OCT4 AND ENVIRONMENTAL SYNERGISM IN CELLULAR REPROGRAMMING

INVESTIGATING THE ROLE OF THE EXTRACELLULAR ENVIRONMENT IN OCT4-MEDIATED CELLULAR REPROGRAMMING

BY

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Abstract

The overexpression of one transcription factor alone – Oct4 – can alter cell fate. In conjunction with lineage-specifying culture conditions, Oct4 can generate multi-lineage blood and neural progenitors without traversing pluripotency. During this conversion, cells achieve a unique cellular status, distinct from that of the somatic or pluripotent states, termed Oct4-induced plasticity, or OiP. This plastic state is characterized by a morphological shift from spindle-elongated to compact-cuboidal cells, which manifests only with culturing in Reprogramming media, suggesting that the extracellular environment plays an key role in this process. However, the precise component(s) of the growth media that inhibit or permit sustained Oct4-expression remains unknown. In direct support of this notion, we revealed that the serum component of Fibroblast media is a crucial factor in preventing OiP, as only serum-free formulations are able to sustain Oct4 expression and induce the plastic state. These results uncover the importance of the extracellular environment, which works collectively with Oct4 to induce OiP, a synergism previously overlooked in the reprogramming field. Identification of these factor(s) may allow for optimization of media formulations as well as the identification of small-molecules that may improve OiP generation or even substitute for the role of ectopic Oct4. As well, the identification of N-cad as a marker of plastic cells as well as the generation of a screening platform to identify chemical activators of endogenous Oct4 will aid in further understanding the nature of OiP as well as the generation of transgene-free cell products. Taken together, elucidation of the larger role of Oct4 in OiP and pluripotency will enable greater control of cell fate conversion, which is essential for this field to evolve.

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To the rest of the Bhatia lab that I have had the pleasure of working with, I must thank you all for your support and encouragement over the past two years, it truly means a lot. You are all truly fantastic and intelligent people who have made my time here full of fun experiences and more laughing then I ever imagined possible. I wish you nothing but good things in your lives. I will certainly miss all of you.

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Although I'm not quite sure what my future has in store, I hope each and every one of you is a part of it in some way. I know I wouldn't be here without you all, and so I must thank you for making me who I am today.

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List of Abbreviations

	Abbreviation	Full Term
#	2i	2 inhibitors (CHIR99021 and PD0325901)
	4F	4 factors (Oct4, Sox2, Klf4, c-Myc)
	7AAD	7-amino actinomycin D
A	AZA	5-azacytidine
B	bFGF	basic fibroblast growth factor
	BMP4	bone morphogenic protein 4
C	ChIP	chromatin immunoprecipitation
	CR	conserved region
D	DE	distal enhancer
	DNA	deoxyribonucleic acid
	DNase	deoxyribonuclease
	DNMT	DNA methyltransferase
E	EC	embryonal carcinoma
	ECE	extracellular environment
	EGC	embryonic germ cell
	eGFP	enhanced green fluorescent protein
	EpiSC	epiblast stem cells
	ESC	embryonic stem cell (h, human; m, mouse)
F	Fibs	fibroblasts (h, human; m, mouse)
	$Fibs^{eGFP}$	eGFP-transduced fibroblasts
	$Fibs^{Oct4}$	Oct4-transduced fibroblasts
	FITC	fluorescein isothiocyanate
H	HBSS	Hank's buffered saline solution
	HDAC	histone deacetylase
	hdF	human ESC-derived fibroblast-like cell
	HMTase	histone methyltransferase
	HUVEC	human umbilical vein endothelial cells
I	ICC	immunocytochemistry
	ICM	inner cell mass
	IGF-II	insulin-like growth factor II
	iPSC	induced pluripotent stem cell (h, human; m, mouse)
	IRES	internal ribosomal entry site
L	LAP	latency-associated peptide
	LIF	leukemia inhibitory factor
M	MEF	mouse embryonic fibroblast/feeder cells
	MET	mesenchymal-to-epithelial transition
N	N-cad	neural (N-) cadherin
	NEAA	non-essential amino acids
	NPC	neural progenitor cell
	NSC	neural stem cell

O	OAC	Oct4-Activating Compound
	OiP	Oct4-induced Plasticity
	OSKM	Oct4, Sox2, Klf4, c-Myc
	OSNL	Oct4, Sox2, Nanog, Lin28
P	PBS	phosphate buffered saline
	PCA	principal component analysis
	PE	proximal enhancer *or* phycoerythrin (context-specific)
	piPSC	partially-reprogrammed iPSC
	POU	Pit-Oct-Unc
	PS	phosphatidylserine
	PSC	pluripotent stem cell
R	RM	Reprogramming media
	RNA	ribonucleic acid
	RNAi	RNA interference
	ROS	reactive oxygen species
	RT	room temperature
S	SCNT	somatic cell nuclear transfer
	SSEA	stage-specific embryonic antigen
T	TE	trophectoderm
	TGF-β	transforming growth factor β
	TF	transcription factor
V	VC6TFZ	the six small-molecules able to induce the formation of mouse ciPSCs (Valproic acid, CHIR99021, E-616452, Tranylcypromine, Forskolin, DZNep)
	VPA	Valproic acid
W	w/ RM	with Reprogramming media (cultured in Reprogramming media)
	w/o RM	without Reprogramming media (cultured in Fibroblast media)

Declaration of Academic Achievement

This thesis was completed by Michelle Jones, with the following contributions from individuals in Dr. Mick Bhatia's lab:

i. Dr. Ryan Mitchell initially characterized the OiP cellular state, helped to formulate key experiments, and assisted in the interpretation of data;
ii. Aline Fiebig-Comyn sacrificed the mice and harvested their dermis, lungs, and cardiac tissue;
iii. Dr. Borhane Guezguez isolated bone marrow from the mice;
iv. Monica Graham, Aline Fiebig-Comyn and Dr. Luca Orlando aided the isolation and culturing of mouse fibroblast cells.

CHAPTER 1: Introduction

1.0 Preamble

The collective findings in this thesis were primarily inspired by the notion that a single transcription factor alone – Oct4 – can alter cell fate. From the first ESC experiments to the findings of induced pluripotency to direct conversion, Oct4 has – and remains – a core factor in cell fate determination. In order to understand the body of work encompassed within this thesis, we must step backward, and discuss the nature of pluripotency and cell fate. I discuss the classical experiments that, in tandem with essential pluripotency findings, led to the pivotal discovery of induced pluripotency that propelled stem cell and regenerative medicine into a new era.

1.1 The Discovery of Pluripotency

Development proceeds through various states of *potency* – from *totipotency*, to *pluripotency*, to *multipotency* – as cells commit to their final cellular fate (Hanna et al., 2010). In this process, cells become increasingly restricted in their developmental potential until they terminally differentiate and become trapped in their cellular fate. Embryonic stem cells capture attention because of their innate properties; they are immortal and have seemingly limitless self-renewal and developmental potential, which has ignited the field of regenerative medicine. The first embryonic stem cells were generated from mice in the early 1980's by two independent groups, the first using whole cultured blastocysts (Evans and Kaufman, 1981) whereas the second derived these cells from the inner cell mass (Martin, 1981). It was not until the late 1990's that human ESCs

were isolated from the inner cell mass of blastocysts (Thomson et al., 1998). Under the correct conditions, ESCs can be maintained indefinitely *in vitro*, which has allowed for identification of several cellular parameters key in developmental and stem cell biology.

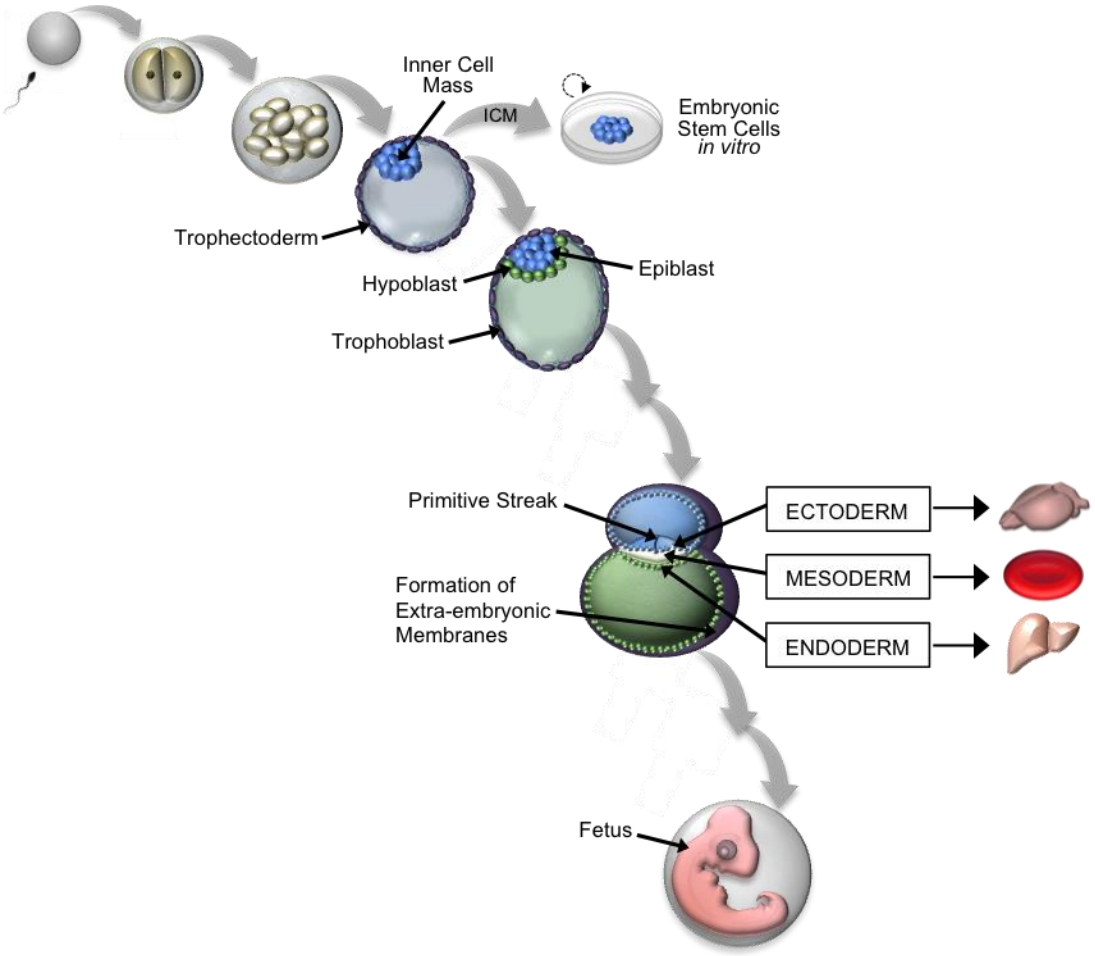


Figure 1. Early human development and the derivation of embryonic stem cells.
The fertilization of an ovum begins a cascade of cell divisions and complex molecular events, which ultimately leads to the generation of an entire organism. In deriving embryonic stem cells, the inner cell mass of the pre-implantation blastocyst is isolated and cultured in pluripotency-supportive conditions that allow for indefinite maintenance *in vitro*.
(Single arrows illustrate few cell divisions between stages, double arrows illustrate multiple cell divisions between stages)

Embryonic stem cells have the inherent ability to both self-renew and differentiate to a specific lineage of one of the three germ layers, ectoderm, mesoderm, or endoderm (Figure 2) (Rossant, 2008). This process of self-renewal follows a hierarchy, beginning with the stem cell at the apex, which can produce multilineage stem and/or progenitor cells, followed by the generation of several differentiated cell types that collectively comprise an entire organism.

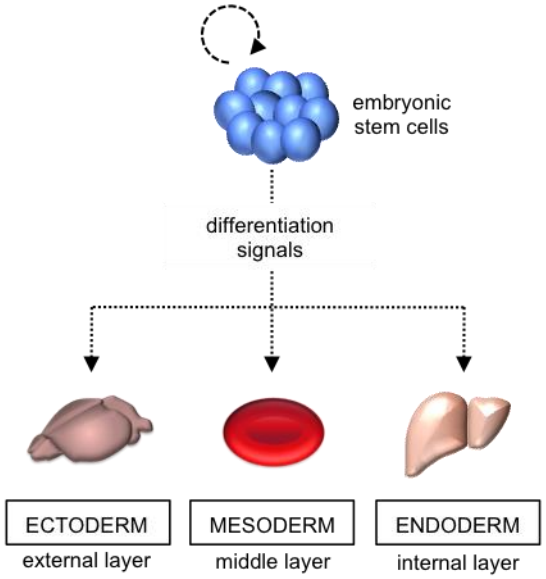


Figure 2. Stem cells display self-renewal and pluripotent properties.
Stem cells can have the unique property of self-renewal, in which they can indefinitely divide to make more stem cells, perpetuating the stem cell pool. These cells are maintained in this pluripotent state through the interplay of a number of key signal transduction pathways, modulated by key transcriptional regulators. With the appropriate cues, stem cells can differentiate into cells of the three developmental germ layers, which include ectoderm, mesoderm, and endoderm.

Due to their high proliferative, self-renewal, and differentiation capacity, ESCs have the potential to be used as a cell therapy in regenerative medicine or to serve as screening

platforms in drug discovery (Rossant, 2008). But very little is known about the mechanisms governing the alteration of cellular fate, hampering the advancement of this technology.

1.2 Nuclear Reprogramming: Alteration of Cellular Fate

Cell fate commitment had been traditionally viewed as an irreversible process, where it was believed that when a cell commits to a particular lineage, it either loses chromosomes or permanently inactivates genes that it no longer requires (Yamanaka and Blau, 2010). This concept is elegantly modeled in Conrad Hal Waddington's "epigenetic landscape model", which depicts a landscape as the developmental hierarchy and a rolling rock as a differentiating cell (Waddington, 1957). In his model, a pluripotent cell, at the peak of a mountain, rolls down the landscape as it differentiates, choosing a specific pathway along its route to determine its final resting state. This is reflective of the natural restriction of cell fate commitment during normal development. As is in nature, this cell cannot "roll upward" back to the pluripotent peak, and is thus permanently fixed at the base.

However, several classic studies have challenged this notion, suggesting that instead cells are *plastic*, and that their cellular fate can change when they are exposed to a different microenvironment (reviewed in Yamanaka and Blau, 2010). Specifically, three approaches to cellular reprogramming – somatic cell nuclear transfer, cell fusion, and transcription-factor transduction – have shown that cell fate may be altered or even reversed to return a somatic cell to a pluripotent state.

1.2.1 Somatic Cell Nuclear Transfer

One of the first notions that committed cells are plastic and can alter their cellular fate came in the early 1950's with Sir John Gurdon. Following the work of Briggs and King, he devised an elegant experiment to test the developmental capacity of differentiated cells using somatic cell nuclear transfer. By introducing nuclei of endodermal cells of various developmental stages into enucleated eggs, Gurdon was able to generate not only swimming tadpoles, but also normal adult frogs, albeit at low frequencies (Gurdon, 1962). Gurdon had demonstrated that the genetic information required for development is contained within the nucleus of *all* cell types, regardless of commitment stage, and is not lost upon differentiation.

Gurdon sought to explain the phenomenon he bore witness to, and postulated two likely possibilities. Firstly, inactivation of certain parts of the genome may occur so that only specific genetic information is available in particular cell types (Gurdon, 1962). Secondly, that the genetic information provided by a nucleus is dependent upon the cytoplasmic environment at any one time, which varies depending on cell type, such that no stable changes are imposed on the genome. In essence, both of Gurdon's ideas were correct, inferring a higher level of genetic control, the epigenome, which not only controls cell fate, but also may be subject to change and/or dynamic regulation upon extrinsic instruction, such as by factors present in the oocyte cytoplasm.

It was more than three decades before this question was addressed in a mammalian context with the successful cloning of Dolly (Wilmut et al., 1997). The remarkable feat clearly demonstrated that during development of mammary and likely

other differentiated cells, there is no irreversible modification or loss of genetic material required for development to term (Wilmut et al., 1997). This provided further evidence for the generally accepted view that differentiation is achieved by changes in nuclear content, but instead by systematic, sequential changes in gene expression brought about by interactions between the nucleus and the changing cytoplasmic environment (Wilmut et al., 1997).

The collective findings of SCNT answered one of the earliest questions of developmental biologists regarding cell fate commitment. For his central role in this, Gurdon would win the 2012 Nobel Prize for Physiology or Medicine alongside the scientist he later inspired, Shinya Yamanaka.

1.2.2 Pluripotent Cell Lines and Fusion Hybrids

Somatic cell nuclear transfer is a powerful tool to prove the developmental potential of a cell, but this technology has its limits (Stadtfeld and Hochedlinger, 2010). Although Gurdon demonstrated that differentiated cells retain all developmental potential within its nucleus, his findings did not speak as to which factor(s) contained within the oocytes cytoplasm harbour the potential to reprogram a cell. This finding came in the 1970's with cell fusion experiments. Cellular fusion, or the merging of two or more cell types to form a single entity, allows the impact of one genome on another to be studied (Yamanaka and Blau, 2010).

The derivation of an immortal pluripotent cell line from teratocarcinomas, termed embryonal carcinoma cells (ECCs), proved to be essential in identifying the mechanisms

underlying cell fate choices (Martin, 1975).

Following the work of earlier cell fusion experiments, Miller and Ruddle, hypothesized that the factors that maintain the differentiated state of mature cells may dominate over those of pluripotent ECCs. To assess this, fusion hybrids of ECCs and thymocytes were generated and assessed for their developmental potential by *in vitro* teratoma formation. Surprisingly, the hybrids displayed no difference in developmental potential compared to the parental ECCs. These results showed that pluripotency in embryonal carcinoma cells was not abolished by the introduction of a somatic genome but instead, ECCs themselves were dominant, apparent by the loss of the somatic cell partner's features (Miller and Ruddle, 1976). Repeated studies using ESCs came to the same conclusion – the pluripotent state can prevail over the somatic state through the action of *trans*-acting factors (Tada et al., 1997; Tada et al., 2001; Stadtfeld and Hochedlinger, 2010). These results are similar to the findings of SCNT, where factors present in the oocytes support early cell development from a mature nucleus. Blau et al. later refined this process by deriving fusion hybrids using naturally multinucleated mouse muscle cells, such that when fused with donor cells, nuclear fusion did not occur (Blau et al., 1983). When these cells were fused with human amniocytes (fetal cells), the expression of four *human* myogenic genes became activated, suggesting that *trans*-acting factors contained in these mouse muscle cells were able to induce muscle gene expression in the human cells (Blau et al., 1983). These studies have provided the first conclusive evidence that previously silent genes could be reactivated in somatic cells by exogenous factors that are capable of influencing gene expression and cellular identity.

1.2.3 Transcription Factor Reprogramming

The third experimental finding leading up to the discovery of iPSCs was the observation that lineage-associated transcription factors, which help to establish and maintain cellular identity can alter the fate of a cell (as reviewed in Stadtfeld and Hochedlinger, 2010).

Experiments by Harold Weintraub and colleagues demonstrated that the overexpression of the myogenic transcription factor MyoD was sufficient to convert fibroblasts into myogenic cells (Davis et al., 1987). The basis for this finding was the observation by Taylor and Jones that treatment of fibroblasts with the nucleotide analogue 5-azacytidine (AZA) resulted in the formation of filamentous structures later identified as syncytial myotubes (Taylor and Jones, 1979).

The observations of cell fate alteration through SCNT, cell fusion, and transcription factor reprogramming provided the rationale for subsequent attempts to reprogram cells beyond the boundaries of their cell lineage and differentiation state, including the pivotal discovery that would ignite the field of stem cell biology.

1.3 The Breakthrough Finding of Induced Pluripotency

1.3.1 Takahashi and Yamanaka

It was the convergence of three scientific principles and technologies over the last six decades that led to an extraordinary discovery that would alter the fundamental ideas of cell fate. In 2006, two Japanese scientists, Kazutoshi Takahashi and Shinya Yamanaka, observed that differentiated cells could be returned to one of the earliest

stages of development – pluripotency – through the ectopic co-expression of transcription factors (Takahashi and Yamanaka, 2006). It was this finding that catapulted forward the field of stem cell biology, offering powerful new opportunities for personalized regenerative cell therapies and modeling of human disease (Robinton and Daley, 2012).

In seeking to circumvent the ethical issues surrounding the derivation of human embryonic stem cells, Takahashi and Yamanaka sought out, and succeeded in, creating embryonic stem cell-like cells from mouse skin cells. The duo performed an elegant screen of 24 pluripotency-associated candidate genes and upon the successful derivation of pluripotent cells, determined the minimally required core set. In this, they identified four genes – *Oct4*, *Sox2*, *Klf4*, and *c-Myc* – that, when collectively expressed in mouse fibroblasts, were sufficient to induce the formation of cell colonies with morphological, functional, and growth properties of ESCs. These properties include the ability to self-renew, or divide to make identical copies for a prolonged period of time without differentiating, as well as pluripotency, which is the ability to generate cells from all three germ layers (endoderm, mesoderm, ectoderm) (Figure 2). This proof-of-principle study clearly demonstrated that the route from a pluripotent cell to a terminally differentiated one is not unidirectional, and can be reversed by overexpression of the Yamanaka factors.

The generation of "induced pluripotent stem cells", or iPSCs, inspired a flurry of follow-up studies, with successful reprogramming quickly translating to human cells (Takahashi et al., 2007; Yu et al., 2007). Intriguingly, this had been accomplished using two distinct sets of transcription factors, with the Thomson group incorporating the alternative pluripotency-associated factors Nanog and Lin28 (OSNL) in place of Klf4 and

c-Myc, suggesting that there could be multiple ways to induce pluripotency in somatic cells (Yu et al., 2007; Zhou et al., 2008).

An advantage of iPSC generation over the previous technologies is its simplicity and reproducibility. As such, multiple groups have sought to explore the underlying mechanisms and interactions between OSKM, co-factors and the chromatin, which ultimately dictate the fate of a cell (David and Polo, 2014).

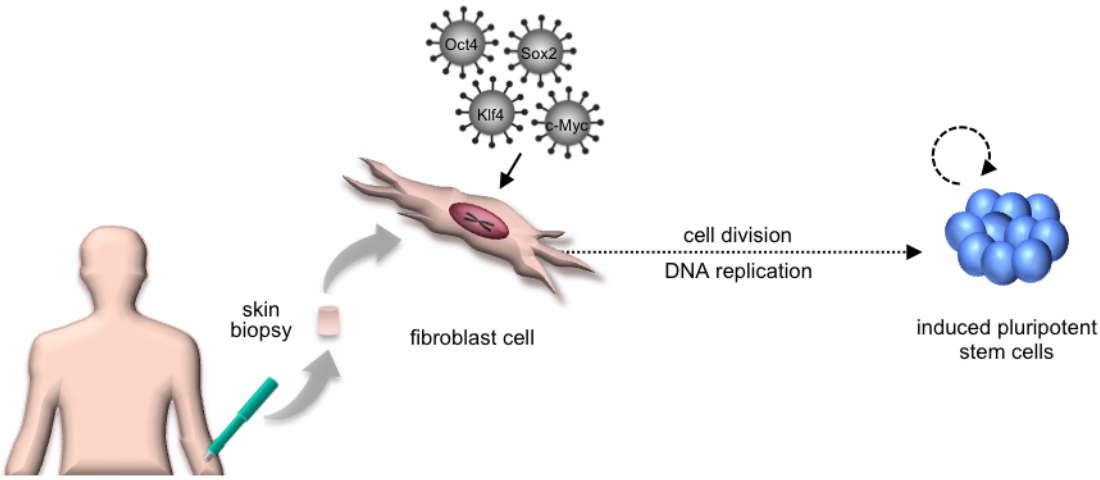


Figure 3. The generation of induced pluripotent stem cells.
The overexpression of four pivotal transcription factors – Oct4, Sox2, Klf4, and c-Myc – can revert somatic cells to pluripotency.

Reprogramming is a slow, gradual process in which somatic cells progressively lose their differentiated identity and assume the growth behaviour, morphology, and transcriptional pattern of embryonic stem cells (Takahashi et al., 2006). Despite the tremendous advances in this field, an understanding of the underlying cellular and molecular mechanisms governing this process is only recently emerging along with key

sequential events required for successful reprogramming, and it remains to be determined exactly how the pluripotency factors control this alteration of cell fate.

1.3.2 The Induced Pluripotent State

The pluripotent state is managed by a network of transcription factors that endow PSCs with their prominent characteristics. Early evidence indicated that pluripotency is critically dependent on the action of three transcriptional organizers – Oct4, Sox2, and Nanog – which function to suppress lineage commitment as a means of preserving pluripotency (Boyer et al., 2005; Silva and Smith, 2008). These factors act in concert with paracrine and other exogenous factors to dictate the intrinsic mechanisms involved in cell fate decisions, including transcriptional programs, signaling pathways, and epigenetic profiles. It is this same network that modulates pluripotency in iPSCs, although the underlying cellular and molecular events governed by these factors remains largely unknown.

While Yamanaka's pioneering study demonstrated that somatic cells can be reprogrammed to pluripotency by the expression of four defined factors, it has been recently determined that these transcription factors are not stringently necessary for the process, as all factors can be substituted for or even eliminated, except for Oct4 (Yu et al., 2007; Nakagawa et al., 2008; Feng et al., 2009). It was recently demonstrated that the requirements to induce pluripotency vary due to the cell of origin, specifically based on endogenous pluripotent factor expression, such that pluripotency factors may be eliminated from the process (Li et al., 2012b). Specifically, it has been recently shown

that neural stem cells, which endogenously express Sox2, Klf4 and c-Myc, can be reprogrammed to pluripotency using exogenous Oct4 alone (Kim et al., 2009b). Since these findings, many groups have made attempts to bypass the requirement for Oct4 using other ectopically-expressed transcription factors or small molecules but despite these efforts, no factor or chemical has been found, as many of these methodologies appear to activate endogenous Oct4 (Radzisheuskaya and Silva, 2014). As such, Oct4 remains essential for the conversion process.

1.3.3 Technical Limitations to Reprogramming

1.3.3.1 Factor Delivery

Induced pluripotent stem cells were first established by the introduction of the four Yamanaka factors by viral transduction, but what soon became obvious about this method is the numerous side effects that can greatly limit their use in downstream applications. Alteration of cellular fate by transgenic methods can result in random integration of the transgene within the host's genome, which may result in issues such as gene mutation, disruption of endogenous gene regulation, or even inappropriate reactivation of the transgene in the downstream terminally differentiated cells (Li et al., 2012b). These caveats not only create heterogeneity among clones, complicating disease modeling, but also increase the risk of tumor formation *in vivo*, rendering them unsuitable for clinical applications. To create a cell fit for downstream applications, researchers have turned to alternate methods of manipulating cell fate. To improve both the efficiency and quality of iPSCs, researchers have turned to alternative methodologies

including non-integrating viruses and episomal vectors, as well as non-DNA methods such as proteins and mRNA molecules (Kaji et al., 2009; Woltjen et al., 2009; Stadtfeld et al., 2008; Zhou et al., 2009a; Fusaki et al., 2009; Okita et al., 2007; Yu et al., 2009; Okita et al., 2011; Zhou et al., 2009b; Kim et al., 2009a). Unfortunately, similar issues of low efficiency and kinetics plague these methods, in addition to innate protocol-specific issues such as inflammatory responses, which limit their widespread use (Robinton and Daley, 2012; Mahendra et al., 2012). The most promising method appears to be the use of small-molecules.

Small molecules used in iPSC generation fall into one of three categories: small molecules that may improve reprogramming efficiency and/or kinetics; compounds that replace one or more reprogramming factors; and compound combinations that alone are sufficient to induce iPSCs (Lin et al., 2009). Small molecules used in reprogramming can also be categorized further depending on their mechanism of action, which may include the following: epigenetic modifiers, signaling moderators, modulators of metabolism, regulators of MET, and governors of cell death/senescence pathways. The first application of compounds in iPSC generation was the epigenetic modifier Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, which was found to increase reprogramming efficiency over 100-fold over that of the traditional OSKM-only protocol (Huangfu et al., 2008). Shortly afterward, small-molecule screens identified multiple chemicals that improve reprogramming, including BIX-01294 and vitamin C (Shi et al., 2008a; Shi et al., 2008b; Esteban et al., 2010). Furthermore, it was found that chemically modulating pathways key in cell identity and pluripotency could replace the need for

certain transcription factors. For example, Ichida et al. identified the TGF-β signaling inhibitor E-616452, as a chemical substitute for *Sox2* in reprogramming (Ichida et al., 2009). Interestingly, this molecule, renamed "RepSox", does not induce *Sox2* expression and instead induces *Nanog* transcription in certain reprogramming intermediates, demonstrating that *Sox2* expression is not required during iPSC reprogramming. These results also demonstrate the feasibility of replacing central reprogramming factors with small molecules to modulate discrete cellular pathways or processes key to altering cellular fate, and that these mechanisms may be discrete from those of the factors that they replace (Ichida et al., 2009).

Given that certain signaling cascades influence the establishment and maintenance of pluripotency, it is not surprising that cytokines or small molecules can be added to the medium to either activate or suppress this transcriptional circuitry. But the question remained as to whether small molecules alone could reprogram cells to pluripotency in the complete absence of exogenous genes. In a landmark study in *Science*, Hou *et al.* pursue a rational, albeit laborious approach to identify cocktails of small molecules whose treatment restored pluripotency in murine fibroblast cells (Hou et al., 2013). In what they termed "chemical reprogramming", six small molecules, Valproic Acid, CHIR99021, E-616452, Tranylcylopromide, Forskolin, and DZNep (VC6TFZ), were sufficient to create chemical induced pluripotent stem cells (ciPSCs) through the upstream activation of *Oct4*, *Sall4*, and *Sox2* and alteration of the epigenetic landscape (Hou et al., 2013). Interestingly, many of these compounds have been previously reported to replace TFs and/or enhance reprogramming by targeting many signaling pathways key in

pluripotency, including the abovementioned RepSox (Ichida et al., 2009). This work confirms the notion that rather than merely serving as modulators of a transcription factor-driven reprogramming process, small-molecule combinations can perturb signaling pathways and epigenetic regulation to allow a desired cell fate to be achieved chemically. Although this recent study has demonstrated transgene-free reprogramming in mouse cells, the chemical reprogramming approach has yet to be applied successfully to human cells. Inherent variances in the extracellular cytokine requirements to induce and maintain pluripotency exist between mouse and human cells, and it is highly likely that a unique chemical cocktail will be required to create human ciPSCs, although this has yet to be ascertained.

The use of small molecules to modulate cellular status has its distinctive advantages over the traditional viral-mediated transgene methods, including higher potency and scalability, greater temporal and spatial control, and most importantly, they do not disrupt the host genome (Hou et al., 2013). Additionally, they are immediately available to cells, are cost-effective, and can be easily manipulated to modulate binding affinity or overall effect (Masuda et al., 2013). It is therefore theoretically appealing to envision the complete replacement of genomic methods with small-molecule compounds, which may improve the safety of human iPSCs.

1.3.3.2 Roadblocks to Reprogramming

The efficiency of iPSC generation is poor – at best, 0.1% of mouse fibroblasts and 0.01% of human fibroblasts reprogram to pluripotency using OSKM (Takahashi and

Yamananka, 2006; Chen et al., 2011c). It was first thought that the low efficiency of reprogramming was due to lack of expression of all four factors in the same cell, as at this time, each transgene was packaged as an individual retrovirus. However, use of the more recent polycistronic lentiviruses argues against this notion as the reason for low efficiency, as only a minimal increase in the number of faithfully reprogrammed cells was observed using this method. If factor expression is truly the cause of low efficiency, the use of an inducible reprogramming cassette in secondary reprogramming systems should theoretically yield 100% efficiency, yet this method is only slightly more efficient (Wernig et al., 2008).

Scientists then raised the possibility that iPSCs are derived from rare stem or progenitor cells within a heterogeneous population, but subsequent studies showed that iPSCs could be derived from sorted, terminally-differentiated pancreatic β cells (Stadtfeld et al., 2008,) lymphocytes (Loh et al., 2009) and even post-mitotic neurons (Kim et al., 2011), therefore demonstrating that most, if not all, somatic cells have the ability to be reprogrammed to iPSCs.

It remains unclear why reprogramming efficiency is so low. Many scientists agree that reprogramming is initiated in more than 1% of cells, yet only a small percentage of these make the full trip to the pluripotent state (Yamanaka, 2012). The collective scientific findings have led have led researchers to postulate that the Yamanaka factors alone are insufficient to permit the transition to pluripotency, and instead, additional events are required to overcome the major barriers that prevent complete reprogramming (Hanna et al., 2009; Smith et al., 2010).

Concerted efforts to elucidate the mechanisms of reprogramming have revealed that the process follows an organized sequence of events that occur between the time of initial transgene expression and the establishment of the pluripotent state (Stadtfeld et al., 2008).

One of the first barriers present to cells is the increase in proliferative capacity, which coincides with the switch to a glycolysis-based metabolism (Smith et al., 2010; Panopoulos et al., 2012). Cells that will successfully reprogram must also abolish their somatic identity by changing their chromatin structure at their promoter and enhancer regions (Samavarchi-Tehrani et al., 2010). Afterward, these small, fast-cycling cells cluster tightly and undergo a MET-like process, with coordinated changes in cell-cell and cell-matrix interactions corresponding with the acquisition of epithelial cell characteristics (Smith et al., 2010; Liu et al., 2010). Instead, most cells fail to successfully induce these changes, remain fibroblast in nature, and often undergo apoptosis, senescence, or cell-cycle arrest (Smith et al., 2010).

Following the initiation phase, the maturation and stabilization phases proceed in only a subset of cells, the criteria for which remain unknown. Recently, the use of stage-specific and/or intermediate cell surface markers have been employed to prospectively identify and isolate the rare cells poised to become iPSCs (Samavarchi-Tehrani et al., 2010). Findings from these studies have identified additional rate-limiting steps, such as the transition from Thy1- to SSEA1+ cells, which may occur due to the depletion of exogenous factors (Yang et al., 2014). In cells that proceed, changes in DNA and histone methylation patterns as well as histone acetylation patterns occur, coinciding with the

upregulation of genes associated with embryonic development and stem cell maintenance (Chen et al., 2013). The final phase of reprogramming involves establishment of stabilized ESC-like colonies that can sustain in the absence of ectopic transgenes, as the reestablishment of the endogenous pluripotency network is sufficient to maintain the pluripotent state (Brambrink et al., 2008; Stadtfeld et al., 2008). It is the reactivation of the pluripotency genes – Oct4, Sox2, and Nanog – that indicate a stable conversion and complete reprogramming.

In addition to the intrinsic barriers of reprogramming, environmental conditions play a significant role during iPSC generation. It has recently become apparent that the extracellular environment influences key rate-limiting events, such as the reactivation of the core pluripotency circuitry (Chen et al., 2013). In this regard, optimizing and fine-tuning the reprogramming protocol, such as the addition of small-molecules to the growth media, provides a powerful tool for adjusting the efficiency and kinetics if the process (Ebrahimi et al., 2015)

1.4 Oct4: The Essential Factor for Lineage Conversion

Oct4, encoded by *Pou5f1*, is a transcription factor identified over two decades ago with specific functions in early embryonic development (Jerabek et al., 2014). Through early studies, it was determined that Oct4 is expressed in primary germ cells, unfertilized oocytes, and the early embryo, with a critical functions in regulating pre-implantation development and maintenance of the pluripotent cell population (Nichols et al., 1998). First identified in mice as an ESC- and germline-specific transcription factor, Oct4

orthologs have since been identified in a wide array of species, including bovine and humans (Medvedev et al., 2008).

Belonging to the POU (Pit-Oct-Unc) family of transcription factors, these proteins are related by their well-conserved homeodomains that play key roles in cell fate specification (Hombria and Lovegrove, 2003). All members contain a bipartite DNA-binding domain, referred to as the POU domain, which is composed of two distinct subunits. The N-terminal subunit is known as the POU-specific subdomain (POU_S), while the C-terminal subunit is the homeobox subdomain (POU_{HD}), and a non-conserved region of 15-55 amino acids tethers these regions (Esch et al., 2013). Studies have determined that the C-terminal domain confers cell type-specific transactivity, regulated through phosphorylation, while the N-terminal domain serves as a direct binding site for cell type-specific regulatory factors, including other transcription factors and chromatin-modifying complexes such as Sall4 and NuRD complex members (Campbell et al., 2007; Babaie et al., 2007).

1.4.1 Oct4 in Early Development and Pluripotency

A germ cell has very different properties from a somatic one, of which the expression of Oct4 is a defining factor required for its survival. Maternal Oct4 is expressed in oocytes as both transcript and protein, and as is typical for most maternal mRNAs, levels of Oct4 drop after fertilization. Instead, zygotic Oct4 becomes activated uniformly in all cells by undetermined maternal regulatory factors during what is termed "zygotic genome activation" (Jerabek et al., 2014). As the outer cells begin to

differentiate toward the trophectoderm lineage, Oct4 expression becomes contained to the inner cell mass of the blastocyst and the *in vitro* derived embryonic stem cells. Cells destined to become the primitive endoderm and mesoderm undergo a transient increase in Oct4 levels, which is then downregulated in the epiblast during implantation and gastrulation. Oct4 expression becomes restricted to primordial germ cells (PGCs), which give rise to gametes that can be fertilized to once again undergo this cycle.

Due to its critical role, it is not surprising that tight control of Oct4 is required to maintain the pluripotency status. Although the mechanism is unclear, it is proposed that Oct4, which functions through the activation of pluripotency and self-renewal associated genes as well as the repression of genes that promote differentiation, may display different affinities towards its partners at different expression levels (Shi and Jin, 2010). As a result, reduced expression of Oct4 results in differentiation into trophectodermal cells, while overexpression results in mesodermal and primitive endodermal lineages (Niwa et al., 2000). This expression level is modulated by multiple factors and mechanisms, and continued efforts are ongoing to elucidate this sophisticated regulatory network.

As development continues, Oct4 expression becomes permanently epigenetically silenced in adult somatic cells through G9a-histone mediated methylation, followed by methylation of the promoter itself through the activity of DNA methyltransferases (Feldman et al., 2006). Other epigenetic regulators as well as *trans*-acting regulators function in concert to silence Oct4 as development progresses. Some groups have reported Oct4 expression in tissue-specific stem cells via immunostaining and flow

cytometry, although improper controls, incorrect Oct4 localization (cytoplasmic vs. nuclear) and background imaging noise cast doubt on these results, and more recent studies have been unable to corroborate these findings (Yu et al., 2007; Matthai et al., 2006; Katona et al., 2007; Lengner et al., 2007; Lengner et al., 2008; Zangrossi et al., 2007; Tai et al., 2005). Specifically, the use of a Cre-lox based recombination approach to achieve tissue-specific inactivation of a conditional *Oct4* allele in the intestine, bone marrow, brain, liver and hair follicles revealed no abnormalities in homeostasis or regeneration capacity upon *Oct4* gene ablation (Lengner et al., 2007). As such, it was conclusively demonstrated that Oct4 is not required for the self-renewal and maintenance of somatic stem cells in the adult mammal. Instead, several reports have argued that detection of *Oct4* in somatic cells are false-positives due to pseudogene transcripts and/or failure to distinguish Oct4 isoforms (Liedtke et al., 2007; Liedtke et al., 2008). Most groups support this conclusion, and Oct4 is therefore believed to be a marker of stemness exclusive to the pluripotent cell population.

1.4.2 Oct4 in Reprogramming: An Indispensable Factor

Takahashi and Yamanaka's pioneering discovery of induced pluripotency drew tremendous interest by the scientific community. Whether it is the Yamanaka's factors (OSKM), the Thomson factors (OSNL), one consistent feature is the requirement of a single factor, Oct4, in order to perform successful reprogramming.

1.4.2.1 Oct4 Structure Dictates Function

Perhaps most interesting about Oct4 is that its role in conferring pluripotency cannot be replaced by other POU family members, such as Oct1, as inclusion of other factors does not lead to the successful derivation of iPSCs. So what is about Oct4 that bestows its pluripotent ability? To understand Oct4's unique role in pluripotency, the group of Hans Schöler characterized the structure of the POU domain in an effort to elucidate the key features that allow Oct4 to act in pluripotency (Esch et al., 2013). The group determined that the linker region, which connects the POU_{HD} and POU_S subdomains, is highly structured in contrast to the unstructured linkers of other family members, such as Oct1 (Esch et al., 2013). Specifically, the amino terminal part of the linker is structured as an α-helix, which allows critical residues to be exposed to the protein's surface. These residues may then bind to and/or interact with specific members of the Oct4 interactome, allowing for recruitment of key epigenetic factors, among others, to sites occupied by Oct4. Point mutations in this α-helical region alter or abolish the reprogramming activity of Oct4 while having no effect on its DNA binding ability, transactivation potential or nuclear localization, confirming its role as a protein-protein interaction interface (Esch, 2013). The Oct4 gene can be alternatively spliced and transcribed, generating at least 5 protein variants that contain differing domain components and linker regions (Figure 4). Specifically, it is the Oct4A isoform that contains the abovementioned elements, and is therefore the only Oct4 protein able to function in inducing and maintaining pluripotency (Esch et al., 2013). From here on in, Oct4A will be referred to simply as Oct4, as is convention in the stem cell field.

During reprogramming, Oct4 functions in concert with a multitude of other transcription factors, such as Sox2 and Nanog, to control the expression of key genes including *Sox2, Fbxo15, Sall4, Dppa3,* and *Fzd5,* while regulating specific cell-signaling pathways including Wnt, TGF-β, MAPK, and Hedgehog, as well as its own autoregulation in conjunction with Sox2.

1.4.2.2 Activation and Regulation of Oct4

The expression of Oct4 is under complex regulation that is specific to both the cell type and developmental stage, although how exactly Oct4 is activated remains an open question (Tiemann et al., 2014; Wu and Schöler, 2014). Upstream of the transcription start site contains three regulatory elements: the distal enhancer (DE), the proximal enhancer (PE), and the TATA-less proximal promoter (PP) (Yeom et al., 1996; Niwa et al., 2007) (Figure 4). These three elements serve as markers for protein binding, histone modifications and/or DNA methylation, which in concert dictate the transcriptional status of the gene. Specifically, these elements are inactive in somatic cells, while active in pluripotent cells to allow for Oct4 transcription. Most studies on these elements have been conducted in the murine system, and have demonstrated a key role for the DE in regulating Oct4 in expression in most pluripotent cell types, including within the ICM, ESCs, and PGCs (Yeom et al., 1996). Alternately, the PE activates Oct4 expression only in epiblast-derived cells. Comparative alignment analysis between murine, bovine, and human Oct4 orthologs have revealed four conserved regions of homology, CR1 – CR4, between these species, the most highly conserved region (CR4) located within the mouse

DE, likely indicating a species-wide role for this regulatory element (Nordhoff et al., 2001). Further investigation is required to fully elucidate the underlying molecular mechanisms through which Oct4 maintains and reinitiates pluripotency.

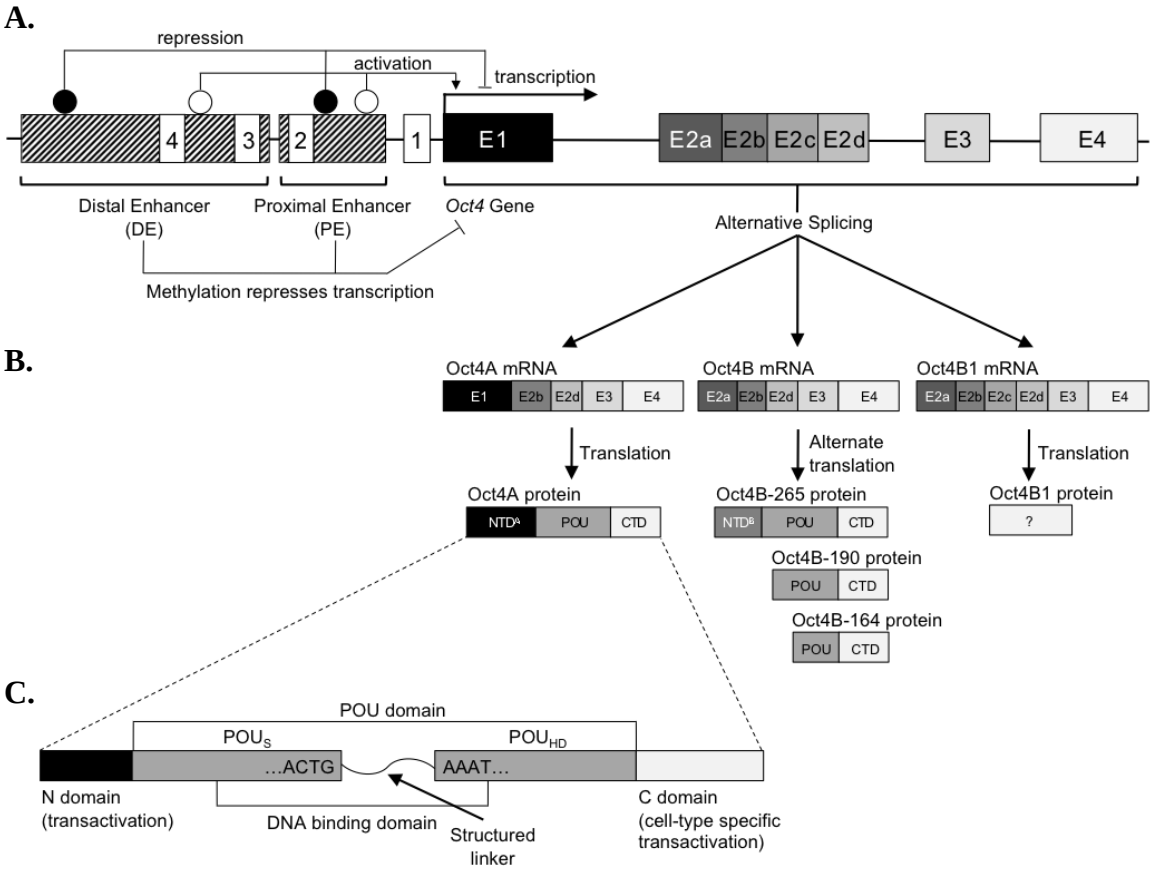


Figure 4. Structure of the Oct4 elements.
A. The Oct4 upstream regulatory elements and gene. The distal enhancer (DE) and proximal enhancer (PE) are important for regulating Oct4 expression. Each enhancer region contains multiple potential binding sites for transcription factors to either activate or repress Oct4 expression. Methylation in these regions represses Oct4 expression in differentiated cells. Four regions upstream of the transcription start site are highly conserved between human, bovine and mouse Oct4, and are shown as white boxes labeled 1-4.
B. Alternative splicing of the Oct4 transcript generates three Oct4 mRNAs, which can produce four known protein constructs.
C. Schematic illustration of the Oct4A protein domains.

1.4.2.3 The Molecular Mechanisms of Oct4 in Reprogramming

In maintaining and inducing pluripotency, Oct4 must directly interact with Sox2 in order to activate target genes, although the overexpression of Oct4 can rescue many functions in the absence of Sox2 (Boyer et al., 2005; Masui et al., 2007). In support of this, it has been demonstrated that other Sox factors or transforming growth factor β (TGF-β) inhibitors can substitute for Sox2's role (Ichida et al., 2009; Nakagawa et al., 2008). Likewise, Klf4 is dispensable for inducing and maintaining pluripotency, as alternative transcription factors and even small-molecules have been shown to replace Klf4 (Jiang et al., 2008; Nakagawa et al., 2008; Chen et al., 2011b). The fact that Oct4 alone can generate iPSCs from MEFs suggests that exogenous Sox2 and Klf4 are indeed dispensable, and that Oct4 may be the only essential factor required to induce pluripotency (Chen et al., 2011b). Interestingly, reprogramming with Oct4 alone displays decreased efficiency and delayed kinetics as compared to OSKM (Chen et al., 2011b). In quantifying the relative contributions of Sox2 and Klf4 in reprogramming, the Pei group estimated that Sox2 and Klf4 increase both the efficiency and kinetics of the process. In a sense, these factors play auxiliary roles as biological catalysts.

Recent experimental evidence suggests that Oct4 participates indirectly in the induction of MET through the suppression of the EMT-regulator Snail (Li et al., 2010). Additionally, Oct4 has been shown to interfere with somatic transcriptional networks in a cell-type-specific manner and without the initiation of a pluripotent gene-expression program in the early phase of reprogramming (Tiemann et al., 2014).

It has been suggested that the Yamanaka factors may act as "pioneer" factors that

open chromatin regions to allow the subsequent activation of those genes essential for the establishment and maintenance of the pluripotent state (Soufi et al., 2012; Zaret et al., 2011; Papp and Plath, 2013). To support this notion, it has been shown that Oct4 can act on the epigenetic state to reprogram cells, specifically via the de-repression of somatic cell chromatin to induce a more transcriptionally-active state (You et al., 2011). Also in support of this idea is the fact that exogenous Oct4 can be replaced by the H3K9 methylase G9a inhibitor BIX-01294, which may occur at least in part through reactivation of endogenous *Oct4* (Chen et al., 2013; Shi et al., 2008b; Feldman et al., 2006).

Based on these findings that Oct4 can conduct multiple cellular functions, it is no wonder that its expression alone can alter cell fate.

1.4.3 Oct4 in Direct Conversion

The desire to generate functional cell types has been a long-standing goal of regenerative medicine and although the conventional approach of directed differentiation of PSCs has been extensively explored, there has been great interest in developing new strategies for obtaining functional cells. Many groups have explored the alteration of cell fate without first establishing pluripotency, directly converting lineages by either overexpressing lineage-specific factors or by a brief expression of the Yamanaka factors followed by lineage-specific culture conditions.

Just as Oct4 alone is sufficient to induce pluripotency in somatic cells, our group was the first to demonstrate that Oct4 could facilitate the direct conversion of cell lineages. Specifically, Szabo et al. demonstrated that overexpression of Oct4 in

fibroblasts followed by culturing in hematopoietic lineage-supportive conditions can generate multilineage blood progenitors (Szabo et al., 2010). Under these conditions, it appears that Oct4 is capable of upregulating critical hematopoietic regulators, resulting in the establishment of sufficient transcriptional programs to support the conversion to the blood lineage. The same methodology was employed to derive neural progenitor cells (NPCs) from fibroblasts using Oct4 alone (Mitchell et al., 2014b). More recently, our lab has reported the direct conversion of an alternative cell type, blood cells, to tri-potent induced neural progenitor cells using Oct4 only (Lee et al., 2015). In this approach, the generation of alternative cell types from an easily obtainable cell source such as blood expands the clinical applicability of direct conversion processes.

Perhaps most importantly, these reports suggest a novel regulatory role for Oct4 outside that of development, primordial germ cells, or pluripotency, and instead as a master regulator of cell fate conversion.

1.4.4 Oct4-induced Plasticity

Orkin and Hochedlinger first used the term "plasticity" to describe cells that are in a confused state and require instruction from the extracellular environment to assume different cellular states, hypothesizing that this cell status manifests as activation of genes of multiple lineages (Orkin and Hochedlinger, 2011). In analyzing the contributions of Oct4 and Reprogramming media on fibroblast cultures, Mitchell et al. described such a cell state (Mitchell et al., 2014a). These cells demonstrated upregulated lineage-specific genes from all three germ layers, indicating that these cells are transcriptionally primed

for multiple cell fates, thereby generating the term "Oct4-induced Plasticity", or OiP, to describe this state. As well, these cells exhibited enrichment for extracellular structure and matrix organization, correlating with a morphological change from that of a spindle-shaped fibroblast cell toward a compact-cuboidal shape (Figure 5A) (Mitchell et al., 2014a). These cells grow amongst seemingly unchanged fibroblasts, pushing them outward to allow the expansion of these rounded, Oct4-expressing cell colonies (Figure 5B). Interestingly, these cells retain certain features of the somatic program, including the expression of the cell surface antigen CD90 (Figure 5B).

Further demonstrating their plasticity, it was demonstrated that culturing these cells in blood and neural lineage-specific conditions, multipotent blood and neural progenitor cells could be established (Szabo et al., 2010; Mitchell et al., 2014b). As well, it was revealed that method is sufficient to generate neural progenitor cells from hematopoietic cells, suggesting that this approach is not limited to fibroblasts (Lee et al., 2015. These findings expand the role of Oct4 in cellular reprogramming that is seemingly distinct from iPSC establishment.

More recent assessment of OiP in our lab has demonstrated pluripotency acquisition through the continued maintenance of these cells in pluripotent-supportive conditions (Salci et al., 2015).

As such, it remains to be determined what the cell fate trajectory of OiP cells is, and if and how they may differ from reprogramming intermediates. My studies, contained in this thesis, address this notion, as well as confirming the role of Oct4 and the extracellular environment in this process.

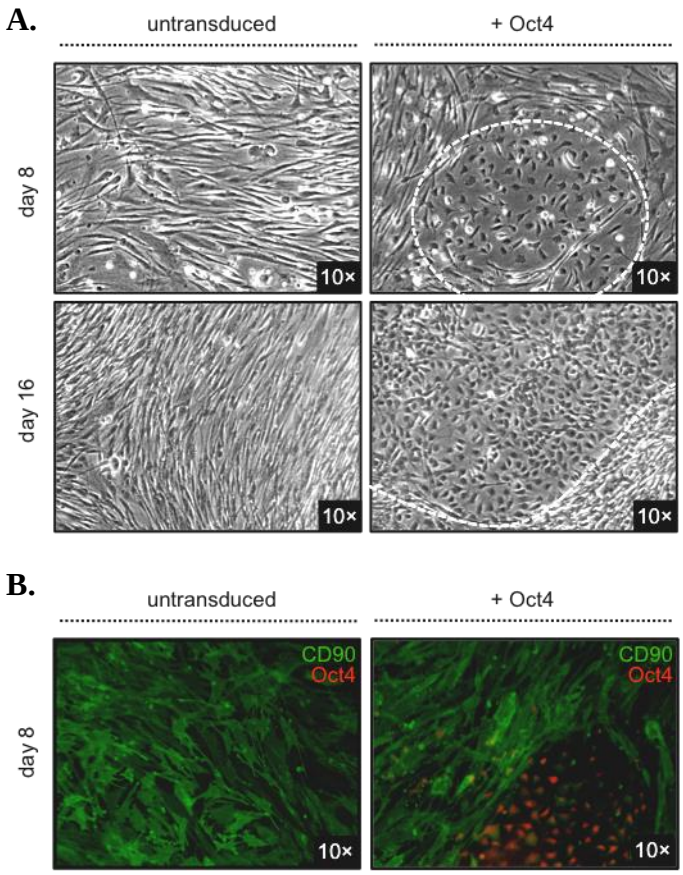


Figure 5. Oct4-induced Plasticity exhibits distinct morphological changes from untransduced fibroblasts.
A. Overexpression of Oct4 in fibroblasts generates cells with a morphological change away from a spindle-shape and toward a compact-cuboidal shape (encircled), which become compacted upon continued culturing.
B. Plastic cuboidal cells are the only cells in the culture to express Oct4 (red), and these cells continue to express the fibroblast marker CD90 (green).

Further research will be required to ascertain precisely the nature of plasticity, including the interplay of Oct4 and media factors, before these cells can be manipulated for therapeutic and industrial purposes.

Summary of Intent

Seminal reprogramming experiments challenged the notion that development is irreversible with the pivotal findings that cellular fate can be altered via the modulation of the epigenome by *trans*-acting factors. It was the convergence of these principles that led to Takahashi and Yamanaka's extraordinary discovery of induced pluripotency via the overexpression of four core pluripotency factors – Oct4, Sox2, Klf4, and c-Myc. This achievement represented a fundamental breakthrough in stem cell biology that began a new era in regenerative medicine.

Since this momentous finding, numerous groups have altered cellular fate using a multitude of cell types and reprogramming cocktails, generating high numbers of specialized cell types. Despite these advances, Oct4 remains a critical and indispensable factor in the alteration of cell fate (Orkin et al., 2008; Sterneckert et al., 2012)

Our lab has contributed to this progression with the demonstration that one factor – Oct4 – is sufficient to alter cell fate. Specifically, the ectopic expression of Oct4 in conjunction with lineage-specific culture conditions promotes the generation of multilineage blood and neural progenitors without traversing pluripotency (Szabo et al., 2010; Mitchell et al., 2014b). During this conversion, fibroblasts achieve a unique cellular status, unique from the somatic or pluripotent states, which acquires morphological and transcriptional profiles. This confused state exhibits upregulation of lineage-developmental genes from all three germ layers, indicating that these cells are transcriptionally primed for multiple cell fates that may be achieved through instruction from the extracellular environment (Orkin and Hochedlinger, 2011; Mitchell et al.,

2014a). As such, this cellular status was termed "Oct4-induced plasticity", or OiP. Furthermore, our group has established a working definition of OiP, which refers to fibroblast cells that express exogenous Oct4 along with displaying morphological changes towards a compact-cuboidal shape. For the purpose of this thesis, these cells will herein be referred to as "plastic cells".

More recent assessment of OiP in our lab has demonstrated pluripotency acquisition through the continued maintenance of these cells in pluripotent-supportive conditions (Salci et al., 2015). It was with this finding that I began to question the nature of the plastic state, specifically whether OiP is a very early reprogramming intermediate, or if its trajectory is separate from that of pluripotency, but may be redirected toward this fate with the proper conditions. Investigation of the literature regarding the early, or induction, phase of reprogramming and the nature of these cells at this time point led me to draw similarities between early iPSCs and plastic cells.

Based upon this knowledge, I hypothesize that Oct4-induced plasticity is a very early stage in the reprogramming process, in which Oct4 and the extracellular environment work synergistically to induce a unique state of cellular and transcriptional plasticity in fibroblasts that is amenable to perturbation via the addition or subtraction of environmental cues. If this idea is true, these cells may exhibit unique molecular and phenotypic characteristics distinct from that of the somatic or pluripotent state, features that may be used for the isolation and further analysis of these cells. As well, elucidation of the role of external stimuli may facilitate an optimal protocol to generate plastic cells, including the use of chemical substitutes for Oct4

expression. To address my hypothesis, this thesis aims to fulfill the following experimental objectives:

1. To determine a reliable cellular surface marker that is unique to the state of Oct4-induced plasticity
2. To identify a small-molecule compound capable of inducing endogenous expression of Oct4 in fibroblast somatic cells
3. To further assess the role of the extracellular environment in Oct4-mediated conversion of fibroblasts to a state of transcriptional plasticity

With the findings of researchers and colleagues before me, I set out to address these objectives in the hopes of elucidating the nature of OiP, as well as the role of the extracellular environment and Oct4 in inducing this state.

CHAPTER 2: Identifying Markers of Plasticity

Overexpression of the pluripotency factor Oct4 alone in fibroblasts is sufficient to confer a unique state onto these cells, termed "Oct4-induced Plasticity", or OiP, as defined by Mitchell et al. (2014). These cells display a distinctive morphological shift away from a bipolar-elongated shape, typical of fibroblasts, toward a compact-cuboidal shape. At the level of the genome, global transcriptional alterations occur in which a subset of lineage-development genes from all three germ layers become upregulated, including enrichment for extracellular structure and matrix organization, correlating with the observed physical changes (Mitchell et al., 2014a). But this process suffers from an inherently low efficiency – that is, few cells continue to express ectopic Oct4 and undergo a morphological alteration. Due to the heterogeneous nature of this process, it would be beneficial to have a selection marker to isolate and purify this population for downstream analysis.

Mitchell et al. examined the global transcriptome of these plastic cells and using untreated fibroblasts as a baseline, identified genes that were up- or down-regulated (Mitchell et al., 2014a). Enrichment was found for extracellular organization and extracellular matrix organization, which correlates with the observed morphological changes observed exclusively in the plastic cell population. With this in mind, it is possible that a related surface marker may be present on these cells.

2.0 Global Gene Expression Profile

In an effort to identify a novel surface marker that is unique to the OiP state, Gene Affymetrix data generated by Mitchell et al. was mined for candidate genes (Mitchell et al., 2014). In this, the top 245 genes that were upregulated in $Fibs^{Oct4}$ w/RM as compared to the two other conditions ($Fibs^{eGFP}$ w/RM and $Fibs^{Oct4}$ w/o RM) were further examined. Each gene was independently analyzed using GeneCards (www.genecards.org) using the following criteria to determine if it may be a candidate gene worthy of further investigation. Briefly, genes were confirmed to be a protein-coding gene, followed by the corresponding protein being expressed on either the plasma membrane or in the extracellular environment (confidence level ≥ 4 on a scale of 1-5) for the ability to identify expression in live cells. Genes for extracellular structure, cell-cell interaction, and cell adhesion were preferentially selected. Thirdly, this protein must have a commercially available purified (non-conjugated) antibody, and lastly, this antibody has been used in published immunocytochemistry (ICC) experiments, as this is the application chosen to be used to identify a unique surface marker. Genes that met these criteria underwent surface marker screening via ICC.

2.1 Surface Marker Screening

2.1.1 Immunocytochemistry-based Antibody Screening

ICC is the preferred procedure for examination of surface markers of a discrete cellular population within a heterogeneous cell culture. Unlike analysis by flow cytometry, in which a heterogeneous population is collected into a single sample, analysis

by ICC retains a culture's *in vitro* heterogeneous characteristics, including cellular morphology. OiP colonies display a vastly different morphology than traditional fibroblast cells, though both are prominent in culture due to the low efficiency of the process. This feature becomes lost upon single-cell collection for flow cytometric analysis, but remains intact during ICC assessment.

Briefly, adult dermal fibroblasts were seeded 2.0×10^4 cells/well in 6-well format on Matrigel-coated tissue culture plates in Fibroblast Media. The following day (day 0), cells were transduced with the pSin-EF1α-OCT4-IRES-Puro lentivirus in Fibroblast Media, to achieve approximately 20% transduction efficiency, as measured by flow cytometry for Oct4, as per the procedure outlined in Mitchell et al. (2014b). Culture media was changed to 50% Fibroblast media/50% Reprogramming media on day 1, followed by a switch to 100% Reprogramming media on day 2. Cells were then cultured for 8 days, passaged onto fresh Matrigel at the same seeding density and format, and allowed to grow for an additional 8 days in order for the emergence of the compact-cuboidal morphology characteristic of OiP cells to occur. For a full outline of the procedure, please see Appendix I: Materials and Methods.

Cultures were prepared for fixed-cell ICC, the BD Cytofix Fixation Buffer (BD Biosciences) for paraformaldehyde fixation, followed by blocking with 3% FBS in PBS for 1 hr. Incubation of primary antibodies (1:1 000) was performed overnight at 4°C, and the following day, secondary antibody incubation was performed for 2 hrs. at 4°C. Lastly, Hoechst nuclear stain (1:1 000) was performed for 10 min. prior to visualization

on the Olympus IX81 Fluorescence microscope.

Initially, three primary antibodies for the following proteins were utilized in ICC: vascular cell adhesion molecule (VCAM1), vascular endothelial growth factor (VEGF), and SERPINB3. Cells were observed for increased fluorescence around and within plastic cell "colonies" as compared to the untransduced controls (Figure 6). No differential expression was seen between plastic cells and fibroblasts. Following, ICC was performed for five additional antibodies (ADAM19, AMIGO2, AZGP1, HILPDA, and GPC6) and has resulted in no differences seen in fluorescence (three are shown in Figure 6).

It was hypothesized that non-specific antibody binding may be occurring for certain candidates, and therefore the blocking procedure was changed from 3% FBS in PBS to animal serum specific to the secondary antibody host species. Upon this change, increased fluorescence was detected across the entire culture, which was confirmed to be independent of the secondary antibody, as this phenomenon was not seen in secondary antibody-only control cultures. It was instead hypothesized that certain primary antibodies may be binding to the Matrigel, a poorly defined basement membrane matrix that is used for cell adhesion and growth. The abovementioned procedure was performed in the absence of Matrigel, but Oct4-transduced cultures failed to establish the conventional OiP morphology, instead remaining fibroblast-like in appearance, therefore this method was discontinued.

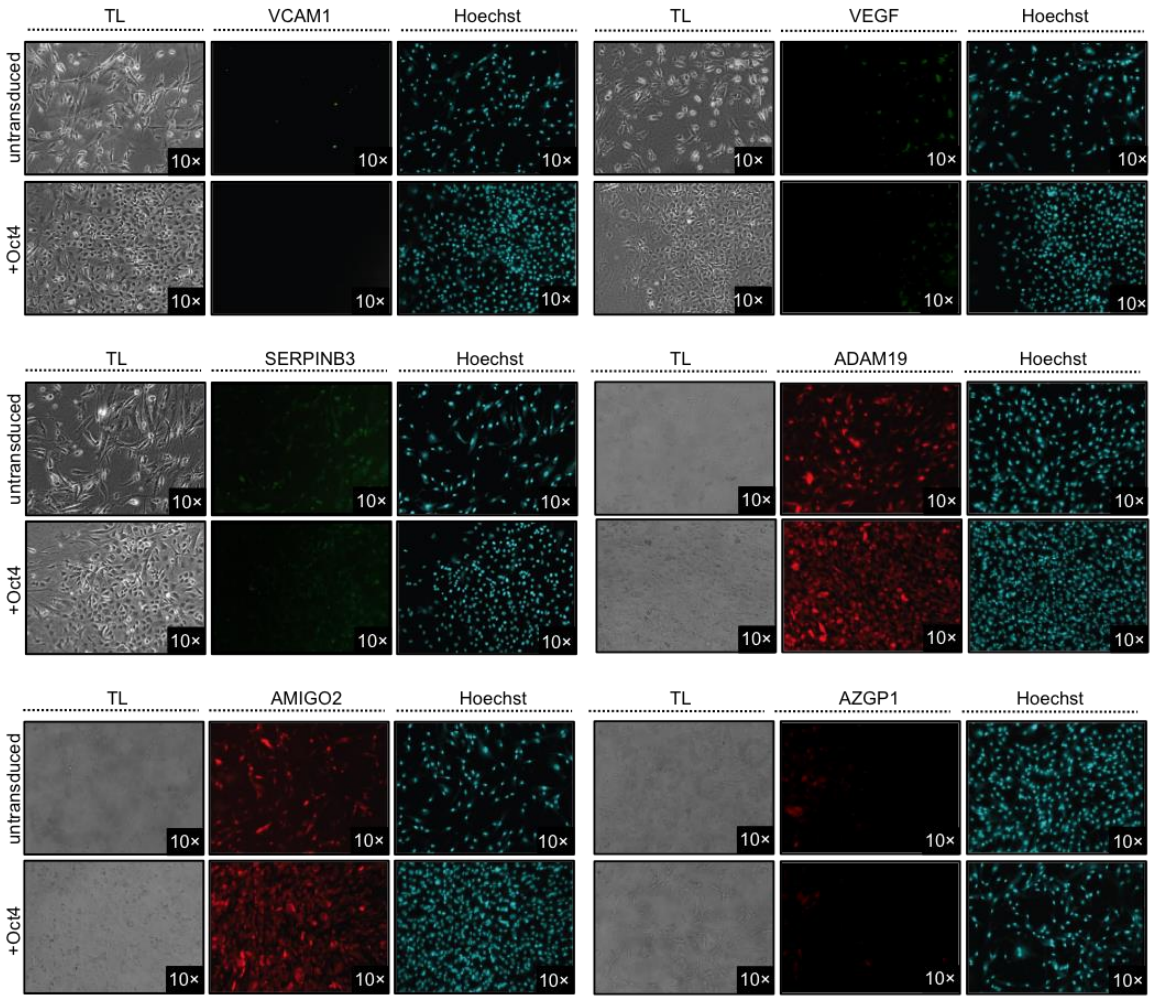


Figure 6. Immunocytochemical analysis of candidate genes for the identification of novel surface markers of the plastic state.

Fixed cell immunocytochemistry (10×) of untransduced fibroblasts and Oct4-transduced fibroblasts after 16 days of culturing in Reprogramming media for candidate gene expression. Transmitted light (TL) and Hoechst nuclear counterstaining performed.

2.1.2 Flow Cytometry-based Antibody Screening

Due to the issues plaguing the ICC procedure, flow cytometry-based antibody screening was instead performed. Although not ideal, due the aforementioned loss of morphological heterogeneity, a crude manual isolation approach was employed in order to analyze OiP colonies separate from their fibroblast-like counterparts, which co-occupy Oct4-transduced cultures. Plastic cell colonies were manually isolated from the bulk heterogeneous culture by scraping and aspirating the surrounding fibroblast-like cells, followed by trypsinization and collection of the remaining colonies. The opposite procedure was performed to isolate the fibroblast-like cells. Due to the large amount of candidate genes identified through Gene Affymetrix data, a different approach was taken in order to identify potential novel surface markers. As plastic cells exhibit upregulation of genes from multiple lineages, antibodies for surface markers from alternate lineages, such as hematopoietic and neural, were screened using the manual isolation flow cytometric approach. All antibodies already in the Bhatia lab inventory were examined, and those that correspond to surface markers, growth factor receptors, cell-cell adhesion proteins, or proteins associated with non-fibroblast lineages were used to identify a potential novel surface marker. Using the aforementioned isolated cell populations, flow cytometry was performed, using untransduced fibroblasts and H9 PSCs as control populations (Figure 7). In this, it was determined that CD49f, an integrin protein implicated in cell adhesion and cell-surface mediated signaling, becomes progressively downregulated with both Oct4 expression and culturing in RM, with very little expression seen in Oct4-transduced cells of plastic morphology, similar to PSCs. As it cannot be

conclusively determined that the OiP cells are the only population lacking CD49f expression, this candidate was not pursued further.

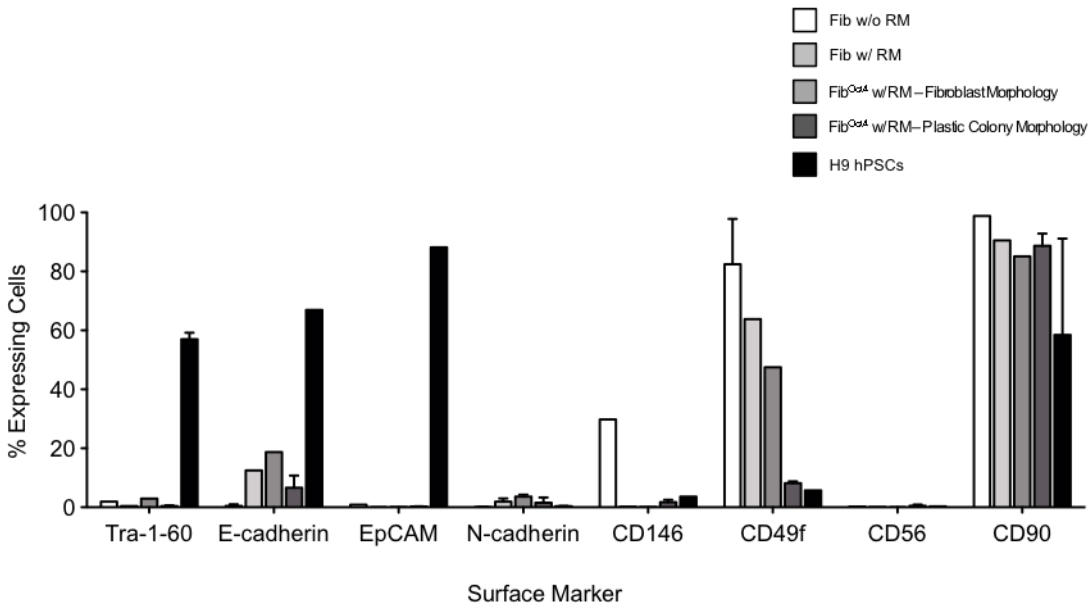


Figure 7. Flow cytometric analysis of candidate genes for the identification of novel surface markers of the plastic state.
Live cell flow cytometry of untransduced and Oct4-transduced fibroblasts after 16 days of culturing in Reprogramming media for candidate gene expression. Analyzed cell populations include untransduced fibroblasts cultured in either Fibroblast media (Fib w/o RM) or Reprogramming media (Fib w/RM); Oct4-transduced cell crudely isolated based on fibroblast-like morphology (Fib^{Oct4} w/RM – Fibroblast Morphology) or plastic colony morphology (Fib^{Oct4} w/RM – Plastic Colony Morphology). H9 hPSCs used as a pluripotency control, and Tra-1-60 expression analyzed.

2.1.3 N-cadherin

Mitchell et al. analyzed $Fibs^{Oct4}$ w/RM for expression of MET genes as well as early pluripotency genes, but it was determined that these cells did not display a bias toward epithelial gene regulators compared to mesenchymal gene regulators, nor did they display upregulation of early pluripotency genes above $Fibs^{eGFP}$ w/RM (Mitchell et al., 2014a).

Originally named for its neural role (neural cadherin), N-cad is a transmembrane glycoprotein belonging to the calcium-dependent cell adhesion molecule family and mediates interactions between adjacent cells in multiple tissues. In development, N-cad functions during gastrulation and is required for the establishment of left-right asymmetry (García-Castro et al., 2000). N-cad expression is sustained through adulthood, with high levels expressed in mesoderm-derived cells. In ESCs, it was found that N-cad can compensate for E-cadherin's function (Hawkins et al., 2012). As E-cadherin is required for STAT3 phosphorylation to result in positive regulation of Klf4 and Nanog to ultimately maintain LIF-dependent mESC pluripotency, the ability to substitute N-cad suggests a role for this marker in pluripotency (Hawkins et al., 2012). Similarly, a recent finding in our group is the demonstration of N-cad as a marker of pluripotent founder cells (manuscript in preparation). N-cad expression can be detected early in the reprogramming process (as early as 8 days post-OSKM transduction in fibroblasts), and precedes Tra-1-60, a well-established marker of pluripotent cells. Additionally, it was found that $N\text{-}cad^{+}$ cells, which mark the PSC colony periphery, express lower levels of pluripotency genes. Considering the characteristics of N-cad cells and the findings that

OiP cells may reach the pluripotent state after continued culturing, the expression of N-cad in OiP cells was assessed (Salci et al., 2015). As per the abovementioned screening procedure, plastic cell colonies were manually isolated from the bulk heterogeneous culture by scraping and aspirating the surrounding fibroblast-like cells, followed by trypsinization and collection of the remaining colonies. Live cells (no fixation or permeabilized) were stained for N-cad (1/200) 1 hr. at 4°C, followed by 7AAD viability staining immediately prior to analysis by flow cytometry. Flow cytometry results showed that N-cad is not expressed on the surface of the plastic cell colonies or the H9 control population via both PE and FITC-conjugated antibodies (Figure 7). Based on the results of Dr. Nakanishi, it has been established that N-cad is expressed on H9 PSCs, therefore these results question the methods used.

Follow-up studies by Dr. Nakanishi corroborated this finding, demonstrating that the current method of cell dissociation by trypsinization may disrupt and/or cleave the N-cad epitope, therefore altering the ability of the antibody to bind and leading to false negative results (unpublished data). As an alternate to this method, live cell in-well staining was performed. Briefly, OiP cell cultures were incubated with N-cad PE-conjugated antibody (1:100) for 2 hrs. at 37°C, followed by staining with Hoechst nuclear stain (1:5 000) for 10 minutes immediately prior to imaging. The results depicted in Figure 7, showing cell cultures grown for 8 days and 20 days, demonstrate that N-cad is expressed on Oct4-expressing plastic cells. These cells are easily identified in Figure 8A, as they are central to the image and are round, compact cells that appear to outwardly push the fibroblast-like cells away from them. In Figure 8B these plastic cells have grown

to a three-dimensional colony, depicted in the upper left-hand corner of the image, that highly expresses both Oct4-E2Crimson transgene and N-cad, with the latter especially prominent within the cell junctions which is typical of N-cad as a cell-cell adhesion molecule (Li et al., 2012a).

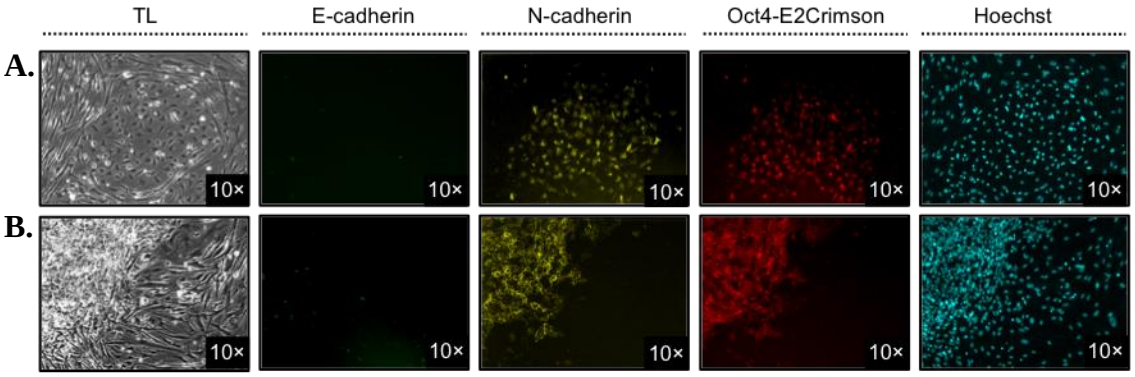


Figure 8. Live cell staining and imaging of Oct4-transduced fibroblasts for the expression of N-cadherin.
Live phase contrast and fluorescence microscopy imaging of fibroblast cells transduced with Oct4-E2Crimson lentivirus. Cells were cultured for **A.** 8 days or **B.** 24 days, and stained for E-cadherin and N-cadherin protein. Hoechst nuclear counterstaining performed.

The expression of N-cad, localized to these plastic cells, may help to elucidate the path that these cells are following. Although Mitchell et. al hypothesized that these cells are distinct from cells undergoing the initiation phase of reprogramming, Salci et al. determined that these cells are capable of iPSC establishment when cultured long-term (45-60 days) (Mitchell et al., 2014a; Salci et al., 2015). Expression of N-cad on this population may indicate this cell population is on the same trajectory as early iPSCs,

although additional follow-up studies are required to confirm this.

Although it has been identified that cells undergo a MET-like process in early reprogramming, it is also not surprising that these early reprogramming intermediates express N-cad, a mesenchymal marker (Samavarchi-Tehrani et al., 2010; Li et al., 2010). This is because a recently study demonstrated that the sequence in which reprogramming factors are introduced into somatic cells can dictate requirements, and that the optimal sequence of introduction using OK + M + S (which yields the highest reprogramming efficiency) induces an early EMT, as indicated by the upregulation of N-cad (Liu et al., 2013). The group hypothesized that EMT may generate a more homogenous population of cells, which may be more amenable to the downstream MET process (Liu et al., 2013).

2.2 Moving Forward with Identifying Markers

Currently, our lab is following up the findings that N-cadherin is expressed on plastic cells. Although not identified as an upregulated gene in Mitchell et al.'s study, it is important to recognize that a cell's transcriptome reflects the genes that are being actively expressed at any given time, which can change depending on transgene expression (such as Oct4) and extracellular environmental conditions. In the central dogma of molecular biology, information is transferred from DNA to mRNA to protein through well-understood processes, with translation being the process that converts mRNA to protein. Several reports find only a weak correlation between mRNA and protein abundances due to the influence of various biological factors, including mRNA stability and protein degradation (Maier et al., 2009). As such, the transcriptome can be

seen only as a precursor for a cell's proteome, and not a direct reflection. Therefore, microarray studies may not provide an accurate reflection of cell surface markers, and a proteomic approach may provide a more accurate method to identify novel surface markers.

The underlying hypothesis of this thesis is that OiP is a very early state in the reprogramming pathway to pluripotency. During the reprogramming process, the somatic program must be abolished prior to the onset of a pluripotent status; therefore it may be worthwhile to explore genes that become downregulated upon the induction of OiP as an additional distinguishing feature of these cells (Theunissen and Jaenisch, 2015). Although it has been established that Thy1/CD90 expression continues to be seen in OiP cells, other notable fibroblast markers, including vimentin, α-smooth muscle actin, fibroblast activation protein, fibronectin, and fibroblast surface antigen may be assessed, as the expression of these markers in may aid in further characterizing OiP and may be used to compare this state to early reprogramming intermediates.

Additionally, the results show that ICC and flow cytometry may not be the best methods for identifying a unique surface marker of these cells, as the trypsinization and/or fixation process may alter or even cleave the protein epitope and thus may affect the antibody binding ability and detection, resulting in false negatives. Additionally, it has been shown that Matrigel may interfere with antibody detection using ICC. Therefore, all additional studies searching for a surface marker should utilize either live cell in-well imaging or flow cytometry using a non-enzymatic cell dissociation method, which is currently being explored in our lab.

CHAPTER 3: Small-Molecules in Oct4-Mediated Lineage Conversion

The generation of autologous and disease-relevant cell types has been a long-standing goal in regenerative medicine. While the derivation of induced pluripotent stem cells has greatly advanced this field, the genetic methods used to alter cell fate limit their therapeutic applications. Although the discovery of OiP has decreased the amount of genetic manipulation (Oct4 transgene vs. OSKM transgenes), this method still harbors a risk of insertional mutagenesis and tumorigenicity (Li et al., 2012b). Recent advancements in the field have come with the use of small molecules to direct cell conversion. Originally used to enhance the reprogramming process, chemical cocktails have been discovered that can modulate cell fate and even induce pluripotency, but the latter has only been demonstrated in mouse cells (Stadtfeld and Hochedlinger, 2010; Hou et al., 2013). Small molecules have distinctive advantages over viral methods, including easier manipulation, higher potency, and the ability to combine chemicals for multiple effects (Hou et al., 2013). Cell screens of synthetic small molecules and natural products have historically proved very useful in determining substitutes of reprogramming factors although to date, no substitute for Oct4 has been described (Ichida et al., 2009). The ultimate goal is to identify a compound that can successfully substitute for Oct4's role in the reprogramming process through activation of endogenous Oct4.

Fibroblasts are a readily available primary somatic cell type that are easily isolated from the skin and therefore represent an attractive source for reprogramming for downstream applications. Unlike their dermal counterparts, keratinocytes, fibroblasts do not endogenously express any the reprogramming factors Klf4 and c-Myc, therefore they

more readily reflect terminally differentiated cell types (Aasen et al., 2008; Patel and Yang, 2010). As such, the identification of a chemical activator of Oct4 in these cells may be more readily applicable to other cell types, such as blood cells. The identification of such a small molecule may provide further insights into the mechanisms that control cell fate, as well as ultimately generating safe and abundant cells for therapeutic and drug discovery applications.

3.0 Reported Activators of Oct4 Expression

Recently, two groups have reported the activating of endogenous Oct4 expression using small-molecules alone, the Oct4-Activating Compounds (OACs), and Reversine. Each compound was evaluated for its ability to induce OiP (via morphological assessment) as well as activate endogenous Oct4 protein expression, as this has not yet been explored in fibroblasts.

3.0.1 Oct4-Activating Compounds

Using cell-based high-throughput screening of chemical libraries, Li et al. identified Oct4-Activating Compound 1, or OAC1, as an activator of both human *Oct4* and *Nanog* promoter-driven luciferase reporter genes (Li et al., 2012c). When added to OSKM, OAC1 enhances both the kinetics and efficiency of MEF reprogramming, independent of either inhibition of the p53/p21 pathway or activation of Wnt/β-catenin signaling. Additionally, two structural analogs of OAC1, OAC2 and OAC3 (Figure 8), can also activate both luciferase reporters and enhanced iPSC formation from MEFs,

albeit at a lower rate than OAC1. As well, it has been recently demonstrated that OAC1 can activate endogenous Oct4 expression in human CB CD34+ cells, leading to Oct4-mediated upregulation of *HoxB4* to enhance *ex vivo* expansion of HSCs (Huang et al., 2015). The effect of OAC1 has yet to be determined in fibroblasts.

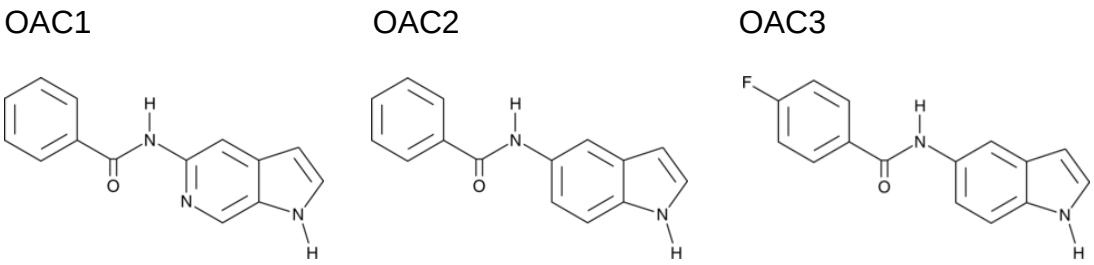


Figure 9. The structure of the Oct4-Activating Compounds.

Adult primary dermal tissue fibroblasts were cultured in Reprogramming media, conditions conducive to Oct4 expression, supplemented with 500 nM of OAC1, OAC2, OAC3, or DMSO as a control. After culturing for 8 days, cells were trypsinized, collected, fixed and permeabilized, and prepared for intracellular flow cytometry to assess for Oct4 and Nanog levels. No significant differences were seen in the expression of either factor upon OAC compound treatment as compared to the DMSO control (Figure 10). Compound concentration was increased to 1 μM, and adult fibroblasts were treated with either the OAC compound alone, or in combination with the histone deacetylase inhibitor Valproic acid (2 mM) to promote a more transcriptionally active chromatin structure, which may increase the OAC compounds' ability to access the Oct4 promoter

(Göttlicher et al., 2001). By flow cytometric analysis, increasing the concentration of OAC compound as well as the addition of the epigenetic modifier VPA did not result in increased Oct4 expression after 8 days. Instead, a decrease in both cell count and cellular viability was seen, likely due to the effects of VPA on cell proliferation and other basic processes (Karén et al., 2011). As such, it was inferred that all three OAC compounds do not induce robust endogenous Oct4 activation in human fibroblasts.

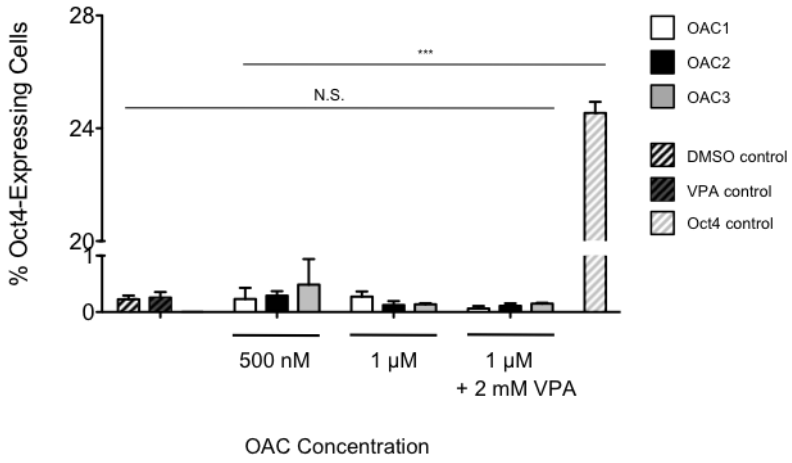


Figure 10. Oct4-Activating Compounds do not activate endogenous Oct4 protein expression in fibroblasts.
Flow cytometric analysis of the percent of $Oct4^+$ cells. Fibroblasts were treated with either 500 nM of OAC1/2/3, 1 μM of OAC1/2/3, or 1 μM of OAC1/2/3 + 2 mM VPA (DMSO and VPA used as negative control, Oct4-transduced cells used as positive control).

3.0.2 Reversine

During a cell-based screen for small molecules that can induce cellular dedifferentiation of lineage-restricted mammalian cells, the heterocyclic purine derivative Reversine was identified due to its ability to increase the plasticity of lineage-committed

murine C2C12 myoblasts (Chen et al., 2004). It is the first synthetic, low molecular weight and permeable compound that can act as a signal to induce lineage committed mammalian cells to regain multipotency (Cabral et al., 2008).

As early as day 4 of compound treatment, striking differences were apparent, specifically the inhibition of multinucleated myotube formation and instead, these cells grew a confluent culture of mononucleated cells (Chen et al., 2004). Phenotypically, expression of the myogenic-specific markers MyoD and myosin were reduced, suggesting that Reversine does not act as a toxin. By day 7 of Reversine treatment, cells acquired the ability to differentiate into multiple nonpermitted cell lineages when exposed to the appropriate conditions, including adipocytes, osteoblasts, and chondrocytes (Chen et al., 2004).

The potential application of these cells faced scrutiny, as C2C12 cells are an immortal, aneuploid and tumorigenic cell line, and do not reflect primary samples. Prompted by this perspective, Anastasia et al. tested Reversine on mouse-derived dermal fibroblasts, which importantly, maintain a normal phenotype and genetic stability (Anastasia et al., 2006). After only 4 days of Reversine treatment, fibroblasts acquired a strikingly different cell morphology compared to control cells, which included distinct cell swelling – up to nine times larger than the control cells – and a flatter, less contrasted shaped that was more adherent to the culture dish (Anastasia et al., 2006). After minor cell loss during the first day of treatment, no significant cell death occurred, although a growth inhibition was noticed. Further studies determined that these cells remain quiescent until either the removal of Reversine, or addition of appropriate extracellular

cues. Anastasia et al. detected no stem cell markers upon Reversine treatment, but recent results from a Chinese group are conflicting, demonstrate selective enhancement of Oct4 expression in these cells (Li et al., 2016).

Adult dermal fibroblasts were treated with 5 μM of Reversine (as per Anastasia et al.) in either Fibroblast media or Reprogramming media on either Matrigel-coated or uncoated tissue culture dishes in order to assess for morphological changes and/or activation of Oct4 expression. After 4 days of compound treatment, fibroblasts acquired the same morphological alteration as described by Anastasia et al., specifically transforming from their long spindle shape to a considerably larger and flatter cell (Figure 11A). It was also evident that growth arrest had occurred, as cell cultures were considerable less dense than untreated cultures. This was confirmed by cell count assays, in which the number of Reversine-treated fibroblasts after 4 days of growth was significantly less than untreated fibroblasts (Figure 11B). Oct4 expression was analyzed by flow cytometry, although there was no expression above untreated controls and as compared to an Oct4-transduced control, in line with previous studies (Figure 11C).

To follow up the findings that Reversine increases the plasticity of lineage-committed cells, assessment of fibroblast plasticity upon Reversine treatment was performed using the direct conversion protocol to neural progenitors (Mitchell et al., 2014b). Specifically, Reversine-treated fibroblasts were cultured on poly-L-ornithine/mouse laminin in neural lineage-specific conditions for neural progenitor formation, as per Mitchell et al. (2014b), but both low numbers of cells and decreased adherence to the tissue culture plate failed to produce any findings.

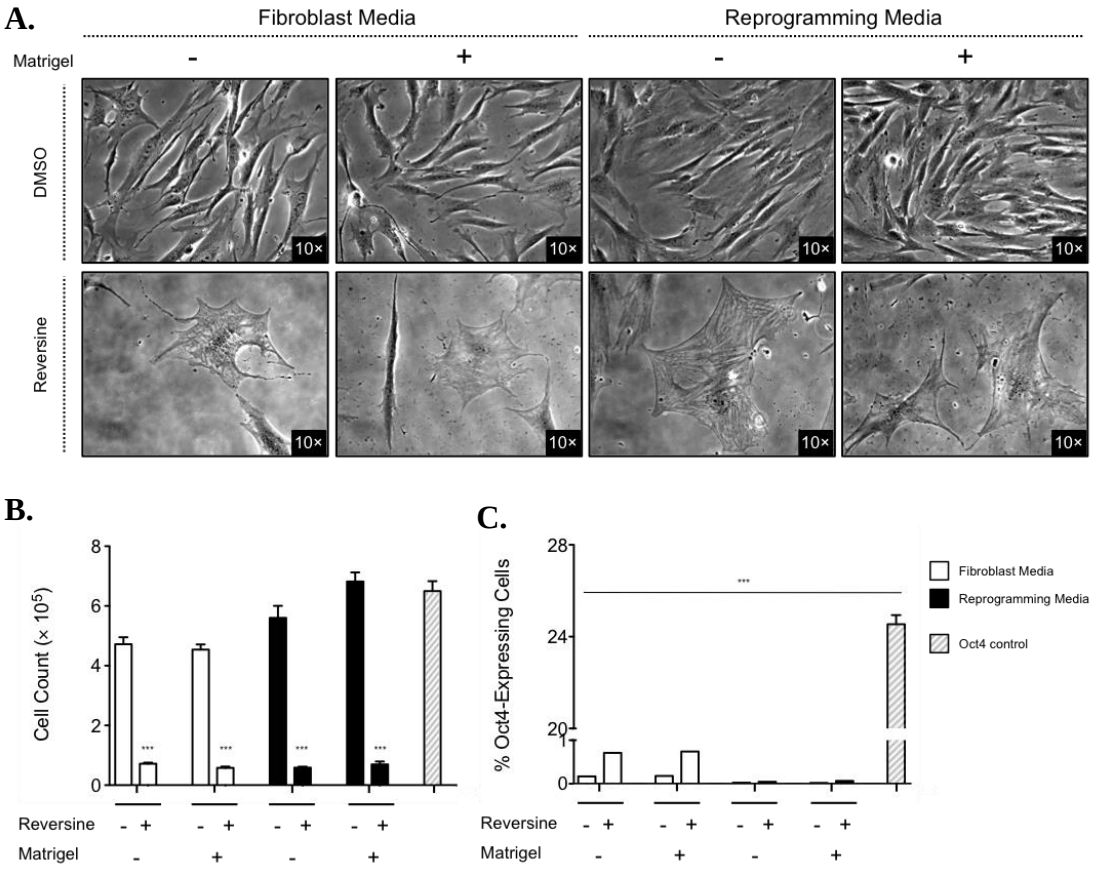


Figure 11. Reversine does not activate endogenous Oct4 protein expression in fibroblasts.

A. Live phase contrast images (10×) of DMSO or Reversine-treated (5 μM) fibroblasts after 8 days of culturing in Fibroblast media or Reprogramming media, on uncoated dishes (-) or Matrigel-coated dishes (+).

B. Cell count assay for total cell culture numbers after 8 days of treatment with DMSO (-) or 5 μM of Reversine (+) in Fibroblast media or Reprogramming media, on uncoated dishes (-) or Matrigel-coated dishes (+), compared to Oct4-transduced control.

C. Flow cytometric analysis of the percent of $Oct4^+$ cells. Fibroblasts were treated with 5 μM of Reversine or DMSO (used as negative control, Oct4-transduced cells used as positive control).

Considering that two previously reported activators of endogenous Oct4 expression, OAC1 and Reversine, failed to induce Oct4 expression in fibroblasts, our group turned to small-molecule screening as a means of identifying a novel robust Oct4 activator in human fibroblasts.

3.1 PL-EOS Vector Reporter-Based Screening

Our lab has developed infrastructure for high throughput and high content screening that has previously been used to monitor human PSCs for the loss of endogenous Oct4 expression. This has been accomplished using imaged-based tracking of an EOS(C3+)-eGFP-transduced human ESC line (Hotta et al., 2009). This vector, produced by James Ellis, encodes a synthetic promoter consisting of repeated Oct4 enhancer elements, which, upon Oct4 expression, results in activation of the downstream GFP gene that is visualized as green fluorescence. The system has been successfully utilized to track the reprogramming of human fibroblasts to iPSCs using exogenously expressed OSKM (Hotta et al., 2009).

The application of a fluorescence image-based protocol to small-molecule assessment provides a robust method for high-throughput screening of large chemical libraries, including those unique to McMaster University, for small-molecules capable of endogenous Oct4 activation. Specifically, the PL-EOS-C(3+)-EGFP-IRES-Puro is a reporter for endogenous Oct4 expression, as binding at the CR4 trimer activates GFP expression, resulting in green fluorescence. As such, the EOS(C3+)-eGFP vector was evaluated for its ability to robustly mark cells expressing endogenous Oct4.

The lentivirus was produced using the second-generation lentiviral packaging system, and lentiviral titration performed using the H9v1 pluripotent cell line due to its endogenous Oct4 activity (Figure 12A). Background levels of fluorescence were examined in fibroblast cells, which contain no endogenous Oct4 expression and should therefore result in no GFP fluorescence seen. As can be inferred from the flow cytometry plots, background levels of GFP expression were seen in fibroblasts, which increased with increasing lentivirus concentration (Figure 12B).

This result was unexpected, and the EOS lentivirus was reassessed in the context of iPSC reprogramming of fibroblasts to determine if expression could be localized to the pluripotent colonies and therefore, Oct4 population. As such, a limiting titre experiment was performed to detect Oct4 endogenous activation via detection of iPSC colonies.

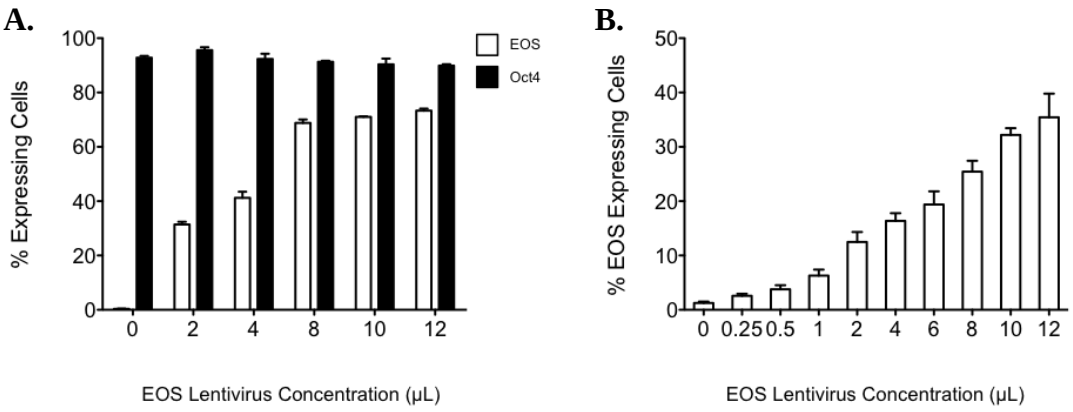


Figure 12. Titration of PL-EOS-C(3+)-EGFP-IRES-Puro lentivirus
A. Flow cytometric analysis of the percent of $Oct4^+$ and EOS^+ cells of PL-EOS-C(3+)-EGFP-IRES-Puro transduced H9v1 cells after fixation and staining for intracellular Oct4.
B. Flow cytometric analysis of the percent of EOS^+ cells of PL-EOS-C(3+)-EGFP-IRES-Puro transduced fibroblast cells after live cell flow cytometry.

In this, the minimum amount of lentivirus required to detect every iPSC colony was determined. Day 4 post-OSKM transduction, cells were assessed by fluorescence microscopy and flow cytometry where minimal amounts of GFP were observed. At day 16 post-OSKM transduction, cultures were assessed by fluorescence microscopy, where it was observed that PSC colonies fluoresced green due to GFP expression (Figure 13A). Following, flow cytometry was performed to quantitatively titre the EOS lentivirus. Perplexingly, results demonstrated that GFP-expressing cells, were in fact, not $Oct4^+$, as evidenced by lack of a $GFP^+/Oct4^+$ double-positive population, which conflicts with the fluorescence microscopy data (Figure 13B). The $Oct4^+$ cells were confirmed to be Tra-1-60^+, therefore it is likely that Oct4 expression was not the issue in these results. In order to account for these results, it has been hypothesized that the EOS lentiviral vector is undergoing lentiviral silencing, where the lentiviral vector is being inserted into a region of chromatin that is open in fibroblasts, but closes upon iPSC induction. The initial paper by Hotta et al. used Puromycin to select for successfully reprogrammed iPSC colonies, which may have caused the chromatin region surrounding the viral integration to remain open (Hotta et al., 2009). Our group has opted not to utilize Puromycin selection due to its potential interference in downstream small molecule screening.

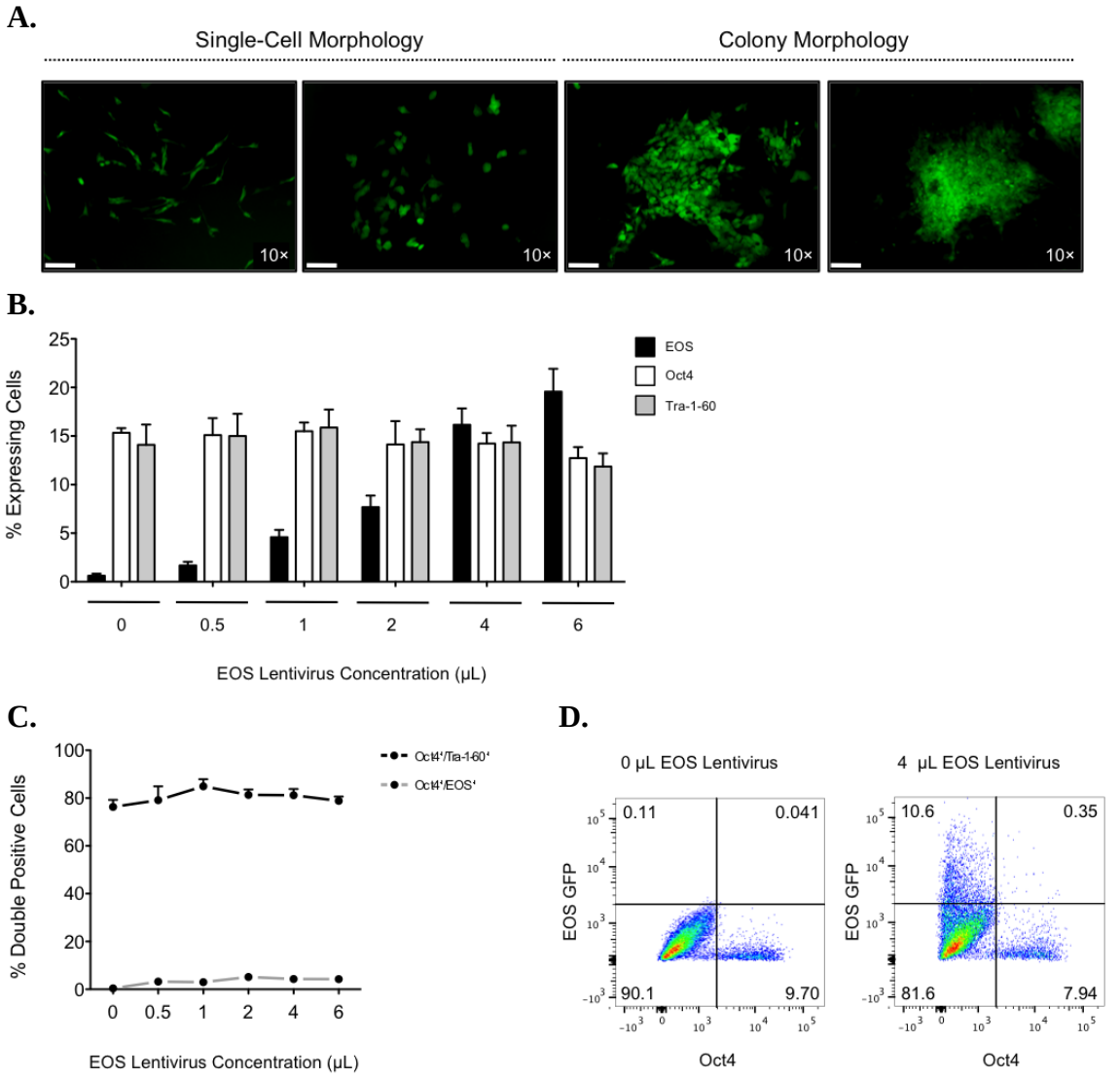


Figure 13. Titration of PL-EOS-C(3+)-EGFP-IRES-Puro lentivirus

A. Live fluorescence microscopy imaging (10×) of PL-EOS-C(3+)-EGFP-IRES-Puro transduced OKSM-transduced fibroblasts (16 days of culturing)

B. Flow cytometric analysis of the percent of $Oct4^+$, $Tra\text{-}1\text{-}60^+$ and EOS^+ cells of PL-EOS-C(3+)-EGFP-IRES-Puro transduced OKSM-transduced fibroblasts (16 days of culturing) after fixation and staining for extracellular Tra-1-60 and intracellular Oct4.

C. Flow cytometric analysis of the percent of $Oct4^+/Tra\text{-}1\text{-}60^+$ and $Oct4^+/EOS^+$ cells of PL-EOS-C(3+)-EGFP-IRES-Puro transfected fibroblast cells after fixation and staining for extracellular Tra-1-60 and intracellular Oct4.

D. Flow plots of flow cytometry data depicting the percent of $Oct4^+/Tra\text{-}1\text{-}60^+$ and $Oct4^+/EOS^+$ cells of PL-EOS-C(3+)-EGFP-IRES-Puro transfected fibroblast cells after fixation and staining for extracellular Tra-1-60 and intracellular Oct4.

3.2 Establishment of a Mouse *Oct4-GFP* Reporter Fibroblast Cell Line

In identifying an alternative method for small-molecule screening, previous prominent chemical library screens were reviewed. It was with this that it was discovered that The Jackson Laboratory has available two genetically modified mice in which an enhanced green fluorescence protein (eGFP) reporter is driven by the *Oct4* promoter. Each strain has been used for the selection of iPSCs and for the general fluorescent labeling of ESCs. The enhanced green fluorescent protein is the ideal reporter for this purpose, as expression of eGFP can be detected in living organisms or cells using fluorescent microscopy without any staining procedure.

The first strain, B6; CBA-Tg(Pou5f1-EGFP)-2Mnn/J, is a transgenic mouse which express eGFP under the control of the *Pou5f1* promoter and distal enhancer (Szabó et al., 2002). Note that this synthetic sequence, inserted into the host genome by microinjection, is separate from the endogenous *Oct4* sequence (Figure 14A). The predecessor of this strain is the *Oct4*-luc reporter, which was employed in the OAC1 small-molecule screen (Yeom et al., 1996; Li et al., 2012c)

Certain caveats may be found when using *Oct4-GFP* transgene reporters as a proxy for endogenous Oct4 expression, as positional effects from the site of integration of the transgene can affect GFP expression, such as the insertion into a transcriptional hot spot. As such, analysis by flow cytometry or fluorescence microscopy may be confounded due to autofluorescence (Lengner et al., 2008). In order to address these issues, a second mouse strain was established. This strain, B6; 129S4-*Pou5f1*^{tmJae}/J, is a targeted mutation in which the host genome harbors an IRES-eGFP fusion cassette

downstream of the stop codon of the *Pou5f1* gene, thereby avoiding any complications related to the site of integration (Lengner et al., 2007) (Figure 14B). As the eGFP reporter is downstream of the endogenous *Oct4* sequence, fluorescence is seen upon activation of all regions of the *Oct4* promoter. This strain was used in the small-molecule screen that identified E-616452 as a replacer of Sox2 (Ichida et al., 2009).

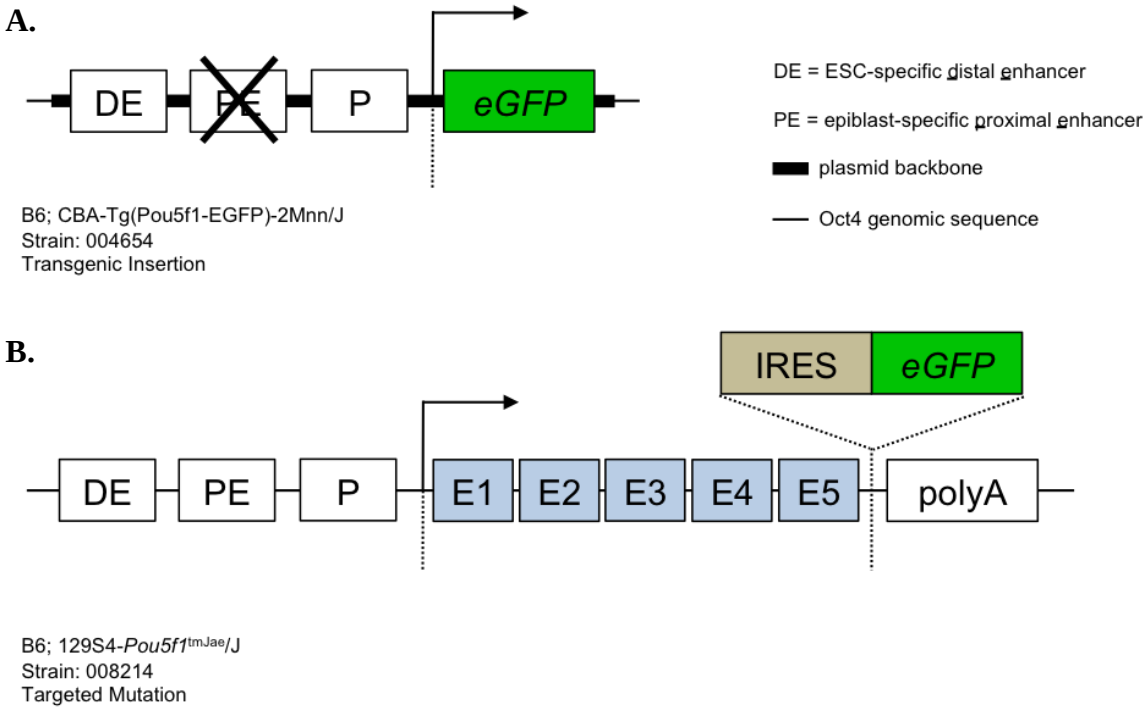


Figure 14. Schematic representation of the reporter locus of *Oct4-GFP* reporter mice.
A. B6; CBA-Tg(Pou5f1-EGFP)-2Mnn/J is a transgenic construct in which an *eGFP* reporter is under the control of a synthetic *Pou5f1* promoter and distal enhancer.
B. B6; 129S4-*Pou5f1*^{tmJae}/J is a targeted mutation in which the *eGFP* reporter is located downstream of the stop codon of the endogenous *Pou5f1* locus.

3.2.0 Transgenic Mice

The abovementioned mice were received from The Jackson Laboratory for the purpose of isolating and culturing *Oct4-eGFP* fibroblasts to be used in a chemical screen to identify potential inducers of endogenous Oct4 expression.

3.2.1 Isolation and Culturing of Adult Mouse Fibroblasts

Although the isolation and culture of mouse embryonic fibroblasts (MEFs) is well established, these cells may retain certain ES-like features in concurrence to their developmental hierarchy and multipotent nature, and have been shown to express SSEA1, Oct4, Nanog, and Sox2 (Yusuf et al., 2013). As such, adult rodent fibroblasts may be a preferred control when comparing to fibroblasts from adult humans (Seluanov et al., 2010). Using a modified protocol from Villegas et al. (2005), mouse chest and abdominal dermal tissue, heart, and lungs, were procured from sacrificed mice and cultured for fibroblast outgrowth (Figure 15). Over the course of three weeks, keratinocytes migrated outward from the dermal tissue pieces, followed by fibroblasts. Heart and lung tissues did not demonstrate substantial fibroblast outgrowth. Upon ~60% confluence (after approximately 2 weeks of outgrowth), cells were collected and were either frozen at P 0, or expanded on 100 mm tissue culture-treated dishes in fibroblast-conducive conditions to enrich for the fibroblast population over keratinocytes. By use of a selective media, keratinocytes were removed from the culture as they have been shown to express endogenous c-Myc and Klf4 (Aasen et al., 2008; Patel and Yang, 2010). Due to their nature, keratinocytes have been shown to reprogram a hundred fold more efficiently and

two fold faster compared to fibroblasts, and thus they do not accurately represent a terminally-differentiated cell population (Aasen et al., 2008; Patel and Yang, 2010).

Isolation and culturing of fibroblasts is an inefficient procedure, as values of 1-10% have been quotes for the number of originally harvested cells from a tissue that survive (Wright et al., 2002). This is also due to the heterogeneous population of cells seen in the skin, heart, and lungs, which can include muscle and blood cells, to name a few. It is also believed that these cells undergo an initial "culture shock" during in vitro propagation, in which many cannot survive (Wright et al., 2002). During the culturing of these samples, no fibroblast outgrowth was observed from heart tissue, and very minimal seen from lung tissue. Dermal skin samples provided the best fibroblast outgrowth, and therefore all fibroblast cell samples ongoing are of dermal skin source (Figure 16A).

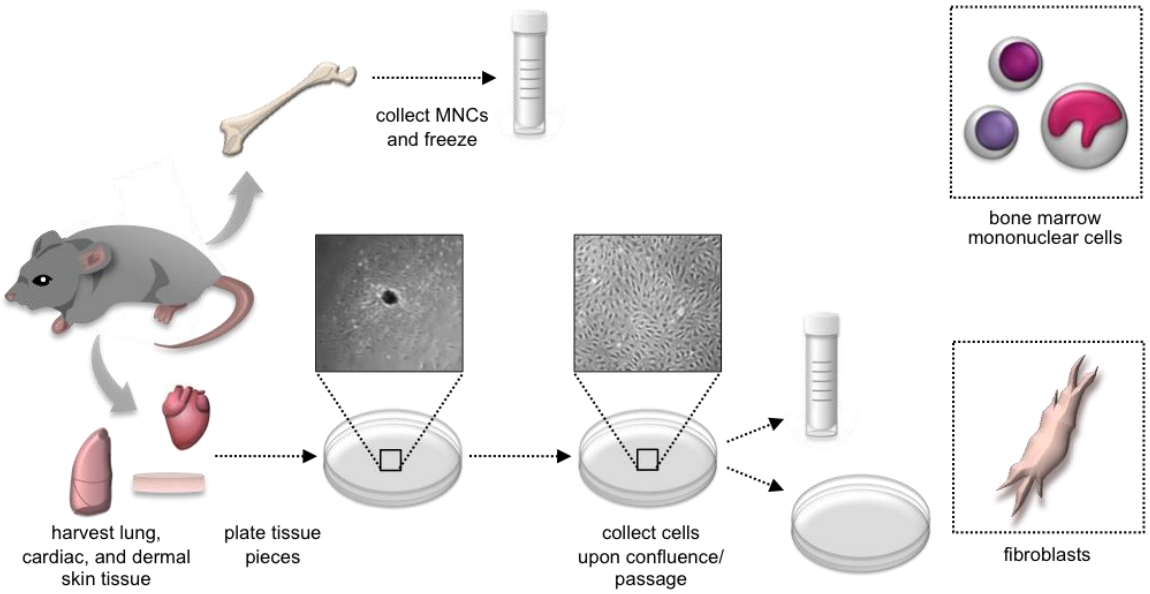


Figure 15. Schematic depiction of the process of harvesting tissue and culturing of cells from *Oct4-GFP* reporter mice.

Lastly, endogenous eGFP expression of fibroblasts was assessed to determine the "leakiness" of the *Oct4-eGFP* reporter, although no expression in the FITC channel was observed (Figure 16B).

3.2.2 Surface Marker Screening

Cell outgrowth from dermal tissue can result in a heterogeneous population, including muscle and blood cells as well as mesenchymal stem/progenitor cells. Culturing in selective media that supports fibroblast cell growth typically reduces this heterogeneity. To ensure that this population was devoid of large numbers of contaminating somatic or stem/progenitor cells, a flow cytometry-based antibody screen was utilized. Negative and positive MSC markers, as proposed by the International Society for Cell Therapy, were used, as well as additional somatic cell markers. These included blood and immune cell markers (CD4, CD11b, CD16, CD19, CD31, CD41, CD45, CD45R, CD71, CD73, CD105) as well as mesenchymal stem/progenitor cell markers (CD73, CD90, CD105, CD117/c-kit, Ter-119, Stro-1). The results demonstrated only minimally positivity for CD19, CD73 and CD105 (< 20%), which may be due to a small amount of contaminating cells (Figure 16C) (Boxall and Jones, 2012). These cells were negative for CD90, which, although a robust fibroblast marker in human cells, has been demonstrated to be a marker of immature and/or stem/progenitor cells in mouse cells (Fries et al., 1994). The finding that these mouse fibroblasts lack CD90 is consistent with the fact that they were derived from *adult* dermal tissue.

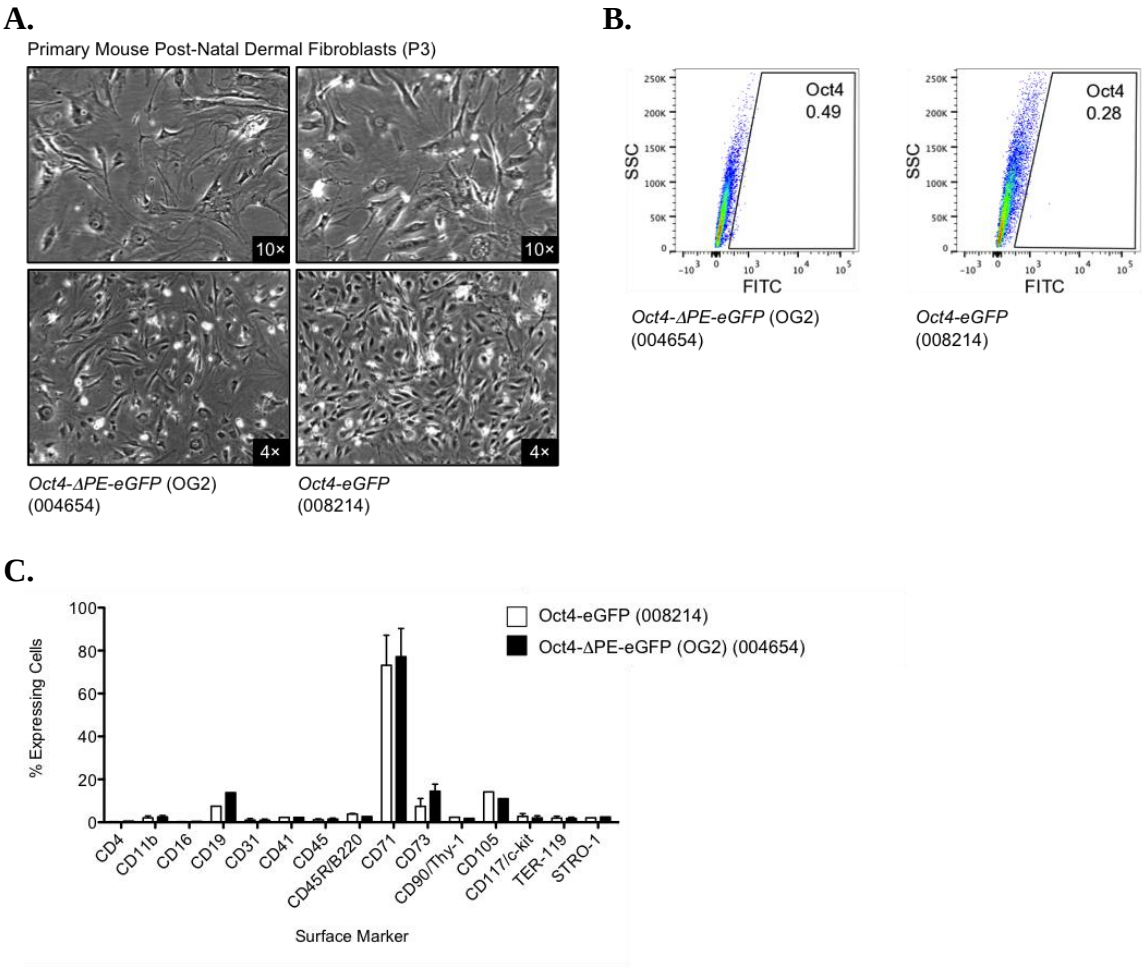


Figure 16. Analyzing fibroblast cells harvested from dermal tissue of *Oct4-GFP* reporter mice.
A. Live phase contrast images (10× and 4×) of P3 primary mouse post-natal dermal fibroblasts.
B. Live cell flow cytometry analysis of mouse dermal fibroblasts for background eGFP (FITC channel) expression
C. Live cell flow cytometry analysis of cell surface markers.

Interestingly, cells were positive for CD71, which is a transmembrane glycoprotein required for iron import from transferrin by endocytosis. Although CD71 is typically used as a marker of early erythroid precursors, it also marks proliferating cells

specifically by facilitating the uptake of iron, which is required for their development (Brekelmans et al., 1994). In this regard, it is not unusual that these cells are positive for CD71. Also noteworthy is that both dermal fibroblast populations were relatively consistent in the expression of all surface markers.

3.2.3 Validation of *Oct4-eGFP* Reporters

In order to begin drug screening, the *Oct4-GFP* reporter must be validated to ensure faithful reporting of all Oct4-expressing cells. It has been well characterized that endogenous Oct4 expression is induced late in the reprogramming process during the stabilization phase (Polo et al., 2012). As reprogramming fibroblasts iPSCs is a long, laborious, and inefficient process, it would therefore be preferable to use a chemical alternative to confirm *Oct4-GFP* reporter activity, which has been shown to induce Oct4 expression earlier in the reprogramming process and with a greater efficiency (Figure 17) (Li et al., 2016).

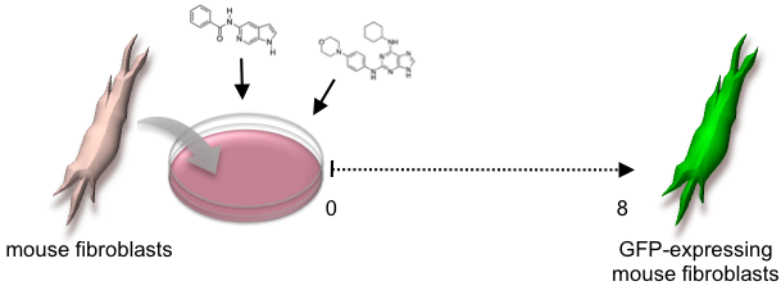


Figure 17. Schematic representation of the validation of the *Oct4-GFP* reporter using small-molecule compounds.

3.2.3.1 Oct4-Activating Compounds

Although the OAC compounds were unable to induce Oct4 expression in human fibroblasts, it is possible that due to the innate differences in the Oct4 promoter and enhancer elements between mouse and human cells, eGFP expression may be seen upon OAC treatment in the *Oct4-eGFP* mouse fibroblasts. Initial studies on Reversine were conducted in the murine system, and due to species-specific differences in the promoter and enhancer regions of mouse and human cells, Oct4 expression, and therefore GFP expression, may be seen upon Reversine treatment of mouse fibroblasts (Chen et al., 2004).

As per section 3.0.1, murine fibroblasts were treated with 1 μM of each OAC molecule for 8 days prior to analysis via fluorescence microscopy. After 8 days, no GFP expression, therefore treatment continued to 16 days, where there continued to be no GFP expression seen.

In all, it can be inferred that the OAC compounds do not activate the *Oct4-eGFP* reporter in these mouse fibroblasts, similar to the results found in human fibroblast cells in section 3.0.1.

3.2.3.2 Reversine

Similar to the OAC compounds, Reversine was unable to induce Oct4 expression in human fibroblasts. As with the above, it is possible that inherent species-specific differences in the Oct4 upstream promoter and enhancer regions are responsible, and therefore murine fibroblasts were treated with Reversine for assessment as per section

3.0.2. After only 4 days of culturing, the treated murine fibroblasts appeared relatively unaffected by Reversine, as the cell number and morphology were comparable to DMSO-treated fibroblasts. No GFP expression was seen at this time. By day 8, Reversine-treated fibroblasts demonstrated drastically slowed cell growth and acquired the same morphology as the human fibroblasts. These large, flattened cells did not express GFP (Figure 18).

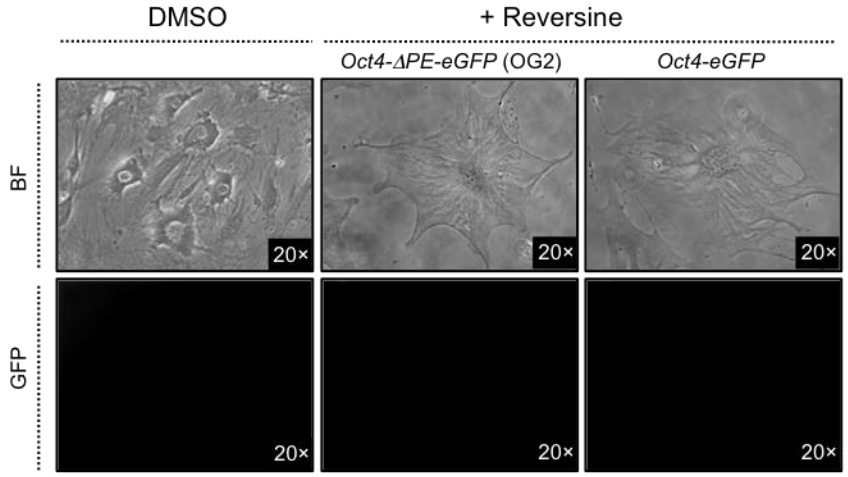


Figure 18. Reversine does not activate the *Oct4-GFP* reporter in mouse dermal fibroblasts.
A. Live phase contrast images (20×) of DMSO or Reversine-treated (5 μM) mouse dermal fibroblasts after 8 days of culturing in Reprogramming media.

3.2.3.3 VC6TFZ

The Deng group identified of a small-molecule combination of VPA (HDAC inhibitor), CHIR99021 (a GSK3 inhibitor), E-616451 (a TGF-β inhibitor), and Tranylcypromine (Parnate; a LSD1 inhibitor), which was shown to enable reprogramming in mouse cells with Oct4 alone (Li et al., 2011). Through additional

small-molecule screening, the same group identified Forskolin (FSK), a cAMP agonist, which could activate an *Oct4*-driven GFP reporter in MEFs (Hou et al., 2013). FSK, in combination with their previous cocktail, VC6T, and the late-stage reprogramming booster DZNep, generated GFP^+ clusters, but these cells lacked robust endogenous pluripotent gene expression. To enhance the expression of these genes, cells were switched to a 2*i*-medium (dual inhibition of GSK3 and MEK) using CHIR99021 and PD0325901, and the resulting cells were validated as pluripotent and subsequently termed chemically-induced pluripotent stem cells, or ciPSCs (Hou et al., 2013). Additional optimization of this process identified TTNPB, a synthetic retinoic acid receptor ligand, as a booster for chemical reprogramming. Using this seven small-molecule method, ciPSCs could be generated from mouse somatic cells with a frequency up to 0.2%, comparable to transcription factor-induced reprogramming.

This reprogramming method was employed in order validate the Oct4-eGFP reporter in the mouse fibroblasts (Figure 19).

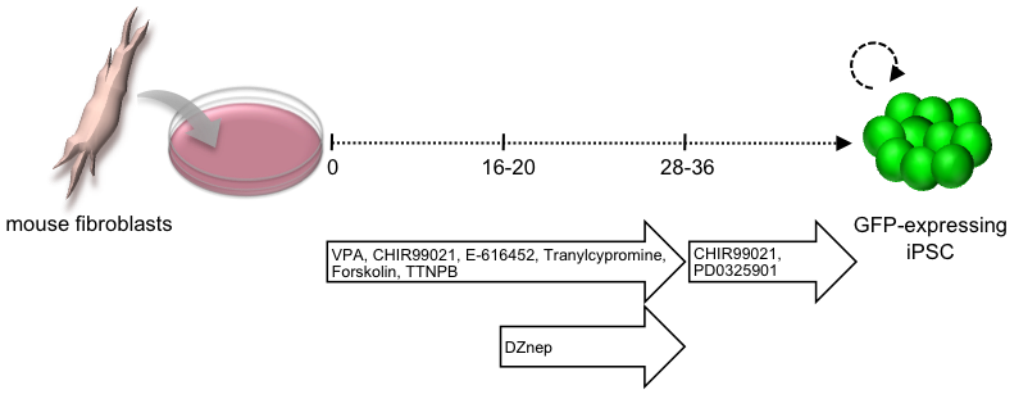


Figure 19. Schematic representation of the validation of the *Oct4-GFP* reporter using the VC6TFZ small-molecule cocktail.

Briefly, cells were treated with VC6TF (see concentrations in Appendix I: Materials and Methods) for 16 days, and analyzed by fluorescence microscopy on day 4, day 8, and day 16. Interestingly, cell death was evident as early as day 4, with large amounts of floating cells and debris present in the culture dish (Figure 20A). By day 8, cell counts had decreased drastically and by day 16, any viable cells remaining were morphologically similar to senescing fibroblasts (Figure 20B). Addition of DZNep at day 16 resulted in complete cell death, in which all cells lifted off the culture dish. At no time was GFP expression detected. As such, this method of reporter validation was discontinued.

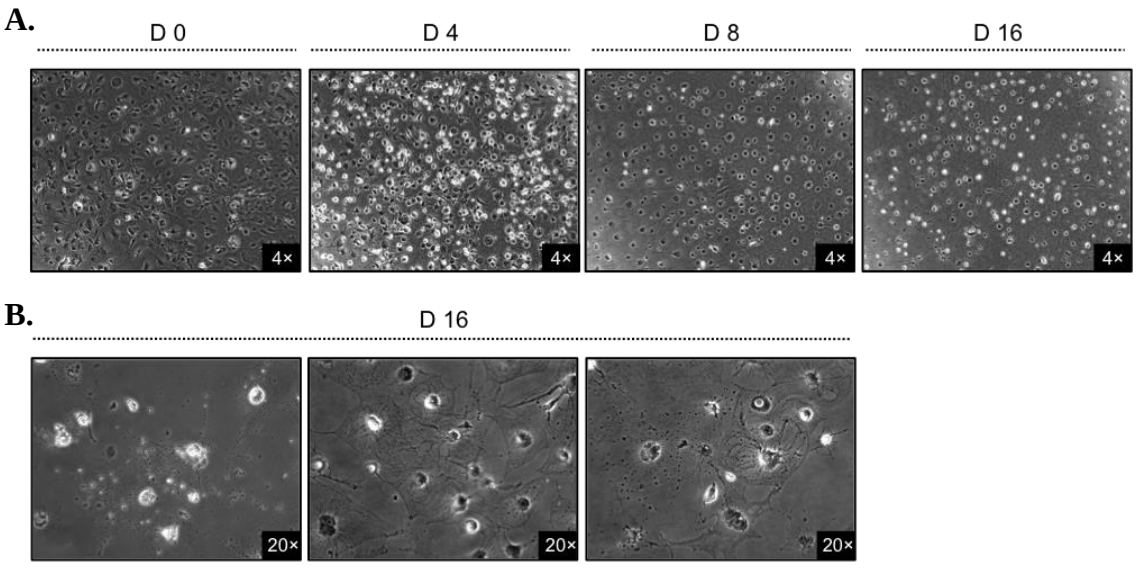


Figure 20. Treatment of mouse dermal fibroblasts with the VC6TF small-molecule cocktail does not activate the Oct4-GFP reporter.
A. Analysis (4×) of pretreated (D 0) and VC6TF-treated cells at day 4 (D 4), day 8 (D 8) and day 16 (D 16) of compound treatment.
B. Analysis (20×) of VC6TF-treated cells at D 16 of compound treatment.

3.2.3.4 Oct4 and OSKM Transgenes

As chemical methods have failed to activate the Oct4-eGFP reporter for both murine fibroblast strains, traditional OSKM-mediated reprogramming to iPSCs will be employed to validate the reporter system. It has been well documented that iPSCs endogenously express Oct4, and this characteristic is a defining feature of all pluripotent cells (Takahashi et al., 2007).

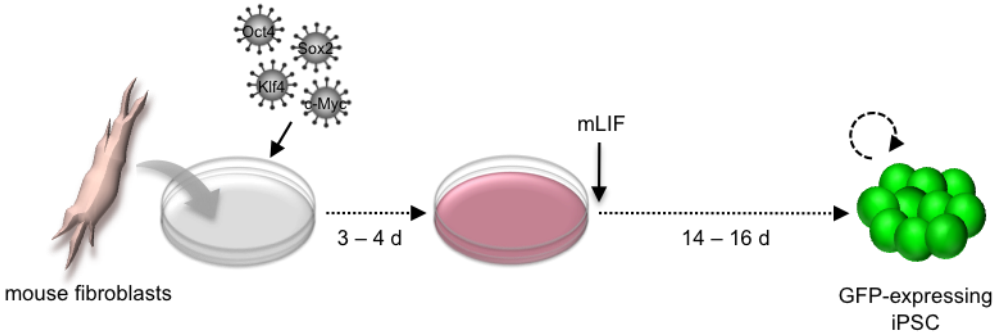


Figure 21. Schematic representation of the validation of the Oct4-GFP reporter using OSKM transduction for iPSC generation.

In order to test this, fibroblasts were seeded onto Matrigel-coated dishes at a seeding density of 1×10^4 cells/well of 12-well format, and transduced with OSKM using the pSIN4-EF2-O2S and pSIN4-CMV-K2M lentiviral vectors, followed by culturing in Reprogramming media for 14 days. Upon successful reprogramming to induced pluripotency, the resulting iPSC colonies should fluoresce green, as endogenous *Oct4* expression at pluripotency should produce the green fluorescent protein. As a control, Oct4 only (pSIN-EF1α-OCT4-IRES-Puro) was ectopically expressed in these fibroblasts, as it alone is insufficient to induce endogenous Oct4 activation in a short time frame

(Mitchell et al., 2014, Salci et al., 2015). As expected, Oct4 overexpression alone failed to activate the *Oct4* promoter as indicated by a lack of green fluorescence, therefore indicating that no endogenous *Oct4* was expressed. Interestingly, these cells underwent a morphological shift very similar to that seen in human Oct4-expressing fibroblasts, consisting of compact-cuboidal cells that coalesce together (Figure 22). This is an important finding, as it indicates that the OiP is not a human-only phenomenon, and instead is an inherent property of Oct4 overexpression in fibroblast cells.

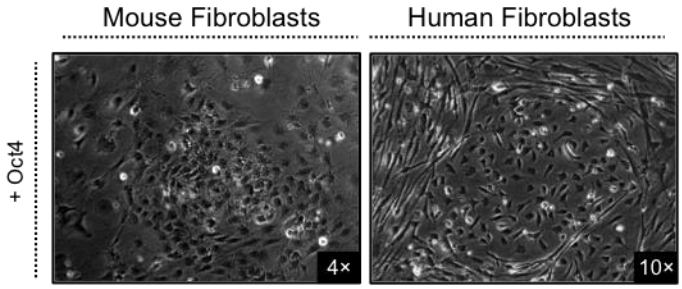


Figure 22. Overexpression of Oct4 in mouse dermal fibroblasts fails to activate the *Oct4-GFP* reporter, but generates cells morphologically similar to OiP

Upon overexpression of OSKM, the mouse fibroblasts failed to establish pluripotency, as demonstrated by a lack of morphological change and green fluorescence. Instead, cells appeared to senesce, as evidenced by a flat, enlarged morphology and failure to proliferate. As a control, human fibroblasts transduced with OSKM and cultured similarly were able to reach pluripotency, as determined through morphological and phenotypic analysis, including the expression of Tra-1-60 and Oct4, indicating that there was no issue with lentivirus concentration (data not shown). This was revisited

using advice from Dr. Jong-Hee Lee. Changes were made in terms of seeding density, cytokine addition, and basement membrane. Specifically, cells were seeded at a higher density, 1×10^5 cells/well of 12-well format, on gelatin-coated plates and the next day, cells were transduced with OSKM in only 0.5 mL of media using 8 μg/mL polybrene as a transduction reagent. After 5 days, cells were trypsinized and replated at 2×10^5 cells/well of 6-well format on either Matrigel or on irradiated MEFs (iMEFs). Half of the cells were cultured in Fibroblast Media, or in Reprogramming media (15% KOSR, 5% FBS) containing (1000 U) mLIF. After two weeks of culturing, it was determined that iPSCs only formed from mFibs cultured on iMEFs in Reprogramming media containing mLIF, as all other conditions failed to establish pluripotent colonies (Figure 23). Establishing pluripotent cultures from these adult mouse fibroblasts has validated the fluorescent reporter and has allowed for screening chemical compounds for activators of endogenous *Oct4* expression to commence.

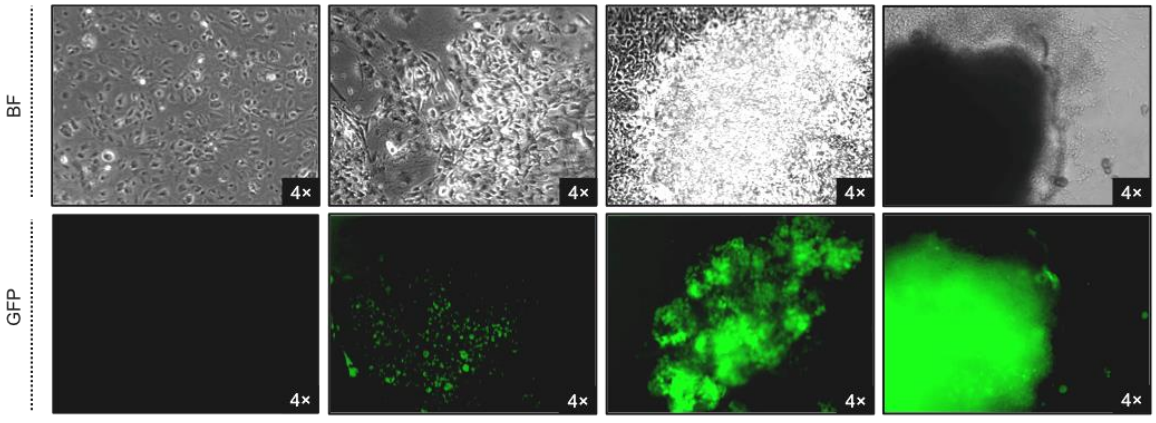


Figure 23. Overexpression of OSKM in mouse dermal fibroblasts generates GFP+ colonies that are morphologically similar to iPSCs.

3.3 Moving Forward With Small-Molecule Screening

As the reporter system has been validated, screening of chemical libraries amounting to 7500 – 10 000 individual compounds unique to McMaster may commence. In this, murine dermal fibroblasts will be seeded in either 96-well tissue-culture or 384-well optical imaging plates and grown in conditions that have been shown to permit iPSC induction and endogenous *Oct4* expression in adult murine fibroblasts (section 3.2.3.4). Individual compounds will be added to the media (starting at 1 μM) and grown for eight days. Afterward, cells will be assessed for morphological changes and imaged using the Operetta/Columbus High Content Imaging platform with both proliferation and eGFP levels per cell quantified using custom, automated image analysis scripts. Compound "hits" will be defined as compounds that induce eGFP fluorescence levels that are three standard deviations above background expression levels. Induced pluripotent cells of each strain, which have been shown to express eGFP due to endogenous *Oct4* expression, will be used as positive controls (section 3.2.3.4). Following, immunofluorescence and flow cytometry for Oct4 protein levels will be performed, as the reporter activity is indicative only of transcriptional activity, not translational. This process will allow for stringent analysis of potential chemical activators of endogenous *Oct4* expression and protein production. Due to the innate differences between mouse and human cells, compound hits will be screened in human fibroblasts in reprogramming conditions that have been shown to sustain *Oct4* expression in these cells (Mitchell et al., 2014a). This is to be followed by antibody-mediated quantification of endogenous Oct4 levels to confirm the hits. Compounds that induce endogenous Oct4 protein production will be evaluated for

their ability to support OIP, followed by direct conversion toward hematopoietic and neural progenitors using lineage-supportive conditions. Auxiliary chemical compounds previously proven to enhance the reprogramming process, such as VPA or CHIR99021, may be employed to improve the efficiency and/or kinetics of this process. Nevertheless, the identification of a chemical-only means of modulating cell fate in human cells would be monumental in furthering the efforts of regenerative medicine.

CHAPTER 4: Extracellular Requirements of Plasticity

An interesting finding by Mitchell et al. regarding OiP is that the induction of this state is critically dependent upon the extracellular environment in which the cells are cultured. Specifically, Oct4-transduced fibroblasts must be maintained in Reprogramming media in order to achieve sustained Oct4 expression and the induction of the plastic state, as culturing in Fibroblast media leads to diminished Oct4 levels and lack of morphological alteration (Mitchell et al., 2014a). Transduction of a control vector demonstrated consistent frequencies of GFP over time in all media conditions, disproving any speculation that this phenomenon was due to a lentivirus anomaly.

Similar findings have been reported in the context of induced pluripotency, in which the reprogramming of somatic cells by defined factors is regulated by the extracellular environment despite being primarily a nuclear process (Chen et al., 2011a). In fact, it was found that extracellular cues modulate the reprogramming process by influencing signaling pathways and even the epigenetic status. As such, there is an underappreciated relationship between reprogramming factors and the extracellular environment that can greatly influence the alteration of cell fate.

In the context-specific nature of OiP, Oct4 expression is independent of its native promoter and enhancer regions, and therefore exogenous expression is mediated through one of two potential elements: positive regulation in the presence of Reprogramming media; or negative regulation likely due to an inhibitory factors present in Fibroblast media. Identification of components that support or inhibit the OiP process in concert with our current understanding may lead to improved generation of these plastic cells as

well as further elucidation of the molecular mechanisms at play in OiP.

Due to the absence of a surface marker to identify this cell population, the determination of media components that enhance or sustain OiP will be based on both sustained Oct4 expression and morphological alteration.

Table 1. Medium formulations for Fibroblast media and Reprogramming media

	Fibroblast Media	Reprogramming Media
Basal Media	DMEM	DMEM/F-12
Growth Factors	-	16 ng/mL bFGF 30 ng/mL IGF-II
Serum Content	10% FBS	20% KOSR
Amino Acids	1× MEM NEAA 1× L-glutamine	1× MEM NEAA 1× L-glutamine
Other Additives	-	0.1 mM β-mercaptoethanol

4.0 The Extracellular Environment

The extracellular environment (ECE), or the conditions outside of a cell's plasma membrane, is a complex and dynamic setting that is an essential component of a cell's milieu (Gattazzo et al., 2014). *In vivo*, cells reside in a highly specialized ECE, denoted as the 'niche', which provides extracellular cues to regulate cellular functions. It is this spatial control that allows the formation of well-organized cell 'communities', such as tissues and organs, which perform one collective function to benefit an organism. *In vitro* culturing requires a specially formulated medium that mimics this niche in order to sustain these cells.

Both the *in vivo* niche and *in vitro* ECE are comprised of various proteins, lipids, ions, cellular metabolites and even other cells, among many other components, which

function to regulate a cell's behaviour, growth, and development through direct and indirect means (Gattazzo et al., 2014). Components such as hormones, growth factors, cytokines and chemokines in the ECE can bind biochemical receptors on cells, influencing intracellular cellular signaling cascades. Conversely, proteins active outside the cell, such as cell attachment and matrix factors, can influence cell or tissue remodeling and migration. Under certain conditions, a cell itself may also contribute to the ECE through the secretion of metabolites or extracellular matrix (ECM) components, such as fibronectin, to influence cell adhesion and structural support. In addition, other physical factors, such as shear stress, oxygen tension, temperature, and pH, also contribute to a cell's behaviour. It is therefore the collective effort of all ECE factors in influencing a cell's behaviour.

4.1 Analyzing the Fate of Oct4-Transduced Cells

Although it has been established that cells require Reprogramming media in order to sustain Oct4 expression, the fate of Oct4-transduced cells in Fibroblast media remains to be determined. This loss or inability to sustain Oct4 expression raises a number of possibilities regarding the fate of these cells, including lentiviral silencing, Oct4 protein degradation, or cell death.

In order to analyze cellular fate depending on culture conditions, adult dermal fibroblasts were transduced with Oct4 lentivirus and cultured either in Fibroblast media or Reprogramming media, and analyzed daily to determine cell count, viability, apoptosis, and Oct4 expression (Figure 24). My assessment of Oct4 expression began 48 hours

post-transduction when both transgene expression and protein levels reach ESC-like levels, and continued for 10 days prior to passaging (Tiemann et al., 2014). Two further measurements were taken 14 and 17 days post-Oct4 transduction to identify differences in these cultures long-term.

As early as 48 hours post-transduction, where cells are cultured in either 100% Fibroblast media or 50% Fibroblast media/50% Reprogramming media, no discernible difference in cell count is seen, yet within these populations, the cells cultured in 50/50 exhibit higher levels of Oct4 transgene expression and Oct4 protein expression (Figure 24). By day 3 post-transduction, cells are now cultured in 100% Fibroblast media or 100% Reprogramming media. At this time, cells cultured in Reprogramming media have drastically expanded, with cell counts roughly double that of Fibroblast media cultures, correlating with higher proliferation via Ki67 analysis (data not shown). Throughout the 10-day experiment, cells cultured in Reprogramming media continue to exhibit much higher cell counts than those cultured in Fibroblast media, even past day 8 when cultures reach confluence and cells begin to die (Figure 24A).

Analysis of the Oct4 transgene levels over 10 days demonstrates a higher level of expression in Reprogramming media, but this does not necessarily correlate to a higher level of protein expression within the first 7 days. It is only on day 8 that Oct4 protein levels drastically fall in Fibroblast media cultures, which is met with a decrease in transgene levels the following day (Figure 24B, C).

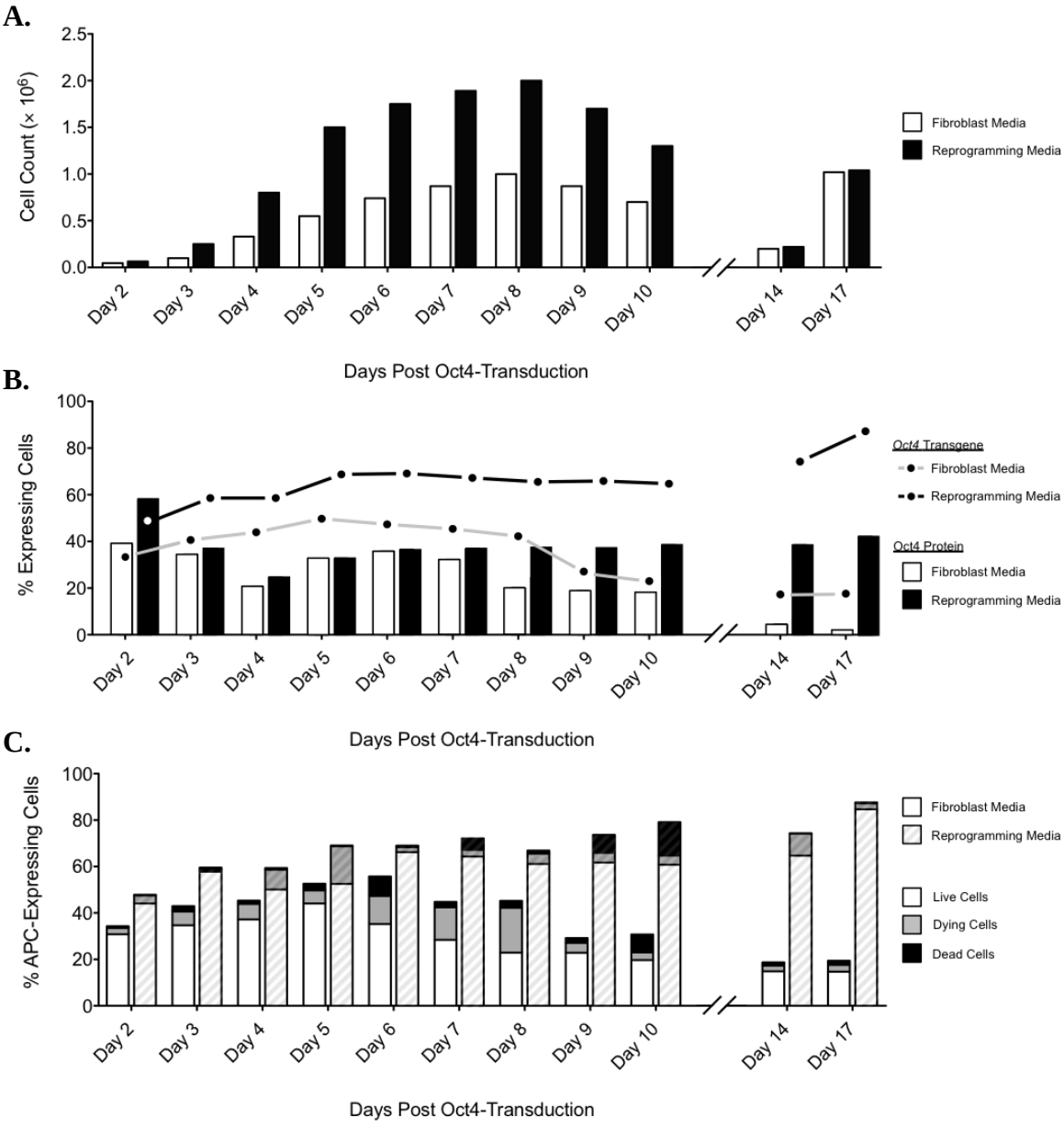


Figure 24. Culturing of Oct4-transduced fibroblasts in Fibroblast media results in lower cell proliferation as well as decreased Oct4 transgene expression and protein levels over time when compared to Reprogramming media.

A. Cell count assay of Oct4-transduced cells cultured in Fibroblast media and Reprogramming media over 17 days.

B. Flow cytometric analysis of the percent of APC^+ (Oct4 transgene) and $Oct4^+$ cells by live cell or fixed cell and intracellular staining analysis, respectively.

C. Annexin V analysis by live cell flow cytometry to identify the fate of Oct4 transgene-expressing cells (APC^+) over time. (Dying cells: Annexin $V^+/7AAD^-$; dead cells: Annexin $V^+/7AAD^+$) (/ / indicates cell passaging at day 10)

Through Annexin V analysis, it is evident that Oct4 transgene-expressing cells cultured in Fibroblast media begin to die by day 6, continuing through to day 8, where much lower levels of APC^+ cells are seen (Figure 24C). This is in contrast to those cultured in Reprogramming media, in which steady levels of transgene expression and protein levels are seen through to day 10. Following, cells were passaged onto fresh Matrigel-coated dishes on day 10, and allowed to grow for 4 additional days prior to continued analysis. During this time, it was observed that most cells that had adhered to the dish in Reprogramming media were smaller, more compact, while those in Fibroblast media remained fibroblast-like in morphology. Interestingly, the growth rate of cells in Reprogramming media decreased upon passaging, with cell counts comparable to those in Fibroblast media seen for days 14 and 17. Remarkably, these passaged cells in Reprogramming media, though low in number, sustained Oct4 transgene expression and protein levels, while these factors were diminished in Fibroblast media upon passaging. By day 17, Oct4 transgene levels were at their highest in Reprogramming media, while simultaneously at their lowest in Fibroblast media.

Through this analysis, it is evident that Oct4 transgene and protein levels decline during culturing in Fibroblast media as compared to cells in Reprogramming media, corresponding to the findings of Mitchell et al. that Fibroblast media does not sustain Oct4 expression (Mitchell et al., 2014a). It is difficult to say if the transgene becomes silenced, or if these cells may be selectively dying in Fibroblast media. In order to conclusively determine if lentiviral silencing is occurring, PCR may be used to determine if day Fibroblast media-cultured cells contain in their genome the integrated Oct4

lentiviral transgene, and if it is expressed as RNA.

As is apparent when viewing the cell count assay (Figure 24A), Oct4-transduced cells cultured in Fibroblast media do not proliferate as well as their Reprogramming media counterparts. Tracking early reprogramming through time-lapse imaging, the Meissner group found that cells that will successfully reprogram demonstrate an increase in proliferation, which occurs as early as 24 hours post-transduction (Smith et al., 2010). Perhaps it is this failure to induce a higher proliferative rate that results in the decline of Oct4 expression in Fibroblast media-cultured cells and ultimately, the inability to induce the plastic state.

The above findings are similar to that of Kim et al., who demonstrated that Oct4 overexpression in porcine fibroblasts results in morphological changes, increased migration abilities, and enhanced proliferation (Kim et al., 2014). Specifically, RT-PCR results showed upregulation of the cell-cycle and apoptosis-related genes *p16*, *Bcl2* and *Myc* in Oct4-overexpressed fibroblasts. The authors suggested the increase in cell proliferation was largely due to increased *Myc* expression, while *Bcl2* reduced apoptosis in these cells. Importantly, this study also noted no endogenous expression of *Oct4*, *Sox2*, or *Nanog* (Kim et al., 2014).

It is therefore important to determine the contribution of media components that are responsible for permitting or inhibiting the sustained expression of Oct4. Extrication of components that support Oct4 expression and OiP induction, in concert with our current understanding of this state, can lead to the improvement of cell fate alteration using Oct4 alone.

4.2 Interrogation of Media Components

The *in vitro* culturing of cells has basic environmental requirements in order for these cells to grow optimally, including controlled temperature, substrate for cell attachment, an incubator that maintains correct oxygen and/or carbon dioxide content (Arora et al., 2013). Arguably the most important and crucial factor is growth media, which is generally composed of a complement of amino acids, vitamins, inorganic salts, glucose, and serum. Certain cell types may have additional requirements above a basic growth media, therefore further components are generally added to the basal medium to help sustain cell proliferation and maintain normal cell metabolism. These factors may include hormones, signaling molecules, and growth factors.

4.2.1 Growth Factors

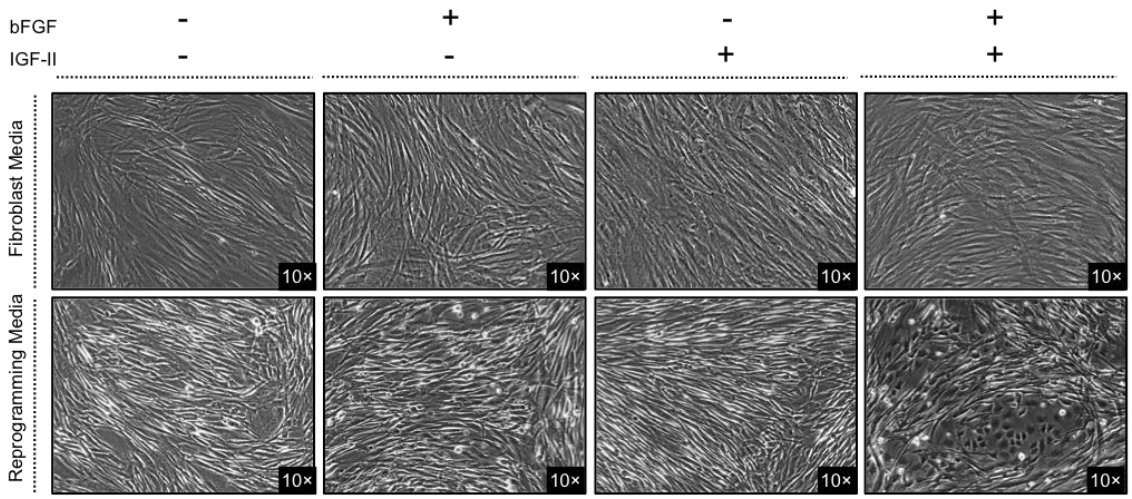
A previous study published in *Nature* from the Bhatia lab demonstrated that pluripotency is under a level of external control from the microenvironment (Bendall et al., 2007). In this study, the observed heterogeneity of PSC cultures – the colonies and the fibroblast-like cells (hdFs) – was in part a result of a dynamic interplay between these cell types, mediated by growth factor production and receptor expression. In particular, these cells were uniquely defined by insulin-like growth factor (IGF) and fibroblast-growth factor (FGF) dependence. IGF1R expression was found to be exclusive to the hESCs, whereas FGFR1 expression was restricted to the surrounding hdFs. Blocking the IGF-II/IGF1R pathway reduced survival of the ESCs, whereas inhibition of the FGF pathway indirectly caused differentiation. Lastly, it was found that IGF-II is expressed by

hdFs in response to FGF, and alone was sufficient in maintaining hESC cultures. A separate study in the lab of James Thomson demonstrated that out of all growth factors tested, bFGF had the greatest effect in promoting hESC self-renewal (Xu et al., 2001).

With this knowledge, it is logical to assume that these two growth factors, key in PSC maintenance and supplemented in Reprogramming media, may play a role in the induction of a different, unique Oct4-dependent state; OiP. In two concurrent experiments, bFGF and IGF-II were added sequentially to Fibroblast Media, while they were similarly removed from Reprogramming media to assess their ability to sustain Oct4 expression.

After 18 days of culturing post Oct4-transduction, flow cytometry analysis proved that neither growth factor significantly affected Oct4 expression (N.S., $p > 0.5$), although the addition of bFGF to Reprogramming media slightly improved the number of Oct4-expressing cells (Figure 25B). Therefore, growth factors alone are unable to sustain Oct4 expression in Fibroblast Media. Thus, there must be additional factors that permit this occurrence in Reprogramming Media, or inhibit this in Fibroblast Media.

A.



B.

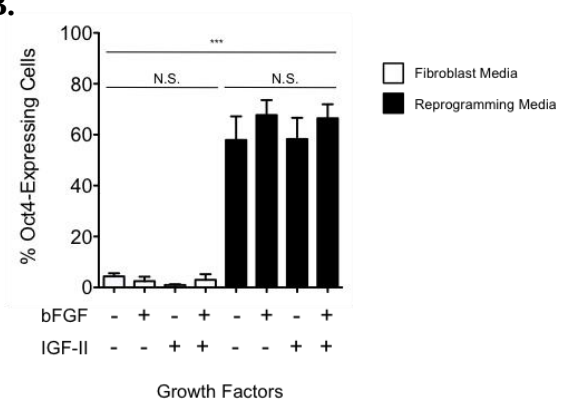


Figure 25. Basic fibroblast growth factor and insulin-like growth factor-II do not enhance Oct4 expression in Fibroblast media

Analysis of Oct4-transduced fetal fibroblasts after 16 days of culturing in either Fibroblast media or Reprogramming media +/- 16 ng/mL bFGF +/- 30 ng/mL IGF-II.

A. Live phase contrast images (10×)

B. Flow cytometric analysis of the percent of $Oct4^{+}$ cells.

Through microscopy analysis, it was noted that bFGF and IGF-II, though not important in sustaining Oct4 expression, is crucial for the induction of the compact-cuboidal cellular morphology characteristic of OiP plastic cells, as cultures devoid of either or both growth factor failed to manifest this morphology (Figure 25A). Therefore,

it is possible that bFGF and IGF-II affect other signaling processes critical to the induction of OiP, consistent with the findings that bFGF is critical for OSKM-mediated reprogramming (Xu et al., 2001). Additionally, although it has been shown that bFGF and IGF-II are not required for sustaining Oct4 expression within the first 16 days of Oct4 introduction, it is possible that these growth factors are required for sustaining Oct4 expression past 16 days in Reprogramming Media.

It has also been noted that bFGF enhances the proliferation rate of fibroblasts, most evident between days 1-5 post-transduction, as determined through microscopy analysis. These findings highlight the importance of multiple methods of scientific evaluation, and that Oct4 expression alone does not necessarily delineate OiP induction.

In the case of my objectives, which are to determine if there is a media component(s) contained in Reprogramming media that is responsible for sustaining Oct4 expression to induce OiP, I can infer that bFGF and IGF-II are not these factors, as they are unable to sustain Oct4 expression in Fibroblast media. Due to the fact that bFGF and IGF-II are required for the induction of plastic cell morphology, both growth factors were used in all subsequent experiments.

4.2.2 Basement Membrane

In vivo, normal tissues contain a thin extracellular matrix, the basement membrane, which contacts a multitude of cell types including epithelial, endothelial, smooth muscle, and fat cells (Benton et al., 2011). Comprised mainly of laminins, collagens, proteoglycans, this biologically active matrix provides mechanical structure,

adhesion and certain growth factors to support the growth and proliferation of specific cells (Saski et al., 2004; Hughes et al., 2010). As a result, the *in vitro* culturing of many cell types requires additional supplementation, including growth factors, serum, or even a biological matrix, the most common being Matrigel.

Derived from an Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in ECM proteins such as laminins, vitronectin and fibronectin, Matrigel provides biological activity and adhesion for the optimal growth and maintenance of many cell types (Kleinman and Martin, 2005; Hughes et al., 2010). In the absence of feeder cells, Matrigel is even used for the culturing of PSCs to maintain stable proliferation, and the undifferentiated state (Xu et al., 2001; Hughes et al., 2010)

Cell attachment factors are often required for serum-free culture, such as Reprogramming media, but not for serum-containing media, such as Fibroblast media. This is because serum-free supplements lack many attachment and spreading factors and as such, a pre-coating of the culture vessels with an extracellular matrix, such as Matrigel, is required for some cells (Kleinman et al., 1987). It is therefore possible that a component contained within Matrigel, which is a complex formulation, is responsible for the sustained Oct4 expression seen in Oct4-transduced cells cultured in Reprogramming media conditions. As such, Oct4-transduced cells were cultured on tissue culture-treated or Matrigel-coated dishes in Fibroblast media to assay for Oct4 expression after 16 days. Interestingly, culturing on Matrigel in Fibroblast media did not enhance Oct4 expression, and therefore this component is not solely responsible for the sustained Oct4 expression in Reprogramming media cultures (Figure 26).

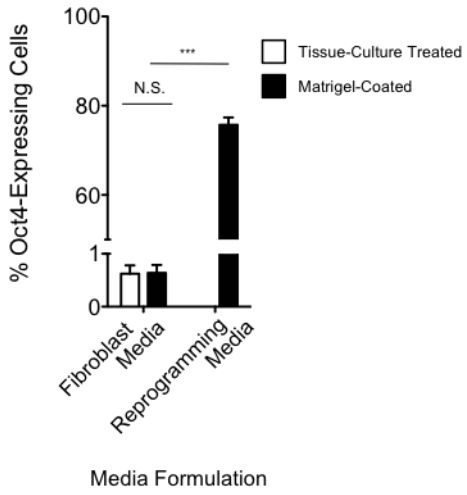


Figure 26. Culturing Oct4-transduced fibroblasts on Matrigel does not enhance Oct4 expression in Fibroblast media
A. Flow cytometric analysis of the percent of $Oct4^+$ cells in Oct4-transduced fetal fibroblasts after 16 days of culturing in Fibroblast media on uncoated tissue culture plates of Matrigel-coated tissue culture plates.

Although shown not to enhance Oct4 expression in Fibroblast media, all subsequent experiments were performed on Matrigel-coated dishes to increase the diversity of ECE components, which may work in concert with yet-unidentified factors in inducing OiP, as seen in Reprogramming media.

4.2.3 Basal Media

The largest component of growth media, by volume, is the basal medium, which in general, supplies standard inorganic salts, vitamins, glucose, essential amino acids, and a buffering system. Fibroblast media is composed of DMEM, while Reprogramming media has a base of DMEM/F-12, which accounts for 90 and 80% of the total growth

media volume, respectively. Therefore, this component is the largest difference between media formulations, by volume, and may account for the variation in Oct4 expression seen.

DMEM, or Dulbecco's Modified Eagle's Medium, is a modification of the original Eagle's Medium adapted for serum supplementation, and contains a four-fold higher concentration of amino acids and vitamins, as well as supplementary components (Dulbecco and Freeman, 1959). This medium is routinely used to culture a wide variety of cells including primary fibroblasts, neurons, glial cells, and smooth muscle cells. DMEM/F-12, containing a 50/50 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 media, has been developed more recently for growing cells in more defined, serum-free conditions (Mather and Sato, 1979). This media combines the higher concentrations of the components in DMEM with the wider range of Ham's F-12 ingredients, including biotin, putrescine, lipoic acid, glycine, proline, copper, and zinc, which are not present in DMEM (Ham, 1965). One paper has explored the effect of basal media in reprogramming cultures in order to formulate an optimal media for human iPS cell derivation, TeSR-E8 (Chen et al., 2011a; Chen et al., 2011c). In this study, 12 different basal media were examined for their effect on hESC survival and proliferation, and the result was that DMEM/F-12 had the best performance. Important to note is that these measurements were on PSCs and not early iPSC or fibroblast cultures, therefore it is difficult to say whether these results hold true for OiP cells. As a result, we investigated the effect of the basal media, notably DMEM and DMEM/F-12, on both cell survival and proliferation, as well as sustained Oct4 expression 8 days post-transduction.

Table 2. Basal medium formulations for DMEM and DMEM/F-12

	DMEM (11965)	DMEM/F-12 (11320)
Amino Acids	Glycine	Glycine
		L-**Alanine**
	L-Arginine hydrochloride	L-Arginine hydrochloride
		L-Asparagine H2O
		L-Aspartic acid
		L-Cysteine hydrochloride H2O
	L-Cysteine 2HCl	L-Cysteine 2HCl
		L-Glutamic Acid
	L-Glutamine	L-Glutamine
	L-Histidine hydrochloride H2O	L-Histidine hydrochloride H2O
	L-Isoleucine	L-Isoleucine
	L-Leucine	L-Leucine
	L-Lysine hydrochloride	L-Lysine hydrochloride
	L-Methionine	L-Methionine
	L-Phenylalanine	L-Phenylalanine
		L-**Proline**
	L-Serine	L-Serine
	L-Threonine	L-Threonine
	L-Tryptophan	L-Tryptophan
	L-Tyrosine disodium salt dihydrate	L-Tyrosine disodium salt dihydrate
	L-Valine	L-Valine
Vitamins		**Biotin**
	Chlorine chloride	Chlorine chloride
	D-Calcium panthothenate	D-Calcium panthothenate
	Folic acid	Folic acid
	Niacinamide	Niacinamide
		Para-Aminobenzoic Acid
	Pyroxine hydrochloride	Pyroxine hydrochloride
	Riboflavin	Riboflavin
	Thiamine hydrochloride	Thiamine hydrochloride
		Vitamin B12
	i-Inositol	i-Inositol
Inorganic Salts	Calcium chloride	Calcium chloride
		Cupric sulfate
	Ferric nitrate	Ferric nitrate
		Ferric sulfate
		Magnesium chloride
	Magnesium sulfate	Magnesium sulfate
	Potassium chloride	Potassium chloride
		Potassium nitrate
	Sodium bicarbonate	Sodium bicarbonate
	Sodium chloride	Sodium chloride
		Sodium phosphate dibasic
	Sodium phosphate monobasic	Sodium phosphate monobasic
		Zinc sulfate

Other Components	D-Glucose Phenol Red	D-Glucose **Hypoxanthine Na** **Linoleic Acid** **Lipolic Acid** Phenol Red **Putrescine 2HCl** **Sodium Pyruvate** **Thymidine**

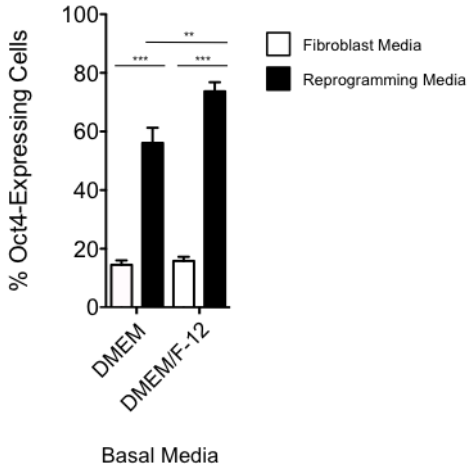


Figure 27. Formulation of Fibroblast media using DMEM/F-12 does not enhance Oct4 expression
A. Flow cytometric analysis of the percent of $Oct4^+$ cells in Oct4-transduced fetal fibroblasts after 16 days of culturing in Fibroblast media (DMEM or DMEM/F-12 base) or Reprogramming media (DMEM or DMEM/F-12 base)

Both Fibroblast and Reprogramming Media were produced with only a change in the basal media, while all other components remained the same. Eight days post Oct4-transduction in all media formulations, Oct4 expression levels were assessed by flow cytometry (Figure 27). While DMEM/F-12 permitted a slightly greater percentage of Oct4-expressing cells in the culture as compared with DMEM ($p < 0.01$), this media component was unable to achieve sustained Oct4 expression in Fibroblast media. As

such, the basal media does not contain factors to permit OiP, and it is therefore another component in the media responsible for the OiP phenomenon. It is possible that slight variations in these media formulations, including the addition of certain amino acids, vitamins, or lipids, may allow for DMEM/F-12 to slightly enhance Oct4 expression, and therefore following experiments were performed using DMEM/F-12 as the basal medium, unless specifically stated otherwise.

4.2.4 Serum Composition

Serum is arguably the most important components of cell culture medium, as it supplies all essential nutrients for cell metabolism, growth, and proliferation (Gstraunthaler, 2003). A complex mixture of albumins, hormonal and growth factors, and growth inhibitors, this element is key to the *in vitro* growth and maintenance of a wide-range of cell types, despite the varying requirements of different cultures (Zheng et al., 2006). Due to its rich content of growth factors and its low gamma-globulin content, fetal bovine serum, or FBS, is the most widely used serum supplement.

FBS is the liquid fraction obtained from the clotted blood of a bovine fetus (Gstraimtjaler, 2003). It contains numerous factors required for the survival and propagation of mammalian cells in culture, the major constituent being bovine serum albumin (BSA), although its precise composition remains a "black box".

However, the use of animal serum in culture has a number of disadvantages. In particular, its ill-defined, ambiguous nature coupled with significant lot-to-lot variations can introduce several unknown variables into the tissue culture system. Additionally, the

large quantities of undefined proteins and/or adverse factors, such as hemoglobin, can lead to unwanted stimulation of or inability to support the growth of specialized cell types (Gstraunthaler, 2003). Specifically, serum may be unable to prevent the overgrowth of the culture by fibroblasts.

Biotechnology companies have recognized the growing trend away from products of animal origin and as a result, multiple animal-free and/or serum-free formulations have been created. With the recent identification of essential growth factors, hormones, transport proteins, co-factors and minerals required for cell culturing, chemically defined media have been formulated to replace FBS. A conventional serum replacement comprises albumin, lipids, amino acids, vitamins, transferrin, antioxidants, insulin, and other trace elements (Garcia-Gonzalo et al., 2008). Perhaps most importantly, serum-free media exhibit a vastly reduced variability in qualitative and quantitative culture medium composition.

The most widely used serum-free formulation is knock-out serum replacement, or KOSR. Originally formulated to prevent spontaneous mESC differentiation, KOSR is a defined, serum-free formulation optimized to grow and maintain undifferentiated ESCs in culture that can replace FBS in media used for blastocyst injection, embryo culture, embryoid-body formation, and *in vitro* differentiation studies (Price et al., 1998). As a result, it has been widely used in ESC and reprogramming cultures (Xu et al., 2001). Additionally, the kinetics and efficiency of reprogramming is greatly improved using KOSR instead of FBS, maintaining full developmental potential, indicating that culture conditions significantly affect iPSC generation (Zhao et al., 2010)

To test the effect of serum content on Oct4 expression, Fibroblast media was formulated with DMEM/F-12 (due to the enhanced Oct4 expression seen in Reprogramming media, Figure 27), and supplemented with either the traditional 20% KOSR, or 10% FBS as a serum substitute, and used to culture the cells for 8 days post Oct4-transduction and Oct4 expression analyzed via flow cytometry. The results showed that FBS-containing Fibroblast media had significantly less Oct4 expression than KOSR-containing Fibroblast media ($p < 0.001$), indicating that there is either an inhibitory factor in FBS preventing sustained Oct4 expression, or a permissive factor in KOSR that allows for sustained Oct4 expression (Figure 28).

4.3 Dissecting Serum Content

In an effort to determine which factor(s) in either FBS or KOSR affect the sustained expression of Oct4, serum contents were investigated, and are listed in Table 3. As can be noted, FBS contains a vast array of components, many still undefined, while KOSR is a much more standardized formulation. Importantly, FBS contains an array of growth factors, signaling ligands, and animal hormones that are known to affect cell growth and proliferation (Brunner et al., 2009). As such, further investigation into these factors commenced to determine their effect on Oct4 expression in the context of OiP.

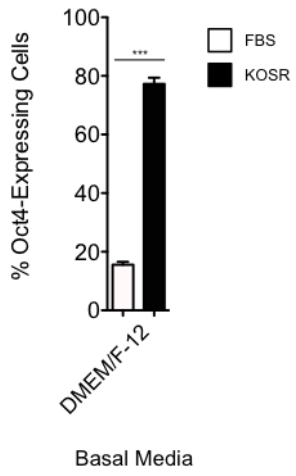


Figure 28. FBS in Fibroblast media does not sustain Oct4 expression.
A. Flow cytometric analysis of the percent of $Oct4^+$ cells in Oct4-transduced fetal fibroblasts after 8 days of culturing in Fibroblast media (formulated with DMEM/F-12 base) formulated with their serum (FBS) or serum-free supplementation (KOSR).

It is important to note that from this point forward, adult dermal patient-derived fibroblast were used in place of fetal fibroblasts, as these cells more closely resemble those that would be used for downstream autologous cell therapy and/or drug screening of diseased tissue. Additionally, fetal fibroblasts may retain characteristics or pluripotent cells due to their developmental hierarchy, and have demonstrated higher levels of exogenous Oct4 expression and more easily develop the classic OiP morphology than their adult counterparts, despite equal lentivirus amount and identical culturing conditions (Yusuf et al., 2013).

Table 3. Composition of FBS and KOSR.

	Fetal Bovine Serum (FBS)	Knockout Replacement Serum (KOSR)
Key Proteins	insulin albumin (BSA)	insulin albumin (BSA) transferrin
Amino Acids	N/A	glycine L-histidine L-isoleucine L-methionine L-phenylalanine L-proline L-hydroxyproline L-serine L-threonine L-tryptophan L-tyrosine L-valine
Hormones	cortisol growth hormone parathormone triiodothyronine thyroxine thyroid-stimulating hormone follicle-stimulating hormone testosterone progesterone luteotropic hormone lutenizing hormone	-
Antioxidants/Vitamins	vitamin A vitamin E	thiamine reduced glutathione ascorbic acid 2-PO_4
Signaling Ligands	prostaglandin E prostaglandin F TGF-β1	
Trace Elements	Na^{+} K^{+} Ca^{2+} Cl^{-} P_i SeO_3^{2-}	Ag^{+} F^{-} $Al3^{+}$ Mn^{2+} Ba^{2+} Si^{4+} Cd^{2+} V^{5+} Co^{2+} Mo^{6+} Cr^{3+} Ni^{2+} Ge^{4+} Rb^{+} Se^{4+} Sn^{2+} Br^{-} Zr^{4+} I^{-}
Other	fibronectin urea acid creatine hemoglobin bilirubin	

4.3.1 TGF-β

4.3.1.1 Optimized Media: TeSR-E7 and TeSR-E8

In analyzing the composition of FBS, one component stood out – TGF-β1. The TGF-β superfamily of cytokines contributes to an array of cellular functions, ranging from adhesion to apoptosis, cellular proliferation, and differentiation (Moses et al., 1990). Perhaps most importantly in this context, TGF-β signaling has been shown to be inhibitory to the reprogramming process, as it is widely viewed as a growth stimulatory factor for mesenchymal cells while inhibiting epithelial cell growth. Specifically, it has been demonstrated to promote EMT and prevent MET, a recently identified key step in reprogramming (Li et al., 2010; Samavarchi-Tehrani et al., 2010; Chen et al., 2011b). Furthermore, it has been demonstrated that inhibition of TGF-β signaling not only increases the efficiency of the reprogramming process, it can bypass the requirement for exogenous c-Myc or Sox2 (Ichida et al., 2009; Maherali and Hochedlinger, 2009). Further research has suggested that c-Myc downregulates the effect of the ligand TGF-β1 to allow for MET, therefore in the absence of c-Myc, media conditions may be critical in facilitating the early reprogramming process (Chaudhry et al., 2008). As the prevailing hypothesis is that OiP cells are in the early stages of MET, TGF-β1, present in FBS but not KOSR, may be inhibiting this process.

In formulating a chemically defined culture system for hES and hiPS cells, Chen et al. examined the individual components required for growth and proliferation (Chen et al., 2011a; Chen et al., 2011c). Using the previously developed mTeSR media, which

contains 18 components added to a DMEM/F-12 base, the group developed a xeno-free formulation free of BSA and BME that contains only eight defined components shown to be critical for the induction and maintenance of pluripotency (Chen et al., 2011c). This final formula, named TeSR-E8, consists of just insulin, selenium, transferrin, L-ascorbic acid, bFGF and TGF-β, in DMEM/F-12 with pH adjusted with $NaHCO_3$ (Table 4) (Chen et al., 2011c). This simplified medium demonstrates improved reprogramming efficiencies over the previous mTeSR formula. Most importantly, removing TGF-β resulted in further improvement in efficiency, as TGF-β was shown to inhibit fibroblast overgrowth of iPS cells. As such, TeSR-E7 was formulated specifically for reprogramming, while TeSR-E8 is recommended for maintaining the pluripotent status.

The simplest approach to determine the contribution of TGF-β1 is a contributing factor to FBS's inability to sustain Oct4 expression, is the use of TeSR-E7 and TeSR-E8, which are identical in composition with the exception of TGF-β1 content. Briefly, cells were seeded onto Matrigel-coated dishes, transduced with Oct4 lentivirus in Fibroblast media, transitioned to 50/50 the following day, and finally 100% either TeSR-E7 or TeSR-E8 on day 2 post-transduction. As a control, Oct4-transduced cells cultured in Fibroblast media or Reprogramming media were conducted simultaneously.

Table 4. Medium formulations for TeSR-E7 and TeSR-E8.

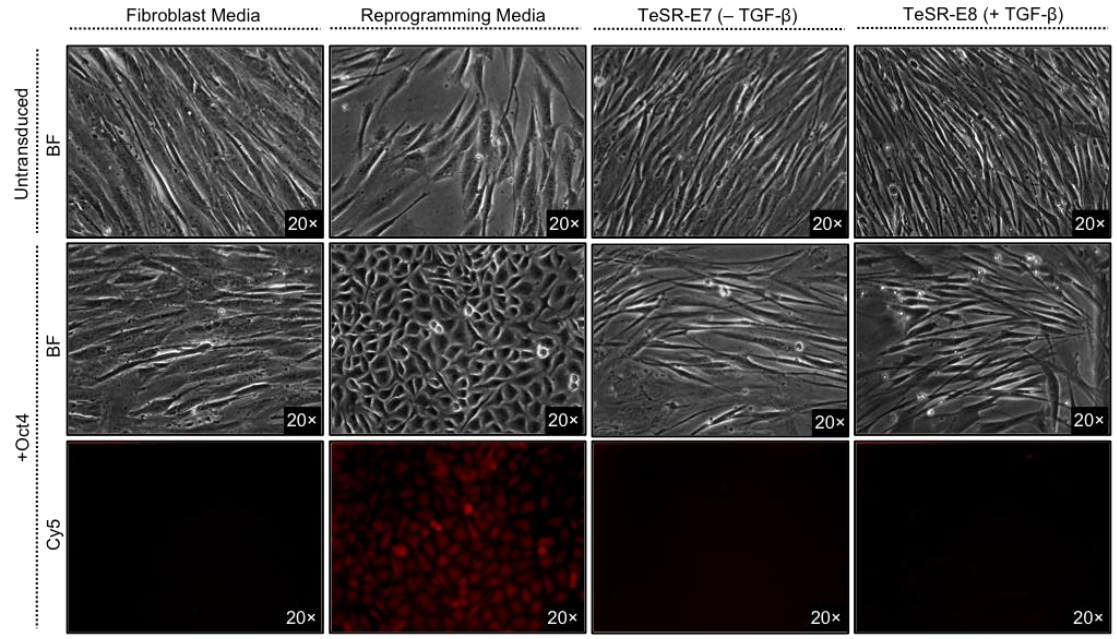
A.

		Maintain	Reprogram	Differentiate	
Cytokine	Function	mTeSR1 mTeSR 2 TeSR-E8	TeSR-E7	TeSR-E6	TeSR-E5
Insulin	- important for cell survival and proliferation - inhibits differentiation to specific lineages (eg. cardiomyocytes)	•	•	•	
bFGF	- important for hPSC self-renewal and expansion	•	•		
TGF-β	- important for maintenance of pluripotency of hPSCs - inhibits reprogramming	•			

B.

	TeSR-E7	TeSR-E8
Basal Media	DMEM/F-12	DMEM/F-12
Buffering	$NaHCO_3$ (543 mg/L)	$NaHCO_3$ (543 mg/L)
Additional Components	L-ascorbic acid-2-phosphate magnesium (64 mg/L)	L-ascorbic acid-2-phosphate magnesium (64 mg/L)
	sodium selenium (14 μg/L)	sodium selenium (14 μg/L)
	transferrin (10.7 mg/L)	transferrin (10.7 mg/L)
	insulin (19.4 mg/L)	insulin (19.4 mg/L)
	bFGF (100 μg/L)	bFGF (100 μg/L)
	-	TGF-β1 (2 μg/L)

A.



B.

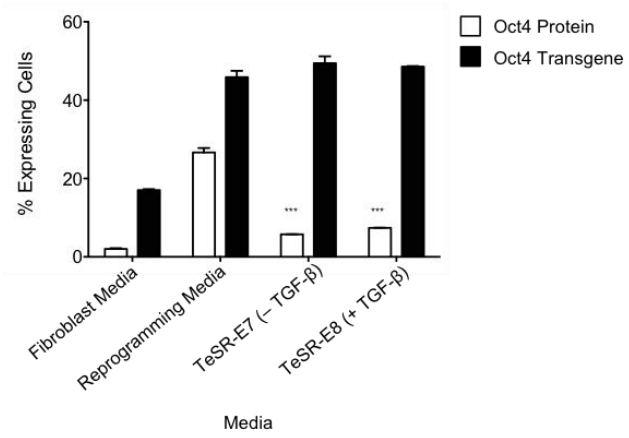


Figure 29. TeSR-E7 and TeSR-E8 minimal media formulations sustain Oct4 transgene expression, but not protein levels

Analysis of untransduced and Oct4-transduced adult fibroblasts after 16 days of culturing in Fibroblast media (negative control), Reprogramming media (positive control), TeSR-E7 (– TGF-β) or TeSR-E8 (+ TGF-β)

A. Live fluorescence microscopy imaging (20×)

B. Flow cytometric analysis of the percent of $Oct4^{+}$ cells

After 16 days of growth, with one passage at day 8, only Oct4-transduced cells in Reprogramming media demonstrated OiP morphology, while in all other conditions cells remained fibroblast-like (Figure 29A). Through fluorescence microscopy, no Oct4-E2Crimson transgene expression was detected in either TeSR cultures. Analysis by flow cytometry showed high levels of the Oct4-E2Crimson transgene (comparable to levels seen in Reprogramming media), although interestingly, very little Oct4 protein was detected in either TeSR-E7 or TeSR-E8 cultures (~7%) compared to Reprogramming media (~26%) (Figure 29B).

Due to the discrepancy seen between transgene expression and Oct4 protein levels, it is difficult to determine the effect of TGF-β on OiP. In an effort to explain this, it is possible that the TeSR minimal essential media do not contain sufficient factor(s) to translate the Oct4 mRNA or sustain Oct4 expression, or perhaps Oct4 protein stability is compromised in these media. Further investigation is required to determine the nature of this disparity.

4.3.1.2 SB-431542

As an alternative approach to determining the contribution of TGF-β1 on Oct4 expression, inhibition of TGF-β1 was performed using the small-molecule SB-431542. This small molecule is a selective and potent inhibitor of the TGF-β/Activin/Nodal pathway through the inhibition of ALK4, ALK5, and ALK7, but does not affect the BMP type I receptors ALK2, ALK3 and ALK6 (Iman et al., 2002; Laping et al., 2002).

Briefly, adult fibroblasts were transduced with Oct4 lentivirus and cultured in the presence or absence of 10 μM SB-431542 for 16 days prior to analysis by microscopy and flow cytometry. Microscopic analysis showed no difference in morphology as compared to untreated and untransduced controls (Figure 30A). Additionally, addition of SB-431542 did not increase expression of Oct4 beyond the untreated and untransduced controls (Figure 30B). Therefore, inhibition of TGF-β1 alone is unable to sustain Oct4 expression to levels seen in KOSR-cultured cells.

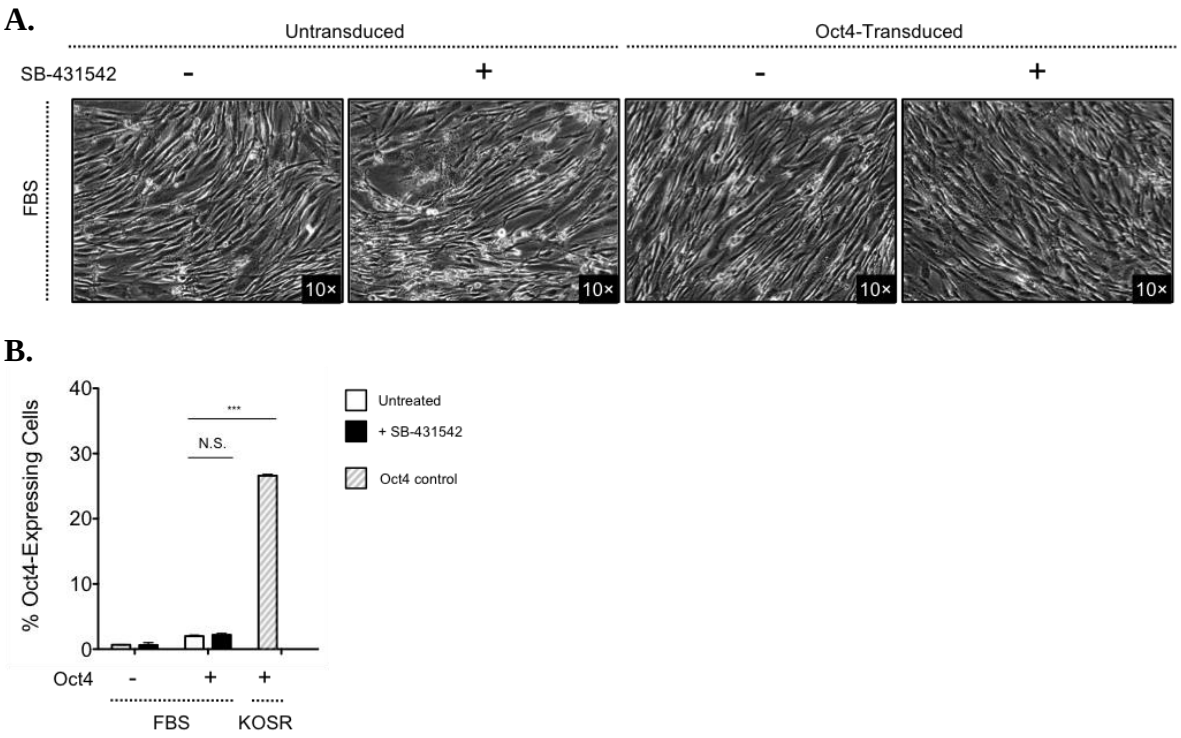


Figure 30. TGF-β inhibition by SB-431542 does not enhance Oct4 expression in Fibroblast media

Analysis of untransduced (-) and Oct4-transduced (+) adult fibroblasts after 16 days of culturing in Fibroblast media +/- 10 μM SB-431542 (Oct4-transduced cells cultured in Reprogramming media used as positive control)

A. Live fluorescence microscopy imaging (20×)

B. Flow cytometric analysis of the percent of $Oct4^{+}$ cells

Therefore, it remains to be determined if TGF-β1 in FBS contributes to the inability to sustain Oct4 expression compared to KOSR. A protocol to deplete TGF-β from FBS been published, although the requirement for specialized anti-latency-associated peptide monoclonal antibodies is costly, and the full process is time consuming, although it may be worthwhile to answer this question (Oida and Weiner, 2010).

4.4 Probing Plasticity-Enhancing Compounds

Just as Klf4 and Sox2, although dispensable, are powerful boosters for reprogramming, it has been shown that small-molecules can also be used to enhance the reprogramming process (Figure 31). Chemicals used to improve the efficiency and kinetics includes epigenetic modifiers (eg. VPA), signaling pathway modulators (eg. CHIR99021), metabolic modifiers (eg. PS48), and cell death and stress alleviators (eg. Rapamycin) (Feng et al., 2009). In fact, it has been recently demonstrated that iPSCs can be generated using only Oct4 in combination with appropriate growth factors and signaling ligands (Chen et al., 2011b). These findings suggest that one can formulate an optimized culture system for inducing pluripotency.

With this knowledge in mind, it was hypothesized that the addition of certain small-molecules may enhance the efficiency and kinetics of OiP to further enrich for plastic colonies for downstream analysis. Identification of a small-molecule able to aid in the abovementioned processes may assist in the identification of Oct4-mediated reprogramming roadblocks, allowing for not only more efficient cellular conversion, but

elucidate the role of Oct4 in reprogramming. Improving the efficiency of this process may also alleviate efforts in identifying a novel surface marker of OiP.

Considering the goal of this work is to ultimately identify a transgene-free approach to reprogramming, auxiliary transcription factors were not considered. Instead, chemical compounds that may enhance the OiP efficiency or even facilitate the formation of iPSCs from OiP were explored. In searching for chemicals that may facilitate OiP, I began with compounds that have been shown to modulate signaling pathways previously shown to be important in the initiation phase of reprogramming.

4.4.1 BMP-4

The BMP pathway has been implicated as a key regulator of reprogramming initiation via the induction of MET, accelerating the progression to the maturation phase (Samavarchi-Tehrani et al., 2010). Interestingly, BMP supplementation can even functionally replace Klf4 in the reprogramming process, activating the expression of epithelial genes such as *Cdh1* and *Occludin*, and can also suppress the TGF-β pathway to inhibit EMT (Li et al., 2010). Additionally, Oct4 alone with BMP is sufficient to convert MEFs and mouse tail-tip fibroblasts into iPSCs in the optimized iCD1 media (Chen et al., 2011c).

BMPs can be derived from serum or cocultured MEFs, neither of which is used in the induction of the plastic state. Based on the abovementioned findings, I added BMP4 to the serum-free Reprogramming media in an effort to determine if this factor can enhance Oct4 expression and the occurrence of OiP colonies.

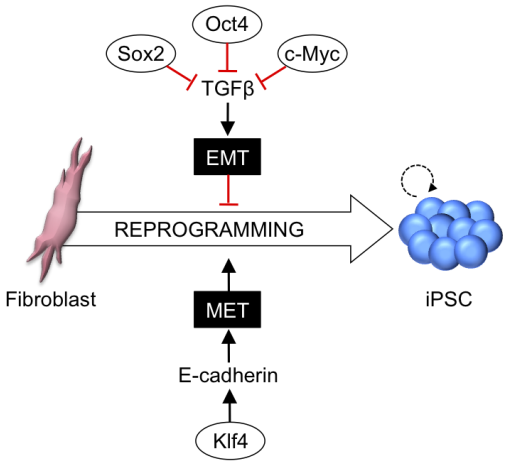


Figure 31. The pluripotency factors suppress EMT and permit MET
BMPs can replace the role of Klf4 in the reprogramming process, permitting MET, while inhibition of TGF- β signaling suppresses EMT to promote reprogramming.

After 8 days of treatment, cells cultured in the presence of 10 ng/mL BMP4 exhibited similar levels of Oct4 expression to untreated samples (Figure 32B). It should be noted that large amounts of cell death were apparent in Oct4-transduced samples treated with BMP4, but not in untransduced cultures treated with BMP4, suggesting a combinatorial effect of Oct4 expression and BMP4. Despite the apparent cell death, cultures remained at comparable cell counts to untreated Oct4-transduced samples, suggesting that high proliferation was occurring. By day 16 (after one passage), expression of Oct4 was slightly lesser in BMP4-treated cultures. Analysis by fluorescence microscopy demonstrated the induction of the OiP phenotype much like untreated samples (Figure 32A). Long-term culturing to day 48 in the presence of BMP4 showed no improvement on Oct4 expression above the untreated samples (Figure 32B).

4.4.2 SB-431542

TGF-β signaling has a critical and dominant role in EMT, a process inhibitory to the reprogramming of somatic cells (Samavarchi-Tehrani et al., 2010). Inhibition of this pathway by small-molecules has been shown to enhance iPSC generation and even bypass the requirement for exogenous c-Myc or Sox2 (Ichida et al., 2009; Maherali and Hochedlinger, 2009).

Many cell types secrete TGF-β1 including fibroblasts, which can autoregulate its own production as well as affect multiple targets (Kelley et al., 1991; Silvera et al., 1994). As c-Myc downregulates the ligand TGF-β1, the small-molecule SB-431542, which inhibits the corresponding receptors, was chosen for further investigation (Chaudhry et al., 2008). As a result, I added the small-molecule inhibitor of TGF-β1, SB-431542 to the serum-free Reprogramming media to investigate the effect of TGF-β1 on Oct4 expression and OiP induction. After 8 days of treatment, Oct4-transduced cells cultured in the presence of 10 μM SB-431542 demonstrated significantly less Oct4 expression than the untreated control but by day 16, the difference was not as drastic (Figure 32B). SB-431542-treated cells exhibited typical OiP morphology by day 16 (Figure 32A). Remarkably, long-term culturing to day 48 in the presence of SB-431542 demonstrated an increased prevalence of OiP colonies over the untreated control. Flow cytometric analysis at this time confirmed a greater Oct4 expression in SB-431542-treated samples (~48%) over untreated controls (~23%) (Figure 32B). These findings suggest a potential role for TGF-β signaling in the induction of OiP.

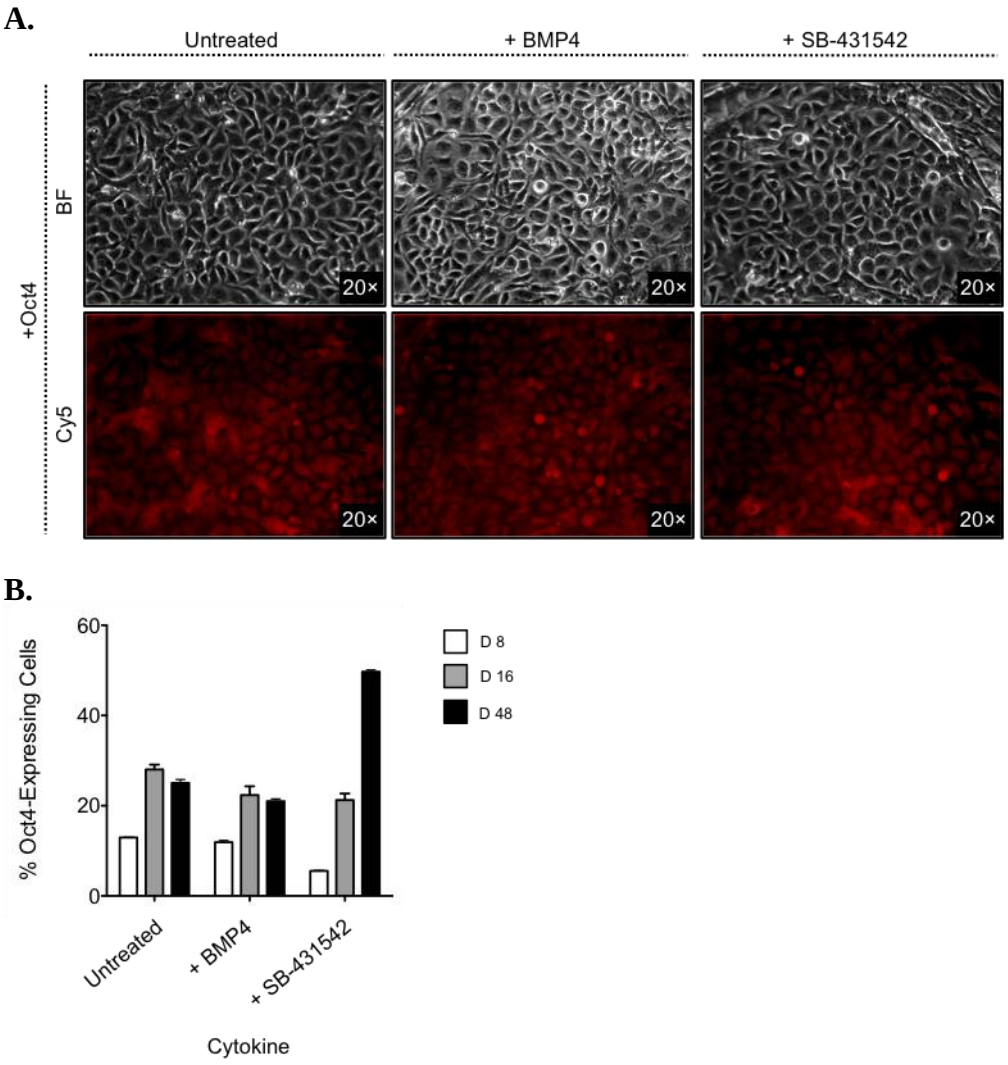


Figure 32. Inhibition of TGF-β but not BMP4 supplementation increases Oct4 expression long-term

Analysis of Oct4-transduced adult fibroblasts after culturing in Reprogramming media +/- 10 ng/mL BMP4 or +/- 10 μM SB-431542 (untreated Oct4-transduced cells cultured in Reprogramming media used as positive control)

A. Live fluorescence microscopy imaging (20×) of day 16 cells

B. Flow cytometric analysis of the percent of $Oct4^{+}$ cells at day 8 (D 8), day 16 (D 16), and day 48 (D 48) post Oct4-transduction.

As long-term culturing (up to 93 days) of OiP cells in Reprogramming media or MEF-conditioned media (MEF-CM) on Matrigel or iMEFs has been shown to induce pluripotency, it would be interesting to determine if SB-431542-treated hFibs^{Oct4} could also generate iPSCs, and at what efficiency and kinetics in comparison to untreated samples (Salci et al., 2015).

CHAPTER 5: Discussion

5.0 What is Oct4-Induced Plasticity?

Oct4-induced plasticity is a unique cell state that is amenable to extracellular influences to alter cell fate. Although it is characterized by a distinctive morphology, sustained Oct4 expression, and a novel transcriptome profile, little is known about the nature of this state, specifically regarding the plastic cell fate trajectory. Is the ability to induce pluripotency after long-term culturing secondary to the induction plasticity, or were these cells originally fated to become pluripotent? In order to address this question, it is important to research cells in the early stages of pluripotent reprogramming so that we may compare this state to OiP.

The first notion of plasticity in reprogramming came from Orkin and Hochedlinger with the suggestion that early in the OSKM-mediated reprogramming process, cell chromatin becomes sufficiently plastic, and may assume different cellular states dependent on the extracellular signals provided (Orkin and Hochedlinger, 2011). In fact, when these cells are exposed to the appropriate culture conditions, non-iPSC fates can be established, a process referred to as direct reprogramming, or transdifferentiation. Due to their generation, it was suggested that these cells might be similar to pre- or partially reprogrammed iPSCs. In fact, these cells exhibit similar characteristics, including the silencing of certain somatic programs, activation of unrelated lineage factors, but do not display activation of pluripotent genes. These intermediates can appear transiently before converting to iPSCs, or may be trapped in this state, unable to overcome intrinsic reprogramming barriers. These cells may stably propagate, but will

not attain an altered cell fate without additional transgene expression or the addition of small-molecules to overcome these roadblocks (Mikkelson et al., 2008; Hanna et al., 2009). Analysis of cells in the early stages of reprogramming revealed that these cells first undergo a rapid shift in their proliferative rate that coincides with a reduction in their cellular area, occurring as early as the first cell division post transgene expression (Smith et al., 2010).

By now, it is evident that the state of Oct4-induced plasticity shares many of these same features – morphological alteration to a compact-cuboidal shape with reduced size, increase proliferative rate, activation of genes of multiple lineages, all without the acquisition of pluripotent gene expression. Also noteworthy is the finding that the continued culturing of OiP cells in pluripotent-supportive conditions (exogenous Oct4 transgene expression and extracellular, pluripotency cues) is sufficient to generate iPSCs (Salci et al., 2015). By definition, it appears as though plastic cells are in the early stages of reprogramming, and are thus fated to become pluripotent. Regardless of the status of these cells, it is evident that the induction of OiP is an infrequent and inconsistent process, making it difficult to assess the molecular mechanisms as well as the role of Oct4. This highlights the critical importance of identifying a surface marker of the plastic state in order to enrich and isolate this population for downstream characterization. It is only then that we can begin to make conclusions regarding this state and its fate trajectory.

Why, though, is the phenomenon of OiP, and reprogramming in general, so rare? Numerous hypotheses and explanations have been put forth to address this question,

which converge as two models of cell fate conversion.

This first idea, termed the elite or deterministic model, presupposes that only a few cells are competent for reprogramming. In this, only a particular subset of cell within a starting population, for example those in a more undifferentiated state such as adult stem or progenitor cells, possess the ability to reach the iPS cell status. In support of this model, cellular reprogramming by SCNT achieves higher efficiency when using nuclei from less-differentiated, progenitor-like cells, than from the nuclei of terminally differentiated cells (Yamanaka, 2009). Similarly, hematopoietic stem and progenitor cells give rise to iPSC colonies with a much higher efficiency and faster kinetics than differentiated cells of the same lineage (10–28% versus 0.03–0.5%) (Emelini et al., 2009; Hanna et al., 2008). In the case of OiP, this would suggest that adult stem cells may contaminate the starting fibroblast population, and that these are the cells that generate plastic colonies. Although multipotent stem cells are known to exist in adult organs such as the skin, reprogramming efficiencies have achieved efficiencies greater than the average percentage of adult cells in a population (Yamanaka, 2009). Furthermore, reprogramming has been confirmed to occur in fully differentiated populations including B and T cells (Emelini et al., 2009; Hanna et al., 2008). This finding argues against the model that only a subset of cell types, particularly more undifferentiated states, are susceptible to reprogramming, but instead suggests that this factor only influences the efficiency and kinetics of the process. Specifically, it may be possible that more undifferentiated cells in a population have a higher probability to overcome innate reprogramming barriers due to their more plastic epigenetic landscapes, and that this

factor may account for their greater efficiency in reprogramming.

Instead of this notion, the majority of cell fate evidence points to the second model of reprogramming – the stochastic model. Based on Waddington's epigenetic landscape model, this idea proposes that most, if not all, differentiated cells have the capacity to successfully reprogram to pluripotency, and that the low efficiency of reprogramming is due to the ability of cells to respond to transgene and cellular cues as well as overcoming reprogramming obstacles.

Somatic cells who achieve pluripotency must undergo a multitude of changes in order to assume this state, including a metabolic shift, progressive gene inactivation/activation, epigenetic alterations (including histone modifications, DNA promoter demethylation), as well as endogenous pluripotent transgene reactivation (Yamanaka, 2009). During the process, cells cycle through distinct states based on their stochastic gene expression and epigenetic modifications, which then render these cells susceptible to other changes. Essentially, reprogramming is a multifactorial process, where multiple fundamental processes act synergistically and in a sequential manner to reach pluripotency (Hanna et al., 2009; Stadtfeld, 2008). Therefore in this model, achieving pluripotency is dependent upon a cell's ability to make these appropriate changes and overcome potential obstacles in timely fashion, and not based on their developmental status.

Consistent with this notion is the finding that chemicals can substantially enhance reprogramming, with the general consensus being that these molecules lower the reprogramming barriers, seemingly acting as biological catalysts to this process. Also in

support of this model is the finding that multiple, distinct cell types can reprogram, although they may have different requirements to achieve cell fate conversion or induce pluripotency. For example, although Mitchell et al. demonstrated the derivation of tri-potent neural progenitor cells from fibroblasts using Oct4 and neural-supportive conditions, this proved insufficient for blood cell conversion (Mitchell et al., 2014b; Lee et al., 2015). Instead, these cells required activation of Wnt signaling and dual inhibition of SMAD signaling through TGF-β and BMP receptors, suggesting unique barriers and requirements for cell fate conversion based on the starting cell that may be overcome by the appropriate extracellular cues (Chambers et al., 2009). Nevertheless, additional research is required to fully elucidate the true nature of reprogramming as well as OiP.

So how may the extracellular environment, specifically serum, contribute to OiP generation? If you recall, FBS contains many factors that promote the growth and differentiation of multiple cell types, while KOSR contains fewer factors, therefore having a lesser ability to support the growth of cells other than PSCs (Zhao et al., 2010). As a result, the presence of certain factors in FBS may induce early-stage reprogramming cells to develop into other cell types or remain as fibroblasts rather than achieve plasticity. Although the total number of live cells in culture is lower in KOSR than in FBS after 8 or 16 days, Oct4 expression is higher in KOSR cultures, suggesting that Oct4-transduced fibroblasts that do not become plastic cells proliferate much more slowly in KOSR than in FBS. As such, KOSR may be selectively supporting the growth of Oct4-transduced cells and thus directly enriching for them in culture while also selecting against non-reprogramming cells, thereby indirectly promoting proliferation by reducing

differentiation signals from other co-cultured cells. Identification of the specific factor(s) contained within the serum formulations that work in conjunction with Oct4 to govern OiP would be essential in uncovering the underlying mechanisms governing this state.

5.1 OiP is Not a Human-Only Phenomenon

An important finding in my thesis is the ability to generate plastic cells from mouse fibroblasts, as this suggests that the role and targets of Oct4 may be similar in mouse and human fibroblasts. Further research is required in order to determine if Oct4 as well as the contributing ECE factors work through the same mechanisms in both species.

Taken together, these results have confirmed that OiP can be applied to multiple somatic cell starting populations. Additional groups have also recently developed Oct4-based reprogramming methods using a variety of staring cells including hair follicle mesenchymal stem cells, $CD34^+$ and $CD34^+CD45^+$ umbilical cord cells, although the identification of a plastic-like state during this process has yet to be reported (Liu et al., 2015; Meng et al., 2013; Liao et al., 2015). It would be interesting to compare the extracellular conditions of these conversions to determine cell-specific requirements and perhaps elucidate the synergistic role of Oct4 and these factors.

5.2 Oct4 as a Master Regulator of Cell Fate

The multiple roles of Oct4 during cell fate conversion has led researchers to suggest that this factor is not only a versatile reprogramming factor, but a master

regulator of cell fate. Specifically in the context of OiP, the ability of Oct4 to act on multiple cell types of different species and direct broad changes suggests a role as a pioneer factor. It has previously been established that in the early stages of reprogramming to pluripotency, Oct4 can bind to closed chromatin located at distal sites of genes and lacking epigenetic marks, proceeding to occupy different regions of the chromatin as reprogramming progresses (Soufi et al., 2012; Jerabek et al., 2014). Oct4 may then recruit other essential factors, such as chromatin remodelers or transcriptional co-activators, to establish complex gene regulation programs. It is only when the pluripotent state is reached that Oct4 assumes its role in the core transcriptional network to maintain pluripotency.

Taken together, these findings suggest that a single small molecule may not be able to substitute for the multiple roles of Oct4, and instead, it is more feasible to envision a molecule that can activate endogenous Oct4 expression in order to chemically alter cell fate. To this end, it must be noted that the levels of Oct4 protein expression may influence the induction of OiP such that this phenomenon only occurs at supraphysiological levels of Oct4, which may be seen only in lentiviral overexpression. It is then possible that small-molecule induction of endogenous Oct4 expression at normal, physiological levels may be insufficient to induce OiP. In support of this, it has been well characterized that precise levels of Oct4 are required in order to maintain pluripotency, as reduced or increased expression results in differentiation toward the trophectodermal lineage, or mesodermal and endodermal lineages, respectively (Niwa et al., 2000). However, the induction of pluripotency in murine fibroblasts by a small-

molecule cocktail suggests that chemicals may induce endogenous pluripotent gene expression at levels that are sufficient to alter cell fate (Hou et al., 2013). Nonetheless, the identification of a small molecule capable of inducing endogenous Oct4 expression is required first before assessing its ability to induce OiP.

5.3 Moving Forward with Oct4-induced Plasticity

The identification of medium composition as a critical regulator of OiP has provided further insight into the role of the extracellular environment in generating OiP. But considering that most of what we know about OiP is related to morphological and transcriptional analyses at the whole population level, the confirmation of N-cadherin as a surface marker will be important to move forward with isolation and/or enrichment of this population for clonal level analysis to reveal the trajectory of these cells. In this way, it may be determined whether these cells are primed for pluripotency, therefore harboring full developmental potential, or if they are restricted toward a specific cell fate. This may also enable further dissection into the manner by which Oct4 and the ECE enable this cell fate alteration. Technologies such as chromatin immunoprecipitation, genome-wide sequencing, and protein-protein interaction mapping can elucidate the role of Oct4 in this process, providing valuable help in our efforts to better understand the mechanisms responsible for the induction and maintenance of the plastic state. Generation of the Oct4 protein-protein interaction network in OiP may identify intrinsic gene regulatory networks that are subject to control by extracellular signals to induce signaling cascades, culminating in the modulation of both transcription factor activity and the epigenetic

landscape. This may provide new insights into the role of Oct4, and what roles it can and cannot fulfill without its accessory pluripotency factors – *Sox2*, *Klf4* and *c-Myc*. Elucidation of the factors essential in this process may allow for the optimization of OiP generation. This knowledge may also help to determine classes of small molecules that may be able to enhance this process or to accelerate this route to pluripotency, or to even substitute for Oct4 itself. This may enable greater control of cell fate conversion, important for this field to evolve. An important consideration, though, is the safety of small-molecules in cell fate conversion. Although chemical reprogramming alleviates certain risks intrinsic to viral methods, the ability of these small-molecules to alter epigenetics and initiate transcriptional programs may produce aberrant gene expression, abnormal protein levels and/or products, as well as chemically induced mutations (Solanki and Lee, 2010). As such, there is a clear need to discover a small molecule that can induce endogenous Oct4 expression but at the same time, does not impact cells at the epigenetic level. Therefore, the use of chemical libraries of molecules with known targets and functions for screening may alleviate these risks. Nonetheless, the safety of any small-molecule on target cells must be assessed prior to use in a clinical setting.

As previously mentioned, the findings in this thesis suggest that OiP may not be limited to human fibroblasts and may instead be extended to murine fibroblasts. In moving forward with OiP, it would be important to determine if the induction of the plastic state can be extended to other starting cell populations, such as blood cells. Recently, Lee et al. made a critical finding that the reprogramming capacity of Oct4 can be extended toward blood cells through the generation of tri-potent induced neural

progenitor cells using Oct4 alone (Lee et al., 2015). In these studies, the intermediate stages of reprogramming were not analyzed, and the authors did not note whether a plastic-like state might have been reached. In moving forward, it would be important to assess any potential plastic state(s) conferred by Oct4 and the ECE on blood cells for morphological alteration and transcriptional profiles. Comparing any potential blood-derived plastic state with the fibroblast-derived plastic state may help to elucidate whether the role of Oct4 in OiP is specific to a given starting cell population, such as fibroblasts, or whether Oct4 functions universally to induce one similar plastic state for the alteration of cell fate.

Although this technology is still in its infancy, the potential is nevertheless enormous. Currently, the treatment of many diseases, such as Parkinson's or Alzheimer's, is limited by the accessibility of affected cells and tissues. Reprogramming has the potential to generate patient-specific cells, providing unprecedented sources for cell therapies. As well, these cells can be used to produce disease-specific cells for disease modeling so that the progression and pathogenesis of genetic disorders can be studied *in vitro* and used for drug screening purposes. Many studies have already shown the faithful *in vitro* recapitulation of diseased phenotypes and cell abnormalities as they are seen in patients (Stadtfeld and Hochedlinger, 2010). Direct induction of these cell types by OiP would limit the genomic manipulation in these cells in which genetic aberrations define many diseases. The use of a small-molecule to achieve this fate would further improve this technology.

Concluding Remarks

The findings in this thesis collectively represent a critical step toward understanding the role of the ECE in Oct4-mediated reprogramming. It also provides insight into how small-molecules may further modulate this state to contribute to optimized OiP derivation. Furthermore, the generation of a screening platform for the identification of a small-molecule inducer of Oct4 expression provides a unique means of further elucidating and improving upon the induction of the plastic state, specifically the production of specialized cellular products without any genetic modifications.

It is hoped that the future application of these cells in drug screening or cell therapy may make an impact in the treatment of genetic and degenerative disorders, such as Parkinson's or Alzheimer's, but further research is required in order to safely and efficiently generate these cell products. It is my belief that the continued pursuit for understanding OiP will enable greater control of cell fate conversion, an essential requirement for this field to progress.

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Appendix I: Materials and Methods

i Lentivirus Production

1. The Oct4 lentiviral vector, either pSin-EF1α-Oct4-IRES-Puro (Addgene #16579) or pHIV-OCT4-IRES-E2Crimson, was cotransfected with the pMD2.g (Addgene #12259) and psPAX2 (Addgene #12260) packaging plasmids (1:1.4:2.4 ratio) into the HEK293FT cell line at 90% confluence in 1.5 mL OptiMEM (Gibco) and 45 μL Lipofectamine transfection reagent (Thermo Fisher Scientific) to initiate viral particle production.
2. Ten hours later, fresh media was added to the HEK293FT cells.
3. Viral supernatants were harvested 72 hours post transfection and ultracentrifuged at 25,000 rpm for 2 hours to concentrate the virus.
4. Lentivirus was titred on adult dermal fibroblast cells to achieve a transduction efficiency of ~45% $Oct4^+$ cells, as assessed by fixed cell flow cytometry.

ii Isolation and Expansion of Mouse Dermal Tissue

Animal Facility

1. Mouse was euthanized by cervical dislocation, and the carcass immediately submerged in 70% ethanol
2. Upper chest and lower abdomen was shaved using a razor, and 1 cm × 1 cm pieces of dermal tissue were removed per region and immediately submerged in a 50 mL conical tube containing cold Fibroblast Media, and kept on ice
3. The chest cavity was opened and the heart and lungs removed and immediately submerged in separate 50 mL conical tubes containing cold Fibroblast Media, and kept on ice
4. Animal carcass was disposed

Tissue Culture Lab

1. Tissues and organs were removed from the 50 mL conical tubes, and rinsed in cold PBS + 1% Penn/Strep (P/S)
2. Tissue or organ was transferred into a 100 mm tissue culture-treated dish using forceps
3. Tissue and organs were into ~ 1 mm^2 pieces using a sterile scalpel and forceps
4. Using forceps, tissue and organ pieces were transferred onto a new, sterile 100 mm tissue culture-treated dish, with 3-4 tissue pieces plated per dish and separated by at least 2 cm
5. Tissue pieces were left to dry within the BSC, with the lid of the plate removed, (approx. 10-15 min.)
6. 1 mL of warmed Fibroblast media containing 1% P/S was added on top of each tissue piece, such that all tissue was covered

7. Cultures were incubated at 37°C, and let to grow for 4 days
8. Plates were checked on day 4 and every other day thereafter for fibroblast outgrowth and a change in media colour. Keratinocytes and Fibroblasts began to outgrow from tissue fragments and attach to the plate after approximately 4 days.
9. Upon cell outgrowth to ~60% confluence, cells were collected as follows:
    i Media was carefully aspirated, 1 mL of Trypsin-EDTA was added to cells, and cultures incubated at RT for ~10 min.
    ii Trypsin-EDTA solution was aspirated and 1 mL of warm Fibroblast media + 1% P/S was added to the top of skin fragments
    iii Media was continuously pipetted up and down to rinse the tissue pieces to dissociate all outgrown fibroblasts from the plate
    iv Fibroblasts were collected in a 15 mL conical tube, and centrifuged at 1200 rpm for 5 min., and cell count assay performed
    v Cells were frozen at P1 (5×10^5 cells/1 mL), or re-seed onto Gelatin-coated tissue-culture plates at 2.0×10^4 cells/cm^2 and left to continue fibroblast outgrowth for an additional 7 days (by day 14, all viable fibroblasts have exited the tissue fragments)
10. Cells were aliquoted at 5.0×10^5 cells/vial in 1 mL of Freezing media (90% Fibroblast media, 10% DMSO), placed in a CoolCell (biocision) cryo container and chilled to -80°C over 24 h prior to placement in long-term storage in liquid nitrogen at -150°C.

iii Cell Culture

Human Fibroblasts

Primary human dermal fibroblasts derived from either breast dermal tissue (adult) or foreskin (fetal) were maintained in Fibroblast media [DMEM (Gibco), 10% v/v Fetal Bovine Serum (HyClone), 1 mM L-glutamine (Gibco), 1% v/v non-essential amino acids (NEAA; Gibco)] on tissue-culture treated dishes.

Mouse Fibroblasts

Primary murine dermal fibroblasts derived from the dermal tissue of either B6; CBA-Tg(Pou5f1-EGFP)-2Mnn/J or B6; 129S4-*Pou5f1*^{tmJae}/J mice (The Jackson Lab) were maintained in Mouse Fibroblast media [DMEM (Gibco), 10% v/v Fetal Bovine Serum (HyClone), 1 mM L-glutamine (Gibco), 1% v/v Sodium Pyruvate (Gibco), 1% v/v NEAA (Gibco) supplemented with 0.1 mM β-mercaptoethanol] on gelatin-coated tissue-culture treated dishes.

iv Lentiviral Reprogramming

1. Fibroblasts were seeded at 2.0×10^3 cells/cm^2 on Matrigel-coated tissue culture plates in Fibroblast media

2. The following day, cells were transduced with pSin-EF1α-Oct4-IRES-Puro or pHIV-OCT4-IRES-E2Crimson in Fibroblast media (day 0).
3. On the following day (day 1), media was changed to 50% Fibroblast media, 50% Reprogramming media [DMEM/F-12 (Gibco), 20% v/v KOSR (Gibco), 1 mM L-glutamine (Gibco), 1% v/v NEAA (Gibco), supplemented with 0.1 mM β-mercaptoethanol, 16 ng/mL bFGF (BD Biosciences), and 30 ng/mL IGF-II (Sigma)],
4. On the following day, media was changed to 100% Reprogramming media, and media replaced every other day
5. Upon culture confluence (~90%), cells were collected, counted, and re-seeded onto Matrigel-coated tissue culture plates at the same seeding density on day 8, and grown to day 16-18 for the emergence of "plastic" cellular colonies.

v Chemical Reprogramming

1. Mouse dermal fibroblasts were seeded at 2.0×10^4 cells/cm^2 on Matrigel-coated tissue culture plates in Fibroblast Media.
2. The following day, cells were changed to 50% Fibroblast media, 50% Reprogramming media.
3. The next day, cultures were changed to 100% Reprogramming media supplemented with 500 μM VPA, 20 μM CHIR99021, 10 μM E-616452, 10 μM Tranylcypromide, and 50 μM Forskolin.,
4. Cells were cultured for 16 days, with media replaced every other day.

vi Immunocytochemistry (ICC) Staining

Live Cell

1. Cells were washed 1× with RT Phosphate Buffered Saline (PBS; Gibco) + 3% FBS, and incubated with the appropriate fluorophore-conjugated antibody for 2 hours at 37°C
2. Cultures were washed 2× with RT PBS + 3% FBS to remove unbound antibody.
3. Cells were stained 1:10 000 with the nuclear dye Hoechst 33342 (Thermo Fisher) for 5 minutes RT
4. Cells were washed 2× with RT PBS + 3% FBS to remove unbound stain.
5. Cells were visualized by an Olympus IX81 Fluorescence microscope and Volocity software, and analyzed using ImageJ (NIH).

Fixed Cell

1. Cells were washed 1× with RT PBS, then fixed using 2% paraformaldehyde via the BD Cytofix Fixation Buffer (BD Biosciences) for 10 min. at RT.
2. Following, blocking was performed for 1 hr. in PBS + 3% FBS, supplemented with the appropriate animal serum at 5% v/v (Sigma-Aldrich)

3. If permabilization was required (for intracellular staining), cells were instead fixed using 1× BD Cytofix/Cytoperm Solution (BD Biosciences) for 10 min. at RT, and permeabilization followed using either 1× BD Perm/Wash Buffer (BD Biosciences), or 0.5% Triton X-100 (Sigma-Aldrich) for 30 min. at RT.
4. Cells were stained overnight in PBS, containing 5% animal serum only for the surface staining procedure.
5. The following morning, 3× washes in PBS were followed by nuclear staining via 1:1 500 staining in Hoechst 33342 (Thermo Fisher) for 3 min.
6. Following, an additional 3× washes in PBS to remove Hoechst was performed.
7. Cultures were visualized and imaged using the Olympus IX81 Fluorescence microscope and Volocity software, and analyzed using ImageJ (NIH).

vii Flow Cytometry

1. Cells were dissociated as single cells using TrypLE (Gibco) for 10 minutes at 37°C
2. Following, cultures were gently scraped to remove all cells.
3. Upon collection, cells were washed 1× with RT PBS + 3% FBS.
4. Cells were counted using the Countess™ Automated Cell Counter (Invitrogen) using the live/dead discrimination dye Trypan Blue (0.4%).

Live Cell

1. Live cells were stained with a surface marker antibody at the appropriate dilution in 200 μL PBS + 3% FBS for 1 hr. in the dark at RT.
2. Cells were washed 1× in PBS + 3% FBS
3. Cells were resuspended in PBS + 3% FBS, and stained at 1:50 with 7-amino actinomycin (7AAD) live/dead exclusion dye immediately prior to analysis.
4. Cell acquisition was performed using the BD LSR-II flow cytometer (BD Biosciences) using FACSDiva software. Collected events were analyzed using FlowJo 10.0.0 Software (Tree Star).

Fixed Cell

1. Fixed cells were first stained at 1:7 000 with Live/Dead Violet discrimination dye (BD Biosciences) for 30 min. at 4°C in the dark.
2. Following 1× washing in PBS, cells were fixed using 400 μL of 1× BD Cytofix/Cytoperm Solution (BD Biosciences) for 20 min. at 4°C in the dark.
3. Cells were then washed in 1 mL of 1× BD Perm/Wash Buffer (BD Biosciences).
4. Following, cells were stained with fluorophore-conjugated monoclonal antibodies in the abovementioned permeabilization solution overnight at 4°C.
5. The following morning, cells were washed 1× in PBS and resuspended in 200 μL PBS
6. Cell acquisition was performed using the BD LSR-II flow cytometer (BD Biosciences) using FACSDiva software. Collected events were analyzed using

FlowJo 10.0.0 Software (Tree Star).

Optimal working dilutions were determined for individual antibodies and are delineated in the brackets as follows:

Oct4-PE (1:200)
Oct4-Alexa Fluor 647 (1:200)
Oct4-FITC (1:50)
Ki67-PE (1:400)

viii Live Cell Microscopy and Image Analysis

1. Live cells were stained with a surface marker antibody at the appropriate dilution in 1 mL PBS + 3% FBS for 2 hrs. in the dark at RT.
2. Cells were washed 3× in PBS + 3% FBS, and stained at 1:10 000 with the nuclear dye Hoechst 33342 (Thermo Fisher) for 5 minutes RT
3. Cells were then washed 2× with RT PBS + 3% FBS to remove unbound stain.
4. Cells were visualized by an Olympus IX81 Fluorescence microscope and Volocity software, and analyzed using ImageJ (NIH).

Optimal working dilutions were determined for individual antibodies and are delineated in the brackets as follows:

Nanog-PE (1:200)
Hoechst (1:5 000)

ix Data & Statistical analysis

All results were expressed as the mean ± S.D. and generated from at least three independent experiments and managed using Prism GraphPad software. Statistical significance was determined using either the unpaired Student *t*-test or groups analyzed using the 1-way ANOVA. Results were considered significant or highly significant when $p < 0.05$ * or $p < 0.01$ **, or $p < 0.001$ ***, respectively. $p > 0.05$ is N.S.

Appendix II: Additional Reference Materials

i The Jackson Lab - Mice

	Oct4-ΔPE-eGFP (OG2)	Oct4-eGFP
Supplier	The Jackson Lab	The Jackson Lab
Donating Investigator	Jeff Mann, Murdoch Children's Research Institute	Dr. Rudolf Jaenisch, Whitehead Institute (MIT)
Strain	B6; CBA-Tg(Pou5f1-EGFP)2Mnn/J	B6; 129S4-*Pou5f1*^{tm2Jae}/J
Stock No.	004654	008214
Mutation/Allele Type	Transgenic (reporter)	Targeted mutation (reporter)
Date of Birth/ Age	03 Aug 15/ 39 d (5 w)	16 Sept 15/ 38 d (5 w)
Gender	F	F
Reference	Szabó et al., 2002.	Lengner et al., 2007.