OCT4 FACILITATED CELLULAR REPROGRAMMING OF HUMAN SOMATIC CELLS

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Abstract

OCT4 is one of four transcription factors known to induce pluripotency when expressed together in somatic cells. However, brief expression of these pluripotency inducing factors in somatic skin fibroblasts followed by treatment with lineage specific culture conditions results in direct conversion towards alternative lineage specific cell types. Our group has previously shown that expression of OCT4 alone in adult human fibroblasts followed by treatment with hematopoietic supportive conditions resulted in the generation of multi-potent blood progenitors without transitioning through a pluripotent intermediate. Despite never having been associated with transcriptional regulation within the hematopoietic compartment, expression of OCT4 induced expression of hematopoietic factors in skin fibroblasts. As such, I hypothesized that *ectopic expression of OCT4 in human somatic cells can induce changes in transcription that bestow the potential to make cell fate choices in response to external stimuli.*

In direct support of this notion, we revealed that expression of OCT4 in adult human fibroblasts, followed by culturing in neural supportive conditions resulted in the generation of tri-potent neural progenitors, suggesting the effects of OCT4 were not specific or limited to activation of hematopoietic programs. In an effort to understand how OCT4 bestows the potential to make cell fate choices, we assessed the individual vs combined impact of OCT4 and the extracellular environment on transcription during direct conversion of fibroblasts to both blood and neural progenitors. In doing so, we have started to define an induced state of transcriptional activity that is distinct from cells transitioning to pluripotency, and instead characterized by expression related to lineage

development that is responsive to changes in the extracellular environment that we have termed OCT4 induced plasticity (OiP). Moreover, we revealed that OCT4 mediated direct conversion can facilitate the reprogramming of hematopoietic progenitor cells towards neural progenitor cells, suggesting that this cellular reprogramming approach is not limited to the use of differentiated fibroblasts. In summary, this thesis expands our current knowledge on both the use and understanding of OCT4 as a facilitator of cellular reprogramming in human somatic cells.

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List of Abbreviations

Abbreviation	Long Form
iPSC	Induced pluripotent stem cell
PSC	Pluripotent stem cell
ESC	Embryonic stem cell (h designates human, m designates mouse)
ICM	Inner cell mass
ECC	Embryonal carcinoma
MEF	Mouse embryonic fibroblast
bFGF	Basis fibroblast growth factor
hDF	Human ESC derived fibroblast like cells
IGFII	Insulin like growth factor two
TGF-β	Transforming growth factor β
SCF	Stem cell factor
Flt3L	FMS related tyrosine kinase 3 ligand
IL-3	Interleukin 3
IL-6	Interleukin 6
G-CSF	Granulocyte colony stimulating factor
BMP4	Bone morphogenetic factor 4
DNA	Deoxyribonucleic acid
SCNT	Somatic cell nuclear transfer
RNA	Ribonucleic acid
5-Aza	5-Azacytidine
cDNA	Complimentary DNA
BAM	Brn-2, Ascl1, Myt1l
iN	Induced neuron
iCM	Induced cardiomyocytes
PCR	Polymerase chain reaction
POU	Pit Oct Unc
NPC	Neural progenitor cell
hFib-NPC^{OCT4}	OCT4 reprogrammed human fibroblast derived NPC
CD45^{+}Fibs^{OCT4}	OCT4 reprogrammed human fibroblast derived blood progenitor
hFib	Human fibroblast
iNPC	Induced neural progenitor cell
eGFP	Enhanced green fluorescent protein
EGF	Epidermal growth factor
NSC	Neural stem cell
O4	Oligodendrocyte marker 4
MAP2	Microtubule associated protein 2
GFAP	Glial fibrillary acid protein
NOD/SCID	Non-obese diabetic severe combined immunodeficiency
RM	Reprogramming media
hFibR^{OCT4}	OCT4 expressing human fibroblasts cultured in RM (Chapter 2)
hFibR^{eGFP}	eGFP expressing human fibroblasts cultured in RM (Chapter 2)

Abbreviation	Long Form
$hFibR^{SOX2}$	SOX2 expressing human fibroblasts cultured in RM (Chapter 2)
FACS	Fluorescence activated cell sorting
OSNL	OCT4 SOX2 NANOG LIN28
PCA	Principle component analysis
$Fibs^{OCT4}$	OCT4 expressing human fibroblasts
$Fibs^{EGFP}$	eGFP expressing human fibroblasts
$Fibs^{4F}$	OSNL expressing human fibroblasts
MET	Mesenchymal to epithelial transition
CNS	Central nervous system
PNS	Peripheral nervous system
BD-iNPC	Blood derived OCT4 induced neural progenitor cell
NC	Neural crest
DA	Dopaminergic
TH	Tyrosine hydrolase
NEFH	Neurofilamin heavy chain peptide
CLACA	Calcitonin related peptide α
CIPN	Chemotherapy-induced peripheral neuropathy
MSC	Mesenchymal stem cell
CB	Umbilical cord blood
MB-PB	Mobilized peripheral blood
BDNF	Brain derived neurotrophic factor
GDNF	Glial cell line derived neurotrophic factor
SHH	Sonic Hedgehog
PDGF	Platelet derived growth factor
VPA	Valproic acid

Chapter 1: Introduction

1.0 Preamble

The body of work presented in this thesis was primarily inspired by the findings of Szabo et al. and Kim et al. and the opinions formed in the cellular reprogramming community with regards to the nature of direct conversion reprogramming facilitated by transcription factors known to support induction of pluripotency. Importantly, the work of Szabo et al. and Kim et al. were both inspired by the findings of Takahashi and Yamanaka regarding the induction of pluripotency within somatic cells, resulting in the formation of induced pluripotent stem cells (iPSCs). According to Yamanaka, the driving force behind the discovery of iPSCs was a convergence of ideas and concepts from the study of *in vitro* pluripotency using embryonic stem cells and historical examples of cellular reprogramming. As such, this chapter provides the reader with an overview of both *in vitro* pluripotency and cellular reprogramming in a manner which facilitates the understanding of Yamanaka's idea of convergence that led to the discovery of induced pluripotency. I then discuss the critical chain of events within the cellular reprogramming field that led to my own postulations regarding the nature of OCT4 mediated direct conversion. Lastly, I briefly discuss current opinions on the role of OCT4 as a transcriptional regulator within the context of pluripotency, induced pluripotency, and direct conversion reprogramming.

1.1 Capturing pluripotency *in vitro*

1.1.0 Teratoma, teratocarcinoma, and embryonal carcinoma cells

Pluripotency describes a status of cellular developmental potential that is characterized in mammals as the ability to generate all of the downstream cells that make up the embryo through the process of differentiation towards the three germ layer lineages. The concept for deriving a pluripotent cell line to study development was born out of an observation of a very rare form of tumor called teratomas (Stevens and Little, 1954). Teratomas are tumors that present as a disorganized collection of adult tissues and organs such as hair, teeth, neural tissue, and bone. Leroy Stevens conducted a great deal of early teratoma research, which was enabled by a particular strain of mice that had a natural incidence of testicular teratoma formation in the testis of approximately 1% (Stevens and Little, 1954). Stevens found that a rare fraction of the naturally occurring teratomas was serially transplantable, suggesting that a subset of teratoma cells harbored self-renewal capacity (Stevens, 1958). The composition and multi-lineage differentiation propensity of teratomas suggested that the tumors were derived from pluripotent early embryonic like cells (Stevens, 1960). Stevens confirmed this idea by demonstrating that injection of early embryos intraperitoneally or intratesticularly gave rise to teratocarcinomas; teratomas consisting of a large proportion of undifferentiated self-renewing cells (Stevens, 1970). This evidence suggested that teratomas were derived from primitive embryonic like cells, and, furthermore, that serially transplantable teratocarcinomas likely maintained a fraction of these embryonic like cells in the form of a self-renewing stem cell. Continued interest in teratocarcinoma biology resulted in the derivation of *in vitro* cultured cell lines from putative teratocarcinoma stem cells called embryonal carcinoma

cells (ECC) (Martin, 1975). ECCs are capable of self-renewal and differentiation towards lineages that were present in the parental tumor. Given these capabilities, ECCs were thought to be a suitable surrogate to study the mechanisms that underlie cell fate choices during development *in vitro* (Martin, 1975). However, the pluripotent potential of ECCs was unclear as they demonstrated poor contribution to chimera formation when injected into mouse blastocysts as well as a high incidence of tumor formation, a phenomena likely related chromosomal abnormalities (Martin, 1975; Solter, 2006).

1.1.1 Embryonic stem cells

As the pluripotent nature of ECCs was unclear, the generation of mouse embryonic stem cells (mESCs) by Evans and Kauffman as well as Gail Martin, shifted a great deal of attention away from the use of ECCs in the study of normal development (Evans and Kaufman, 1981; Martin, 1981). Both of these groups set out to derive an *in vitro* pluripotent cell line directly from the early embryo of mice, as opposed to tissue explants of teratomas or teratocarcinomas. The mouse embryonic stem cell lines generated by Evans and Kaufman were derived from whole cultured blastocysts, whereas those produced by Martin were derived from isolated inner cell masses (ICM). In both cases the cells were confirmed to harbor self-renewal and pluripotent differentiation potential evidenced by serial passaging without a loss in competence for the production of teratomas consisting of all three germ layers. Taken together, these studies confirmed that true pluripotent developmental potential could be captured in the form of an immortalized cell line that maintained a karyotypically normal set of chromosomes. More than 10 years later Andras Nagy and Janet Rossant, demonstrated that mESCs were

capable of supporting the development of an entire mouse through a process called tetraploid complementation. With the knowledge that tetraploid blastocysts can only support the development of extraembryonic tissues such as the placenta, Nagy and Rossant injected the mESCs into a tetraploid blastocyst and observed the formation of an entirely ESC derived mouse (Nagy et al., 1993). This demonstration provided definitive proof that ESCs could give rise to every single cell in the mouse, and highlighted their potential as vehicles to create genetically modified strains of mice (Nagy et al., 1993).

Human embryonic stem cells (hESCs) were first described by Jamie Thomson approximately 17 years after the discovery of mESCs (Thomson et al., 1998). Like mESCs, hESCs were derived through *in vitro* culture adaptation of isolated ICMs of human blastocysts. The resulting cell lines were confirmed to be pluripotent based on their ability to culture for multiple months without losing their potential to form teratomas that consisted of all three germ layers when injected into immune deficient mice (Thomson et al., 1998). Ethical and legal issues prevent the testing of human chimera formation in most jurisdictions, and as such the definitive assays that have been performed using mESCs cannot be employed in the human. Nevertheless, to date multiple hESC lines have been derived that are used to study the process of self-renewal and differentiation.

1.1.2 Regulation of hESC Self-renewal

Human ESCs are routinely cultured in the presence of a mouse embryonic feeder (MEF) support layer, or in a feeder free environment with or without MEF conditioned media (Levenstein et al., 2006; Xu et al., 2005). However, each of these different conditions has

a unifying requirement: the addition of basic fibroblast growth factor (bFGF) to the culture environment (Bendall et al., 2007). bFGF was first introduced to hESC culture medium upon adaptation to serum free conditions, where it was found to be essential to block differentiation and sustain self-renewal (Xu et al., 2005). Although many studies demonstrated the importance of bFGF supplementation for the sustainment of self-renewal of hESCs (Levenstein et al., 2006; Xu et al., 2005), the functional relationship between the addition of bFGF to the culture environment and hESC self-renewal was initially described by our group in 2007. In this study, Bendall at al. demonstrated that hESCs produce an autologous fibroblast like support layer (hDFs) that respond to bFGF administration through production of insulin like growth factor 2 (IGFII) and TGF-β family members (Bendall et al., 2007). Through careful analysis of pluripotent colony initiation capacity, proliferation, survival, and differentiation, Bendall et al. were able to discern that inhibition of TGF-β or bFGF signaling at the level of hDFs induced differentiation of hESCs, whereas blocking of IGF signaling within the pluripotent cells reduced their proliferation and colony initiation capacity while concomitantly inducing apoptosis. These results not only described the functional relationship between critical extrinsic regulators of hESCs and pluripotency, but also provided evidence for the existence of a supportive autologously derived niche.

In addition to extrinsic regulators, hESCs are governed by an intrinsic molecular circuitry known as the pluripotency network of transcription factors. This network was largely worked out in studies of murine development and mESCs, and includes (but is not limited to) Oct4, Sox2 and Nanog. Reducing the expression of any one of these factors in

ESCs, results in a loss of self-renewal and subsequent differentiation. Over-expression of Oct4 or SOX2 also results in differentiation (Adachi et al., 2010; Niwa et al., 2000), whereas over-expression of Nanog promotes self-renewal in the absence of required extrinsic signaling (Chambers et al., 2003). In addition to genetic approaches, required extrinsic signaling molecules such as bFGF and TGF-β have been shown to directly regulate and sustain the expression of NANOG, OCT4, and SOX2 (Xu et al., 2008). Genomic localization analysis of OCT4, SOX2, and NANOG within hESCs using ChIP-on-chip arrays revealed that these three factors co-localize to a large number of target genes, including their own enhancer/promoter regions (Boyer et al., 2005). Cross comparison of the observed genomic localization data to gene expression analyses suggested that the complex was bound at both active and inactive genes, pointing to a dual role in activation and repression within the context of hESCs (Boyer et al., 2005). This analysis revealed a subset of genes that was transcriptionally active and bound by the pluripotency factor complexes that are associated with chromatin remodeling, histone-modification, and additional transcriptional activators of ESC self-renewal including their own promoters (Boyer et al., 2005). Conversely, a subset of transcriptionally inactive genes that displayed regulatory binding of the complex had been implicated with differentiation and lineage decision making in the early embryo (Boyer et al., 2005). Taken together these results suggested that these particular members of the pluripotency network act to co-regulate each other as well as transcriptional mediators at the level of enhancer/promoter regions to promote self-renewal and suppress differentiation in response to extrinsic regulation within hESCs (Boyer et al., 2005; Xu et al., 2008).

1.1.3 Regulation of hESC differentiation

As hESCs are derived from the ICM of developing human embryos, they theoretically harbor the potential to generate all cell types of the human body through the process of differentiation (Thomson et al., 1998). The ultimate goal of controlling differentiation of hESCs was set by Jamie Thomson in his original body of work that described their discovery, and still remains today as an intense area of focus. hESCs readily differentiate in culture in the absence of extrinsic growth factor regulation, if they become too confluent and compacted together, or upon injection into immune deficient mice where they form teratomas (Thomson et al., 1998). As such, it would seem that differentiation of hESCs is no difficult task. However, difficulty arises in guiding the cell fate choices made by hESCs that have exited the pluripotent state. This problem has been in part answered by the development of directed differentiation schemes that involve the application of soluble growth factors and signaling molecules, which serve to guide (or select) hESCs along particular pathways of differentiation resulting in the formation of specialized cells.

One of the earliest protocols developed to guide cell fate choices made by hESCs was designed by Melissa Carpenter of Geron Corp. Carpenter developed a differentiation protocol based on previous findings in the mouse system, whereby hESCs were first aggregated into free floating clumps called embryoid bodies in order to induce general differentiation (Carpenter et al., 2001). Embryoid bodies consisting of hESCs begin to exit pluripotency as they are cultured in the absence of required extrinsic regulators (and/or in the presence of differentiation stimulators), and experience similar physical

stimulation to that of over-confluence (Carpenter et al., 2001; Thomson et al., 1998). After approximately 4 days of embryoid body culture, cells were transferred into conditions previously shown to support the expansion of primary neural tissue; serum replacement medium supplemented with bFGF (antagonist of BMP driven inhibition of EGF receptor activity) and epidermal growth factor (EGF) (agonist of EGF receptor based stimulation of mitosis in multipotent neural stem cells) (Carpenter et al., 2001; Lillien and Raphael, 2000). Supplemented after only 3 days of adherent culture with these growth factors, the cells up-regulated markers of primary neural progenitor cells (Carpenter et al., 2001). Continued culture in bFGF/EGF conditions confirmed that they were able to proliferate, whereas withdrawal of bFGF/EGF led to their differentiation towards more mature cells of the neural lineage; neurons, and glia (Carpenter et al., 2001). As such, these results confirmed that differentiation of hESCs could, to some extent, be controlled like that of mESCs by the application of soluble factors and substrates that are known to support the growth and culture of particular cell types.

In a similar effort to guide differentiation, our group was one of the first to develop a novel protocol to direct hESC fate towards hematopoietic progenitors using a soluble growth factor strategy (Chadwick et al., 2003). In this approach, Chadwick et al. formed hESCs into embryoid bodies and then treated them with hematopoietic cytokines (Stem Cell Factor (SCF), FMS related tyrosine kinase 3 ligand (Flt3LG), Interleukin-3 (IL-3, Interleukin-6 (IL-6), and Granulocyte- Colony Stimulating Factor (G-CSF)) with or without the mesoderm inducer bone morphogenic protein 4 (BMP4). Differentiating hESCs were monitored over time using flow cytometry to assess for the expression of the

hematopoietic cell surface markers CD34 and pan-leukocyte marker CD45. Treatment of hESCs with cytokines +/- BMP4 resulted in the formation of CD34+CD45+ cells, which under further investigation were shown to harbor hematopoietic progenitor function using clonogenic colony forming assays. Although limited hematopoietic differentiation capacity was present in control conditions devoid of cytokines and/or BMP4, addition of BMP4 to cytokine treated cells was shown to significantly increase the self-renewal potential of resulting hematopoietic progenitors (Chadwick et al., 2003). Taken together, these results defined the strategy for the generation of hematopoietic cells from hESCs, which is still used by groups studying hematopoiesis from hESCs today (Vo and Daley, 2015).

Collectively, these studies serve as two examples of hESC differentiation protocols that utilize soluble factors that are known to play a functional role in the regulation of the desired endpoint cell type, as modulators of hESC cell fate.

1.2 Cellular Reprogramming

In its simplest form, cellular reprogramming can be defined as the transition from one specific cell type to another outside of the context of classic developmental paradigms. The phenomenon of reprogramming has been studied in multiple different model systems over the last 50 years, and in doing so has yielded information that has helped answer some of the most basic questions regarding development and differentiation and has provided the means for creating cutting edge personalized disease models and potential products for transplant therapies. The following section will provide a selection of what

I believe are the most powerful and insightful examples of cellular reprogramming that have influenced the field and guided it to where it is today.

1.2.0 Somatic cell nuclear transfer

In the mid to late 1950's, Briggs and King began devising a method to test a long standing question in the field of developmental biology: "does differentiation impose changes on the nuclei that are as permanent in nature as the fate of the given cell?" Their idea was as follows; if one could remove the nucleus from an egg, replace it with the nucleus from a more mature cell, and artificially simulate fertilization, they could test whether that nucleus would support normal healthy development. This test would provide insight into the nature of nuclei of differentiated cells compared to those in the undifferentiated state found during early embryogenesis. In order to demonstrate that the experiment was technically feasible, Briggs and King first tested a positive control situation, where they used cells from blastulas (early stage of frog development) considered to be equipotent to single cell embryos, as the source for donor nuclei. By transplanting nuclei from blastula cells into an enucleated oocytes, they could test whether their manipulations prevented normal development from occurring, prior to assessing normal development from nuclei from differentiated cells. Using micropettes and a microscope, Briggs and King mastered the art of removing nuclei from *Rana pipiens* frog eggs, and injecting them with partially ruptured blastula cells such that the nuclei were free to enter the cytoplasm of the oocyte (Briggs and King, 1952). In the end, they were able to demonstrate that the process of nuclear transfer afforded normal development, and therefor they could go on to test whether nuclei at different stages of

development were capable of the same feat. In order to ensure technical consistency regarding cell size, and also due to their easily identifiable morphology, Briggs and King chose endoderm cells as the donor for nuclear transplant experiments that would assess whether differentiation imposed irreversible changes on the chromosomes contained within the nucleus (Briggs and King, 1957). In doing so, Briggs and King concluded that the developmental potential of DNA within endoderm cells decreases as they differentiate in the developing tadpole, as nuclei from cells taken from mid-neurulae stage (prior to heartbeat development) failed to support the generation of swimming tadpoles (Briggs and King, 1957). At this point, it seemed that Briggs and King had answered one of the earliest questions in developmental biology, a question that one year prior had become the focus of a newly appointed graduate student named John B. Gurdon. Gurdon was also very interested in whether or not differentiation imparted irreversible changes on DNA, and had been positioned to answer this using a different species of frog, *Xenopus laveis* (Gurdon, 2013). Despite the work of Briggs and King having "essentially completed his thesis for him", Gurdon felt that it would still be important to repeat the work in *Xenopus laveis* to determine if it would work the same, in which case he could then work on the mechanism by which differentiation was permanently altering DNA. Alternatively he might discover a conflicting result (Gurdon, 2013). Upon establishing reliable methods for performing SCNT in *Xenopus laveis*, Gurdon generated an extremely controversial data set that was in direct conflict with the hypothesis of Briggs and King. Gurdon's data clearly showed that nuclei derived from the intestinal epithelium of feeding tadpoles could support the formation of sexually mature adult frogs through the use of SCNT

(Gurdon and Uehlinger, 1966). These results provided undeniable proof that differentiation within particular amphibian tadpoles was not entirely driven by mandatory permanent changes to the DNA.

Assessing whether mammalian differentiation was a result of permanent alterations to DNA would not be answered for an additional 40 years after the early work of Gurdon. In 1997 Ian Wilmut became famous for the cloning of Dolly the Sheep. Wilmut successfully performed SCNT using adult udder cells, which confirmed that like amphibians, mammalian differentiation did not proceed through permanent changes in DNA sequence that would preclude them from supporting the generation of other cell types (Wilmut et al., 1997). Follow up studies in the mouse went to great lengths to reinforce the notion that donor nuclei of terminally differentiated cells such as B and T lymphocytes were equally competent to support development by tracking inherent genomic reorganization of immunoglobulin alleles and T-Cell receptor genes in B and T cells respectively (Hochedlinger and Jaenisch, 2002).

Through the collective work of some of the most influential and successful biologists of the last century, somatic cell nuclear transfer succeeded in answering one of the earliest questions raised in the study of developmental biology. For his pivotal role in demonstrating maintenance of developmental potential within the nuclei of mature cells, Sir. John Gurdon shared the 2012 Nobel Prize in Physiology and Medicine, with Dr. Shinya Yamanaka. However, both 'the search for' and 'the understanding of' the distinct cytoplasmic factors that are responsible for this reprogramming process are still a subject of intense study today (Gurdon, 2013).

1.2.1 Somatic cell hybrids

SCNT demonstrated the powerful ability of egg cytoplasm to reset the biological clock of somatic nuclei, but did not speak to whether cells other than the egg contained factors within their cytoplasm that harbored equal reprogramming potential. In an effort to address this question, Harris and colleagues began to study the consequences of fusing cells of distinct origin and function together as one (Harris, 1965). Two or more cells that have fused together but maintain distinct nuclei are referred to as heterokaryons. After a varying number of hours or days (depending on the cellular nature of the fusion product) the nuclei of the heterokaryon may also undergo fusion, forming a synkaryon. The fusion of these nuclei more often than not led to deviations in normal DNA replication, and sometimes to lose partial or whole chromosomes (Blau et al., 1983). Harris' initial studies of cell fusion, consisted of hybridizing HeLa cells with highly differentiated mature cells such as lymphocytes and erythrocytes (Harris, 1965). In their native environment, rat small lymphocytes display appreciable levels of RNA synthesis but no detectable level of DNA synthesis, whereas hen erythrocytes demonstrate little to no RNA synthesis as well as no detectable levels of DNA synthesis (Harris, 1965). Using the process of fusion, Harris was able to demonstrate that factors presumably contained within HeLa cells were sufficient to restore detectable levels of DNA synthesis in rat lymphocytes, as well as both RNA and DNA synthesis in hen erythrocytes. The factors contained within HeLa effectively reprogrammed the mature cells to an active state of nucleic acid production that they otherwise would never have re-entered (Harris, 1965). Conversely, Weiss was able to show the suppression of hepatic specific alcohol

dehydrogenase production in hybrids of hepatoma cells with mouse fibroblast cells lines (Bertolotti and Weiss, 1972). These studies clearly demonstrated that a gene product from the fibroblast nuclei was in part responsible for this suppression, as after sufficient chromosome loss post synkaryon formation, alcohol dehydrogenase was once again detected (Bertolotti and Weiss, 1972). Armed with the knowledge that cell fusion experiments could facilitate both activation and repression of gene elements in a dominant fashion, Miller and Ruddle questioned whether these signals would be influential in directing the fate of primitive developmentally potent cells that had not undergone differentiation, such as ECCs (Miller and Ruddle, 1976). They hypothesized that the factors responsible for maintaining the differentiated state of mature cells, may act in a dominant fashion to guide or at least influence the developmental potential of pluripotent ECCs to a greater extent than mature cells. Hybrids of ECCs and thymocytes were formed and heterokaryons were selected that displayed equal contribution of chromosomal content. These hybrids were then assayed for *in vivo* teratoma formation alongside the parental ECCs and analyzed histologically. Surprisingly, the hybrids displayed no difference in developmental potential compared to that of parental ECCs, producing teratomas that consisted of cells from all three germ layers. These results suggested that the programs that govern pluripotency in ECCs were dominant in comparison to the programs governing mature somatic differentiation (Miller and Ruddle, 1976). Interestingly, these results are not unlike that of SCNT, where the nuclei of a mature cell does not reprogram the oocyte cytoplasm with factors more consistent with

that of the donor cell, but instead is hi-jacked by the egg cytoplasm and used to support normal development.

Despite their clear utility, early examples of cell-fusion derived hybrids commonly included a parental cell that was aneuploid, of tumor origin. They displayed unwanted characteristics such as perpetual DNA synthesis that resulted in the formation of synkaryons, limiting their use in the study of normal gene regulation (Blau et al., 1983). Blau et al. would later refine the process of deriving heterokaryons using mouse muscle cells that are naturally multinucleate in their differentiated state. When fused with donor cells, the muscle cells did not undergo nuclear fusion (Blau et al., 1983). Blau determined that the factors contained within mouse muscle cells were able to activate the expression of 4 myogenic genes in human amniocytes (non-muscle cells) including one gene that was entirely specific to human muscle, never before having been reported to be expressed in the mouse. This provided clear evidence for a trans-acting factor that must have travelled through the cytoplasm in order to come into contact with the nuclei from the non-muscle cells, as the heterokaryons never transitioned to synkaryons (Blau et al., 1983).

Taken together, these studies provided clear evidence that differentiated cells contained factors, present in both the nucleus and the cytoplasm that were capable of influencing gene expression related to cellular identity. Moreover, these studies provided further evidence to support the notion that differentiation does not take place through physical loss or gain of DNA content, but instead occurs through regulation of transcription and subsequent translation, in part mediated by soluble factors.

1.2.*2 MyoD and the birth of transcription factor reprogramming*

The initial discovery that would eventually lead to the first example of transcription factor based cellular reprogramming came about during a chemotherapeutic drug screen on mouse fibroblasts in the lab of Peter Jones. Jones had noticed that cells treated with 5-aza-cytidine (5-Aza) underwent a bizarre transformation resulting in the formation of multinucleate striated muscle fibers that spontaneously contracted (Constantinides et al., 1977). Further work by Taylor and Jones would not only confirm this observation, but also expand the reprogramming potential of 5-Aza through demonstrating that individual single cell derived clones of mouse fibroblasts formed both striated muscle and fat cells post treatment with the molecule (Taylor and Jones, 1979). These results confirmed that the transformed cells were truly a product of cellular reprogramming, as opposed to the proliferation of dormant muscle or adipocyte cells (Taylor and Jones, 1979). Initial thoughts regarding the mechanism of action detailed two possible scenarios: 1) the genes corresponding to the observed muscle specific proteins were subject to direct demethylation (cis-activation), or 2) muscle specific gene regulator(s) were demethylated resulting in activation of the measured muscle specific genes (trans-activation) (Lassar et al., 1986). Deciphering whether either, or both, of these scenarios were the cause of the reprogramming event became the central question of Andrew Lassar, who at the time was working with Harold B Weintraub. Lassar reasoned that if 5-Aza directly acted at the level of the observed muscle specific proteins, then simple administration of non-methylated DNA coding for these known genes into the starting population of non-treated fibroblasts should result in their subsequent activation. If however, 5-Aza demethylated a

transcriptional activator of muscle specific genes, which subsequently went on to establish a muscle program, Lassar reasoned that this DNA region would likely be retained in an unmethylated state in the established myogenic lines created from the initial 5-azacytidine treated fibroblasts. In order to test both situations, Lassar transfected both unmethylated DNA coding for muscle proteins that were found to be upregulated after treatment with 5-Aza, as well as genomic DNA collected from converted myoblasts. In the end, only the transfected DNA from established myoblasts lines converted fibroblasts to muscle cells, and so began the search for the factor that was responsible for activating the muscle program in response to 5-Aza treatment (Lassar et al., 1986). By screening a cDNA library for muscle reprogramming competency, Davis, Wientraub, and Lassar discovered the single factor capable of substituting for 5-Aza in the conversion of fibroblasts to striated muscle cells, which they named MyoD (Davis et al., 1987). Ectopic expression of MyoD is, to the best of my knowledge, the first example of targeted transcription factor-based cellular reprogramming, and has served as the inspiration for countless experiments and projects, and likely, programs of study. Thomas Graf would be one of the first to try and apply this notion of transcription factor mediated reprogramming within a different cellular system, by over-expressing Gata-1 in transformed myelomonocytic cell lines (Kulessa et al., 1995). Expression of Gata-1 resulted in the reprogramming of myelomonocytic cells towards both eosinophils and thromboblasts, but perhaps more interesting was that the ratio of this conversion was mediated by dosage of Gata-1 expression (Kulessa et al., 1995). Graf would later go on to show that mature hematopoietic cells such as B cells could also be reprogrammed by

ectopic expression of C/EBPα into macrophages, once again demonstrating the power of transcription factors to activate programs of genes responsible for cellular fate (Xie et al., 2004).

Taken together, these early examples of reprogramming highlighted the power of transcription factors in the regulation of cellular identity, and in doing so would inspire one of the most powerful demonstrations in current biology.

1.3 Convergence of ESC biology and cellular reprogramming: induced pluripotency

Two years after being accredited as an orthopedic surgeon, Dr. Shinya Yamanaka made a choice to pursue research, as he felt that he was not a particularly talented surgeon and there was a great need to try to further our understanding of conditions that physicians were not able to treat or cure through basic research (Yamanaka, 2013). Through his PhD and postdoctoral work, Yamanaka came to learn and master the art of genetically engineering mice through manipulation of mouse embryonic stem cells (Yamanaka, 2013). His initial use of mESCs as a tool (and later human ESCs) progressed into a fascination with studying the underlying mechanisms of pluripotency, which ultimately led Yamanaka's long-term research goal for his lab. Instead of attempting to differentiate ESCs towards interesting and useful somatic cells for downstream studies, Yamanaka wanted to find a way to create ESCs from somatic cells, a method that would circumvent the ethical issues surrounding the derivation of human embryonic stem cells. Yamanaka considered three main observations that led him on this pursuit 1) somatic cell nuclear transfer experiments pioneered by Gurdon, 2) cell fusion experiments with ESCs and somatic cells by Tada, and 3) transcription factor control of cell fate by Weintraub. With

these observations in mind, he hypothesized that imposing ESC-specific transcriptional regulation on somatic cells would lead to the derivation of ESC-like cells (Yamanaka, 2013). Through comparison of transcriptional databases from both ESCs and somatic cells, Yamanaka's group generated a list of ESC-specific factors that he began to functionally interrogate through derivation of knockout mESCs cells and knockout mice (Yamanaka, 2013). This process eventually led to the identification of 24 factors that appeared to be critical for mESC function, which were then tested for their ability to induce pluripotency (Takahashi and Yamanaka, 2006). Yamanaka's pluripotency assay had inadvertently been created during the functional analysis of pluripotency associated gene expression, whereby neomycin resistance cassettes knocked into pluripotent factor loci (as a means of knocking out gene function) served as a means of selecting for cells that had activated pluripotent gene expression (Takahashi and Yamanaka, 2006; Yamanaka, 2013). Takahashi, the post doc assigned to test the 24 factors, introduced each factor, one by one, through retroviral delivery and observed no activation of pluripotency gene expression or changes in morphology, suggesting that no single factor (under these specific experimental conditions) was able to induce pluripotent status within somatic cells (Takahashi and Yamanaka, 2006). However, when all 24 factors were introduced together, Takahashi noticed discrete colony formations that were stable under neomycin drug selection. In order to discern which factors were critical to the colony induction process, Takahashi removed one factor at a time from the pool of 24 and observed whether this resulted in colony formation loss. Eventually, Takahashi identified four critical factors (Oct4, Sox2, Klf4, C-Myc) that when removed from the process,

drastically lowered colony formation or stability. These putative reprogrammed colonies of cells (approximately 0.02% frequency of formation) were assayed for pluripotency using a battery of molecular (endogenous activation of gene networks present in ESCs) and functional tests (*in vitro* differentiation towards the three germ layers, *in vivo* teratoma formation, and chimeric mouse formation post blastocyst injection) that ultimately led to the conclusion that they were pluripotent (Takahashi and Yamanaka, 2006). After completing this rigorous assessment, Yamanaka shared his results with the world, and so was the birth of induced pluripotent stem cells. Only one year later, both Yamanaka and Jamie Thomson (the developer of hESCs) translated the process to the human system, giving rise to human iPSCs (Figure 1) (Takahashi et al., 2007; Yu et al., 2007). Interestingly, Thomson had not only reproduced the phenomenon of iPSC reprogramming, but he had done it using a different set of factors that incorporated NANOG and LIN28, suggesting that there could be multiple ways to generate iPSCs through the expression of pluripotency associated factors (Yu et al., 2007; Zhou et al., 2008). The advent of human iPSCs circumvented two critical drawbacks regarding the use of hESCs: 1) ethical boundaries surrounding the use of human embryos, and 2) immunological complications of using hESC derived tissues in a transplant setting. As such, not only did Yamanaka enable human pluripotent cell research for hundreds of labs that otherwise could not have performed legal research, but the technology also attracted the interest of labs due to the potential clinical utility of hiPSCs in the development of products and procedures within the area of personalized medicine.

In the nearly 10 years that has passed since the discovery of iPSC reprogramming, an astonishing volume of research has been reported regarding novel means of achieving iPSC formation as well as elucidating the mechanistic basis of the reprogramming process. Retroviral transduction of somatic cell starting material as a delivery method for reprogramming factors has been successfully substituted with excisable transposon vectors (Woltjen et al., 2009), non-integrating episomal DNA vectors (Yu et al., 2009), synthetic mRNA (Warren et al., 2010), and synthetic proteins (Zhou et al., 2009), to facilitate the generation of iPSCs. Furthermore, when utilizing the Yamanaka factors for reprogramming, both the factor stoichiometry (Papapetrou et al., 2009) and length of expression (Brambrink et al., 2008) have been linked to successful derivation of stable iPSC. Moreover, additional factors that contribute to the induction of pluripotency have been identified (Brambrink et al., 2008; Feng et al., 2009; Gao et al., 2013; Redmer et al., 2011; Tsubooka et al., 2009) and in one case, iPSC formation has been described in the absence of ectopic pluripotency factor expression through expression of lineage specifying factors (Montserrat et al., 2013). Moving in the other direction, reduction in the number of ectopically expressed pluripotency factors has been achieved through the use of somatic cells that harbor endogenous factor expression (Kim et al., 2009), as well as through substitution of factors with microRNAs and small molecule inhibitors (Anokye-Danso et al., 2011; Hou et al., 2013). Taken together, these studies and others have demonstrated that there are many ways to induce the process of reprogramming towards pluripotency (Hu, 2014), but whether the reprogramming process itself is unique to each methodology, or shared across the board, remains to be elucidated.

The fundamental nature of iPSC reprogramming has been debated with regards to whether the process is stochastic or deterministic. Early studies of iPSC reprogramming supported the notion of a stochastic process that results in very few stable iPSC alongside partially reprogrammed cells (Mikkelsen et al., 2008; Sridharan et al., 2009). However later studies were successful in identifying rate limiting steps that could be overcome with targeted intervention leading to a model that predicted a bi-phasic process consisting of an early stochastic phase and a late deterministic phase (Buganim et al., 2012). Additional studies have provided detailed molecular road maps that intricately describe the stages of pluripotency induction from the earliest time points after initial factor expression, through to stable iPSC formation (Buganim et al., 2012; Li et al., 2010; Polo et al., 2012; Samavarchi-Tehrani et al., 2010). Collectively, these studies have described early and late waves of expression related to pluripotency, as well as a tipping point whereby cells must undergo a mesenchymal-to-epithelial transition prior to advancing to the later stages of the process. Moreover, through stepwise unbiased analysis of cells undergoing iPSC reprogramming a recent study has described the formation of an entirely unique class of pluripotent cells that results from continued expression of the Yamanaka transgenes in combination with endogenous Nanog (Tonge et al., 2014). Interestingly, whether the molecular roadmap of iPSC formation, or the generation of unique pluripotent states are consistent and reproducible amongst all of the different methodologies in which pluripotency can be induced remains to be investigated. Despite any differences with regards to reprogramming methodology, or descriptions of the events that precede pluripotency, all of these studies are unified by the generation of

functional iPSCs. However, if the major goals of iPSC research are to be aligned with those put forth by Jamie Thomson upon discovering human ESCs, then the continued pursuit of understanding the process of reprogramming should be tightly linked to differentiation propensity, in order to ensure the realization of the full scientific potential of iPSC technology.

1.4 Transcription factor reprogramming in a post iPSC world

Yamanaka's demonstration of induced pluripotency resulted in an eruption of not only follow up studies on iPSC generation but also innovative investigations using unique combinations of lineage specific transcription factors from countless labs around the world (Sancho-Martinez et al., 2012). These investigations were unified by the idea that transcription factor reprogramming could be used to interconvert somatic cells of interest without first forming a pluripotent cell, which then required further differentiation. To try and remark on each of these studies would be beyond the scope of this thesis, so instead I will discuss those works arising soon after the discovery of iPSCs that served as a framework for the establishment of two divergent classes of direct conversion based on their use of lineage specific transcription factors (class 1) or transient expression of pluripotency factors (class 2), which served as the foundation for my hypothesis.

1.4.0 Direct conversion of exocrine cells to endocrine cells in the pancreas (Class 1)

In 2008, two years after Yamanaka stunned the scientific community with the discovery of iPSCs, Douglas Melton's group reported the direct conversion of pancreatic exocrine cells into insulin producing β-cells *in vivo* via expression of ectopic transcription factors *(Zhou et al., 2008)*. Similar to Yamanaka's approach, the report detailed that the

transcription factors used in the study were selected through a developmental prioritization, as factors critical to β-cell development (in place of pluripotency development) would be more likely to induce a reprogramming process. Moreover, Melton felt it would be advantageous to perform the conversion using a related tissue resident cell *in vivo* to ensure that the converted cells would reside in their natural habitat, which he hypothesized could play a supportive role in the process. After performing an n-1 factor reduction approach (again, similar to Yamanaka), Melton's group narrowed down the list of 9 original factors to 3: Neurog3, Pdx1, and Mafa. Adenoviral transduction of these three factors into exocrine cells, resulted in the direct conversion of exocrine cells towards insulin-producing β-cells. This was one of the first reports of successful *in vivo* reprogramming using forced expression of lineage-specific transcription factors that resulted in the production of cells that were functional (albeit modestly). Despite improvements over previous attempts to perform *in vivo* reprogramming, the cells did not perform at a level equal to resident β-cells, nor did they organize into islets (Zhou et al., 2008). However, by focusing on the generation of clinically relevant cell types such as β-cells, which could aid in the study and fight against diabetes, Melton's work provided clear support for the continued investigation into cellular interconversions using transcription factor mediated reprogramming.

1.4.1 Direct conversion of fibroblasts to functional neurons (Class 1)

In 2010 Marius Wernig and Tommy Vierbuchen questioned whether application of transcription factor reprogramming could be used to facilitate direct conversions beyond that of related cells of a given tissue (Zhou et al., 2008) by attempting to generate

ectodermal neurons from mesodermal fibroblasts (Vierbuchen et al., 2010). The reprogramming was initiated through the introduction of 3 neurodevelopmental transcription factors that were identified from an n-1 screen of 19 initial genes whose combined expression bestowed a neuronal phenotype to fibroblasts. Forced expression of Brn2, Ascl1, and Myt1l (BAM), in both neonatal and adult mouse fibroblasts, resulted in conversion towards neurons without first establishing pluripotency, a neural progenitor state, or undergoing cell division. Moreover, unlike induction of pluripotency, Wernig's induced neuron (iN) reprogramming efficiency neared 20%, suggesting that fibroblasts are either more competent for neural reprogramming compared to iPSC, or that the conditions for neural reprogramming are more supportive than those for iPSC generation. The production of functional neuronal cells from fibroblasts without first establishing pluripotency represented a monumental reduction in time and reagent costs for potentially producing a cell type that is otherwise impossible to obtain given the obvious ethical and technical issues with obtaining brain biopsies in the human (Vierbuchen et al., 2010).

1.4.2 Direct conversion of fibroblasts to functional cardiomyocytes (Class 1)

Soon after Wernig shared his work on converting fibroblasts to neurons, Deepak Srivastava reported the successful conversion of cardiac and skin derived fibroblasts to cardiomyocyte like cells (Ieda et al., 2010). Using the now well established n-1 transcription factor approach, Srivastava's team started with 14 different cardiac development related transcription factors before arriving at the trio (Gata4, Mef2c, and Tbx5) capable of inducing the cardiomyocyte fate in fibroblasts. Analogous to the description of iN cells, Srivastava's team detailed the morphological, phenotypic marker

expression, and electrochemical similarities between induced cardiomyocytes (iCM) and those derived from fetal murine hearts in order to confirm that the cellular conversion had taken place. However, unlike Wernig, they also included a global transcriptome analysis in addition to targeted interrogation of the epigenetic status of critical cardiomyocyte regulator genes (Ieda et al., 2010). Much like Yamanaka observed the activation of the pluripotent transcription factor network post expression of OSKM, so too, did the expression of Gata4, Mef2c, and Tbx5 initiate endogenous expression of cardiomyocyte specific gene networks (Ieda et al., 2010). Taken together, it appeared that Yamanaka, Wernig, and Srivistava were all performing a similar technique, whereby expression of a small number of critical regulators specific to cell type "A" (ESCs, neurons, cardiomyocytes) were able to hi-jack the transcriptional networks of cell type "B" (fibroblasts) , resulting in reprogramming towards "A" (iPSC, iN, iCM).

1.4.3 Direct conversion of fibroblasts to hematopoietic progenitors (Class 2)

At the time of both Wernig and Srivastava's initial publications of direct conversion in the mouse using lineage specific factors, our group was in the midst of revising a direct conversion study of our own that would challenge the current understanding of pluripotent transcription factor reprogramming (Szabo et al., 2010). During efforts to generate human iPSCs, Szabo et al., noticed an abundance of non-iPSC colonies that would consistently appear in their tissue culture dishes. One colony type was especially identifiable, as it presented as a tight bundle of round cells, with a similar size and shape to that of blood. Expression analysis for the pan-leukocyte marker CD45, confirmed that the cells had upregulated the blood-specific marker that is not expressed on any other

cells in the human body. Isolation of the CD45+ colonies and analysis by PCR for expression of the reprogramming factors used to drive iPSC formation, revealed that the cells preferentially expressed OCT4 above all other factors used. This prompted a re-transduction of fresh human fibroblasts with OCT4 alone which resulted in the formation of rounded colonies of cells that expressed CD45. It appeared that expression of OCT4 alone in fibroblasts was sufficient to up-regulate some level of hematopoietic related expression (Szabo et al., 2010). In order to test whether OCT4 was only activating phenotypic markers of blood cells versus activation of functional hematopoietic transcriptional regulators, Szabo et al. treated the CD45+ colonies with hematopoietic cytokines. Treatment with SCF and Flt3LG resulted in a significant increase in the number CD45+ colonies. These results suggested that OCT4 expression was activating functional transcriptional programs that were competent for responding to hematopoietic signaling molecules (Szabo et al., 2010). Further culturing of CD45+ colonies in hematopoietic cytokine rich media known to support the development of hematopoiesis from hESCs (Chadwick et al., 2003) resulted in the emergence of a subset of cells that expressed the primitive CD34+CD45+ progenitor phenotype. Functional assessment of the putative progenitor cells confirmed their ability to proliferate and differentiate towards the myeloid and erythroid classes of hematopoietic cells, and demonstrated some ability to engraft into immune compromised mice (the ultimate test of stem/progenitor activity). Moreover, analysis of globin proteins (proteins responsible for carrying oxygen within the blood stream) within erythroid populations of cells revealed a biased expression of β-globin compared to fetal globin, a characteristic of adult hematopoiesis

not normally found in blood cells derived from PSCs (McIntyre et al., 2013). From a biological standpoint, Szabo et al. had demonstrated that human fibroblasts could be reprogrammed into multipotent hematopoietic progenitors through combined expression of OCT4 and treatment with cytokines known to support the growth and differentiation of hematopoietic cells (Figure 2) (Szabo et al., 2010). Given that OCT4 had not (and to this day, still has not) been implicated in normal hematopoietic development, these results were considered to be odd. In order to address mechanistically how OCT4 could achieve such a cell fate conversion, Szabo and colleagues considered that it may have been acting in place of OCT1 and OCT2, two POU-domain containing homologues that play a role in hematopoietic development and share the same DNA binding sequence motif as OCT4 (Tantin, 2013). In order to test this theory , Szabo et al. performed chromatin immunoprecipitation to assess for OCT4 occupancy at both known and predicted, POU domain binding sites within a series of genes related to pluripotency versus hematopoietic development. Within the context of this reprogramming phenomena, OCT4 displayed preferential binding within hematopoietic gene regulatory regions compared to that of pluripotency genes. This chromatin level information fit well with a clear transcriptional up-regulation of hematopoietic genes, in the absence of pluripotency gene activation (Szabo et al., 2010). Given these results, Szabo et al. reported that under these specific conditions, it appeared that OCT4 was capable of up-regulating critical hematopoietic regulators through direct binding of octamer motifs within their enhancer/promoter regions, resulting in the establishment of sufficient transcriptional programs to support the conversion of fibroblasts to blood. This report was not only the first demonstration of

functional direct conversion using adult human cells, but also suggested a novel regulatory role for OCT4 outside that of development, primordial germ cells, or pluripotency. Moreover, generation of a somatic progenitor cell would enable scalable culture of hematopoietic progenitors in order to produce the large numbers of cells required for future mechanistic studies or even pre-clinical modelling.

1.4.4 Direct conversion of fibroblasts to neuronal progenitors (Class 2)

Soon after Szabo et al. reported the direct conversion of human fibroblasts to blood progenitors using OCT4 without establishing pluripotency, Kim et al. from the laboratory of Sheng Ding reported the conversion of mouse fibroblasts to neural progenitors using a modified expression strategy that included the four Yamanaka factors (Oct4, Sox2, Klf4, c-Myc) (Kim et al., 2011). Kim et al., like Szabo et al., suggested that the generation of progenitor cells in the absence of establishing pluripotency was a fast and cost effective means of performing cellular reprogramming. They believed that the expression of OCT4 alone had achieved this goal for the generation of hematopoietic progenitors, but that it was doing so in a similar fashion to the over expression of lineage specific factors (Kim et al., 2011). In order to establish a more general approach to generating progenitor cells, Kim et al. expressed the Yamanaka factors for a brief period time followed by exposure to culture conditions shown to support neural development from PSCs. This approach resulted in the generation of neural progenitor cells that were capable of proliferating and differentiating towards functional neurons (Kim et al., 2011). Kim et al. proposed that the expression of the four Yamanaka factors induced a state of epigenetic instability or early developmental plasticity that could then be influenced by the

extracellular environment through physical manipulation of culture conditions. Despite a complete lack in understanding of the cellular state induced by brief expression of the Yamanaka factors, this approach was further utilized by Ding and colleagues as well as others to generate cardiomyocytes, neural progenitor cells, endothelial cells, pancreatic β-cells, and hepatocytes from mouse and human fibroblasts without first establishing stable pluripotency (Zhu et al., 2015).

1.5 OCT4: modulator of cell fate in pluripotency and reprogramming

The Oct proteins belong to the POU (Pit-Oct-Unc) domain containing family of transcription factors (specifically class 2, 3 and 5) that have been characterized by their ability to bind the DNA-based octamer motif (ATGCAAAT) (Tantin, 2013). Oct proteins are expressed in numerous tissues including blood (Oct1, Oct2), brain (Oct2, Oct7, Oct8, Oct9), skin (Oct11), pancreas (Oct9), as well as embryonic and primordial germ cells (Oct4) (Tantin, 2013). Oct4 was discovered 25 years ago in three independent studies that demonstrated the binding of novel protein species within the extracts of ECCs, mESCs, primordial germ cells and unfertilized oocytes to DNA containing the octamer consensus sequence (Lenardo et al., 1989; Okamoto et al., 1990; Scholer et al., 1989). The human POU5F1 gene encoding OCT4 is regulated through a distal and proximal enhancer element, and a proximal promoter sequence (Jerabek et al., 2014). The coding sequence is made up of 4 exons, that, as a result of alternative splicing within exon 2, leads to 3 possible isoforms: OCT4A, OCT4B, and OCT4B1, where full length OCT4A is of greatest importance regarding development and cellular reprogramming (Jerabek et al., 2014). The full length OCT4A protein consists of 360 amino acids organized into 3

domains: N-terminal domain, POU domain, and C-terminal domain (Figure 3). The N- and C- terminal portions function as transactivation domains, whereas the POU domain is further subdivided into the POU specific domain (POU_S) and the POU homeodomain (POU_{HD}), which are connected via a flexible linker region forming a helix-turn-helix (POU_s-linker-POU_{HD}) DNA binding structure. The POU domain binds the octamer sequence in a bipartite fashion where, the POU_s and POU_{HD} bind ATGC and AAAT nucleotides respectively (Jerabek et al., 2014). The following section will provide a description of regulation of, and regulation by, OCT4 within the context of early mammalian development, pluripotent stem cells, the induction of pluripotency and direct conversion cellular reprogramming.

1.5.0 OCT4 in Pluripotent stem cells

OCT4 is a critical transcriptional regulator of the pluripotent state in both mouse and human ESCs (Solter, 2006). As a member of the pluripotency network, OCT4 binds to hundreds of target genes in hESCs, including chromatin remodelers, histone modifiers, and transcription factors related to both self-renewal and differentiation (Boyer et al., 2005). Functionally, suppression of Oct4 expression within mESCs results in differentiation towards trophoblast giant cells, whereas over-expression leads to endoderm and mesoderm differentiation (Niwa et al., 2000). Within somatic cells, expression of OCT4, in combination with additional pluripotency factors, is known to induce pluripotency through a process of reprogramming (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Since this initial discovery, many groups have made attempts to replace OCT4 with other ectopically expressed transcription factors,

epigenetic modifiers, and small molecule compounds (Radzisheuskaya and Silva, 2014). Despite these efforts, no one has found a single or combination of factor(s) that can functionally replace OCT4s role in the regulation of pluripotency, and as such, all of these methodologies are simply different means to activate OCT4 in order to achieve reprogramming towards the pluripotent state. As an inducer of pluripotency and in the context of the Yamanaka factors, Oct4 is thought to behave primarily as an activator of transcription (Hammachi et al., 2012). Oct4 fusion proteins containing well-characterized transcriptional activation domains not only maintained their ability to support pluripotency, but were also more efficient activators of pluripotency during reprogramming (Hammachi et al., 2012). Conversely, Oct4 fusion proteins containing repressor regions were not able to support the maintenance or induction of pluripotency (Hammachi et al., 2012). In the absence of the Yamanaka factors, Oct4 was shown to actively repress somatic-related gene expression across multiple different cell types, without inducing pluripotency-related expression (Tiemann et al., 2014). Surprisingly, comparison of the expression changes across each individual cell type expressing Oct4 revealed limited, to no, overlap in up- or down-regulated genes, suggesting Oct4 transcriptional regulation is cell context dependent (Tiemann et al., 2014). Taken together, these studies illustrate the complex nature of Oct4 transcriptional regulation, and suggest divergent activities in the context of other reprogramming factors versus a native somatic cell environment.

1.5.1 OCT4 in Direct Conversion Reprogramming

In 2010, our group had published the only report using OCT4 alone to facilitate direct conversion cellular reprogramming (Szabo et al., 2010). As of today, there are multiple examples that make use of OCT4 to facilitate reprogramming of fibroblasts and hematopoietic cells towards alternative cellular identities (Liao et al., 2015; Liu et al., 2015; Meng et al., 2013; Wang et al., 2014; Yamamoto et al., 2015; Zhu et al., 2014). Interestingly, groups that previously required the use of multiple lineage transcription factors or brief expression of all four Yamanaka factors to achieve direct conversion have republished their findings using OCT4 alone (Wang et al., 2014; Zhu et al., 2014). Moreover, recent studies have expanded on the role of OCT4 in facilitating hematopoietic direct conversion through the use of hair follicle cells as a starting material, and biasing the conversion process towards erythropoiesis using specially tailored culture conditions (Liu et al., 2015). Going in the other direction, hematopoietic derived CD34+ cells have been converted to mesenchymal stem cells (Meng et al., 2013), and CD45+ human umbilical cord blood cells have been converted to neural progenitor cells (Liao et al., 2015), in both cases through OCT4 mediated reprogramming approaches. These studies, including my own which appear in this thesis, confirm that OCT4 is a unique regulator of cellular reprogramming that cannot be attributed to one cellular starting material or resulting identity.

Figure 1. Generation of induced pluripotent stem cells (Takahashi et al.)

Fibroblasts are transduced with lentiviral particles containing the "Yamanaka Factors" (OCT4, SOX2, KLF4, and CMYC) and cultured in media known to support the growth of pluripotent stem cells (Reprogramming media). After approximately 3 weeks, colonies of compact cells begin to form among fibroblasts that do not undergo morphological changes. Colonies that successfully reprogram into iPSCs become functionally pluripotent and are able to self-renew and differentiate towards cells belonging to the three major germ layers: mesoderm (ex. blood cells), endoderm (ex. liver cells), and ectoderm (ex. Brain cells).

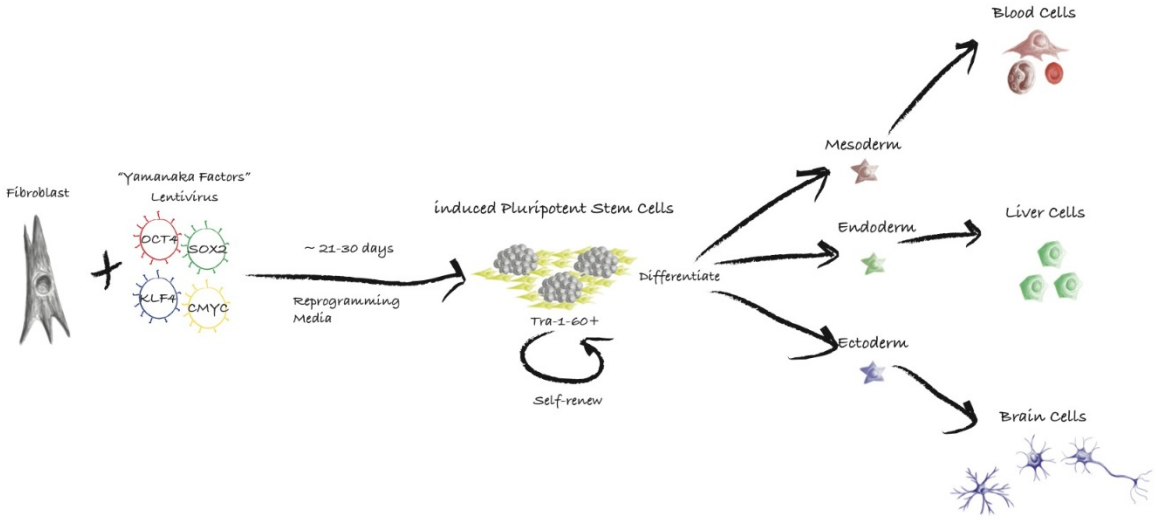

Fibroblast
"Yamanaka Factors"
Lentivirus
OCT4
SOX2
KLF4
CMYC
~ 21-30 days
Reprogramming
Media
Induced Pluripotent Stem Cells
Tra-1-60+
Self-renew
Differentiate
Mesoderm
Blood Cells
Endoderm
Liver Cells
Ectoderm
Brain Cells

Figure 2. Direct conversion of fibroblasts to blood progenitors (Szabo et al.)

Fibroblasts are transduced with lentiviral particles containing OCT4 and cultured in reprogramming media. After approximately 1 week, cells are treated with hematopoietic growth factors (SCF and FLT-3LG) resulting in the formation of colonies that express the blood specific marker CD45. CD45+ cells respond to further stimulation with hematopoietic cytokines and growth factors producing multi-potent hematopoietic progenitors capable of differentiation towards myeloid, erythroid, and megakaryocytic lineages.

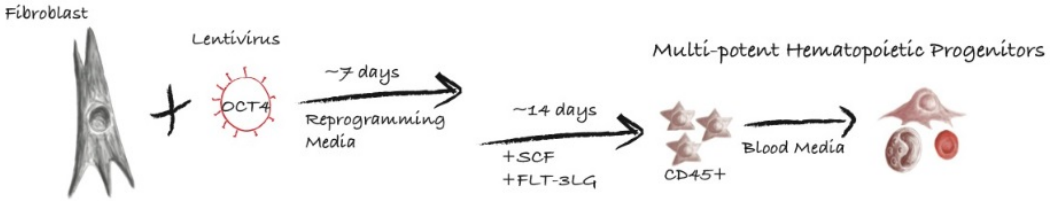
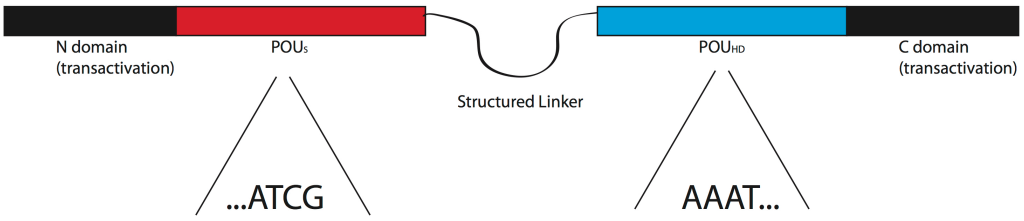

Fibroblast
Lentivirus
OCT4
~7 days
Reprogramming
Media
~14 days
+SCF
+FLT-3LG
Multi-potent Hematopoietic Progenitors
CD45+
Blood Media

Figure 3. Schematic of OCT4A Protein Domains

Schematic illustrates the 4 domains of human OCT4A protein, as well as the individual motif recognition for the octamer consensus sequence by the POU_S and POU_{HD} domains.


N domain
(transactivation)
POUS
Structured Linker
POUHD
C domain
(transactivation)
...ATCG
AAAT...

Summary of Intent

Development is considered unidirectional, as mature organisms do not readily recede back towards their primitive embryonic beginnings with the passing of time. In spite of this, the practice of experimentally testing the permanence of the differentiated state of cells has been an area of intense focus across multiple model systems for decades (Graf, 2011). Foundational studies demonstrating the ability of frog egg cytoplasm to reprogram the nuclei of differentiated endoderm suggested that DNA is not permanently altered during the process of differentiation, and that powerful developmental regulators existed within the egg (Gurdon and Uehlinger, 1966). Knowledge of such soluble regulators inspired numerous studies that aimed to alter the differentiated state through targeted approaches, such as exposure to forced expression of critical transcriptional regulators (Graf, 2011). Our lab entered the field of cellular reprogramming by demonstrating that one of the four Yamanaka factors, OCT4, when introduced alone into human fibroblasts, resulted in the appearance of cell types that were not consistent with iPSCs (Figure 1), but rather, CD45 expressing blood cells (Figure 2) (Szabo et al., 2010). Based on this work, we hypothesized that OCT4 was able to transcriptionally rewire human fibroblasts to express key hematopoietic regulator genes that likely supported the conversion towards blood without first establishing pluripotency (Szabo et al., 2010).

Soon after publishing this work, Kim et al. demonstrated that a short period of controlled expression of the four Yamanaka factors within mouse fibroblasts allowed for the generation of “unstable pluripotency” or “plasticity” that could then be guided to generate neuronal progenitor cells through the addition of supportive culture conditions

Figure 3) (Kim et al., 2011). Much like our work using OCT4, Kim et al. had also demonstrated that the fibroblast starting population did not traverse a stable pluripotent intermediate prior to ending at the destination cell type. Despite the clear differences in the transcription factor requirements, and the nature of the resulting cell types between these two studies, the reprogramming field opined that the two approaches shared the same mechanistic basis, whereby brief expression of pluripotency associated factors gave rise to an unstable state of pluripotency or plasticity (Chambers and Studer, 2011; Orkin and Hochedlinger, 2011). This state could theoretically be guided using suitable culture conditions that contained growth factors and cytokines tailored to the desired endpoint cell type. It was these postulations that prompted me to ask a series of key questions regarding the nature of OCT4 based cellular reprogramming.

1) *Does expression of OCT4 in human fibroblasts support cellular reprogramming towards cell types other than blood?*
2) *What is the role of OCT4 expression versus the extracellular environment during the process of converting fibroblasts towards alternative cellular identities?*
3) *Is OCT4 based cellular reprogramming a fibroblast restricted phenomenon?*

Understanding whether OCT4 based reprogramming can support the generation of cell types other than blood, and the extent to which the conversion process depends on OCT4 expression versus manipulation of the extracellular environment will aid in determining its potential utility as a cellular reprogramming technology and will provide insight into the underlying mechanism of the proposed plastic state. Moreover, understanding

whether OCT4 based reprogramming is restricted to fibroblast populations will help to realize whether historically banked tissues such as blood could serve as an access point to construct disease models for difficult to obtain tissues such as the brain.

Based upon this knowledge, **I hypothesize that ectopic expression of OCT4 in human somatic cells can induce changes in transcription that bestow the potential to make cell fate choices in response to external stimuli.**

If this idea is true, then expression of OCT4 should support the conversion of multiple somatic cell types towards unique endpoints through simple manipulation of the extracellular culture environment. To address my hypothesis, I defined the following specific objectives:

1) *Investigate whether expression of OCT4 can facilitate direct conversion of fibroblasts towards alternative progenitor cells, such as those of the neural lineage (Figure 4).*
2) *Assess the individual vs combined impact of OCT4 and the extracellular environment on transcription during direct conversion (Figure 5).*
3) *Investigate whether expression of OCT4 can facilitate direct conversion using alternative somatic cell populations, such as those of the hematopoietic lineage (Figure 6).*

Through implementation of targeted changes to the OCT4 reprogramming strategy used by our group to generate multi-potent hematopoietic progenitors, I was able to successfully generate tri-potent neural progenitors from adult human fibroblasts (Chapter 2). The generated neural progenitor cells displayed transcript and protein level markers

of neural phenotype, as well as proliferative and differentiation capacity similar to that of NPCs derived from pluripotent cells. Importantly, OCT4 derived NPCs did not transition from a pluripotent intermediate, nor were they tumorigenic when injected into immune deficient mice. Molecular analysis revealed that expression of OCT4 in combination with neural specific culture conditions activated regulators of neural development that had previously been shown to induce cellular reprogramming of fibroblasts towards the neural lineage. These results confirmed that OCT4 expression in human fibroblasts, in combination with lineage specific culture conditions, was capable of generating multiple progenitor cell types, suggesting that this reprogramming process was not limited to generation of blood.

Following this body of work, we then sought to characterize the mechanism by which OCT4 expression was able to bestow the ability to make cell fate choices, through global transcriptional analysis of the individual versus, combined, role of OCT4 and extracellular environment (Chapter 3). We first analyzed the initial stage of the direct conversion process, as it was shared among the protocols used to generate both hematopoietic and neural progenitors. Surprisingly, we found that OCT4 expression was not sustained in fibroblast populations unless the cells were cultured in reprogramming media (conditions shown to support self-renewal of PSCs (Bendall et al., 2007)). Moreover, global gene expression analysis revealed that the combination of OCT4 expression and reprogramming media induced transcriptional changes that were not equal to the sum of OCT4 and reprogramming media alone. Further analysis confirmed that the transcriptional state of cells in the early stages of direct conversion were distinct from

those induced by the combination of multiple pluripotency inducing factors, unlike what had been postulated by the cellular reprogramming community. In the end, we revealed that fibroblasts undergoing direct conversion facilitated by OCT4 are subjected to transcriptional changes that result in the activation of developmental regulator genes belonging to all three major germ layers. We have since termed this unique state, OCT4 induced plasticity (OiP). This state of OiP bestows the ability for fibroblasts to make cell fate choices in response to the addition of lineage specific culture conditions that results in conversion towards progenitor cell types.

As one of the major goals of cellular reprogramming is to enable the development of personalized disease models, and given that fibroblasts are not routinely sampled from patients in clinical settings, we evaluated whether OiP could be applied to readily accessible cell types from tissue banks, such as blood (Chapter 4). Through further implementation of targeted changes to the reprogramming strategy applied to fibroblasts in Chapter 2, we were successful in deriving NPCs from defined populations of hematopoietic progenitor cells from both human umbilical cord blood, and mobilized adult human peripheral blood. Interestingly, we found that OCT4-expressing blood cells did not respond to neural lineage extracellular conditions that facilitated fibroblast direct conversion towards NPCs. Instead, OCT4 expressing blood cells required the addition of small molecule inhibitors, shown to enhance neural specification of pluripotent cells, to achieve conversion towards NPCs. Moreover, global gene expression analysis revealed that this treatment regimen of small molecule inhibitors resulted in thousands of transcript level changes related to both neural (up-regulated) and blood (down-regulated) programs

during the conversion of blood to NPCs, whereas these same molecules had little to no effect on the transcriptome of fibroblasts. These results strongly suggest that fibroblasts and blood undertake distinct reprogramming pathways towards NPCs, which was further evidenced by identifying of pre-enrichment of neural crest related transcription in fibroblasts compared to that of blood.

Overall, this thesis has advanced the field of cellular reprogramming by providing novel insight into the role of OCT4 as a transcriptional regulator during direct conversion and its interplay with the extracellular environment. We have: expanded on the role of OCT4 as a facilitator of reprogramming towards blood cells by demonstrating the successful conversion of adult human fibroblasts to tri-potent neural progenitor cells (Chapter 2); created a working definition of OCT4 induced plasticity during direct conversion reprogramming (Chapter 3); and demonstrated that OCT4 based reprogramming is not restricted to the use of fibroblasts, evidenced by the conversion of hematopoietic progenitors towards neural progenitors (Chapter 4). Overall, these findings expand on the role of OCT4 as a regulator of reprogramming and cell fate.

Figure 4. Objective 1

Fibroblasts will be transduced with lentiviral particles containing OCT4 and cultured in reprogramming media. Instead of treating OCT4 expressing fibroblasts with hematopoietic growth factors, the cells will be treated with culture conditions known to support the generation and growth of neural progenitor cells. Putative neural progenitor cells will be functionally assayed through testing for tri-potent differentiation towards neurons, astrocytes, and oligodendrocytes.

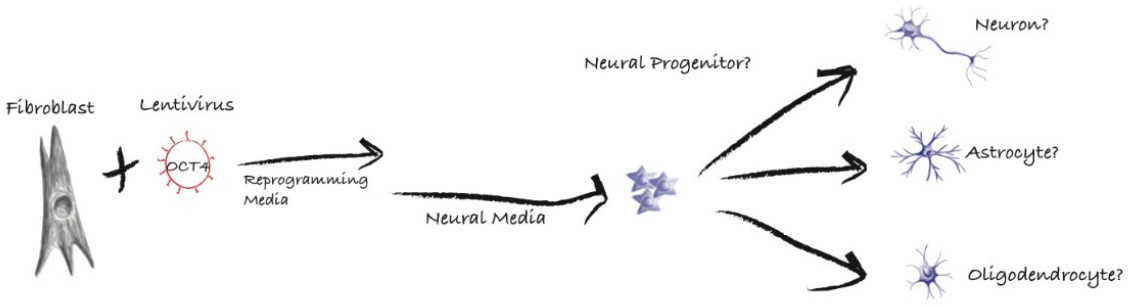

Fibroblast
Lentivirus
OCT4
Reprogramming Media
Neural Media
Neural Progenitor?
Neuron?
Astrocyte?
Oligodendrocyte?

Figure 5. Objective 2

The transcriptional impact of OCT4 expression vs reprogramming media will be evaluated by performing global expression profiling on fibroblasts transduced with OCT4, fibroblasts treated with reprogramming media, and fibroblasts transduced with OCT4 and treated with reprogramming media. The schematic illustrates potential outcomes of transcriptional regulation where OCT4 and reprogramming media regulate independent genes (red arrow for OCT4 and yellow arrow for RM), but in the presence of both OCT4 and RM, unique gene regulation takes place (Green arrow for OCT4+RM). Each of these experimental scenarios will be evaluated for direct conversion competency as well as additional transcriptional changes imposed by lineage-specific culture conditions (addition of blood or neural media).

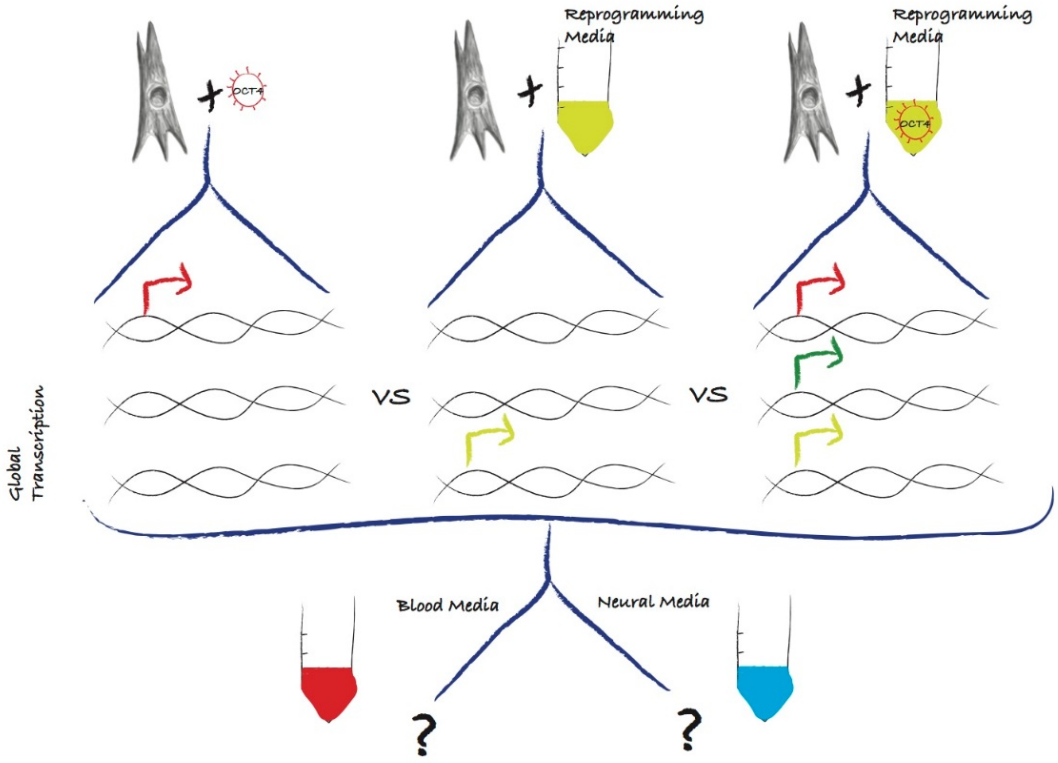
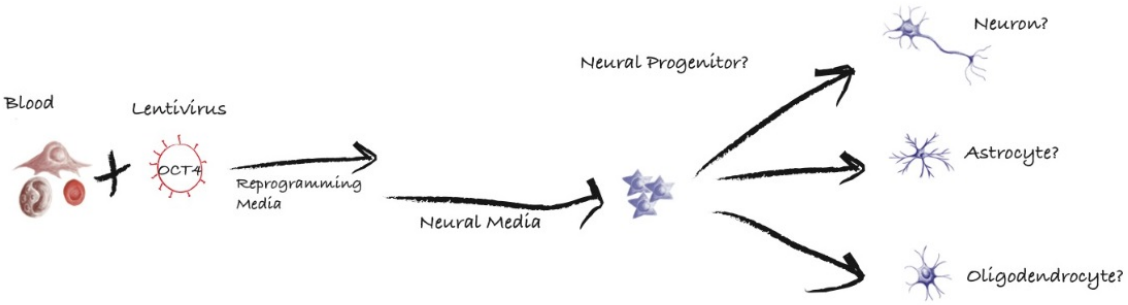

Reprogramming Media
Reprogramming Media
OCT4
OCT4
VS
VS
Global Transcription
Blood Media
Neural Media
?
?

Figure 6. Objective 3

Blood cells will be transduced with lentiviral particles containing OCT4 and cultured in reprogramming medium. Similar to objective 2 OCT4 expressing blood cells will then be cultured in neural supportive culture conditions and evaluated for generation of neural progenitor cells. Putative neural progenitors will be functionally evaluated for tri-potent differentiation.


Blood
Lentivirus
OCT4
Reprogramming Media
Neural Media
Neural Progenitor?
Neuron?
Astrocyte?
Oligodendrocyte?

Chapter 2

Activation of neural cell fate programs towards direct conversion of adult human fibroblasts to tri-potent neural progenitors using OCT-4

Preamble
This Chapter is an original published article. It is presented in its published format.

"This research was originally published in Stem Cells and Development. Mitchell Ryan R., Szabo Eva, Benoit Yannick D., Case Daniel T., Mechael Rami, Alamilla Javier, Lee Jong Hee, Fiebig-Comyn Aline, Gillespie Deda C., and Bhatia Mickie. Activation of neural cell fate programs towards direct conversion of adult human fibroblasts to tri-potent neural progenitors using OCT-4. Stem Cells and Development. August 15, 2014, 23(16): 1937-1946. Doi:10.1089/scd.2014.0023. Copyright© Mary Ann Liebert"

I designed the study and wrote the paper along with input from Dr. Eva Szabo and my supervisor Dr. Mick Bhatia. I performed all cell culture and reprogramming experiments in order to generate hFib-NPC^{OCT4}. I performed all flow cytometric and immunocytochemistry experiments. Dr. Eva Szabo, Dr. Yannick D Benoit and Rami Mechael performed qRT-PCR experiments. Daniel T Case and Dr. Javier Alamilla performed the electrophysiology. Dr. Jong Hee Lee performed immunocytochemistry on human embryonic stem cell derived neural precursor differentiated derivatives. Aline Fiebig-Comyn provided technical support with teratoma generation and analysis. Dr. Deda C Gillespie oversaw the electrophysiology. Dr. Mick Bhatia oversaw the entire study and assisted in manuscript preparation.

The central question behind this body of work was prompted by observations made during the reprogramming of human skin cells towards multipotent hematopoietic progenitors through the use of exogenous OCT4 expression (Szabo et al., 2010). In the same manner that $CD45^{+}Fibs^{OCT4}$ were discovered in cultures of fibroblasts undergoing iPSC reprogramming, multiple unidentified colonies arise during direct conversion of fibroblasts to $CD45^{+}Fibs^{OCT4}$. Morphological assessment by eye suggested that a portion of the unidentified colonies could be neural in nature due to their resemblance to hESC derived neural progenitor cells. After making these observations, I questioned whether modification of our OCT4 reprogramming procedure could result in the generation of functional neural progenitors. In support of this idea, Kim et al. had recently reported that brief expression of the Yamanaka factors combined with neural lineage supportive culture conditions was sufficient to induce neural progenitors from mouse fibroblasts. As such, I expressed OCT4 in human fibroblasts and treated them with conditions shown to support neural development from hESCs, which resulted in the formation of tri-potent neural progenitor cells.

Activation of neural cell fate programs towards direct conversion of adult human fibroblasts to tri-potent neural progenitors using Oct-4

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Running Title: OCT4 induced neural fate conversion

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R.R.M designed and performed experiments and wrote the paper. E.S. designed and performed experiments and wrote the paper. Y.D.B. performed experiments. D.T.C performed patch clamp experiments. R.M. performed experiments. J.A. performed patch clamp experiments. J.H.L. helped design critical elements of the study. A.F-C. performed teratoma assays. D.C.G designed electrophysiology experiments and wrote the paper. M.B. designed experiments and wrote the paper.

Abstract

Several transcription factors and methods have been used to convert fibroblasts directly to neural fate and have provided insights into molecular mechanisms into how each of these required factors orchestrate neural fate conversion. Here we provide evidence and detailed characterization of the direct conversion process of primary adult human fibroblasts (hFib) to neural progenitor cells (NPC) using OCT4 alone. Factors previously associated with neural cell fate conversion were induced during hFib-NPC^{OCT4} generation, where OCT4 alone was sufficient to induce neural fate conversion without the use of promiscuous small molecule manipulation. Human Fib-NPC^{OCT4} proliferate, express neural stem/progenitor markers, and harbour developmental potential that gives rise to all three major subtypes of neural cells: astrocytes, oligodendrocytes, and neurons with functional capacity. We propose a de-convoluted reprogramming approach for neural fate conversion where OCT4 is sufficient for inducing neural conversion from human fibroblasts for disease modeling as well as the fundamental study of early neural fate induction.

Introduction

Direct conversion transcription factor reprogramming of somatic cells holds great potential for the generation of patient specific disease models, and possibly cells for transplantation therapy. Although first reported in the 1980s [1], this reprogramming method has been the basis for a plethora of studies, all of which achieve alteration of cell fate in the absence of establishing pluripotency [2-4]. The catalyzing study that lead to the revisiting of direct conversion/transdifferentiation methods, demonstrated that expression of neural lineage associated transcription factors in mouse fibroblasts lead to the activation of endogenous neural genes[5]. Through a process of elimination, Brn-2, Myt1-l, and Ascl-1 were identified as a key set of factors that could initiate a change in cell fate from fibroblast to functional neuron (iN), in the presence of neural supportive culture conditions [5]. Soon after, with the addition of NEUROD1, this same combination of genes was found to induce direct conversion of human fibroblasts to neurons. Generation of neural tissue for study or transplantation is of great value, as the physical and ethical barriers regarding biopsies of the brain are numerous. To date, the iN approach has been harnessed to produce multiple neuron subtypes by introduction of additional lineage specific transcription factors, from both mouse and human starting cell populations of varied tissue origin [6-10]. In addition to directly converting somatic cells to post mitotic neurons, Kim et al. demonstrated the conversion of mouse fibroblasts to neuronal progenitor cells. This transformation was achieved through brief expression of transcription factors followed by addition of neural supportive culture conditions [11]. Many groups have adapted this strategy by expressing the pluripotency factors alone, or in combination with neural lineage specifying factors to produce both mouse and human

induced neural progenitor cells (iNPCs) [12-15]. iNPCs are capable of proliferating and differentiating to all three families of neural cells including, neurons, astrocytes, and oligodendrocytes. Despite the large number of studies demonstrating direct conversion of somatic cells to neural progenitor cells, the reprogramming mechanism behind these cellular transformations remains to be completely understood. Here we demonstrate direct conversion of adult human fibroblasts to neural progenitor cells through the use of a single factor OCT4, and provide insights into the minimal essential mechanisms of reprogramming induction.

Materials and Methods

Cell Culture

Dermal skin biopsies (5mm x 5mm) were obtained from the forearm of consenting donors in accordance with Research Ethics Board-approved protocols at McMaster University. Primary human fibroblast cultures were established as described [16]. Human dermal adult fibroblasts (Sciencell and Donor derived) were initially maintained in fibroblast medium: DMEM (Gibco) supplemented with 10% v/v FBS (Neonatal Bovine Serum, HyClone), 1 mM l-glutamine (Gibco), and 1% v/v non-essential amino acids (NEAA; Gibco) before transduction with OCT4 lentivirus vector.

Cell Viability

Viable cells were determined by Trypan Blue exclusion assay performed using Countess automated cell counter (Life Technologies).

Lentivirus Preparation and Transduction

pSIN-EF1α-OCT4-Puro (or pHIV-EF1α-IRES-eGFP (Addgene 21373) sub cloned with OCT4 sequence from Addgene 16579) was obtained (Addgene 16579) and co-transfected with pMD2.G (Addgene 12259) and psPAX2 (Addgene 12260) plasmids into the 293-FT cell line (Invitrogen) in order to initiate virus particle production. Viral supernatants were harvested 48hr after transfection and ultracentrifuged to concentrate the virus. Human fibroblasts were transduced in 50%Fibroblast medium 50% reprogramming medium (F12 DMEM 20% Knockout Serum Replacement (Gibco), l-glutamine (Gibco), and 1% v/v non-essential amino acids (NEAA; Gibco), 0.1mM β-mercaptoethanol, 16ng/mL basic

fibroblast growth factor (bFGF), 30ng/mL insulin growth factor 2 (IGF2), supplemented with 8µg/mL polybrene (SIGMA).

Reprogramming, Neural Progenitor and Differentiation Culture

Adult human fibroblasts were seeded on tissue culture treated plates at approximately $2x10^4$ cells per well of a 6 well standard plate. Fibroblasts were transduced in 50% fibroblast media: 50% reprogramming media containing 8µg/ml polybrene with sufficient eGFP or OCT4 expressing lentivirus to achieve approximately 20% transduction efficiency as measured by flow cytometry for eGFP or OCT4 respectively. Human fibroblasts expressing eGFP and/or OCT4 were cultured for 8 days post transduction in reprogramming media before being trypsinized and seeded as single cells in ultra-low attachment plates at a concentration of $1x10^5$ cells per mL of progenitor culture media: F12:DMEM supplemented with 1X N2 and 1X B27 20ng/mL bFGF, 20ng/mL epidermal growth factor (EGF). Spheres were fed fresh media and growth factors after 7 days of culture. After 14 days sphere clusters were dissociated using Accutase (Gibco) and seeded on to POLY-L-ORNITHINE/Mouse laminin coated tissue culture plates in neural progenitor media for continued maintenance or in differentiation culture media consisting of F12 DMEM 1XN2 1XB27. For best differentiation, cells were cultured adherently in progenitor media until dense colony clusters appeared such as those depicted in figure 1K prior to passaging into differentiation conditions. For neuronal differentiation, media was supplemented with 5nM forskolin. For Astrocyte differentiation, media was supplemented with 5% FBS. For Oligodendrocyte differentiation, media was supplemented with 100ng/mL insulin growth factor 1 (IGF1),

200nm Ascorbic Acid (Sigma), 5nM forskolin (Tocris). NSC from H9 were derived and cultured as previously described [17].

Flow Cytometry

Intracellular staining for OCT4 (BD OCT3/4-PE [1:100], OCT3/4-647[1:100]), SOX2 (BD SOX2-647[1:1000]), Ki67 (BD Ki67-PE[1:400]), and Live Dead Discrimination Dye (BD Live Dead Violet[1:7500]) was performed on cells having undergone fixation and permeabilization using the BD Fix/Perm kit. Briefly, cells were washed in PBS and staining for 30 min at 4°C in Live Dead Violet. Cells were then washed in PBS and fixed for 20min at 4°C in Fix solution. Cells were then washed in Perm solution, and left to block in Perm solution for 1hr at 4°C. Cells were stained overnight at 4°C, prior to washing in PBS and acquisition. Live cell visualization of eGFP expression was performed on cells treated with 7AAD (BD [1:50]) live dead discriminator. Acquisition was performed using LSRII (BD Biosciences), and analysis was performed using FlowJo 9.2 Software.

Immunocytochemistry

For surface marker staining cells were stained directly in PBS 3%FBS or fixed using BD Fixation buffer for 40min 4°C. Cells were washed in 3%FBS HBSS (Gibco). For intracellular staining cells were fixed using BD Fixation/Permeabilization buffer for 40min at 4°C. Cells were washed in BD 1X Perm solution. Neural lineage cells were identified by staining with monoclonal antibodies OCT4-PE (BD), SOX2-647 (BD), SOX2 anti-human (BD), NESTIN anti-Human (R&D), β-III Tubulin /TUJ1 anti-Human

(R&D), Microtubule associated protein 2/MAP-2 anti-Human (Abcam), Oligodendrocyte marker 4/O4 anti-Human (R&D), Glial fibrillary acidic protein/GFAP anti-Human (SIGMA), and CD133-PE (Miltenyi). Antibodies were diluted in BD 1X wash buffer and incubated overnight at 4°C. Non-conjugated antibodies were visualized using appropriate Alexa-Flour secondary reagents (Life Technologies). Optimal working dilutions were determined for individual antibodies.

Teratoma Assay

5 x 10^5 iPSCs or hFib-NPC^{OCT4} were injected intratesticularly into male NOD/SCID mice. Teratomas/testis were extracted 10-12 weeks after injection and analyzed as previously described [17]. Samples were stained with Haematoxylin-Eosin and OCT4, mounted using Permount and imaged by scanning slides using Aperio Scan Scope. Tissue typing was performed based on stringent histological and morphological criteria specific for each germ layer subtype. The presence of the germ layers and tissue typing was confirmed by McMaster Pathology department.

Quantitative Polymerase Chain Reaction

Total RNA was isolated using the Qiagen mini RNA isolation kit. RNA was then subjected to cDNA synthesis using superscript III (Invitrogen). Quantitative PCR (qPCR) was performed using Platinum SYBR Green–UDP mix (Invitrogen). Threshold was set to the detection of Gus-B (β-glucuronidase)46 and then normalized to GAPDH. Primer Sequences: ASCL1: 5’ caagagagcgcagccttag, 5’ gcaaaagtcagtgctgaacg BRN2: 5’ aataaggcaaaaggaaagcaact, 5’ caaaacacatcattacacctgct MYT1L: 5’

caatggaaagggattttaagca, 5' tttgagattatgtaccaacgttagatg NEUROD1: 5' gttattgtgttgccttagcacttc, 5' agtgaaatgaattgctcaaattgt MSI2: 5' ggcagcaagaggatcagg. 5'ccgtagagatcggcgaca NCAN: 5' gccttgggccttttgatgc, 5' ccttggtccactttatccgagg COL1A1: 5' gagggccaagacgaagacatc, 5' cagatcacgtcatcgcacaac DKK3: 5' aggacacgcagcacaaattg, 5' ccagtctggttgttggttatctt SNAI1: 5' tcggaagcctaactacagcga, 5' agatgagcattggcagcgag

Cytosolic Calcium Imaging

Measurement of cytosolic calcium was performed by monitoring Fluo-4 fluorescence of cells, adhered to plastic 35mm dishes or 6well plastic plates using an Olympus IX81 inverted epi-fluorescence microscope (Olympus, Markham, ON) coupled to a xenon arc lamp (EXFO, Quebec, QC). Cells were washed and incubated in Hanks' balanced salt solution HBSS, supplemented with 25 mM HEPES buffer, 5.5 mM Glucose. Indicated agonists, diluted in the aforementioned solution, were washed over the cells at the indicated time points, using a custom applicator and aspirator system. Fluo-4 was loaded into the cells by incubation with 1 μM Fluo-4 (Invitrogen, Carlsbad, CA) acetoxymethyl ester (45 min incubation followed by a 45 min period for de-esterification). Fluorescent images were collected using an intensified charge-coupled device video camera (Photometrics, Tucson, AR) every 2s through a GFP filter cube (Semrock, Rochester, NY). Off-line analysis of the intensity pattern of Fluo-4 signal, was performed in ImageJ (NIH, Bethesda, MD).

Action Potential/Patch Clamping

To determine whether cells could fire action potentials, the cells were transferred on a coverslip to an upright microscope and were continuously perfused with artificial cerebrospinal fluid (ACSF, pH 7.2) containing (in mM): 125 NaCl, 1 MgSO4, 5 KCl, 1.25 KH2PO4, 10 dextrose, 26 NaHCO3, 2 CaCl2•2H2O. ACSF was heated to ~33°C and superfused with 95% O2/5% CO2. Electrodes for cell-attached and whole-cell voltage clamp recordings had resistances of 2-4 MΩ and were filled with a K-gluconate solution (pH 7.3) containing (in mM): 100 K-gluconate, 20 KCl, 10 Na2-phosphocreatine, 10 HEPES, 0.3 GTP-Na, 4 ATP-Mg•3.5H20. Recordings (MultiClamp 700B or Axopatch 200B amplifier; Molecular Devices) were sampled at 5 or 10 kHz, filtered at 2 or 5 kHz, and saved for offline analysis with Origin or custom Matlab software. Cell-attached and whole-cell recording configuration were achieved in voltage-clamp mode, and recordings were subsequently made in current-clamp mode. Whole-cell recordings were compensated by a minimum of 80% with <10 μs lag, and were discarded if series resistance changed by more than 15% from its initial value. In the attempt to initiate action potentials, current injections were made in 6 steps (1nA to 6nA) via the patch electrode. To ensure that voltage-sensitive sodium channels were not in a chronic inactivated state, the recording configuration was periodically turned back to voltage-clamp mode and the cell was held at a potential of -60mV or less in order to de-inactivate sodium channels. If reliable action potential-like firing was observed in a cell, the broad-spectrum antagonist zonisamide (10 μM; Ascent) or the fast acting sodium channel tetrodotoxin (1uM) was added to the perfusate.

Results

OCT4 direct conversion reprogramming facilitates generation of a proliferative neural progenitor phenotype from fibroblasts

In an effort to assess the functional conversion capacity of OCT4 expressing cells, we devised a culture strategy incorporating elements from our previous direct conversion approach using OCT4, and the derivation of neural progenitors (NPCs) from pluripotent stem cells (Figure 1A) [18,19]. Adult human fibroblasts (hFib) transduced with lentivirus expressing OCT4 were cultured in reprogramming media ($hFibR^{OCT4}$) for one week to allow for sufficient expansion of transduced cells (Figure 1B). $hFibR^{OCT4}$ were then trypsinized to form a single cell suspension and seeded in neural progenitor culture media. Single cell suspensions of $hFibR^{OCT4}$ maintained in neural progenitor culture formed neural sphere like clusters similar to those described from pluripotent cells (Figure 1c) [19]. To address whether these spheres were a result of proliferation rather than coalescence of cells we dissociated the spheres and performed cell cycle analysis using Ki67, a marker of all stages of the active cell cycle. Ki67 expression was evident in a defined subset of sphere-derived cells, of which nearly all cells co-expressed OCT4 (Figure 1D). As proliferation is a property of NPCs (Supplementary Figure 1A), we asked whether $hFibR^{OCT4}$ sphere derived cells also expressed key neural progenitor regulators. Given that recent reports suggest SOX2, a key regulator of NPCs (Supplementary Figure 1A), is sufficient to induce direct conversion of mouse and human fetal fibroblasts to neural progenitors [20], we analyzed adult derived $hFibR^{OCT4}$ cells for SOX2 expression. A small but distinct population of cells expressed SOX2, of which nearly all co-expressed OCT4, indicating that OCT4 expression in conjunction with

neural progenitor culture can induce hallmarks of a neural progenitor phenotype (Figure 1E). Further analysis of hFibR^{OCT4} for the expression of additional neural progenitor markers revealed a subset of OCT4 positive cells that co-expressed the cell surface marker CD133 (Figure 1F). In addition to these protein level analyses, hFibR^{OCT4} also displayed up-regulation of MSI2 and NCAN transcript compared to hFibs (Supplementary Figure 1C). Importantly, hFibR^{OCT4} down-regulated key fibroblast genes COL1A1, DKK3, and SNAI1 in response to the reprogramming process (Supplementary Figure 1D). Taken together these results demonstrate that hFibR^{OCT4} sphere derived cells have initiated a molecular shift from fibroblastic programs towards that which reflect well characterized primary human neural progenitor cells [21].

To further characterize these emerging neural like cells from hFibR^{OCT4} cells, we cultured them on the neural substrate poly-ornithine/laminin (POL). hFibR^{OCT4} sphere-derived cells cultured on POL displayed a similar morphology to NPCs derived from pluripotent cells (Figure 1F and Supplementary Figure 1B) [22]. In agreement with flow cytometric analysis, Immunofluorescence for OCT4 and SOX2 revealed co-expressing populations of cells organized in colony like regions consistent with an emerging progenitor phenotype (Figure 1G). Continued passaging on laminin resulted in linear growth on an exponential scale, indicating that the proliferating population of cells had become abundant (Figure 1H). Consistent with this observation as well as expansion of the neural progenitor phenotype, nearly all of the cells in culture expressed the proliferation marker Ki67 in combination with SOX2 (Figure 1I). Moreover, the vast majority of cells co-expressed OCT4 and SOX2, a clear indication that passaging of

hFibR^{OCT4} on laminin had enriched for this phenotype (Figure 1J). Despite being highly proliferative and expressing the pluripotency associated transcription factors OCT4 and SOX2, hFibR^{OCT4} cells failed to produce teratomas (or tumors of any kind) when injected into immune deficient mice at cell concentrations well above the tumor initiating requirement of iPSC (Supplementary Figure 2A and 2B). Having verified the absence of pluripotency, we assessed whether the expansion of this phenotype occurred alongside up-regulation of additional neural stem/progenitor marks. Accordingly, we performed immunofluorescence for SOX2 in combination with the neural filament NESTIN, a marker of primitive neural stem/progenitor cells. This analysis revealed co-expression of both SOX2 and NESTIN within hFibR^{OCT4} cells (Figure 1K). Cumulatively these results demonstrate that culturing of hFibR^{OCT4} cells in neural progenitor culture is sufficient to confer both growth and phenotypic properties of neural stem/progenitor cells. As such, we termed these cells hFib-NPC^{OCT4}.

To determine whether OCT4 expression was required to induce direct conversion of adult human fibroblasts to NPCs, we cultured eGFP expressing fibroblasts using the strategy highlighted in Figure 1A. Similar to hFibR^{OCT4}, hFibR^{eGFP} were propagated in reprogramming media to expand eGFP expressing cells (Supplementary Figure 3A). hFibR^{eGFP} were then seeded as single cells in progenitor culture media and analyzed for sphere formation. Surprisingly, hFibR^{eGFP} in these conditions formed sphere like clusters similar in morphology to hFibR^{OCT4} (Supplementary Figure 3B). However, cell cycle analysis of the resulting spheres revealed a comparatively small number of Ki67 positive cells to that of hFibR^{OCT4} spheres (Supplementary Figure 3C). In addition to the lack of

proliferation, hFibR^{eGFP} sphere derived cells failed to up-regulate the neural stem/progenitor transcription factor SOX2 (Supplementary Figure 3D), and displayed fibroblastic morphology after culturing on laminin (Supplementary Figure 3E). Together, these results indicate that culturing of adult human fibroblasts in neural progenitor media is insufficient to sustain growth and phenotypic characteristics of induced human neural progenitors.

OCT4-NPCs differentiate to all three neural subfamilies including functional neurons

Although neural progenitor phenotype is defined by the expression of specific transcription factors and structural proteins, progenitor function is assessed in part by the ability to differentiate to the three major families of neural cells: neurons, astrocytes, and oligodendrocytes. Differentiation of neural stem/progenitors can be achieved by withdrawal of factors shown to support proliferation and addition of those that support the generation of specific lineages of neural cells (Supplementary Figure 4) [22]. Accordingly, culturing of hFib-NPC^{OCT4} in differentiation conditions shown to support the generation of glial cells produced glial fibrillary acidic protein expressing cells resembling astrocytes (Figure 2A) and O4 expressing cells resembling oligodendrocytes (Figure 2B). Moreover, culturing of hFib-NPC^{OCT4} in neuronal differentiation media was able to induce the generation of a neuronal phenotype, marked by expression of the neuron specific class III β-tubulin protein or TUJ1 (Figure 2C). Further exposure of hFib-NPC^{OCT4} derived neurons to differentiation media resulted in the formation of a subset of TUJ1 positive neurons that upregulated the mature neuronal marker MAP2 (Figure 2D). Importantly, cells that expressed high levels of TUJ1 also demonstrated

little to no expression of OCT4, suggesting that differentiation towards mature functional cell types occurs concomitantly with silencing of OCT4 expression (Supplementary Figure 5A and 5B).

To assess the functional capacity of hFib-NPC^{OCT4} derived mature neurons, we evaluated their response to the neurotransmitters ATP and glutamate via calcium transient formation using a FLUO-4 fluorescence assay. Nearly all hFib-NPC^{OCT4} derived neurons produced calcium transients in response to ATP, while a distinct subset responded to glutamate. Importantly, hFib-NPC^{OCT4} neurons did not respond to histamine, a known inducer of calcium release in fibroblasts (Figure 3A and 3B). Furthermore, hFib-NPC^{OCT4} derived neurons fired repetitive action potentials in response to current injection, and exhibited sensitivity to sodium channel inhibitor (Figure 3C-3E). These collective results suggest that hFib-NPC^{OCT4} possess all of the defining features of tri-potent neural progenitor cells, confirming successful cell fate transition through expression of OCT4.

OCT4 expression activates a hierarchy of neural conversion transcription factors during reprogramming of adult human fibroblasts to NPCs

Having confirmed that hFib-NPC^{OCT4} possess both phenotypic and developmental characteristics of neural progenitor cells, we next aimed to better understand the molecular events underlying the reprogramming process. Given that hFib-NPC^{OCT4} express SOX2, a neural fate conversion factor, we investigated whether other genes shown to induce neural fate were similarly activated during the reprogramming process. We performed qRT-PCR on Fibs, hFibR^{eGFP} and hFibR^{OCT4} sphere derived cells to

measure for the expression of genes shown to induce the neural fate from fibroblasts: BRN-2, ASCL1, MYT1L, and NEUROD1 (Figure 4A-D). With the exception of ASCL1, hFibR^{OCT4} progenitor culture demonstrated elevated expression of neural conversion genes compared to naïve fibroblasts, and hFibR^{eGFP} progenitor culture. Based on these results and the induced expression of SOX2, we hypothesized that the combination of OCT4 and neural progenitor culture may regulate a hierarchy of established transcriptional networks for converting fibroblasts to the neural lineage. Considering recent reports, we postulated that SOX2 should act downstream of OCT4, and therefore should be able to convert adult human fibroblasts to neural progenitors in the absence of OCT4. To test this hypothesis we transduced adult human fibroblasts with lentivirus expressing SOX2 and cultured them according to the strategy in Figure 1A. hFibR^{SOX2} were cultured in reprogramming media to allow for expansion of SOX2 expressing cells (Figure 5A). hFibR^{SOX2} cells were seeded as single cells in neural progenitor culture media and analyzed for sphere formation. After two weeks in neural progenitor culture hFibR^{SOX2} single cells had given rise to neural sphere like clusters similar to hFibR^{OCT4} (Figure 5B). Although forced expression of OCT4 was able to up-regulate expression of SOX2 (Figure 1E), forced expression of SOX2 did not up-regulate OCT4 (Figure 5C). Similar to hFibR^{eGFP}, hFibR^{SOX2} sphere like clusters contained low percentages of Ki67 positive proliferating cells (Figure 5D). Moreover, propagation of hFibR^{SOX2} sphere derived cells on the neural substrate laminin resulted in the generation of cells with fibroblastic morphologies (Figure 5E), and a sharp decline in cell viability (Figure 5F). Despite the failure of hFibR^{SOX2} to propagate in neural supportive culture

conditions, qRT-PCR analysis displayed a similar pattern to hFibR^{OCT4} regarding up-regulation of BRN2, MYT1L, and NEUROD1 (Figure 5G-J). These results suggest that SOX2 expression is activated in response to OCT4 expression, and that SOX2 expression is sufficient to activate neural fate conversion factors BRN-2, MYT1L, ASCL1 and NEUROD1. However, forced expression of SOX2 alone is not sufficient to induce neural progenitor fate from adult human fibroblasts suggesting a unique additional role for OCT4 during the reprogramming process.

Reprogramming technologies are seen to hold great potential for the construction of patient specific disease models and cells for drug screening. However, in order to ensure applicability of a given reprogramming technology to patient specific endeavors, freshly derived cell cultures from donors must prove to be a suitable starting material for successful cell fate transition. To ensure that hFib-NPC^{OCT4} reprogramming was applicable to donor derived fibroblasts, we generated hFib-NPC^{OCT4} cells from a freshly isolated skin biopsy (hFib(2)). hFibR(2)^{OCT4} formed spheres in neural progenitor culture media, and upon dissociation were able to propagate on the neural substrate laminin (Supplementary Figure 6A). Laminin adapted hFibR(2) sphere derived cells displayed similar morphology to hFibR^{OCT4} sphere derived cells and NPCs derived from ESCs (Figure 1G and Supplementary Figure 1B). Importantly, hFibR(2)^{OCT4} sphere derived cells activated established neural conversion factors in an OCT4 dependent manner, suggesting that they had established similar neural conversion regulatory networks as hFib-NPC^{OCT4} (Supplementary Figure 6B). Furthermore, when cultured in neural differentiation conditions, hFibR(2)^{OCT4} cells successfully differentiated to all three major

subfamilies of neural cells including astrocytes, oligodendrocytes and neurons (Supplementary Figure 6C). Taken together these results confirm that hFib-NPC^{OCT4} reprogramming is applicable to multiple lines of adult fibroblasts, illustrating the robust nature of OCT4 to alter cell fate.

Discussion

Derivation of hFib-NPC^{OCT4} is simple, rapid, and produces a proliferative, non-tumorigenic population of progenitor cells capable of differentiating to all three major neural subtypes: neurons, astrocytes, and oligodendrocytes. Importantly, hFib-NPC^{OCT4} derived neurons functionally respond to both current injection and neurotransmitters, positioning hFib-NPC^{OCT4} as an appealing reprogramming tool for the generation of neuronal based disease models in a manner that is not convoluted by the use of other factors associated with stages of neural development [23] or promiscuous chemical manipulation *in vitro* [24]. This reprogramming technology is sufficiently robust to allow for the use of donor derived adult fibroblasts as a starting material for cell fate conversion, positioning it as a candidate for the construction of relevant patient specific disease models for use in drug screening.

Our study represents the only example where a single exogenous factor, OCT4, has directly reprogrammed adult human somatic cells to tri-potent neural progenitors without other manipulations. Although co-expression of SOX2 hallmarks the emergence of hFib-NPC^{OCT4}, expression of SOX2 alone in adult human fibroblasts using our culture system, does not result in successful reprogramming to neural progenitors. However, forced expression of SOX2 in the presence of a feeder support layer, has been shown to induce direct conversion of human fetal fibroblasts to tri-potent neural progenitor cells[20]. Therefore, it is likely that the required (yet undefined) regulation bestowed by the feeder layer on cells undergoing SOX2 neural cell fate conversion is intrinsic to cells undergoing OCT4 induced neural cell fate conversion. The up-regulation of key neural

conversion factors during reprogramming of fibroblasts to hFib-NPC^{OCT4} in this study is not unlike the up-regulation of key hematopoietic transcription factors reported during the conversion of fibroblasts to multipotent blood progenitors using OCT-4 alone[18]. Together these findings highlight the acquisition of crucial fate inducing transcriptional programs as a result of forced OCT4 expression in human fibroblasts that may also allow other cell fates to be induced. These and other lineage-based studies are currently on-going in our lab.

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Disclosure

We declare no conflicts

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Figure 1. Expression of OCT4 in combination with neural progenitor culture induces conversion of fibroblasts to a proliferating neural progenitor like cell (hFib-NPC^{OCT4}).

A, Schematic of conversion strategy. B, Flow cytometric plot of intracellular OCT4 expression in adult human fibroblasts after 8 days of culture in reprogramming media culture (hFibR^{OCT4}). C, Phase contrast image of hFibROCT4 neural sphere like clusters after 14 days in neural progenitor culture. D, Flow cytometric plot of OCT4 vs Ki67 expression in hFibR^{OCT4} sphere derived cells. E, Flow cytometric plot of OCT-4 vs SOX2 expression in hFibR^{OCT4} sphere derived cells. F, Flow cytometric plot of eGFP expression from hFibR^{OCT4} cells generated with pHIV-ef1α-OCT4-IRES-eGFP vs CD133 on live cells. G, Phase contrast image of hFibR^{OCT4} sphere derived cells cultured on poly-ornithine/laminin (POL). H, Immunofluorescence results for OCT4 and SOX2 expression in hFibR^{OCT4} sphere derived cells culture on POL. I, Cell growth curve illustrating cumulative number of viable trypan blue excluding cells with increasing passage number. J, Flow cytometric plot of SOX2 vs Ki67 expression in passage 5 POL cultured hFibR^{OCT4} sphere derived cells. K, Flow cytometric plot of OCT4 vs SOX2 expression in passage 5 POL cultured hFibR^{OCT4} sphere derived cells. L, Immunofluorescence results for NESTIN and SOX2 expression in passage 5 POL cultured hFibR^{OCT4} sphere derived cells. Representative results from n=4 rounds of hFib-NPC^{OCT4} conversion. Scale bar = 100μM. White arrows denote areas of interest.

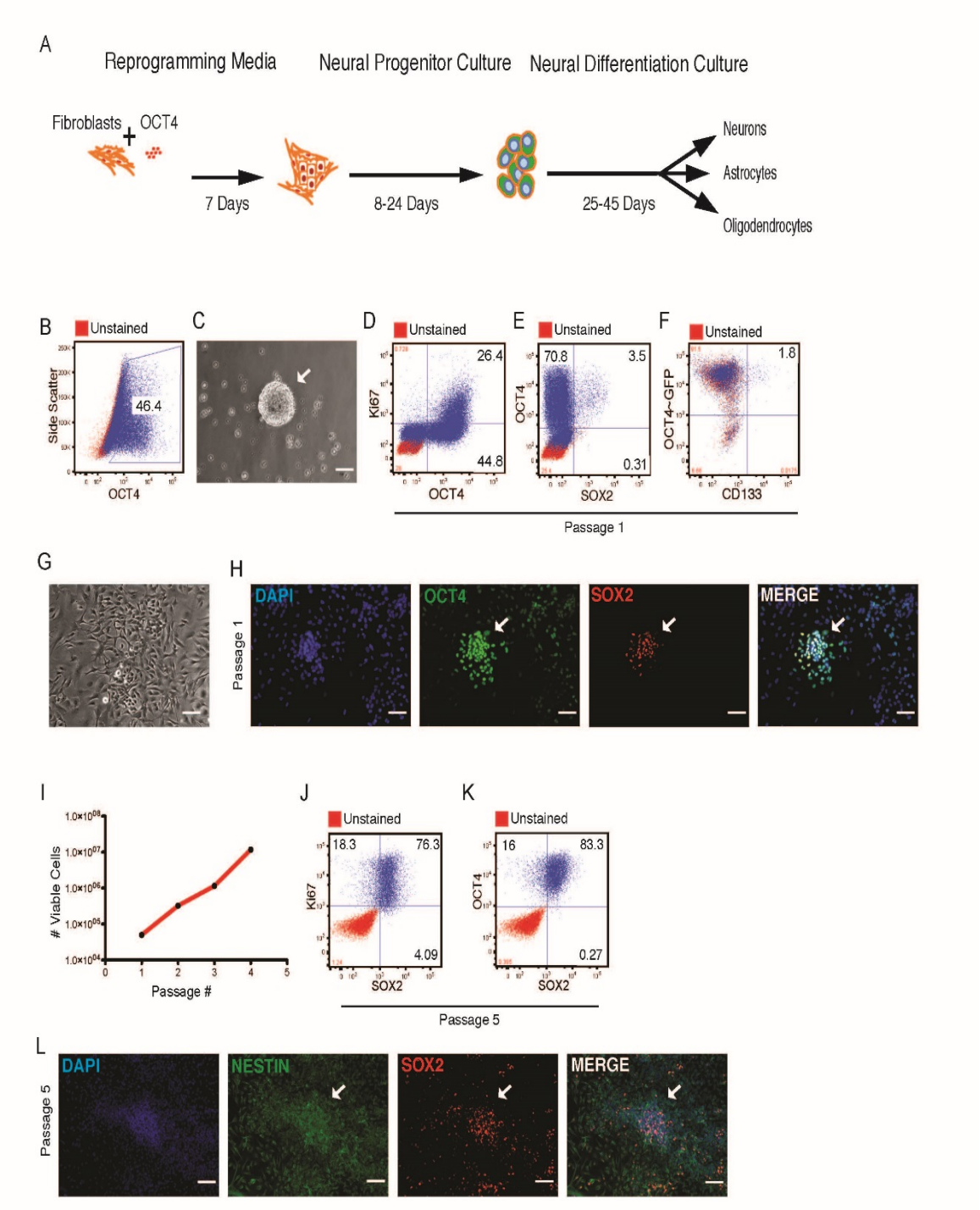

A
Reprogramming Media
Neural Progenitor Culture
Neural Differentiation Culture
Fibroblasts
OCT4
7 Days
8-24 Days
25-45 Days
Neurons
Astrocytes
Oligodendrocytes
B
Unstained
Side Scatter
46.4
OCT4
C
D
Unstained
Ki67
26.4
44.8
OCT4
E
Unstained
OCT4
70.8
3.5
0.31
SOX2
F
Unstained
OCT4-GFP
1.8
CD133
Passage 1
G
H
Passage 1
DAPI
OCT4
SOX2
MERGE
I
Viable Cells
Passage #
J
Unstained
Ki67
18.3
76.3
4.09
SOX2
K
Unstained
OCT4
16
83.3
0.27
SOX2
Passage 5
L
Passage 5
DAPI
NESTIN
SOX2
MERGE

Figure 2. hFib-NPC^{OCT4} display tri-potent differentiation potential

A-D, Immunofluorescence images of neural lineage protein expression A, GFAP expressing astrocytes. B, O4 expressing oligodendrocytes. C, TUJ1 expressing neurons. D, TUJ-1 MAP-2 co-expression in a subset of neurons. Representative images from n=4 rounds of hFib-NPC^{OCT4} conversion. Scale bar = 100μM.

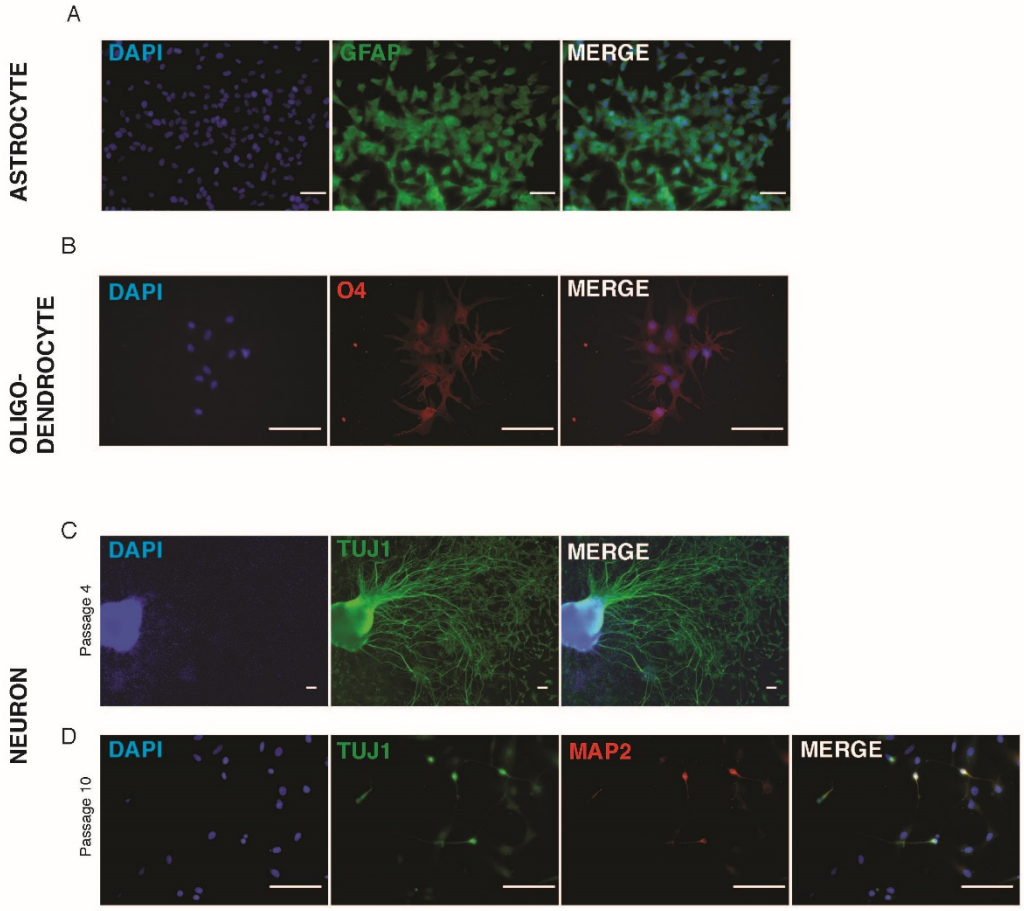

A
ASTROCYTE
DAPI
GFAP
MERGE
B
OLIGO-
DENDROCYTE
DAPI
O4
MERGE
C
NEURON
Passage 4
DAPI
TUJ1
MERGE
D
Passage 10
DAPI
TUJ1
MAP2
MERGE

Figure 3. Differentiated hFib-NPC^{OCT4} derived neurons demonstrate functional response to neurotransmitters and current injection.

A, hFib-NPC^{OCT4} differentiated cells treated with calcium reactive compound FLUO-4 and various calcium release stimuli. Representative fluorescent microscopy images of FLUO-4 dye reacting with calcium after administration with neurotransmitter glutamate. Fluorescent intensity cell trace diagrams of individual cells and cell-cell junctions during treatment with intracellular calcium release stimulants. B, Fibroblasts cultured in neural progenitor and neural differentiation medium treated with FLUO-4 and assayed for calcium release using stimulants. Representative fluorescent microscopy images of FLUO-4 dye reacting with calcium after administration with histamine. Fluorescent intensity cell trace diagrams of individual cells and cell-cell junctions during treatment with intracellular calcium release stimulants. C, Action potential measurements of hFib-NPC^{OCT4} derived neurons. D, Action potential measurement during administration of fast acting sodium channel inhibitor tetrodotoxin (TTX). E, Averaged traces of evoked repetitive action potentials in 4 separate cells.

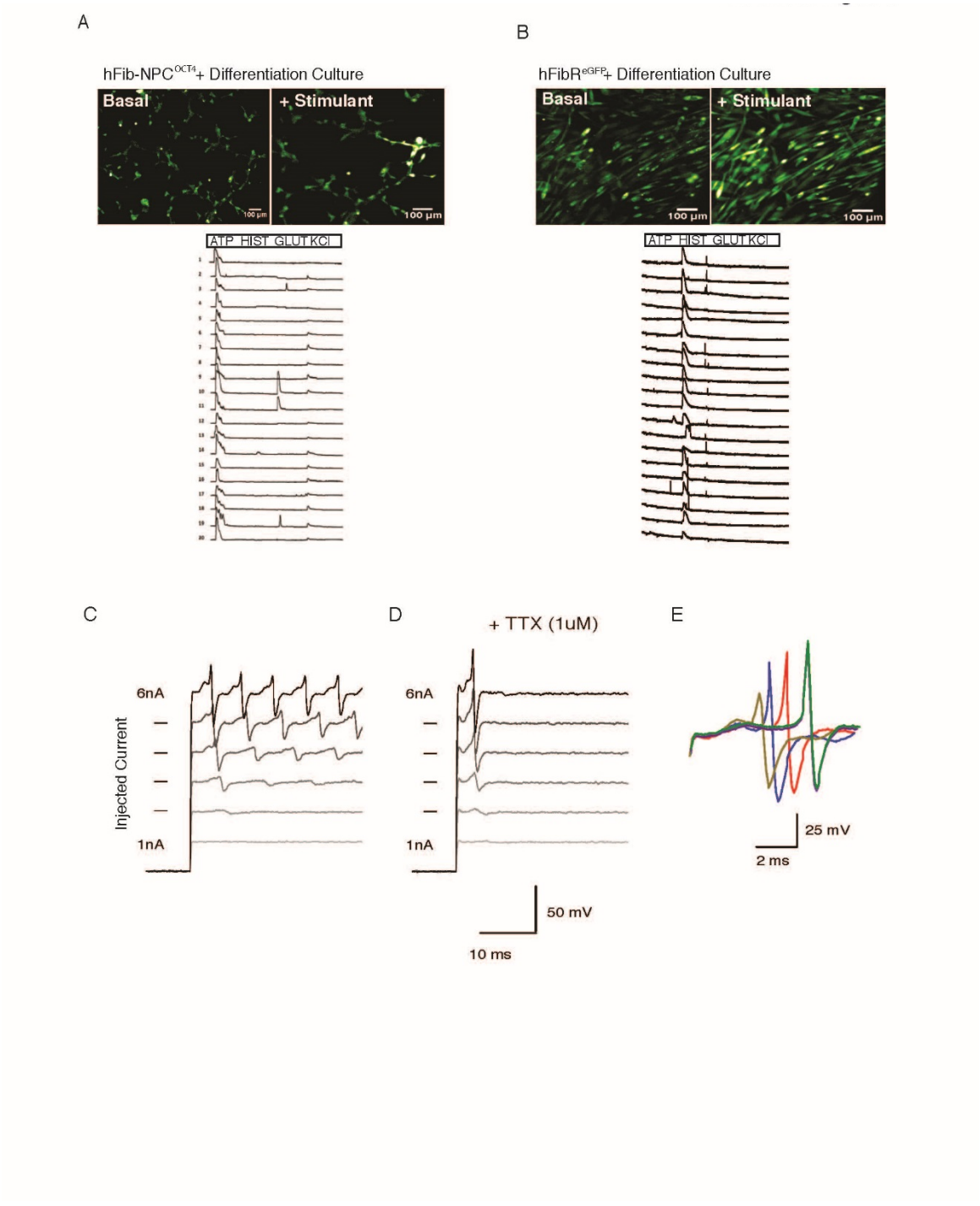

A
hFib-NPC^{OCT4}+ Differentiation Culture
Basal
+ Stimulant
100 µm
ATP HIST GLUT KCl
B
hFibR^{eGFP}+ Differentiation Culture
Basal
+ Stimulant
100 µm
ATP HIST GLUT KCl
C
6nA
1nA
Injected Current
D
+ TTX (1uM)
6nA
1nA
50 mV
10 ms
E
25 mV
2 ms

Figure 4. hFib-NPC^{OCT4} activate neural conversion transcription factors

A-D, qRT-PCR results for expression of neural conversion transcription factors in hFibR^{eGFP} cultured in neural progenitor culture, and hFibR^{OCT4} cultured in neural progenitor culture. Expression levels are normalized to expression in hFibs.

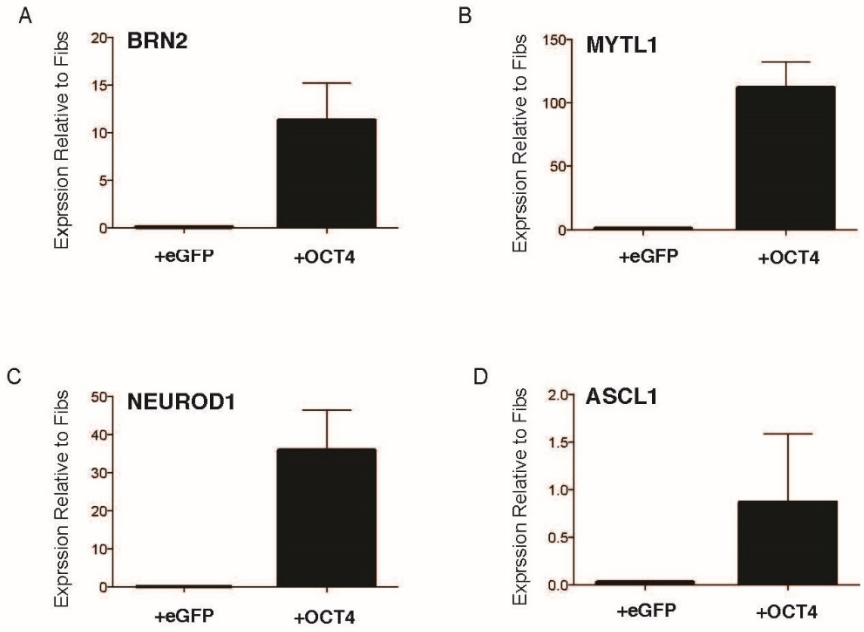
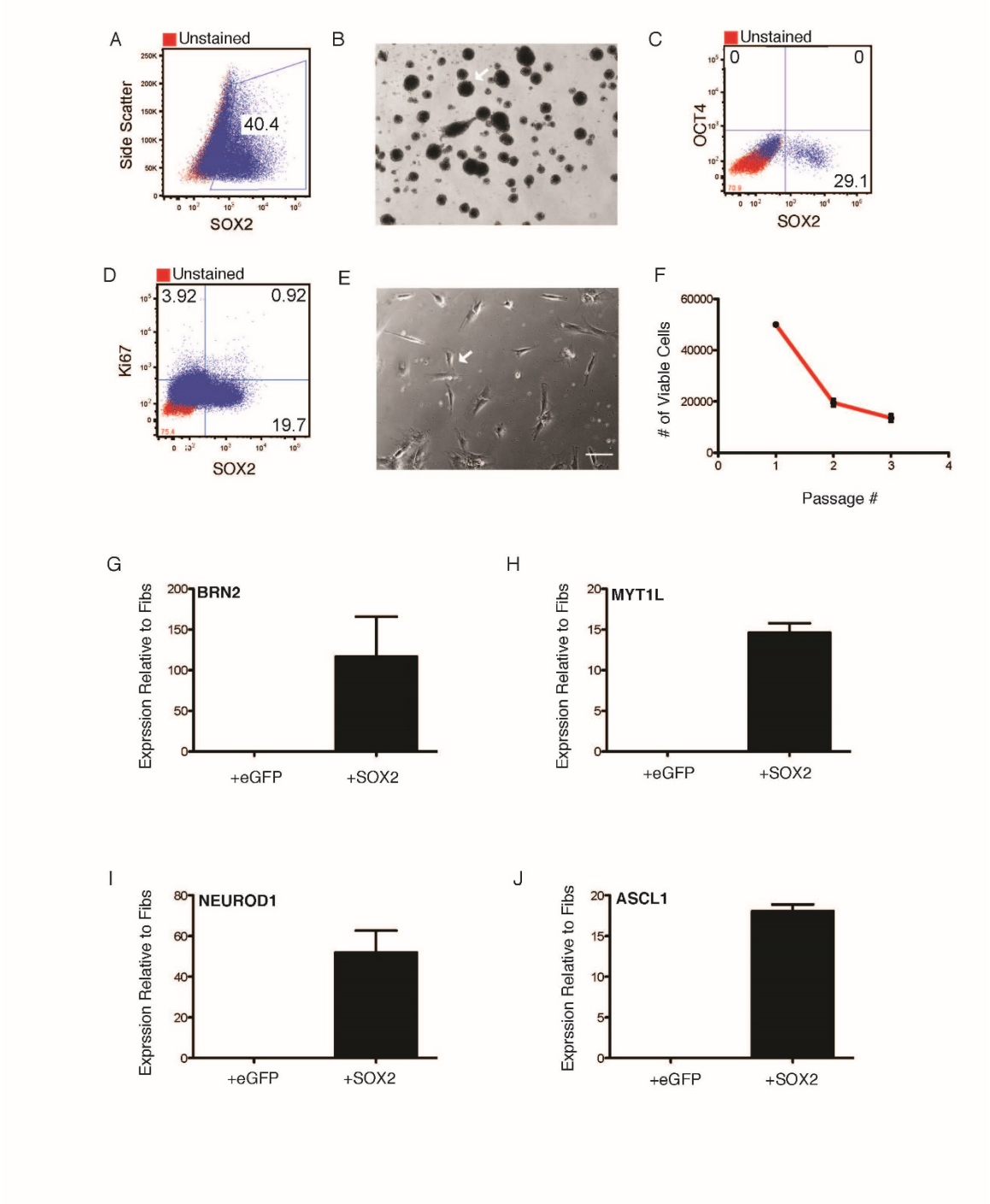

A
BRN2
Exprssion Relative to Fibs
20
15
10
5
0
+eGFP
+OCT4
B
MYTL1
Exprssion Relative to Fibs
150
100
50
0
+eGFP
+OCT4
C
NEUROD1
Exprssion Relative to Fibs
50
40
30
20
10
0
+eGFP
+OCT4
D
ASCL1
Exprssion Relative to Fibs
2.0
1.5
1.0
0.5
0.0
+eGFP
+OCT4

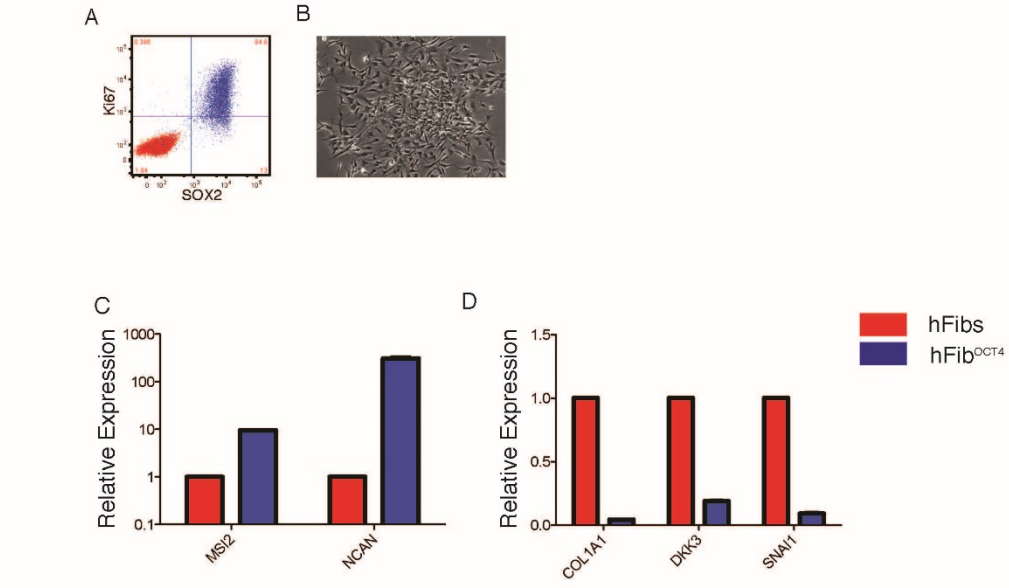
Figure 5. Expression of SOX2 in combination with neural progenitor culture fails to induce conversion of adult fibroblasts to neural progenitors, despite successfully activating downstream neural conversion transcription factors.

A, Flow cytometric plot of intracellular SOX-2 expression in adult human fibroblasts after 8 days in reprogramming media ($hFibR^{SOX2}$). B, Phase contrast image of $hFibR^{SOX2}$ sphere like clusters after 14 days in neural progenitor culture. C, Flow cytometric plot of SOX2 vs OCT4 expression in $hFibR^{SOX2}$ sphere derived cells. D, Flow cytometric plot of SOX2 vs Ki67 expression in $hFibR^{SOX2}$ sphere derived cells. E, Phase contrast image of $hFibR^{SOX2}$ sphere derived cells cultured on POL. F, Cell growth curve illustrating cumulative number of viable trypan blue excluding cells with increasing passage number. G-J, RT-qPCR results for expression of neural conversion transcription factors in $hFibR^{eGFP}$ cultured in neural progenitor culture, and $hFibR^{SOX2}$ cultured in neural progenitor culture. Expression levels are normalized to expression in hFibs (n=2). Representative results from n=3 attempts of $hFibR^{SOX2}$ conversion. White arrows indicate areas of interest.


A
Unstained
Side Scatter
40.4
SOX2
B
C
Unstained
0
0
OCT4
29.1
SOX2
D
Unstained
3.92
0.92
Ki67
19.7
SOX2
E
F
60000
40000
20000
0
# of Viable Cells
0
1
2
3
4
Passage #
G
BRN2
Exprssion Relative to Fibs
200
150
100
50
0
+eGFP
+SOX2
H
MYT1L
Exprssion Relative to Fibs
20
15
10
5
0
+eGFP
+SOX2
I
NEUROD1
Exprssion Relative to Fibs
80
60
40
20
0
+eGFP
+SOX2
J
ASCL1
Exprssion Relative to Fibs
20
15
10
5
0
+eGFP
+SOX2

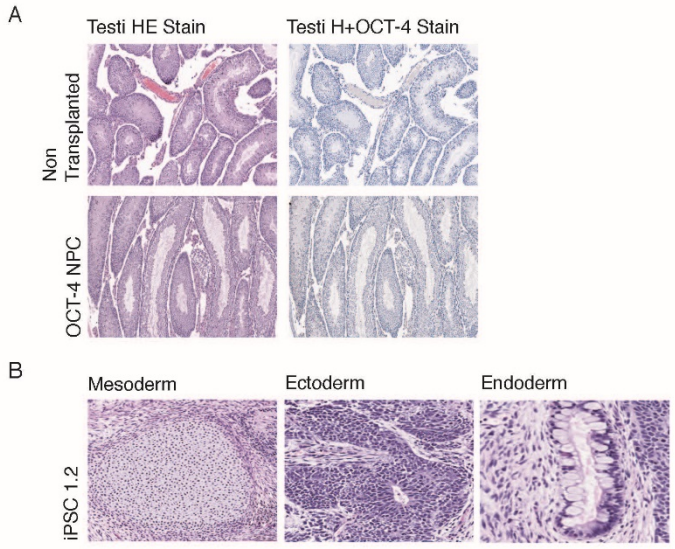
Supplementary Figure 1. Properties of pluripotent derived NPCs and reprogramming induced changes in both neural and fibroblast gene expression

A, Flow cytometric plot of SOX2 expression vs Ki67 expression in neural stem/progenitor cells (NPCs) derived from H9 ESCs. B, Phase contrast image of H9 derived NPC colony morphology when cultured on POL. C, RT-qPCR results for expression of neural progenitor genes MSI2 and NCAN in hFibR^{OCT4} relative to fibroblasts (n=3). RT-qPCR results for expression of fibroblast genes COL1A1, DKK3, and SNAI1 in hFibR^{OCT4} relative to fibroblasts (n=3).


A
B
Ki67
SOX2
C
Relative Expression
1000
100
10
1
0.1
MSI2
NCAN
D
Relative Expression
1.5
1.0
0.5
0.0
COL1A1
DKK3
SNAI1
hFibs
hFibOCT4

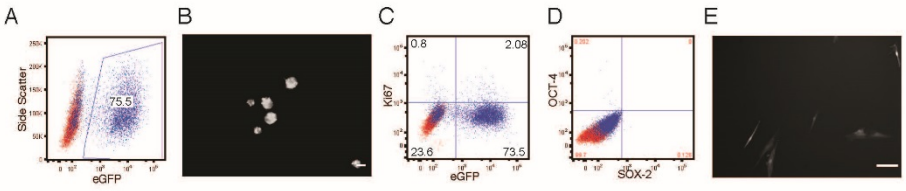
Supplementary Figure 2. hFib-NPC^{OCT4} lack pluripotency associated teratoma potential

A, Histological analysis of hFib-NPC^{OCT4} and non-injected mouse testis that failed to develop teratomas. OCT4 staining is presented alongside haematoxylin eosin staining. B, iPSC teratoma histological analysis of mesoderm, ectoderm and endoderm tissue formed in NOD SCID mice testis.


A
Testi HE Stain
Testi H+OCT-4 Stain
Non Transplanted
OCT-4 NPC
B
Mesoderm
Ectoderm
Endoderm
iPSC 1.2

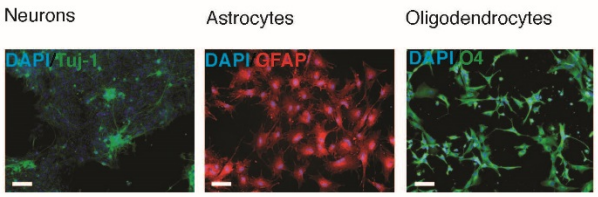
Supplementary Figure 3. Culturing of adult human fibroblasts in reprogramming media and neural progenitor culture fails to induce conversion to proliferating neural progenitor like cells

A, Flow cytometric plot illustrating eGFP expression in adult human fibroblasts after 8 days of culture in reprogramming media ($hFibR^{eGFP}$). B, fluorescence microscopy image of $hFibR^{eGFP}$ sphere like clusters after 14 days in progenitor culture media. C, Flow cytometric plot of eGFP vs Ki67 expression in $hFibR^{eGFP}$ sphere derived cells. D, Flow cytometric plot of OCT4 vs SOX2 expression in $hFibR^{eGFP}$ sphere derived cells. E, Immunofluorescence image of $hFibR^{eGFP}$ sphere derived cells cultured on POL. Representative results from n=3 attempts of $hFibR^{eGFP}$ conversion. Scale bar = 100μM


A
B
C
D
E
Side Scatter
75.5
eGFP
Ki67
0.8
2.08
23.6
73.5
eGFP
OCT-4
SOX-2

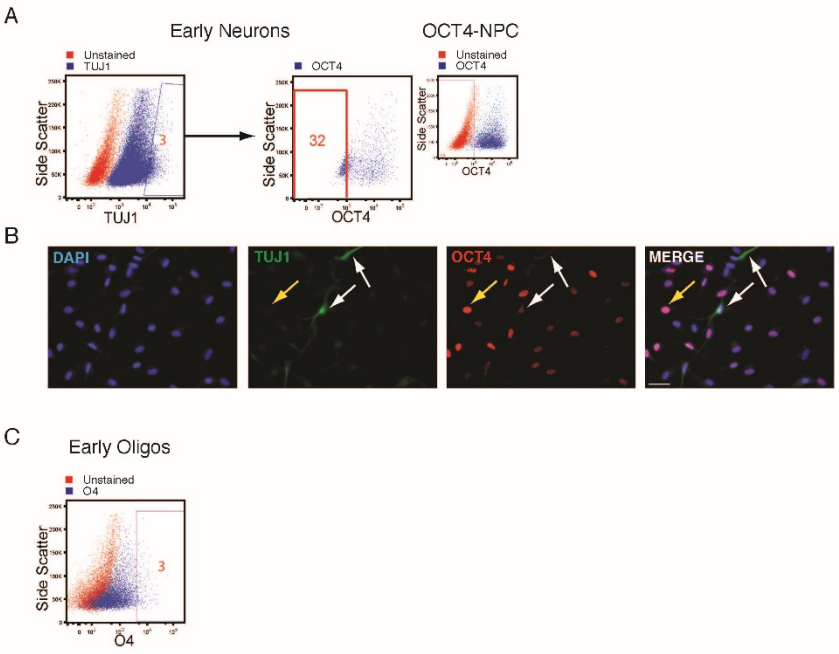
Supplementary Figure 4. Tri-potent differentiation of pluripotent derived NPCs

Immunofluorescence images of TUJ-1 positive neurons, GFAP positive astrocytes, and O4 positive oligodendrocytes derived from H9 NPCs.


Neurons
Astrocytes
Oligodendrocytes
DAPI/Tuj-1
DAPI/GFAP/
DAPI/O4

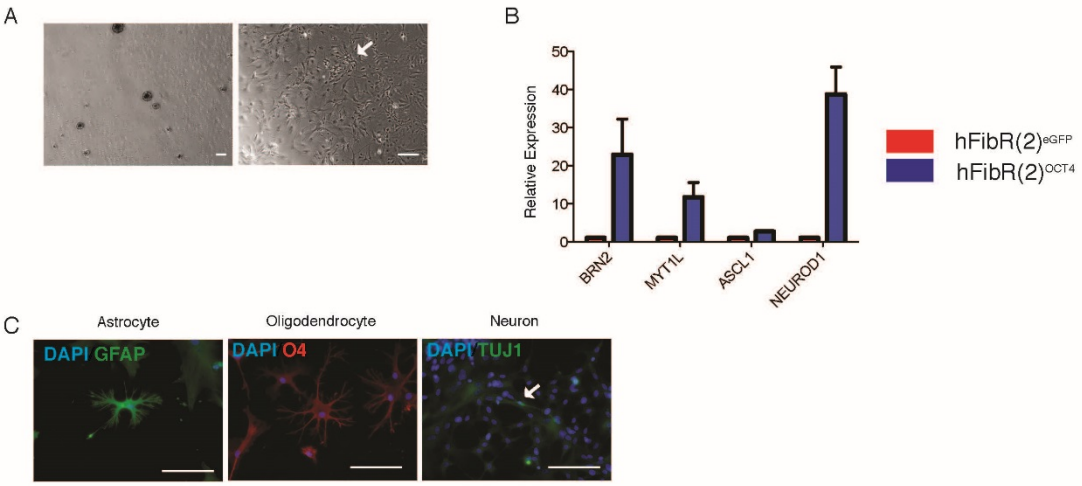
Supplementary Figure 5. Acquisition of differentiation markers correlates with loss of OCT4 expression in hFib-NPC^{OCT4}

A, Flow cytometry analysis of TUJ1 expression in differentiated hFib-NPC^{OCT4}. Flow cytometry plots illustrating OCT4 expression in high TUJ1 expressing cells compared to OCT4 expression in non-differentiated hFib-NPC^{OCT4}. B, Immunofluorescence for TUJ1 and OCT4 on newly differentiated neurons. White arrows denote cells that are expressing TUJ1 but not OCT4. Yellow arrows denote cells that are expressing OCT4 but not TUJ1. C, Flow cytometry plot illustrating frequency of O4 expressing cells during early stages of oligodendrocyte differentiation.


A
Early Neurons
OCT4-NPC
Unstained
TUJ1
Side Scatter
3
TUJ1
OCT4
32
OCT4
Unstained
OCT4
Side Scatter
OCT4
B
DAPI
TUJ1
OCT4
MERGE
C
Early Oligos
Unstained
O4
Side Scatter
3
O4

Supplementary Figure 6. Generation of hFib-NPC^{OCT4} is applicable to multiple adult cell lines

A, Phase contrast images of hFibR(2) derived spheres and laminin adapted cells in neural progenitor culture. B, RT-qPCR results for expression of neural conversion factors in hFibR(2)^{OCT4} vs hFibR(2)^{eGFP} sphere derived cells relative to fibroblasts (n=2). C, Immunofluorescence for GFAP, O4, and TUJ1 in astrocytic, oligodendrocytic, and neuronal differentiation cultures respectively. Scalebar=100µm


A
B
Relative Expression
50
40
30
20
10
0
BRN2
MYT1L
ASCL1
NEUROD1
hFibR(2)eGFP
hFibR(2)OCT4
C
Astrocyte
Oligodendrocyte
Neuron
DAPI GFAP
DAPI O4
DAPI TUJ1

Chapter 3

Molecular Evidence for OCT4 Induced Plasticity that Facilitates Direct Conversion of Adult Human Fibroblasts

Preamble
This chapter is an original published article. It is presented in its published format.

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I designed the study and wrote the paper with input from Dr. Eva Szabo and my supervisor Dr. Mick Bhatia. I performed all cell culture and reprogramming experiments. I performed all flow cytometric and immunocytochemical analyses. Zoya Shapovalova provided technical support during affymetrix array analysis. Lili Aslostovar sub cloned OCT4 into the pHIV-ef1α-IRES-eGFP lentiviral backbone. Kennedy Makondo provided technical support during cell sorting experiments.

This study was designed to investigate how OCT4 expression was able to support direct conversion towards two independent cellular endpoints. Specifically, we chose to look at the molecular state of cells in the early stages of the reprogramming process where the experimental conditions were identical, as well as after the administration of lineage specific culture used to support hematopoietic and neural cells. Through dissection of global transcriptional changes, we developed a working definition of OCT4 induced plasticity or OiP facilitated direct conversion towards multi-potent progenitors.

Molecular evidence for OCT4 induced plasticity that facilitates direct conversion of adult human fibroblasts

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Running Title: OCT4 induced Plasticity

Key Terms: Reprogramming, OCT4, Transdifferentiation

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Abstract

Here we characterize the molecular and biological requirements for OCT4 plasticity induction in human skin derived fibroblasts (hFibs) that allows direct conversion of cell fate without iPSC formation. Our results indicate that adult hFibs not only require OCT4 but also short-term exposure to reprogramming media (RM) to successfully undergo direct conversion to early hematopoietic and neural progenitor fates. RM was found to be essential in this process and allowed for unique changes in global gene expression specific to the combined effects of OCT4 and treatment with reprogramming media to establish a plastic state. This molecular state of hFib plasticity was distinct from transient expression of a full complement of iPSC reprogramming factors consistent with a lack in molecular hallmarks of iPSC formation. Human Fib-derived OCT4 plastic cells display elevated levels of developmentally related genes associated with multiple lineages, but not those associated with pluripotency. In response to changes in the extracellular environment, plastic OCT4-expressing hFibs further activate genes involved in hematopoietic as well as tri-potent neural progenitor biology that allow cell fate conversion. Our study provides a working definition of hFib induced plasticity using OCT4 and a deconvoluted system to elucidate the process of direct cell fate reprogramming.

Introduction

The first reported evidence that a transcription factor could alter cell fate was the observation that ectopic expression of MYOD resulted in up-regulation of muscle-specific genes in non-muscle cells [1]. Years later, independent studies by Yamanaka and Thomson identified combinations of pluripotency associated transcription factors that yield induced pluripotent stem cells (iPSCs) when expressed in human skin derived fibroblasts (hFibs) [2, 3]. Although generation of stable iPSC lines has obvious utility, concerns regarding tumorigenicity, incomplete differentiation and prohibitive costs associated with iPSC generation, characterization and validation, represent a significant limitation toward practical applications [4]. As such, many groups have revisited previously established concepts of altering cell fate without establishing pluripotency, forcing cells to turn from cell type A directly into cell type B [5]. One successful re-demonstration of this cellular reprogramming process characterized the direct conversion of mouse fibroblasts to post-mitotic neurons [6] where Fibs transduced with several specifying transcription factors and cultured in previously established neural supportive conditions lead to an observed conversion of fibroblasts to mature mouse neurons [6]. In humans, direct conversion of hFibs to blood progenitors has been shown using a single factor, OCT4, known to be associated with pluripotency and early embryonic development [7]. Although not known to be involved in normal hematopoiesis, OCT4 was able to promote expression of key hematopoietic regulators in the presence of supportive culture conditions optimized for *in vitro* maintenance of adult human hematopoietic progenitors. Following the demonstration of direct conversion using OCT4 alone, mouse fibroblasts were shown to directly convert to cardiomyocyte-like cells after

brief expression of a full complement of pluripotency factors and exposure to cardiomyocyte supportive culture conditions without achieving pluripotency. Based on these key initial observations, many groups developed direct reprogramming strategies generating cell types of interest through forced expression of transcription factors without first establishing iPSCs [5].

As a result, direct conversion reprogramming techniques have been divided into two groups based on generally accepted but largely unsubstantiated thoughts regarding overall mechanism. The first, overexpression of lineage specifying transcription factors is thought to flood the parent cell with instructions to activate alternative regulatory programs, eventually leading to a transition from cell type A into cell type B [5, 8–10]. The second suggests that brief expression of the pluripotency factors or OCT4 alone confers a "plastic state" onto somatic cells allowing subsequent differentiation [5, 8–10]. Despite these discussed ideas, no study to date has defined the molecular properties of cell "plasticity" achieved [5, 8–10]. Here, we provide a foundation toward molecularly defining and understanding the potential of the plastic state induced from hFibs. The molecular state induced by OCT4 expression alone is distinct from cells partially or fully reprogrammed to iPSC and induces activation of developmental genes corresponding to multiple cell lineages that is dependent on culture conditions. Furthermore, by providing evidence for the generation of tri-potent neural progenitor cells, we demonstrate that the utility of OCT4 for direct conversion is not limited to the study of hematopoietic cells.

Methods

Fibroblast Cell Culture

Human dermal adult fibroblasts derived from breast dermal tissue (Sciencell, Corte Del Cedro Carlsbad, CA, http://http://www.sciencellonline.com) were initially maintained in fibroblast medium: Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, http://www.invitrogen.com) supplemented with 10% v/v FBS (Neonatal Bovine Serum, HyClone, Logan, UT, http://www.hyclone.com), 1mM L-glutamine(Gibco), and 1% v/v nonessential amino acids (NEAA; Gibco)before transduction with OCT4 lentivirus vector.

Lentivirus Transduction and Reprogramming Culture

Lentiviral plasmids (Addgene, Cambridge, MA http://www.addgene.org) pSIN-EF1α-Oct4-Puro and pSIN-EF1α-IRES-eGFP-Puro were obtained and co-transfected with pMD2.G and psPAX2 plasmids into the 293-FT cell line (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) in order to initiate virus particle production. Viral supernatants were harvested 48 hours after transfection and ultra-centrifuged to concentrate the virus. hFibs were transduced in 50% fibroblast medium 50% reprogramming media (RM) (RM-F12 DMEM 20% knockout serum replacement (Gibco),1mM L-glutamine (Gibco), and 1% v/v NEAA (Gibco), 0.1 mM β mercaptoethanol (Sigma Aldrich, St. Louis, MO, http:/www.sigmaaldrich.com), 16 ng/mL basic fibroblast growth factor (bFGF), 30 ng/mL insulin growth factor 2 (IGF2), supplemented with 8 μg/mL polybrene (Sigma Aldrich). 2 days post-transduction with

either enhanced green fluorescent protein (eGFP) or OCT4, medium was replaced with 100% RM and cells were cultured for 8 days, replacing the medium every other day.

Hematopoietic Progenitor Generation and Culture

Human dermal adult fibroblasts were directly converted to multipotent hematopoietic progenitors as previously described [7]. Colony forming unit assay was performed using Metho-Cult, Stem Cell Technologies, Vancouver, BC, http://www.stemcell.com, and colony morphology was assessed as previously described [7]. For assessment of transcriptome modulation by hematopoietic culture conditions, hFibR^{OCT4} were cultured in RM supplemented with 300 ng/mL stem cell factor and 300 ng/mL Fms related tyrosine kinase 3 ligand for 14 days.

Neural Progenitor Generation and Culture

hFibs expressing OCT4 (or GFP) were cultured for 8 days post-transduction in RM before being trypsinized and seeded as single cells in ultra-low attachment plates (Corning, NY, http://www.corning.com) containing progenitor culture media: F12 DMEM supplemented with 13 N2 (Gibco) and 13 B27 (Gibco) 20 ng/mL bFGF, 20 ng/mL epidermal growth factor (EGF). After 14 days sphere clusters were dissociated using Accutase (Gibco) and seeded on to poly-L-ornithine/mouse laminin-coated (Sigma Aldrich) tissue culture plates in neural differentiation culture media consisting of F12 DMEM 1X N2 1X B27. For neuronal differentiation, media was supplemented with 5 nM forskolin (Sigma Aldrich). For astrocyte differentiation, media was supplemented with 5% FBS. For oligodendrocyte differentiation, media was supplemented with 100 ng/mL

IGF1, 200 nm ascorbic acid (Sigma Aldrich), 5nM forskolin (R&D Systems,Minneapolis, MN, http://www.rndsystems.com).

Endoderm Cell Culture

hFibR and hFibR^{OCT4} were differentiated to the endodermal lineage with high dose (100 ng/mL) Activin-A (R&D Systems) in IMDM plus 1mM L-glutamine for more than 5 days [11]. Serum concentrations were 0% for day 1, 0.2% for day 2, and 2% for days 3–5. For generation of hepatocytes, cells were cultured in F12DMEM containing 1 lM dexamethasone (Sigma Aldrich), 1 lg/mL insulin (Sigma Aldrich), 1mM L-glutamine (Gibco), 20 ng/mL EGF (R&D Systems), and 20 ng/mL hepatocyte growth factor (R&D Systems) for 7 days post-activin A treatment.

Episomal iPSC Reprogramming Vector Transfection

Stemcircle episomal vector (Stemcell Technologies) was obtained and transfected into human adult fibroblasts using LTX (Life Technologies, Rockville, MD, http://www.lifetech.com). Transfected cells were cultured in RM for 8 days prior to FACS sorting on eGFP expression.

Flow Cytometry and Fluorescence-Activated Cell Sorting

Cell surface staining for CD45 (BD Bioscience, Mississauga ON, http://www.bdbiosciences.com, CD45PE [1:100]) was performed at RT for 30 minutes prior to washing in 3% FBS, phosphate buffered saline and staining with 7-Aminoactinomycin D (BD Biosciences [1 in 50]) live/dead discriminator dye. Acquisition

was performed using LSRII (BD), and analysis was performed using FlowJo 9.2 Software. Fluorescence activated cell sorting (FACS) was performed using FACS AriaII as previously described [12].

Microarray Analysis

Total RNA was extracted (Norgen Biotek, Thorold, Ontario, http://www.norgenbiotek.com) and hybridized to the Affymetrix Gene Chip Human Gene 1.0 ST arrays (London Regional Genomics Centre, ON, Canada). Output data were normalized using Robust Multichip Averaging algorithm and baseline transformation to the median of all samples using Partek. Principle component analysis (PCA) was generated based on normalized expression values of all entities. To create Venn diagrams, differentially expressing gene lists were generated using unpaired t test with multiple test correction and fold change ≥1.5. Normalized arrays were hierarchically clustered based on all entities or selected gene sets, which were subsequently compared using Pearson Centered similarity measure and Centroid linkage rule in order to generate dendrograms. In case when the samples were from different technologies, Partek was used to find common gene entities, followed by normalized values being imported in dChip for clustering analysis. Publicly available samples were also used for clustering analysis: hNSC Sox21 (GSE27505). Gene Ontology (GO) analysis was performed using DAVID bioinformatics resource 6.7 functional annotation tool suite. The top three GOTERM_BP_- FAT annotations for a given set of gene inputs were listed as annotation terms [13].

Immuncytochemistry

For surface marker staining, cells were fixed using BD Biosciences Fixation buffer for 40 min at 4°. Cells were washed in 3% FBS Hanks buffered saline solution (Gibco). For intracellular staining, cells were fixed using BD Biosciences Fixation/Permeabilization buffer for 40 min at 4°. Cells were washed in BD Biosciences 13 Perm solution. Lineage specific cells were identified by staining with monoclonal antibodies SOX-2-647 (BD Biosciences), NESTIN anti-Human (R&D Systems), β-III Tubulin/Tuj1 anti-Human (R&D Systems), Oligodendrocyte marker 4/O4 anti-Human (R&D Systems), and glial fibrillary acidic protein/anti-Human (Sigma, Aldrich), FOXA2, NKX2.1. Antibodies were diluted in BD 13 wash buffer and incubated overnight at 4°C. Non-conjugated antibodies were visualized using appropriate Alexa-Flour secondary reagents (Life Technologies). Nuclei were counterstained with 1:1000 dilution of 40,6- diamidino-2-phenylindole (BD Biosciences). Optimal working dilutions were determined for individual antibodies.

Quantitative Polymerase Chain Reaction

Total RNA was isolated using the Qiagen mini RNA isolation kit. RNA was then subjected to cDNA synthesis using superscript III (Invitrogen). Quantitative polymerase chain reaction (qPCR) was performed using Platinum SYBR Green-UDP mix (Invitrogen). Threshold was set to the detection of Gus-B (b-glucuronidase) and then normalized to GAPDH.

Results

Direct Cell Fate Conversion to Both Blood and Neural Progenitors from Human Fibroblasts Is Dependent on OCT4 as Well Culture in RM

Similar to previously shown [7], hFibs transduced with OCT4 expressing lentivirus that are cultured in RM and then stimulated with hematopoietic cytokines undergo direct conversion to $CD45^{+}Fibs^{OCT4}$ (Fig. 1A). However, when either OCT4 expression is induced in the absence of RM or RM treatment is applied in the absence of OCT4 expression, hFibs stimulated with hematopoietic cytokines fail to generate $CD45^{+}Fibs^{OCT4}$ (Fig. 1B, 1C). These observations demonstrate that fibroblasts require both OCT4 expression along with RM treatment in order to respond to hematopoietic cytokines and successfully drive direct cell fate conversion to $CD45^{+}Fibs^{OCT4}$. To determine if this requirement for both OCT4 and RM to elicit direct conversion was specific to hematopoietic cytokine stimulation, we tested equally well established neural stem/progenitor culture medium [14]. OCT4 expressing hFibs treated with RM underwent direct conversion to tri-potent neural progenitor cells ($Fibs\text{-}NPC^{OCT4}$) (Fig. 1D) [15]. Similarly, if either OCT4 expression or RM is removed from this process, hFibs stimulated with neural stem/progenitor culture medium fail to generate $Fibs\text{-}NPC^{OCT4}$, as evidenced by the maintenance of fibroblast morphology and inability to propagate on laminin (Fig. 1E) along with failure to upregulate the neural progenitor marker SOX2 (Fig. 1F). We conclude that hFibs also require OCT4 and RM treatment in order to respond to neural stem/ progenitor culture conditions and undergo direct conversion to $Fibs\text{-}NPC^{OCT4}$. Taken together, these results uncover the combined importance of both

OCT4 expression and short term RM treatment that allows for direct conversion of adult hFibs.

RM Is Required to Sustain OCT4 Expression in Adult Human Fibroblasts

To better understand the relationship between OCT4 expression and RM required for direct conversion of hFibs, we analyzed the initial stages of direct conversion prior to the treatment with lineage-specific culture conditions. As early as 1 week after transduction with OCT4 expressing lentivirus ($Fibs^{OCT4}$) and treatment with RM (w/RM), hFibs display morphological changes distinct from elongated fibroblast morphology that resemble a more cuboidal shape (Fig. 2A i) not detectable in untreated control hFibs (Fig. 2A ii). These morphological differences failed to manifest in hFibs expressing OCT4 in the absence of RM (w/o RM), and in hFibs expressing eGFP ($Fibs^{eGFP}$) treated with RM (Fig. 2A iii, iv). These results demonstrate unique changes in fibroblast morphology only present in $Fibs^{OCT4}$ w/RM that serve as a predictor for subsequent direct conversion competency.

To evaluate whether the observed differences in morphology between $Fibs^{OCT4}$ w/ RM and $Fibs^{OCT4}$ w/o RM were a result of differential OCT4 expression, we analyzed intracellular OCT4 expression by flow cytometry. $Fibs^{OCT4}$ w/RM maintained consistent frequencies of OCT4 expressing cells over time, whereas $Fibs^{OCT4}$ w/o RM demonstrated a surprising decline in the frequency of cells expressing OCT4 (Fig. 2B). As control transduced $Fibs^{eGFP}$ w/RM or $Fibs^{eGFP}$ w/o RM demonstrated consistent frequencies of eGFP expressing cells over time (Fig. 2C), the decline in OCT4 was not due to lentiviral silencing in the absence of RM. Furthermore, the decline in OCT4 expressing cell

frequency was not due to selective degradation of OCT4 protein, as fibroblasts expressing OCT4 and eGFP displayed a similar phenomenon to OCT4 expressing hFibs, as OCT4/eGFP double positive frequency levels were only maintained during treatment with RM (Fig. 2D). These combined results reveal that RM is required in order to maintain stable OCT4 expressing adult hFibs.

The Combination of OCT4 Expression and RM Induces Unique Molecular Profile Changes in Adult Human Fibroblasts

To better understand the contributions of OCT4 expression and RM on the early transcriptional changes during direct conversion of hFibs, we performed global gene expression profiling on cells that were in the early stages of direct conversion prior to treatment with lineage-specific culture conditions. First, we applied PCA to profiles collected from Fibs, $Fibs^{OCT4}$ w/RM, $Fibs^{OCT4}$ w/o RM, and $Fibs^{eGFP}$ w/RM to determine the molecular impact of OCT4 expression and RM on the transcriptome of adult hFibs (Fig. 3A). This analysis revealed that the combination of OCT4 expression and RM induces distinct global transcription changes compared to that of OCT4 expression or RM alone. Furthermore, these results highlight that RM-induced transcriptional changes exhibit a dominant effect over OCT4-induced changes, as $Fibs^{OCT4}$ w/RM clustered closer to $Fibs^{eGFP}$ w/RM than to $Fibs^{OCT4}$ w/o RM.

To identify genes associated with the observed clustering via PCA for use in downstream biological annotation, we performed differential gene expression analysis. Untreated hFibs were used as a baseline in these analyses in order to identify genes that were upregulated and downregulated. Comparison of $Fibs^{OCT4}$ w/o RM to $Fibs^{OCT4}$ w/RM

allowed for identification of unique OCT4 dependent regulation that only occurs in the presence of RM (1,123 UP 1 671 DOWN regulated genes) (Fig. 3B, 3C). GO analysis of $Fibs^{OCT4}$ w/RM specific upregulated genes revealed an enrichment for extracellular structure organization and extracellular matrix organization within the top three returned annotations ranked by p value (3D). These results correlate with observed morphological changes that were observed exclusively in $Fibs^{OCT4}$ w/RM prior to the addition of lineage supportive culture conditions. Analysis of shared and unique upregulated genes within $Fibs^{OCT4}$ w/o RM (2,454 UP 1 2,051 DOWN regulated genes in common, 977 UP 1 791 DOWN regulated genes specific to FibsOCT4 w/o RM) by GO revealed enrichment for cell cycle and RNA processing related biological processes (Fig. 3B–3D). These findings confirm that the transcriptional changes induced by OCT4 in combination with RM are unique from those induced by OCT4 alone. In order to identify transcriptional changes that were specific to RM alone, we compared $Fibs^{eGFP}$ w/RM to fibroblasts cultured in RM that had not undergone lentiviral transduction (Fibs w/RM) (Fig. 3E, 3F). GO analysis of RM dependent upregulated genes (2,598 11,190 UP 1 2,348 1 1,077 DOWN regulated genes) revealed strong enrichments for translation- and transcription-related biological processes (Fig. 3G). Our results indicate that transcriptional changes induced by the combination of OCT4 expression and RM are unique from the sum of their individual contributions. These unique gene regulation events likely contribute to the early observed morphological changes in adult hFibs that precede successful direct conversion to multipotent progenitors, thereby providing a foundation to define plasticity at the molecular level.

Expression of OCT4 versus OSNL Induces Distinct Transcriptional Regulation in Combination with RM in Adult Human Fibroblasts

The process of directly converting somatic cells to progenitor states has been achieved by using brief expression of the same pluripotency factors used to generate human iPSCs [16– 18], leaving it unclear as to whether subsequent lineages derived from this approach are a product of partially reprogrammed iPSCs or other cellular processes. Having established an initial description of the transcriptional status of hFibs undergoing OCT4 reprogramming, we asked whether this status was in fact similar to that found in cells undergoing direct conversion facilitated by temporary expression of a complete set of pluripotentcy reprogramming factors.

Human Fibs were transfected with a single vector containing four reprogramming factors 1 EGFP (OCT4, SOX2, NANOG, Lin28) (Supporting Information Fig. 1) and cultured in RM (Fibs4F w/RM). To reduce impurities, we enriched for GFP positive hFibs and compared the resulting expression profiles to hFibs and $Fibs^{OCT4}$ w/RM. PCA revealed the added expression of SOX2, NANOG, and LIN28 ($Fibs^{4F}$ w/RM) resulted in an expression profile that formed a cluster distinct from cells expressing OCT4 alone ($Fibs^{OCT4}$ w/RM) (Fig. 3H). Differential gene expression analysis highlighted almost equal numbers of independently regulated genes by SOX2, NANOG, and LIN28 (1,112 UP 1 738 DOWN regulated genes) and OCT4 (1,068 UP 1 807 DOWN regulated genes), in addition to a set of commonly regulated genes (2,509 UP 1 1,915 DOWN regulated genes) (Fig. 3I, 3J). Ontology analysis of commonly upregulated genes revealed enrichment for transcription related processes (Fig. 3K), similar to the effects of culturing

fibroblasts in RM on its own (Fig. 3G). Ontology analysis of genes upregulated only in the presence of all four reprogramming factors ($Fibs^{4F}$ w/RM) displayed enrichment for early development related biological processes, whereas $Fibs^{OCT4}$ w/ RM displayed enrichment for metabolic related processes (Fig. 3K). These results indicate that both $Fibs^{4F}$ w/RM and $Fibs^{OCT4}$ w/RM exhibit unique global gene expression patterns and are molecularly distinct, whereby $Fibs^{4F}$ w/RM are enriched for early developmental programs compared to $Fibs^{OCT4}$ w/RM.

To further investigate whether OCT4 direct conversion displayed transcriptional hallmarks of early iPSC reprogramming, we analyzed $Fibs^{OCT4}$ w/RM for expression of mesenchymal to epithelial transition genes (MET- a process known to precede iPSC formation [19]), as well as early and late pluripotentcy genes [20]. $Fibs^{OCT4}$ w/RM neither display a bias toward epithelial gene regulators compared to mesenchymal gene regulators (an indication of MET) nor did they display up-regulation of early or late pluripotentcy genes above that achieved by culturing hFibs in RM alone (Supporting Information Fig. 2A–2D). These findings indicate that $Fibs^{OCT4}$ w/RM are distinct from cells undergoing initialization, early or late stages of pluripotentcy as defined by recently reported molecular models [19, 20].

RM and OCT4 Expression Combined Induce Plasticity in Adult Human Fibroblasts

Orkin and Hochedlinger [8] have recently shared the term plasticity to describe cells that are in a "confused state" and require "instruction" from the extracellular environment. It has been hypothesized that this state of confusion may manifest as activation of lineage genes corresponding to multiple cell types or germ layers [8]. Accordingly, we

investigated whether the unique transcriptional status of Fibs^{OCT4} w/RM prior to treatment with lineage supportive culture conditions displayed gene expression patterns corresponding to multiple cell lineages. We filtered the gene expression profile of Fibs^{OCT4} w/RM on established lineage development gene lists (Supporting Information Tables 1–3) corresponding to hematopoiesis, neurogenesis, and general endoderm development in order to represent all three germ layers. Treatment of OCT4 expressing fibroblasts with RM upregulated (1.5 fold increase compared to Fibs) a subset of lineage development genes from all three germ layers (Fig. 4A), indicating that Fibs^{OCT4} w/RM are transcriptionally primed for multiple fates prior to the addition of lineage supportive culture. The results of this analysis support the observation that Fibs^{OCT4} w/RM are not only competent for direct conversion to both the hematopoietic and neural fate but also present the possibility for direct conversion toward endoderm. To assess whether Fibs^{OCT4} w/RM were capable of direct conversion to endodermal derivatives, we used conditions shown to support endoderm differentiation from pluripotent stem cells (PSCs). Culturing of Fibs^{OCT4} w/RM in conditions shown to support early endoderm specification from PSCs did not result in emergence of early endoderm derivatives as marked by expression of FOXA2 and NKX2.1 (Fig. 4B i, ii). However, these same culture conditions were able to induce markers of early endoderm in H9 PSCs (Fig. 4B iii). These results suggest that the level of endodermal lineage gene activation in Fibs^{OCT4} w/RM may not have been sufficient to support endodermal direct conversion using our current supportive culture conditions. Collectively, Fibs^{OCT4} w/RM induce a transcriptional status that displays

elevated expression of genes corresponding to multiple cell lineages. From herein, we refer to this unique status as "OCT4 plasticity."

OCT4 Plasticity Facilitates Direct Conversion through Activation of Stem/Progenitor Gene Expression in Response to Lineage Supportive Culture Conditions

In an effort to understand the molecular events leading toward the conversion of $Fibs^{OCT4}$ w/RM to both hematopoietic and neural progenitors, we performed global gene expression profiling on $Fibs^{OCT4}$ w/RM treated with hematopoietic and neural lineage supportive culture media. Resulting gene profiles were filtered on the aforementioned lineage development gene lists corresponding to hematopoiesis and neurogenesis. Treatment with hematopoiesis-supportive culture conditions resulted in an increase in the number of upregulated hematopoiesis genes in $Fibs^{OCT4}$ w/RM (Fig. 5A i). Similarly, treatment with neurogenesis-supportive culture conditions resulted in an increase of the number of neurogenesis genes in $Fibs^{OCT4}$ w/ RM (Fig. 5A ii). These results indicate that OCT4 induced plasticity allows for the subsequent activation of additional lineage genes in response to lineage supportive culture conditions.

Based on these observations, we aimed to determine whether the lineage genes activated during OCT4 plasticity were akin to those found in true somatic stem/progenitor cells derived from adult sources, and/or whether this activation was unique to $Fibs^{OCT4}$ w/RM. We performed hierarchal cluster analysis using lineage development gene lists on expression profiles corresponding to $Fibs^{OCT4}$ w/RM, $Fibs^{OCT4}$ w/o RM, and $Fibs^{eGFP}$ w/RM exposed to lineage supportive culture as well as a corresponding somatic stem/progenitor cell. Only $Fibs^{OCT4}$ w/RM clustered with somatic hematopoietic

progenitors when exposed to hematopoietic supportive culture conditions, and with neural progenitors when exposed to neural supportive culture conditions (Fig. 5B i, ii). Taken together these results support the notion that coordinated activation of lineage-specific gene expression in response to changes in the extracellular environment is a unique and defining property of $Fibs^{OCT4}$ w/RM and OCT4 plasticity.

Discussion

Although it has been intimated that OCT4 is capable of inducing a state of plasticity, our results now provide the first evidence to support this supposition. Importantly, these results highlight the previously unappreciated relationships between reprogramming factors and the extracellular environment. We demonstrate that RM is required to maintain stable populations of adult hFibs expressing OCT4 for use in direct conversion reprogramming. The relationship between OCT4 expression and RM can be described by unique changes in fibroblast transcriptome that are not achievable through singular exposure to either of these required elements.

These findings expand the role of OCT4 in cellular reprogramming that is distinct from iPSC establishment. Expression of OCT4 in somatic cells cultured in RM induces a state unlike that of a complete set of reprogramming factors. OCT4 induced plasticity is capable of responding to changes in the extracellular environment that ultimately lead to alteration of cell fate. This state does not share hallmarks of cells progressing along the path to become iPSC but instead displays up-regulation of a mixture of lineage genes spanning the three germ layers. The treatment of hFibs with culture conditions for both the hematopoietic and neural lineages resulted in the further stimulation of lineage development genes facilitating the direct conversion process. Although conversion to the endodermal lineage was not achieved, this is not unprecedented. To date we are unaware of any reports of endodermal lineage conversions that do not rely on the expression of fate specifying transcription factors. Our study does not rule out the possibility of

achieving endodermal conversion using OCT4 induced plasticity but instead suggests the requirement to revisit different endodermal supportive culture strategies.

OCT4 induced plasticity represents a simplified means of dedifferentiating adult human somatic cells for the purpose of redirecting their fate to desirable progenitor states. Progenitor cells are capable of producing the large cell numbers required for downstream studies in disease modeling. Importantly, isolation of progenitor clones will remove genetic heterogeneity found in mixed populations of cells having received integrative viral vectors. It is unlikely that direct conversion studies using fate specifying transcription factors will be able to circumvent these issues, as each individual cell will be genetically different from its neighbor given the nature of the cell division independent process. The description of OCT4 induced plasticity we have provided here serves as a step forward in deconvoluting the process of cellular reprogramming by providing a new working definition of the molecular state of hFib induced plasticity and forms the basis to identify the critical signaling pathways that support OCT4 expression in non-pluripotent cells. These and other studies are ongoing in our lab based on the findings reported here.

Summary

Culturing of OCT4 expressing adult human fibroblasts in reprogramming media induces a state of unique transcriptional plasticity. These cells are able to respond to changes in the extracellular environment through activation of lineage development genes that leads to the alteration of cell fate towards both hematopoietic and neural progenitors. Our work

highlights OCT4 direct conversion as a unique and versatile reprogramming methodology.

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Author Contributions

R.M. and E.S.: conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing; Z.S.: data analysis and assembly of data; L.A.: provision of study materials; K.M.: collection and assembly of data; M.B.: conception and design, data analysis and interpretation, and manuscript writing.

Disclosure

The authors declare no potential conflicts of interest.

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Figure 1. OCT4 expression and reprogramming media (RM) are required for direct conversion to hematopoietic and neural progenitors.

(A): Phase contrast image of human fibroblasts expressing OCT4 and treated with reprogramming media ($Fibs^{OCT4}$ w/RM) after addition of hematopoietic cytokines (i). Flow cytometry plot demonstrating CD45 pan hematopoietic marker expression from $Fibs^{OCT4}$ w/RM (ii). Colour image of $CD45^{+}Fibs^{OCT4}$ derived mixed lineage colony forming unit in hematopoietic cytokine enriched methyl cellulose (iii). (B): Phase contrast image of human fibroblasts treated with RM alone ($Fibs^{eGFP}$ w/RM) after addition of hematopoietic cytokines (i). Flow cytometry plot demonstrating lack of CD45 expression from $Fibs^{eGFP}$ w/RM (ii). (C): Phase contrast image of human fibroblasts expressing OCT4 in the absence of RM ($Fibs^{OCT4}$ w/o RM) after addition of hematopoietic cytokines (i). Flow cytometry plot demonstrating lack of CD45 expression from $Fibs^{OCT4}$ w/o RM (ii). (D): Phase contrast image of neural sphere structure derived from $Fibs^{OCT4}$ w/ RM after addition of neural progenitor culture conditions (i). Phase contrast image of Fibs-NPC^{OCT4} cultured as colonies on laminin. Immunocytochemistry for neural progenitor markers NESTIN and SOX2 on Fibs-NPC^{OCT4}. Immunocytochemistry for neural differentiation markers TUJ-1 (i), GFAP (ii), and O4 (iii) on differentiated Fibs-NPC^{OCT4}. (E): Phase contrast image of neural sphere structure derived from $Fibs^{eGFP}$ w/RM after addition of neural progenitor culture conditions (i). Phase contrast image of $Fibs^{eGFP}$ w/RM derived spheres failing to propagate on the neural substrate laminin (ii). (F): Phase contrast image of neural sphere structure derived from $Fibs^{OCT4}$ w/o RM after addition

of neural progenitor culture conditions (i). Phase contrast image of $Fibs^{OCT4}$ w/o RM derived spheres propagating on laminin (ii). Flow cytometry plot demonstrating failure of $Fibs^{OCT4}$ w/o RM derived spheres to express neural progenitor marker SOX2 (iii). All representative images and flow plots are from n=3 experimental replicates. Scale bar= 100 μM. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; Fibs, fibroblasts; GFAP, glial fibrillary acidic protein.

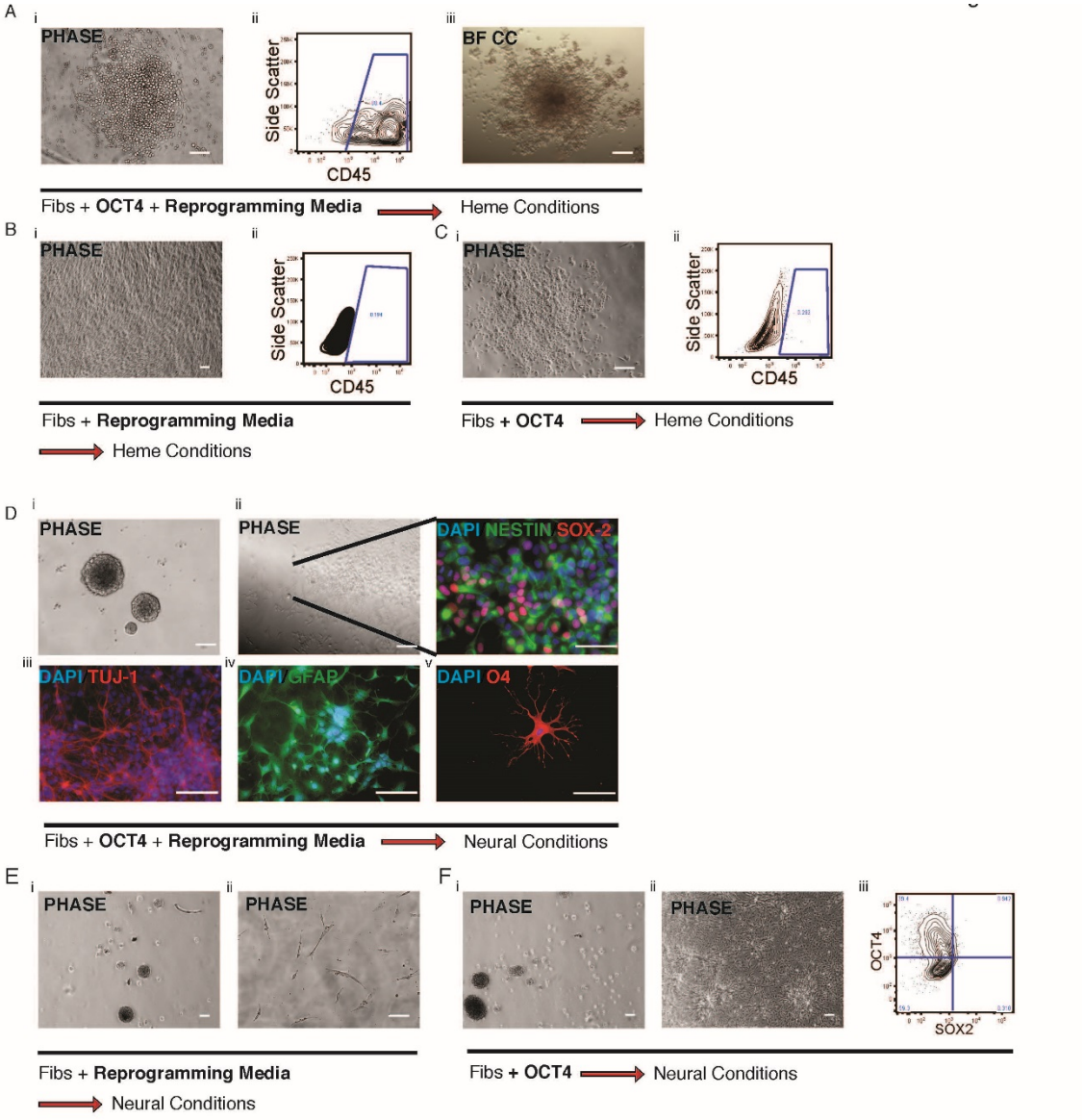

A
i
PHASE
ii
Side Scatter
CD45
iii
BF CC
Fibs + OCT4 + Reprogramming Media
Heme Conditions
B
i
PHASE
ii
Side Scatter
CD45
Fibs + Reprogramming Media
Heme Conditions
C
i
PHASE
ii
Side Scatter
CD45
Fibs + OCT4
Heme Conditions
D
i
PHASE
ii
PHASE
DAPI NESTIN SOX-2
iii
DAPI TUJ-1
iv
DAPI GFAP
v
DAPI O4
Fibs + OCT4 + Reprogramming Media
Neural Conditions
E
i
PHASE
ii
PHASE
Fibs + Reprogramming Media
Neural Conditions
F
i
PHASE
ii
PHASE
iii
OCT4
SOX2
Fibs + OCT4
Neural Conditions

Figure 2. RM is required to sustain OCT4 expressing adult human fibroblasts for use in direct conversion reprogramming.

(A): Representative phase contrast images of (i) $Fibs^{OCT4}$ w/RM—[yellow dashed circles indicate area of interest], (ii) hFibs, (iii) $Fibs^{OCT4}$ w/o RM, and (iv) $Fibs^{eGFP}$ w/RM. (B): Flow cytometry plots illustrating OCT4 expression in $Fibs^{OCT4}$w/ versus w/o RM over a 5 day time course. (C): Flow cytometry plots illustrating eGFP expression in $Fibs^{eGFP}$w/ versus w/o RM over a 5 day time course. (D): Flow cytometry plots illustrating OCT4 and eGFP co-expression in $Fibs^{OCT4+eGFP}$w/ vs w/o RM over a 5 day time course. All representative images and flow plots are from n=3 experimental replicates. Scale bar=100 μM. Abbreviations: eGFP, enhanced green fluorescent protein; Fibs, fibroblasts; RM, reprogramming media.

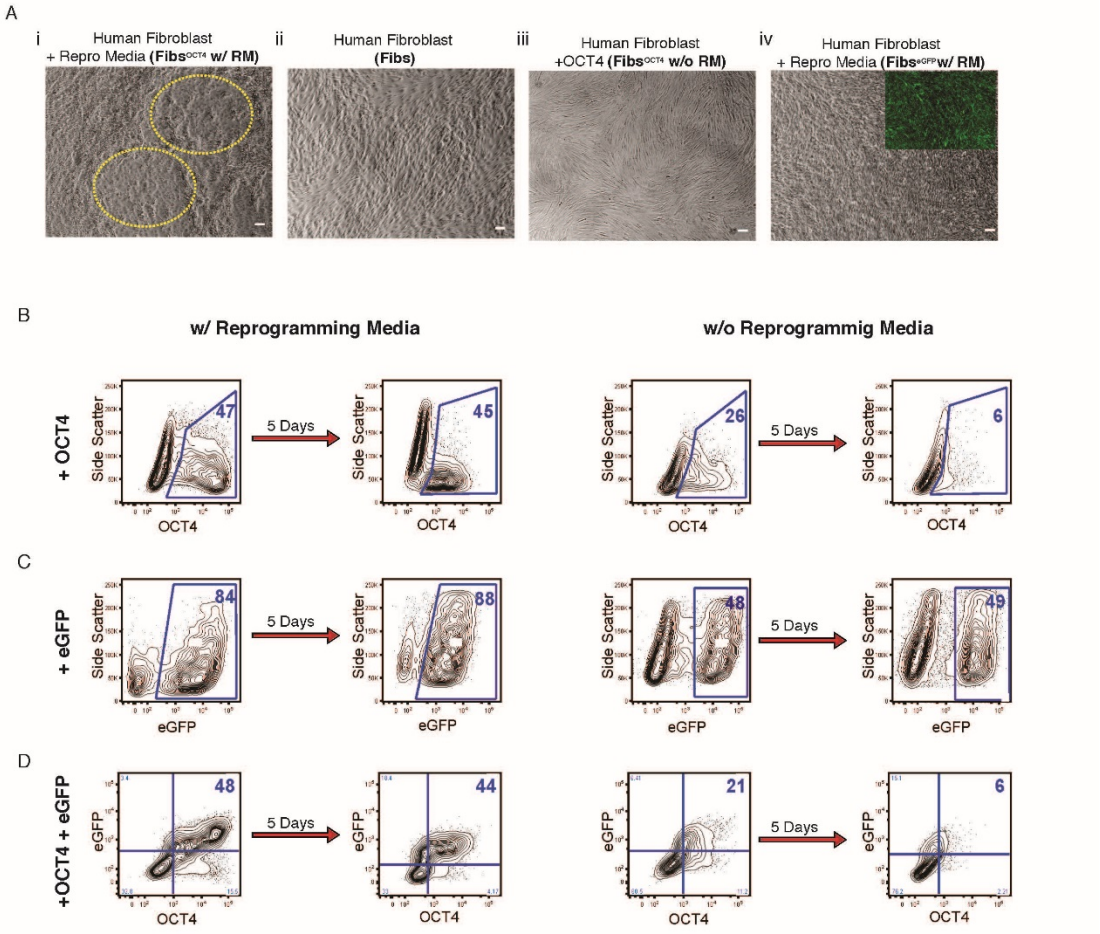

A
i Human Fibroblast + Repro Media (Fibs^OCT4 w/ RM)
ii Human Fibroblast (Fibs)
iii Human Fibroblast +OCT4 (Fibs^OCT4 w/o RM)
iv Human Fibroblast + Repro Media (Fibs^eGFP w/ RM)
B
w/ Reprogramming Media
w/o Reprogrammig Media
+ OCT4
Side Scatter
OCT4
47
45
26
6
5 Days
C
+ eGFP
Side Scatter
eGFP
84
88
48
49
5 Days
D
+OCT4 + eGFP
eGFP
OCT4
48
44
21
6
5 Days

Figure 3. Characterization of unique global transcription changes in Fibs^{OCT4}w/ RM.

(A): Principle component analysis (PCA) on all entities from global gene expression profiling of fibroblasts, Fibs^{eGFP}w/ RM, Fibs^{OCT4}w/ RM, and Fibs^{OCT4}w/o RM. (B): Venn diagram illustrating differential gene upregulation between Fibs^{OCT4}w/o RM and Fibs^{OCT4}w/ RM compared to fibroblasts. (C): Venn diagram illustrating differential gene downregulation between Fibs^{OCT4} w/o RM and Fibs^{OCT4}w/RM compared to fibroblasts. (D): GO analysis of differentially expressed upregulated genes between Fibs^{OCT4} w/o RM and Fibs^{OCT4}w/ RM. (E): Venn diagram illustrating differential gene upregulation between Fibs^{eGFP} w/RM and Fibs w/RM compared to fibroblasts. (F): Venn diagram illustrating differential gene downregulation between Fibs^{eGFP} w/RM and Fibs w/RM compared to fibroblasts. (G): Gene ontology analysis of differentially expressed genes between Fibs^{eGFP} w/RM and Fibs w/RM. (H): PCA analysis on all entities from global gene expression profiling of fibroblasts, Fibs^{4F} w/RM, and Fibs^{OCT4} w/RM. (I): Venn diagram illustrating differential gene upregulation between Fibs^{4F} w/RM and Fibs^{OCT4} w/RM compared to fibroblasts. (J): Venn diagram illustrating differential gene downregulation between Fibs4F w/RM and Fibs^{OCT4} w/RM compared to fibroblasts. (K): GO analysis of differential gene expression between Fibs^{4F} w/RM and Fibs^{OCT4} w/RM. All gene expression analysis was based on n=2 or n=3 experimental replicates per sample. Abbreviations: Fibs, fibroblasts; GO, gene ontology; RM, reprogramming media.

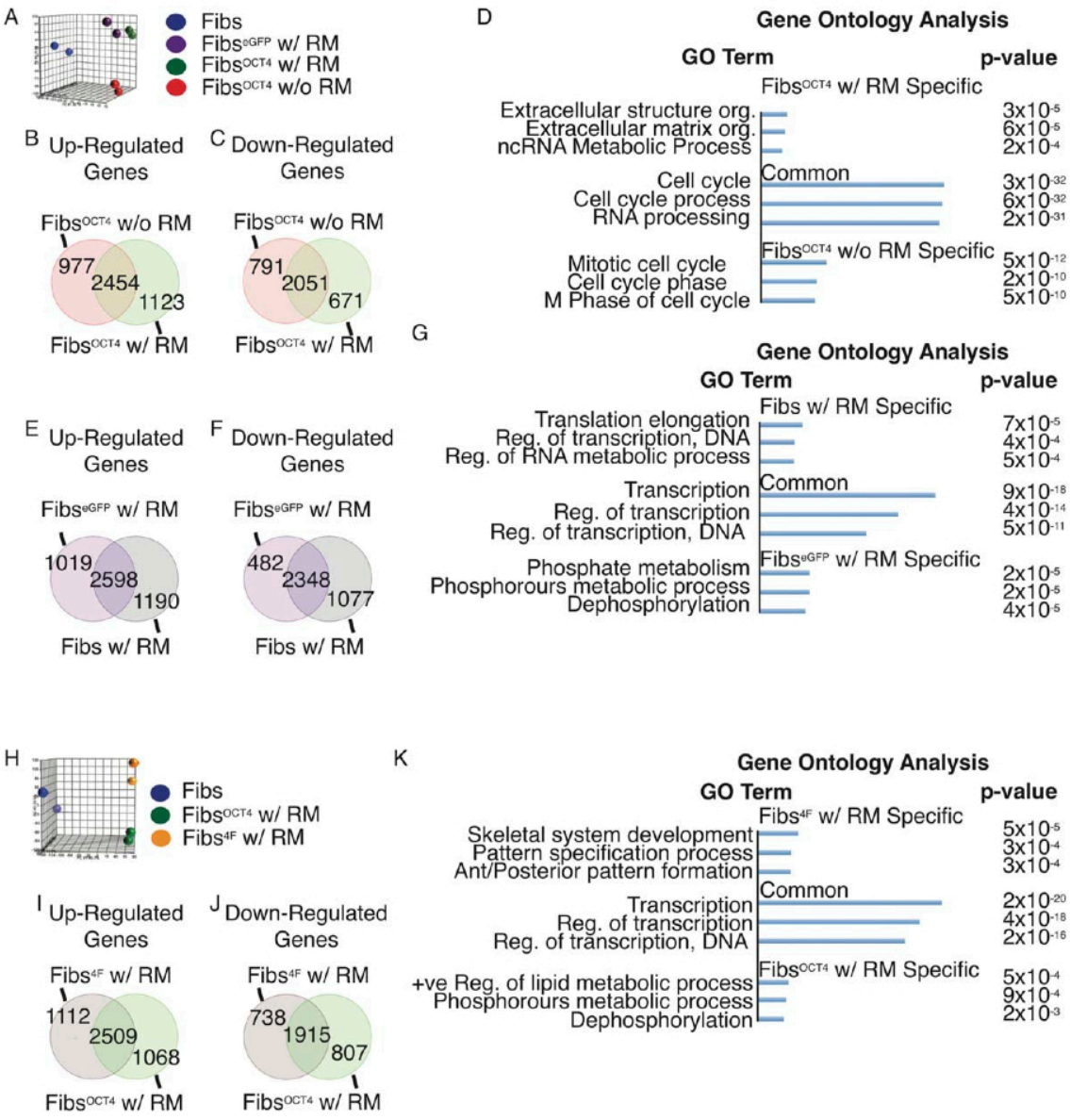

A
Fibs
Fibs^eGFP w/ RM
Fibs^OCT4 w/ RM
Fibs^OCT4 w/o RM
B Up-Regulated Genes
Fibs^OCT4 w/o RM
977
2454
1123
Fibs^OCT4 w/ RM
C Down-Regulated Genes
Fibs^OCT4 w/o RM
791
2051
671
Fibs^OCT4 w/ RM
D
Gene Ontology Analysis
GO Term
p-value
Fibs^OCT4 w/ RM Specific
Extracellular structure org.
Extracellular matrix org.
ncRNA Metabolic Process
3x10-5
6x10-5
2x10-4
Common
Cell cycle
Cell cycle process
RNA processing
3x10-32
6x10-32
2x10-31
Fibs^OCT4 w/o RM Specific
Mitotic cell cycle
Cell cycle phase
M Phase of cell cycle
5x10-12
2x10-10
5x10-10
E Up-Regulated Genes
Fibs^eGFP w/ RM
1019
2598
1190
Fibs w/ RM
F Down-Regulated Genes
Fibs^eGFP w/ RM
482
2348
1077
Fibs w/ RM
G
Gene Ontology Analysis
GO Term
p-value
Fibs w/ RM Specific
Translation elongation
Reg. of transcription, DNA
Reg. of RNA metabolic process
7x10-5
4x10-4
5x10-4
Common
Transcription
Reg. of transcription
Reg. of transcription, DNA
9x10-18
4x10-14
5x10-11
Fibs^eGFP w/ RM Specific
Phosphate metabolism
Phosphorours metabolic process
Dephosphorylation
2x10-5
2x10-5
4x10-5
H
Fibs
Fibs^OCT4 w/ RM
Fibs^4F w/ RM
I Up-Regulated Genes
Fibs^4F w/ RM
1112
2509
1068
Fibs^OCT4 w/ RM
J Down-Regulated Genes
Fibs^4F w/ RM
738
1915
807
Fibs^OCT4 w/ RM
K
Gene Ontology Analysis
GO Term
p-value
Fibs^4F w/ RM Specific
Skeletal system development
Pattern specification process
Ant/Posterior pattern formation
5x10-5
3x10-4
3x10-4
Common
Transcription
Reg. of transcription
Reg. of transcription, DNA
2x10-20
4x10-18
2x10-16
Fibs^OCT4 w/ RM Specific
+ve Reg. of lipid metabolic process
Phosphorours metabolic process
Dephosphorylation
5x10-4
9x10-4
2x10-3

Figure 4. RM and OCT4 expression combined induce plasticity in adult human fibroblasts.

(A): Fibs^{OCT4} w/RM gene expression filtered through three curated developmental gene lists (hematopoiesis, neurogenesis, and endoderm formation), where genes that displayed ≥1.5-fold change compared to fibroblasts were plotted as a heat map. Heat maps are plotted as normalized expression values in fibroblasts compared to Fibs^{OCT4} w/RM. Count = # of significantly upregulated genes. (B): Immunocytochemistry for endodermal markers FOXA2 (i) and NKX2.1 (ii) in Fibs^{OCT4} w/RM after addition of endodermal culture conditions. Representative immunocytochemistry for FOXA2 and NKX2.1 in H9 embryonic stem cells differentiated toward endoderm (iii). All representative images and flow plots are from n = 3 experimental replicates. Scale bar=100 μM. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; Fibs, fibroblasts; RM, reprogramming media.

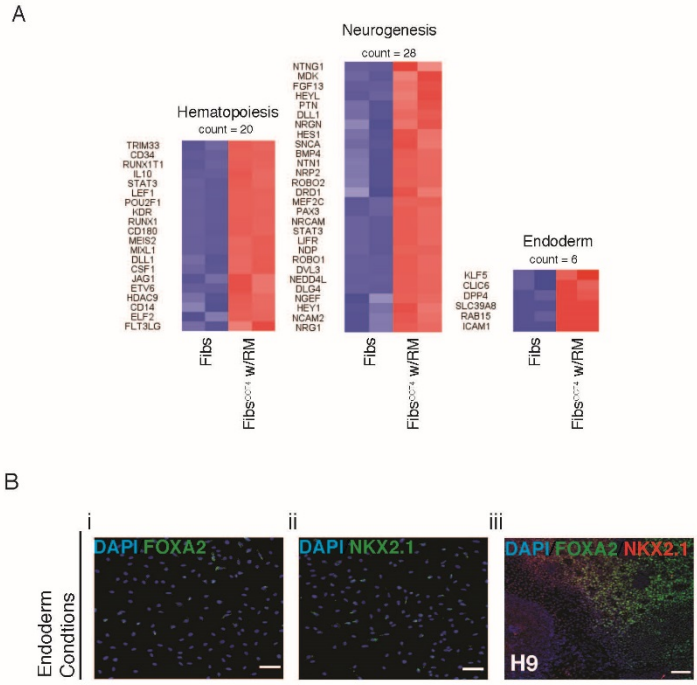
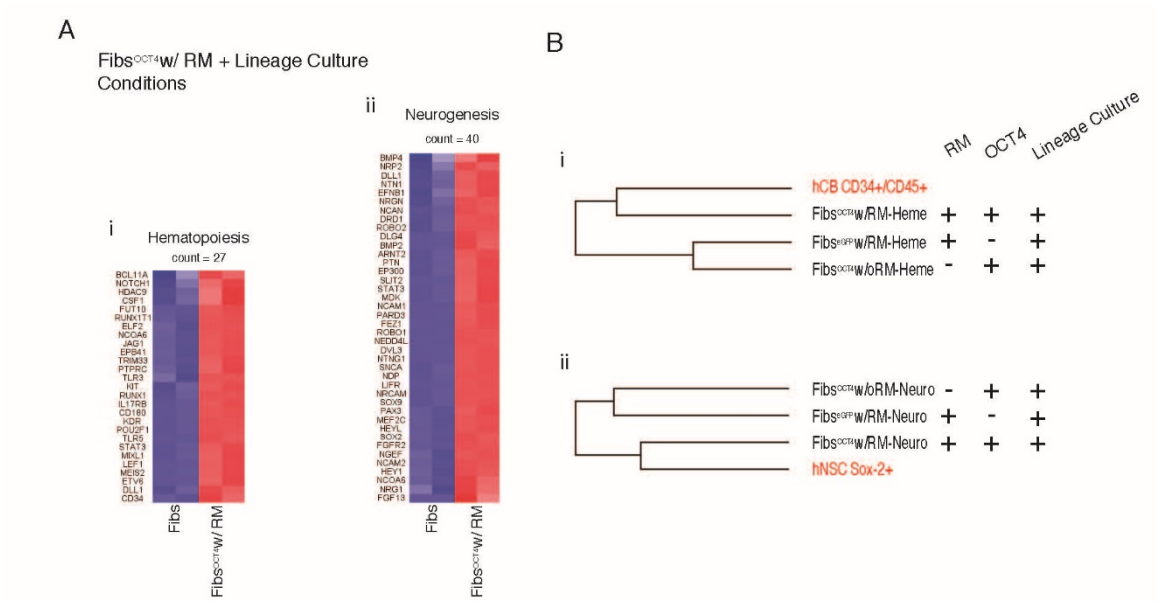

A
Hematopoiesis
count = 20
TRIM33
CD34
RUNX1T1
IL10
STAT3
LEF1
POU2F1
KDR
RUNX1
CD180
MEIS2
MKL1
DLL1
CSF1
JAG1
ETV6
HDAC9
CD14
ELF2
FLT3LG
Fibs
Fibs OCT4 w/RM
Neurogenesis
count = 28
NTNG1
MDK
FGF13
HEYL
PTN
DLL1
NRGN
HES1
SNCA
BMP4
NTN1
NRP2
ROBO2
DRD1
MEF2C
PAX3
NRCAM
STAT3
LIFR
NDP
ROBO1
DVL3
NEDD4L
DLG4
NGEF
HEY1
NCAM2
NRG1
Fibs
Fibs OCT4 w/RM
Endoderm
count = 6
KLF5
CLIC6
DPP4
SLC39A8
RAB15
ICAM1
Fibs
Fibs OCT4 w/RM
B
i
ii
iii
Endoderm Condtions
DAPI FOXA2
DAPI NKX2.1
DAPI FOXA2 NKX2.1
H9

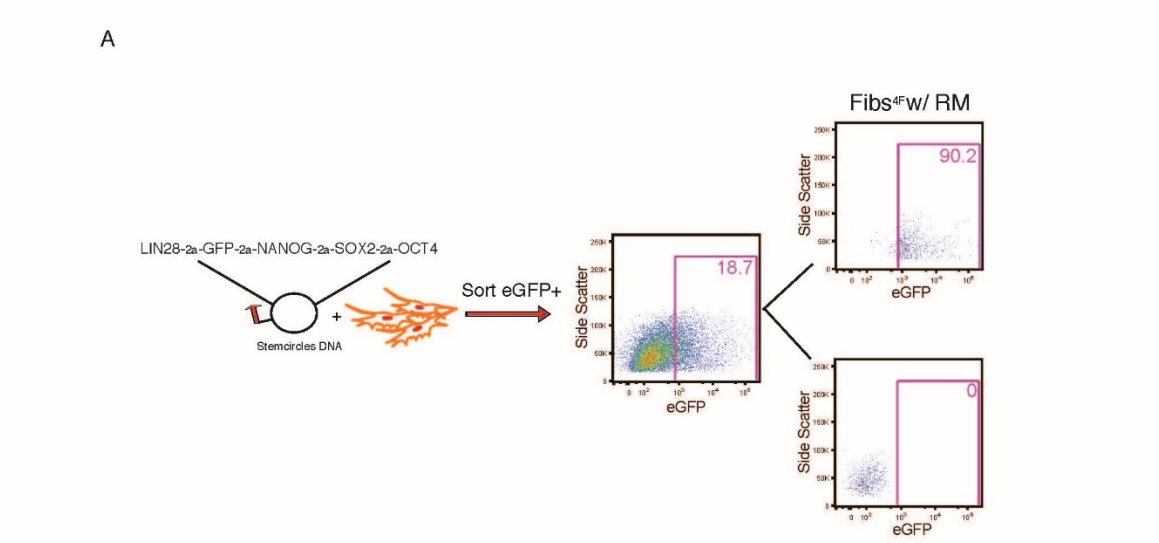
Figure 5. Fibs^{OCT4} w/RM respond to changes in the extracellular environment through activation of lineage development transcription present in somatic stem/progenitor cells.

(A): Global gene expression from Fibs^{OCT4} w/RM cultured in (i) hematopoietic supportive conditions or (ii) neural supportive conditions filtered through hematopoiesis and neurogenesis gene lists respectively, where genes that displayed ≥1.5-fold change compared to fibroblasts were plotted as a heat map. Heat maps are plotted as normalized expression values in fibroblasts compared to Fibs^{OCT4}w/ RM after culture in supportive conditions. (B): Hierarchal cluster analysis on Fibs^{eGFP} w/RM, Fibs^{OCT4} w/o RM, Fibs^{OCT4} w/RM and neural (hNSC SOX2+) or hematopoietic (hCB CD34+CD45+) somatic stem/progenitor cells. Cluster analysis of cells treated with hematopoietic supportive conditions and hCB CD34+CD45+ stem/progenitor cells, filtered on hematopoietic gene list (i). Cluster analysis of cells treated with neural supportive culture conditions and hNSC SOX2+ stem/progenitor cells, filtered on neurogenesis gene list (ii). All gene expression analysis was based on n = 2 or n = 3 experimental replicates per sample. Abbreviations: Fibs, fibroblasts; RM, reprogramming media.

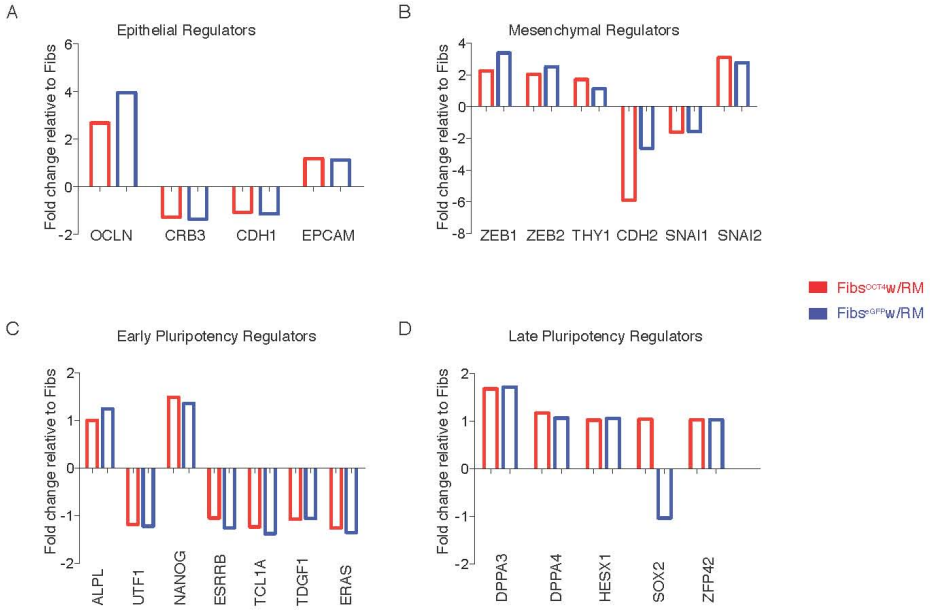

A
Fibs^OCT4w/ RM + Lineage Culture Conditions
i
Hematopoiesis
count = 27
BCL11A
NOTCH1
HDAC9
CSF1
FUT10
RUNX1T1
ELF2
NCOA6
JAG1
EPB41
TRIM33
PTPRC
TLR3
HIT
RUNX1
IL17RB
CD180
KDR
POU2F1
TLR6
STAT3
MKL1
LEF1
MEIS2
ETV6
DLL1
CD34
Fibs
Fibs^OCT4w/ RM
ii
Neurogenesis
count = 40
BMP4
NRP2
DLL1
NTN1
EFNB1
NRGN
NCAN
DRD1
ROBO2
DLG4
BMP2
ARNT2
PTN
EP300
SLIT2
STAT3
MDK
NCAM1
PARD3
FEZ1
ROBO1
NEDD4L
DVL3
NTNG1
SNCA
NDP
LIFR
NRCAM
SOX9
PAX3
MEF2C
HEY1
SOX2
FGFR2
NGEF
NCAM2
HEY1
NCOA6
NRG1
FGF13
Fibs
Fibs^OCT4w/ RM
B
RM
OCT4
Lineage Culture
i
hCB CD34+/CD45+
Fibs^OCT4w/RM-Heme + + +
Fibs^eGFPw/RM-Heme + - +
Fibs^OCT4w/oRM-Heme - + +
ii
Fibs^OCT4w/oRM-Neuro - + +
Fibs^eGFPw/RM-Neuro + - +
Fibs^OCT4w/RM-Neuro + + +
hNSC Sox-2+

Supplementary Figure 1. FACS enrichment of hFibs^{4F} w/RM

A, Fluorescence activated cell sorting strategy for isolation of RM cultured fibroblasts expressing OCT4, SOX2, NANOG, LIN28, and eGFP. Representative flow cytometry plots illustrating sort purity. Representative flow plots are from n=3 technical replicates.


A
LIN28-2a-GFP-2a-NANOG-2a-SOX2-2a-OCT4
Stemcircles DNA
Sort eGFP+
Side Scatter
eGFP
18.7
Fibs4Fw/ RM
90.2
0

Supplementary Figure 2. Fibs^{OCT4}w/ RM lack hallmarks of early iPSC reprogramming A, Epithelial regulator gene expression of Fibs^{eGFP}w/ RM and Fibs^{OCT4}w/ RM shown as fold change in normalized expression relative to standard cultured fibroblasts. B, mesenchymal regulator gene expression of Fibs^{eGFP}w/ RM and Fibs^{OCT4}w/ RM shown as fold change in normalized expression relative to standard cultured fibroblasts. C, Early pluripotency regulator gene expression of Fibs^{eGFP}w/ RM and Fibs^{OCT4}w/ RM shown as fold change in normalized expression relative to standard cultured fibroblasts. D, late pluripotency regulator gene expression of Fibs^{eGFP}w/ RM and Fibs^{OCT4}w/ RM shown as fold change in normalized expression relative to standard cultured fibroblasts. Values derived from n=2 experimental replicate.


A
Epithelial Regulators
Fold change relative to Fibs
OCLN
CRB3
CDH1
EPCAM
B
Mesenchymal Regulators
Fold change relative to Fibs
ZEB1
ZEB2
THY1
CDH2
SNAI1
SNAI2
FibsOCT4w/RM
FibsEGFPw/RM
C
Early Pluripotency Regulators
Fold change relative to Fibs
ALPL
UTF1
NANOG
ESRRB
TCL1A
TDGF1
ERAS
D
Late Pluripotency Regulators
Fold change relative to Fibs
DPPA3
DPPA4
HESX1
SOX2
ZFP42

Chapter 4

Single Transcription Factor Conversion of Human Blood Fate to NPCs with CNS and PNC Developmental Capacity

Preamble
This chapter is an original published article. It is presented in its published format.

"This research was originally published in Cell Reports. Jong-Hee Lee, Ryan R. Mitchell*, Jamie D. McNicol, Zoya Shapovalova, Sarah Laronde, Borko Tanasijevic, Chloe Milsom, Fanny Casado, Aline Fiebig-Comyn, Tony J. Collins, Karun K. Singh, Mickie Bhatia. Single Transcription Factor Conversion of Human Blood Fate to NPCs with CNS and PNS Developmental Capacity. http://dx.doi.org/10.1016/j.celrep.2015.04.056. © 2015 The Authors. Published by Elsevier Inc."*

**Co First Author*

Together with Dr. Lee and Dr. Bhatia I conceived the idea for the study and its basic design. I co-wrote the paper with Dr. Lee. I performed all fibroblast based reprogramming and prepared all samples for transcription array analysis. I designed and implemented the global gene expression analysis with technical assistance from Zoya Shapovalova. Jamie D. McNicol provided technical assistance with calcium imaging. Sarah Laronde provided assistance with blood derived NPC conversion. Dr. Borko Tanasijevic provided technical assistance with RT-PCR. Dr. Chloe Milsom provided technical assistance with immunocytochemistry of mouse xenograft tissue samples. Dr. Fanny Casado provided technical assistance with HPLC. Aline Fiebig-Comyn provided

technical assistance with animal work. Dr. Tony J Collins performed calcium imaging and designed the taxol neurite assay. Dr. Mick Bhatia oversaw the entire study and co-wrote the paper.

Understanding that expression of OCT4 in combination with RM was sufficient to induce a state of plasticity in fibroblasts that facilitates reprogramming, we questioned whether we could apply this methodology to alternative somatic cells. Moreover, as one of the major goals of cellular reprogramming is to develop personalized medicine disease models and transplant products, we felt it would be advantageous to retool our reprogramming strategy to make use of a somatic tissue source that is routinely banked in the clinic, such as blood. In executing this study, we were able to define specific extracellular requirements for blood versus fibroblast reprogramming, and performed a comparative transcriptional analysis in order to molecular describe these differences. Although not central to this thesis, we demonstrated the generation of a novel NPC that was competent for differentiation towards both central nervous system and peripheral nervous system related progeny. Using this method, we generated sensory neurons that modeled chemotherapy induced neuropathy in a microwell format suitable for screening.

Single transcription factor conversion of human blood fate to NPCs with CNS and PNS developmental capacity

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Summary

The clinical applicability of direct cell fate conversion depends on obtaining tissue from patients that is easy to harvest, store, and manipulate for reprogramming. Here we generate induced neural progenitor cells (iNPCs) from neonatal and adult peripheral blood using single factor OCT4 reprogramming. Unlike fibroblasts that share molecular hallmarks of neural crest, OCT4 reprogramming of blood was facilitated by SMAD+GSK-3 inhibition to overcome restrictions on neural fate conversion. Blood derived (BD)-iNPCs differentiate *in vivo*, and respond to guided differentiation *in vitro* producing glia (astrocytes and oligodendrocytes) and multiple neuronal subtypes including dopaminergic (CNS related) and nociceptive neurons (PNS). Furthermore, nociceptive neurons phenocopy chemotherapy induced neurotoxicity in a system suitable for high throughput drug screening. Our findings provide an easily accessible approach to generate human NPCs that harbor extensive developmental potential, enabling the study of clinically relevant neural diseases directly from patient cohorts.

Highlights

• Human blood can be directly converted to tri-potent iNPCs with a single factor

• BD-iNPCs uniquely differentiate to neurons with properties of both CNS and PNS

• Conversion process towards iNPCs from blood differs from fibroblasts

• Nociceptive neurons recapitulate chemo-induced neuropathy in a screening format

Introduction

The reprogramming of adult cells into alternative tissue cell fates holds promise for regenerative medicine and drug discovery, especially for human cell types that are difficult to procure such as neural tissue (Sancho-Martinez et al., 2012). However, significant limitations remain using current technology as it relates to human sources, thus novel approaches that allow generation of large numbers of renewable neural cells from easily accessible tissues derived from donors is required. Complete cellular reprogramming to the pluripotent state has gone some way to realize this promise (Takahashi and Yamanaka, 2006). However, although transformative, this advance is limited by costly and time consuming methods over several months to first derive skin fibroblasts and then generate and characterize resulting iPSCs (Stacey et al., 2013). Furthermore, resulting iPSCs acquire inefficiencies in lineage specific differentiation from pluripotent state that limits reproducible production of specific mature cell types (Lee et al., 2014). Similarly, use of hiPSCs in cell replacement therapy continues to precipitate barriers and concerns that require laborious measures to assure resulting cells are free from tumor forming pluripotent cells has yet to be resolved (Cunningham et al., 2012).

More recent studies have established a paradigm whereby forced expression of lineage-specific factors allows direct reprogramming into differentiated somatic cells, including cardiomyocytes, hepatocyte-like cells, blood and neurons without iPSC formation (Efe et al., 2011; Pang et al., 2011; Szabo et al., 2010). However, direct cell fate reprogramming of human cells is accompanied by other limitations and remains

inefficient, requiring multiple transcription factors to be ectopically expressed in every cell, and is largely based on difficult to obtain human skin biopsies that are not available from historical clinical studies. Alternatively, blood cells can be readily obtained from patients, require no culture derivation prior to reprogramming, and have been stored and banked (Broxmeyer, 2010) from large cohort patient trials in the past such as those suffering from neurological disorders (http://brainbank.ucla.edu; http://www.clsa-elcv.ca/). Here we applied our unique OCT4 induced plasticity reprogramming approach (Mitchell et al., 2014a) in combination with previously identified neural potentiating small molecules(Chambers et al., 2009; Li et al., 2011) to directly convert human blood progenitors derived from both cord blood and adult sources to neural progenitor cells (NPCs). We demonstrate that these human Blood derived (BD) NPCs are capable of *in vivo* differentiation and survival as well as tri-potent neural differentiation *in vitro* that includes neuronal differentiation towards clinically relevant CNS and PNS subtypes.

Results

Generation of iNPCs from neonatal and adult blood cells using OCT4 and SMAD+ GSK-3 inhibition

In an effort to make use of readily accessible hematopoietic cells as a starting material to generate neural derivatives, we employed OCT4 based reprogramming (Mitchell et al., 2014a; Mitchell et al., 2014b) to both cord blood and adult peripheral blood progenitors (Fig.S1A). Human blood cells from both sources were negative for pluripotent markers (SSEA3, TRA1-60), early neural markers (NESTIN, PAX6) as well as neural crest (NC) markers (p75, CD57) (Fig.S1B), thereby excluding the presence of contaminating cells with pluripotent or NPC features within the starting blood samples. Transduction with OCT4 alone has previously been shown to induce human skin fibroblast conversion to tri-potent neural progenitors (Mitchell et al., 2014a), despite reports suggesting the requirement for a chemically diverse cocktail of inhibitors in addition to OCT4 (Zhu et al., 2014). However, transduction of human blood with OCT4 alone failed to induce production of iNPCs (Fig.1A-C). As both inhibition of SMAD and glycogen synthase kinase-3 (GSK-3) signaling have been independently reported to efficiently neuralize hPSCs (Chambers et al., 2009), we examined whether dual inhibition with SMAD and GSK-3 chemical inhibitors could facilitate iNPC generation from blood coupled with OCT4 induced plasticity (Fig.1A). When human blood progenitors obtained from neonatal cord blood or adult peripheral blood expressing OCT4 were transferred to SMAD+GSK-3 inhibition conditions (SB431542, LDN-193189, Noggin, CHIR99021), iNPC-like clusters appeared within as little as 8-10 days and showed the expression of the neural stem cell marker, NESTIN (Fig.1B and Fig.S1C). Addition of these same

molecules to human fibroblasts had no effect on NPC generation (Mitchell et al., 2014a; Mitchell et al., 2014b) (data not shown). As SOX2 has also been implicated in direct-fate reprogramming towards the neural lineage (Ring et al., 2012), we tested whether SOX2 transduction alone or in combination with OCT4 enhanced iNPC-like cluster formation. We found no detectable iNPC-like clusters upon expression of SOX2 alone, as well as reduced iNPC formation when used in combination with OCT4 (Fig.1C). The use of OCT4 expression combined with chemical inhibitors was a highly efficient process, and up to 12 putative iNPC-like colonies could be generated from as few as 50,000 (50K) human blood progenitors (Fig.1C). OCT4-dependent generation of human iNPC colonies could not be established from more mature blood cells devoid of CD34 expression (CD34-) and was restricted to the hematopoietic progenitor-containing compartment (Fig.1D). Furthermore, individual iNPC-like colonies demonstrated robust survival that allowed subsequent collection and re-culturing to promote cell proliferation and expansion into primary neurospheres using suspension culture (Ring et al., 2012) conditions known to support human NPCs (Fig.1E). The absence of pluripotent markers (TRA1-60 and SSEA3) demonstrated that OCT4 induced iNPCs were not products of intermediate pluripotent states (Fig.S1D), which was further supported by the failure to give rise to teratomas when transplanted into immunodeficient mice (Fig.S1E). The complete absence of a pluripotent cell from human blood derived OCT4-induced iNPC also removes safety concerns regarding potential future use of BD-iNPCs. BD-iNPCs derived from either neonatal cord blood or adult peripheral blood consistently expressed neural stem cell associated markers including PAX6, NESTIN, SOX2 and CD133 similar

to control human NPCs (Fig.1F,G and Fig.S1F,G). Moreover, cultured BD-iNPCs contained ki67 expressing proliferative cells (Fig.S1F) that enabled serial passaging without the loss of NPC marker expression, neural transcriptional programs, or genomic integrity (Fig.S1H-J and S2A,B). As a testament to their robust practical utility, with an average of as few as 12 iNPC colonies consistently generated from 50K human blood progenitors, we have determined that we can generate as many as 100 million progenitor cells over 10 passages (Fig.1H) using this direct conversion approach from human blood samples.

SMAD+GSK-3 inhibition facilitates NPC generation from human blood

In order to gain a better understanding for the requirement of dual SMAD+GSK-3 inhibition during OCT4 mediated conversion of human blood cells to iNPCs, we assembled molecular profiles of blood cells expressing OCT4 that were either treated or not treated with inhibitors, and compared them to profiles of recently described Fibs-iNPC^{OCT4} that were derived in the same fashion. To evaluate the molecular profiles, we performed hierarchal cluster analysis of global gene expression profiles and included SOX2 expressing primary neural stem/progenitor cells isolated from human brain tissue as a base of reference (Fig.2A). As expected, Fib-iNPCs were highly related to primary human NPCs regardless of inhibitor addition (Mitchell et al., 2014b), whereas BD-iNPCs required SMAD+GSK-3 inhibition in order to cluster together with primary NPCs (Fig.2A). Investigation of differential gene regulation between +/- inhibitor treated fibroblasts and blood cells during generation of NPCs displayed minimal changes in fibroblast transcriptome compared to blood cells, suggesting a unique role for

SMAD+GSK-3 inhibition during blood based OCT4 reprogramming (Fig.2B). In order to classify the gene programs that were specifically regulated in blood cells undergoing OCT4 iNPC reprogramming, we performed gene set enrichment analysis on blood cells +/- inhibitor treatment during derivation of NPCs. The addition of SMAD+GSK-3 inhibition resulted in the enrichment of multiple neural related gene sets that were otherwise not activated in the presence of OCT4 expression alone (Fig.2C). Furthermore, filtering of both up- and down-regulated genes using the Tissue Expression analysis tool on DAVID Bioinformatics Resource revealed enrichment of down-regulated genes within hematopoietic programs and up-regulated genes within neural programs (Fig.S3). In order to validate the trends from our molecular profiling studies, we performed candidate qPCR on BD-iNPCs for potent hematopoietic and neural progenitor regulatory genes, which confirmed a successful molecular switch from blood to neural progenitors (Fig.2D). These detailed analyses indicate the processes involved in conversion of human skin fibroblasts to NPCs vs. blood derived NPCs are also molecularly distinct, and reveal a complete conversion of human blood progenitors to NPC fate that is not limited to phenotypic alternations alone.

BD-iNPCs expand and functionally respond to in vivo and directed in vitro differentiation cues

Having established the role for SMAD+GSK-3 inhibition during the initial generation of BD-iNPCs from human blood progenitors, we next examined the direct effects on proliferative expansion and developmental potential of the resulting BD-iNPCs. SMAD+GSK-3 inhibition resulted in enhanced proliferation of BD-iNPCs compared with

inhibitor-withdrawn cultures (Fig.S4A). However, enhanced proliferation came at the expense of differentiation, as BD-iNPCs maintained in the presence of SMAD+GSK-3 that were transferred to culture conditions conducive for neuronal-differentiation (Fig.S4B), displayed continued PAX6 expression but failed to upregulate Tuj1 compared to BD-iNPCs where inhibitors were withdrawn (Fig.S4C,D). Despite a clear indication that inhibitor treatment imposed differentiation block, this phenomena was rapidly reversed within one round of passaging in the absence of inhibitors, indicative of successful maintenance of differentiation potential throughout proliferative cycles (Fig.S4E, F). Interestingly, quantified levels of PAX6 in long-term cultures revealed the expression of the NPC marker was higher in the presence of SMAD+GSK-3 inhibitors, although the frequencies of positive cells were comparable (Fig.S4G, H). These results reveal that modulation of SMAD+GSK-3 signaling plays an important role in the regulation of proliferation and differentiation potential of BD-iNPCs.

We next set out to evaluate the developmental potential of OCT4 induced BD-iNPCs by assessing their ability to functionally differentiate *in vivo* towards the three main neural lineages. BD-iNPCs were transduced with a GFP expressing lentiviral vector and then injected into the brains of p2-p4 mouse pups and allowed to engraft for 3 weeks (Zhu et al., 2014). Analysis of GFP signal from sectioned brain tissue as a surrogate of human engraftment revealed multiple sites containing intact human cells (Fig.3A). Investigation for differentiated BD-iNPC progeny revealed populations of GFP positive cells that co-expressed both TUJ1 and MAP2 with clear neuronal morphology (Fig.3B). Moreover, we identified GFP positive human cells that also co-expressed glial fibrillary

acidic protein (GFAP), consistent with the presence of astrocytes (Fig.3B). Despite confirming *in vivo* differentiation potential towards both neurons and astrocytes, we did not find evidence of *in vivo* differentiation towards oligodendrocytes, a finding not unlike that of other human iNPC studies which have relied on murine xenograft assays (Zhu et al., 2014).

Although *in vivo* xenograft studies are considered to be the gold standard for many assays of human biology that can otherwise not be measured, *in vitro* differentiation allows for the directed production of specific cell types that will likely be useful in near term personalized medicine applications of drug screening/testing rather than cellular transplantation. Despite limited detection of oligodendrocytes in our *in vivo* tests, BD-iNPCs possessed astrocyte and oligodendrocyte differentiation potential *in vitro* as evidenced by GFAP and O4 expression, respectively, with characteristic morphology similar to differentiated cells from human PSCs (Fig.3C, D and Fig.S4I). Furthermore, culture conditions for the specification for neuronal development resulted in mature neurons expressing canonical markers Tuj1 and MAP2 (Fig.3E and Fig.S4J), with the majority expressing high levels of glutamate, consistent with excitatory glutamatergic neurons. Importantly, prior to differentiation BD-iNPCs express OCT4 transgene at observable levels, however similar to previous reports (Mitchell et al., 2014b), OCT4 expression is silenced upon complete differentiation towards mature functional cells types (Fig.S4K). Using specific conditions for GABAergic neurons, we successfully generated GABA-positive inhibitory neurons (Fig.3E), suggesting BD-iNPCs harbored broad neuronal developmental potential. Moreover, BD-iNPC derived neurons also exhibited a

punctate pattern of synapsin expression suggesting the development of synapses (Fig.3F), which was confirmed using electrophysiological analysis (Fig.3G-I). Specifically, upon positive current injection, spontaneous repetitive action potential firing was induced (Fig.3G) and voltage-dependent transient Na+ and sustained K+ currents were detected (Fig.S4L). Application of tetrodotoxin (TTX) blocked rapidly activating and inactivating inward currents, further demonstrating that the differentiated neurons expressed voltage-activated sodium channels associated with primary neurons (Fig.3I). Thus, neurons derived from iNPCs appear to exhibit the functional membrane properties and activities of mature neurons. Having observed robust functional neuronal differentiation activity, we investigated whether BD-iNPCs neuronal differentiation capacity could be expanded into more specialized neurons, such as dopaminergic (DA) neurons, in response to specific instructions. We found that treatment with Sonic Hedgehog (SHH) and FGF8b (Li et al., 2011) further differentiated BD-iNPCs into neurons expressing tyrosine hydrolase (TH), the rate-limiting enzyme in the synthesis of DA (Fig.3J). These neurons also expressed the nuclear receptor NURR1 (a.k.a. NR4A2), a key regulator of the dopaminergic system (Fig.3J). Moreover, the detection of secreted DA in culture medium further supported the presence of functional dopaminergic neurons *in vitro* (Fig.3K).

Taken together these results confirm that BD-iNPCs are capable of robust expansion without sacrificing their broad developmental potential, and thereby exhibit the most critical features of bonafide human neural progenitors.

BD-iNPC generate functional nociceptors that model chemotherapy induced neuropathy

Based on the broad neuronal developmental potential of BD-iNPCs, we further analyzed the transcriptome of BD-iNPCs. These analyses revealed an enrichment of neural crest cell related gene activity compared to that found in blood progenitors (Fig.2C). Recent work has demonstrated the conversion of human fibroblasts to both putative neural crest (Kim et al., 2014), as well as sensory neurons (neural crest derived peripheral neurons) using typical lineage specifying transcription factor reprogramming strategies (Blanchard et al., 2015; Wainger et al., 2015). Despite a lack of neural crest or sensory neuron functional activity in starting populations of fibroblasts, these human fibroblasts were found to be enriched for neural crest related genes compared to that of human blood cells, suggesting a transcriptionally primed state of neural potential for conversion towards the neural lineage within skin fibroblast cultures not seen in blood progenitors (Fig.3L). Therefore, in contrast to skin fibroblasts, BD-iNPC conversion involves de novo acquisition of neural crest related gene expression (Fig3M). Based on this observation, we hypothesized that their developmental potential may extend to the peripheral nervous system derivatives, such as sensory neurons.

Recent work has demonstrated that combined small-molecule inhibition (SU5402, DAPT and CHIR99021) converts human pluripotent cells into sensory neurons (nociceptors) (Chambers et al., 2012). Based on previous reports (Chambers et al., 2012; Guo et al., 2013), we made modifications to this procedure (Fig.S5A) and tested whether these small-molecule inhibitors could induce generation of nociceptive sensory neurons from directly converted human neonatal and adult BD-iNPCs. We found that the

canonical sensory neuronal markers ISL1 and BRN3A were expressed within differentiated neuron preparations from cord blood and adult peripheral blood BD-iNPCs, in a similar fashion as hESC-derived cells shown previously (Fig.4A). In addition, sensory culture derived neurons expressed glutamate, consistent with an excitatory glutamatergic neuronal phenotype (Fig.S5B) and demonstrated transcript level expression of sensory neuron related genes such as NTRK1, 2, and 3 receptors, neurofilamin heavy chain peptide (NEFH) and calcitonin related peptide α (CALCA) (Fig.S5C). These results confirmed that upon treatment with appropriate culture conditions, BD-iNPCs were capable of generating putative sensory neurons; supportive of our idea that BD-iNPC developmental potential extends to PNS related progeny.

Given strong clinical interest for furthering our understanding of neurological pain and neuropathy conditions (Bennett and Woods, 2014; Pino, 2010b), combined with the notion that nociceptive neurons (NTRK1 expressing) can be functionally assayed (Blanchard et al., 2015; Wainger et al., 2015), we decided to focus our efforts on nociceptive (NTRK1) neuron generation from BD-iNPCs for further characterization and optimization for use. Analysis of NTRK1 expression at the protein level, revealed approximately 50% of differentiated cell positivity of putative nociceptors (Fig.4B). Over 14 days, analysis of ISL1, BRN3A and NTRK1 expression indicated that putative nociceptive sensory neurons could be sustained and continually generated from differentiating BD-iNPCs over time (Fig.4C). Induced neurons were often organized into ganglia-like structures in long-term culture and expressed Substance P (TAC1) indicating the presence of peptidergic nociceptors (Fig.4D). Moreover, the expression of nociceptor-

specific channels and receptors were upregulated during sensory neural induction (Fig.4E). Expression of the purinergic receptor, P2RX3, considered a unique phenotype of human sensory neurons (Jarvis et al., 2002), was confirmed by immunofluorescence analyses (Fig.4F).

Functionally, human cord blood and adult BD-iNPC differentiated neurons were evaluated using calcium flux in response to α,β-methylene-ATP, a selective agonist of P2X3 (Fig.4G,H) (Jarvis et al., 2002). Although neurons at day 7 post-induction showed expression of putative sensory neuron markers (Fig.4C), only a minimal response to α,β -methylene-ATP was detectable, whereas continued culture to day 14 allowed a robust response to manifest (Fig.4I,J and S5D). This indicates that the development of phenotype alone does not suggest functional capacity in terms of ligand responsiveness- an important caution in pragmatic use of converted human neuronal cell types. Furthermore, both the TRPV1 vanilloid receptor agonist capsaicin and P2X3 agonist α, β-methylene ATP (α,β-meATP) could evoke calcium transients in BD-iNPC derived neurons (Caterina et al., 1997), demonstrating functional activity of nociceptive sensory neurons (Fig.4I,J and S5D). Importantly, A-317491, a selective P2X3 inhibitor, significantly decreased this response (Fig.4K), providing evidence that the α,β -methylene-ATP mode of action was indeed through activation of P2X3 receptors (Fig.4K). Our findings demonstrate the differentiation potential of BD-iNPCs into nociceptive sensory neurons, which, when combined with their expansion capacity, raises the possibility of generating sufficient quantities of these specialized cells for drug discovery, toxicity and screening applications. We have estimated that a single round of

reprogramming could support the generation of as many as 100 million sensory neurons (Fig.4L) from 50K human blood cells.

Approximately 30 to 40 percent of cancer patients experience the cancer treatment complication of chemotherapy-induced peripheral neuropathy (CIPN) (Pino, 2010a) through the direct impact of the drug on nerve fibers causing nerve degeneration and axon dieback (Boyette-Davis et al., 2011; Burakgazi et al., 2011; Liu et al., 2010; Wang et al., 2012). We tested whether BD-iNPC derived sensory neurons showed similar response to chemotherapy treatment *in vitro*. Forty-eight hours after treatment with Taxol, neurites of sensory neurons generated from human blood were quantified and showed a dose-dependent reduction in length without concomitant loss of viability (Fig.4M). This miniaturized and automated approach illustrates the potential utility of these converted cells as an *in vitro* model of human axonopathy for drug-discovery and form the basis for future development to expand to other PNS and CNS disorders.

Discussion

We provide evidence that small molecule inhibitors targeting SMAD+GSK-3 enable ectopic expression of OCT4 to directly convert human blood progenitors into proliferative, non-tumorigenic neural precursors with unique multipotent developmental properties that includes generation of both dopaminergic and sensory neurons. Unlike skin fibroblasts with hallmarks of neural lineages, purified CD34+CD45+ blood is devoid of ectoderm derived cells, and as such BD-iNPCs represent evidence for epigenetic conversion of cell fate state from one developmentally distinct cell type to another (Rieske et al., 2005). Within the context of fibroblast reprogramming, expression of OCT4 and the addition of basal neural progenitor culture conditions is sufficient to support conversion towards iNPCs (Mitchell et al., 2014b) whereas generation of BD-iNPCs shown here is highly dependent on the usage of SMAD+GSK-3 inhibition. As inhibition of these pathways has already been established as a means of specifying neural identity within cells undergoing cell fate changes (Chambers et al., 2009; Li et al., 2011), we hypothesize OCT4 induced plasticity within hematopoietic cells similarly responds to these cues in order to support direct conversion towards the neural lineage. Previous attempts to convert human hematopoietic tissue towards the neural lineage were restricted to the use of neonatal cord blood derived MSCs (Yu et al., 2015) or have resulted in the production of neuronal restricted progenitors with limited proliferative potential (Castano et al., 2014). To the best of our knowledge, our current study defining BD-iNPCs are the first example of tri-lineage neural progenitor cells produced from direct conversion of adult human blood.

Our report uniquely provides a practical and simple approach for generating neural progenitor cells capable of nociceptive neuron differentiation. Although recent work using fibroblasts has demonstrated successful conversion towards pain sensing neurons, these studies require a multi-factor trans-differentiation strategy that bypasses the neural progenitor state (Blanchard et al., 2015; Wainger et al., 2015). As such, each resulting cell is unique from one another given the heterogeneity of fibroblast populations and complex multi-vector integration. BD-iNPCs could aid in realizing goals of better understanding the peripheral-neuropathy component of pain associated with complex disorders such as diabetes and chemotherapy, as well as primary pain that often precedes motor-dysfunction in Parkinson's patients by several years (Reichling and Levine, 2011; Tesfaye et al., 2013). For the purpose of drug screening or replacement therapy, prospective isolation or generating pure populations from single progenitor clones will be critical to moving forward. Moreover, it will be critical to understand how application of selectable inducible expression systems that have been widely used during mouse direct reprogramming strategies may elucidate the required epigenetic transformations (Wapinski et al., 2013) that take place during human direct conversion reprogramming. Thus further studies are required in order to develop appropriate scale-up protocols to allow BD-iNPCs to better serve as a personalized neurological model system, where the conversion process detailed here provides the foundation for patient specific analyses.

Materials and Methods

Cell Culture and derivation of iNPCs

Mononuclear cells from cord blood (CB) or mobilized peripheral blood (MB-PB) were isolated by density gradient centrifugation and lineage depleted (Miltenyi Biotec). CD34+ cells were further selected by immunomagnetic separation (Miltenyi Biotec) and purification efficiency was verified by flow cytometric (FACS) analysis with CD34-PE and CD45-APC antibodies. pSIN-EF1α-Oct4 plasmid (Addgene 16579) was used for lentivirus production with packaging plasmids, pMD2.G (Addgene 12259) and psPAX2 (Addgene 12260). To derive iNPCs, purified CD34+ blood cells were transduced with generated OCT4 lentivirus in the presence of SCF, Flt-3L, IL3, and TPO cytokines (R&D System). After 48 h, CD34+ blood cells were cultured on Matrigel (BD Biosciences) or irradiated MEFs with reprogramming media and 10 ng/mL bFGF (R&D System) for 5 days. The cells were then switched to neural basal medium consisting of DMEM/F12, 1X N2, 1X B27 (Invitrogen) supplemented with, 10 μM SB431542, 100 nM LDN-193189 (Stemgent), 100 ng/mL Noggin (R&D System) and 3 μM CHIR99021 (Stemgent). After 10-14 days neural precursor-like colonies were manually picked, transferred to Poly-ornithine /Laminin (POL)-coated culture plates for propagation with neural induction medium supplemented with 20 ng/mL bFGF and 20 ng/mL EGF (R&D System). Primary neurosphere culture was used to further enrich iNPCs.

Teratoma assay

iNPCs or undifferentiated hPSCs (1 x 10^6 cells/mouse) were IT injected into NOD/SCID mice as described previously (Werbowetski-Ogilvie et al., 2009). 8 weeks post-injection,

mouse testicles were harvested, sectioned and stained with haematoxylin and eosin. Images were acquired using ScanScope CS digital slide scanner (Aperio, CA, USA).

iNPC differentiation

For neuronal differentiation, basal media was supplemented with 2 μM retinoic acid (Sigma), 5 μM forskolin (Stemgent), 10 ng/mL BDNF, 10 ng/mL GDNF (R&D System) and 200 μm ascorbic acid (Sigma). For Astrocyte differentiation, basal media was supplemented with 5% FBS. To derive oligodendrocytes, cells were cultivated in basal medium supplemented with 200 ng/mL SHH C25II, 20 ng/mL bFGF and 20 ng/mL PDGF (R&D System) for 7 days. Afterwards, PDGF and bFGF were replaced by 40 ng/mL 3,3,5-triiodothyronine (T3) hormone (Sigma), 100 ng/mL Noggin, 100 ng/mL IGF1, 10 ng/mL NT3 and 5 μM forskolin as previously reported with modifications (Lujan et al., 2012; Najm et al., 2013).

Generation of neuronal subtypes from iNPC

For GABA neuron induction, we modified previous protocols (Barberi et al., 2003; Ma et al., 2012); iNPCs were cultivated with neural basal medium supplemented with 200 ng/mL SHH C25II without EGF. After 7 days, media was supplementation was changed to 10 μM VPA, 20 ng/mL NT4, 20 ng/mL BDNF, 10 ng/mL GDNF, 10ng/mL IGF1 and 5 μM forskolin for 21 days. For DA neuron induction, we followed previous described protocols with modifications (Kriks et al., 2011; Li et al., 2011); iNPCs were cultured in neural basal medium supplemented with 100 ng/mL SHH C25II and FGF8 (R&D System) without bFGF/EGF. After 7 days, supplementation was changed to 20 ng/mL

BDNF, 20 ng/mL GDNF, 1 ng/mL TGFβ3, 200 μM ascorbic acid, 5 μM forskolin and 10 μM DAPT (Sigma) for 21 days. For nociceptive sensory neurons, we modified previous reports (Chambers et al., 2012; Guo et al., 2013; Lee et al., 2012). Briefly, iNPCs in neural basal medium were supplemented with 1 μM SU5402, 10 μM DAPT and 3 μM CHIR99021. After 4 days, supplementation was switched to 10 ng/mL BDNF, 10 ng/mL GDNF, 10 ng/mL NGF, 10 ng/mL NT3 (R&D System), 200 μM ascorbic acid and 5 μM forskolin for 7-14 days until the desired maturation stage for a given experiment.

Flow cytometry

Cells were fixed using the BD Cytofix/Cytoperm kit (BD bioscience), including 4% (vol/vol) paraformaldehyde fixation step. Fixed cells were stained using the following antibodies: SSEA3, TRA1-60, PAX6, p75, CD57 (BD Biosciences), Nestin, NTRK1 (R&D Systems). Unconjugated antibodies were visualized with appropriated fluorochrome conjugated secondary antibody. FACS analysis was performed on a FACS Calibur cytometer (Becton Dickinson Immunocytometry Systems) and analyzed using FlowJo software (Tree Star Inc).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and stained with appropriate antibodies. If permeabilization was required, cells were treated with 0.1% saponin (BD Biosciences) prior to staining. Appropriate primary and fluorochrome-conjugated secondary antibodies were used. Cells were then counterstained with Hoechst 33342 (Invitrogen). The following antibodies were used: SSEA-3, TRA-1-60, OCT4, PAX6, p75, CD57 (BD

biosciences), Nestin, TuJ1, MAP2, O4 (R&D System), Synapsin, TH, BRN3A, ISL1, P2X3R (Millipore), Glutamate, GABA, GFAP (Sigma), Nurr1 (Santa Cruz), vGluT1 (Abcam).

Reverse transcriptase PCR and quantitative PCR (RT-PCR and RT-qPCR)

Total RNA purification was performed using RNeasy Mini Kit (Qiagen), including DNase I on-column digestion step, according to manufacturer's instructions. Purified RNA was quantified on a Nanodrop 2000 Spectrophotometer (Thermo Scientific). For RT-PCR, cDNA was synthesized from 500ng of total RNA using iScriptTM cDNA Synthesis Kit (BioRad). RT-PCR was performed using Recombinant Taq DNA Polymerase (Thermo Scientific). Random-primed Human Reference cDNA (Clontech) was used as a putative positive control. For RT-qPCR, cDNA was synthesized from 1µg of total RNA using SuperScript III First-Strand Synthesis (Life Technologies). RT-qPCR was carried out using Platinum SYBR Green qPCR SuperMix-UDG (Life Technologies) utilizing manufacturer's recommended cycling conditions on an Mx3000P QPCR System (Stratagene). To find a list of primers used in this study refer to Supplemental Tables 1 and 2.

Calcium imaging

Differentiated cells at 1-2 weeks were loaded with Fluo-4-AM fluorescence dye (Invitrogen, CA) for 1 hr incubation followed by 45 mins period for de-esterification. Cells were washed and incubated in Hanks' balanced salt solution (HBSS), supplemented with 25 mM HEPES buffer, 5.5 mM Glucose. Calcium flux was monitored using an

Olympus IX81 inverted epi-fluorescence microscope (Olympus, Markham, ON) coupled to a xenon arc lamp (EXFO, Quebec, QC). Indicated agonists, α,β-methylene-ATP or capsaicin, were diluted in the aforementioned solution and added to the well to give the final stimulation concentration (30 μM α,β -methylene-ATP, 1 μM capsaicin) using a dropping pipette and aspirator system. Fluorescence images were collected using an EMCCD camera (Photometrics, Tucson, AR) every 2s through a GFP filter cube (Semrock, Rochester, NY). In a subset of wells, ionomycin was added as a second stimulation for the dye loading control. For experiments using the selective P2X3 antagonist A-317491, the indicated concentration of compound was added to the wells 15 min before calcium imaging, and then calcium flux was measured as above. Off-line analysis of the intensity pattern of Fluo-4 signal was performed in ImageJ (NIH, Bethesda, MD).

Electrophysiology

Patch-clamp recordings were conducted at room temperature (~21˚C) using an Axopatch 200B amplifier (Axon Instruments Inc., USA) from Cerebrasol (Montreal, Canada). Electrodes had a resistance of 2-4 MΩ when filled with recording solutions. The external recording solution contained 140 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl2, 2.5 mM CaCl2, and 10 mM HEPES (pH 7.3), adjusted to 320 mOsm/l with glucose. Internal solutions. The intracellular solution contained 100 mM CsF, 45 mM CsCl, 10 mM NaCl, 5 mM EGTA, 1 mM MgCl2, 10 mM HEPES (pH 7.3) adjusted to 300 mOsm with sucrose. For current-clamp recordings pipette solution of the following composition was used: 130 mM KCl, 0.5 mM EGTA, 10 mM HEPES, 1 mM MgCl2, 5 mM Mg-ATP and

3 mM Na-GTP (pH 7.3), adjusted to 310 mOsm/l with glucose. Data were filtered at 1 KHz and digitized at 10 kHz. 25mm coverslips with adhered cells were transferred to a recording chamber and cells visualized on an inverted Nikon microscope. Cells were continuously perfused at a slow perfusion rate of approximately 0.5 mL/min. For assessment of electrical excitability, experiments were conducted using the current-clamp recording configuration. Cells were held at approximately -60 mV and a series of hyperpolarizing and depolarizing current steps injected to characterize voltage gated currents and action potential initiation. For the assessment of voltage-activated sodium conductance (NaV), experiments were conducted using the voltage-clamp recording configuration. The presence of NaV conductances was determined using a simple step protocol from a holding potential (HP) of -120 mV to 0 mV for 30 ms, then back to -120 mV repeated at a frequency of 0.1 Hz.

In vivo Transplantation

In vivo transplantation of cells into the neonatal mouse cortex has been described elsewhere (Zhu et al., 2014). Briefly, P2 to P4 old Nod.Scid (NOD.CB17-Prkdcscid/J) neonatal mice were injected with a total of 4 x 10^5 BD NPC (2 injections into each right and left hemisphere, 1 x 10^5 cells each site). Four weeks after injection, the mice were sacrificed and perfused with lactated ringers followed by 10% formalin. Brains were removed, incubated in 4% PFA for 24 hours and stored in PBS. All mice were bred and maintained in the Stem Cell and Cancer Research Institute (SCC-RI) animal barrier facility at McMaster University. All animal procedures received the approval of the animal ethics board at McMaster University.

Gene Expression Analysis

Total RNA from hFib-iNPC^{OCT4} and hBD-iNPC^{OCT4} with or without SMAD/GSK-3 inhibitors was hybridized to Affymetrix Human Gene 1.0 ST arrays (London Regional Genomics Centre). Normalized expression data was applied to create hierarchical clustering and statistically significant gene lists (multiple test corrected $p \leq 0.05$, fold change ≥ 1.5) using Partek Genomics Suite 6.6 (Partek Inc., St Louis, MO, USA). For hierarchical clustering, primary human neural stem/progenitor cells were obtained from publicly available GEO source (GSE27505). Using Gene Set Enrichment Analysis software (Mootha et al., 2003; Subramanian et al., 2005), samples from hBD-iNPC^{OCT4} with or without inhibitors were compared to CD34+ cord blood, and statistically significant (FDR q-value ≤ 0.05) enriched gene set lists were generated. Tissue expression analysis was done using DAVID Bioinformatics database (Benjamini adjusted $p \leq 0.01$).

Comparative Genomic Hybridization Array

Genomic DNA from samples was isolated using DNeasy kit (Qiagen) and concentrations were measured using NanoDrop. Sample DNA was hybridized to Agilent human CGH 4x44k microarrays (Princess Margaret Genomics Centre, Toronto, ON). Standard human genomic DNA was hybridized to arrays as a reference. Partek Genomic Suite 6.6 software was used for analysis. Criteria of diploid copy number higher than 2.5 being as amplification and lower than 1.5 being as deletion was used, as well as statistical

segmentation parameters with minimum genomic markers of 10 to specify genomic region and p-value threshold 0.001.

Analysis of catecholamines in culture media.

1mL of culture medium was collected from culture wells. The oxidation status of the catecholamines was stabilized with 0.02 mL of an EGTA and glutathione buffer, and the sample was frozen at -30oC. Before analysis, the internal standard (3,4-Dihydroxybenzylamine) was added to the thawed medium for further processing using solid phase extraction cartridges as per manufacturer's recommendations (ChromSystems, Grafelfing, Germany). The samples were eluted into 0.12 mL and injected within 24h in a High Performance Liquid Chromatographic System (HPLC, Waters 2695) coupled to an Electrochemical Detector (Waters 2465). The HPLC system used an analytical reverse phase column (Atlantis dC18; 5 micron; 4.6x150mm; Waters) and an organic mobile phase (ChromSystems). Three physiological tyrosine-derived catecholamines (noradrenaline, adrenaline, and dopamine) were used as standards. The concentration of catecholamines was calculated using the average area under the curve (n=3 injections) of the chromatograms of the calibration standards.

Statistical Methods

Unless otherwise noted standard deviation was used in performing a students t-test (two tailed) where * p=0.05 ** p=0.01

Author Contributions

JHL and RRM designed the study, performed experiments and co-wrote the paper. JDM provided technical support for calcium imaging. ZS provided technical support for microarray analysis. SL and BT provided assistance with RT-PCR and qRT-PCR. CM performed immunofluorescence on xenografted brain slices. FC provided technical assistance for HPLC measurements of dopamine. AFC provided technical assistance for all animal work. TJC designed experiments and assisted with calcium imaging and wrote the paper. KKS designed and assisted during *in vivo* xenotransplantation experiments. MB designed and oversaw the entire study, and co-wrote the paper.

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Fig. 1. Generation of iNPC from neonatal and adult blood cells.

(A) Schematic strategy for deriving iNPCs from lineage depleted CD34+CD45+ blood cells. (B) Representative phase-contrast images of iNPCs generated by exogenous expression of OCT4 along with inhibition of SMAD and GSK-3. Scale bars represent 300 μm. (C) Quantification of iNPCs-like colonies after induction with Oct4, Sox2 or both transcription factors in the presence or absence of inhibitors. (D) Number of iNPCs colonies generated from CD34+ progenitors or CD34- matured cells transduced with OCT4 and treated with inhibitors. (E) Phase-contrast image of neural spheres generated through suspension culture of iNPCs. Scale bars represent 300 μm. (F) Immunofluorescence analysis of iNPCs with antibodies to neural stem cell markers, PAX6, NESTIN and SOX2. As a positive control, NPCs derived from hESC lines were used. Scale bars represent 100 μm. (G) Intracellular FACS analysis for PAX6 and NESTIN, neural stem cell markers, from iNPCs (n=4). (H) Extrapolation of the number of neural precursor cells can be generated from 50K human blood progenitors.

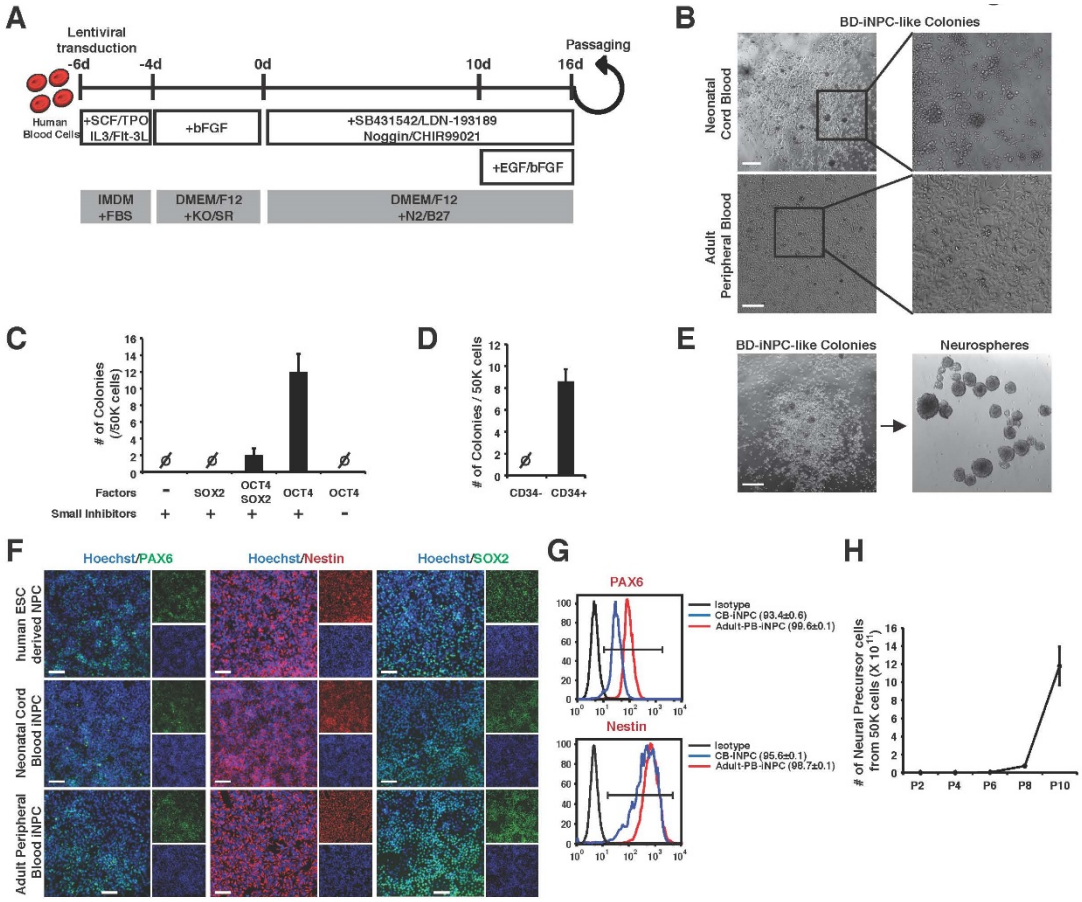

A
Lentiviral transduction
-6d
-4d
0d
10d
16d
Passaging
Human Blood Cells
+SCF/TPO IL3/Flt-3L
+bFGF
+SB431542/LDN-193189 Noggin/CHIR99021
+EGF/bFGF
IMDM +FBS
DMEM/F12 +KO/SR
DMEM/F12 +N2/B27
B
BD-iNPC-like Colonies
Neonatal Cord Blood
Adult Peripheral Blood
C
# of Colonies (/50K cells)
Factors
Small Inhibitors
SOX2
OCT4 SOX2
OCT4
D
# of Colonies / 50K cells
CD34-
CD34+
E
BD-iNPC-like Colonies
Neurospheres
F
Hoechst/PAX6
Hoechst/Nestin
Hoechst/SOX2
human ESC derived NPC
Neonatal Cord Blood iNPC
Adult Peripheral Blood iNPC
G
PAX6
Isotype
CB-iNPC (93.4±0.6)
Adult-PB-iNPC (99.6±0.1)
Nestin
Isotype
CB-iNPC (95.6±0.1)
Adult-PB-iNPC (98.7±0.1)
H
# of Neural Precursor cells from 50K cells (X 10^11)
P2
P4
P6
P8
P10

Fig. 2. Molecular profiling of OCT4 BD-iNPC generation.

(A) Hierarchal cluster analysis on global gene expression of Fib-iNPC and BD-iNPC with or without inhibitors with primary human neural stem/progenitor cells. (B) Number of genes changing in response to inhibitors in Fib-iNPC versus hBD-iNPCOCT4 (FDR p<= 0.05, fold change >= 1.5). (C) Geneset enrichment analysis shows neural related gene sets are highly activated in BD-iNPC upon SMAD+GSK-3 inhibitor treatment. (D) Expression of hematopoietic or neural specific genes in BD-iNPCs and control NPCs derived from hPSCs.

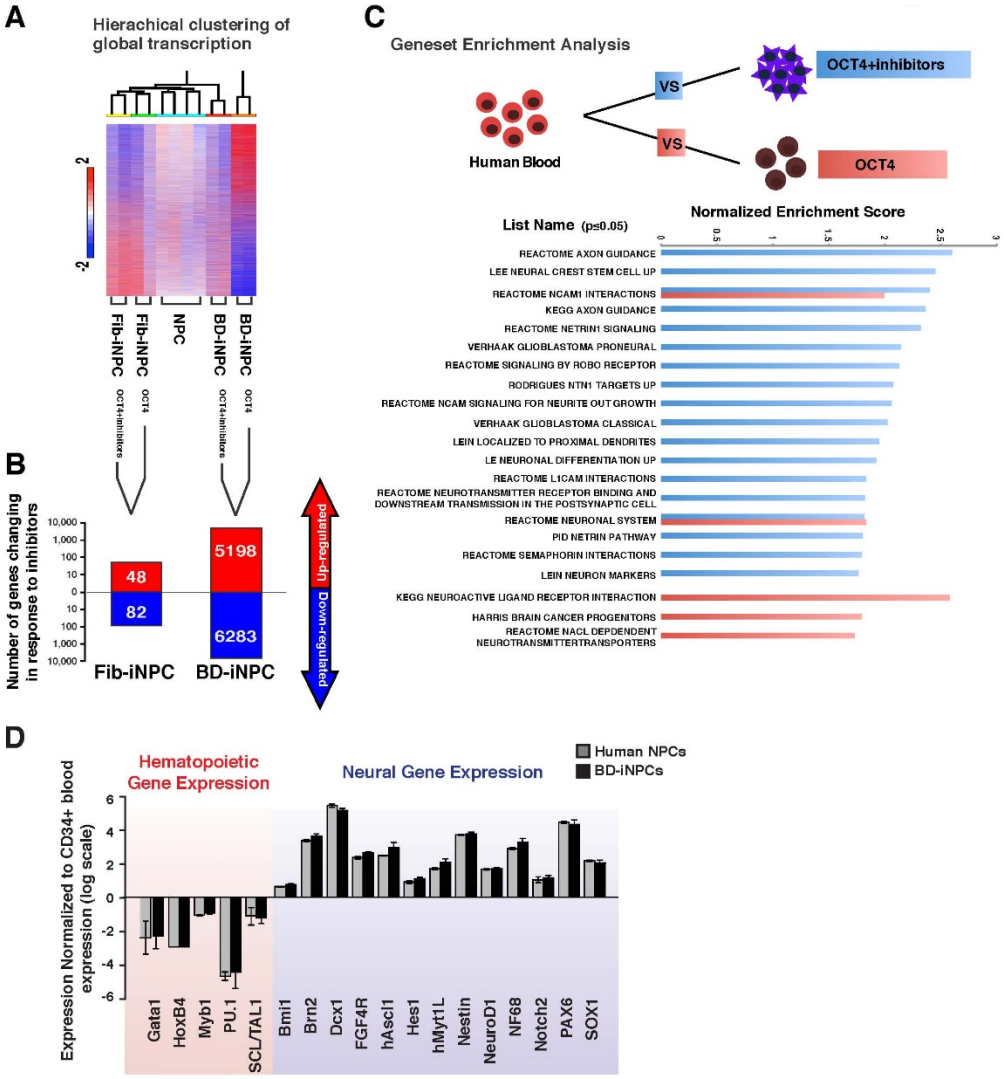

A
Hierachical clustering of global transcription
2
-2
Fib-iNPC
Fib-iNPC
NPC
BD-iNPC
BD-iNPC
OCT4+inhibitors
OCT4
OCT4+inhibitors
OCT4
B
Number of genes changing in response to inhibitors
10,000
1,000
100
10
0
10
100
1,000
10,000
48
82
5198
6283
Fib-iNPC
BD-iNPC
Down-regulated
Up-regulated
C
Geneset Enrichment Analysis
Human Blood
VS
VS
OCT4+inhibitors
OCT4
List Name (p≤0.05)
Normalized Enrichment Score
0
0.5
1
1.5
2
2.5
3
REACTOME AXON GUIDANCE
LEE NEURAL CREST STEM CELL UP
REACTOME NCAM1 INTERACTIONS
KEGG AXON GUIDANCE
REACTOME NETRIN1 SIGNALING
VERHAAK GLIOBLASTOMA PRONEURAL
REACTOME SIGNALING BY ROBO RECEPTOR
RODRIGUES NTN1 TARGETS UP
REACTOME NCAM SIGNALING FOR NEURITE OUT GROWTH
VERHAAK GLIOBLASTOMA CLASSICAL
LEIN LOCALIZED TO PROXIMAL DENDRITES
LE NEURONAL DIFFERENTIATION UP
REACTOME L1CAM INTERACTIONS
REACTOME NEUROTRANSMITTER RECEPTOR BINDING AND DOWNSTREAM TRANSMISSION IN THE POSTSYNAPTIC CELL
REACTOME NEURONAL SYSTEM
PID NETRIN PATHWAY
REACTOME SEMAPHORIN INTERACTIONS
LEIN NEURON MARKERS
KEGG NEUROACTIVE LIGAND RECEPTOR INTERACTION
HARRIS BRAIN CANCER PROGENITORS
REACTOME NACL DEPENDENT NEUROTRANSMITTERTRANSPORTERS
D
Hematopoietic Gene Expression
Neural Gene Expression
Human NPCs
BD-iNPCs
Expression Normalized to CD34+ blood expression (log scale)
6
4
2
0
-2
-4
-6
Gata1
HoxB4
Myb1
PU.1
SCL/TAL1
Bmi1
Brn2
Dcx1
FGF4R
hAscl1
Hes1
hMyt1L
Nestin
NeuroD1
NF68
Notch2
PAX6
SOX1

Fig. 3. *In vivo* and *in vitro* differentiation potential of BD-iNPCs.

(A) Montaged image from sectioned brain tissue 3 weeks after injection of BD-iNPCs expressing GFP. (B) *In vivo* differentiation of BD-iNPCs into neurons (expression of Tuj1, MAP2 and NEUN) and astrocytes (expression of GFAP). C-E. *In vitro* differentiation of BD-iNPCs in to GFAP-positive astrocytes (C) and O4-positive oligodendrocytes (D). (E) Tuj1 and MAP2 positive neurons and glutamatergic and GABAergic neuronal subtypes. Scale bars represent 100 μm. (F) Expression of Synapsin, presynaptic marker, and indicative of synaptic formation *in vitro*. Scale bars represent 50 μm. (G) Raw traces of membrane potential changes to stepwise current injection of equal increment. (H) Repetitive action potential firing was induced upon depolarizing current injection. (I) TTX-sensitive fast inward currents on depolarization further verified voltage-dependent Na+ channels. (J) TH and Nurr1 positive dopaminergic (DA) neurons derived from BD-iNPCs. Scale bars represent 100 μm. (K) Dopamine detected in cultures of DA neurons differentiated from iNPCs. Representative HPLC chromatogram in a sample (left) and levels in multiple DA cultures (right). (L) Gene set enrichment analysis for LEE_NEURAL_CREST_STEM_CELL gene list between human fibroblasts and human blood. (M) Gene set enrichment analysis for LEE_NEURAL_CREST_STEM_CELL gene list between human blood and BD-iNPCs.

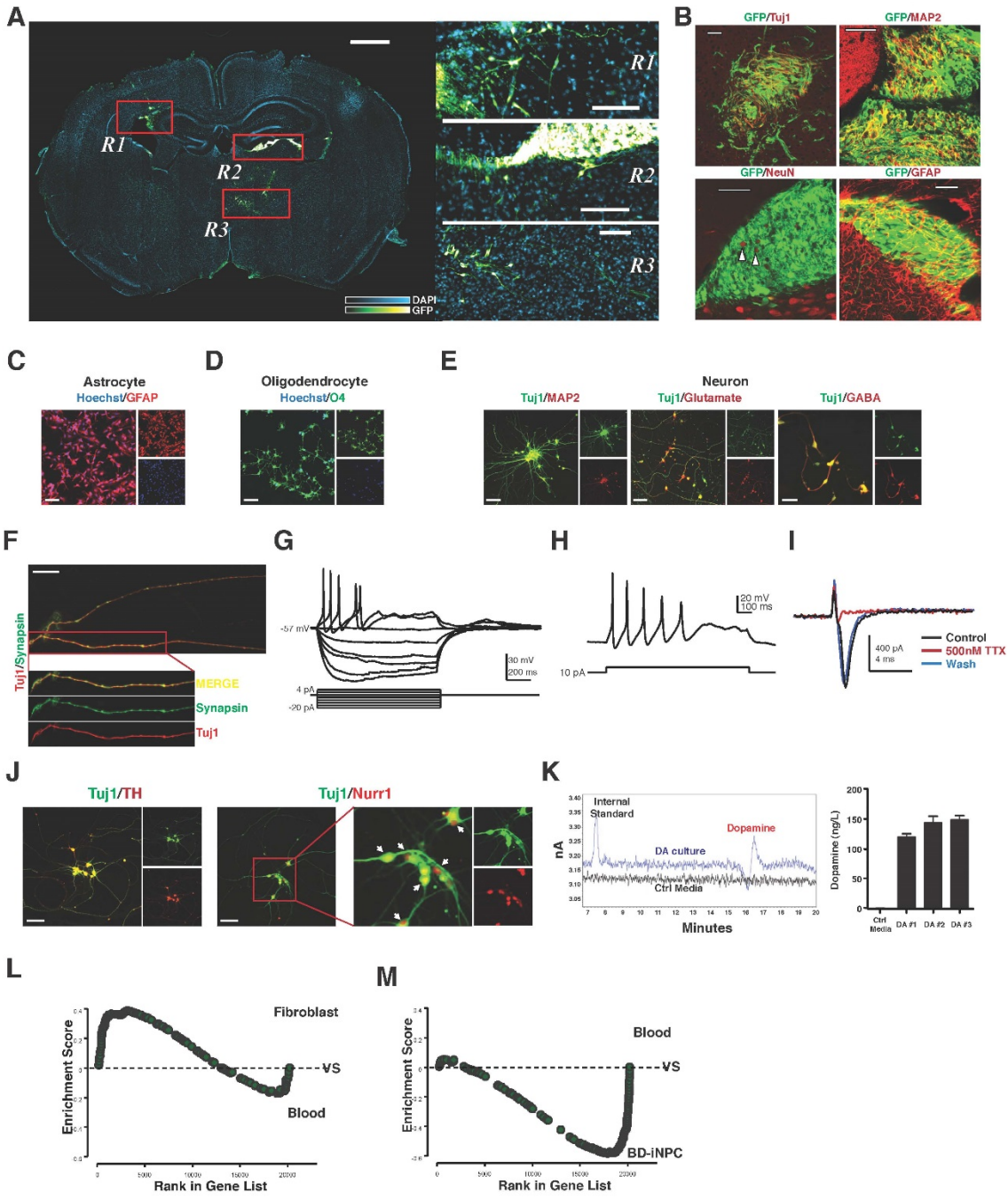
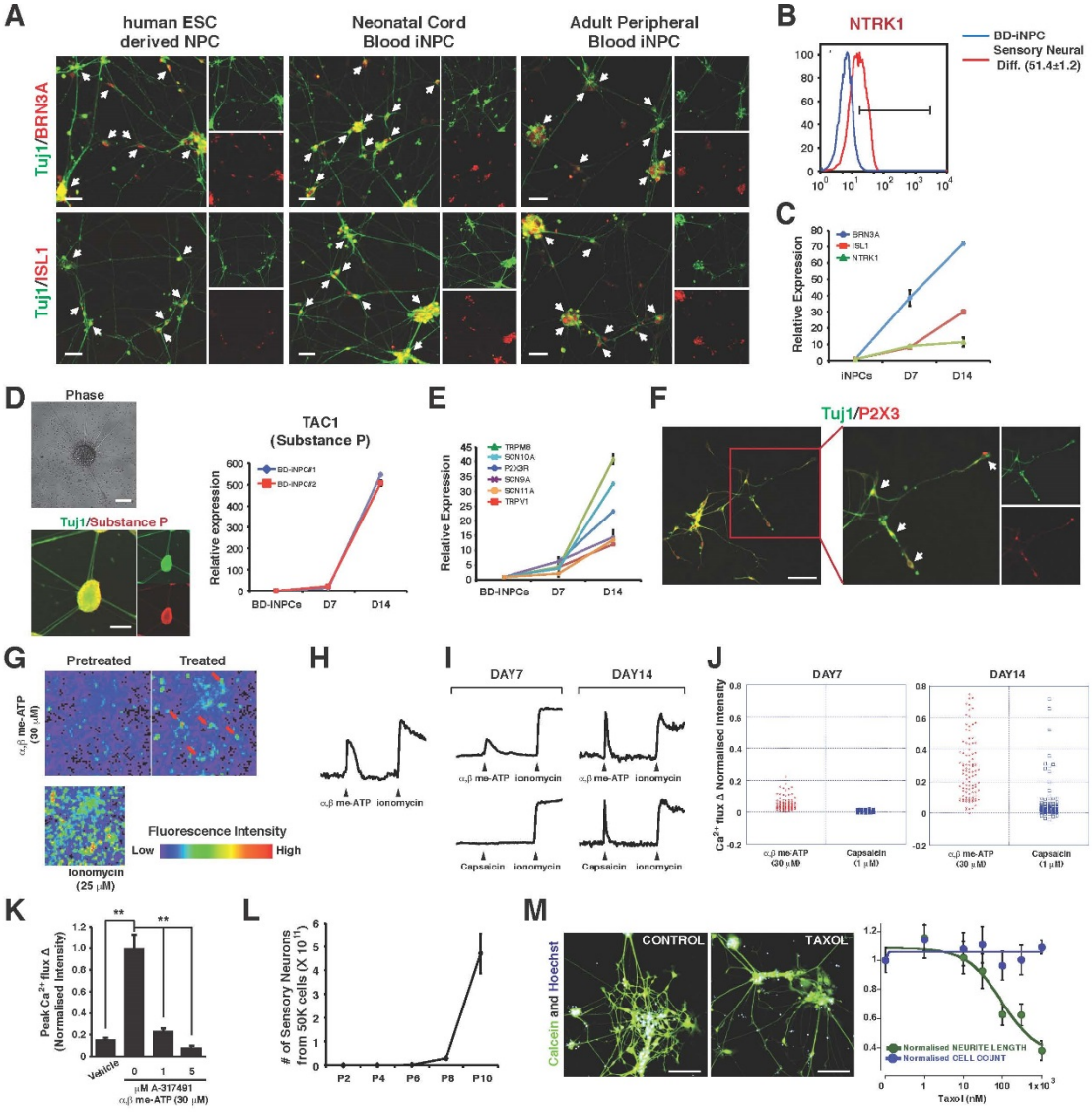

A
R1
R2
R3
DAPI
GFP
B
GFP/Tuj1
GFP/MAP2
GFP/NeuN
GFP/GFAP
C
Astrocyte
Hoechst/GFAP
D
Oligodendrocyte
Hoechst/O4
E
Neuron
Tuj1/MAP2
Tuj1/Glutamate
Tuj1/GABA
F
Tuj1/Synapsin
MERGE
Synapsin
Tuj1
G
-57 mV
30 mV
200 ms
4 pA
-20 pA
H
20 mV
100 ms
10 pA
I
400 pA
4 ms
Control
500nM TTX
Wash
J
Tuj1/TH
Tuj1/Nurr1
K
Internal Standard
Dopamine
DA culture
Ctrl Media
nA
Minutes
Dopamine (ng/L)
Ctrl Media
DA #1
DA #2
DA #3
L
Fibroblast
VS
Blood
Enrichment Score
Rank in Gene List
M
Blood
VS
BD-iNPC
Enrichment Score
Rank in Gene List

Fig.4. Generation of functional nociceptive neurons that model chemotherapy induced neuropathy.

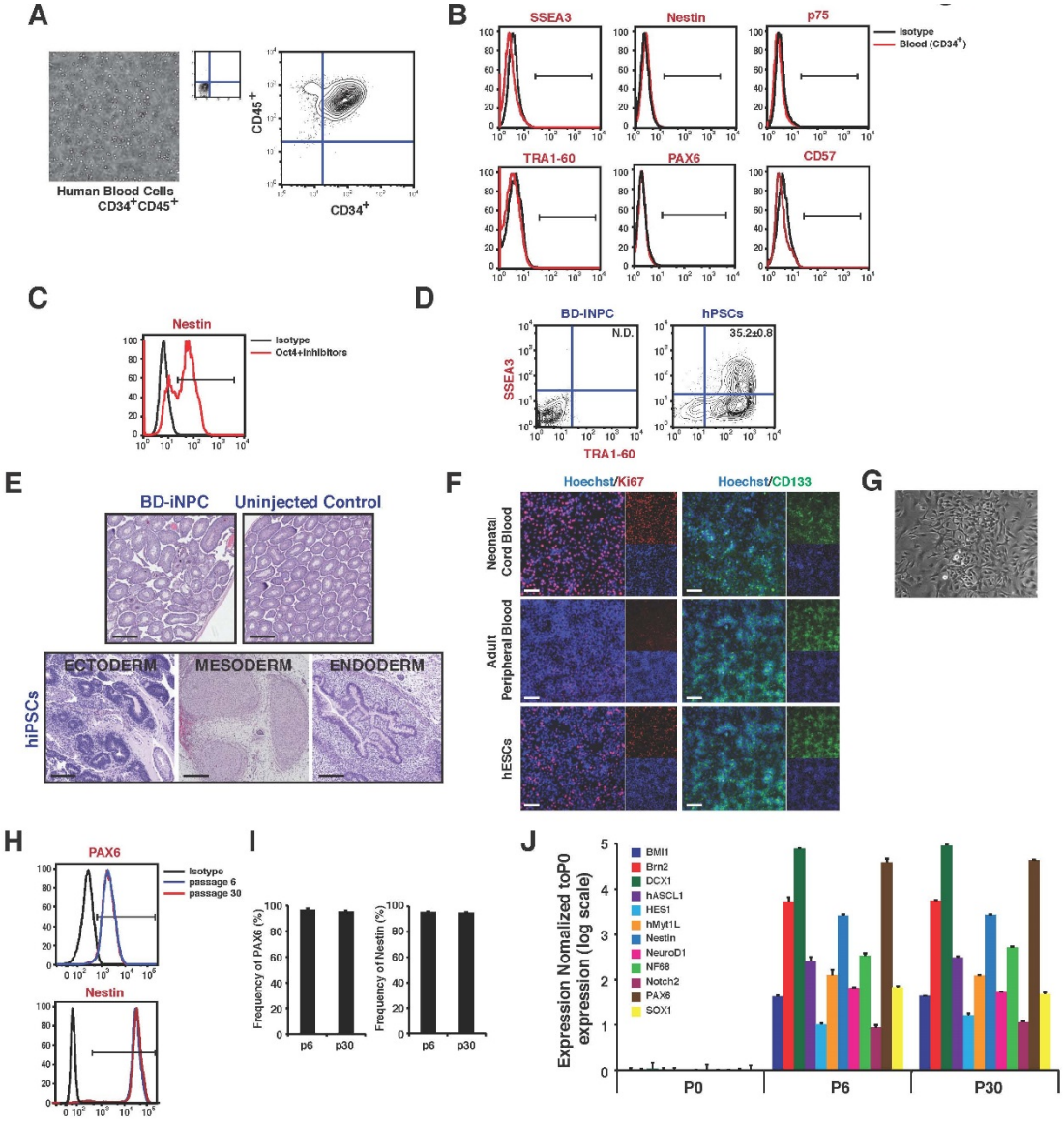
A-C. Differentiation potential into nociceptive sensory neurons. (A) Tuj1+ neurons express BRN3A and ISL1, canonical markers of sensory neurons. As a positive control, sensory neurons derived from hESC lines were used. Scale bars represent 50 μm. (B) Expression of NTRK1 as measured by FACS analysis. (C) Gene expression analysis of BRN3A, ISL1 and NTRK1 during differentiation from iNPCs toward sensory neurons. (D) Neuronal cell bodies arranged as clusters (top left) and expressed Substance P, indicative peptidergic nociceptors (low left). Scale bars represent 100 μm. (right) RT-PCR further supports expression of TAC1 (Substance P). (E) Expression of channels, channel subunits and receptors, specific to nociceptor, in neurons generated from BD-iNPCs. (F) Expression of P2X3 was further confirmed by immunocytochemistry. Scale bars represent 100 μm. (G, H) Calcium flux in response to 30 μM α,β-methylene ATP treatment of day-14 sensory neurons derived from adult PB-iNPCs. (G) Shows representative calcium trace. (I, J) Calcium flux in response to 30 μM α,β-methylene ATP or 1 μM capsaicin treatment. Modest calcium signals were induced only by α,β -methylene ATP at day 7 of differentiation. By day 14, both capsaicin and α,β -methylene ATP stimulation elicited robust calcium response. (I) shows representative calcium trace and (J) indicates distribution of cells responsive to 30 μM α,β -methylene ATP and 1 μM capsaicin. (K) P2X3 antagonist A-317491 significantly inhibited the calcium-response to α,β -methylene ATP, verifying action of agonist through the P2X3 receptor. (L) Extrapolative number of nociceptive sensory neurons can be generated from 50K human

blood progenitors. (M) *In vitro* response of BD-iNPC derived sensory neurons to chemotherapeutic drug, Taxol, indicating a dose-dependent reduction in neurite length without loss of viability. Representative image of calcein and Hoechst stained viable neurons forty-eight hours after treatment (left) and normalized dose-dependent neurite length and cell count (right).


A
human ESC derived NPC
Neonatal Cord Blood iNPC
Adult Peripheral Blood iNPC
Tuj1/BRN3A
Tuj1/ISL1
B
NTRK1
BD-iNPC
Sensory Neural Diff. (51.4±1.2)
C
BRN3A
ISL1
NTRK1
Relative Expression
iNPCs
D7
D14
D
Phase
Tuj1/Substance P
TAC1 (Substance P)
BD-iNPC#1
BD-iNPC#2
Relative expression
BD-iNPCs
D7
D14
E
TRPM8
SCN10A
P2X3R
SCN9A
SCN11A
TRPV1
Relative Expression
BD-iNPCs
D7
D14
F
Tuj1/P2X3
G
Pretreated
Treated
αβ me-ATP (30 μM)
Ionomycin (25 μM)
Fluorescence Intensity
Low
High
H
αβ me-ATP
Ionomycin
I
DAY7
DAY14
αβ me-ATP
Ionomycin
Capsaicin
J
DAY7
DAY14
Ca2+ flux Δ Normalised Intensity
αβ me-ATP (30 μM)
Capsaicin (1 μM)
K
Peak Ca2+ flux Δ (Normalised Intensity)
Vehicle
μM A-317491
αβ me-ATP (30 μM)
L
# of Sensory Neurons from 50K cells (X 10^3)
P2
P4
P6
P8
P10
M
Calcein and Hoechst
CONTROL
TAXOL
Normalised NEURITE LENGTH
Normalised CELL COUNT
Taxol (nM)

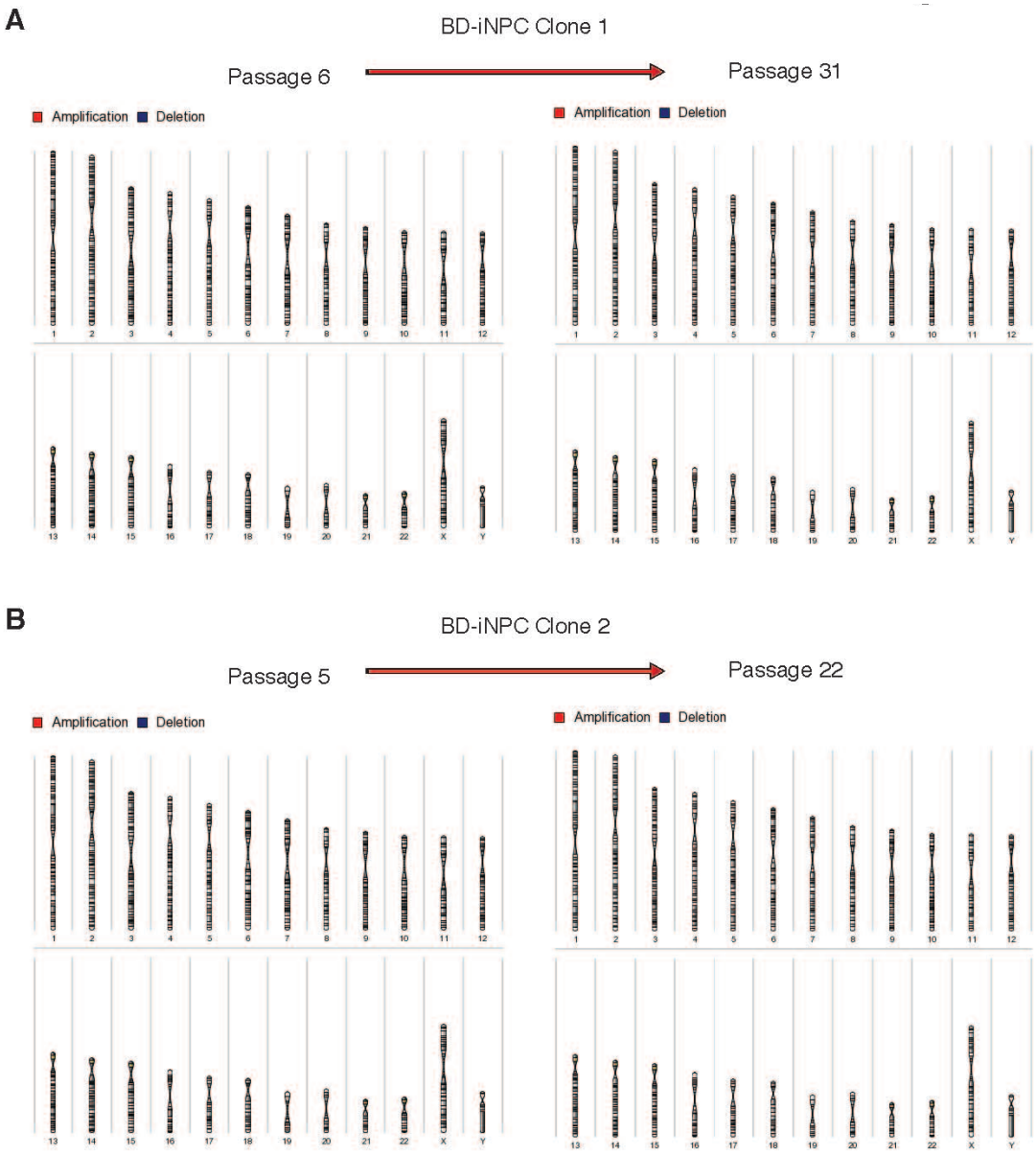
Supplementary Fig. 1. Generation of iNPC from neonatal and adult blood cells.

(A) Preparation of CD34+CD45+ blood cells from lineage depleted mononuclear cells from adult peripheral blood or umbilical cord blood shown in representative panel. (B) FACS analysis shows CD34+CD45+ human blood cells are devoid of pluripotent markers (SSEA3, TRA1-60), early neural markers (NESTIN, PAX6) and neural crest (NC) markers (p75, CD57). (C) Upon exogenous expression of Oct4, along with inhibition of SMAD and GSK-3, human CD34+CD45+ blood cells acquire neuronal marker, NESTIN as shown in representative flow cytometry plot. (D) Human BD- putative iNPCs do not express hallmark pluripotent markers, SSEA3 and TRA1-60 as compared to hPSCs, suggesting iNPCs generated bypass pluripotent states as measured by phenotypical alterations. (E) *In vivo* transplantation of BD-iNPCs fail to generate teratomas upon intratesticular injection into NOD/SCID recipients (top). As a positive control, hiPSCs lines were used and induced teratoma formation (bottom). (F) Immunofluorescence analysis of iNPCs with antibodies to neural stem cell marker, CD133. Expression of Ki67 indicated proliferation property of iNPCs. (G) Phase contrast image of H9 hESC-derived NPCs. (H-J) Stable expansion of human blood derived iNPCs over long-term passages *in vitro*. (H)Flow cytometric analysis showed that BD-iNPCs stably expressed makers associated with adult human neural stem cells, PAX6 and NESTIN after from 6 passages up to long-term *in vitro* expansion up to 30 passages (p30). (I) Quantification of flow analysis represented in H. (J) Comparative analyses of expression of genes associated with neural lineage development over long-term culture from p6 to p30.


A
Human Blood Cells
CD34+CD45+
CD45+
CD34+
B
SSEA3
Nestin
p75
Isotype
Blood (CD34+)
TRA1-60
PAX6
CD57
C
Nestin
Isotype
Oct4+Inhibitors
D
BD-iNPC
hPSCs
N.D.
35.2±0.8
SSEA3
TRA1-60
E
BD-iNPC
Uninjected Control
hiPSCs
ECTODERM
MESODERM
ENDODERM
F
Hoechst/Ki67
Hoechst/CD133
Neonatal Cord Blood
Adult Peripheral Blood
hESCs
G
H
PAX6
Isotype
passage 6
passage 30
Nestin
I
Frequency of PAX6 (%)
Frequency of Nestin (%)
p6
p30
J
Expression Nomalized toP0 expression (log scale)
BMI1
Brn2
DCX1
hASCL1
HES1
hMyt1L
Nestin
NeuroD1
NF68
Notch2
PAX6
SOX1
P0
P6
P30

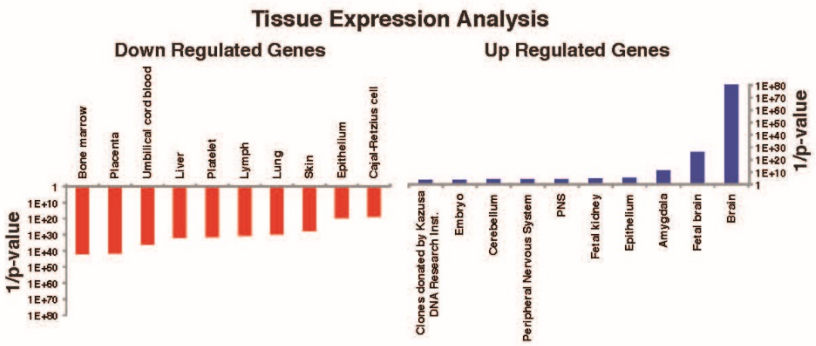
Supplementary Fig. 2. Genomic integrity of BD-iNPCs over passaging.

aCGH analysis of 2 different clones of NPCs (A and B) in the early passage was compared to late passage number and no statistically significant (minimum genomic markers of 10 to specify genomic region and p-value < 0.001) chromosomal aberrations were found.


A
BD-iNPC Clone 1
Passage 6
Passage 31
Amplification
Deletion
Amplification
Deletion
B
BD-iNPC Clone 2
Passage 5
Passage 22
Amplification
Deletion
Amplification
Deletion

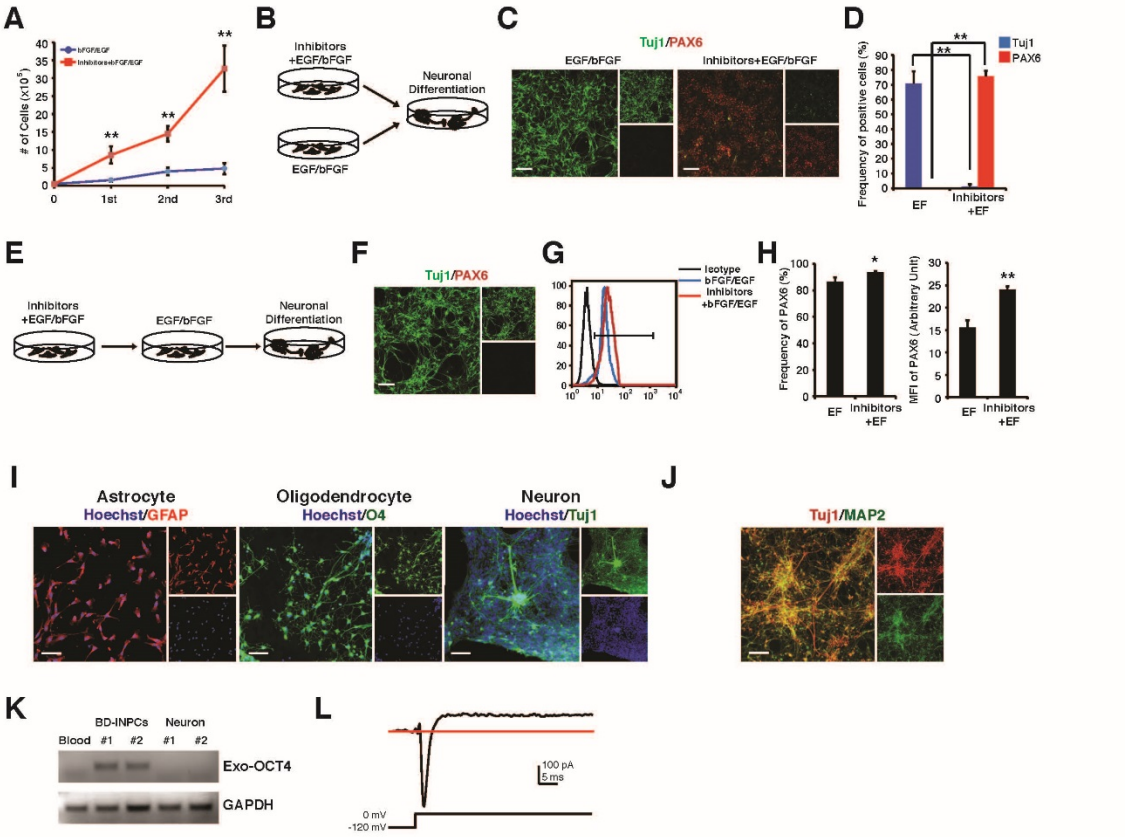
Supplementary Fig. 3. Loss of hematopoietic and gain of neural transcriptional programming during BD-iNPC generation.

Tissue Expression analysis on DAVID Bioinformatics Resource tool shows enrichment of up-regulated genes within neural programs and down-regulated genes within hematopoietic programs.


Tissue Expression Analysis
Down Regulated Genes
Up Regulated Genes
1/p-value
1
1E+10
1E+20
1E+30
1E+40
1E+50
1E+60
1E+70
1E+80
Bone marrow
Placenta
Umbilical cord blood
Liver
Platelet
Lymph
Lung
Skin
Epithelium
Cajal-Retzius cell
Clones donated by Kazusa DNA Research Inst.
Embryo
Cerebellum
Peripheral Nervous System
PNS
Fetal kidney
Epithelium
Amygdala
Fetal brain
Brain

Supplementary Fig. 4. *In vivo* and *in vitro* differentiation potential of BD-iNPCs.

A-H. Effects of small molecule inhibitors in the self-renewal and developmental potential of human BD-iNPCs. (A) Quantitative analysis of BD-iNPCs expansion in the presence or absence of inhibitors. (B) Schematic strategy for neuronal differentiation of BD-iNPCs cultured in the presence or absence of small molecule inhibitors. (C) Immunofluorescence analysis with antibodies to neuron marker Tuj1, and to neural stem cell marker, PAX6. (D) Quantitative analysis of representative images shown in L. (E) Schematic strategy for differentiating BD-iNPCs into neurons after removing inhibitors in the cultures. (F) Immunofluorescence analysis with antibodies to Tuj1 and PAX6, after culturing BD-iNPCs based on schematic strategy shown in N. (G) Intracellular analysis by flow cytometry for PAX6 from BD-iNPCs cultured in the presence or absence of small molecule inhibitors with EGF and bFGF. (H) Frequency of PAX6 positive cells (left) and mean fluorescent intensity (MFI) (right) of from flow cytometry analysis shown in P for human BD-iNPCs. (I) GFAP-positive astrocytes (left), O4-positive oligodendrocytes (middle), and Tuj1 positive neurons (right) derived from hPSCs. (J) Tuj1 and MAP2 positive neurons derived from adult PB-iNPCs. (K) Expression of residual exogenous OCT4 in established hiNPCs and silencing in mature neurons. GAPDH used as control. (L) Voltage-clamp recordings reveal both fast inactivating inward and outward currents indicating functional voltage-dependent Na+ and K+ channels.


A
B
C
D
E
F
G
H
I
J
K
L
Inhibitors +EGF/bFGF
EGF/bFGF
Neuronal Differentiation
Tuj1/PAX6
EGF/bFGF
Inhibitors+EGF/bFGF
Tuj1
PAX6
EF
Inhibitors +EF
Isotype
bFGF/EGF
Inhibitors +bFGF/EGF
Astrocyte
Hoechst/GFAP
Oligodendrocyte
Hoechst/O4
Neuron
Hoechst/Tuj1
Tuj1/MAP2
BD-INPCs
Neuron
Blood
#1
#2
#1
#2
Exo-OCT4
GAPDH
100 pA
5 ms
0 mV
-120 mV

Supplementary Fig. 5. Generation of functional nociceptive neurons that model chemotherapy induced neuropathy.

(A) Scheme of protocol used to generate nociceptive sensory neuronal development from human BD-iNPCs. (B) Differentiated nociceptive sensory neuron shows high levels of glutamate, consistent with an excitatory glutamatergic neuron. (C) RT-PCR for sensory neuron marker genes (D) Photomontage of calcium flux images of neurons derived from BD-iNPC calcium response at day 7 (left) and day 14 (right) upon treatment with 30 μM α,β -methylene-ATP or 1 μM capsaicin. The calcium ionophore ionomycin was used as a dye loading control.

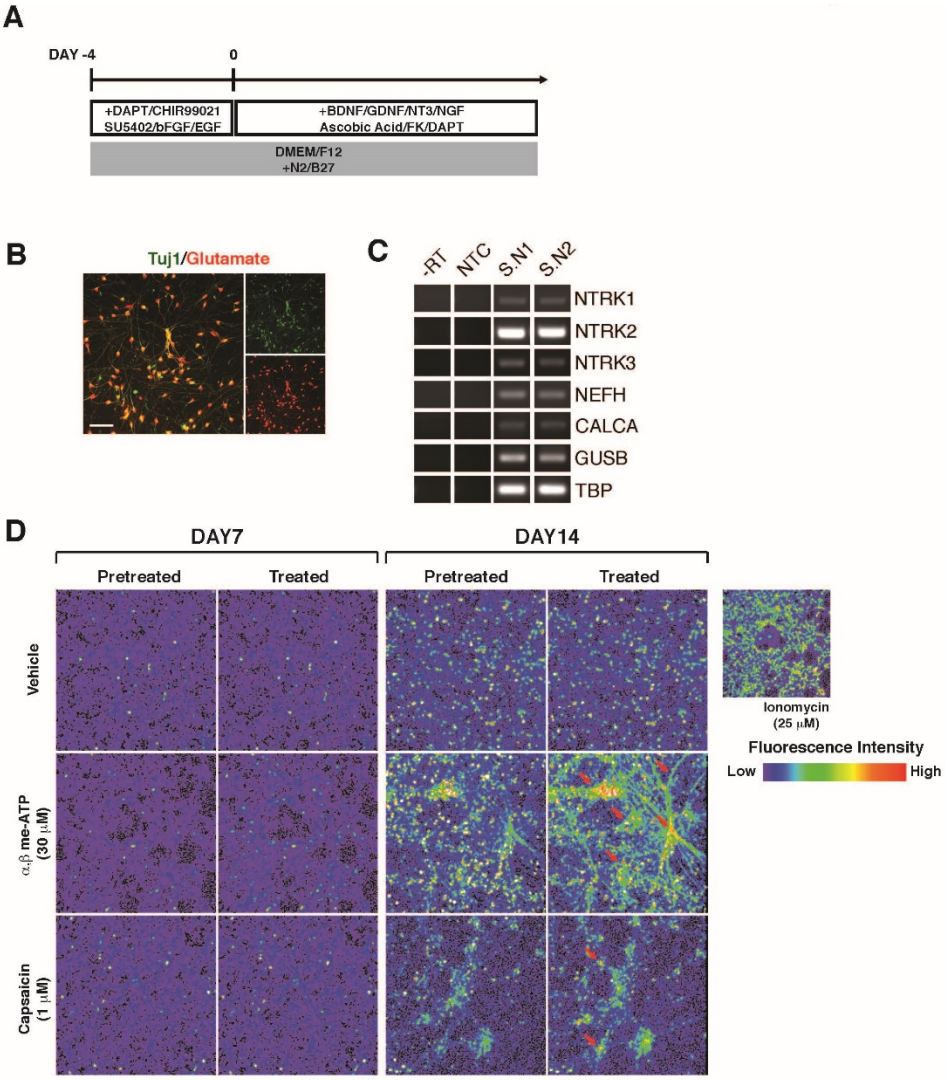

A
DAY -4
0
+DAPT/CHIR99021
SU5402/bFGF/EGF
+BDNF/GDNF/NT3/NGF
Ascobic Acid/FK/DAPT
DMEM/F12
+N2/B27
B
Tuj1/Glutamate
C
-RT
NTC
S.N1
S.N2
NTRK1
NTRK2
NTRK3
NEFH
CALCA
GUSB
TBP
D
DAY7
DAY14
Pretreated
Treated
Pretreated
Treated
Vehicle
αβ me-ATP (30 μM)
Capsaicin (1 μM)
Ionomycin (25 μM)
Fluorescence Intensity
Low
High

Table 1. qRT-PCR Primer List

Gata1	F: 5'-GGGATCACACTGAGCTTGC
	R: 5'-ACCCCTGATTCTGGTGTGG
HoxB4	F: 5'-CCTGGATGCGCAAAGTTCA
	R: 5'-AATTCCTTCTCCAGCTCCAAGA
PU.1	F:5'-ACGGATCTATACCAACGCCA
	R:5'-GGGGTGGAAGTCCCAGTAAT
Bmi1	F:5'-CAGAACAGATTGGATCGGAAA
	R:5'-CCGATCCAATCTGTTCTGGT
Brn2	F:5'-AATAAGGCAAAAGGAAAGCAACT
	R:5'-CAAAACACATCATTACACCTGCT
Dcx1	F:5'-AGACCGGGGTTGTCAAAAAACTCTAC
	R:5'-TCAGGACCACAGGCAATAAACACATC
hAscl1	F:5'-CAAGAGAGCGCAGCCTTAG
	R:5'-GCAAAAGTCAGTGCTGAACG
Hes1	F:5'-GAGCACAGAAAGTCATCAAAGC
	R:5'-TCCAGAATGTCCGCCTTC
hMyt1L	F:5'-CAATGGAAAGGGATTTTAAGCA
	R:5'-TTTGAGATTATGTACCACGTTAGATG
Nestin	F:5'-TCCAGGAACGGAAAATCAAG
	R:5'-GCCTCCTCATCCCCTACTTC
NeuroD1	F:5'-GTTATTGTGTTGCCTTAGCACTTC
	R:5'-AGTGAAATGAATTGCTCAAATTGT
NF68	F:5'-CAGACCGAAGTGGAGGAAAC
	R:5'-CCTCTTCCTTGTCCTTCTCCT
Notch2	F:5'-ACATCATCACAGACTTGGTC
	R:5'-CATTATTGACAGCAGCTGCC
PAX6	F:5'-CCGGCAGAAGATTGTAGAGC
	R:5'-CGTTGGACACGTTTTGATTG
SOX1	F:5'-AACACTTGAAGCCCAGATGGA
	R:5'-GCAGGCTGAATTCGGTTCTC
BRN3A	F:5'-GTACCCGTCGCTGCACTC
	R:5'-GGCTTGAAAGGATGGCTCTTG
ISL1	F:5'-TACAAAGTTACCAGCCACC
	R:5'-GGAAGTTGAGAGGACATTGA
NTRK1	F:5'-TTGGCATGAGCAGGGATATCT
	R:5'-ACGGTACAGGATGCTCTCGG
TAC1	F:5'-GCAGAAGAAATAGGAGCCAATG
	R:5-CGATTCTCTGCAGAAGATGCTC
TRPV1	F:5'-GGCTGTCTTCATCATCCTGCTGCT
	R:5'-GTTCTTGCTCTCCTGTGCGATCTTGT
P2RX3	F:5'-CCCCTCTTCAACTTTGAGAAGGGA
	R:5'-GTGAAGGAGTATTTGGGGATGCAC
SCN9A	F:5'-GCTCCGAGTCTTCAAGTTGG
	R:5'-GGTTGTTTGCATCAGGGTCT
SCN10A	F:5'-CAAATCTGAAACTGCTTCTGCCACA
	R:5'-CTAGGGCCCAGGGGCAATCAGCTCC
SCN11A	F:5'-CCCAGCAGCTGTTAAAGGAG
	R:5'-CTGGGACAGTCGTTTGGTTT
TRPM8	F:5'-CAGCGCTGGAGGTGGATATTC
	R:5'-CACACACAGTGGCTTGGACTC

Table 2. RT-PCR Primers

NTRK1	F: GGCAGAGGTCTCTGTTCAGG
	R: TGAACTCGAAAGGGTTGTCC
NTRK2	F: GTGGCGGAAAATCTTGTAGG
	R: CCCCATTGTTCATGTGAGTG
NTRK3	F: CAACTGCAGCTGTGACATCC
	R: GCCCAGTGACTATCCAGTCC
NEFH	F: GGTGAACACAGACGCTATGC
	R: TCTCCCACTTGGTGTTCCTC
CALCA	F: TGCACTGGTGCAGGACTATG
	R: AAGGCTTTGGAACCCACATT
GUSB	F: ACGACACCCACCACCTACAT
	R: TACAGATAGGCAGGGCGTTC

Chapter 5: Discussion

5.0 Cellular reprogramming: from natural occurrences to *in vitro* models

Although prominent figures in the field of stem cell biology have referred to the process of reprogramming as alchemy (Daley, 2012; Western and Surani, 2002), cellular reprogramming has been shown to occur naturally in amphibians and mammals. Amphibians harbor an incredible regenerative potential that is facilitated through a process of reprogramming (Endo et al., 2004). Upon amputation of salamander limbs, resident tissue cells undergo dedifferentiation and collectively form a heterogeneous bundle of undifferentiated progenitor cells referred to as a blastema (Kragl et al., 2009). The cells of the blastema then participate in the development of an entirely new limb, through a process that mimics embryonic limb generation. This process has been demonstrated to be both injury and nerve dependent through engineered formation of ectopic blastema (Endo et al., 2004). Surgical re-routing of nerves in the limb to an area of freshly resected skin, followed by grafting of a skin patch, results in the formation of an ectopic limb at the site of the graft. Through the study of induced limb formation, researchers aim to identify the minimal set of molecules and transcriptional responses that initiate dedifferentiation and blastema formation in order to fully elucidate the mechanism behind this process of regeneration (Endo et al., 2004). Although mammals do not harbor the same level of limb regeneration capacity as that of amphibians, differentiated cells of mouse proximal tubule (Kusaba et al., 2014) and lung airway epithelium (Tata et al., 2013) undergo a similar process of dedifferentiation towards a progenitor state to facilitate repair in response to injury. In addition to dedifferentiation, the adrenal gland

(Freedman et al., 2013) and, more notably, the pancreas (Chera et al., 2014) both harbor cell populations that undergo direct conversion or trans-differentiation from one differentiated cell to another in response to injury and to maintain normal tissue homeostasis. Collectively, these examples illustrate that cellular reprogramming is not the product of alchemy, but is perhaps a useful tool developed by Mother Nature for tissues and organs that either do not contain, or cannot appropriately mobilize, stem cells to facilitate homeostasis and/or repair.

Although naturally occurring, this process of direct conversion within a tissue (*in vivo)* has been engineered using forced expression of transcription factors in the pancreas (Zhou et al., 2008), heart (Qian et al., 2012), and brain (Torper et al., 2013) as a proof of principle that reprogramming can be harnessed to induce endogenous repair. Interestingly, the transcription factors used to achieve *in vivo* direct conversion were originally discovered performing *in vitro* investigations (Ieda et al., 2010; Vierbuchen et al., 2010), suggestive of a common mechanistic basis between both phenomena (Qian et al., 2012; Torper et al., 2013). Therefore, the generation and study of simplified models of *in vitro* direct conversion will likely further our overall understanding of this powerful mediator of tissue homeostasis and repair. This thesis provides a description of such a simplified model of reprogramming that encompasses elements of both dedifferentiation and direct conversion phenomena through the use of a single transcription factor, OCT4. Moreover, it begins to describe the molecular contribution of the elements used to facilitate OCT4 based reprogramming in order to establish a basic mechanistic definition of the process. Lastly, evidence is provided to suggest that this reprogramming approach

is applicable to multiple somatic cell types, providing further evidence for its utility in the study of the direct conversion process. Overall, this body of work allows multiple conclusions to be made regarding the nature and utility of OCT4 to bestow cell fate potential.

5.1 Expansion of reprogramming trajectories for OCT4 expressing fibroblasts

Through modification of our previously established OCT4 reprogramming conditions (Szabo et al., 2010), I was able to establish a method that allowed direct conversion of adult fibroblasts towards NPCs (Figure 1) (Chapter 2). Similar to our approach of applying hESC differentiation paradigms suited for hematopoiesis, I applied a simplified set of culture conditions previously shown to support the differentiation of hESCs towards NPCs (Chapter 2). In the most basic sense, these results served to answer my first question regarding whether OCT4 reprogramming could support alternative direct conversion outcomes. More importantly, this study revealed that OCT4 expressing fibroblasts responded to treatment with neural lineage culture conditions by up-regulating neural developmental programs, in the same manner in which they up-regulated hematopoietic regulators when treated with hematopoietic supportive conditions in our original study (Szabo et al., 2010). This outcome informed me in two ways: 1) OCT4 was not specifically imposing a dominant hematopoietic transcriptional program on fibroblasts, and 2) the extracellular environment was likely a critical regulator of the impact of OCT4 expression. The second of these two postulations was the driving force of the study presented in chapter 3, where I dissected the individual versus combined

molecular impact of OCT4 expression and the extracellular environment during direct conversion to both blood and neural progenitors.

By transitioning OCT4 expressing fibroblasts into neural supportive culture conditions containing bFGF and EGF, I was able to induce direct conversion towards NPCs. Immediately prior to publishing this work, Zhu et al. reported similar findings where the combination of OCT4 expression and treatment with small molecule inhibitors in the absence of bFGF and EGF facilitated direct conversion towards NPCs (Zhu et al., 2014). Initially, Zhu and colleagues discovered that expression of OCT4 in fetal fibroblasts combined with a chemical cocktail consisting of GSK-3 (CHIR99021) and TGF-β (A8301) inhibitors, conditions known to enhance neural specification of hESCs (Li et al., 2011), was sufficient to generate NPC like colonies. However, substitution of the fetal fibroblasts for adult tissue resulted in a loss of colony formation. This prompted Zhu et al. to perform a chemical screen for small molecules that would restore NPC colony formation, which resulted in the identification of 3 additional inhibitors and a lipid which they deemed were critical to support the reprogramming process. Interestingly, Zhu et al. did not report whether they attempted to perform the reprogramming process in the absence of GSK-3 and TGF-β inhibition and presence of bFGF/EGF (neural inducers utilized in chapter 3), and therefore their report and that of my own can be considered independent processes. Despite these differences, this work still supports our hypothesis that the combination of OCT4 expression and lineage specific culture conditions can facilitate the direct conversion of human somatic cells.

To date, OCT4 based reprogramming has been utilized to support direct conversion towards multiple different somatic progenitor cells, such as MSCs (Meng et al., 2013), erythrocyte progenitor cells (Liu et al., 2015), osteoblasts (Yamamoto et al., 2015), and NPCs (Liao et al., 2015). Similar to our reports, each of these studies applies unique lineage specific culture conditions to cells expressing OCT4, further stressing the importance of the extracellular environment in directing the reprogramming process. Taken together these studies provide ample evidence to suggest that OCT4 reprogramming is not restricted to the generation of hematopoietic progenitors, but instead can be used as a multipurpose reprogramming factor. The use of a single transcription factor technically simplifies the practice of reprogramming, and provides a more tractable system to perform mechanistic studies in order to understand the impact of the individual elements that comprise the process.

5.2 The impact of OCT4 expression is context dependent

Expression of OCT4 in fibroblasts cultured in reprogramming media (RM) leads to a state of transcriptional plasticity that bestows the ability to make cell fate choices in response to changes in the extracellular environment (Figure 1) (Chapter 3). We have termed this reprogramming process OiP as it is a molecularly unique state from that induced by expression of a full set of pluripotency factors. This demonstration of OiP provides evidence to suggest that both the regulation and impact of OCT4 expression in somatic cells is highly context specific. Our study has also revealed a regulatory mechanism of OCT4 expression that is independent of its native enhancer/promoter regions, and mediated through two potential elements: 1) an inhibitory factor present in serum (serum

is present in fibroblast medium but not RM), or 2) positive regulation in the presence of bFGF/IGF2 (growth factor supplements in RM). As it stands, assessment of OCT4 expression under the influence of combinations of these growth factors in both the presence and absence of serum will be required in order to fully elucidate the nature of this regulatory event. Most importantly, this study provided clear evidence that OCT4 expression impact is regulated by the extracellular environment, and this regulation ultimately supports the direct conversion of fibroblasts towards multipotent progenitors. Unlike reports of cellular reprogramming that focus solely on the role of transcription factors during the induction of lineage conversion (Ieda et al., 2010; Vierbuchen et al., 2010), OiP clarifies the potential inductive role of the extracellular environment as opposed to it serving as a passive landing pad for converted cells.

Similar to our interest in understanding the role of OCT4 during OiP, other groups have designed studies aimed at uncovering the role of Oct4 during iPSC generation (Tiemann et al., 2014). Tiemann et al., found that ectopic Oct4 expression within mouse somatic cells results in the repression of somatic specific gene expression related to the cell of origin in the absence of any up-regulation of pluripotency associated transcription (Hammachi et al., 2012; Tiemann et al., 2014). Moreover, expression of Oct4 induced unique transcriptional regulation across differing somatic cell types, supporting the notion that the impact of Oct4 expression is highly reliant on both the cell and environmental context. However, upon expression of additional pluripotency regulating transcription factors, Oct4 contributes to the activation of transcriptional circuitry related to the induction of pluripotency (Hammachi et al., 2012; Tiemann et al., 2014), where even

brief expression leads to the generation of transient iPSCs (Bar-Nur et al., 2015; Maza et al., 2015). The induction of pluripotency via forced expression of the Yamanaka factors appears to be a dominant program that can initiate in multiple extracellular environmental conditions, as *in vivo* activation of Oct4, Sox2, Klf4, and c-Myc in mice resulted in the formation of teratomas in multiple organs and tissue types, suggesting the successful formation of pluripotent cells (Abad et al., 2013). These results were unlike those reported after the whole body expression of Oct4 alone, which only resulted in some levels of neoplasia in the gut, but otherwise induced no cellular transformations consistent with pluripotency (Hochedlinger et al., 2005). These results once again support the differing role of OCT4 expression alone versus when it is combined with additional regulators of pluripotency.

Despite clearly demonstrating that OCT4 induced plasticity is unique from that of cells passing through the early stages of pluripotency, continued culture of OCT4 expressing fibroblasts in RM for extended periods of time (approximately 70 days) results in the formation of putative iPSC colonies (Salci et al., 2015). Upon transferring these colonies to enriched media in the form of MEF-CM, they form stable iPSCs that possess all the hallmark features of hESCs. Given that OCT4 iPSCs take up to 5 times longer to generate than conventional iPSCs, it is likely that OCT4 is capable of inducing the expression of pluripotency associated factors in the presence of RM, but that this process is secondary to induction of plasticity (Figure 1).

Constructing a working definition of OiP has provided the field with clarity regarding the nature of reprogramming induced by expression of OCT4 alone versus

OCT4 combined with additional reprogramming factors. As we intimated in our description of OiP (Chapter 3), brief expression of a full set of pluripotency factors is sufficient to induce a transient state of pluripotency (Bar-Nur et al., 2015; Maza et al., 2015). The absence of transient iPSC formation during OiP is consistent with our recent report of OCT4 iPSCs, where formation of pluripotent cells occurred on average 60 days after induction of plasticity. Therefore, OiP can be considered a unique means of facilitating cellular reprogramming that unifies the previously distinct methodologies of direct conversion between somatic cell states and the induction of pluripotency, where iPSCs, like hematopoietic or neural progenitors, are simply cell fate trajectories available to cells that have induced a state of transcriptional plasticity via expression of OCT4 (Figure 1) (Salci et al., 2015).

5.3 Reprogramming capacity of OCT4 is not limited to fibroblasts

Armed with the understanding that OCT4 in combination with RM induced a state of plasticity in human fibroblasts that bestowed the ability to make cell fate choices towards somatic progenitor cells (Chapter 3), we next aimed to identify if our technology was applicable to alternative somatic cell starting populations. To address this final question, we expressed OCT4 in both neonatal and adult blood progenitor cells cultured in RM, followed by additional treatment with neural supportive culture conditions in order to generate tri-potent neural progenitor cells (Figure 1) (Chapter 4). Initially, OCT4 expressing blood progenitors cultured in RM did not reprogram when stimulated with conditions that supported NPC generation from fibroblasts (Chapter 2&3). To overcome this, we supplemented our neural lineage culture conditions with molecules based on

previous studies that applied developmental paradigms to enhance neural specification from hESCs mediated by simultaneous activation of Wnt signaling and dual inhibition of SMAD signaling via TGF-β and BMP receptors (Chambers et al., 2009; Li et al., 2011). It should be noted, that for non-scientific or purposeful reasons, IGF2 was omitted from RM during this process (personal communication Dr. J.H. Lee). As such, these results informed us of the following: 1) IGF2 may be a critical regulator of OiP regarding activation of neural lineage regulator gene expression, and 2) activation of Wnt and inhibition of SMAD is sufficient to induce neural regulator gene expression in OCT4 plastic blood cells devoid of IGF2 influence. In a broader sense, these results may also represent a reprogramming barrier inherent to blood cells with regards to activation of neural regulator gene expression that is not present in fibroblasts. Alternatively, fibroblasts may be more receptive or even biased to activation of neural regulator transcription. In support of this latter idea, we demonstrated that fibroblasts, although devoid of neural reprogramming capacity in the absence of OCT4 and/or RM (Chapter 3), are enriched for basal levels of primitive neural crest related gene expression compared to that of blood cells (Chapter 4). Moreover, unbiased analysis of gene set enrichment upon addition of small molecule inhibitors of GSK-3 and SMAD to the neural lineage culture conditions used to induce neural fate from OCT4 expressing blood cells, revealed neural crest gene expression to be the most prominently enriched neural related gene set. These results suggest that this particular combination of small molecule inhibitors activates transcriptional programs already present to some extent in fibroblasts, providing a

potential explanation for their specific requirement during OCT4 reprogramming of human blood.

Our observations of neural crest related gene expression activation correlates with the developmental potential of blood derived NPCs (Chapter 4). These results are not unprecedented, as the combination of Wnt activation and SMAD inhibition has also been shown to support the generation of neural crest from hESCs (Menendez et al., 2011). This early specification event has recently been modeled in hESCs, whereby induction of Wnt signaling results in the binding of β-catenin to both inducers of neural crest as well as OCT4- and SMAD-occupied primitive streak genes (Funa et al., 2015). Binding of β-catenin results in expression of both neural crest and primitive streak genes, however inhibition of SMAD is required to prevent nodal signaling from repressing neural crest gene expression. Given that OiP is not transcriptionally or functionally consistent with hESCs, it will be of interest to determine whether the observed differences in fibroblast versus blood cell OiP facilitated reprogramming are hinged upon similar early specification pathways, or make use of these regulators in a novel manner.

Taken together, these results have confirmed that OiP can be applied to multiple somatic cell starting populations (Figure 1). In support of this notion, additional groups have recently developed OCT4 based reprogramming approaches that utilize a variety of starting cell populations such as hair follicle mesenchymal stem cells (Liu et al., 2015), CD34+ (Meng et al., 2013) and CD34+CD45+ (Liao et al., 2015) umbilical cord blood cells. These studies not only provide evidence to support the utility of OCT4 as a versatile reprogramming factor, but also provide a framework to perform comparative

studies in order to understand the differential impact of OCT4 on somatic transcriptional regulation in the context of unique cellular and extracellular environments. Such an analysis would further our understanding of the powerful function of OCT4 as a regulator of transcription and cellular reprogramming.

5.4 Moving forward with OCT4 induced plasticity

The construction of an OiP based reprogramming model has provided a biological framework in the form of robust assays that will enable further dissection into the manner in which OCT4, in concert with the extracellular environment, bestows the potential on somatic cells to make new or alternative cell fate choices (Figure 1). In addition to the specific points addressed above, the development of a working definition of OiP has raised a number of additional questions regarding the nature of this unique reprogramming process. Most of what we know regarding the mechanism of OiP is related to transcriptional responses that have been measured at the whole population level (Chapter 3&4). At this point, we have yet to identify a protein level marker of OiP that could serve as a means to isolate or enrich for cells participating in the reprogramming process, similar to that of Tra-1-60 for iPSCs. Isolation of this discrete population would enable clonal level analysis that could reveal whether cells that have initiated OiP harbor equal developmental potential, or instead are restricted towards specific cell fate trajectories. Moreover, development of a reliable marker would enable the collection of cells under the influence of OiP in large numbers for use in down-stream molecular analysis with reduced noise from non-reprogramming cells. Similar to studies of iPSC reprogramming (Soufi et al., 2012; Soufi et al., 2015), cells that have initiated OiP could

be analyzed for the binding of OCT4 to the genome, and verify whether OCT4 behaves as a pioneer factor in a similar fashion to when it is expressed in tandem with the Yamanaka factors. OCT4 localization data could be compared with that in fibroblasts containing all four Yamanaka factors to establish whether OCT4 displays unique genome targeting that facilitates OiP, or binds to similar locations found in cells destined to become iPSCs. Furthermore, paired analyses for changes in histone level regulation and DNA methylation status could be applied to both naïve fibroblasts and those at different time points post induction of OiP to establish whether this process truly results in epigenetic alterations to OCT4 target gene loci prior to transcriptional reprogramming. In addition, protein-protein interaction maps of Oct4 in pluripotent cells have established an Oct4 centric network that includes members of chromatin remodeling complexes and additional transcriptional regulators (van den Berg et al., 2010). Deciphering whether OCT4 forms a similar network within somatic cells or one that is unique to OiP would serve as a means to generate a list of potentially required factors that could be further assayed through controlled expression/repression experiments to evaluate their role in the initiation of the reprogramming process. In depth molecular analyses such as those listed above could equally be applied to OiP cells that have been treated with lineage specific culture conditions in order to evaluate whether OCT4 itself continues to play a role in the reprogramming process beyond that of initiation of plasticity. Moreover, these studies could be performed on both fibroblast and blood cells undergoing OCT4 reprogramming in order to understand the molecular basis for their differential requirements to achieve direct conversion to NPCs.

Taken together, these questions will further our understanding of how OiP is mechanistically established within somatic cells, and bestows the ability to make cell fate choices towards alternative multi-potent progenitor states or even iPSCs.

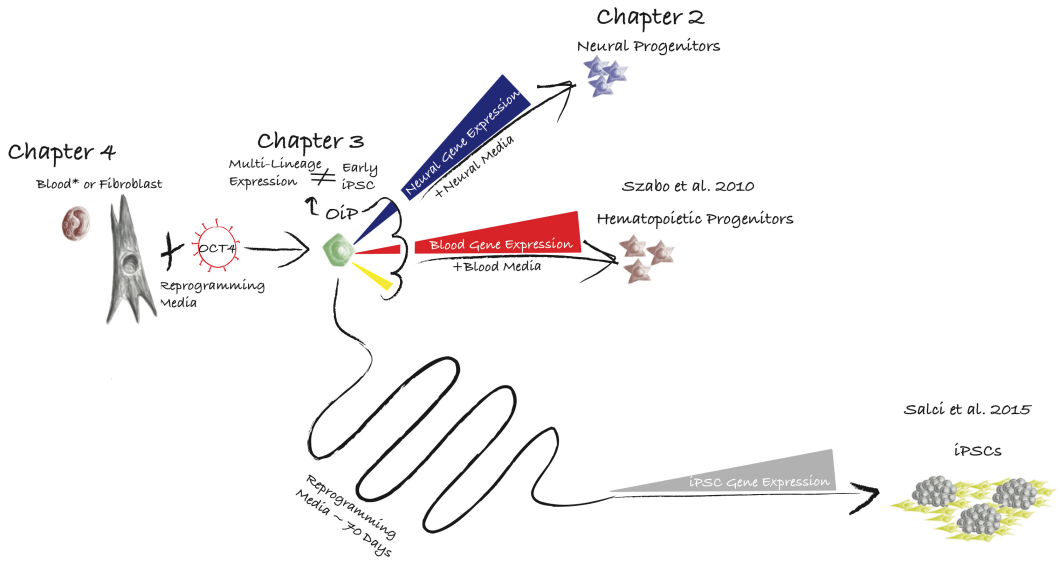
5.5 Concluding Remarks

Within the context of regenerative medicine, the current overarching goals for cellular reprogramming are similar to those of the stem cell community and can be broken down into three major pursuits: construction of clinically relevant disease models, generation of autologous cells for transplantation therapy, and activation of endogenous repair. Although these pursuits are fundamentally different in their approach to addressing degenerative disease, they are unified by the process of reprogramming. Evidence from studies of interconverting cells of the pancreas and adrenal gland (Chera et al., 2014; Freedman et al., 2013), dedifferentiating cells of the kidney and lung (Kusaba et al., 2014; Tata et al., 2013), as well as the awesome regenerative power of salamanders (Endo et al., 2004; Kragl et al., 2009) informs us that Sir John Gurdon's principles regarding the nature of differentiation are similarly applied in nature as they are during early SCNT experimentation (Gurdon and Uehlinger, 1966). The studies within this thesis highlight the role of OCT4 as a mediator of inducing a cellular status capable of responding to soluble factors that are known to stimulate developmental regulators and pathways critical to cellular differentiation (Chapters 2-4). This status is unique from the induction of pluripotency, and instead supports both the interconversion of somatic progenitors (Chapter 4) as well as their formation from differentiated mature cell populations (Chapter 2 & 3). This process is not unlike the initiation of salamander blastemas, where

mature cells of the skin, bone, and nervous system dedifferentiate and form a heterogeneous mix of undifferentiated progenitor like cells (Kragl et al., 2009). Both OCT4 plastic cells and salamander blastemas respond to developmentally related growth factors and signaling events that guide further specification and subsequent differentiation (Endo et al., 2004). Moreover, transcription factors known to interact with OCT4 such as CMYC and SP1 are expressed during blastema formation and form highly connected networks with both up- and down- regulated proteins during the onset of regeneration (Jhamb et al., 2011). Although expression of OCT4 has not been directly implicated in salamander regeneration (Jhamb et al., 2011), the underlying principles of direct conversion towards somatic progenitor cells may still share common mechanistic elements. It is my belief that the continued pursuit for evolving our understanding of the fundamental molecules and signaling events that underlie both the induction of OCT4 plasticity and naturally occurring reprogramming events such as salamander blastemas will aid in the application of these phenomena in an effort to combat degenerative disease.

Figure 1. Model of OCT4 induced Plasticity (OiP)

Expression of OCT4 in somatic cells cultured in reprogramming media induces a state of transcriptional plasticity that we have named OCT4 induced plasticity or OiP. Cells that have entered a state of OiP gain the ability to make new or alternative cell fate choices in response to stimulus from the extracellular environment. This process enables the generation of both neural and hematopoietic progenitors from skin fibroblasts and the conversion of hematopoietic progenitors into neural progenitors. Lastly, OiP is a molecularly distinct state from that of cells destined to become iPSCs, but it maintains the choice to adopt iPSC fate upon appropriate environmental stimulation. *Blood cells were only utilized to generate NPCs.


Chapter 2
Neural Progenitors
Neural Gene Expression
+Neural Media
Chapter 4
Blood* or Fibroblast
Chapter 3
Multi-Lineage Expression
Early iPSC
OCT4
OiP
Reprogramming Media
Szabo et al. 2010
Hematopoietic Progenitors
Blood Gene Expression
+Blood Media
Salci et al. 2015
iPSCs
iPSC Gene Expression
Reprogramming Media – 30 Days

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Appendix II: List of Scientific Publications, Abstracts, and Patents

Published Referred Papers

Ryan R. Mitchell, Szabo Eva, Benoit Yannick D., Case Daniel T., Mechael Rami, Alamilla Javier, Lee Jong Hee, Fiebig-Comyn Aline, Gillespie Deda C., and Bhatia Mickie. (2014). Activation of neural cell fate programs towards direct conversion of adult human fibroblasts to tri-potent neural progenitors using Oct-4. **Stem Cells and Development**. 23(16): 1937-1946. Doi:10.1089/scd.2014.0023.

Mitchell, R., Szabo, E., Shapovalova, Z., Aslostovar, L., Makondo, K. and Bhatia, M. (2014), Molecular Evidence for OCT4-Induced Plasticity in Adult Human Fibroblasts Required for Direct Cell Fate Conversion to Lineage Specific Progenitors. **STEM CELLS**, 32: 2178–2187. Doi: 10.1002/stem.1721

Jong-Hee Lee,Jung Bok Lee,Zoya Shapovalova,Aline Fiebig-Comyn,Ryan **R. Mitchell**, Sarah Laronde,Eva Szabo,Yannick D. Benoit& Mickie Bhatia. (2014), Somatic transcriptome priming gates lineage-specific differentiation potential of human-induced pluripotent stem cell states. **Nature Communications** 5, Article number: 5605 doi:10.1038/ncomms6605

Jong-Hee Lee*, **Ryan R. Mitchell***, Jamie D. McNicol, Zoya Shapovalova, Sarah Laronde, Borko Tanasijevic, Chloe Milsom, Fanny Casado, Aline Fiebig-Comyn, Tony J. Collins, Karun K. Singh, Mickie Bhatia. (2015). Single Transcription Factor Conversion of Human Blood Fate to NPCs with CNS and PNS Developmental Capacity.

http://dx.doi.org/10.1016/j.**celrep**.2015.04.056. © 2015 The Authors. Published by Elsevier Inc. ***Co-First Author**

Kyle R. Salci, Jung Bok Lee, **Ryan R. Mitchell**, Luca Orlando, Aline Fiebig-Comyn, Zoya Shapovalova, Mickie Bhatia Acquisition of pluripotency through continued environmental influence on OCT4 induced plastic human fibroblasts. **Stem Cell Research** 06/2015; 25(1). DOI:10.1016/j.scr.2015.06.005

Kyle R. Salci, Jung Bok Lee, **Ryan R. Mitchell**, Luca Orlando, Aline Fiebig-Comyn, Zoya Shapovalova, Mickie Bhatia. Derivation of human induced pluripotent stem cells through continued exposure of OCT4-induced plastic human fibroblasts to reprogramming media. **Stem Cell Research** 06/2015; 25(1). DOI:10.1016/j.scr.2015.06.005.

Manuscripts Under Peer Review

Yannick D. Benoit,*, **Ryan R. Mitchell**,*, Ruth M. Risueño,*, Angelique Schnerch, Allison L. Boyd, Lili Aslostovar, Linda May, Mio Nakanishi, Zoya Shapovalova, Uyen M. Dang, Masakatsu Eguchi, Monica Graham, Aline Fiebig-Comyn, Michelle Jones, Anargyros Xenocostas, Michael R. Trus, Brian Leber, Tony J. Collins, Eva Szabo and Mickie Bhatia. Sam68-mediated disruption of CBP/β-catenin neoplastic transcriptional programming allows selectivity against human cancer stem cells. **Nature 2015. *Co-first Author**

Manuscripts Awaiting Resubmission

Jong-Hee Lee, Sarah Laronde, **Ryan Mitchell**, Tony J. Collins, Jung Bok Lee, ZoyaShapovalov, Jamie D. McNicol, Aline Fiebig-Comyn, Borko Tanasijevic, Sean C.Bendall, and Mickie Bhatia. Lineage specific differentiation is prejudiced by state of human pluripotency. **Cell Press 2015**.

Abstracts

Ryan Mitchell, Eva Szabo, Tony Collins, Zoya Shapovalova, and Mickie Bhatia. Altering Cell Fate with OCT4. TMM 2014.

Patents (Inventor Status)

Type: US provisional application

Title: Generating Induced Neural Progenitor Cells from Blood

Serial Number: 62/164,222

Filing Date: May 20, 2015