Changing Fate: Cellular Reprogramming

by

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ABSTRACT OF THE THESIS

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Tissue and organ transplantations have historically been limited to allogenic donor grafts. Allografts can restore some degree of functionality to the transplant site, but they are a less-than-optimal substitute for patient-derived materials, as they require patient immunosuppression. The ideal clinical approach is to replace damaged tissue and organs with grafts grown directly from cells harvested from the patient. The induction of pluripotency in fibroblasts was one of the first experiments to show that cell fate could be reprogrammed, to a pluripotent state, by overexpressing four transcription factors – Oct4, Sox2, Klf4, and Nanog. Later research by other groups discovered that cells could also be directly reprogrammed into other cell types, without going through a pluripotent intermediate step. Direct cell reprogramming presents a method to generate a source of patient-specific graft tissues and organs. In this thesis, I aim to provide a molecular mechanistic model for direct cell reprogramming. Evidence suggests a model in which pioneer molecules make a cell competent for reprogramming by gaining a foothold at key promoter sites, thereby making the binding site accessible to epigenetic modification by chromatin remodeling complexes.

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ABBREVIATIONS

Abbreviation	Meaning
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
CpG	Cytosine-Guanine island
DNA	Deoxyribonucleic acid
EGFP	Enhanced green fluorescence protein
ENCODE	Encyclopedia of deoxyribonucleic acid elements
ES	Embryonic stem cell
GABA	γ-Aminobutyric acid
HSC	Hematopoietic stem cells
ICM	Inner cell mass
iN	Induced neuronal cell
iPS	Induced pluripotent stem cell
JAK	Janus kinase inhibitor
MEFs	Mouse emybronic fibroblasts
miRNA	Micro-ribonucleic acid
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MSCs	Mesenchymal stem cells
mV	Millivolts
NHEKs	Neonatal human epidermal keratinocytes
OPC	Oligodendroglial progenitor cell

OSKM	Oct4, Sox2, Klf4, c-Myc
pM	Motor neuron progenitor
pMN	Motor neuron progenitor domain
PRC	Polycomb repressive complex
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SON	Sox2, Oct4, Nanog
TF	Transcripton factor
Thr118	Threonine at position 118 in primary amino acid sequence
V2 interneurons	Ventral interneuron group 2

1. INTRODUCTION

1.1 Historical Review

The 2012 Nobel Prize in Physiology or Medicine was recently awarded to Drs. Yamanaka (Japan) and Gurdon (U.K.) for their pioneering studies on cell reprogramming. The ability to reprogram somatic cells into a novel cell type has tremendous potential for regenerative medicine. Historically, the majority of tissue and organ transplants have come from genetically non-identical donors (i.e. allografts). However, the use of allogeneic donor tissues for transplantation in humans has some significant drawbacks including immune rejection. To decrease that risk, patients are required to take immunosuppressant medications that make them susceptible to infection. Hematopoietic stem cell transplantation, a procedure where a patient's immune system is replaced by an allogeneic transplant, is generally performed in high-risk with severe disease (e.g., leukemia). The graft scenario ideal is to replace a tissue with an identical match – and cellular reprogramming allows us to do that. Autologous transplant tissue is preferable because it eliminates the risk of tissue rejection.

It wasn't long ago that many biologists believed cell fate was permanent and irreversible. In 1952, Briggs and King published a study in which they transferred embryonic cell nuclei of various developmental stages into enucleated *Rana pipiens* frog eggs. When nuclei were taken from embryos in the blastula stage, the animals showed normal development. However, when nuclei were taken from later stage gastrula embryos, there were significant developmental abnormalities. These results led the team to conclude that as cells differentiate, they undergo permanent changes to the nucleus

(Briggs and King, 1952; Gurdon and Melton, 2008). Several years later, Gurdon and Uehlinger performed a similar study in *Xenopus laevis*, but observed a very different phenomenon. In their experiment, they transplanted into the egg nuclei cells that were taken from the intestinal epithelia of tadpoles and observed developmentally normal animals from the embryos. This led them to conclude that in the genomic makeup of the nucleus hasn't changed and that even fully differentiated cells still have the genetic content necessary to make all cell lineages (Gurdon and Uehlinger, 1966; Gurdon and Melton, 2008).

It is also possible to take multiple nuclei and inject them into an oocyte during the first meiotic prophase. Interestingly, transplantation alone results in the reprogramming of the nuclei to express pluripotency markers -- and importantly, no cell divisions are necessary (Gurdon and Melton, 2008).

A third nuclear reprogramming technique involves the fusion of two cells into a heterokaryon. Strikingly, when treated with cell-cycle inhibitors, the larger of the two cell nuclei actually reprograms the gene expression of the smaller cell. Just as with the multiple nuclei transfer experiments, cell division was not necessary for reprogramming to occur. These data indicate that there must be some trans-acting regulatory factors from the dominant cell that are able to control the genome of the subordinate cell (Gurdon and Melton, 2008).

1.2 Recent Developments in Nuclear Reprogramming

In 2006, Takahashi and Yamanaka pioneered a new frontier in cellular reprogramming. In a seminal paper, they identified four transcription factors (*Oct3/4,*

Sox2, Klf4, and *c-Myc*) that trigger a dedifferentiation of fibroblasts to induced pluripotent stem (iPS) cells (Takahashi *et al*., 2006). An important precursor experiment by a different group showed that the overexpression of one factor, MyoD, was sufficient to reprogram some non-muscle cell types into muscle cells, but the transformation was not always permanent and required selection for MyoD expression (Weintraub *et al.*, 1989). However, Yamanaka's study was the first demonstration that transgenes can permanently redirect the phenotype of a fully differentiated cell type. These iPS cells share key features of embryonic stem (ES) cells, including the ability to self renew and the ability to generate any germ layer cell type. Thus, the resultant iPS cells have reverted to a very early embryonic cell type, representing a true lineage reversion that is different from the abnormal transformation seen in neoplasia. This iPS cell reversion process is slow, inefficient, and poorly understood. The process can take up to 3-4 weeks *in vitro* and less than one percent of cells that express these transgenes become true iPS cells.

Individual roles for each of the four key factors are starting to be revealed. c-Myc is not necessary for iPS cell induction to occur, although it increases the efficiency (Wernig *et al*., 2008). c-Myc may act as proliferation enhancer as well as promote the autoregulatory loop of the endogenous *Oct4*, *Sox2*, and *Nanog* genes (Wernig *et al*., 2008; Boyer *et al*., 2005). c-Myc has well over 1,000 cis-regulatory gene target sites and it is not known which, if any, are critical to this process. Likewise, *Klf4* is not essential, as Huangfu and colleagues induced pluripotency in human fibroblasts using only *OCT4* and *SOX2* overexpression (Huangfu *et al*., 2008). Klf4 may also contribute to conversion efficiency by promoting proliferation. Finally, a follow-up experiment showed that neonatal human epidermal keratinocytes (NHEKs) could be reprogrammed by combining

exogenous Oct4 with specific pharmaceutical inhibitors. These results also indicate that the developmental 'age' of the target cell (*i.e.*, embryonic versus adult fibroblasts) is also a determining factor (Zhu *et al*., 2010).

Thus, in the sixty years since Briggs and King, molecular biology has developed a transgene-mediated approach to reprogram somatic nuclei, or intact somatic cells, into pluripotent stem cells. The field is now moving forward in order to directly reprogram fibroblasts into any defined cell type and this emerging technology has tremendous implications for regenerative biology and clinical therapeutics. This thesis will explore the mechanistic details of transgene-mediated cell reprogramming in order to construct a model of how a differentiated cell goes through the process of fate change.

1.3 Mechanistic Model

This thesis hypothesizes that direct cell lineage conversion represents epigenetic chromatin modifications at cis-regulatory sites (*e.g.*, transcription enhancers, promoters) of key and specific "master regulator" genes. The targets of these chromatin alterations include helix-loop-helix transcription factors, microRNAs, chromatin remodeling complexes and histone chaperones.

Cell state maintenance requires the selective activation and repression of state-specific genes. For example, for an ES cell to remain as an undifferentiated cell, pluripotency genes must be expressed and germ layer-specific genes must be silenced. Likewise, differentiated cells must silence pluripotency genes and activate lineage-specific genes. In 1969, Britten and Davidson proposed a model in which cell maintenance cues are propagated in top-down gene regulatory pathways. External signals received by sensor genes (master regulators) pass on the signal to integrator genes

that regulate the activity of downstream producer genes (slave factors) that produce differentiated structural and functional cell characteristics (Britten and Davidson, 1969). For ES cells, we have now identified the cell maintenance cues, their master regulator targets, and factors that can reprogram fibroblasts to begin expressing this hierarchy of ES cell-specific genes. In the next section, we will explore the specific mechanisms and molecular players involved in cell maintenance and cell fate.

2. UNDERSTANDING PLURIPOTENCY

2.1 Ground State Pluripotency

Embryonic stem cell pluripotency serves as a convenient model for studying the regulatory mechanisms involved in the maintenance of cell phenotype. The characterization of key molecular players important for ES cell self-renewal, and maintaining their competence for pluripotent differentiation during embryogenesis, has identified key transcription factors needed for ES cell phenotype maintenance (Fig. 1). Likewise, understanding the process of differentiation during iPS cell reprogramming is a particularly important model to study in order to harness the potential of direct cell reprogramming (transdifferentiation).

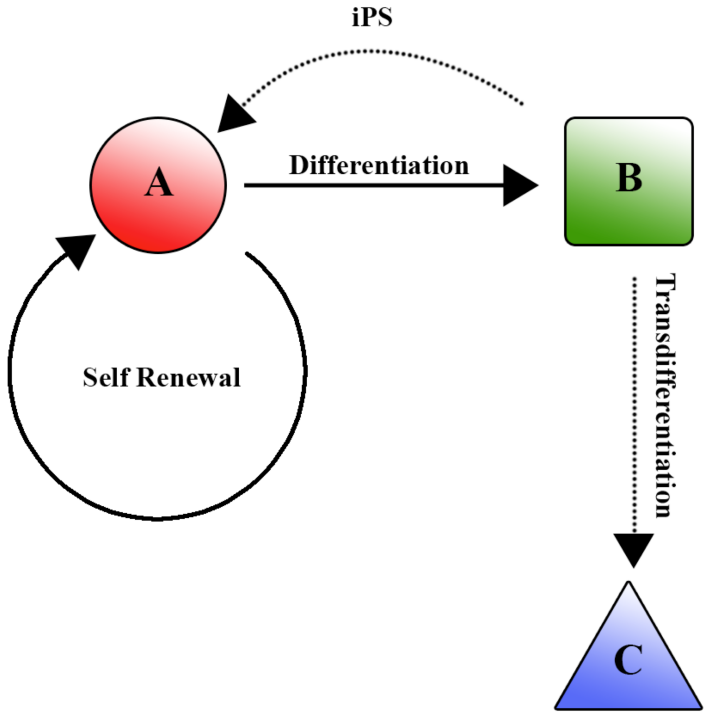

Figure 1. Cell differentiation and reprogramming. Pluripotent cells (A) can undergo either self-renewal or differentiation. Differentiated cells (B) can converted back into pluripotent cells via iPS or can be directly converted into other cell types (C) via transdifferentiation.

Embryonic stem cells in the ground state actively maintain pluripotency and self-renewal pathways while ensuring readiness to activate differentiation pathways. A host of regulatory factors maintain this state by silencing differentiation-specific regulators and promoting the expression of pluripotency regulators. Cellular regulatory mechanisms include transcription initiation and elongation, mRNA stability, signal transduction, and chromatin modification.

Expression of Nanog is one of the earliest markers for epiblast cells in the inner cell mass and plays an important role in maintaining pluripotency (Silva, *et al.* 2009). During very early stages of mammalian embryogenesis, the zygote forms the blastocyst, which consists of a spherical layer of extraembryonic cells called the trophoblast

surrounding a inner cavity containing the inner cell mass (ICM) (Selwood *et al*. 2006). The ICM then develops into the extraembryonic hypoblast and embryonic epiblast cells, with Nanog expression completely localized to the epiblast (Silva, *et al.* 2009). Silva and colleagues concluded that Nanog acts as a line of demarcation between ICM cells destined for pluripotency and those that will differentiate into extraembryonic tissue. In female mice, the paternal X chromosome is inactive during the very early stage of embryogenesis, and its subsequent reactivation in ICM cells is also a marker for pluripotency (Silva *et al.,* 2009). ICM cells that are positive for X reactivation also show co-localized expression of Nanog, and when Nanog is deleted, the ICM do not mature into pluripotent epiblast cells, but either differentiate into trophoblast or undergo programmed cell death (Silva *et al*., 2009).

A second master regulatory factor for pluripotent ES cells is Oct4. During gastrulation, the embryonic epiblast cells generate extraembryonic mesoderm, which combines with the trophoblast and the hypoblast to create the placenta and the yolk sac, respectively (Selwood *et al.*, 2006). Oct4 is initially expressed in all ICM cells along with Nanog. Oct4 is also expressed in trophoblast cells and hypoblast cells expressing the markers Gata4 and Gata6, whereas Nanog does not co-localize at all with Gata4/6 positive cells (Silva *et al*., 2009; Okamoto *et al.*, 2004). Thus in the ICM, the Nanog-positive and Oct4-positive cells are the pluripotent founders that generate the embryonic germ layer.

Pluripotent ES cells are perhaps the most thoroughly studied mammalian cell type, and can serve as a model for understanding how cell identity is established and maintained. At the highest level, the transcription factors Sox2, Oct4, and Nanog (SON)

act as master regulators for maintaining pluripotency and self-renewal in ES cells (Chen *et al.*, 2008). Together, they coordinately regulate 353 known genes that include both protein coding and non-coding RNAs (Medvedev *et al.,* 2008). Sox2, Oct4, and Nanog also positively regulate their own promoters, creating positive-feedback loops for promotion of an undifferentiated state (Fig. 2A) (Young, 2011). Oct4 knockout cell lines containing an inducible transgene differentiate into trophectodermal cells when the expression of Oct4 was either below 50% or above 150% of endogenous levels, indicating a crucial role for Oct4 in ES cell ground state pluripotency (Niwa, *et al.,* 2000; Medvedev, *et al.,* 2008). Similarly, knockout of Nanog in primate ES cells resulted in a loss of pluripotency and expression of endoderm and trophectoderm markers (Yasuda *et al.,* 2006). Thus, these master regulatory genes control and coordinate the expression of a battery of effector genes that determine ES cell identity. As we will discuss below, the introduction of these master regulators can also co-opt (reprogram) fibroblasts into the ES cell phenotype.

2.2 On-Off Regulation

Gene expression is regulated through the actions of trans-acting factors (*e.g.*, activators and repressors) that interact with chromatin. Sox2, Oct4, and Nanog serve as site-specific gene activators by binding to cis-regulatory DNA enhancer sites located at a distance from the promoter of their target genes, then recruiting cofactors including RNA polymerase II (Young, 2011; Kagey *et al.,* 2010). These large protein complexes activate transcription by bridging the gap between the promoter and enhancer regions of SON-

regulated genes (Young, 2011; Kagey *et al.,* 2010). They indirectly recruit cohesin and create a looped chromosomal region between the enhancer and promoter.

The silencing of non-essential genes is a second level of transcriptional control of cell identity. For ES cells, this means silencing the expression of genes that are not required for maintaining pluripotency and self-renewal (Fig. 2B). For example, Cdx2 is a trophectoderm lineage factor whose repression is important for the maintenance of pluripotency (Yeap *et al.,* 2009; Young, 2011). Gene silencing mechanisms include CpG methylation of promoter and enhancer sequences, and histone protein modifications (e.g., methylation, acetylation). Proteins including SetDB1 and the Polycomb Repressive Complex (PRC) are two examples of gene repressors. SetDB1, which methylates histone subunit 3 (H3) on a specific lysine residue (H3K9me3), represses Cdx2. Oct4 is involved in this repression via an interaction between the sumoylated form of SetDB1 and the SUMO-interacting motif (SIM) of Oct4.

For any given cell type, the majority of the genome is maintained as either completely silent heterochromatin or a silenced but actionable 'bivalent' state. The second example of gene repressors, PRCs, can maintain genes in either the "on" or "off" state (Fig. 2C). The PRCs form two repressive histone modifications – mono-ubiquination of histone 2A (K119ub1, by PRC1) and trimethylation of H3 (K27me3, by PRC2) (Gao *et al.*, 2012; Margueron *et al.*, 2009). The PRC also recruits the serine-5 phosphorylated RNA Polymerase II to the silenced genes via the ubiquitination of H2A (Stock *et al.,* 2007; Young, 2011), suggesting that the PRC maintains a bivalent gene that is repressed but poised for activation. When two PRC subunits (Ring1A, Ring1B) were deleted, H2A was no longer ubiquitinated and bivalent genes were no longer poised for

transcription initiation (Stock *et al.*, 2007). This data indicates that the PRC plays a critical role in maintaining the bivalent nature of lineage specific genes in ground state ES cells.

Non-coding RNAs may also play a significant regulatory role in the cell state. These small RNAs are post-transcriptional regulators that control mRNA steady state levels, generating a RNA-induced silencing complex to target mRNAs for dicer-dependent destruction. In particular, miRNAs have been identified as important factors for self-renewal, pluripotency, and cell differentiation. Moreover, Sox2, Oct4, and Nanog regulate a subset of miRNA genes expressed in ES cells (Young, 2011). Cell fate miRNA genes remain in a bivalent state and are repressed in ES cells by the PRC (Marson *et al.,* 2008). During differentiation, lineage-specific miRNAs become expressed and may have a role in post-transcriptional removal of ESC mRNAs (Marson *et al.,* 2008).

Together, a small core of master regulators orchestrate the many molecular slaves necessary for ES cells to either remain pluripotent or to respond to external cues and initiate cell lineage differentiation.

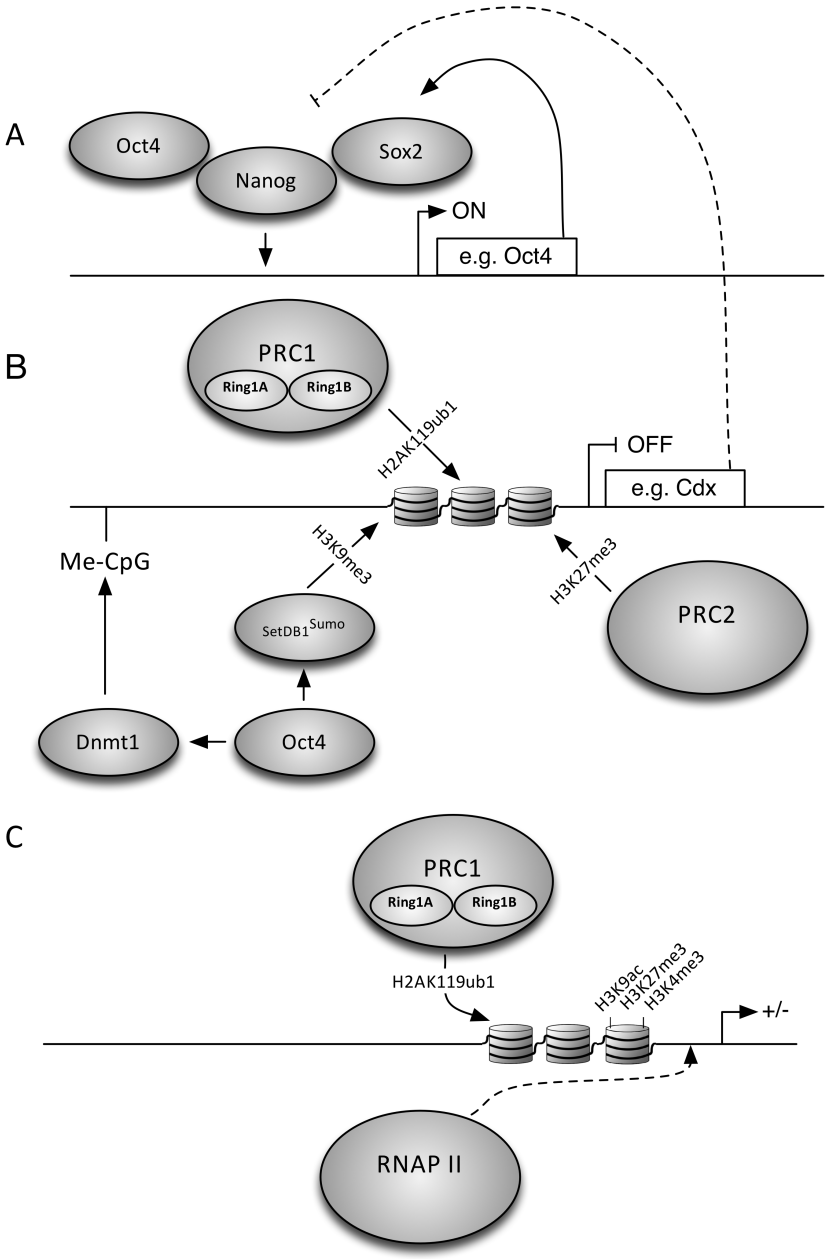

Figure 2. Cell fate gene regulation mechanisms. Oct4, Sox2, and Nanog positively regulate genes necessary for maintaining pluripotency and self-renewal in ES cells (A). In order to remain pluripotent, ES cells must silence lineage-specific genes. Oct4 coordinates the methylation of CpG islands and H3K9 via Dnmt1 and the sumoylated form of SetDB1, respectively. Additionally, PRC1 and PRC2 ubiquinate and methylate H2AK119 and H3K27 residues, respectively (B). The ubiquitination of H2AK119 by Ring1A/B of PRC1 is necessary to recruit serine-5 phosphorylated RNA polymerase and to promote a poised and ready bivalent state in genes necessary for cell differentiation (C).

2.3 Induced Pluripotency

Takahashi and Yamanaka identified four factors (*Oct3/4, Sox2, c-Myc,* and *Klf4*) that trigger a dedifferentiation of fibroblasts to a pluripotent state; the resultant cells were termed induced pluripotent stem (iPS) cells (Takahashi *et al*., 2006). These represent a subset of a group of 24 factors that had been identified as regulatory proteins expressed by ES cells. Before this study, transgenes had never been used to reprogram a differentiated cell. Earlier studies had reported cases of differentiated cells changing fates, but most did not survive critical review for several reasons. One of the issues was that cell identification methods did not include a functional analysis, but rather relied on morphological and immunohistochemical analyses. Because the latter two methods are not always cell-type specific, the data can be unreliable and false positives can't be ruled out (Raff, 2003). An additional issue was the use of mixed populations of donor cells for transplantation (Raf f, 2003). Before it was known that hematopoietic stem cells (HSC) circulate through the blood and are present systemically, three groups all reported that skeletal muscle stem cells were able to generate blood cells (Gussoni *et al*., 1999; Jackson *et al.*, 1999; Pang, 2000; Raff, 2003). However, it turned out that it was because the donor cells were a mixed population consisting of both HSC and skeletal muscle cells; it was actually HSC that generated the blood cells (Raff, 2003). A third weakness in the stem cell plasticity studies was that cell fusion had not been considered as a possible explanation. HSC are able to give rise to all types of blood cells. It has been shown that macrophages, which are derived from hematopoietic stem cells, are able to readily fuse with other cells (Parwaresch *et al*., 1986; Raff, 2003). One study reported that transplantation of hematopoietic stem cells into a diseased mouse liver generated

donor-type hepatocytes (Lagasse *et al.*, 2000). However, a follow up study by the same group discovered that the production of donor-type hepatocytes was actually the result of cell fusion between the donor and host cells and not differentiation of the hematopoietic stem cells (Wang *et al*., 2003). Cell fusion still remains a useful technique for investigating *trans*-acting factors that play critical roles in the reprogramming process (Vierbuchen, *et al.*, 2012).

The underlying mechanism of transcription factor (TF) iPS reprogramming remains an unanswered question. Subsequent studies have now begun to unravel the core functions of these four genes. Oct4 and Sox2 serve as master pluripotency inducers by acting as pioneer molecules that displace histones at target gene promoters. For example, Sox2 induces expression of Sox21, which then represses the cell identity gene Cdx2. Among its targets, Oct4 induces DNA (cytosine-5-)-methyltransferase (Dnmt1) expression, which then works to silence tissue-specific promoters. Cell division is necessary for induction to occur, and the proto-oncogenes Klf4 and c-Myc appear to promote self-renewal and fix any modifications to the chromatin regulatory regions. Consistent with this, these oncoproteins can be substituted by a mutation in the gene encoding the tumor suppressor protein p53 (Sarig *et al*., 2010; Krizhanovsky and Lowe, 2009). The absence of p53 acts like a gain-of-function proto-oncogene to promote cell proliferation and neoplasia, comparable to the effects of c-Myc and Klf4.

Reprogramming using a retroviral-mediated transgene delivery mechanism requires an initial overexpression of the exogenous Oct4, Sox2, Klf4, and c-Myc (OSKM) transgenes (Takahashi *et al*., 2006). However, after pluripotency has been induced, OSKM transgene expression is almost completely silenced in cells expressing

the pluripotency marker Nanog (Okita *et al.*, 2007; Stadtfeld *et al.*, 2010; Takahashi *et al*., 2006). Importantly, transgene overexpression activates endogenous expression of Oct4 and Sox2, which serve as pluripotency master regulators. While overexpression of the exogenous transgenes is critical for the initiation of dedifferentiation, it is not necessary for the maintenance of pluripotency once the switch has occurred.

In a noteworthy advancement, a protocol was recently developed for isolating exfoliated renal epithelial and mesenchymal cells from urine and reprogramming them to iPS cells (Zhou *et al*., 2012). Cells were harvested from a urine sample and plated in dishes containing 10% fetal bovine serum to promote adhesion along with a proliferation medium to support cell division. Cells were transfected with retroviruses containing Oct4, Sox2, c-Myc, and Klf4 and iPS cells were generated at an efficiency of 4%. While this protocol is still subject to the issues associated with iPS cells (e.g., tumorigenesis, low efficiency), it does represent a significant improvement for potential clinical applications since harvesting cells from urine is non-invasive and cheap compared to alternative methods (Zhou *et al*., 2012).

Finally, pluripotency can be induced with only exogenous expression of Oct4 in several cell types including adult neural stem cells, neonatal human epidermal keratinocytes, and hair follicle dermal papilla cells (Kim, *et al*., 2009; Zhu, *et al*. 2010; Tsai *et al*., 2011). This discovery is important because it could refine the induction process by simplifying the requirements for reprogramming. Some cell types (*e.g.*, adult neural stem cells, NHEKs, and keratinocytes) lend themselves well to single-factor reprogramming with Oct4 alone because they have high endogenous expression of the other necessary factors (Kim *et al.*, 2009; Zhu, *et al.*, 2010). In the case of NHEKs, Oct4

induces pluripotency when combined with a cocktail of small molecule inhibitors and activators of regulators and signaling pathways (Zhu *et al*., 2010). It is important to note that although the use of these other cell types allows for simpler manipulation, it does not reduce the risk of iPS-associated tumorigenesis since the process still involves high gene expression of the proto-oncogenes.

It is still unknown if reprogramming occurs in a deterministic or a stochastic manner and why the efficiency and speed are very low (0.5%-10% and several weeks, respectively). In a deterministic pathway, there are defined start and end points with intermediate steps that are fixed and predictable. If the process is stochastic, there is some indeterminacy and randomness such that there is a defined start point, but the way in which the steps are carried out can vary significantly. There is an increasing amount of evidence to suggest that induction occurs through an initially stochastic process during the early phase of ectopic overexpression, followed by a shift to a more deterministic process in the later stages when the cell switches on endogenous transcription factor expression (Buganim *et al*., 2012; Yamanaka, 2009). Yamanaka proposed a model in which the early stages of reprogramming iPS cells is stochastic and is like a ball rolling down the slope of a valley where exogenous reprogramming factors act to push cells up the slope to pluripotency. Sometimes, cells reach the plateau at the top of the hill and remain pluripotent. Other times, there is not enough momentum to move up the pluripotency hill and they get caught at intermediate terraces along the way and are not completely reprogrammed (Yamanaka *et al.,* 2009). At some point in the process, it is necessary to turn off the exogenous transgenes and switch on the endogenous Oct4 and

Sox2 regulatory genes. How and when this occurs still remains unknown and more work needs to be done to elucidate the details of that mechanism.

There are some significant limitations in the practical use of iPS. Specifically, there is a high risk of tumorigenicity. Young cells carry a lower risk of tumorigenesis than older cells. Another significant problem is very low transformation efficiency. Although iPS cells serve as invaluable models for studying differentiation mechanisms, they are not suitable for clinical applications (Takahashi *et al*., 2006; Vierbuchen *et al*., 2011).

2.4 Chromatin Regulators of Cell Identity

In 1979, Taylor and Jones demonstrated that fibroblasts could be reprogrammed into myocytes and adipocytes when cells were treated with 5-azacytidine, which inhibits DNA methylation – thus indicating that chromatin modification plays an important role in nuclear reprogramming (Vierbuchen and Wernig, 2012; Taylor and Jones, 1979).

New evidence from *S. cerevisiae* shows that the intrinsic binding sequence preference of yeast transcription activators positively correlates with histone binding sequences (Charoensawan *et al*., 2012). If that proves translatable to higher eukaryotes, it is plausible that SOX2 and OCT4 act as pioneers to prime the nucleosomes of target genes by displacing histone octamers. If this histone-transcription factor competition is stoichiometric, it would follow that overexpression of select transcription factors – specifically, SOX2 and OCT4 -- would create enough steric hindrance to tip the scale in favor of their binding to target promoters.

Recent experiments have started to elucidate the regulatory roles for both SOX2 and OCT4 in reprogramming (Huangfu *et al*., 2008; Rizzino, 2009). In a mouse intestinal stem cell model, induced expression of Sox2 increased the number of stem cells and repressed endodermal differentiation (Kuzmichev *et al*., 2012). Sox2 also induced the expression of Sox21, a regulatory protein that suppresses expression of the endoderm cell fate regulator protein Cdx2 (Kuzmichev *et al*., 2012). Experiments in mouse ES cells revealed that SOX2 is stabilized when phosphorylated by AKT1 at Thr118 (Jeong *et al*., 2010). Moreover, mouse embryo fibroblasts (MEFs) infected with viral vectors encoding Oct4, Klf1, c-Myc, and a mutated Sox2 (Thr118 → Ala; $Sox2^{T118A}$) had a lower conversion rate compared to the wild-type Sox2. When Oct4 and Nanog are knocked down in mesenchymal stem cells (MSCs), the cells begin expressing tissue-specific markers, indicating an important role in maintaining an undifferentiated state (Tsai *et al*., 2012). Oct4 and Nanog were found to upregulate the DNA methytransferase Dnmt1 by binding to its promoter (Tsai *et al*., 2012). Dnmts are important for maintaining CpG methylation of tissue specific genes, which indicates a role for Dnmt1 in ES cells in the silencing of differentiation-related genes (Tsai *et al*., 2012; Fouse *et al*., 2008). This evidence suggests a mechanism whereby Dnmt1-mediated silencing is transcriptionally regulated by Oct4 and Nanog.

3. TRANSDIFFERENTIATION

3.1 Introduction

Regenerative medicine relies on knowledge from the study of embryogenesis and the factors involved in the differentiation of embryonic stem cells into tissue-specific cell types. Due to the oncogenic risks involved in using cell types derived from pluripotent cells, there is a large incentive to develop a method of reprogramming that does not involve the use of pluripotent cells as an intermediate cell type. To reduce the risk, the ideal would be to skip the pluripotency step altogether, bypassing the oncogenic transcription factors and pluripotent intermediates that can form teratomas.

More recent research suggests a process called direct reprogramming, where one cell type is transformed directly into another without going through a transient pluripotent intermediate (Vierbuchen *et al*., 2010; Marro *et al*., 2011; Lujan *et al*., 2012; Yoo *et al*., 2009; Yoo *et al*., 2011; Efe *et al*., 2011). If a specific set of transcription factors can be used to reprogram fibroblasts to a pluripotent state, it stands to reason that one could also use transcription factors involved in cell phenotype maintenance to reprogram a fibroblast into another cell-type – and that is precisely what has been done with direct programming. Since direct reprogramming is still in its infancy, there are currently some technical limitations with the method. The transformation efficiency is still quite low and is not as efficient as methods like cell fusion. Much of the current knowledge in the field has been derived from studies using mouse cells (e.g., mouse embryonic fibroblasts) and there have only been a small number of experiments that have used human somatic cells. While some groups have shown evidence that functional cells can be created using direct reprogramming, there are still questions about whether or not the cells remain only partially functional (Vierbuchin *et al*., 2010). A variety of cell lineages have been reprogrammed to other types by using specific sets of transcription factors associated

with differentiation in the target cell type. Some examples include the creation of β-cells from adult pancreatic exocrine cells (Zhou *et al*., 2008), multipotent haematopoietic cells from human fibroblasts (Szabo *et al*., 2010), hepatocyte-like cells from mouse fibroblasts (Sekiya, *et al.*, 2011), and cardiomyocytes from mouse fibroblasts (Efe *et al*., 2011).

Transcription factors suitable to reprogram adult pancreatic exocrine cells to β-cells were narrowed down from over 1,000 factors associated with pancreas cell fate, then further dwindled to three factors – *Neurog3*, *Pdx1*, and *Mafa*. Expression of these factors was sufficient to create induced β-cells that resemble pancreatic islet β-cells in terms of morphology, function (*e.g.*, produce insulin), and expression of cell-specific genes (Zhou *et al.*, 2008).

Similarly, transcription factors involved in the normal differentiation of hepatocytes from hepatic progenitor cells were screened and a set of transcription factors were identified – Hnf4α and any one of three Foxa factors (*i.e.*, Foxa1, Foxa2, and Foxa3). When expressed in any of the three combinations, it was sufficient to induce fibroblasts to cells closely resembling hepatocytes (Sekiya *et al.*, 2010).

MEFs can be reprogrammed to cardiomyocytes by first tricking the cells to undergo epigenetic activation by transducing with Oct4, Sox2, and Klf4 (c-Myc was dispensable) and in the absence of leukaemia inhibitory factor (LIF), which prevents the induction of pluripotency but leaves them competent for reprogramming (Efe *et al.*, 2011). After cells reached this state – as detected by a lacZ reporter assay – they were first transferred to media containing a JAK inhibitor along with very specific amounts of fetal bovine serum for nine days then switched to media containing the growth factor

BMP4. After this process, cells were obtained that were functionally and morphologically consistent with a cardiomyocyte phenotype (Efe *et al.*, 2011).

MEFs can be reprogrammed to induced neuronal (iN) cells by combinatorial expression of select transcription factors associated with neuronal lineage. Vierbuchen and colleagues demonstrated that expression of *Mash1*/*Ascl1* alone was sufficient to induce cells positive for markers of immature neurons (e.g., Tuj1, marker for neuron-specific β-tubulin; and TauEGFP, an axonal marker). *Ascl1* co-expression with either *Brn2* or *Myt1l* was sufficient to induce cells with complex neuronal morphologies and functional synapses, thus indicating conversion to mature neuronal cells. The majority of the iN cells were excitatory, although a small percentage expressed inhibitory GABA receptor markers (Vierbuchen et al., 2010).

Expanding on the work by Vierbuchen *et al*., it was later demonstrated that human fibroblasts could be reprogrammed to generate induced dopaminergic neurons (i.e., neurons expressing synaptic receptors for the dopamine neurotransmitter). The factors Ascl1, Brn2, and Mytrl1 along with Lmx1a and FoxA2, which are two transcription factors known to be involved in the developmental pathway for dopaminergic neurons, were necessary and sufficient for conversion. Induced cells were positive for tyrosine hydroxylase, an essential enzyme in the biosynthesis of dopamine, and showed action potential activity when electrophysiology assays were performed (Pfisterer *et al*., 2011).

MEFs can be reprogrammed into induced motor neurons (iMNs) using a set of defined transcription factors including those used to generate iN cells. When *Ascl1*, *Brn2*, and *Mytl1* were combined with *Lhx3*, a subset of cultured MEFs expressed a

transgenic reporter gene specific for motor neurons (*Hb9*:GFP), however they were not sufficient to generate functional motor neurons. When *Hb9*, *Isl1*, and *Ngn2* were co-expressed with *Ascl1*, *Brn2*, *Mytl1*, and *Lhx3*, motor neurons were induced at an efficiency between 5% and 10% (Son *et al*., 2011). These iMNs express characteristic markers of motor neurons including motor neuron-specific transcription factors (e.g., *NeuroD* and *Isl1*) and *ChAT* which codes for an essential enzyme required for the biosynthesis of the acetylcholine neurotransmitter, which is produced in motor neurons. iMNs exhibit action potentials and electrophysiological responses consistent with what one would expect for motor neurons, and they are able to form synapses with muscle cells. Thus, these combined data suggest that certain transcription factors are sufficient to reprogram fibroblasts into neurons, but additional manipulation is required to generate neurons that express specialized characteristics (e.g., acetylcholinergic, dopaminergic, GABAergic).

While fibroblasts serve as a very useful donor cell type for reprogramming due to ease in harvesting, it is still limited in some applications where invasive surgery would be required to treat an affected area. In sensitive regions like the brain and spinal cord, it may instead be advantageous to use an i*n vivo* approach by directly reprogramming cells that neighbor an affected region containing a different cell type. Pericytes are found in the central nervous system as cells that play an important role in maintaining the integrity of the endothelial cell semi-permeable blood-brain barrier (Daneman *et al*., 2010; Armulik *et al.*, 2011). In an important step for cellular reprogramming, it was demonstrated that human cells taken from the cerebral cortex that express pericyte markers (e.g., platelet-derived growth factor receptor-β, neuron-glial antigen 2, smooth

muscle action, CD146, and CD13) can be reprogrammed to iN cells using only *SOX2* and *MASH1* (Karow *et al.*, 2012).

MASH1 is a transcription factor protein that has been previously shown by other groups to play an important role in neuronal identity and somatic reprogramming (Lo, *et al.*, 1998; Vierbuchen *et al.*, 2010). When pericytes were transduced with *MASH1* alone, there was a decrease in expression of the pericyte marker PDGFRβ and an increase in the neuronal marker β-tubulin in a subset of the transfected cells. Transfection with *SOX2* alone had no significant effect on β-tubulin expression. Importantly, expression of *MASH1* and *SOX2* together resulted in a marked increase in the number of cells expressing β-tubulin of approximately 48% (Karow *et al.*, 2012). In addition, ~28% of the cells showed neuronal morphology and ~46% expressed MAP2, a marker for dendrites which may indicate some degree of polarization. It is also important to note that when single-cell time-lapse imaging was performed, there was no evidence to suggest any cell division occurred, which is consistent with what has been observed by other groups doing direct cell reprogramming.

Thus, it seems that while the specific factors required for transdifferentiation may vary from cell-type to cell-type, the mechanism for reprogramming is the same – the epigenetic landscape of a host cell is radically and forcibly altered when exposed to defined sets of transcription factors.

3.2 Transdifferentiation: A Direct Reprogramming Model

Unanswered questions remain about the actual mechanism(s) involved in the process of directed transdifferentiation. The forced overexpression of exogenous defined

factors may either activate or de-repress transcriptional activity at epigenetically silenced promoters and enhancers. It is still unclear whether these factors are serving as master regulators or are merely activators of downstream cell lineage-specific regulators. One proposed mechanism suggests that these ectopic factors serve as pioneer molecules by first gaining a foothold at the promoter sites and then recruiting chromatin remodeling complexes and other transcription factors to the binding site (Vierbuchen *et al*., 2011; Smale, 2010). In a way, each specific locus may need to be reset from the previous hereditary setting and transition to the chromatin marks associated with the target cell type. As this is still a burgeoning field, these mechanisms will be elucidated over the coming few years.

By studying neural development, Yoo and colleagues (2009) were able to provide insight into a mechanism of post-transcriptional gene silencing during the decision for neural progenitor cells to either proliferate or differentiate into neurons -- and those discoveries were later used to directly transdifferentiate fibroblasts into neurons (Yoo *et al*., 2011). The Swi/Snf-like BAF complex contains the BAF53a and BAF53b subunits; BAF53b activity is essential for neurogenesis, while BAF53a inhibits neurite outgrowth by antagonizing BAF53b (Yoo *et al*., 2009). The 3' UTR of *BAF53a* mRNA contains binding sites for specific miRNAs (*e.g.,* miR-9/9*, and miR-124), which suggests that BAF53a expression is repressed by the activity of these miRNAs. Experimentation by the group confirmed that miR-9/9* and miR-124 do repress BAF53a expression and their activity is essential for dendritic morphogenesis. Using this knowledge, they infected human neonatal foreskin fibroblasts with a lentiviral vector containing miR-9/9* and miR-124. Interestingly, the fibroblasts had a marked decrease in proliferation and began

to express neuronal markers (MAP2-positive) and morphological characteristics within thirty days post-infection. In order to optimize transdifferentiation efficiency, miR-9/9* and miR-124 were co-expressed with the previously known neurogenic transcription factors NEUROD2, ASCL1, and MYT1L (Yoo *et al.*, 2011). The results from this experiment show a clear role for chromatin remodeling complexes and their regulators in the mechanism of cell conversion.

4. FUTURE DIRECTION: ENCODE PROJECT, DATA MINING, AND UNBIASED BIOCHEMICAL SCREENS

Direct cell reprogramming techniques have been used to generate several varieties of cell types found in the nervous system, including tripotent neural stem cells (Ring *et al.*, 2012; Thier *et al*., 2012) as well as acetylcholinergic, dopaminergic, and GABAergic neurons (Vierbuchen *et al*., 2010; Pfisterer *et al*., 2011; Son *et al*., 2011). Induced neural stem cells were capable of generating neurons, astrocytes, and oligodendrocytes. To date, an efficient method for generating induced oligodendrocytes from fibroblasts has remained elusive.

Neurons are specialized cells found in the nervous system and generally have three common structures – the cell body (soma), branch-like projections (dendrites), and a single long projection (axon). In a resting state, excitatory neurons maintain a membrane electrical potential of around -60 mV via an electrochemical gradient of sodium and potassium ions (Barnett *et al*., 2007). Neuronal signaling is carried out through a process known as synaptic transmission. Chemical input signals are received

by neurons via the binding of a neurotransmitter molecule ligand to transmembrane receptors that localize to dendrites (Barnett *et al.*, 2007). Some classes of neurotransmitters bind to ionotropic receptors, which are ligand-gated ion channels. Other neurotransmitters bind metabotropic receptors, which utilize a signal transduction mechanism.

When a signal is received by the post-synaptic neuron, an initial depolarization of the selectively permeable membrane occurs as ion channels open and allow ion influx (e.g., sodium ions) and efflux (e.g., potassium ions) across the cell membrane. When the membrane potential has depolarized to a critical threshold, the signal is interpreted at the axon hillock, where the cell body interfaces to the axon (Barnett *et al.*, 2007).

Voltage-gated sodium ion channels will open when the critical threshold is reached and trigger a wave of signal propagation via localized depolarization. The axon of a neuron is sheathed in defined intervals by a lipid-rich layer of myelin. In the peripheral nervous system, the myelin sheath is produced by Schwann cells. In the central nervous system, it is produced by oligodendrocytes, which are a type of glia. Myelin acts as electrical insulator by preventing leakage of ions across the membrane (Barnett *et al.*, 2007). The myelin sheath is broken at intervals called Nodes of Ranvier which contain a high density of voltage-gated sodium channels. The action potential wave travels down the axon by having localized depolarization at one node which then travels through the cytoplasm to the next node – this process is known as saltatory conduction (Barnett *et al.*, 2007; Baumann *et al*., 2001). Thus, the myelin sheath is necessary for fast action potential propagation down the axon.

Disruption of oligodendrocyte function can have very pronounced neurological outcomes. In particular, demyelination of axons has been associated with diseases including multiple sclerosis (MS) and cerebral palsy (Káradóttir *et al.*, 2007). MS is characterized by a host of systemic symptoms including dysphagia, ataxia, pain, and various degrees of vision loss (Poser *et al.*, 1983; McDonald *et al.*, 2001).

Currently, there have been no reports demonstrating the successful direct reprogramming of fibroblasts or other somatic cells into oligodendrocytes. Previous attempts to reprogram fibroblasts into myelin gene-expressing cells using six oligodendrocyte-specific transcription factors (Olig1, Olig2, Sox10, Mash1, E47, and Nkx2.2) were unable to produce cells that endogenously express the myelin genes in sufficient amounts or express an oligodendrocyte-specific transcription pattern (Liu *et al.*, 2010). During embryonic development, embryonic stem cells in the motor neuron progenitors domain (pMN) give rise to both motor neurons (pM) and oligodendroglial progenitor cells (OPC) (Wu *et al.*, 2005). Olig2 is a helix-loop-helix transcription factor gene that is expressed in myelin-producing cells of the central nervous system and in the progenitors from which they are derived. In $Olig2^{-/-}$ knockout mice, neither motor neurons nor oligodendrocytes were produced in the spinal cord, indicating that Olig2 is essential for the development of both cell fates (Takebayashi *et al.*, 2002; Wu *et al.*, 2005). It is important to note that while a deletion of Olig2 halted production of motor neurons and oligodendrocytes, there was a significant increase in the generation of both astrocytes and V2 interneurons (Takebayashi *et al.*, 2002; Wu *et al.*, 2005). It is also noteworthy to mention that astrocytes and motor neurons share a common lineage (Leber *et al*., 1990; Masahira, *et al.*, 2006).

To identify which set of transcription factors is sufficient and necessary to reprogram somatic cells to oligodendrocytes, we propose an unbiased screening method. According to a recent genome analysis, there are 20,687 protein-coding genes in the human genome (Pennisi, 2012). A cDNA library derived from the human fetal brain expressed genes can be used to identify genes capable of reprogramming. By dividing the library into several reprogramming clone groups driven by a CMV promoter, we can transfect cells with these constructs and observe for signs of reprogramming. Olig2 gene expression serves as a useful marker for successful reprogramming to oligodendrocytes because it is exclusively expressed in myelin-producing cells. To detect a phenoconversion, we can use quantitative polymerase chain reaction (qPCR) to measure Olig2 gene expression. Therefore, if reprogramming is observed, a reprogramming group can be divided into smaller groups to narrow down the list of genes that are necessary and sufficient for reprogramming.

While qPCR is useful for measuring changes in a particular gene's expression (e.g., Olig2), the nature of reprogramming seems to heavily involve epigenomic changes. Therefore, additional assays could help to identify those changes in chromatin marks. The ENCODE (Encyclopedia of DNA Elements) consortium has been an ongoing bioinformatics project that has now found that the majority of DNA that was previously characterized as 'junk DNA' actually have important regulatory functions (Pennisi, 2012). Using a computational approach, a potential regulatory region upstream of Olig2 with a high transcription factor binding site density has been identified (Chen *et al*., 2008). Moreover, the ENCODE data has predicted several CpG islands within the potential regulatory region upstream of the Olig2. These regulatory sites may serve as

useful target identifiers to measure epigenetic changes to the Olig2 gene during a reprogramming event.

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