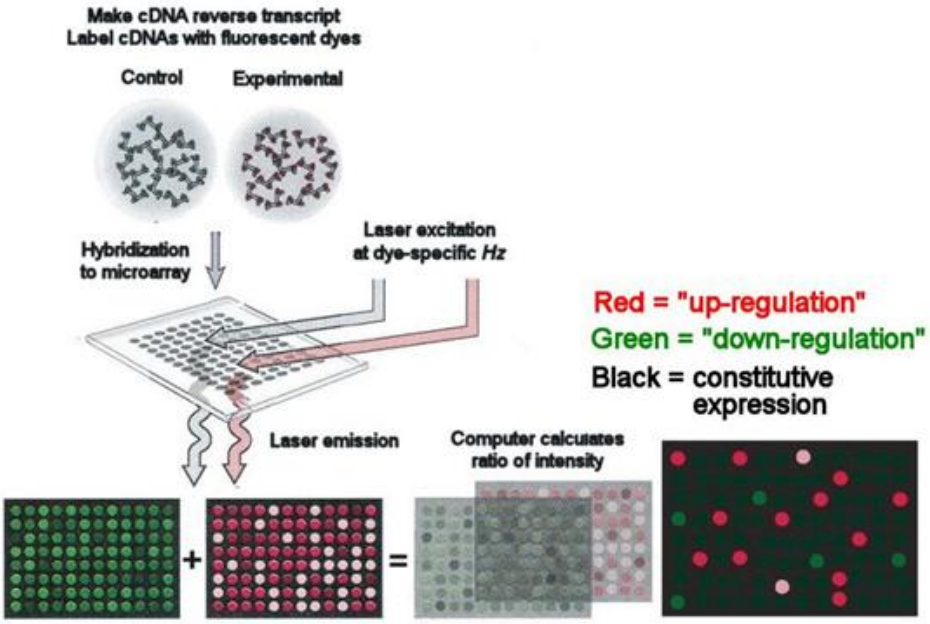


Figure 10 - Principle of cDNA Microarray Assay of Gene Expression
A complete set of **mRNA** transcripts (the **transcriptome**) is prepared from the tissue of an **experimental** treatment of condition [iPSCs]. **Complementary DNA** (**cDNA**) **reverse transcripts** are prepared and labelled with a [**red**] fluorescent dye. A **control** library is constructed from an untreated source [ESCs or somatic cells]; this library is labelled with a different fluorescent [**green**] dye. The experimental and control libraries are hybridized to the **microarray.** A **Dual-Channel Laser** excites the corresponding dye, and the fluorescence intensity indicates the degree of hybridization that has occurred. Relative gene expression is measured as the ratio of the two fluorescence wavelengths. Increased expression or "***up-regulation***" of genes in the experimental transcriptome relative to the control will be visualized as a "***hotter***" red "***pseudo-colour***," and decreased expression or "***down-regulation***" shows as a "cooler" green. Intensity of color is proportional to the expression differential. Unchanged, **constitutive expression** (**1:1** ratio of experimental to control) shows as a neutral **black**. (Reprinted from Carr 2013 with permission)[102]

they may lead to an incomplete analysis of the cells, since important information relative to other non-target genes may be missed. That said, serial analysis using different commercially available microarrays of pluripotency and non-pluripotency genes could maximize the data obtained.

Massively parallel signature sequencing (MPSS) [103-105], serial analysis of gene expression (SAGE) [106-108], and RNA-sequencing [109, 110] have also been used to create profiles without limitation of predetermined genes of interest. MPSS & SAGE techniques couple RT-PCR and hybridization to measure mRNA, however DNA fragment size, tag length and DNA sequence analysed are determined by the restriction enzymes used to cleave the DNA and make the tags. Although the two techniques can provide non-specific mRNA data that could be missed by specific arrays, sections of the genome can still be missed since restriction enzymes have specific cut sites. Again, serial analysis using different restriction enzymes would be necessary to thoroughly investigate gene expression, however this could make for a lengthy process [36]. Since using a smaller number of shorter tags in SAGE decreases fidelity of the results [103, 104], a modified SAGE, long SAGE, using longer probes, was successfully use in the characterization of nine ESCs lines [111]. MPSS has also been used in the characterization of signalling pathways activated in ESCs and therefore could potentially enable identification and characterization of iPSC colonies [108]. RNA sequencing also allows for a non-specific analysis of total RNA, small RNA, microRNA (miRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). This technique can provide interesting information from sources not usually analysed by other methods [109, 112].

To produce a more complete transcriptome analysis, scientists should utilize a variety of techniques that can provide information on many pluripotency-related and somatic-related genes, as well as non-coding segments of the genome.

Interesting computational tools, for the analysis of large scale experimental data, are being developed in order to facilitate identification of iPSCs. Using identical culture conditions for all cells types, DNA profiling of over 15 000 genes was accomplished via microarray for 20 previously derived hESC and 12 hIPSC lines as well as 6 fibroblast cell lines. By comparing each line to one another and to a reference ESC line, Bock *et al*. created reference maps (similarities and differences) between the three cell types [51]. Their results showed that iPSCs were more similar to ESCs in gene expression profiles than to the 6 fibroblast cell lines examined, and that iPSCs showed a higher global deviation then ESCs, but that most of the genes that deviated were similar among these two cell types. Using bioinformatics data, they integrated the results into a scorecard that could measure the quality of iPSCs lines [51]. They created a deviation scorecard with the outlier information and a lineage scorecard with lineage-specific outliers which were then incorporated into the "Q-RT-PCR TaqMan® hPSC Scorecard™ assay" used in further studies to target self-renewal and differentiation genes [52]. This study was unique in that it developed an *in silico* tool that can aid other scientists in the identification and characterization of their iPSC lines. It was also unique because the group maintained culture conditions therefore, discrepancies noted between cell lines are attributable to the cells and not the medium in which they were maintained. Other computational methods developed by merging experimental and bioinformatics data include PluriTest [113], and CellNet [114], which were developed to help identify differentiated cells from PSCs.

Large scale studies like these can help gain insight on cell-specific behaviours during reprogramming. In order for these methods to be successful, large working databases of experimental and bioinformatics information are necessary. There are many bioinformatics databases accessible through PubMed, however not as many experimental databases such as

Embryonic Stem Cell Atlas from Pluripotency Evidence (ESCAPE) database [115, 116] which is still being kept current (last update in august of 2014). Unfortunately, without scientists to share information, these bases cannot grow and may become stagnant.

Transcriptome analysis can lead to the development of helpful computational methods for identification and characterization of iPSCs, however they are not common practice. This lack of broad-scale information can be problematic because there is conflicting evidence as to which pluripotency markers are efficient for iPSC characterization. Chan's study indicates that expression of TRA-1-60, DNMT3B and REX1 can be used to identify fully reprogrammed iPSCs, whereas AP, SSEA-4, GDF3, hTERT and NANOG are insufficient as markers since they may appear in incompletely reprogrammed cells [117], yet all of these markers have been validated by either the ISCI or the ISCBI [34, 35]. Chan's study demonstrates the importance of a more thorough transcriptomic analysis when identifying and characterizing iPSC lines.

2.4.2 Epigenome analysis

Epigenetics is another area of study to be considered when characterizing iPSC lines. It has been shown that somatic cells and stem cells have different DNA methylation and histone modification (mostly acetylation and methylation) patterns [118-120]. For example, repressive Histone 3 Lysine 9 (H3K9) and H3K27 trimethylation ($H3K9me^3$) of pluripotency genes is lower in ESCs as opposed to their differentiated counterpart [121]. Although iPSC characterization is usually based on similarities to ESC features, like common $H3K4me^3$ and $H3K7me^3$ of Oct 4 and Nanog [35, 82, 122], as well as characteristic X-chromosome reactivation in female cells [35, 92], it has been noted that iPSCs and ESCs have minor epigenetic differences [120, 123, 124]. These differences are often thought to be caused by somatic memory (somatic cell epigenetic

pattern), however studies showed that somatic memory could only explain a small fraction, 0,01% to 0.001%, of the patterns exhibited by iPSCs [125]. Many methods exist for identifying methylation patterns.

The simplest methods for analysing DNA methylation patterns are: methylated DNA immunoprecipitation (MeDIP) [126, 127], followed by array-based hybridization (MeDIP-ChIP) and high-throughput sequencing (MeDIP-seq)[128, 129]. During these procedures, DNA is sonicated, and denatured to create small fragments of single-stranded DNA that will be treated with an antibody for 5methylcytosine (5meC), then isolated by immunoprecipitateion. In MeDIP-ChIP, methylated and input DNA are differentially stained and hybridized to arrays and quantified for comparison, while in MeDIP-seq, both methylated and input DNAs are sequenced and compared [129]. Another method that could be useful in the comparison of iPSCs with somatic and ESCs, is MeDIP with array-based comparative genomic hybridization (array CGH) [130]. This method couples MeDIP with arrays that target the whole genome as well as specific loci to produce a broader view of the methylation patterns.

The most common techniques to analyse the epigenome are based on bisulphite conversion. In direct bisulphite sequencing (DBS) [131, 132], DNA treatment with bisulphite converts cytosines (C) to uracils (U), while methylated Cs remain unaffected [133]. DNA is then amplified, sequenced, and compared to controls [134, 135]. A more advanced technique called reduced-representation bisulphite sequencing (RRBS) is based on the same principles as DBS. In this method, DNA is first treated with restriction enzyme Msp1 in order to create segments of DNA (150-175bp and 175-225bp) that will be separated by electrophoresis, treated with bisulphite, amplified then sequenced [125]. Bisulphite padlock probes (BSPP) is a method that is more specific than DBS and RRBS since it allows for targeting of specific gene promoters by using

probes that are constructed prior to bisulphite treatment [118, 136]. Comprehensive high-throughput array-based relative methylation (CHARM) analysis [120, 137], makes use of a methylation-sensitive restriction enzyme McrBC as well as array technology to create methylation libraries for somatic, stem and iPS cells that can be compared. Immuno-based techniques are limited by the efficiency of the antibodies used and have been shown to be less precise than bisulphite conversion methods [137]. Restriction enzyme methods are also limited to the restriction sites in the genome [138]. Furthermore, since CHARM is not biased to the CpG islands like immunoprecipitation and bisulphite conversion methods are, it can give a broader view of the epigenome.

Histone modification analysis in iPSCs is usually performed by chromatin immunoprecipitation (ChIP)[139]. In this procedure, chromatin is conjugated with an antibody that can recognize DNA/protein/RNA complexes that can then be separated by precipitation. These samples can then be analysed by PCR, q-PCR (ChIP-PCR/q-PCR) or coupled to next generation sequencing (NGS) in ChIP-seq [55, 66, 121, 122, 139, 140].

Epigenetic studies can also lead to *in silico* methods for future studies. Bock's thorough transcriptome analysis of iPSCs included an epigenetic analysis by RRBS [51, 125]. Their results showed that iPSCs were more similar to ESCs in DNA methylation patterns than with the 6 fibroblast cell lines tested. As with the DNA profiling patterns, the group was able to create scorecards that could be used to identify appropriately reprogrammed iPSCs [125].

Since epigenetics studies are limited in iPSCs, the ISCBI does not suggest that epigenetic analysis be used as a criteria to classify iPSC lines at this time [35]. Even though iPSCs and ESCs have more distinct epigenetic patterns then transcriptome patterns [51], making it difficult

to assess reprogramming based on epigenetics, these patterns can help identify preferences and limitations for differentiation lineages [125], and may provide insight as to molecules that could enhance reprogramming protocols [119, 141, 142].

2.5 Banking and Extended Use Analysis

When cells are maintained for a prolonged period of time, experiments may need to be repeated to ensure cell line integrity, particularly since studies have shown that the number of passages can affect aneuploidy rates and genomic stability [91]. Typical analyses include pluripotency marker analysis, EB assay, as well as karyotyping or other genetic stability testing (Figure 3)[143].

Furthermore, if the iPSC cell lines are to be banked, testing becomes even more elaborate since scientists need to ensure that cellular identity can be verified before and after banking. Proper testing for contamination of the cell line should also be performed. Human Leukocyte Antigen (HLA) typing and microbial testing are usually performed prior to storage and after banking, along with other characterization experiments such as marker analysis, karyotyping, and DNA profiling.

HLA typing is a test performed prior to organ transplants in order determine the histocompatibility of a donor with the recipient and is therefore done on iPSCs to ensure their proper identification prior to cell therapy usage [144-146]. HLA typing can be done in order to identify a particular cell line [39, 147] or to identify its histocompatibility with another cell line [148]. A series of low and high resolution HLA typing tests are commercially available [149-152]. DNA profiling, used in the identification of individuals, or in paternity/maternity identification [153], could also be used to identify iSPC lines that have been stored. This involves

amplification of short tandem repeats (STR) of the iPSCs DNA using STR primers. Once amplified, the STRs can be separated by gel electrophoresis and banding patterns can be saved for future reference when verifying the cell's identity after being removed from storage. This test is not common practice, but authors suggest that it could become a quick and efficient method of identification since reagents are already commercially available and used in forensic casework for identification purposes [154, 155] and have been used for identification of ESC lines [148]. Josephson's group also suggested that mitochondrial DNA sequencing could be used to identify cell lines as it is already used in identification of individuals [148, 156], however this technique has a lower statistical power than STR analysis of nuclear DNA, and has a limited potential for excluding identity [156].

Microbial testing is also an elaborate process that should be undertaken prior to banking, especially if there is anticipation of usage for cell therapies since cross-contamination can lead to potential dangers for patients [157, 158], and inaccurate results [159]. Test not only include those for fungi, or mycoplasma, for which a series of different methods exist [34, 160-163], but some banks may require tests for hepatitis C, human immunodeficiency virus (HIV), human papillomavirus (HPV), herpes simplex virus, and human herpes viruses [36]. Prior to utilization of previously banked iPSCs, retesting of iPSCs should be performed to ensure 1) identification of the cells (via marker analysis, DNA profiling), 2) integrity of the cells (via karyotyping), and 3) safety of the cells (via microbial testing)[36]. Cell lines that have been altered or contaminated should not be used in further studies or clinical studies.

2.6 Reprogramming efficiency

Once treated cells have been characterized and identified as fully reprogrammed, they are termed iPSCs. Efficiency of reprogramming is represented by the number of iPSC colonies that appear on a growth plate per number of cells that were seeded ($1 \times 10^4 - 5 \times 10^8$)[32, 164]. In some cases, an efficiency percentage is provided. Often times, efficiency is a relative term comparing new results to the original induction experiments performed in mice [32] or in humans [37]. Some groups simply indicate if modifications to their protocols have enhanced reprogramming compared to their own previous protocols. What is certain of the reprogramming process is that as indicated by the low percentage of colonies formed, it is not an efficient process. To date, percentages have failed to exceed 11% efficiency, however once established, iPSC lines have the approximate doubling time as ESCs [37, 56].

Another aspect of efficiency is the time needed in order to produce iPSCs. This is not always measured in the same manner and is sometimes difficult to compare from one study to the next. For example, some authors will give a length of time needed before seeing morphological changes on the plates, or when cells were reseeded for expansion [38, 165]. This time period is usually within two weeks [37, 48], however some techniques have taken up to 40 days to show ESC-like colonies on a plate [165]. Some studies do not indicate time at all [166], and unfortunately, total time to characterize and obtain completely reprogrammed usable cell lines is not noted in reprogramming studies. All this considered, comparing efficiency of reprogramming in terms of time is a difficult task.

For the simplicity of this paper, when sufficient data is provided, efficiency of reprogramming will be shared by two means; the number of colonies (completely reprogrammed) per number of

cells plated and by percentage. Although time to reprogram is an important factor to consider when working in the lab, since not all studies include all necessary times data to formulate valid conclusions, only time differences within the same study may be noted.

Thus far, techniques for characterizing iPSCs during, after reprogramming, prior to and after banking have been detailed. The next section of this paper will review: the variety of somatic cell types used in induction experiments, the different factors and small molecules used to reprogram or enhance reprogramming, as well as the different methods used to deliver factors that induce somatic cell reprogramming. Efficiency of each particular method will be provided when data is available.

Chapter 3 – Reprogramming Methods

Since Takahashi and Yamanaka's first successful induction experiments, the procedure has been repeated in different cell types and animal species, using different factors and methods. This chapter will summarize the variety of cells and factors used and give a detailed description of the different methods of reprogramming developed along with their advantages, limitations and efficiencies.

3.1 Cell Types and Species

iPSC technology has given much liberty to the stem cell field since scientists are no longer restricted to isolating adult stem cells with limited differentiation potential. They can now choose from a larger pool of available differentiated cells from the body to produce iPSCs then redifferentiate them into the cell lines of interest. The biggest challenge to choosing an adult cell for reprogramming is selecting an easily accessible cell that demonstrates a high reprogramming efficiency.

Although fibroblasts (tail tip fibroblast (TFFs), foreskin fibroblast, dermal fibroblast, human fetal lung fibroblasts) are the most commonly used cells for reprogramming [32, 37, 38, 42, 55, 143], many other cell types have been used to produce iPSCs. These include but are not limited to: mouse and human hepatocytes [42, 167-169], adult mouse stomach cells [167], mouse pancreatic beta cells [170], neonatal human epidermal keratinocytes (NHEKs)[38], adipocyte stem cells (ASCs) [171-173], neural progenitor cells (NPCs)[174-176], astrocytes [177], cancer cell lines [178], as well as other commercially available cell lines.

Although each cell type was reprogrammed successfully, there are limitations to the use of certain cells lines mentioned above. Firstly, using commercially available cell lines removes the risk of an invasive procedure, and can give insight on reprograming ability, however the experimental information gathered from them may not be clinically relevant if they are not species and/or patient specific. Ideally, isolating cells from each patient for testing would ensure the highest probability of transferability to the patient. Commercially available cancer cell lines could be used in research for disease modeling purposes only, since they have been shown to retain pathophysiological features from original cells after reprogramming and differentiation [178]. Furthermore, isolation, from a patient, of some of the cell lines mentioned above, would be problematic. Fetal tissue would be impossible to harvest from an adult. Furthermore, harvesting of neural progenitor cells and astrocytes would require the use of invasive biopsy techniques that could cause irreparable damage to the patient. Hepatocytes, pancreatic β cells, and stomach cells could be harvested from a donor, but the procedure involves an invasive biopsy that requires medical supervision, anesthesia and may require a significant amount of recovery time [47, 179].

Dermal fibroblasts remain the most common source of cells used for induction of human iPSCs (hiPSCs) and mouse iPSCs (miPSCs) [32, 37, 38, 48, 166, 180, 181]. Skin punch biopsies are considered invasive procedures that require local anesthetic, a medical follow up and also bring on the risk of bleeding and infection [182], however the patient can heal more quickly from this procedure than a biopsy needed for isolation of cells from visceral organs (ex. hepatocytes and stomach cells) [47, 179]. Keratinocytes from skin biopsies have also been shown to be efficiently reprogrammed. Interestingly, keratinocytes and fibroblasts can be isolated from the same sample simultaneously [143]. This practice could make better use of the cells provided in one sample and decrease the need for multiple biopsies.

Cells for reprogramming can also be obtained from procedures that would normally discard useful tissues. For example, HSCs (sometimes identified as $CD34^+$ or $CD133^+$ cells) from umbilical cord blood [174, 183-188] or amniotic tissue [189-191] that are normally discarded after birth have been used to produce iPSCs. The limitation is that they must be harvested at the time of birth for future use in order to match the patient. Banking of these cells could also allow their use for a recipient for which histocompatibility has been established. Having banked cells that have already been characterized would facilitate the process of iPSC production and utilization for cell therapies in histocompatible persons. Granulosa cells (normally discarded) retrieved from the egg follicles of women undergoing infertility treatment [192] are another example of cells that have been used to produce iPSCs. Although the fertility treatments are invasive, harvesting of the cells does not add to the procedure, and the possibility of banking granulosa cells could provide a potential access point for histocompatible recipients with no other options. iPSCs have also been produced using the human dental pulp progenitor/stem cells [193] isolated from the dental pulp of the third molar, also known as a wisdom tooth [194, 195]. In most cases, removal of wisdom teeth is a relatively standard procedure, for which isolation of the stem cells post-removal would not add to the recovery time. Lastly, human foreskin fibroblasts or keratinocytes used for iPSC production [143] could be harvested from the foreskin normally discarded after the surgical or non-surgical circumcision shortly after birth or in adulthood.

The best option for cell harvesting is obtaining cells in a non-invasive fashion. Obtaining a peripheral blood sample is a relatively simple procedure that usually takes less than a minute, and requires no medical follow up. iPSCs have been produced from a mixture of the mononuclear [196-198], B-lymphocytes alone [199], and T-cells alone [187, 200] isolatable from a simple blood sample. Adult keratinocytes from plucked hair have also been reprogrammed [143, 201].

Since humans can shed up to 200 hairs a day, this source of cells is abundant. Most interesting, urine-derived progenitor cells (UDC) from a simple 100mL urine sample [202] have been used to produce iPSC lines [203, 204]. Since humans can produce up to 2L of urine a day which can contain from 2000- 7000 cells [205], this is also a good source for obtaining reprogrammable cells. These are three excellent examples of non-invasive methods of obtaining reprogrammable cells from a patient. Since they are not invasive, they could also be easily donated from the general public in order to conduct broad-scale studies on iPSC reprogramming or disease modelling.

Unfortunately, accessibility does not necessarily correlate with high reprogramming efficiency. Table 1 lists some of the reprogramming efficiencies by cell type. Efficiency comparisons are difficult to make since the methods used varied from one cell type to another. From the studies listed, we can see that HDF are the cells with the highest efficiency of reprogramming, reaching a very high 11% [206]. It would be interesting to see the efficiency of this same method in reprogramming more easily obtainable UDC, mononuclear cells or keratinocytes from plucked hair. Some reviews claim that terminally differentiated cells are more resistant to reprogramming than adult progenitors; however it is difficult to come to this conclusion with the diversity of results from different studies. More thorough, wide-scale investigations of a variety of cells using identical culture conditions and methodology would be necessary in order to determine the most easily reprogrammed cell type. For example, Giorgettii *et al* in 2009, showed that their method could reprogram $CD133^{+}$ cells, while it was inefficient for the more commonly used fibroblasts and keratinocytes [183]. Zhu's and Panopoulos' studies both showed that their methods were more efficient in reprogramming human umbilical vein endothelial cells (HUVECs) than adult keratinocytes and amniotic fluid derived cells [207, 208](Table 3.1). Efficiency comparisons

Cell type	Species	Efficiency	Accessibility	Reference
Adult stem/progenitors				
Adipocyte stem cells (ASCs)	Human	0.0002-0.02% (1/5x10^3 cells-1/5x10^5 cells)	Very invasive	Aoki 2010 [172]
Neural progenitor cells (NPCs)	Mouse Mouse Mouse Mouse Mouse	0.014% 0.11% - 0.36% (counting only GFP+ cells/ cells calculated to be efficiently transfected and not clones/cells seeded) 0.004-0.006% 0.034% 0.0125% 0.001- 0.002%	Patient derived Impossible Nasal swab slightly invasive	Kim J.B. 2009 [209] Kim J.B. 2008 [175] Kim 2008 [175] Shi 2008 [210] Silva 2008 [211] Eminli 2008 [174]
Dental pulp progenitor cells	Human	0.03 – 0.085% (no colony numbers noted, article states a few colonies, and # of cells seeded. To obtain the lesser % noted, there would have to be 900 colonies formed / 3 x 10^4 cells)	Does not add to invasiveness of procedure	Takeda-Kawaguchi 2014 [193]
Urine-derived progenitor (UDP) cells	Human	0.1 – 4% (# of colonies seen not given) (# of colonies picked (2-11) and # of cells seeded (5 x 10^4) gives 0.008-0.02%)	Non-invasive	Zhou 2011 [203]
Mesenchymal stem cells (MSC) from bone marrow	Human	0.07% (7 colonies/ 1 x 10^4 cells seeded)	Very invasive	Streckfuss-Bömeke 2013 [201]
$CD34^+$ (HSC) cells from umbilical cord blood $CD133^+/CD34^+$ (HSC) cells	Human	0.9% (90 colonies/1 x 10^4 cells) 0.09% (49 colonies/5.5 x 10^4 cells) 0.45% (#colonies unknown/10^4 cells)	Non-invasive	Meng 2013 [186] Okita 2013 [187] Giorgetti 2009 [183]
Adult Somatic Cells				
Fibroblasts				
Human fetal lung fibroblasts	Human	0.01% (10/1 x 10^5 cells)	Impossible	Warren 2010 [38]
Dermal fibroblasts	Human Human Human Human Human	0.01% (10/1 x 10^5 cells) 0.02% (10/ 5x10^4 cells) 0.14% (14/1 x 10^4 cells) 11% (11 579/1 x 10^5 cells) 0.14% (#colonies unknown/10^4 cells)	invasive	Warren 2010 [38] Takahashi 2007 [37] Strechfuss-Bomeke 2013 [204] Anokye-Danso 2011 [206] Giorgetti 2009 [186]
Foreskin fibroblasts	Human Human	0.01% (10/1 x 10^5 cells)	Moderately invasive	Warren 2010 [38] Aasen 2010 [143]
Human fetal skin fibroblasts	Human	0.01% (10/1 x 10^5 cells)	Invasive	Warren 2010 [38]
Tail tip fibroblast (TTF)	Mouse Mouse	0.0016% (13/8 x 10^5 cells) 0.04% (# of colonies unknown)	Invasive	Takahashi 2006 [32] Eminli 2008 [177]
Mouse embryonic fibroblasts	Mouse	0.02% (160/8 x 10^5 cells) 0.14 – 0.35% (# of colonies unknown) 0.1 – 0.3% (# of colonies unknown) 0.11% (# of colonies unknown) 9.14% (1600 colonies/1.75 x10^4 cells)	Invasive	Takahashi 2006 [32] Li 2012 [212] Judson 2009 [213] Moon 2011 [214] Anokye-Danso 2011 [209]
Keratinocytes				
Keratinocytes from plucked hair	Human Human	A few colonies from 1 plucked hair (no other numbers provided) 0.05% (5 colonies/1 x 10^4 cells seeded) No numbers provided (increase 3.5 fold by adding small molecules) mention a few colonies that were expanded	Non-invasive	Aasen 2010 [146] Strechfuss-Bomeke 2013 [204] Petit 2012 [215]

Keratinocytes from human foreskin	Human Human	0.08% (8 colonies/ 1 x 10^4 cells seeded) 100x more efficient then fibroblast a few hundred colonies (300 as opposed to 4 with fibroblasts) but no other cell # provided)	Mildly invasive	Ruiz 2010 [177] Aasen 2010 [146]
Adult human epidermal keratinocytes (AHEKs)	Human	0.45% (#colonies unknown/10^4 cells) 0.0003% (3/1x10^6 cells) 1x efficiency of HUVEC cells from same study	Invasive	Giorgetti 2009 [186] Zhu 2010 [207] Panopoulus 2011 [211]
Other Somatic Cells				
Stomach cells	Mouse	No colony or cell numbers provided 0.01%	Invasive	Aoi 2008 [167]
Astrocytes	Human	0.07 and 0.1% (7 & 10 colonies/1x10^4 cells)	Very invasive	Ruiz 2010 [180]
Granulosa cells	Human	0.1% (100 colonies/ 1x10^5 cell)	Does not add to invasiveness of procedure	Chuang 2015 [192]
Mononuclear cells T-lymphocytes B-lymphocytes	Human Human Human	Efficiency numbers unavailable 0.004 – 0.1% (42/1 x 10^6 – 101/1 x10^5 cells) 0.002 – 0.01% (number of colonies and cells not provided)	Mildly invasive	Churko 2013 [198] Hubbard 2014 [196] Okita 2013 [187] Choi 2011 [199]
Hepatocytes	Mouse Mouse	0.0001 – 0.0029% (1-29/ 1 x 10^6 cells) 0.01% (# of colonies unknown)	Very invasive	Okita 2008 [42] Aoi 2008 [170]
Pancreatic beta cells	Mouse	0.16% (4/2500 cells) (Should be 4/4 x 10^4) they considered how many cells they believed were actually transfected from the 40 000 cells – 2500	Very invasive	Standfeld 2008 [170]
Cancer cell lines	Human Human	Numbers not available 2-5% (# of colonies unknown)	Commercially available non-invasive Patient-derived very invasive	Moore 2015 [178] Lin 2008 [216]
Adipose stromal cell	Mouse Human	0.0001% (# of colonies unknown) 0.0002% (# of colonies unknown)	Invasive	Miyoshi 2011[217]
Neonatal epidermal keratinocytes	Mouse	0.01% (10/1 x 10^5 cells)	Invasive	Warren 2010 [38]
Neonatal human epidermal keratinocytes (NHEKs)	Human	0.0004% (4/4x10^6 cells) – 0.104% (104/1x10^5 cells)	Invasive	Zhu 2010 [210]
Human umbilical vein endothelial cells (HUVECs)	Human	0.0035% (7/2x10^5 cells) 0.012% (23/2x10^5 cells) 2.5 – 3% higher than keratinocytes from the same study	Non-invasive	Zhu 2010 [210] Panopoulos 2011 [208]
Amniotic fluid derived cells (AFDCs)	Human	0.0011% (11/1x10^6 cells)	Non-invasive	Zhu 2010 [207]

Table 1 - Reprogramming Efficiencies of Different Cell Types

would also need to be species-specific since it has already been shown that handling as well as reprogramming techniques in different species must be adapted [37]. Lastly, efficiency is not the only factor to consider when choosing a cell type. If the cells are going to be used for cell therapy, patient-derived cells are more clinically-relevant, and the least invasive method should be used to obtain them in order to avoid trauma to the patient, even if the reprogramming efficiency may not be quite as elevated.

Since most studies to date have been completed in mice and humans [218], most of the methods discussed will have been conducted using mouse or human cells. It is worth mentioning that some iPSC studies have been conducted in pig [219, 220], rabbit [221], rat [222] marmoset [223, 224] as well as rhesus monkeys [225-227], Pigtailed macaque [228] cynomolgus monkey [229, 230], and chimpanzees [66, 231].

Next we move on to evaluate the different methods that have been used in order to reprogram somatic cells. This will include looking at different transcription factors, methods, small molecules, microRNAs, and bacteria used to create iPSCs.

3.2 Reprogramming Factors

In order to attempt the reprogramming of somatic cells to an embryonic state, it is imperative to understand the properties of ESCs as well as the factors that regulate their pluripotency and self-renewal. The elucidation of these factors over the years was key in the first induction experiments and will briefly be discussed. mESCs were isolated and cultured for the first time in 1981 by Kaufman's and Martin's groups [232, 233], while hESCs were only isolated in 1998 by Thompson's group [3]. Although many differences exist between the two cell types, both share

the same core pluripotency factors, notably, Oct4, Nanog, and Sox2 [92, 234-236] (Figure 11). Oct4 encodes for a transcription factor (TF) that belongs the POU homeodomain DNA binding family [237] that must be tightly regulated to ensure pluripotency and self-renewal, as an increase or decrease by half will induce differentiation into either trophoectoderm or primitive endo/mesoderm respectively [238, 239]. Sox2 TFs have the high mobility group (HMG) DNA-binding domain, known for its importance in the later stages of embryogenesis and in neural progenitor self-renewal [33, 240]. Oct 4 and Sox2 have shown to bind together to regulate transcription of other pluripotency genes such as Fbx15, fibroblast growth factor 4 (Fgf4) and undifferentiated embryonic cell transcription factor 1 (Utf1) [241-244]. Nanog is also part of the homeobox domain TFs essential to pluripotency as Nanog-deficient ESCs differentiate [245]. These three TFs have been shown to bind one another to regulate their own promoters, forming an auto-regulatory loop [246, 247](Figure 11). They also co-operate to promote the expression of other genes responsible for pluripotency, self-renewal and proliferation such as: krüpple-like factor 4 (Klf4), cellular myelocytomatosis (cMyc), signal transducer and activator of transcription 3 (Stat3), Klf2, sal-like protein 4 (Sall4), Rex1, dosage-sensitive sex reversal, adrenal hypoplasia critical region, chromosome X, gene1 (Dax1), transcription factor 4 (TCF4), Sox18, zinc-finger protein 281 (Zfp281), and repress differentiation genes [236, 246-253] (Figure 11).

Although mESCs and hESCs have been studied extensively since their isolation, and we now know much about the regulation of their pluripotency and self-renewal, much of this information was not available prior to the first induction experiments. As experiments were performed to reprogram cells, more knowledge was gained pertaining to the efficiency, safety, and necessity of different factors.

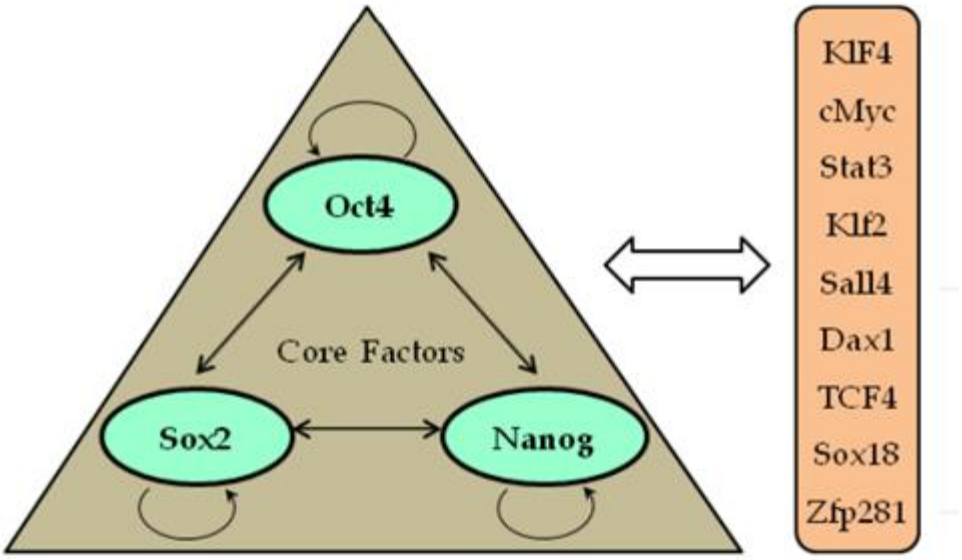


Figure 11 - Schematic Representation of the Transcriptional Regulation of Core Pluripotency Factors and the Extended Factors

(Reprinted from Bieberich and Wang 2013 with permission) [254]

In the initial experiment, Takahashi and Yamanaka selected 24 genes to test based on previous experiments showing their involvement in pluripotency, self-renewal, and/or cell survival [32]. They developed an assay that would allow them to determine which factors were primordial in creating iPSCs. This system consisted of inserting a beta geo (βgeo) cassette into the promoter of the proliferation-associated gene Fbx15. This cassette included the β-galactosidase as well as the neomycin resistance gene. Insertion of this reporter construct into the cellular DNA, allowed scientists to identify cells with an active Fbx15 promoter (reprogramming cells) by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining of gentamycin resistant cells [32]. With their system, individual withdrawal testing identified 10 factors necessary for reprogramming. Subsequent single factor withdrawal experiments led to the identification of four primordial inducing factors. These have now been termed the four Yamanaka factors and consist of; OCT4, Sox2, cMyc, and Klf4, two core pluripotency factors, and two secondary factors known to regulate proliferation. These factors produced iPSCs with an efficiency of 0.0016-0.02% (13 colonies/$8x10^5$ cells -160 colonies/$8x10^5$ cells) using mouse embryonic fibroblasts and tail tip fibroblasts [32] and 0.02% (10 colonies/$5x10^4$ cells) using human dermal fibroblasts [37]. These four factors have acquired the abbreviation (OKSM) and their use in different studies has been cited multiple times in many reviews [179, 218, 255, 256]. Many other factors have been used in reprogramming. At approximately the same time as Yamanaka's group, Thompson's group successfully reprogrammed human fibroblasts using their four factors; OCT4, Sox2, NANOG and lineage protein 28 (LIN28), selected from their initial 14, with 0.022% efficiency (198 colonies/$9x10^5$ cells) [50]. These include three core proliferation factors and LIN28, which encodes a microRNA-binding protein that inhibits processing of microRNA (miRNA) in ESCs [257]. Shortly after, an extensive study was done, showing that Sox2, Klf4, cMyc could be replaced using family members, Sox1, Klf2 or Klf5, lung-Myc (LMyc) or neuroblastoma-Myc

(NMyc) respectively [47] to produce iPSCs with different efficiencies. The use of cMyc and Klf4, however have revealed to be problematic. Following germ-line competency testing of iPSCs, it was discovered that the cMyc transgene reactivation, a known oncogene, was responsible for the production of tumors in mouse pups [48]. Furthermore Klf-4 is also an oncogene associated with the pluripotency, and migration of cancer cells [258]. Member of the Klf and cMyc family used in Nakagawa's study are also oncogenic and carry the same potential dangers [259, 260]. The use of any of these oncogenes is a safety concern for cells that would potentially be used in clinical applications. For this reason, production of iPSCs by transfection of OS, LIN28 and NANOG are more suitable for cells that may be used in clinical applications. Efforts to identify non-oncogenic factors, led Nakagawa's group to modify their initial protocol and produce iPSCs with only OKS [47]. Giorgetti *et al* were also successful in inducing iPSCs utilising only SOX2 and Oct4 factors, although at much lower efficiencies (0.00625%: 5 colonies/$8x10^4$ cells)[183].

The most frequently used factors remain OSKM, LIN28 and NANOG [261], however other factors have been identified to either enhance reprogramming with these most common factors or to replace one or several of them. For example, Sall4 is part of a TF family known for their cysteine 2 histidine 2 (C2H2) zinc-finger motifs suggested to contribute to the maintenance of pluripotency in ESCs by regulation of Oct4 gene expression [262, 263]. Sall4 co-expression with OSK, showed a 2 fold increase of production of iPSCs (0.15% - 300 colonies/$2x10^5$cells) from fibroblasts as compared to OSK alone (0.08% -160 colonies/$2x10^5$cells)[264]. Glioma-associated oncogene similar transcription factor 1 (Glis1) was used to enhance OKSM (0.074%- 37 colonies/$5x10^4$ cells) as well as replace cMyc (0.07% - 35 colonies/$5x10^4$ cells) in reprogramming [265]. Nuclear receptor 5a2 (Nr5a2) was used with SKM (0.024%- 24

colonies/1x10^5 cells) or SK (0.003% - 3 colonies/1x10^5 cells) to produce iPSCs [266], while Esrrβ was used to replace Klf4 and cMyc in OS reprogramming (≈ 80 colonies)[267]. E-cadherin (E-cadh) was also used to replace Oct4 in KSM reprogramming [268]. Authors note the decreased efficiency in reprogramming; however they did not provide colony or cell numbers. Sall4, Esrrβ, and LIN28 were used in combination with developmental pluripotency associated factor 2 (Dppa2) or Nanog and produced iPSCs [269]. More recently, Bugamin also showed that Sall4, NANOG, Esrrβ and LIN28 (SNEL) could be used to reprogram somatic cells albeit less efficiently (0.001-0.005% - 1-5 colonies/1x10^5 cells) than the OKSM factors [76]. The group went on to show that cMyc and Klf4 caused genomic instability, since they are responsible for an increase in gamma histone 2AX (γ-H2A.X) phosphorylation, a marker for double stranded DNA breaks [270].

It is clear that reprogramming efficiency varies with the use of different factors; however it is unclear that this is the only variable implicated since the methods and cell types used in the studies mentioned were not consistent and may also affect results. Studies showing negative effects of TFs like cMyc and Klf4 bring up safety concerns associated with choosing factors for reprogramming. Although OKSM factors are commercially available for reprogramming with many different methods (which will be discussed shortly), possible negative effects, as well as future use of the cell lines need to be considered before choosing reprogramming factors. The safety of the factors needs to be considered especially if cells will be later used in a clinical setting. Furthermore, since using different cell lines or exposing cells to different factors could potentially generate different results, using identical cells and factors in both the laboratory cell lines and clinical cell lines would increase the transferability of lab results to a clinical setting.

3.3 Delivery Methods

While some groups work on identifying easily obtainable and reprogrammable cells and/or safe and efficient TFs, much work has been done to introduce different methods by which to deliver these factors to different cells. Since the initial induction experiments [32], a variety of methods have been developed and used to reprogram somatic cells to an embryonic state. This section will describe the principles behind the techniques currently in use, as well as their advantages and disadvantages. These include a variety of viral, non-viral DNA, and DNA-free delivery methods, as well as small molecules, and miRNAs identified for enhancement or *de novo* reprogramming. Lastly, a bacterial reprogramming method recently discovered will be presented.

3.3.1 Viral Delivery Methods

3.3.1.1 Integrative Viral Methods

The first induction experiments were completed by retroviral transduction (Figure 12) of OKSM factors by Yamanaka's groups [32, 37, 48]. Their pMXs-gw vectors are based on the Maloney murine leukemia virus (MMLV). At approximately the same time, Yu's group did their first induction experiments using lentivirus' [50](Figure 12), which are commonly derived from the human-immunodeficiency virus (HIV)[255]. Both of these viral systems infect in approximately the same manner, however retrovirus are only taken up by replicating cells while lentivirus can be incorporated into dividing and non-dividing cells [255, 271, 272]. They are recognized by the host cell via cell-surface receptors, and then internalized. Reverse transcription into cDNA of the viral RNA is achieved via their own retrotranscriptase, after which the cDNA is shuttled into the nucleus (Figure 12). Prior to integration into the host genome, the viral cDNA

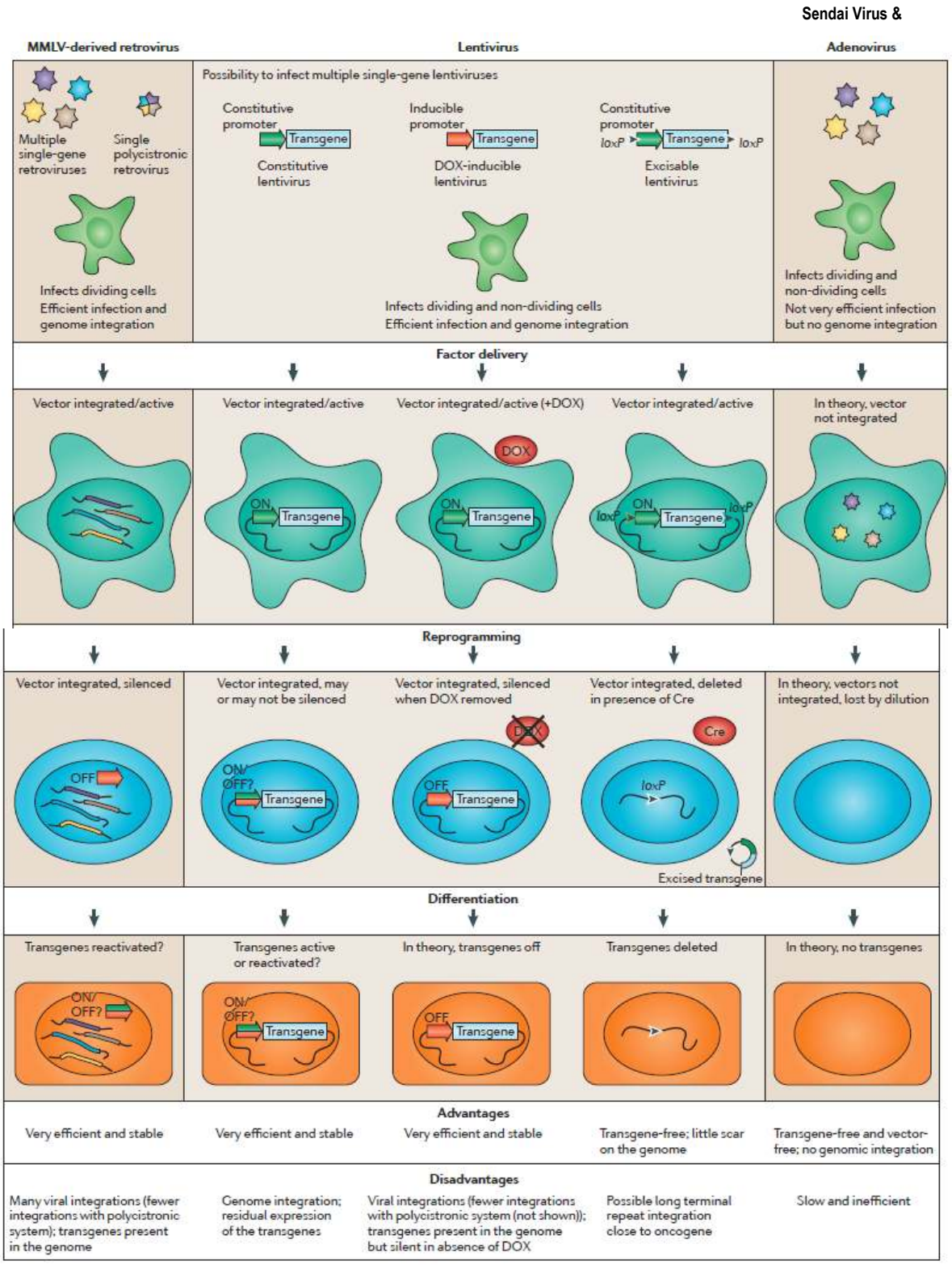


Figure 12 - Viral Methods in Reprogramming
(Modified from Gonzalez *et al*. 2011 with permission)[255]

undergoes a deletion of its 3' untranslated region (3'UTR) region, the 3' long terminal repeat (LTR) and internal promoter rendering it self-inactivated and incapable of self-expression [271]. Since these viruses lack their replication machinery and 3' promoter, they will not replicate and produce new viable viruses, but their cDNA can be expressed by the host machinery using the viral promoter in the 5'UTR region of the virus [273, 274]. They may be transcribed multiple times before being silenced through a variety of host epigenetic silencing mechanisms [275]. Although these methods are effective in producing iPSCs (0.02% retroviral system [37] and 0.022% lentiviral system [50]), viral transgene integration is permanent [276], causing changes in copy number variation and genomic instability. It was established that each iPSC clone had 3-6 retroviral integrations for each factor transfected [37]. Although epigenetically silenced, their permanency opens the possibility of reactivation of the transgenes, which is especially problematic in the case of oncogenes cMyc and Klf4 that could induce tumorigenesis [48]. Even in the absence of oncogenes, like in the case of induction with OS, LIN28 and NANOG, integration can still disrupt expression (downregulate or upregulate) of nearby genes [255].

3.3.1.2 Inducible Viral Methods

To address transgene reactivation, a Doxycycline (DOX)-inducible lentivirus was produced by replacing the ubiquitin C promoter of the plasmid with a tetracycline operator (tetracycline response element (Tet)) also known as a doxycycline (DOX)-inducible promoter and minimal cytomegalovirus (CMV) promoter [170, 277-280] (Figure 12). Only cell cultures treated with DOX are successfully transformed into iPSCs. After removal of doxycycline, transgenes are no longer expressed. Furthermore, studies showed that decreased expression of these transgenes allowed for a better differentiation potential after reprogramming [277].

Although this technique addresses reactivation concerns, it does not address genomic instability from viral transgene integration.

3.3.1.3 Excisable Viral Methods

In order to address the genomic instability caused by these first two methods, excisable viral methods were developed. Cre-recombinase excisable lentivirus have been developed [234] by inserting LoxP sequence into the 3' deleted untranslated (dU3) long terminal repeat (LTR) region of the lentivirus. During reverse transcription of the viral RNA, the 3' loxP sequence is replicated and inserted into the 5' LTR, rendering the inserted transgenes "FLOXED" with loxP sites on either side. Two groups showed that generation of iPSCs was successful after transfection with the Cre-loxP lentiviruses [234, 281]. iPSCs are then treated with Cre-recombinase, either by transfection of an adenoviral vector expressing Cre-recombinase [281], a Cre-encoding plasmid [278], Cre-mRNA [282] or even Cre-protein [234]. Cre-recombinase is an enzyme that recognizes and binds to a 34 base pair sequence in the FloxP repeat, then excises the floxed gene [283]. Successful removal of transgenes is confirmed by PCR amplification [234, 281] and/or Southern blotting of particular transgene sequence within the iPSCs DNA [281]. This system has been used to produce iPSCs with higher efficiency (0.5% - colony numbers not given) [280] than the retroviral system (0.02%)[37]. Other than avoiding transgene reactivation, excision of the transgene increases differentiation potential of the iPSC colonies [281]. A DOX-inducible Cre-excisable lentivirus system expressing OKSM (also known as STEMCCA-LoxP vector) was also developed [234, 278, 280, 281], and showed to produce hiPSCs that have gene expression profiles more similar to those of hESCs than other hiPSCs produced by retroviral transfection with the same four factors, suggesting a more complete reprogramming [278]. Although the

removal of the transgene is an improvement on the previous viral delivery methods, it can still create genomic instability since the loxP sites remain integrated into the host genome [283].

3.3.1.4 Non-Integrative Viral Methods

There are two non-integrative viral delivery methods available, which are based on the adenovirus [42, 284-288], and sendai virus (SeV)[164, 185, 289]. The Adenovirus is a DNA virus while the SeV is an RNA virus. Both will be incorporated into dividing and non-dividing cells, and produce proteins from their respective nucleic acids independently of the host genome while remaining in the cytoplasm. Although adenoviral methods were successful, and without tumor formation after transplantation, efficiency is very low (0.0001- 0.001%)(0.0002% - 2 colonies/1x10^6cells)(0.0011 - 0.006% - colony numbers not provided)[284, 285, 287]. The Sendai viral system was effective in producing iPSCs in a variety of cells (0.001-1%),(0.002-0.22% - 2-22 colonies/1x10^4cells)[164, 185]. Non-integration of the viral vectors was confirmed by PCR [42, 284, 285] and Southing blotting [185, 284, 285] and absence of viral gene expression was confirmed in iPSC colonies by RT-PCR [185]. iPSC production with these two systems may be lengthy since cells are sometimes put through a heat treatment to minimize SeV expression after induction [185]. Furthermore it may take several passages for cells to be completely virus free [185, 285]. However, the adenoviral and SeV methods have shown to be the safest viral methods since they address genomic integration and reactivation of oncogenic transgenes.

3.3.2 Viral Free DNA Delivery Methods

Non-viral DNA delivery methods of have also been used to reprogram somatic cells. This method consists in transfecting linear or circular DNA vectors into the host cell without any other viral or bacterial packaging. These include linear viral vector, bacterial episomal non-replicative or replicative vectors [42, 166, 290], as well as DNA minicircles [291] [292](Figure 13). Theoretically, these vectors do not integrate into the host genome, and are expressed because they contain constitutively active promoters. They are degraded and lost by dilution rather quickly, and may need multiple transfections to induce reprogramming as opposed to the higher transfection rate of viral methods. In order to ensure that exogenous factors are expressed, some groups use integrative-excisable plasmid vectors (PB transposon and LoxP-Cre)[281, 293] (Figure 13). We will first discuss non-integrative, then integrative-excisable vector methods.

3.3.2.1 Non-Integrative plasmid vector DNA Methods

Yamanaka's group created two plasmid vectors from adenoviral sources to produce iPSCs; both harbouring a constitutively active CAG promoter [180]. The CAG promoter consists of the cytomegalovirus enhancer fused to the chicken beta actin promoter. Their first vector was polycystronic, containing three OKS factors separated by a 2A self-cleaving peptide. The second contained only the single cMyc factor. Since they are non-replicative, these vectors had to be repeatedly transfected in order to induce reprogramming (2 to 4 times each). Although the PCR and Southern blot experiments in 2008 were negative for transgene integration, PCR experiments in 2010 confirmed that12 of 15 clones showed positive banding for genomic integration using this method [42, 180]. It therefore maintains the genomic instability and transgene reactivation concerns of the previous integrative viral systems.

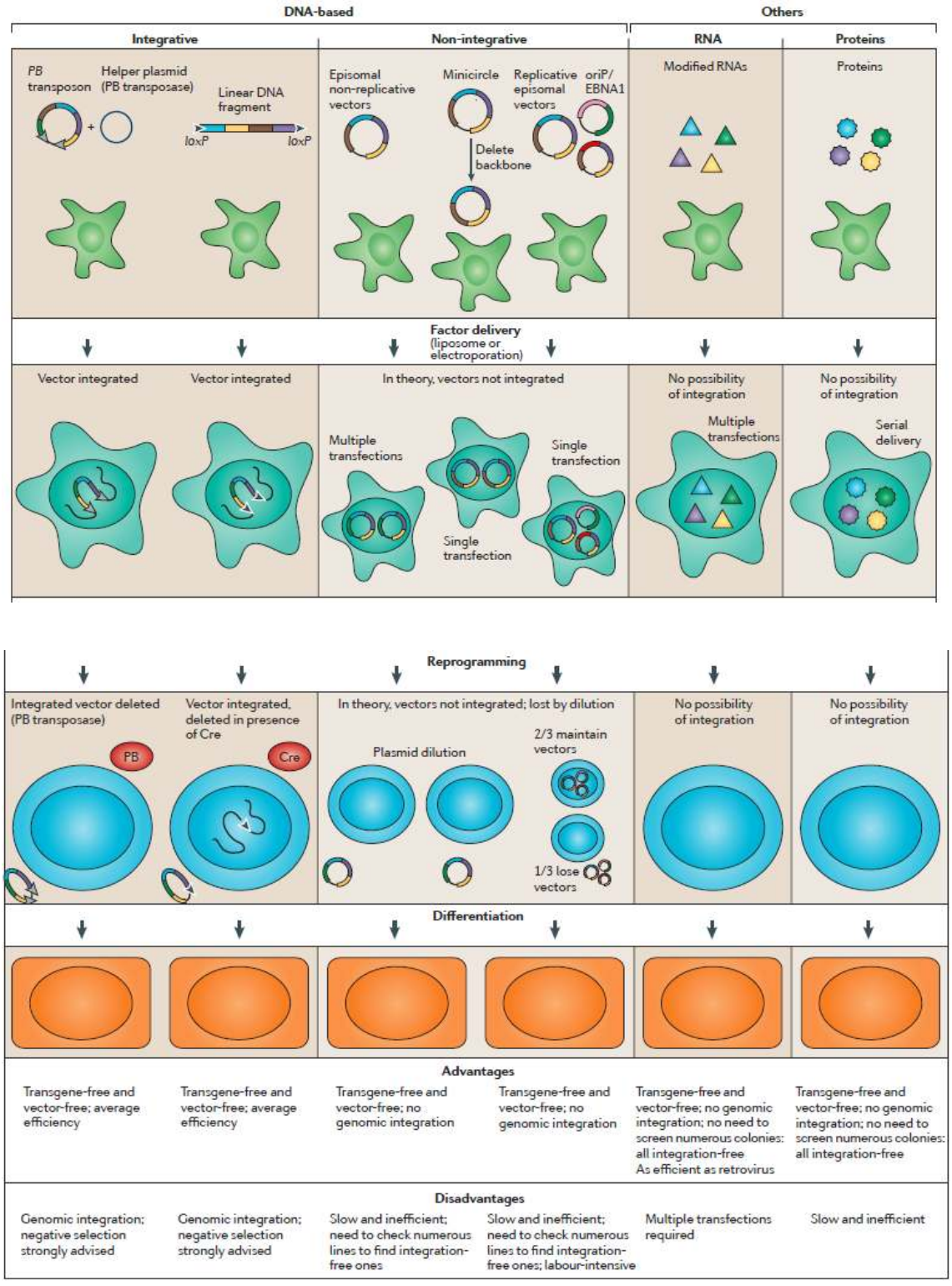


Figure 13 - Viral Free Methods in Reprogramming
(Reprinted from Gonzalez *et al*. 2011 with permission)[255]

3.3.2.2 Non-Integrative Replicative and Non-Replicative Episomal Vector DNA Methods

Replicative and/or non-replicative episomal vectors of bacterial origin have also been used to reprogram somatic cells [42]. These plasmids have the ability to replicate and/or express their genes autonomously and therefore should potentially integrate less than other plasmid vectors. Replicative vectors include the origin of replication/Epstein-Barr nuclear antigen 1 (OriP/EBNA-1) replication sites, while non-replicative vectors do not. The advantage of replicative vectors is that they may decrease the number of transfections necessary to induce reprogramming; however they may also increase the time necessary to dilute vectors from iPSCs after reprogramming. These vectors have been used to express OKSM factors and modified to allow expression of other reprogramming factors [50]. Yu's original system used a total of three vectors with the OriP/EBNA-1 sites which introduced six reprogramming factors in total: Oct3/4, Sox2, cMyc, Klf4, LIN28 as well as NANOG [166]. This system was not successful [50] until the group added the proto-oncogenic Simian Vacuolating Virus 40 Tag large T gene (SV40LT) to the vector. Its role in efficiency is not quite known, but it is postulated that the SV40LT increases reprogramming efficiency by inhibiting tumor suppressors such as p53, p107 and retinoblastoma (RB), hence promoting cell survival [290], yet it also counteracts the oncogenic effect of cMyc [166, 294, 295] which allows cell survival during reprogramming. Yu's group also modified the vector to allow for drug selection. This allows for quicker, more effective dilution of the plasmid from the iPSC clones after induction. Measurements of exogenous mRNA levels via RT-PCR experiments after cellular reprogramming confirmed that, in the absence of drug selection, approximately 5% of the episomal vectors are lost each cell cycle due to silencing of the viral promoter driving EBNA-1 expression. Although PCR experiments showed transgene integration,

they also showed that subclones of the original iPSCs had lost this integration, demonstrating that subclones may not harbour the same integration concerns as their parental iPSC lines [166].

In 2012, Meng's group created a replicative episomal vector that did not require the use of SV40 or cMyc. It consisted of the Spleen focus-forming virus (SFFV) promoter that had been shown to be more efficient in HSCs than other promoters [296, 297]. The vector contained the Sox2 and Oct 4 genes, separated by the 2A self-cleaving peptide, the OriP/EBNA1 sites to allow replication, and the SV40 poly adenylate (polyA) tail only. This first attempt was unsuccessful in producing iPSCs. The group modified the vector by adding the woodchuck transcriptional regulatory element (Wpre) after the second gene of interest. This is a technique often used in the lentivirus system. This change allowed the group to produce 20 iPSC colonies from 1 x 10^5 cord blood (CB) $CD34^+$ cells (0.02%). What was even more interesting is that the group was able to induce up to 90 iPSC colonies with two Wpre-vectors containing OS and MK respectively from 1 x 10^4 CB $CD34^+$ cells (0.9%). These efficiencies surpass Takahashi's retroviral OKSM system (0.02%) as well as those previously stated for all other methods when reprogramming cord blood cells [186].

3.3.2.3 Non-Integrative Minicircle Vector DNA Methods

Another viral-free DNA method of reprogramming is that of DNA minicircle vectors developed by Wu and Longaker in 2010 [291]. This system allowed reprogramming of human adipose stem cells (hASCs). Minicircle vectors are essentially the DNA of the reprogramming genes once the plasmid backbone (origin of replication, antibiotic resistance cassette) has been removed. Since it is smaller and contains only the expression cassette of the reprogramming genes, it is more resistant to host silencing than other plasmids, increasing transgene expression and avoiding the

need for daily transfections [298]. Since they are still lost by dilution quite easily, cells are usually transfected on days 1, 4 and 6 [291]. Minicircles have shown to be more efficient for transfection and transgene expression then other full-length DNA plasmids [291, 299]. However minicircles containing the four factors (Oct4, Sox2, LIN28 and NANOG) showed an efficiency of 0.005% [291] which is much lower than that reported by viral-based methods with the same four factors (0.022%) [50]. The benefits of this method are said to outweigh the decreased efficiency since the protocols are simple, using a single non-integrative vector, without the use of oncogenic cMyc, Klf4 or SV40. These non-integrative, non-replicative, non-tumorigenic methods are a good advancement in the field of iPSC production. Furthermore, minicircles are FDA approved therefore could have clinical applications [291].

3.3.2.4 Inducible-Excisable Vector DNA Methods

In order to address the repeated transfections necessary with non-integrative and non-replicative vectors, inducible-excisable vector systems can be used. The Dox-piggyBac transposon system requires the transfection of a minimum of two plasmid vectors. The first vector contains the transgenes of interest and two piggybac (PB) transposons, which are short sequences of DNA containing two inverted terminal repeat (ITR) sequences upstream and downstream of the gene [300-303]. The second vector contains the DOX-inducible promoter with the gene coding for the protein transposase. In a first step, both vectors are transfected simultaneously [293, 304]. Upon treatment with DOX, the transposase gene will be expressed. Transposase cuts the PB transposon at ITR sites, excises the transgene and inserts it into the host genome at specific TTAA sites [300]. A second transfection with the PB transposase and DOX treatment, allows for recognition and excision of the ITR and transgenes previously inserted leaving the host genome relatively

void of the transgene and PB transposon that was inserted [304]. Although this method has shown to be effective at inducing iPSCs (0.22% - 1250 colonies/5x10^5 cells), PB transposase insertion and excision activity cannot be specifically controlled. Therefore a second transfection of PB transposase could lead to the excision and reinsertion of PB transposon elsewhere in the genome [293]. Although this system has shown to allow removal of transgenes from iPSCs, the fact that PB transposase preferentially target palindromic TA-rich sequences in the human genome remains a concern [305]. Since these sections are known for a higher rate of gene expression, integration could have various effects on expression of endogenous genes, reprogramming, or other unknown effects on the cells. Furthermore this technique is labour intensive and requires many steps not needed in other methods that have been and will be described[306].

As previously discussed with the lentiviral system, the loxP-Cre excisable system can be applied to the plasmid vector method or linear DNA fragment method (Figure 13). As a brief review, this method consists in inserting loxP segments on either side of the transgene that will allow for excision of the transgene upon exposure to Cre-recombinase. Once excised, the transgene segments are lost by dilution [255] (and should not pose a threat to the remaining cells), however the remaining loxP sites could cause some genomic scaring and instability as opposed to the more effective removal by the PB transposon method.

3.3.3 DNA Free Delivery Methods

Although the methods mentioned above have been successful in reprogramming cells and showing lack of integration, using DNA based methods inherently carry the possibility of integration into the host genome. In order to avoid the possibility of integration of exogenous

DNA, DNA-free induction methods have been developed. These methods include the use of messenger RNA (mRNA) [38, 73, 272, 307-311], or proteins [49, 312] to deliver the factors to the somatic cells.

3.3.3.1 Reprogramming via messenger RNA (mRNA)

Reprogramming of somatic cells by mRNA was attempted by many groups [38, 73, 307, 313]. The process begins with the preparation of synthetic mRNA encoding for different factors by a process termed *in vitro* transcription (IVT) (Figure 14). RNA purification is achieved via spin-column as well as DNase and phosphatase treatment. This removes any DNA in the mix as well as the 5' triphosphates that can cause immunogenicity [38, 314]. The integrity of RNA is measured via agarose electrophoresis prior to transfection. A detailed protocol of mRNA induction of iPSCs with fibroblasts has been published by Mandal & Rossi [309]. mRNAs are then transfected into cell lines using Lipofectamine cationic lipid delivery vehicles [38, 313] or microporation [307]. Since mRNA is not self-replicating, transfection must be repeated. Although Plew's study was unsuccessful in producing iPSCs with their OKSM[SV40] combination [307], Yakubov *et al* were successful in producing IPSCs with 5 transfections of mRNAs 24 hours apart with an efficiency of 0.05% (50 colonies/1x10^5 cells)[313]. Warren *et al*. transfected for two weeks and had efficiencies of 2% (500colonies/2.5x10^4cells) with OKSM and 2.8% (700 colonies/2.5x10^4 cells) with OKSML [38, 313]. Both groups were able to visualize iPSCs in approximately 2 to 3 weeks with higher efficiency then the OKSM retroviral methods of 0.02% [37, 38, 313].

This method has many advantages. Since there is no risk of integration, it favours the generation of genetically stable iPS cell lines. Furthermore there is no need for subclone isolation, or drawn

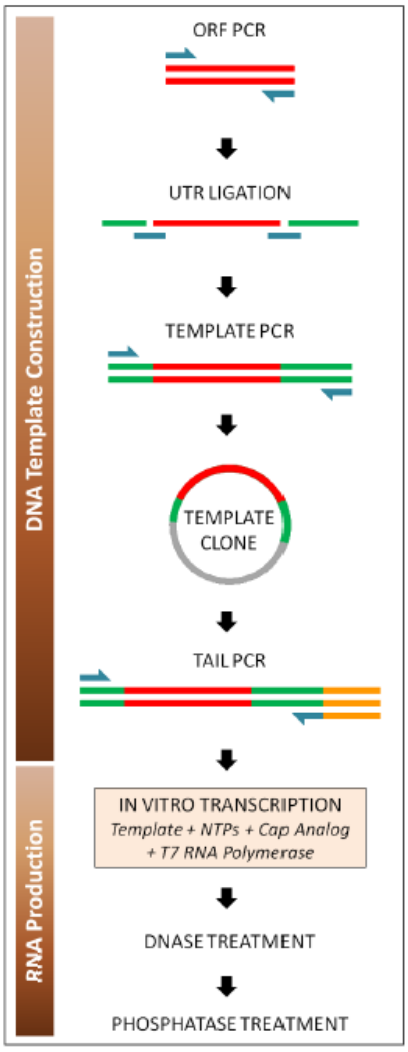


Figure 14 - RNA Production Flowchart
To construct a template for RNA transcription reactions, the ORF of a gene of interest is first PCR amplified from a cDNA. Long oligonucleotides containing UTR sequences are then joined to the top strand of ORF amplicons by a thermostable DNA ligase, mediated by annealing to splint oligos which bring the desired single-stranded DNA (ssDNA) ends together. A T7 promoter is incorporated in the 5' UTR fragment. The ssDNA product is amplified using generic primers and TA cloned. A polyA tail is added with a PCR reaction using a T120-heeled reverse primer and the amplicons are used to template IVT reactions. Modified and unmodified nucleobases are used in the IVT reaction. An anti-reverse di-guanosine cap analog (ARCA) is included in the IVT reaction at four-fold higher concentration than guanosine triphosphate (GTP), as a result of which an estimated 80% of the product is capped. Spin-column purified IVT product is DNase-treated to eliminate the DNA template. Treatment with a phosphatase is used to remove immunogenic 5' triphosphate moieties from the uncapped RNA fraction. The completed modified-RNA is then re-purified for use in transfections. (Reprinted from Warren 2010 with permission)[38]

out PCR evaluation of the iPSCs for integration. Even though it necessitates multiple transfections, it is relatively quick since it does not require any steps for treatment with antibiotics (such as DOX) or passages to allow dilution of exogenous factors. It is essentially a quick, simple, footprint free, efficient method of producing iPSC. Since Yukobov's method does not use oncogene cMyc, it is more appealing to clinical work applications [313]. The prospect of using mRNA derived iPSCs in cell therapies has been made more realistic with the development use of mRNA in pre-clinical and clinical studies for cancer immunotherapy [315].

3.3.3.2 Reprogramming Via Proteins

Reprogramming of somatic cells can also be achieved using proteins [49, 165, 312]. This method relies on the in frame fusion of proteins coding regions with coding regions of cell-penetration peptide (CPPs) or protein transduction domains [316] that allows the protein to be incorporated into the cell. Zhou's group created a system that would allow preparation of recombinant OSKM proteins by using the HIV transactivator of transcription (Tat)/poly-arginine system expressed in *E.coli* inclusion bodies [317-319]. Once solubilized, refolded and purified, the proteins were used to prepare a protein transduction media with which the somatic cells were treated overnight four times at 48 hour intervals. The group was successful in producing three colonies per 5 x 10^4 cells (0.006%) when transduced with four factors, and one colony per 5 x 10^4 cells (0.002%) but only when valproic acid (VPA) was included (VPA is a histone deacetylase inhibitor that will be discussed later)[49]. Without VPA, efficiency with OSKM proteins was reduced to 0.001% (13colonies/number of cells not provided)[312], however iPSCs were capable of being differentiated into dopaminergic neurons used in the treatment of Parkinson disease (PD)[320].

More recently, Nemes *et al* modified the HIV/arginine system by adding a nuclear localization signal (NLS) that would potentially increase nuclear localisation of the recombinant proteins and therefore reprogramming efficiency[165]. Transfection of cells was done four times on a 48 interval as in Rhee's study [320]. The experiments were performed on two types of mice, outbred (Imprinting Control Region (ICR)) mice and with inbred C57 black 6 (C57BL6) mice. Although both experiments yielded 8 and 6 primary iPSC colonies, respectively, only those stemming from outbred mice could be fully reprogrammed. These results indicate the importance of cell and species choice when studying iPSC technology for clinical applications. Although the reprogramming efficiency for Nemes studies could not be calculated, other studies show that reprogramming via proteins is less efficient and also takes longer for the appearance of iPSC colonies (on average 43 days)[49, 165, 312] than other methods. Efficiency aside, since the proteins eliminate the risk of DNA damage via integration, and genetic instability, as well as transgene reactivation, iPSCs derived via exogenous proteins expression are a safer source of cells for clinical applications.

All the reprogramming methods discussed above focused on the delivery of exogenous pluripotency factors. Next we will discuss reprogramming via small molecules that target cellular changes and pathways to induce an embryonic stem cell state.

3.3.4 Reprogramming Via Small Molecules

An important part of the progress in the reprogramming of somatic cells has come from the study of the cellular processes that occur within the cells during this process. Time-based studies of the reprogramming process have allowed the elucidation of common gene expression patterns, and pathways that are upregulated or downregulated in a time sensitive fashion. From these studies,

scientists have been able to identify molecules and/or chemicals that can promote or inhibit pathways in order to enhance their reprogramming methods. Next, we will look at the cellular events of the different phases of reprogramming, as well as the small molecules that have been used to target these cellular mechanisms in order to enhance existing methods, or induce reprogramming on their own.

3.3.4.1 Phases in Reprogramming

A review by David & Polo in 2014 looked at different studies published on the creation of iPSCs from mouse embryonic fibroblasts (MEFs) using the classic OKSM factors [321]. Although this review was specific to mouse species, and OKSM factors, these types of studies give insightful information on possible commonalities of the internal cellular processes occurring which must be targeted in order to enhance reprogramming protocols. Their findings are summarized in Figure 15 [321]. An explanation of these findings will be supplemented with recent data from other studies. Essentially, cellular processes leading to the reprogramming can be grouped into three phases; 1) Initiation: initial epigenetic and gene expression changes due to ectopic expression of OKSM factors, 2) maturation: a slow transition for preparation to independent pluripotency, and 3) stabilization: activation of self-sufficient pluripotent cell populations [322]. Specific hallmarks, morphological features, transcriptome and cellular markers have been identified for each phase. Since time studies include specific time points (days post infection (dpi)), some reviews use time frames of 0-3, 3-9, and 9-12 days post induction (dpi) to represent the three phases [323, 324](Figure 15 and Figure 16, Panel A). Other studies have less defined time points, for example showing only changes occurring during the initial ten days of reprogramming [324, 325] (Figure 16, Panel B).

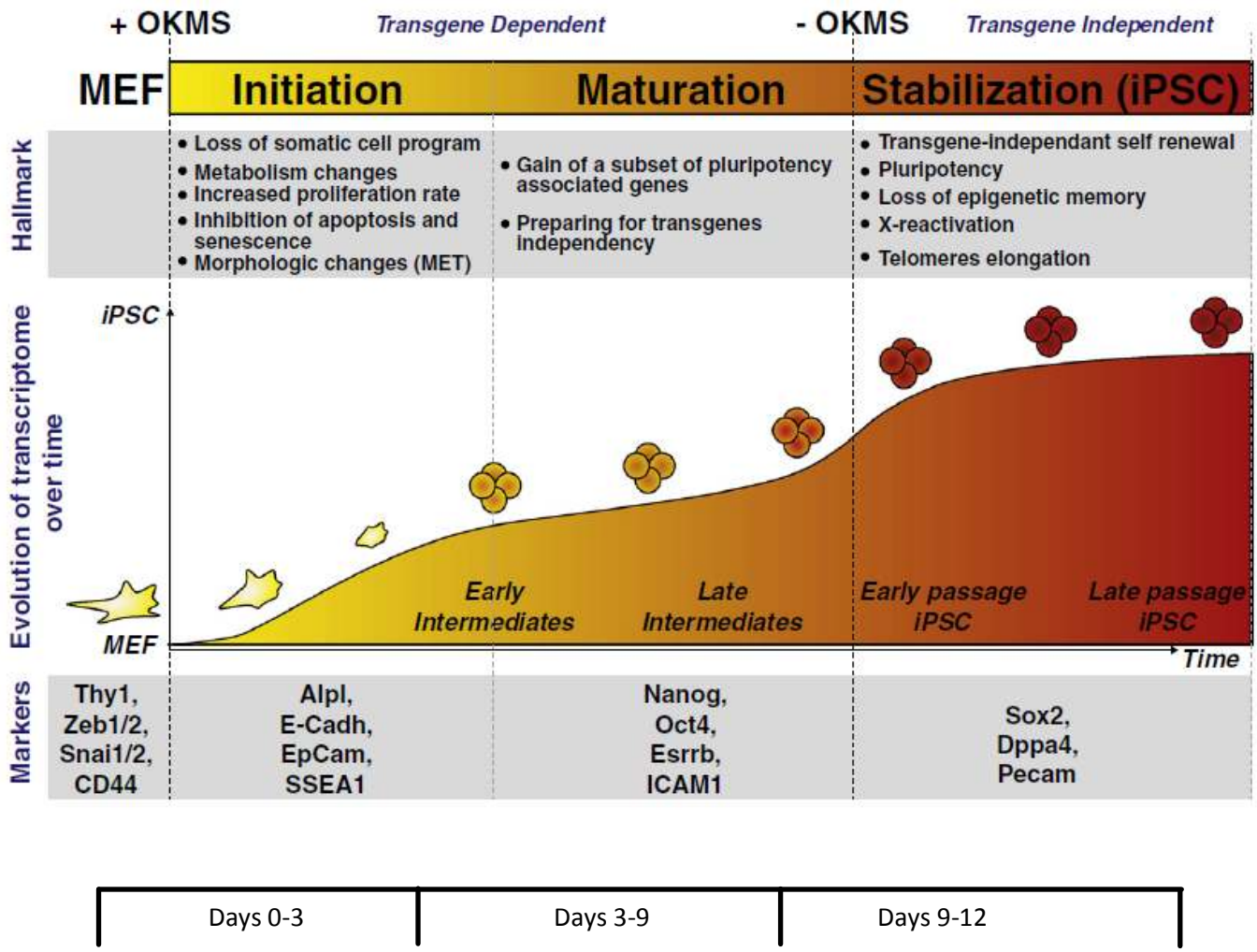


Figure 15 - Phases in Reprogramming
Sequential events occurring during somatic cell reprogramming. Markers and specific hallmarks of each phase of reprogramming are indicated. The iPSC transcriptome is acquired in two major steps. Abbreviations: Thy1: thymocyte antigen 1, Zeb1/2: zinc-finger E-box homeobox protein 1 and 2, Snai1/2: small zinc-finger protein 1/2 , CD44: Cluster of differentiation 44 , MET: mesenchymal to epithelial transition, Alpl: alkaline phosphoatase, E-cadh: E-cadherin, EpCam: Epithelial cell adhesion molecules, SSEA1: species-specific embryonic antigen 1, ICAM1: Intercellular adhesion molecule 1 , Dppa4: Developmental pluripotency associated factor 4 , Pecam: Platelet endothelial cell adhesion molecule. (Modified from David 2014 with permission)[321]

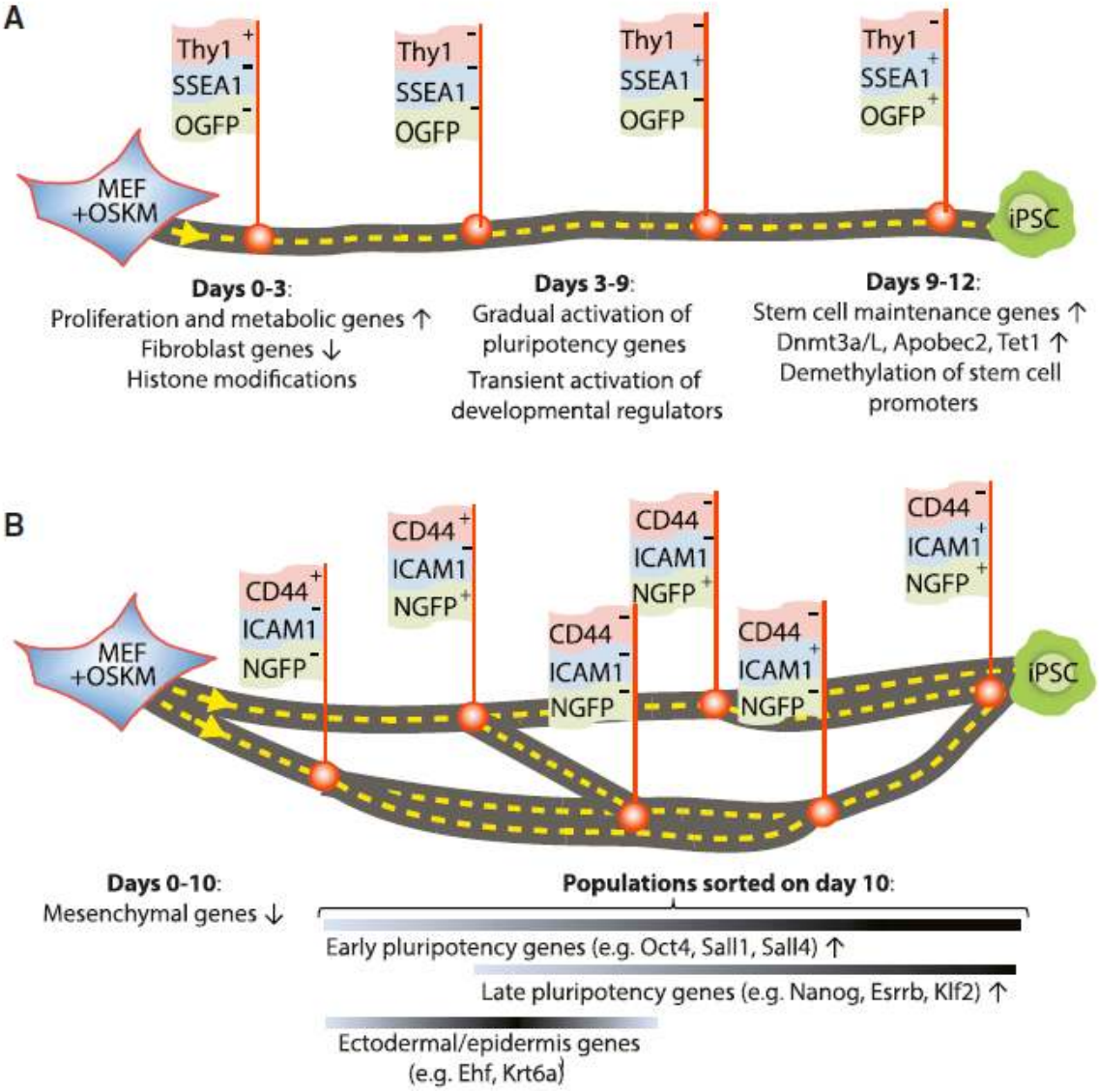


Figure 16- Roadmaps of Epigenetic Reprogramming
[A] Trajectory of reprogramming intermediates defined by Thy1, SSEA1, and Oct4-GFP (OGFP) expression (Polo *et al*., 2012). [B] Trajectories of reprogramming intermediates defined by CD44, ICAM1, and Nanog-GFP (NGFP) expression (O'Malley *et al*., 2013). Double lanes indicate transitions that occur at a higher frequency. iPSC, induced pluripotent stem cell; MEF, mouse embryonic fibroblast; MET, mesenchymal-epithelial transition; OSKM, Oct4, Sox2, Klf4, and cMyc. (Repinted from Theunissen & Jaenisch 2014 with permission)[324]

3.3.4.1.1. Initiation phase

During the initiation phase, when transgenes are expressed, there is a general consensus that this ectopic expression of factors induces a change in endogenous gene expression patterns, more particularly for genes responsible for cell morphology, motility, signalling, proliferation, and metabolism [326]. The initiation phase is characterized by hallmarks like the loss of the somatic cell program, which has been shown to come about mostly by histone modifications as opposed to DNA methylation [323]. There is a redistribution of the H3K4 dimethylation (H3K4me2) associated with accessible DNA [327]. Furthermore, some cases included suppression of somatic marker genes (like thymocyte antigen 1(Thy1) and cluster of differentiation 44 (CD44)) via transcriptional repressive H3K27me3, and activation of pluripotency genes (AP and SSEA1 (mice only)) via loss of suppressive H3K27me and gain of activating H3K4me3 [141, 277, 322, 323, 325, 327, 328]. It has been postulated that OKS most likely bind to promoters of inactive DNA regions promoting activation of these regions, while cMyc likely binds to accessible, active somatic gene promoters to promote loss of somatic cell identity and promote the mesenchymal-to-epithelial transition (MET) [329, 330], yet another hallmark of the initiation phase [331-333]. MET is demonstrated by the loss of expression of mesenchymal fibroblast TFs small zinc-finger proteins 1 and 2 (Snai1/2) and zinc-finger E-box homeobox proteins 1 and 2 (Zeb1/2) [141, 170, 330] and the gain of expression of epithelial markers such as E-Cadh, epithelial cell adhesion molecule (Epcam), or miRNA-200 family [322, 334, 335].

Other hallmarks of the initiation phase include the change in proliferation rate, closer to that of embryonic stem cells, which is accompanied by decrease in apoptosis and senescence markers [141, 331-333, 336, 337]. Shortly after reprogramming is initiated, the cell cycle p53 pathway is activated due to increase levels in p53, cell cycle inhibitor p21, and p16. Furthermore, there is a

decrease in levels of some cell cycle facilitators such as cyclin dependant kinase 1 (cdc2a or CDK1), cyclin B (Ccnb1), as well as cyclin dependant kinase 2 (Cdk2)[326](Figure 17). These changes in expression should lead to an increase in cell death and senescence. However increased levels of mouse double minute 2 (Mdm2), a p53 suppressor, and increase in cyclin D, a cell cycle facilitator, seem to counteract p53s effects and instead activate proliferation in reprogramming cells [212, 326](Figure 17).

Metabolic shift is another hallmark of the initiation phase. Although some studies indicate that metabolic changes from oxidative phosphorylation to a more glycolytic pathway of energy production occurs in the initiation phase, other studies show that different metabolic changes occur at different time points in the process. For example, early reprogramming cells are most metabolically active at day 9pdi (late maturation) and decrease to a glycolytic/gluconeogenesis pathway in the pluripotent self-renewing iPSCs (late stabilization)[326, 338]. A study by Park *et al* showed that there was an increase in the expression of hypoxia-related genes early after induction which often correlates to a conversion to a more glycolytic, anaerobic metabolic pathway [326]. A more recent study has indicated that although both hypoxia inducible factor 1 alpha (HIF1α) and HIF2α are necessary to initiate the metabolic switch, HIF2α can inhibit iPSC generation [339] in a manner that has yet to be elucidated. The initiation phase is one which most cells can complete, however the maturation phase is a bottleneck in the process of iPSC generation [340].

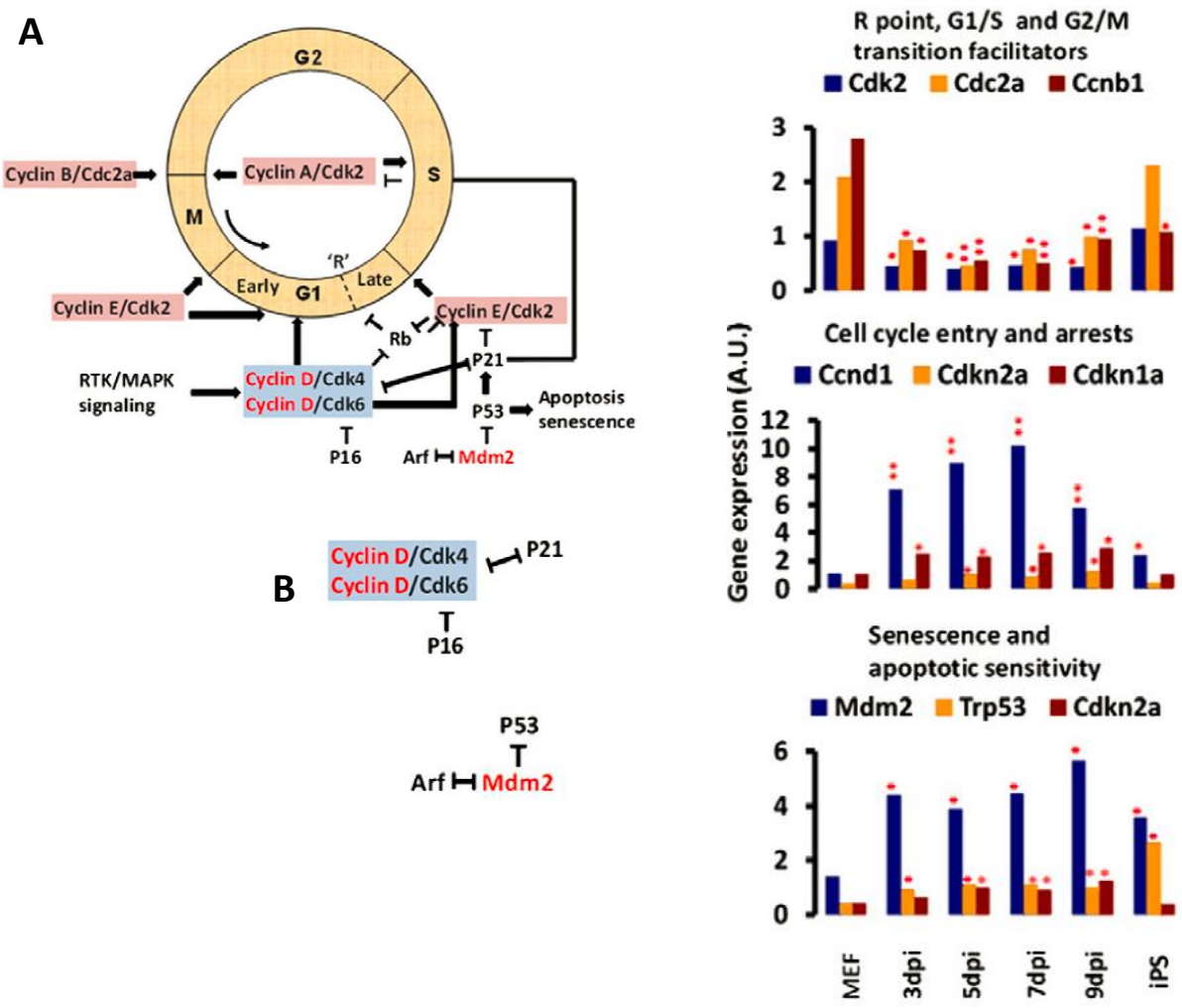


Figure 17 - Transitional Rewiring of Global Signaling Network during Early Reprogramming

[A] Diagram depicting molecular mechanisms involved in regulating proliferation by various pathways. It explains the effect of key genes in the p53 pathway on proliferation. Blue boxes indicate Cdk activities during reprogramming while red boxes indicate Cdk activities in both reprogramming and final iPS cells. [B] Key DEGs and control modules of the p53 pathway affect cell cycle facilitation, cell cycle arrests as well as senescence & apoptotic sensitivity during reprogramming. '*' and '**' represent a change in expression of at least two-folds and four-folds over MEF respectively. Although p53 pathway activators (Cdkn1a, Cdkn2a and Trp53) undergo significant increase and some cell cycle facilitators (Ccnb1, Cdc2a and Cdk2) are down-regulated, the constitutive increase in cyclin D and Mdm2 negates the effects of cell proliferation inhibitors in successfully reprogramming cells. (Reprinted from Park *et al*. 2014 with permission)[341]

3.3.4.1.2. Maturation Phase

The major hallmark of the maturation process is the expression of endogenous pluripotency genes. Although analysis was performed differently (single cell transcriptomics followed by clonal analysis and fluorescent in situ hybridization (FISH) or FACs cell sorting), many studies have shown that cells acquire specific markers in a sequential way; Fbx15, Sall4, Oct4 in a first phase, then Nanog, and Esrrβ in a later stage [322, 323, 342, 343]. The cell surface marker intercellular adhesion molecule 1 (ICAM1) can also be identified during this later phase [325]. Studies done by Galipour showed the importance of endogenous Oct4 and NANOG expression in the preparation of transgene independency [277, 343] by their capability of binding promoters of inactive primary and secondary pluripotency-associated factors and inducing the DNA methylation changes characteristically seen in the stabilization phase [323].

3.3.4.1.3. Stabilization Phase

The changes that occur after iPSCs have acquired transgene-independent self-renewal and pluripotency are grouped together in the stabilization phase [81, 82, 277, 344]. This phase is marked by changes in DNA methylation patterns, due to changes in the expression pattern of methylation regulatory genes. These include: activation-induced cytidine deaminase (AID), deaminase apolipoprotein B mRNA editing enzyme catalytic polypeptide (Apobec), 5-methylcytosine hydroxylase ten-eleven translocation 1 (Tet1), and DNA methyltransferases 3a and 3L (Dnmt3a, Dnmt3L). By changing inhibitory and activating methylation patterns, these enzymes allow the transcription of another wave of endogenous pluripotency markers such as Sox2, and platelet endothelial cell adhesion molecule (Pecam) (also known as CD31), and Dppa4 [284, 322, 323, 342]. AID has also been shown to be involved in re-establishing the ESC

epigenetic memory in iPSCs [345, 346]. Other epigenetic changes such as telomere elongation and X-chromosome reactivation in female cell lines are visible in fully reprogrammed iPSCs [284, 347]. Other changes that may play a role in the transgene-independent self-renewal of iPSCs is the attenuation of p53, p21, and p16 levels, and the return of cell cycle facilitators levels (cdc2a, Ccnb1 (Cyclin B1), to their normal state, allowing progression through the cell cycle [326].

3.3.4.2 Reprogramming Via Small Molecules

These time-based studies have allowed scientists to enhance reprogramming using small molecules or chemicals that can target pathways and hallmarks, thus increasing efficiency. They have also used small molecules in combination to replace one, two, three, or all reprogramming TFs. Small molecules can be categorized based on their function as: 1) epigenetic modifiers, and 2) signalling modulators that target pathways and/or reprogramming hallmarks (MET, metabolic shift). Figure 18 depicts the roles of a multitude of small molecules in cellular reprogramming.

3.3.4.2.1. Epigenetic Modifiers

The first category of molecules consists of epigenetic modifiers. As seen previously, DNA methylation and hypermethylation is associated with the silencing of gene expression, while DNA demethylation is associated with reactivation of gene expression [141, 142]. In the case of reprogramming, studies have looked at increasing methylation of somatic genes and the demethylation self-renewal and pluripotency genes. Examples of molecules used to modify the epigenetic state include DNA methyltransferases inhibitors (DNMTi) like 5-Azacytidine

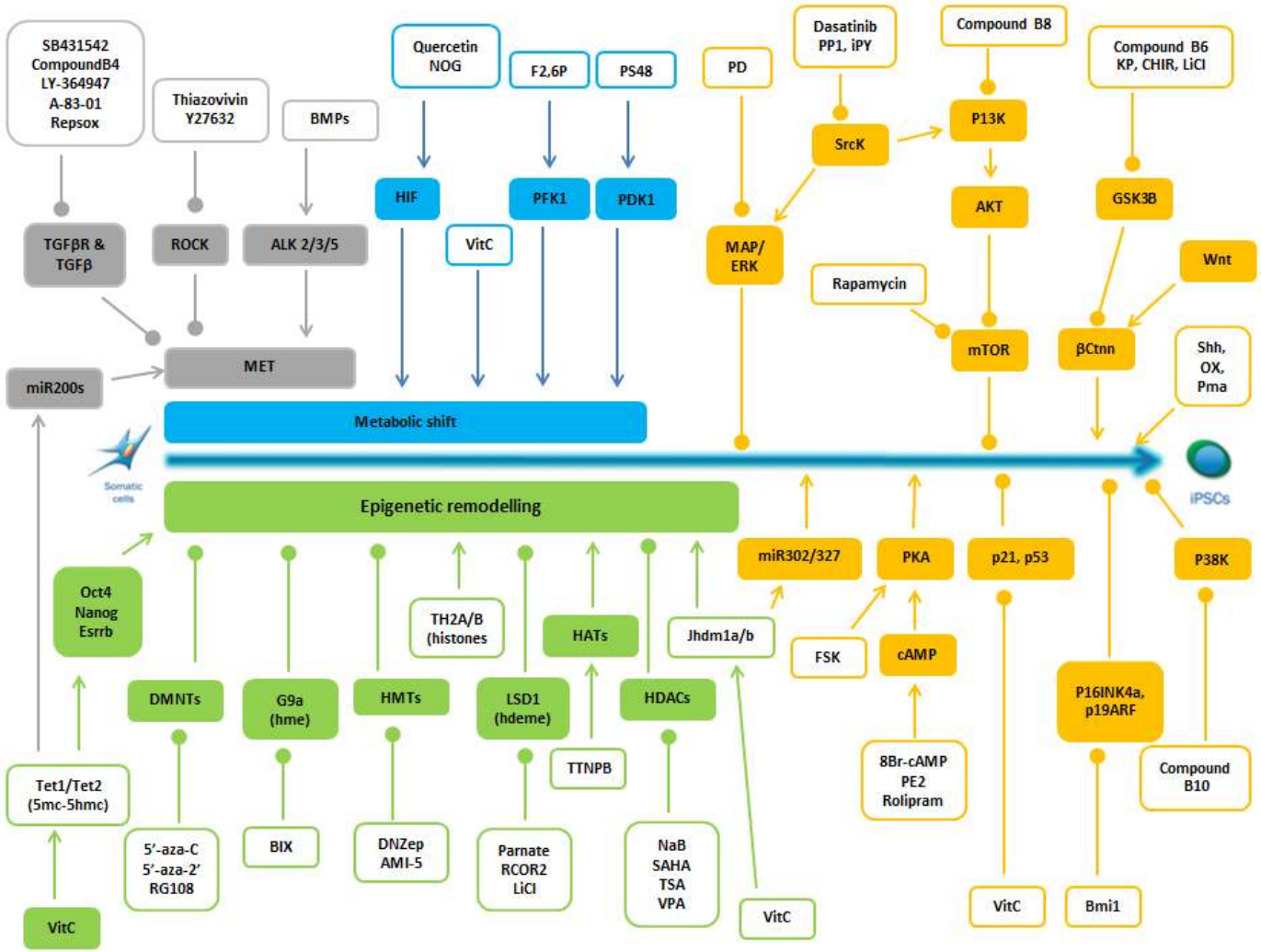


Figure 18 - Small Molecule-regulated Reprogramming
Small molecules are indicated by the white boxes, while their targets are in coloured boxes. Arrow heads indicate activation while circles indicate inhibition. (Format reprinted from Su *et al*., 2013 with permission [348] and information from [73, 212, 214, 307, 322, 336, 345, 346, 348-368]

(5-aza-C) [119, 141, 369], 5-Aza-2'-deoxycytidine (5'aza-2') [307], and RG108 [210, 352]. Tet1, a 5-methylcytidine (5mC) hydroxylase, has also been used to increase the efficiency of reprogramming. This enzyme works by hydroxylating the 5-methylcytidine at the Oct4 loci, which promotes its demethylation and reactivation during OSKM reprogramming. The use of this molecule has enabled the elimination of Oct4 in TSKM reprogramming [351]. Tet2 was also shown to induce the 5mC – 5-hydroxymethylcytidine (5hmC) conversion at the NANOG and Esrrβ loci to enhance somatic cell reprogramming [351]. Furthermore, treatment with Tet family members is believed to improve reprogramming by promoting 5mC – 5hmC conversion of the miRNA-200 cluster which plays an important role in the MET-associated transition of early reprogramming in fibroblasts [322, 351].

Molecules that induce histone modifications and change accessibility of DNA have also been used to enhance reprogramming. Histone acetylation (usually on arginine or lysine residue) removes positive charges on the histone. This decreases the attraction between the histone and the negatively charged phosphate groups of the DNA backbone allowing for a more relaxed and accessible DNA for transcription [370]. Deacetylation allows DNA to return to a supercoiled heterochromatin essentially silencing transcription in this area [371]. Histone deacetylase inhibitors (HDI or HDACi) therefore allow for a more accessible DNA. HDACis used in reprogramming experiments include: sodium butyrate (NaB) [372], suberanilohydroxamic acid (SAHA), Trichostatin A (TSA) [353, 354] and VPA [206, 355]. 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNBP), a retinoic acid receptor agonist which promotes histone acetylation, has also been used to enhance reprogramming of somatic cells [356].

Another epigenetic change targeted is histone methylation. Histone methylation can be repressive or activating depending on the histone and the number of methyl groups added. Monomethylation such as H3K9me, H3K27me, H3K20me, H3K79me, and di or trimethylation of H3K4m2/3 are common activation methylations. Conversely, H3K9me2/3, H3K27me2/3, H3K20me3, and H3K79me3 are known inhibitory methylations [373, 374]. Rest compressor 2 (RCOR2), lithium chloride (LiCl), and Parnate (tranylcypromine), enhance reprogramming by inhibiting lysine-specific histone demethylase 1's (LSD1) activity [357, 358]. RCOR2 was used in conjunction with OKSM to increase efficiency to 0.47% producing 235 colonies for 5 x 10^4 cells seeded. RCOR2 also eliminated the need for Sox2 in OKM reprogramming, albeit with lower efficiency [358]. 3-deazaneplanocin A (DZNep) and histone arginine methyltransferase inhibitor 5 (AMI-5), are two histone methyltransferase inhibitors (HMTi) that have been used to enhance reprogramming [356, 359]. G9a histone methylase inhibitor BIX [210] as well and H3K36 demethylase Jumanji/ARID containing histone demethylation 1a and 1b (Jhdm1a/1b) [360, 375] have also been used to enhance reprogramming with one, two, or three of the original OKSM factors. Jhdm1a and 1b were found to enhance reprogramming by promoting Oct4 dependent expression of microRNA302/327 cluster responsible for regulating the cell cycle [360, 375, 376]. Interestingly, Shinagawa was able to produce iPSCs by using histone variants present in oocytes (testicular histone 2B and 2B (TH2A and TH2B)) in conjunction with Klf4 and Oct3/4. Efficiency was also 18 fold higher with TH2A and TH2B with OKSM, compared to their OKSM controls [361].

3.3.4.2.2. Signalling Modulators

The next category of molecules used to enhance reprogramming is that of signalling modulators. These can target particular cellular pathways, their receptors or intermediates that are involved in reprogramming or specific hallmarks of the reprogramming phases. Transforming Growth Family Beta (TGF-β), Wingless-related integration (Wnt), Sonic Hedgehog (Shh), their ligands, and/or downstream effectors have been at the center of many studies. Since TGF-β pathway inhibits the epithelial-mesenchymal transition necessary in the early stages of reprogramming [334], molecules inhibiting the receptor or intermediates in the pathway have been used to overcome this obstacle and enhance reprogramming. These include 616452 (Repsox)[362], A83-01 [207], LY-364947 [357, 364, 365], and activin-like kinase 4 (ALK4) receptor inhibitor compound B4 [212]. Lin *et al* were able to enhance OKSM reprogramming with the use of TGF-β inhibitor SB431542 [208, 365], and mitogen activated phosphate kinase/Extracellular signal-regulated kinase (MAPK/ERK) inhibitor PD0325901 [365]. Bone Morphogenic Proteins (BMPs) are members of the TGF-β family that promote MET during reprogramming [322], and have been shown to replace Klf4 and induce reprogramming when used with Oct4 alone to produce iPSCs [377]. Thiazovivin and Y27632, which are Rho-associated protein kinase inhibitors (ROCKi), have also shown to enhance reprogramming by promoting MET [38, 365-367].

CHIR, a glycogen synthase kinase-3 beta inhibitor (GS6K-3βi), enhances reprogramming by inhibiting the Wnt signaling pathway [357]. Other than CHIR, GSK-3βis Kenpaullone (KP)[363], compound B6 [212] and lithium chloride (LiCl) [360] have also been used in conjunction with OKSM factors to enhance reprogramming. Also notable, the activation of the Shh pathway via Shh, purmorphanime or oxysterol in conjuction with Oct4 was used to produce iPSCs with 0.11% efficiency [214]. Sarcoma (Src) family tyrosine kinase inhibitors such as

Dasatinib, iPY razine (iPY), protein phosphatase 1 (PP1) have been successful in replacing Sox2 during reprogramming [364]. Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, Compound B8, an inositol triphosphate 3-kinase (IP3K) inhibitor, and Compound B10, a P38 kinase inhibitor are all kinase inhibitors that have enhanced reprogramming [212, 377]. 8-Bromoadenosine 3'-5'-cyclic monophosphate (8-Br-cAMP), prostaglandin E2 (PE2), Forskolin (FSK), and Rolipram are all cAMP-dependent protein kinase activators [356, 378] that can target signalling pathways to promote reprogramming with at least 1 of the original OKSM factors.

Metabolic shift has also been a target of small molecules used to enhance reprogramming. Fructose 2,6-bisphosphate (F2,6P) activates phosphofructokinase 1 (PFK1), an enzyme in the glycolytic pathway, inducing glycolysis directly. Targeting glycolysis indirectly through activation of hypoxia-inducible factor (HIF) pathway using hydroxylase inhibitors such as N-oxalylglycine and Quercetin has also shown to enhance reprogramming efficiency. PS48, a 3'phosphoinositide-dependant kinase (PDK)-1 activator enhanced reprogramming 15 fold, while PS48 and NaB (HDACi) enhanced reprogramming 25 fold compared to controls in Zhou's study [207]. Other groups have produced iPSCs by using hypoxic growth conditions. Takahashi and Yamanaka's group showed that hypoxic conditions could produce 2.5x – 3x the number of iPSC colonies by OKSM retroviral transfection and DOX-inducible PB transposon transfection of MEFs [75]. Panopoulous's group noticed a 0.5 – 1.5 % increase in relative reprogramming efficiency in 5% oxygen conditions compared to 20% oxygen controls using OKSM retrovirus without and with TGFβi SB431532 [208].

The use of ascorbic acid (VitC) has shown to improve efficiency when used with OKS [350], Jhdm1a/OS, Jhdm1a/O [368], or Jhdm1a/1b [375]. Jhdm1a is a histone demethylase that functions in a VitC dependent fashion to promote DNA accessibility [375]. VitC may also play a

role in the metabolic shift during reprogramming since its involvement in the regulation of gluconeogenesis in hepatic cells has already been confirmed [368]. VitCs enhancement capabilities may also stem from its ability to decreased p21 and p53 levels, allowing cell cycle progression and proliferation of cells [350]. Further investigations have also shown that ascorbic acid functions as a cofactor for Tet family members, promoting DNA demethylation and activation of the MET-associated miRNA-200 cluster, Oct4, NANOG, and Esrrβ loci, therefore enhancing reprogramming of somatic cells [322, 349, 379].

3.3.4.2.3. Reprogramming Enhancement and *de novo* Reprogramming with Small Molecules

Of all the categories of molecules, those that enhanced OKSM reprogramming the most were small molecules named compound 4 (a TGFβi), compound 6 (a GSK3βi), compound 8 (a PI3Ki), and compound 10 (a p38i) with efficiencies of 0.42%, 0.28%, 0.25%, and 0.25% respectively. CHIR (GSK3βi) enhanced OSK to its highest efficiency at 0.18% [357].

Most of the experiments mentioned above used three or four OSKM factors; however some groups were able to produce iPSCs using one or a combination of small molecules with only Oct4 or no factors [207, 214, 355, 359, 379] with varying efficiencies. Oct4 in combination with binding to microtubules 1 (Bmi1) was shown to induce iPSCs with an efficiency of 0.17% [214]. Bmi1 is postulated to work by inhibiting cell cycle repressors p16, and apoptosis inducer p19. The same group replaced SKM using Sonic hedgehog agonists (oxysterol, purmorphanime or oxysterol) in combination with Oct4 to produce iPSCs with an efficiency of 0.11% [214]. Yuan *et al* were able to induce reprogramming with Oct4, A-83-01 (TGFβi) and AMI-5 (methyltransferase inhibitor) with an efficiency of 0.02% [359]. Oct4 with a combination of VPA

(HDACi), CHIR (GSK-3βi), 616452 (TGFβi), and parnate (LSD1i) carried an efficiencies of 0.05-0.08% (15-25 colonies/3x10^4cells) in MEFs [355], while Parnate, CHIR, A8301 (TGFβi), PS48 (PDK1i), NaB (HDACi), and PD0325901 (MEKi) showed an efficiency of 0.0003% - 0.012% (3 colonies/10^6 cells – 23 colonies/2x10^4cells) depending on the human cell type [207]. The use of VPA, CHIR, 616452, parnate, Forskolin (FSK), DZNep without and with TTNPB reprogrammed MEFS with efficiencies of 0.04 % (20 colonies/ 5x10^4 cells) and 0.16% (80 colonies/5x10^4 cells)[356]. Most impressively, RG108 (DNMTi) has shown to be able to reprogram mouse somatic myoblasts with an efficiency of 0.12% (57-60 colonies/5x10^4cells) without any factors [352].

Although in some cases, the addition of small molecules and chemicals did enhance the efficiency of reprogramming compared to the original Takahashi method (0.02%)[37], efficiency remained under 1%. The highest level observed was 0.42% with OKSM and compound 4 [212], followed by 0.18% using a combination of seven small molecules [356]. Although this is not as efficient as mRNA-mediated reprogramming (2.8%) [308], small molecules alone can reprogram somatic cells with higher efficiency than retroviral transfection of OKSM, and is comparable to the highest efficiencies obtained with viral (0.5%) [281] and plasmid vectors (0.9%) [186].

Other advantages to using small molecules should be considered when choosing a method for iPSC induction. First and foremost, studies have shown that exposure of cell to exogenous OKSM factors via mRNA can be cytotoxic and therefore decrease the number of viable cells for reprogramming. This cytotoxic effect does not occur with the use of small molecules such as BIX, VPA, and 5'aza-2' [307]. Therefore using small molecules could increase the amount of viable cells available for reprogramming. Furthermore, treatment of cells with small molecules is a much simpler process since there are no viral/plasmid vectors or mRNA preparation phases

needed. Small molecules are simply added to the media with which the cells are treated. Although initial appearance of ESC-like colonies may take somewhat longer (40 days vs. 7 to 30 days via other methods), there is no clean up (excision of transgenes) phase necessary after reprogramming is complete, as small molecules are degraded by the cell, and also diluted out in a few passages. Again since small molecules are a footprint free method, PCR or Southern blotting experiments post-reprogramming to verify non-integration or excision of plasmids are not necessary. Lastly, there is lower risk of DNA damage with these methods since small molecules act on a signalling level, or on an epigenetic level to change expression patterns of endogenous pluripotency genes to induce reprogramming.

It is evident that the use of small molecules in reprogramming is a vast field of study that is expanding. The next section will focus on the use of microRNA (miRNA) to enhance reprogramming or somatic cells.

3.3.5 Reprogramming via microRNA (miRNA)

Previous methods of reprogramming discussed focused on the ectopic expression of different transcription factors that have been identified as having important roles in pluripotency and self-renewal such as OKSMLN [32, 48, 92], or targeting of cellular pathways and hallmarks that are recalled during reprogramming. The methods that will now be described are based on studies analysing microRNAs (miRNAs) levels within ESCs, iPSCs and somatic cells. miRNAs are small single-stranded non-coding RNAs approximately twenty-two nucleotides (nt) in length that regulate translation by binding to and targeting mRNAs for cleavage [380] or translational repression [381, 382]. Sometimes the DNA sequences of multiple miRNAs' are found in the same genomic region and are therefore named as clusters or families [383]. Furthermore, it is

postulated that miRNAs with similar seed sequences function to have the same targets [335].

By comparing results from qPCR, northern blot, microarray and deep sequencing of ESCs and somatic cells, miRNAs associated with pluripotency, self-renewal, and differentiation have been identified [110, 384-387]. From miRNA analyses, scientists have been able to modify/enhance already existing reprogramming protocols as well as develop new individual protocols using miRNAs alone to produce iPSCs [192, 206, 388]. This next section will provide information on miRNAs responsible for pluripotency, self-renewal, and differentiation. It will further discuss miRNAs that have been used in somatic cell reprogramming.

One study categorized hESC-enriched miRNAs into five major groups: miR-302, miR-17, the miR-371/373, as well as miR-130, and miRNAs from C19MC (chromosome 19 miRNA cluster) [335]. Each cluster contains at least two miRNAs stemming from the same chromosome (Table 2). Another group confirmed miR-302d role in ESCs and identified the miR290s cluster (miR-290-295) as important regulators in ESCs [386]. Qi's group also confirmed the role of miR-372 and discovered the potential for miR-195 [387]. Although the roles of all miRNAs individually have not yet been elucidated, since the majority of them exert their function by acting upon cell cycle regulators, they have been termed ES cell-specific cell cycle-regulating (ESCC) miRNAs [387]. miR290s (particularly 291a-3p/294/295) and miR302d function by inhibiting G1/S transition inhibitors p21, retinoblastoma like 2 (Rbl2), and large tumor suppressor (Lats2) [386](Figure 19). miR-17 (particularly 92b) promotes ESC proliferation by inhibiting the G1/S transition inhibitor p57 [389], while miR-195 was shown to target G2/M transition inhibitor WEE1 protein kinase [387]. Not only are the ESCC miRNAs upregulated by pluripotency genes Sall4, Oct4, NANOG, Sox2 and cMyc [390], miR-302s in particular stops epigenetic silencing of

Family/miRNA	*Chromosome*	*Seed Seq.*	*Family/miRNA*	*Chromosome*	*Seed Seq.*
miR-302 Cluster			**C19MC Cluster**		
miR-302a	4	aagugcu	miR-512-5p	19	acucagc
miR-302a*	4	cuuaaac	miR-498	19	uucaagc
miR-302b	4	aagugcu	miR-515-3p	19	agugccu
miR-302b*	4	cuuuaac	miR-519e*	19	ucuccaa
miR-302c	4	aagugcu	miR-520f	19	agugcuu
miR-302d	4	aagugcu	miR-519c	19	ucuagag
miR-367	4	auugcac	miR-520a-3p	19	aagugcu
			miR-526b	19	ucuugag
			miR-519b	19	ucuagag
miR-371–373 Cluster			miR-525	19	uccagag
miR-372	19	aagugcu	miR-523	19	aacgcgc
miR-373	19	aagugcu	miR-518f	19	aaagcgc
			miR-518f*	19	ucuagag
			miR-520b	19	aagugcu
miR-17 Family			miR-518b	19	aaagcgc
miR-17	13	aaagugc	miR-526a	19	ucuagag
miR-17*	13	cugcagu	miR-520c	19	ucuagag
miR-18a	13	aaggugc	miR-524-3p	19	aaggcgc
miR-19a	13	gugcaaa	miR-517a	19	ucgugca
miR-20a	13	aaagugc	miR-519d	19	aaagugc
miR-19b-1	13	gugcaaa	miR-521	19	acgcacu
			miR-520d	19	uacaaag
miR-106a	X	aaagugc	miR-517b	19	cgugcau
miR-18b	X	aaggugc	miR-520g	19	caaagug
miR-20b	X	aaagugc	miR-518e	19	aagcgcu
miR-19b-2	X	gugcaaa	miR-518a	19	ugcaaag
miR-363	X	auugcac	miR-517c	19	ucgugca
			miR-527	19	ugcaaag
miR-106b	7	aaagugc	miR-516a-5p	19	ucucgag
miR-130 Family					
miR-130a	11	agugcaa			
miR-301	22	agugcaa			

Table 2 - Human Embryonic Stem Cell-Enriched microRNAs
Members of microRNA (miRNA) families enriched in human embryonic stem cells (hESCs) as determined in our microarray profiling analyses are further annotated to describe in which chromosome they reside and their seed sequence (nucleotides 2–8 of miRNA). Highly similar seed sequences are similarly colored. All miRNA annotation data are obtained from miRBase, Release 14.0, September 2009 (http://microrna.sanger.ac.uk/)(Reprinted from Stadler 2010 with permission)[335]

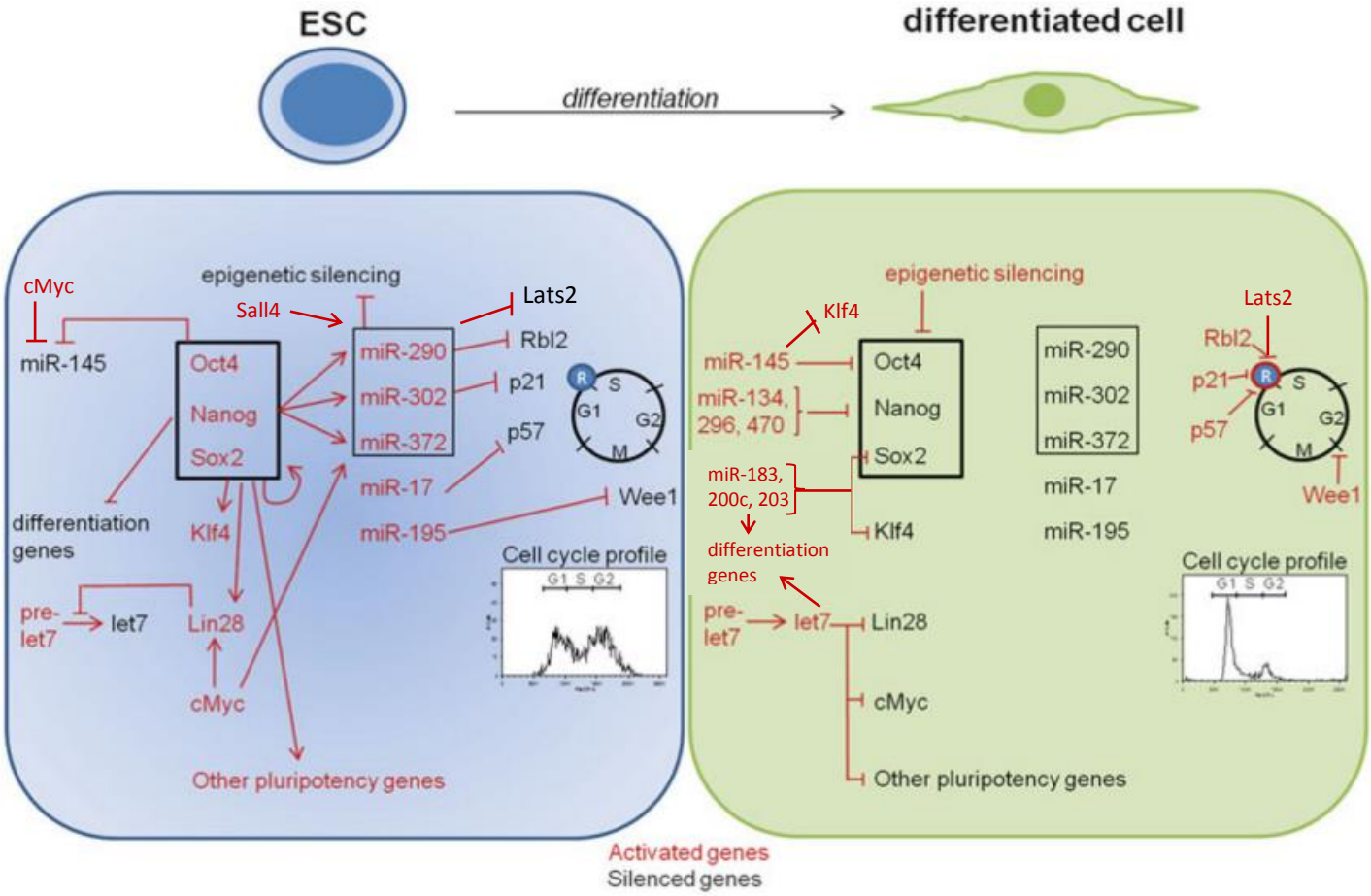


Figure 19 - Role of miRNAs in ESC Self-Renewal, Proliferation and Differentiation
ESCs express a unique signature of miRNAs whose transcription is regulated by a core pluripotency factors (Oct4, Sox2, Nanog). ESC-enriched miRNAs control the specific ESC cell cycle by targeting regulatory proteins involved in G1/S and G2/M transitions. ESC-enriched miRNAs maintain self-renewal capacities of ESCs as well as their pluripotency potential. Differentiated cells express miRNAs such as miR-145 and let-7 that target pluripotency factors and activate differentiation genes. Moreover, cell cycle inhibitors are expressed and cells exhibit a cell cycle dependent of the restriction point (R) (Modified from Mathieu et Ruohola-Baker 2013 with permission)[376]

Oct4, Sox2 and Nanog by inhibiting DNMTs [391](Figure 19). C19MC are the least studied of the ESCC miRNAs, however it has been postulated that they may play similar roles in establishing ESC pluripotency and self-renewal as the other ESCC miRNAs since their seed sequence is quite similar [335].

Other miRNAs have been identified for their role in differentiation [386, 387, 392]. For example, miR-145 is significantly upregulated upon differentiation of hESCs [335]. miR-145 controls ESC differentiation by directly targeting the stem cell factors, thereby silencing the self-renewal program. Interestingly, miR-145 promoter is repressed by Oct4 in hESCs. Its loss therefore impairs differentiation and allows the expression of Oct4, Sox2, and Klf4 [393](Figure 19). miR200c, miR183 and miR203 have been identified as stemness-inhibiting miRNAs since they cooperate in the repression of pluripotency genes Sox2 and Klf4 and the expression of differentiation genes in differentiated cells [394], while miR134, miR296 and miR470 repress expression of Oct4, Sox2 and Nanog [395]. Mature lethal-7 (let7) miRNA is also responsible for expression of differentiation genes in differentiated cells, once Lin28 can no longer repress its conversion from an immature pre-let7 [396, 397]. Let7 is also responsible for suppressing Lin28, cMyc and Sall4 expression in differentiated cells [397](Figure 19).

Since fully reprogrammed iPSCs have a similar miRNA profile as ESCs, scientists have also studied the expression of miRNA during the reprogramming process and noted that ESC-enriched miRNAs are expressed during the early phases of reprogramming [390, 392, 398, 399]. Furthermore, since there is a functional link between miRNAs activation and Oct4, Sox2, cMyc, Lin28, Klf4, Nanog expression levels [390, 400], the role of miRNAs in reprogramming is of great interest. miRNAs have now been used in conjunction with other factors to enhance reprogramming [213] as well as on their own to induce reprogramming [206, 217, 391]

(Figure 20, Panel A).

miR-200s and miR-205, usually triggered by the BMP pathway [322], enhanced reprogramming by inhibiting Zeb1 and 2 mesenchymal-associated factor, therefore allowing expression the MET-associated marker E-cadherin [334, 401, 402](Figure 20, Panel B). Judson's group studied the ability of miRNA-290 cluster (ESCC) to enhance reprogramming of MEFs by lentiviral vectors of OKSM factors [213]. The group co-transfected the miRNAs on day zero with other factors, then again on day 6. Their results indicated that miR-291/3p, miR-294, and miR-295 significantly enhanced reprogramming with the OKS factors[213]. Similar experiments showed that miR-302 and miR-372 clusters, enhanced OKS and OKSM retroviral reprogramming in human cells (Figure 20)[213, 403]. Subramanyam's group further showed that human miR-302b and human miR-372 inhibit expression of genes that regulate the cell cycle (p21), and epithelial to mesenchymal transition (TGF-βRII), enhancing reprogramming by inducing proliferation and MET [403](Figure 20, Panel B). miR-106 and miR-93 have been shown to enhance OSK and OSKM reprogramming in the same way as miR-302 and miR-372 [399](Figure 20, Panel B). Liao's study confirmed Subramanyam's results and further showed that miR-302 and miR-367 increase MET [404]. Lin et al's group studied the epigenetic effects of miR-302s on reprogramming factors since this particular miRNA targets lysine-specific histone demethylases such as: amine oxidase family (AOF 1 and 2), LSD1, and methyl CpG-binding protein (MECP)[391]. The group was successful in producing iPSCs by transfecting human hair follicle cells (hHFCs) with a DOX-inducible plasmid vector containing the miRNA by electroporation. Further analysis by bisulfite DNA sequencing, immunostaining, and northern blotting showed that iPSC induction by miR-302 involved suppression of AOF2 which

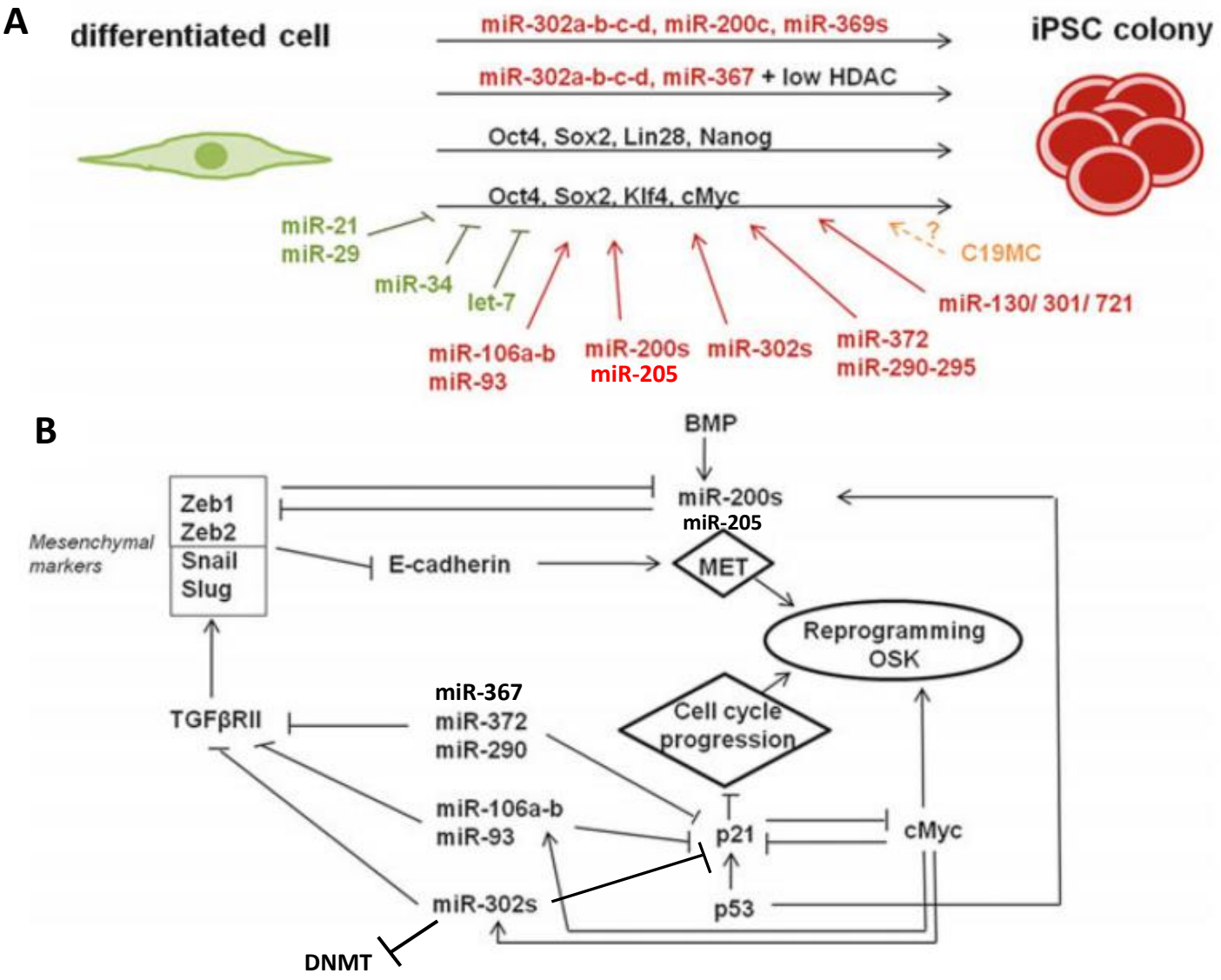


Figure 20 - Functions of miRNAs in Cellular Reprogramming
[A] Overview of the effects of miRNAs on iPSC formation. miRNAs beneficial for iPSC induction are represented in red while miRNAs shown to repress iPSC formation are in green. In orange are the microRNAs whose function has not been tested yet during reprogramming of somatic cells into pluripotent stem cells. [B] Mechanisms of action of miRNAs during the reprogramming process. OSK: Oct4, Sox2, Nanog. MET mesenchymal to epithelial transition. (Modified from Mathieu et Ruohola-Baker 2013 with permission)[376]

destabilizes DNA methyltransferase 1 (DNMT1) and inhibits its ability to methylate DNA. This demethylation enables activation of Oct3/4, Sox2, and NANOG transcription factors required for reprogramming [391](Figure 20, Panel B). Anokye-Danso's group also efficiently produced mouse (from MEFs) and human (from human foreskin and dermal fibroblasts) iPSCs by transfecting miRNA-302/367 via lentiviral vectors without other exogenous factors [206](Figure 20, Panel A). Interestingly, reprogramming was successful in both species; however mouse somatic cells required the use of VPA. Furthermore, miPSCs showed positive results of viral integration, whereas the human hiPSCs did not. These two species-specific differences in the results reiterate the importance of species-specific studies in the field of iPSCs. This method reached an efficiency of 9.14 % (1600 colonies for 1.75 x 10^4 cells seeded) with MEFs, and 11% (11579 colonies/10^5 cells) with HDFs. It is the method that has the highest rate of efficiency thus far [206].

While some group used ESC-enhanced miRNAs to enhance reprogramming, others looked at inhibiting differentiation-inducing miRNAs by antagomirs, genetic ablation, or antisense inhibitors to enhance reprogramming. For example, inhibition of miR-21 (TGFβ agonist) [169], miR-29 (p53 agonist)[173], miR-34 (p53 target)[405, 406], and Let-7 (LIN28 repressor) [397, 407] were all shown to enhance OSK and OKSM reprogramming.

These studies were successful in demonstrating that miRNAs can be used to enhance reprogramming or induce it on their own. The downfall to these past experiments is that they require the use of vector-based gene transfer to introduce the miRNAs which carry an inherent concern for integration. Miyoshi's experiments addressed these concerns by transfecting mature double stranded miRNAs into mice and human cells at concentrations of 10–50 nM using Lipofectamine[217]. When transfecting all seven miRNAs (200c, 302a, 302b, 302c, 302d, 369-

39, 369-5p), the group was able to reprogram mouse cells with an efficiency of 0.003% (5 clones for 1.5 x 10^5 cells seeded), and human cells with an efficiency of 0.002% (2 colonies per 1 x 10^5 cells) [217]. The decreased efficiency with a direct miRNA transfection could possibly be due to the decreased expression of miRNA, or possibly to an increase HDAC activity in the cells since they were not treated with VPA as in Anokye-Danso's method [206]. Testing this method with the addition of VPA would give a better indication of the efficiency differences between the Miyoshi and Anokye-Danso's methods. Furthermore, although miR-200 clusters are ESC-enhanced [335], and enhance MET in reprogramming cells [322, 334, 402], miR-200c has specifically been identified as a stemness-inhibiting miRNA by a different group [394]. miR-200c may be responsible for the decrease in reprogramming efficiency. Even though efficiency was lower with the direct miRNA method, this method shows potential for clinical application since it does not show the same integration concerns as methods using viral or plasmid vectors [217].

3.3.6 Bacterial Reprogramming

An interesting discovery was made in 2012 while a group was studying the influence of lactic acid bacteria (LAB) on cells other than those of the gastrointestinal epithelium, to which they are usually associated in a symbiotic fashion [408, 409]. The group incorporated LAB into human dermal fibroblast (HDF) cells by trypsinization and co-cultivation. The cells formed clusters within 2-3 days that resembled embryoid bodies formed from hESCs differentiation. Although the cells did not have proliferative capabilities, they had some of the characteristic features of hESCs and hiPSCs. They stained positive for ALP, NANOG, OCT3/4, SOX2 and SSEA-4. The clusters were multipotent, thus differentiating into cells of all three germ layers in culture, and when injected into immunodeficient mice. Authors speculate that the bacteria may be initiating reprogramming of HDF by secreting molecules that stimulate immune system activation, however further studies need to be completed to elucidate the mechanism. The efficiency of reprogramming was not noted in this study, however authors suggest that, with further research and development, bacterial reprogramming with symbiotic bacteria like LAB may present a safe alternative for producing cells needed for cell therapy [410].

Chapter 4 - Applications and Advantages

As is the case with other stem cells, iPSCs have many applications. Their role in basic science, disease modeling, drug development and screening, toxicology testing as well as therapeutics will now be discussed.

4.1 Basic Science

iPSCs are of particular value in developmental biology research since they have the potential to differentiate into all cell types. For example their ability to differentiate into all types of pyramidal neurons made it possible to study cortical development in mice [411]. iPSCs have also been used in basic research to compare phenotypic differences between species. Marchetto *et al* explored the differences in gene expression profiles between humans and chimpanzees by using iPSCs generated from human dermal fibroblasts and frozen non-human primate tissues. Their objective was to elucidate evolutionary trends in gene expression that could explain the phenotypic differences between the two species [66].

4.2 Disease Modeling

An important use for pluripotent stem cells is disease modeling. Essentially, analysis of diseased cells (proteomic, transcriptomic, epigenetic etc.) and comparison to a normal control can help determine the underlying mechanisms of the disease. Elucidating the molecular mechanism of a disease can allow medical professionals to give a molecular diagnosis as well as a clinical diagnosis to their patients. Furthermore, gaining molecular insight about the disease can lead to

the development of appropriate target-based treatments. Prior to the discovery of iPSCs, hESCs were the most commonly used cells in disease modeling. ESC-derived diseased cells can be prepared by genetically modifying existing hESC lines, or by isolating pre-implantation embryos carrying the mutation of a genetic disorder [412-414]. The use of hESCs however is limited in disease modeling since genetic modifications have shown to be difficult, and embryos with pre-implantation genetic diagnosis (PGD) only exists for monogenic diseases [415]. Furthermore, there is a very negative stigma attached to the use of cells that require the destruction of potentially viable embryos. Other cells that could be used for disease modeling include pathological somatic cells however they may be difficult to isolate from tissues and are difficult to maintain in culture [415].

iPSCs have several advantages over hESCs as well as pathological somatic cells. Since they can be generated from a variety of easily accessible somatic cells (from blood, urine, and hair), they eliminate the need for invasive isolation of diseased cells (hepatocytes, neurons or others) and the destruction of embryonic material. Furthermore, iPSCs have been used to produce disease-relevant cells to study monogenic disorders like epidermolysis bullosa [416], and more complex disorders such as Rhett syndrome [417] that ESCs have not been able to model. Using human-specific diseased cells is also advantageous since previous studies have shown that the cellular mechanisms can be species-specific [51], and thus so can be the molecular mechanisms of diseases. One example of this is a study by Seok that showed that inflammatory response to tissue damage was quite different in mice than humans [418]. The use of human cells is important in elucidating the appropriate dysfunctional mechanism in order to target the disease properly.

There have been many diseases modeled using iPSCs since the first induction experiments. In 2014, a review showed that there has been a dramatic increase in the amount of studies published on disease modeling with disease-relevant cells derived from iPSCs. From 2008 to 2011 there were 2, 7, 14, and 43 studies published respectively in disease modeling, while in 2012 and 2013 there were 122 studies published on this topic. The most frequent diseases modeled were neurological (Alzheimer's disease, Amyotrophic lateral sclerosis, spinal muscular atrophy, Huntington's disease and Parkinson's disease)(71 studies), cardiac (long QT syndrome 1, 2 and 3, Catecholaminergic polymorphic ventricular tachycardia type 1 and 2)(26 studies), hematological (sickle cell disease, Fanconi anemia, Chronic myeloid leukemia)(23 studies), and metabolic (Wilson's disease, Diabetes type 1&2)(18 studies). Other pathologies modeled include muscular (Duchenne muscular dystrophy) (6), ocular (Retinitis pigmentosa)(7), skin (Dyskeratosis congenita)(5), imprinting (Pradder-Willi syndrome)(2) conditions, and 30 other random diseases such as: Down syndrome (trisomy 21), Turner syndrome (monosomy X), Hutchinson Gilford Progeria Syndrome, and Schizophrenia [419]. There has also been work done by Liu and Ding on modeling tumors [420], and by Gamm *et al* on modeling retinal degeneration [421]. This review is a good indicator of the growing implication of iPSCs in disease modeling. Albeit the difficulty of establishing appropriate controls for disease modeling using iPSCs [415], many studies have been successful in recapitulating disease phenotypes [422]. One factor to be considered when using iPSCs in disease modeling is the choice of reprogramming method. Integrative methods could potentially cause genomic instability as well as change expression patterns within the cells. To decrease the possibility that results are caused by reprogramming itself, non-integrative methods should be used in disease modeling.

4.3 Drug Development and Screening

Drug development and screening is another avenue for iPSCs use. In 2012, iPSCs-derived motor neurons generated from patients led to the discovery of a chemical compound capable of treating amyotrophic lateral sclerosis (ALS) [423]. A compound that could treat familial dysautonomia was also identified using patient-derived iPSCs [424]. Having human-based in vitro testing of drugs is important since previous studies have shown that the effectiveness of some medications can vary between species and even between individuals of the same species. For example, Lovastatin, a cholesterol-lowering agent was inefficient in rats, however proved quite successful in human clinical trials [425, 426].

iPSCS can also be used in an even more specific manner in drug screening. Different drugs could be tested on patient-derived cells to determine the most efficient drug for each patient. With this information, researchers and medical practitioners can stratify clinical studies and trials into cohorts depending on the responses of each patient's cells to different medications. When only one drug is available, in vitro testing of patient-specific cells allows identification of positive responders and elimination of non-responders prior to clinical trials. Furthermore, analysis of non-responder's cells can lead to the identification of possible molecular mechanisms of the lack of response, and elucidation of an appropriate target-based treatment opportunity for the non-responder group. This was done in the clinical study of the effects of VPA on patients with spinal muscular atrophy [427]. The group performed microarray analysis on neurons derived from patient-specific iPSCs and compared gene expression profiles of their responders to those of their non-responders. From this, they identified an increase in the expression of a fatty acid translocase enzyme (FAT/CD36) in non-responders compared to responders. The group then showed that

CD36 was indeed responsible for the non-responsiveness of the cells to the VPA [427]. Lastly, once drugs have been tested for efficiency, dose-response parameters can also be established for pre-clinical work. Implications of iPSCs in drug development and screening can increase success rates of clinical studies and trials, and therefore improve patient outcomes.

Patient-specific iPSCs could also serve in toxicity testing of non-target cells. Essentially, scientist can produce a variety of somatic cells (like neurons, cardiomyocytes, and hepatocytes) to test drug toxicity levels on these non-targets in pre-clinical trials. Not only can this provide information on toxic drugs, but it can also help determine the appropriate dosage for treatment in a patient-specific manner. Although hiPSCs-derived cardiomyocytes for pharmaceutical cardiotoxicity testing were made commercially available in 2011 [428], their characterization is still ongoing [429]. Since toxicity may be patient-specific, it may be wise to do toxicity testing on a patient to patient basis. It is clear that iPSCs can be a definite asset in drug toxicity testing during pre-clinical trial development.

4.4 Therapeutics

A growing field for iPSC technology is their use in therapeutics, like regenerative medicine or cell therapies. The hope is to be able to generate iPSC-derived somatic cells that would be injectable into the patient tissues in order to regenerate and have some kind of restorative effect on the function of the tissue. One big advantage of using patient-derived iPSC-derived cells (autologous) over ESCs-derived cells (heterologous) is that they decrease the risk of rejection of the cells or tissues. Although early studies showed that undifferentiated iPSCs injection in vivo can stimulate an immune response [77, 430], further studies showed that differentiated cells of

iPSC origin did not have the same fate [431, 432]. Furthermore, pre-clinical models have shown that iPSC-derived somatic cells can be transplanted without immune rejection [433, 434]. Although it is not patient-specific, since iPSCs can be banked like ESCs, an "iPSC stock project" was authorized to bank iPSC lines for future use with histocompatible donors with no other transplant options [435].

One concern to be addressed prior to using iPSC-derived cells in cell therapies is their tumorigenic potential. It is well known that iPSCs produce teratomas once injected into a host as well as tumors in pups from germline competent iPSCs injected into blastocyst. Scientists have suggested that this may be due to the proliferative nature of the cells, the use of oncogenes during reprogramming or the genetic instability caused by integrative methods [32, 48]. What has been debated is wether or not the differentiated cells obtained from iPSCs are tumorigenic. There is hope that this is not the case since iPSC-derived cardiomyocytes showed no sign of tumorigenicity after injection into SCID mice [436]. It was made clear by this study that differentiated cells must be carefully purified prior to injection, since non-differentiated and unpurified cells were shown to produce tumors [436]. It will be imperative that iPSC-derived cells follow rigorous tumorigenicity testing prior to use in therapeutic clinical trials [437, 438].

Since the first induction experiments, many cell-based pre-clinical studies have been conducted using iPS-derived cells. The first was in 2007 for the treatment of sickle cell anemia [439]. Table 3 summarizes some of the most notable pre-clinical studies done thus far, the disease implicated, the iPSC derivatives, as well as the restorative effect of transplantation. These include recovery of Parkinson's disease symptoms [320, 440-442], improved muscular function in muscular dystrophy [443], and enhanced recovery of motor function in spinal cord injuries [444], as well as activity of photoreceptors in age-related macular degeneration and retinitis pigmentosa [289].

Disease model	iPS cell derivatives	Restorative effect
Sickle cell anemia	Hematopoietic precursors (genetic defect corrected by gene therapy)	Normal erythrocyte phenotype restored
Parkinson's disease	Midbrain dopaminergic neurons	Recovery of Parkinsonian symptoms in behavioral tests
Muscular dystrophy	Myogenic progenitors (genetic defect corrected by gene therapy)	Improvement of muscle function
Spinal cord injury	Neurospheres	Enhanced recovery of motor function
Ischemic stroke	Neuroepithelial-like stem cells	Improved functional recovery of stroke-damaged brain
	Neural progenitor cells	Improvement of somatosensory and motor symptoms
	Neural progenitor cells	Graft survival and differentiation to neuronal phenotypes but no restorative effect
Intracerebral hemorrhage	Neuro-epithelial-like stem cells	Significant recuperation of neural function
Limb ischemia	Fetal liver kinase-1 positive cells	Revascularization of the ischemic limb accelerated via increased expression of VEGF
	Endothelial progenitors	Neovascularization
	Mesenchymal stem cells	Attenuation of severe ischemia
Myocardial infarction	iPS cells	Regeneration of infarcted tissue and improvement of contractile performance
	Endothelial progenitors	Neovascularization, reduction of fibrosis and infarction site
Cirrhotic liver	Hepatic progenitors	Liver regeneration
Retinitis pigmentosa	Retinal pigmented epithelial cells	Improved visual function
Age-related macular degeneration and retinitis pigmentosa	Developing rod photoreceptors	Neural activity similar to native photoreceptors

Table 3 - Preclinical Studies Using iPS-Derived Cells in Cell-based Therapeutic Approaches
(Reprinted from Lázaro *et al*., 2014 with permission)[256]

The last study mentioned above has led to the approval of the first human clinical study using iPSC derivatives that is being led by Dr. Masayo Takahashi at the RIKEN Center for Developmental Biology in Kobe Japan [445]. The group produced iPSCs by transfecting human dermal fibroblasts of patients with age-related macular degeneration with the SeV harbouring the four Yamanaka factors. In a two-step process, iPSCs were differentiated into retinal pigment epithelium (RPE) by exposure to Left-right determination factor A (Lefty A), Dickkopf WNT signalling pathway inhibitor 1 (Dkk1), and Activin A, then into RPE with photoreceptor capabilities with exposure to Taurin, Shh and retinoic acid [289, 446]. Sheets of cells are then transplanted into the eye to restore vision.

The first patient received her transplantation in September of 2014, however in July of 2015, it was announced that the clinical study had to be put on hold after the second patient's cells did not pass the genetic stability testing needed for transplantation [447, 448]. Although this news may have been a shock to some, from the information presented in this review about the disadvantages of using pluripotency factors, and viral systems for reprogramming, it is not surprising that the cells transfected with OKSM SeV developed genetic mutations. The nature of the genetic instability is not quite clear since one reference states that the mutations that the cells have suffered were induced by the reprogramming process [447], while another states that it is unclear if it was a pre-existing somatic cell mutation [448]. It is an unclear future for the study since it is unknown as of yet if Dr. Takahashi will work on generating patient-specific iPSCs by a different method or simply continue using the allogeneic cell lines that are being produced in Yamanaka's lab [449].

This new development in the world of iPSC technology demonstrates the importance of stringent validation testing during these clinical studies as well as the importance of focusing time and

energy on developing effective reprogramming methods that will generate genetically stable iPSCs that are safe to use in a clinical setting. Many groups still use the original factors, two of which are oncogenic. Furthermore integrative techniques, although useful in achieving high exogenous gene expression, could cause genomic instability and mutations in the host DNA. The use of non-integrative methods, non-oncogenic factors, such as small molecules and/or miRNA may prove to be better options for generating iPSCs that will later be used in therapeutics.

Another component of iPSC generation that has not been discussed is the feeder layer of cells. Mouse embryonic fibroblasts are the most commonly used feeder cells for human iPSC generation; however this could be a possible source of contamination for cells that need to be histocompatible. Many groups have shown that iPSCs can be successfully generated without a MEF feeder layer, using synthetic xeno-free media with different induction methods [185, 308, 450, 451]. Specific protocols for integration free, xeno-free reprogramming would have to meet the Good Manufacturing Practice and clinical-grade requirements prior to clinical trials [377, 452, 453]. Furthermore, the same Good Manufacturing Practice status for differentiation and isolation protocols would need to be achieved prior to the use of iPSC-derived cells in cell-based therapeutic clinical trials.

Chapter 5 – Conclusion

It is clear by this review that the generation of induced pluripotent stem cells less than ten years ago was a considerable factor in many discoveries that would not have been possible with the use of other stem cells. Their induction has also allowed for the expansion of another branch of cells which have been used in many applications in basic science research, disease modeling, drug development and screening, toxicology testing as well as therapeutics.

In the past nine years, significant efforts have been made to properly characterize iPSCs and understand the cellular mechanisms of the reprogramming process to elucidate more efficient and safe induction methods. From many of these studies, particularly the results of the first human clinical study, it is clear that some methods still carry safety concerns that need to be addressed prior to their use in human clinical trials.

Although the elaboration of a plethora of different techniques from different groups has led to progress in cell choice, efficiency and safety, the lack of consistency in methods has also increased the challenge to produce a standard reprogramming protocol that can be applied to all cell types for all cell applications. Scientists have been able to analyse reprogramming cells with time-scale studies, as well as completely reprogrammed cells in order to elucidate what cellular mechanisms have been turned on and off to allow reprogramming; for example, the decrease of expression of somatic-related genes and increase in expression of pluripotency-related genes, as well as the epigenetic changes needed to allow these modifications to occur in the cells. However, they have not been able to explain the minimal requirements needed for cellular reprogramming, nor why only such a small percentage of cells (under 1% in most studies)

actually reprogram. This leaves the scientific community with many questions about the reprogramming process and the use of iPSC-derived cells. Why are only certain cells reprogramming? If the cells are all of the same type, why are they not all transforming uniformly? What is particular to the cells that can successfully reprogram from those of the same colony that cannot reprogram? It can be postulated that only those cells that are unable to protect themselves against these foreign substances will be able to reprogram. It is also possible that only the cells that have been subjected to the appropriate factors or molecules in a specific order, or in sufficient amounts will be susceptible to this internal rewiring. These theories have yet to be verified or even truly examined. To date, there is no magic formula for producing iPSCs. The key to reprogramming has not been elucidated.

There may never be a magic formula to reprogramming, however elucidation of the minimal key components that need to be triggered in the cell, regardless of cell type, need to be discovered if reprogramming efficiency is to be increased significantly. In order to achieve this result, broad-scale transcriptomic, epigenetic, and proteomic analyses will need to be conducted on iPSCs derived from different cell types. Furthermore, it will be of primordial importance that time, energy, and funds be spent on performing these analyses on iPSCs that have been reprogrammed by methods that have shown to pass all appropriate validation (genetic stability) and safety criteria. It would be an unfortunate waste for scientists to be using techniques that could affect the validity of their results or would limit the use of cells later on. Since there are multiple elements that still need to be addressed in iPSC technology, it is unclear if or when a safe, efficient, comprehensive method for achieving reprogramming of somatic cells will be developed.

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