Transdifferentiation of Fibroblasts to Neural Stem Cells

By

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Abstract:

The developmental process is carefully controlled by transcriptional and epigenetic changes that occur as a zygote transforms into an adult organism. This process can be reversed by the overexpression of transcription factors Oct4, Sox2, Klf4, and c-Myc, which reprogram a differentiated cell's nucleus to one that is transcriptionally and epigenetically indistinguishable from an embryonic stem (ES) cell. However, it is still unclear if transcription factors can completely convert the nucleus of a differentiated cell into that of a distantly related somatic cell type with complete transcriptional and epigenetic reprogramming maintained in the absence of exogenous factor expression. To test this idea, we generated doxycyline (dox)-inducible vectors encoding neural stem cell-expressed factors. We found that stable, self-maintaining NSC-like cells could be induced under defined growth conditions. These cells were characterized in the absence of exogenous factor induction and were shown to be transcriptionally, epigenetically, and functionally similar to endogenous embryonic cortical NSCs. Additionally, a cellular system was created for reproducible generation of dox-independent iNSCs without additional factor transduction. Our results show that a transcriptionally and epigenetically reprogrammed somatic nucleus can be stabilized *in vitro* and provides a tool to study the mechanism of somatic cell conversion.

Thesis Supervisor: Rudolf Jaenisch
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Dedication:

I would like to dedicate this work to my father Johnny, whose unconditional support and undying love will forever guide me through life.

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I have a lot of people to thank:

First, I would like to thank Rudolf for letting me explore this exciting, yet incredibly challenging project that stretched my brain in ways I didn't realize existed! I was given freedom to pursue the problems that I found most interesting. Looking back, I wanted to continue at MIT for my graduate training because I felt it would give me a graduate experience that no other place could provide. And it did. Rudolf and the members of the Jaenisch Lab gave me experiences that I will never forget. I am deeply grateful for the opportunity that Rudolf has given me.

Also, I want to thank Rudolf for being there when I needed him the most; scientifically, but more importantly, personally. I had only been in the lab for six months when I developed serious medical problems. I quickly learned why the Germans call their advisors "doctor-fathers." Rudolf was among the first to call me when I was admitted to the hospital. He said, "John, I'm in the airport in New York and thought I would call you." He called regularly, each time from another city, thinking of me while he was on the road. When he returned to Boston, he visited me in the hospital. I had no idea where they had placed me in the hospital, but Rudolf came walking up, bringing gifts and making my family feel very comfortable. I never expected this when I joined one of the most respected labs in the world. Thank you!

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My parents have done everything possible to make sure that I had the opportunity to get where I am in life. I want to thank my mom, who made it clear growing up that there is nothing she would not sacrifice to help me. My dad—I decided in sixth grade that I wanted a PhD, just so I could be like him. I know how incredibly proud of me he is, even if he can no longer express it. I hope to lead by example one day the same way my parents did for me.

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Chapter 1

Introduction

During mammalian development, the totipotent zygote gives rise to all the cell types and tissues that form the adult organism. As development proceeds, this single totipotent cell begins to divide and progressively loses its potency. By the blastocyst stage, two distinct cell types have emerged—a compact cluster of pluripotent cells that are the precursors to the embryo and the surrounding trophoblasts that form the trophectoderm. The blastocyst is reorganized for embryonic development during gastrulation, and the cells are further restricted to one of three germ layers: ectoderm, mesoderm, and endoderm. This differentiation process continues, and increasingly specialized cells are generated until terminally differentiated post-mitotic cells are created, which do not have the capability to generate progeny.

Remarkably, the diverse cell types generated during mammalian development contain the exact same genome. This means that cell function and developmental potential are governed entirely by cell type-specific regulation of gene products through chemical modifications placed on the genome. These epigenetic modifications and the resulting gene expression patterns provide unique signaling cues that maintain a cell's identity and ensure proper progression through development.

Normal development is a unidirectional process. However, since the genomes of differentiated cells are equivalent, it raises the intriguing possibility that the epigenetic modifications maintaining cell identity can be manipulated such that a cell can be "converted" or "reprogrammed" to an entirely different type of cell. Indeed, this has been shown convincingly through decades of work on reprogramming differentiated cells to pluripotency. Recently, these studies culminated with the discovery that just

four transcription factors (Oct4, Sox2, Klf4, and c-Myc) are sufficient to reprogram the nucleus of any adult cell type to a state identical to embryonic stem cells. These induced pluripotent stem (iPS) cells are now valuable tools for studying the pluripotent state, understanding the epigenetic regulation of cells, and creating patient-specific therapies.

The surprising discovery that four transcription factors can provoke this dramatic cell fate change to pluripotency has led us to wonder if other cell types can be induced in a similar manner. The work in this thesis focuses on changing the epigenetic identity of fibroblasts to that of neural stem cells through the forced expression of transcription and chromatin-modifying factors. In order to provide proper context for this work, the introduction will discuss three topics: 1) embryonic stem cells and the methods used for nuclear reprogramming, 2) neural stem cells and the factors that drive their biology, and 3) the meaning of “transdifferentiation” and evidence for it in the literature.

Part I: Embryonic stem cells and nuclear reprogramming

Embryonic stem cells

The first pluripotent cells to be grown *in vitro* were embryonal carcinoma (EC) cells, derived from mouse germ cell tumors. Analysis of these transformed cells elucidated growth conditions under which pluripotent cells can be grown *in vitro* (Smith and Hooper, 1983; Kahan and Ephrussi, 1970). Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass (ICM) of pre-implantation blastocyst stage embryos. During *in vivo* development, the cells in the ICM are a transient cell type and differentiate into the epiblast and the hypoblast. However, unlike the cells of the ICM, when explanted *in vitro*, ES cells can grow indefinitely and maintain the ability to differentiate into all of the cell types that make up the embryo (Evans and Kaufman, 1981; Martin 1981; Thomson et al., 1998).

Key intrinsic and extrinsic factors for ES cells

The ease with which ES cells are grown *in vitro* has allowed for careful analysis of this cell state and, along with classical genetic approaches, has led to a detailed understanding of the intrinsic and extrinsic drivers of pluripotency. Below are a few of the factors known to be important for this process.

Oct4/Pou5f1

Oct4, a POU-domain transcription factor encoded by the *Pou5f1* gene, was the first transcription factor found to regulate pre-implantation embryo development (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990). Oct4 expression is closely associated with the pluripotent state. The protein is initially present in the developing embryo as a maternally inherited factor (Scholer et al., 1989a), and the zygotic gene is expressed throughout early development, including in the blastomere, ICM, and epiblast (Yeom et al., 1996). In the adult, Oct4 expression is restricted to primordial germ cells (Pesce and Scholer, 2001; Scholer et al., 1989b). The Oct4 gene is dispensable for all somatic cells (Lengner et al. 2007), but required for early embryogenesis; null embryos display peri-implantation lethality, and their ICM lacks pluripotent cells (Nichols et al., 1998). Oct4 expression in ES cells is tightly regulated to maintain self-renewal: too much expression triggers differentiation to primitive endoderm and mesoderm, and too little expression results in trophectoderm formation (Niwa et al., 2000). Interestingly, Oct4 binds regulatory elements with a number of partner factors to control gene expression (Scholer et al., 1991; Yuan et al., 1995; Ambrosetti et al., 1997; Ben-Shushan et al., 1998; Botquin, et al., 1998).

Sox2

Sox2 is a well-studied binding partner of Oct4, and the two regulate the *Fgf4* gene through cooperative binding (Kamachi et al., 2000; Yuan et al., 1995; Ambrosetti et al., 1997). Sox2 is a member of the SRY-related HMG-box family of transcription

factors. Like Oct4, it is expressed in the ICM, epiblast, and germ cell, but unlike Oct4 it is also expressed in somatic cells. Sox2 is highly expressed in the multipotent stem and progenitor cells in the developing nervous system and extraembryonic ectoderm (Avilion et al, 2003; Zappone et al., 2000), as well as numerous adult stem cell compartments (Arnold et al., 2011). Sox2-deficient embryos die during peri-implantation due to defects in the epiblast (Avilion et al., 2003). Furthermore, Sox2 is required for the proper expression of Oct4 in mouse ES cells, and Sox2-null ES cells cannot be established (Masui et al., 2007).

Nanog

The Nanog homeodomain transcription factor was initially discovered as a factor whose constitutive expression promotes the proliferation of ES cells and the maintenance of pluripotency in conditions that otherwise induce differentiation (Chambers et al. 2003; Mitsui et al., 2003). Nanog is critical for pluripotency *in vitro* and *in vivo*. Nanog-null ES cells proliferate slower than wild-type ES cells and differentiate into extraembryonic endoderm in the absence of cytokine signaling (Mitsui et al., 2003). Embryos deficient for Nanog fail to develop epiblast cells and die shortly after implantation (Mitsui et al., 2003). Interestingly, Nanog levels has been reported to fluctuate within ES cell colonies, suggesting that it may not be crucial for the maintenance of pluripotency, although its down-regulation does predispose ES cells to differentiation (Chambers et al., 2007). Established ES cells in which Nanog is genetically deleted maintain the ability to self-renew and contribute to all three germ

layers of mouse chimeras (Chambers et al., 2007). This suggests that Nanog is important for the establishment of pluripotency, but is dispensable for maintaining pluripotency (Chambers et al., 2007).

Klf4

Klf4, or Kruppel-like factor 4, is a zinc-finger transcription factor expressed in a variety of somatic cell types such as the colon, small intestine, stomach, and skin, among others (Shields et al., 1996; Garrett-Sinha et al., 1996). Prior to the discovery of iPS reprogramming (Takahashi and Yamanaka, 2006), only one study had examined Klf4's role in ES cells. That was a report by Niwa and colleagues showing that Klf4 in conjunction with Oct4 and Sox2 activates the *Lefty1* gene in ES cells (Nakatake et al., 2006). It is now known that Klf4 and its closely related family members Klf2 and Klf4 play important roles in pluripotency, since the simultaneous knockdown of all three results in ES cell differentiation (Jiang et al., 2008).

Lif/Stat3

Leukemia inhibitory factor (Lif) was first cloned as a cytokine that could induce the differentiation of myeloid leukemia cells into macrophages (Gearing et al., 1987). It was later identified as the paracrine factor secreted by fibroblasts that promotes the self-renewal of ES cells (Smith et al., 1987; Williams et al., 1988). Lif is a member of the IL-6 cytokine family, and its binding to the Lif receptor (Lif-R) causes heterodimerization between the Lif-R and Gp130, which in turn activates the STAT3

transcription factor (Ernst et al., 1996). Klf4 is a downstream target of Lif signaling, and it has been shown that forced expression of Klf4 promotes Lif-independent growth of ES cells (Niwa et al., 2009).

The core transcriptional circuitry of ES cells

The key characteristics of ES cells—their self-renewal and pluripotency—are maintained by a unique transcriptional circuitry that ensures the expression of ES cell-associated genes necessary for self-renewal while also maintaining the repression of key developmental regulators necessary for differentiation (Figure 1). Genome-wide analysis of transcription factor binding in ES cells has provided insight into how these factors act globally to maintain pluripotency. In ES cells, three transcription factors—Oct4, Sox2, and Nanog—bind to and regulate their own and each other's promoter, forming a highly redundant interconnected auto-regulatory loop (Loh et al., 2006; Boyer et al., 2005) (Figure 1). This important finding suggests one possible explanation for why the ES state is stable: once the auto-regulatory loop is active, it maintains its own expression. Furthermore, these key transcription factors may continuously buffer the expression of one another, such that variations in one factor's expression can be compensated for by the other factors. This has been shown to be the case for Oct4 and Sox2, where the forced expression of Oct4 can rescue the pluripotency defect of Sox2-null ES cells (Masui et al., 2007).

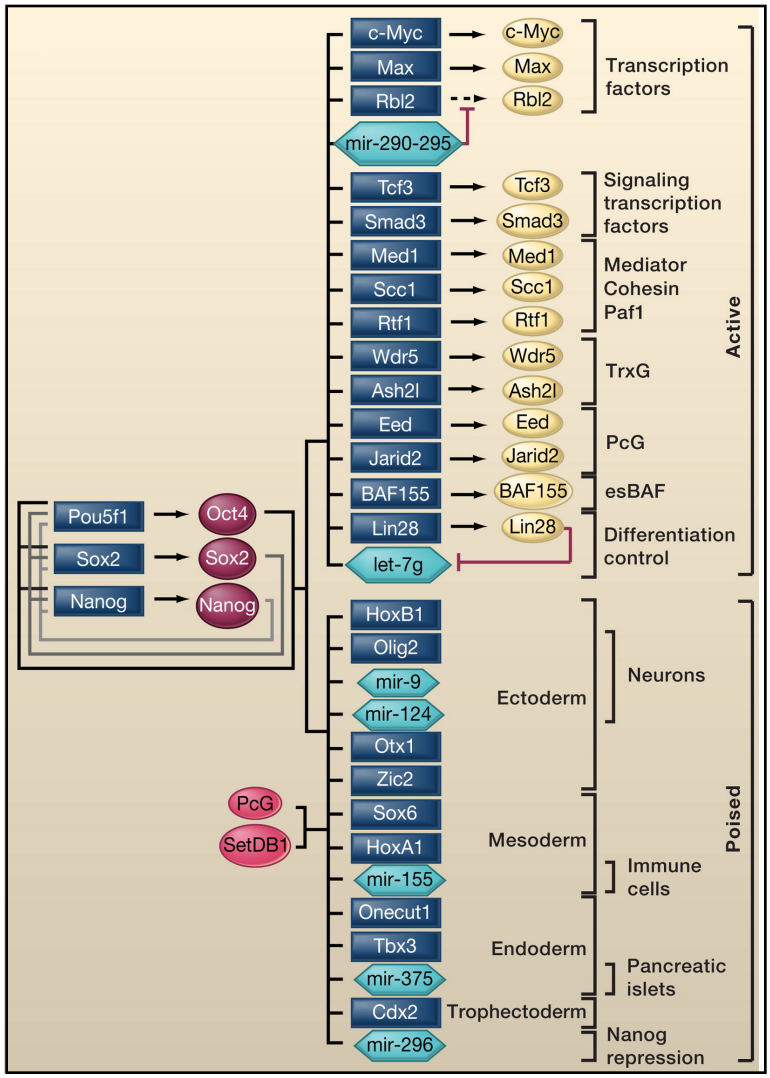


Figure 1. Transcriptional circuitry of ES cells
In ES cells, Oct4, Sox2, and Nanog bind to their own and each other's promoters. This creates a feed-forward auto-regulatory loop that maintains the expression of these genes in ES cells. These transcription factors also bind to many other genes. At active genes, their concerted binding maintains the expression of genes necessary for the ES cell state. Repressed genes are not only bound by Oct4, Sox2, and Nanog, but also by the repressive Polycomb Group proteins (PcG). This binding pattern leaves the genes (often developmental regulators) poised for expression upon differentiation cues. Figure adapted from Young, 2011.

Another important finding from these genome-wide studies is that Oct4, Sox2, and Nanog have coordinated binding on many of their target genes, which include both active and inactive genes (Figure 1). For the active genes, the concerted action of these transcription factors acts to reinforce and sustain the unique transcriptional state of ES cells by maintaining the expression of ES cell-expressed regulators. The repressed genes are not only bound by the three key transcription factors, but also by the Polycomb repressive complex (PRC) 2, which epigenetically marks genes for inhibition by trimethylating histone H3 lysine 27 (H2K27me3) (Boyer et al., 2006; Lee et al., 2006; Cao et al., 2002). Interestingly, these repressed genes are developmental regulators that are not expressed in ES cells, but "poised" for rapid expression in response to developmental cues. (Boyer et al., 2006; Lee et al., 2006). Indeed, disruption of PRC2 in ES cells relieves the repression on poised genes and results in their expression (Boyer, 2006). Thus, the core transcriptional circuitry of ES cells maintains the ES cell-specific phenotype by cooperatively binding to and activating genes necessary for the ES cell state, while simultaneously keeping repressed genes poised for expression upon differentiation (Young, 2011).

Transcription factors and enhancers

Transcription factors bind DNA at enhancers, where they coordinate with histone modifiers, chromatin remodelers, and mediators of transcription to dictate gene expression (Buecker and Wysocka, 2012; Spitz and Furlong, 2011). Since DNA is packaged into nucleosomes, some DNA sites are inaccessible to transcription and other

factors. Cooperative transcription factor binding, like that observed for the core ES factors (Oct4, Sox2, and Nanog) is one way that factors can overcome this barrier (Calo and Wysocka, 2013; Adams and Workman, 1995). Alternatively, some transcription factors have a unique ability to reposition nucleosomes. These so-called "pioneer" factors open enhancer sites so other factors can bind (Calo and Wysocka, 2013; Zaret and Carroll, 2011).

Enhancers are specifically marked throughout the genome. All enhancers (active and poised) are marked by histone 3 lysine 4 mono-methylation (H3K4me1), but active enhancers have an additional mark, histone 3 lysine 27 acetylation (H3K27ac) (Creyghton et al., 2010; Rada-Iglesias et al., 2011). Each cell type has a characteristic enhancer chromatin pattern, and enhancer profiles change during differentiation (Creyghton et al., 2010). In fact, enhancer usage is so specific that a gene expressed in two different cell types can be regulated and marked by different enhancers in the two cell types, such as the case of *Pou5f1* (which encodes Oct4), which is driven by different enhancers in pre- versus post-implantation pluripotent cells (Yeom et al., 1996).

Nuclear reprogramming

Epigenetic marks like H3K4me1 or H3K27ac are placed on the genome to regulate gene expression and to ensure that proper cell identity is maintained throughout development. Since development is a unidirectional process, it was not immediately clear if these marks could be modified such that a cell assumes a different

nuclear identity. We now know that this is possible and that successfully reprogramming of a differentiated cell's nucleus to the pattern present in pluripotent cells gives it properties of self-renewal and pluripotency. This reprogramming event can be achieved by three methods: nuclear transfer, cell fusion, and forced transcription factor expression (Figure 2).

Nuclear transfer

During fertilization, the epigenetic marks of the gametes are reprogrammed to allow for early embryonic development, a process mediated by the cellular components of the oocyte (Morgan et al. 2005). The first hints that the somatic nucleus can be reprogrammed originated from nuclear transfer experiments in *Xenopus*. John Gurdon showed that proper development can proceed even if the nucleus of an egg is replaced by the differentiated nucleus of a tadpole intestinal cell (Jaenisch, 2012; Gurdon, 1960). This demonstrated that all cells have the requisite genetic information for full development.

The cloning of 'Dolly the Sheep' demonstrated that nuclear transfer can be applied to more complex developmental systems (Wilmut et al., 1997). However, nuclear transfer/ reproductive cloning was found to be extremely inefficient: most embryos die soon after implantation, and many of the surviving ones are born developmentally abnormal, likely due to incomplete epigenetic reprogramming

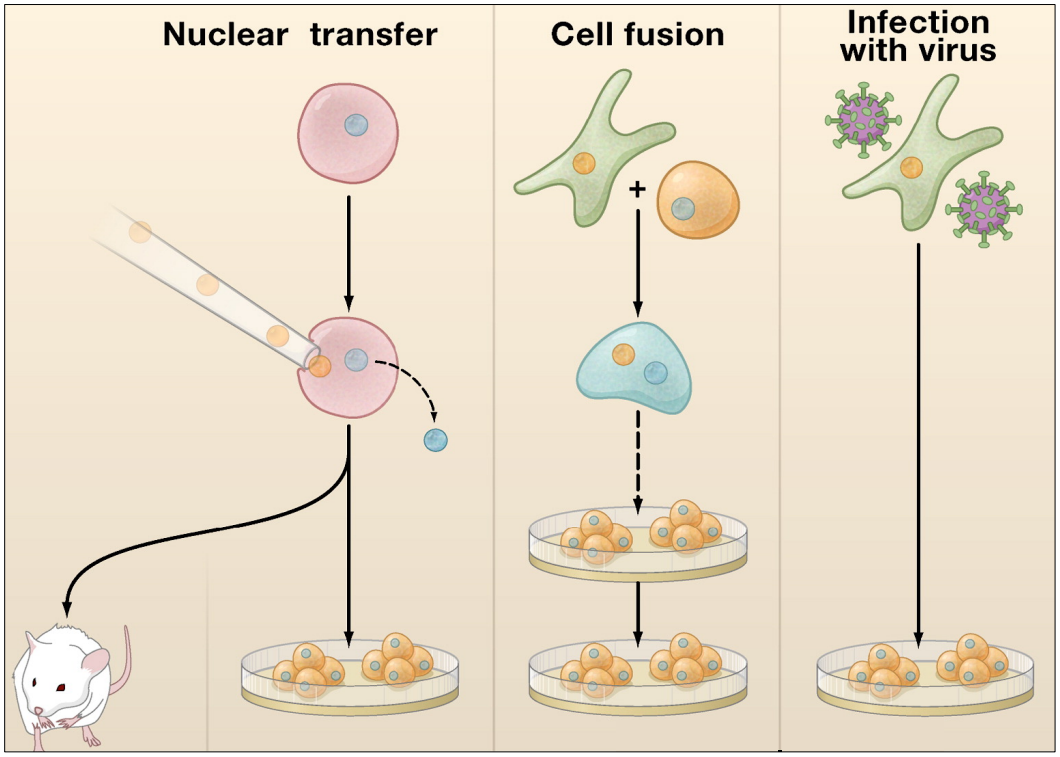


Figure 2. Nuclear reprogramming techniques.
There are three main methods to reprogram somatic cells to pluripotency. Nuclear transfer involves removing the nucleus of an oocyte and replacing it with a somatic nucleus. The factors in the oocyte are sufficient to induce pluripotency (Left). ES cells have dominant *trans*-acting factors that can impart pluripotency of a somatic nucleus upon cellular fusion (Middle). The overexpression of 4 transcription factors (Oct4, Sox2, Klf4 and c-Myc) in somatic cells can reprogram their nucleus to pluripotency (Right). Figure adapted from Jaenisch and Young, 2008.

(Hochedlinger and Jaenisch, 2006). Despite this, the nuclear transfer technology provided unequivocal proof that terminally differentiated cells have the capacity to be reprogrammed to pluripotency (Hochedlinger and Jaenisch, 2002; Eggan et al., 2004).

Despite the therapeutic potential and biological importance of the nuclear transfer-mediated reprogramming, this technique has some major drawbacks that have limited its widespread use. Chief among these are the limited supply of oocytes and ethical concerns of using human oocytes for this process. Additionally, nuclear transfer has proven to be a technically difficult process in the mammalian system—with success in the human occurring nearly 55 years after Gurdon's initial discovery (Tachibana et al., 2013).

Cell fusion

Cell fusion mixes two cells' cellular components and allows the dominant *trans*-acting factors of one cell to exert control over gene expression in the other cell, inducing genes that would otherwise not be expressed. In this way, the resulting hybrid's phenotype is generally associated with the cell that expressed the most dominant acting factors (Graf, 2011). This has been shown to be the case for pluripotent cells, such as embryonal carcinoma cells and embryonic stem cells (Miller and Ruddle, 1976; Tada et al., 2001; Cowan, 2005).

Cell fusion has demonstrated that ES cells, like oocytes, have *trans*-acting factors that can bestow pluripotency on a differentiated nucleus. Unfortunately, the nature of the fusion event makes it extremely difficult to separate the nuclei and recover

diploid cells. For this reason, cell fusion has limited applicability for patient-specific therapy or studies on the mechanisms of nuclear reprogramming

Factor-mediated reprogramming

The concepts of nuclear reprogramming took a dramatic leap forward with Takahashi and Yamanaka's discovery that four transcription factors are sufficient to initiate a cascade of cellular changes that results in a pluripotent, epigenetically reprogrammed nucleus (Takahashi and Yamanaka, 2006). Reprogramming quickly went from being an inefficient, technically-cumbersome phenomenon mediated by obscure factors present in oocytes or ES cells, to a highly reproducible, widely-applicable process mediated by the overexpression of four proteins—Oct4, Sox2, Klf4, and c-Myc. Although the induced pluripotent stem (iPS) cells initially reported were not competent for germline transmission, a hallmark of pluripotent cells, this was later shown to be an artifact of the gene used for iPS selection and not a deficiency in the reprogramming mechanism itself (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). In fact, iPS cells have been shown to fulfill even the most stringent test of pluripotentcy—tetraploid complementation (Zhao et al., 2009).

Many iPS cells have complete epigenetic reprogramming, including the full complement of histone marks and enhancer sites, although vestiges of epigenetic memory remain in some clones (Creyghton et al., 2010; Kim et al., 2010). Although highly reproducible, the generation of fully reprogrammed cells is quite slow and inefficient with just ~0.001-0.1% of transduced cells attaining pluripotency after 3-4

weeks (Wernig et al., 2007, Wernig et al., 2008a). Importantly, iPS reprogramming has been shown to epigenetically reprogram terminally differentiated cells, demonstrating that this process works for all cell types tested (Hanna et al., 2008; Wernig et al., 2008b).

The mechanism by which the four factors induce pluripotency currently is an intensely studied process. The analysis of this process has been greatly aided by the establishment of a 'secondary' reprogramming system (Hanna et al., 2008; Wernig et al., 2008a). In this system (Figure 3), doxycyline (dox)-inducible vectors are used to express the four factors, and fully reprogrammed iPS cells are generated. When the iPS cells are injected into a blastocyst for chimera formation, the iPS cells give rise to cells in the chimera tissue with the exact transgene integration pattern as the original ('primary') iPS cells. Then, addition of dox to the culture can produce 'secondary' iPS cells. Thus, it is a reprogramming system in which genetically homogenous embryonic or adult cells can be reprogrammed without the need for factor transduction.

The secondary reprogramming system has led to multiple important findings about factor-mediated reprogramming. First, it has aided in the reprogramming of terminally differentiated B-cells by circumventing the need for factor transduction, thus increasing the efficiency of the process (Hanna et al., 2008). Second, it has allowed for a careful analysis of reprogramming kinetics, which resulted in the discovery that given unlimited time, all cells will reprogram; the perceived low efficiency stems from analysis

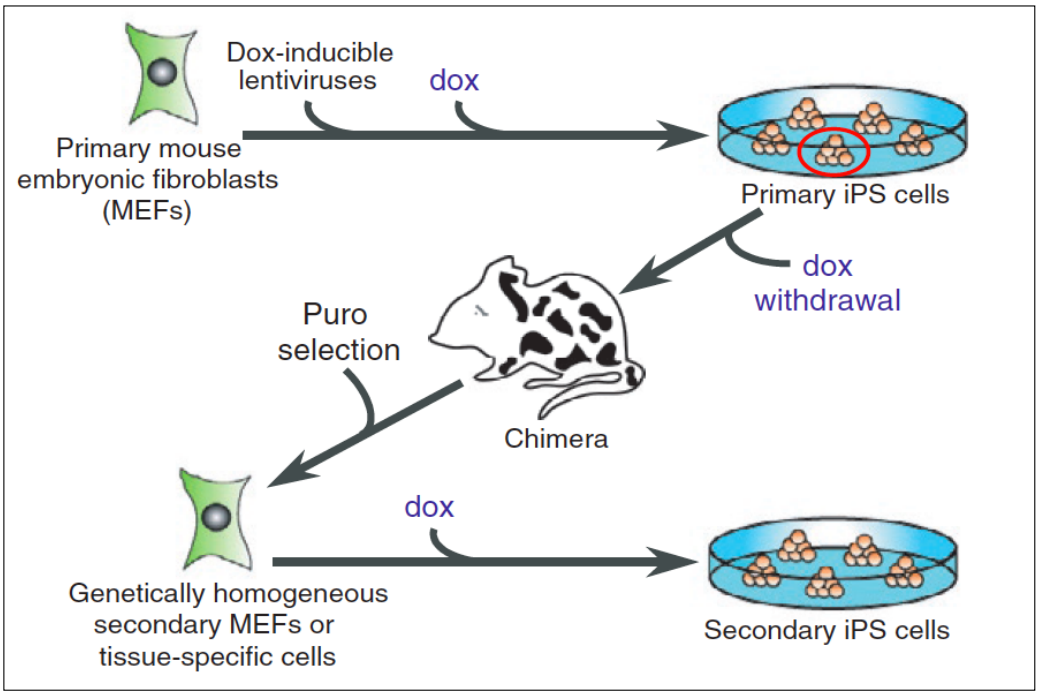


Figure 3. The secondary reprogramming system.
Doxycyline (dox)-inducible lentiviruses are used to express the four factors, and fully reprogrammed iPS cells are generated. When the iPS cells are injected into a blastocyst for chimera formation, the iPS cells give rise to cells in the chimera with the exact factor integrations as the original ('primary') iPS cells. After selection these cells can be reprogrammed with dox induction alone to generate 'secondary' iPS cells.
Figure adopted from Wernig et al., 2008a.

at relatively early time points (Hanna et al., 2009). Third, it makes possible the study of the reprogramming process in genetically identical single cells, thus minimizing differences caused by variable transgene transductions.

Is the pluripotent state uniquely amenable to reprogramming?

Several lines of evidence suggest that ES cells may be unique in their capacity for *in vitro* nuclear reprogramming. As described above, cell fusion experiments show that ES cells can bestow characteristics of pluripotency on a somatic nucleus after cell fusion. This suggests that ES cells have dominant *trans*-acting factors that are able to override somatic cell identity (Tada et al., 2001). Pluripotent cells also express numerous, well-characterized transcription factors that are known to cooperatively regulate genes. Importantly, these factors bind to their own enhancers and maintain the proper expression of each other, thereby forming an endogenous interconnected, autoregulatory gene circuit (Boyer et al., 2005) that may maintain the ES cell state once it is activated. Finally, pluripotency may be further stabilized *in vitro* by its unique epigenetic environment. Experiments on the Moloney murine leukemia virus have demonstrated that pluripotent cells express unique epigenetic regulators that methylate retroviruses and silence their transcription (Barklis et al., 1986; Jahner et al., 1982). It has also been shown that the transcriptional silencer responsible for this process, Trim28, is present in pluripotent cells, but downregulated during differentiation (Wolf and Goff, 2007). Another epigenetic characteristic specific to pluripotent cells is their destabilized nucleosome remodeling and histone deacetylation (NuRD) complex, which

is due to their low expression of NuRD component Mbd3 (J. Hanna, unpublished data). In somatic cells, which express higher levels of Mbd3, the NuRD complex is responsible for widespread transcriptional repression (Xue et al., 1998). Importantly, Mbd3 expression has recently been shown to be a major barrier for nuclear reprogramming (J. Hanna, unpublished data). Finally, the growth advantage that pluripotent cells have over somatic cells and their domed morphology may aid the practicality of detecting rare iPS reprogramming events and isolating them for further study.

This first section has discussed ES cells and mechanisms that govern their characteristic qualities of self-renewal and pluripotency. A differentiated cell can assume these ES cell traits through nuclear reprogramming, such as that achieved by the overexpression of Oct4, Sox2, Klf4, and c-Myc. ES cells have unique properties that stabilize their cell state, but it is currently not known if a non-pluripotent cell state can also be induced by transcription factor overexpression. Since neural stem cells share self-renewal and multipotency features with ES cells, their cell state may also be stabilized *in vitro*.

Part II. Neural Stem Cells

The default model of neural induction

In mammalian development, neural cells originate from the ectoderm germ layer during gastrulation. In addition to the neural cells of the central nervous system (CNS), the ectoderm also gives rise to the neural crest (migratory cells that become melanocytes, facial cartilage, and the peripheral nervous system) and the epidermis (which forms the skin). Early neural induction experiments performed in amphibian model organisms have analyzed how neural cells are specified within the developing embryo. The work of Spemann and Mangold suggested that neural tissue is specifically induced by factors originating in the "organizer," a specific part of the embryo that develops into neural tissue. When presumptive neural tissue, which includes the organizer, was transplanted from one salamander embryo to another embryo, the recipient embryo developed two complete nervous systems—its normal nervous system and a second one at the site of transplantation, which would have otherwise formed the epidermis (Hemmati-Brivanlou and Melton, 1997a). We now know that the signals emanating from the organizer are not inducing neural tissue, but rather repressing other cell fates by inhibiting Bmp4 signaling (Hemmati-Brivanlou and Melton, 1994a; Hemmanti-Brivanlou and Melton, 1994b; Smith et al, 1993). Thus, in the absence of differentiation signals, neural formation is the "default fate" of ectoderm development in certain model organisms (Hemmati-Brivanlou and Melton, 1997a, Hemmati-Brivlanou and Melton, 1997b). However, it has since been shown that Bmp4 inhibition is not

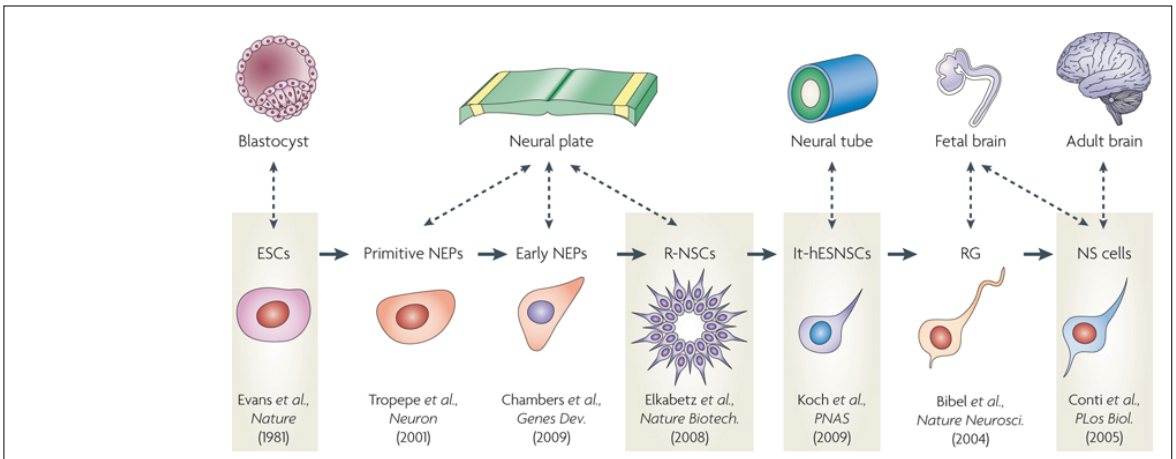
sufficient for neural induction in higher organisms and that instructive signals, such as those provided by fibroblast growth factors, are also required for proper neural development (Gaulden and Reiter, 2008; Munoz-Sanjuan and Brivanlou, 2002). The key factors and signaling pathways for mammalian neural development will be discussed in more detail below.

Types of neural stem cells

Neural stem cells (NSCs) are the somatic progenitor cells that give rise to the entire mammalian CNS. Like all stem cells, NSCs have the capacity to either self-renew or differentiate, and differentiation generates neurons, astrocytes, and oligodendrocytes (Gage, 2000). A number of distinct types of NSCs are generated during CNS formation, and many have been isolated from the brain and explanted for analysis *in vitro* (Cattaneo and Mckay, 1990; Reynolds and Weiss, 1992; Doetsch et al., 1999). The use of ES cells has further aided our understanding of this cell type by allowing for the generation of many NSC types *in vitro* that share the properties of their *in vivo* counterparts (Conti and Cattaneo, 2010) (Figure 4). The following includes a brief description of some well-studied types of NSCs.

Neuroepithelial stem cells

In the developing mouse, the first NSCs are generated during the early stages of neurulation. These neuroepithelial cells (NECs) form a sheet of epithelial-like cells that



Developmental stage	E3.5	E5.5	E7.5	E8.25	E8.5	E9.5-E18.5	Adult
Growth factor dependence	Lif	Lif	SHH	SHH, Notch	EGF, FGF	EGF, FGF	EGF, FGF

Figure 4. NSC subtypes *in vivo* and *in vitro*.

Various types of NSCs are generated during mammalian development. Shown are the NSCs that have been isolated or generated *in vitro*, along with their corresponding developmental stage *in vivo* and growth factor dependence *in vitro.* Figure adapted from Conti and Cattaneo, 2010.

make up the neural tube and are responsible for the first wave of neurogenesis (Smart, 1973). They initially undergo rapid, symmetric cell divisions and later transition to asymmetric division, producing both NSCs and cells that migrate radially outward (Conti and Cattaneo, 2010). Mouse ES cells rapidly differentiate to NEC-like cells in the absence of serum and cell contact (Smukler et al., 2006; Tropepe, 2001). The NEC-like cells express neural markers like Nestin and Sox1, but also retain many characteristics of ES cells such as Oct4 expression, dependence on Lif signaling, and the ability to contribute to all the germ layers of mouse chimeras (Smukler et al., 2006; Tropepe, 2001).

Rosette-stage NSCs

Rosette-stage NSCs (R-NSCs) are an ES cell-derived cell type that corresponds to NSCs present *in vivo* at the late-neural plate stage (Elkabetz et al., 2008; Elkabetz and Studer, 2008). These cells are distinguished by their capability to differentiate into CNS and PNS fates, characteristic rosette morphology, and dependence on Notch and Sonic Hedgehog (SHH) signaling for proliferation (Elkabetz et al., 2008). Additionally, R-NSCs have a distinct gene expression pattern that includes Sox1, FoxG1, PLZF, Nestin, ZO-1, and Forse1 expression (Conti and Cattaneo, 2010). Long-term self-renewing human ES-derived neuroepithelial stem cells (lt-hESNSCs) are another type of rosette-NSC derived from ES cells (Koch et al., 2009). They display many of the properties of R-NSCs including rosette-specific gene expression, broad differentiation

capacity, and rosette morphology, but lt-hESNSCs proliferate with EGF and FGF growth factors.

Radial glial cells

During development, radial glial neural stem cells (RGCs) are generated from NECs at the onset of neurulation and are the main NSC type in the developing brain (Franco and Muller, 2013, Conti and Cattaneo, 2010). A number of qualities distinguish RGCs from NECs: RGCs have fewer epithelial features, which includes the loss of tight junctions (Aaku-Saraste et al., 1996); they express astroglial markers not expressed in NECs, such as Blbp and Glast (Hartfuss et al., 2001); and they have a more restricted differentiation potential, with most being bipotent or unipotent (Conti and Cattaneo, 2010). Basal progenitors, also called intermediate progenitor cells, are unipotent neuronal stem cells that are generated *in vivo* from differentiating RGCs or NECs (Conti and Cataneo, 2010).

Key intrinsic and extrinsic factors for NSCs

NSC subtypes display a multitude of characteristics based on the extrinsic factors they encounter in their niches, as well as their expression of intrinsic factors that control gene expression or modulate the chromatin. The complex interplay of these factors during development gives NSCs their positional and temporal identities, and *in vitro* they determine the cells' developmental potentials. Many key extrinsic factors have been determined by studying the *in vitro* growth properties of NSCs explanted from the brain,

and many intrinsic factors have been elucidated by the genetic analysis of genes that have been found to be required for proper NSC function. Insight into these factors is necessary for a full understanding of the mechanisms governing the induction and maintenance of this cell type. The following is a brief description of many such factors.

EGF and bFGF

Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) are mitogens from the receptor tyrosine kinase (RTK) family that promote the *in vitro* self-renewal of multiple types of NSCs, including RGCs of the subventricular zone (SVZ) and ES-derived NSCs (Conti and Cattaneo, 2010; Reynolds and Weiss, 1992). EGF is the main contributor to NSC proliferation, since removal of bFGF has little effect on proliferation, whereas EGF withdrawal results in slower proliferation and cell death (Conti et al., 2005, Pollard et al., 2006). Furthermore, NSCs derived in bFGF alone have a limited growth potential and become restricted to glial progenitors after several divisions (Conti et al., 2005). The importance of these signaling molecules for brain development *in vivo* was confirmed by genetic knockout experiments; mice lacking the EGF receptor develop neurodegeneration in the frontal cortex (Sibilia et al., 1998; Threadgill et al., 1995) and mice deficient in bFGF have a reduced expansion of the progenitor pool during neurogenesis (Raballo et al., 2000).

Sonic Hedgehog

Sonic Hedgehog (SHH) signaling has important roles during limb and neural development, and its misexpression can lead to developmental malformations and cancer (Chiang et al., 1996, Hahn, et al., 1996). SHH signaling promotes the proliferation and maintenance of embryonic and adult NSCs both *in vivo* and *in vitro* (Ahn and Joyner, 2005; Palma et al. 2005; Lai et al., 2003). In addition to its roles in NSC proliferation and maintenance, SHH also has an instructive role and can induce NSC formation from neural tissue that otherwise does not form NSCs (Scott et al., 2010).

Wnt/β-catenin

Multiple members of the Wnt family are expressed in the CNS (Parr et al., 1993), and this pathway plays a critical role in NSC proliferation (Chenn and Walsh, 2002). For instance, expression of a stabilized version of β-catenin, the downstream transducer of Wnt, results in an enlarged brain due to expansion of the progenitor pool. The converse is also true, where the conditional deletion of β-catenin in the nervous system results in a decreased progenitor pool and smaller brain size (Zechner et al., 2003).

Notch and Notch effectors

Notch is both a receptor and a transcription factor. After binding to its ligand Jagged or Delta-like, Notch is cleaved in its transmembrane domain, and the released intracellular domain (ICD) translocates to the nucleus and induces gene transcription

(Kopan, 2012). Notch signaling maintains the self-renewal of NSCs, and its deletion in NSCs leads to neuronal differentiation and depleted progenitor pools (Shi et al., 2008; Yoon and Gaiano, 2005). A similar phenotype is observed when the redundant Notch effectors Hes1, Hes3, and Hes5 are simultaneously knocked-out (Imayoshi et al., 2010, Ohtsuka et al., 1999). Constitutive expression of the Notch ICD leads to sustained effector gene expression resulting in forced maintenance of the NSC-state and inhibition of neuronal differentiation (Ohtsuka et al., 1999). Interestingly, Notch is also active in the final stages of neural differentiation when it biases multipotent progenitors towards an astrocyte fate as opposed to an oligodendrocyte one (Grandbarbe et al., 2003, Tanigaki et al., 2001).

Sox1, Sox2, and Sox3

The SoxB1 family of transcription factors—Sox1, Sox2, and Sox3—are expressed in the developing nervous system where they have highly redundant functions in the maintenance of NSCs (Bylund et al., 2003; Wood and Episkopou, 1999). Sox1 is one of the earliest transcription factors expressed during the induction of neuroectoderm (Pevny et al., 1998), and its forced expression in ES cells leads to neural differentiation (Pevny et al., 1998). Sox2 is expressed in ES cells, but becomes restricted to the prospective neural plate at the onset of gastrulation (Wood and Episkopou, 1999). Constitutive expression of Sox2 in NSCs inhibits their differentiation and maintains NSC characteristics, whereas loss of Sox2 induces differentiation and loss of NSC characteristics (Graham et al., 2003). Like Sox2, Sox3 also promotes the

maintenance of NSCs when constitutively expressed, and its inhibition causes differentiation (Bylund et al., 2003).

FoxG1

Forkhead-box G1 (FoxG1) is a transcriptional repressor associated with forebrain development (Fasano et al., 2009, Tao et al., 1992). Misexpression of FoxG1 causes mental disorders like Rett syndrome, epilepsy, and microcephaly (Danesin and Houart, 2012). FoxG1 dosage is thought to temporally regulate neurogenesis in the developing cortex (Danesin and Houart, 2012), with increased expression in the more restricted progenitors (Shen et al., 2006). Bmi1, which is important for the self-renewal of many types of somatic stem cells, cooperates with FoxG1 to maintain self-renewal in forebrain NSCs (Fasano et al., 2009).

Brn1 and Brn2

The Brain (Brn) proteins are POU-domain homeobox transcription factors expressed specifically in the developing and adult nervous system (He et al., 1989). Brn1 and Brn2 are expressed in NSCs and in migrating cortical neurons, and mice deficient for both genes display cortical defects (McEvilly et al., 2002; Sugitani et al., 2002). Brn2 and Sox2 co-occupy many distal enhancers in NSCs, suggesting that they may be partner factors (Lodato et al., 2013).

Pax6

Paired-box gene 6 (Pax6) is a highly conserved transcription factor that is critical for the development of the CNS, eyes, and nose (Georgala et al., 2011). In humans, mutations in this gene are associated with multiple disorders, most notably aniridia (Ton et al., 1991). Pax6 is expressed in various NSCs and more restricted progenitor cells, where mutations affect proliferation, multipotency, and neurogenesis (Sansom et al. 2009).

Transcriptional regulation of NSCs

Unlike ES cells, NSCs do not have a well-characterized transcriptional circuitry network, and thus little is known about the precise molecular control of the NSC cell state. One hypothesis for NSC maintenance, however, is the dynamic regulation of Notch effectors and proneural transcription factors. This regulation is precisely controlled in NSCs to strike a delicate balance between NSC self-renewal and neurogenesis, and its misregulation often results in developmental disorders and cancers (Lasky and Wu, 2005; de Ponual et al., 2003; Molofsky et al., 2005; Molofsky et al., 2003).

As mentioned above, Notch is a transmembrane receptor expressed in NSCs. It is activated upon binding its ligand Delta, whose expression in neighboring cells is induced by proneural transcription factors (Castro et al., 2006; Henke et al., 2009). Upon activation, Notch is cleaved and acts as a transcription factor to induce the expression of a number of genes, most notably Hes1 (Figure 5A). Hes1 is a

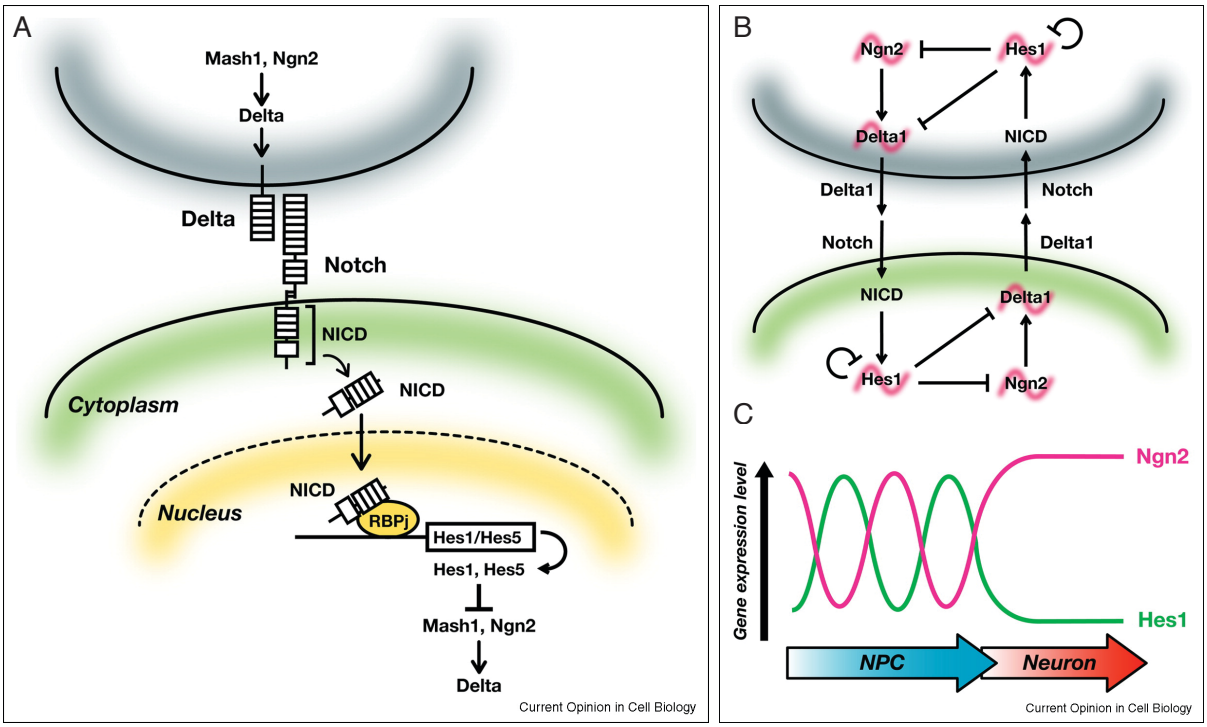


Figure 5. Oscillations in the Notch pathway promote NSC self-renewal.

(A) Notch is a transmembrane receptor that is activated by binding its ligand Delta, which is expressed on the surface of neighboring cells. Upon activation, Notch is cleaved in its transmembrane domain, and the intracellular portion (NICD) translocates to the nucleus where it induces the expression of several genes, including the Hes family of transcriptional repressors. (B) Notch induces Hes1, which represses proneural genes like Ngn2 as well as itself. Autoinhibition of Hes1 then allows for the induction of Ngn2, which induces the Notch receptor Delta. The expression of Delta activates Notch signaling in neighboring cells. Thus, waves of Notch signaling pass between adjoining cells, and the expression of pro-self-renewal factors is maintained. (C) Hes1 and Ngn2 expression oscillates in neural stem/progenitor cells (NPCs) with a periodicity of 2-3 hours to maintain NSC identity. NSCs differentiate when this complementary expression pattern is disrupted.

Figure adapted from Kageyama et al., 2009.

transcriptional repressor that regulates proneural genes like Ngn2 as well as itself. Once Hes1 is activated, it binds to its own promoter, thereby reducing its own expression (Hirata et al., 2002). In the absence of Hes1 repression, proneural genes are activated and induce the expression of Delta in that particular cell (Figure 5B). Thus, neighboring NSCs sustain Notch expression, and therefore NSC maintenance, by oscillating the expression of Notch effectors and proneural transcription factors. When these periodic oscillations are broken, Ngn2 expression is sustained, and neuronal differentiation ensues (Shimojo et al., 2008).

NSC transcription factor Pax6 plays a critical role in regulating the Hes1/Ngn2 gene oscillations responsible for NSC self-renewal. Genome-wide location analysis has revealed that Pax6 not only binds directly to NSC self-renewal genes like Hes1, but also to proneural genes like Ngn2 and Mash1/Ascl1 (Sansom et al., 2009). Its effect on these genes is highly dose-dependent: whereas increased amounts of Pax6 lead to neurogenesis by inducing the proneural genes Ngn2 and Ascl1, decreased expression of Pax6 leads to neurogenesis by reducing the amount of Hes1 (Sansom et al., 2009). Thus, Pax6 preserves NSC self-renewal by regulating the Notch signaling/proneural transcription factor oscillatory network.

This section has explored the nature of NSC formation *in vivo* and *in vitro*, as well as some factors that help to maintain the NSC phenotype. The wealth of knowledge about this cell type, including its defined growth conditions, ability to proliferate

indefinitely *in vitro*, and stable transcriptional network, make NSCs an ideal cell type to test somatic cell conversions using defined factors. Furthermore, although much is already known about NSCs, a detailed molecular understanding of their core transcriptional circuitry remains elusive. Given the insight that the iPS reprogramming process has provided regarding the factors and pathways important for pluripotency, NSC regulatory mechanisms may be further elucidated by studying the induction of NSC characteristics in distant cell types. Somatic cell conversion, or "transdifferentiation," is a provocative idea, but its concepts are complex, as is its history.

Part III. Transdifferentiation and somatic cell conversion

Definition of Transdifferentiation

Transdifferentiation, also called direct conversion or lineage reprogramming, is defined as the conversion of one somatic cell state directly into a distinctly different somatic cell state without passing through pluripotency and re-differentiating (Wagers and Weissman, 2004; Graf and Enver, 2009; Hanna et al., 2010).

This definition requires the following three criteria to be fulfilled to demonstrate transdifferentiation. First, it requires a defined starting cell type. Many primary cell cultures contain a heterogenous mix of cell types representing the tissue of origin. For instance, although 80% of the adult liver is made up of hepatocytes, there are also a variety of non-epithelial cells present (Blouin et al., 1977), as well as the blood that innervates the organ. When a tissue is explanted for growth *in vitro*, specific growth factors are often used to promote the survival and expansion of the desired cells. Since some conversion experiments occur at a low frequency, a contaminating cell could confound the results. The true origin of a starting cell population can only be ensured by using genetic markings, like the DNA rearrangements found in blood cells or cell type-specific Cre-induced recombination, for retrospective analysis of the converted cell.

Transdifferentiaton also requires the validation and functional characterization of a resulting cell type independent of exogenous perturbations. The resulting cell must (1) express the unique markers of the endogenous target cell type; (2) fulfill the functional requirements of the endogenous cell type, such as differentiation or a cell

type-specific process; and (3) reprogram its nucleus such that the gene expression and epigenetic state are similar to the endogenous cell type. Additionally, all of these cell characterizations should be analyzed independent of the exogenous transgenes or chemicals that were used to impose the conversion to minimize any effect these perturbations may have had on the state. Since naturally occurring cell states are stable, a cell that has been truly converted would be equally stable and would not need exogenous perturbations to maintain its properties. Analysis in the absence of exogenous factors allows for the characterization and assessment of the intrinsic properties of the converted cell.

A third requirement for transdifferentiation is that the process should not go through a pluripotent intermediate, which would be considered reprogramming with subsequent differentiation. Reprogramming to pluripotency is a well-studied phenomenon, and differentiation is a natural property of pluripotent cells, so a conversion that goes through a pluripotent state would not need to acquire the properties of the target cell *de novo* and therefore would not be a "direct" conversion. Since pluripotency can happen transiently, a genetic mark is one way this could be shown. For instance, the pluripotent state could be assessed by a Cre allele driven by a key pluripotency gene (such as *Pou5f1*), which would genetically mark any cells that induce expression of that gene. Another option would be to genetically knock out a key pluripotency gene in the somatic donor cell so that pluripotency cannot be attained.

Although transdifferentiation has a somewhat narrow definition, it falls under a larger category of "cell conversion," which is a more general term used to describe the process of changing a cell's typical fate.

Review of transdifferentiation/direct conversion experiments

Numerous experiments have been performed to test the concept of transdifferentiation. The following is a critical review of these works with an emphasis on the three criteria for transdifferentiation discussed above.

The discovery of MyoD

The discovery that transcription factors can be used to convert somatic cells to pluripotency was a tremendously important finding and has revolutionized the fields of regenerative medicine and stem cell biology. However, the principles of defined factor-mediated conversion are rooted in a series of seminal experiments that occurred decades earlier. In the 1980's, Harold Weintraub and colleagues were interested in identifying factors that regulate cell type-specific gene expression and then determining what effect these factors may have when expressed in an unrelated cell type (Tapscott, 2005). The experimental system they used was the 10T1/2 fibroblast cell line, which had been shown to produce subclones with myogenic, adipogenic, or chondrogenic phenotypes upon exposure to the demethylating agent 5-azacytidine (Constantinides et al., 1977; Taylor and Jones, 1979). Reasoning that this conversion may be due to the random expression of undermethylated genes, the scientists made DNA libraries from

the aza-converted myoblast-like cell lines, as well as from the parental fibroblast line and an immortalized myoblast cell line (Lassar et al., 1986). Upon DNA transfection into fibroblasts, they found that DNA from either the aza-converted cells or the immortalized myoblasts can induce myogenic colonies, whereas DNA from fibroblasts had no effect (Lassar et al., 1986). *Myod* was found to be the single gene responsible for this myoblast determination, and its overexpression had a similar effect on multiple fibroblast cell lines (Davis et al., 1987).

This elegant series of experiments had a profound impact on our understanding of differentiation and gene regulation. First, the discovery that *Myod* transduction was sufficient to initiate a myogenic differentiation pathway indicated that cell type-specific factors may have crucial roles in cell specification (Davis et al., 1987). Indeed, the identification of *Myod* allowed for other tissue-specific transcription factors to be identified by virtue of their homology with *Myod* (Massari and Murre, 2000). Also, subsequent studies on the family of myogenic transcription factors elucidated general principles of mammalian gene regulation, the collaborative and antagonistic relationship between certain transcription factors, and the molecular initiation of the differentiation process (Tapscott, 2005). Importantly, the discovery of *Myod* demonstrated that the overexpression of key transcription factors in certain cell types can override the cell's endogenous gene expression pattern and change its normal fate. Although the effect of *Myod* was induced through its constitutive expression in fibroblasts, and it was not sufficient to fully convert distantly related cell types like neuroblastoma and melanoma

cells (Weintraub et al., 1989), these seminal experiments laid the groundwork for defined factor-mediated reprogramming and somatic cell conversion.

The "plasticity" of bone marrow stem cells

After the discovery of *Myod*, interest grew in exploring the plasticity of adult mammalian cells and testing the limits of somatic cell conversion and transdifferentiation. A number of studies reported an incredible plasticity in bone marrow (*i.e.* mesenchymal) stem cells—when transplanted, these cells could convert into diverse cell types like skeletal muscle (Ferrari et al., 1998), cardiomyocytes and cardiac endothelium (Jackson et al., 2001), pancreatic β-cells (Ianus et al., 2003), neurons (Brazelton et al., 2000; Mezey et al. 2000), and epithelial cells of the skin, liver, lung, and intestine (Krause et al. 2001). Many of these studies relied on activation of donor-specific reporters in the host tissue as evidence for conversion. However, upon closer examination, it was determined that the observed "plasticity" of the bone marrow stem cells was not cell conversion, but the result of a fusion event between the stem cell's natural progeny and host cells (Wagers et al. 2002; Camargo et al., 2003).

In addition to mesenchymal stem cell's (MSC's) purported ability to differentiate into neural cells upon transplantation, they were also reported to have amazing plasticity *in vitro*. MSCs exposed to chemical agents like DMSO or β–mercaptoethanol supposedly could be spontaneously converted to neurons and glia (Sanchez-Ramos et al., 2000, Woodbury et al., 2000). The cells' morphology was consistent with differentiated neural cells, and they stained for the neural differentiation markers

neuron-specific nuclear protein (NeuN), β-III tubulin (Tuj1), and glial fibrillary acidic protein (GFAP). Additionally, these cells were reported to have functional properties like calcium uptake and electrophysiological activity typical of functional mature neurons (Kohyama et al., 2001). However, these experiments were later shown to be an artifact of the experimental system. The cells had not been converted to neuronal cells, but instead had undergone extreme morphological changes. Time-lapse imaging showed that the processes and neurites observed after this neural "induction" were actually the result of cytoplasmic retraction in response to the cytotoxic agents and not from process extension, which happens during neuronal differentiation (Lu et al., 2004; Neuhuber et al., 2004). Furthermore, the protein and gene expression changes were likely due to aberrant gene expression changes in response to stress or to background expression coupled with an extreme change in morphology (Neuhuber et al., 2004).

The studies reporting the *in vivo* and *in vitro* plasticity of bone marrow stem cells highlight the complexities involved with cell conversion experiments. The use of an ill-defined starting population of bone marrow stem cells isolated using slightly different protocols by each group makes it difficult to know which starting cell was responsible for this effect (Theise et al., 2003). Furthermore, many of the reports relied on *in vitro* morphology or *in vivo* location and not on a functional assessment of the "converted" cell; therefore, their results were over-interpreted. Finally, conversion was often quantified by the reactivation of a cell type-specific reporter present but not expressed in the donor cell. However, donor-specific gene expression was not assessed for repression and may have remained active following cell fusion.

The artifactual results of the mesenchymal stem cell experiments cast doubt on the feasibility of *in vitro* transdifferentiation and somatic cell conversion (Graf, 2011). However, interest in the topic was reignited by Takahashi and Yamanaka's discovery that the overexpression of key transcription factors can convert somatic cells into pluripotent stem cells (Takahashi and Yamanaka, 2006).

Hematopoietic Conversions

A number of studies have tried Yamanaka's approach of overexpressing transcription factors to find the factor or factor combination that can induce specific cell types. Transcription factors were shown to re-specify cells within the hematopoietic lineage. Forced expression of the erythroid-megakaryocyte transcription factor GATA-1 in monocytes induced these cells to undergo an erythroid-, eosinophil-, or basophil-like cell fate (Heyworth et al., 2004). Also within the hematopoietic lineage, Graf and colleagues found that overexpression of C/EBPα and PU.1 in B- and T-lymphocytes gives them macrophage properties like cell-surface marker expression, morphology, and phagocytic capacity consistent with macrophages (Xie et al., 2004, Laiosa et al., 2006). However, the extent of conversion is unclear since the converted cells rely on constitutive expression of the exogenous factors, and they also maintain expression of a number of donor-specific markers (Hanna et al., 2010). Furthermore, overexpression of C/EBPα and PU.1 in more distantly related fibroblasts resulted in pseudo-converted cells whose phenotype would revert to fibroblast-like unless they were stabilized through continuous expression of the exogenous transcription factors (Feng et al., 2008).

Other Non-Neural Conversions

Recently, many reports have claimed the induction of particular cell types by overexpressing key transcription factors. However, these studies do not fulfill the criteria for direct conversion because they do not demonstrate that the cell created can function without exogenous factor expression. For instance, studies showing the generation of brown fat cells by overexpressing C/EBPβ and PRDM16 in fibroblasts (Kajimura et al., 2009), as well as the creation of hepatocyte-like cells with factors Gata4, Hnf1α, and FoxA3 (Huang et al., 2011; Sekiya and Suzuki, 2010), used constitutive retroviruses or constitutive lentiviruses for factor transduction, making it impossible to determine the extent of conversion. Buganim et al. (2012) used doxycycline (dox)-inducible lentiviruses to show that embryonic sertoli-like cells can be induced from fibroblasts through the overexpression of factors Gata4, Nr5a2, Wt1, Dmrt1, and Sox9, but their analysis was performed only in the presence of dox, and therefore exogenous factor expression. An elegant study demonstrated that cardiac fibroblasts and tail-tip fibroblasts could be converted into cardiomyocytes through the overexpression of Gata4, Mef2c, and Tbx5 (Ieda et al., 2010). Although the authors showed their induced cells express genes present in cardiomyocytes after exogenous factor withdrawal, they rely on constitutive retroviral expression for the functional cardiomyocyte assays of *in vivo* engraftment, spontaneous contraction, and electrophysiology.

Post-mitotic neural cell conversions

Many direct conversion studies have focused on creating cells of the neural lineage, and both post-mitotic cells and proliferating cells have been generated *in vitro*. Overexpressing three factors in mouse fibroblasts—Brn2, Ascl1, and Mytl1 (BAM)—can create post-mitotic induced neuron-like (iN) cells (Vierbuchen et al., 2010), and human fibroblasts can be converted by supplementing BAM with NeuroD1 (Pang et al., 2011). Within one cell division and 2-3 weeks of factor induction, mouse fibroblasts express multiple neuron-specific proteins, generate action potentials, and form functional synapses; however, nearly all of the analysis was performed in the presence of the exogenous factors. Although Wernig and colleagues later show that the neuron-specific marker TuJ1 persists in the iN cells made from hepatocytes after the de-induction of BAM, they did not assess the functional activities of these cells by measuring action potentials or show functional synaptic properties in cells that do not express the transduced factors (Marro et al., 2011).

In addition to generic neurons, cells with characteristics of subtype-specific neurons have also been generated. Induced motor neurons (iMNs) can be created with forced expression of a pool of 7 transcription factors in mouse or human fibroblasts (Son et al., 2011). iMNs have motor neuron gene expression, electrophysiology, and the ability to induce muscle cell contractions *in vitro*, and they can integrate into developing chick spinal cords (Son et al., 2011). However, the true identity of these cells is ambiguous because there is no epigenetic analysis to show conversion, and the

exogenous factors are expressed at levels 300-1600 fold higher than background in iMN cells (Son et al., 2011).

Cells with qualities of dopaminergic (DA) neurons have also been induced with the overexpression of key transcription factors (Caiazzo et al., 2011; Pfisterer et al, 2011; Kim et al., 2011). The induced dopaminergic (iDA) neurons have properties of endogenous DA neurons, including characteristic morphology, production of tyrosine hydroxylase and other markers of DA neurons, and electrophysiology. The authors use dox-inducible transgene expression to show that most of these qualities persist after exogenous factor withdrawal; however, it is unclear if iDA neurons are molecularly and functionally equivalent to primary DA neurons because the gene expression and the majority of functional assays were only assessed in the presence of dox (Caiazzo et al., 2011; Kim et al., 2011).

Direct conversion into a differentiated, post-mitotic cell type inherently makes it difficult to analyze the molecular features of the cells. Since the cells do not divide, it is impossible to generate a large, clonal population of cells needed for accurate gene expression, epigenetic, or biochemical analyses. Instead, many of the assays are either performed on single cells, which may or may not represent the larger population, or on a heterogeneous mix of cells that have different integrations and expression levels from the exogenous factors, which can be misleading especially since many studies analyze the cell in the presence of the factors. Moreover, modulating the epigenetic status of cells during iPS reprogramming requires multiple cell divisions (Hanna et al., 2009), and with limited epigenetic analyses performed on “converted” cell types—and none in the

absence of exogenous factors—it is impossible to know if these post-mitotic cells were able to modulate their epigenomes to be like the endogenous cells without dividing. Furthermore, it is not immediately obvious what would happen to an incompletely reprogrammed post-mitotic cell in the absence of the exogenous factors, since it would not have subsequent cell divisions to establish or revert its epigenome. Although it has been demonstrated that the post-mitotic cells have the morphology and express structural proteins consistent with their target cell types, it should be noted that transcription factor expression, genome-wide gene expression or epigenetic analysis, and assays showing cell functionality have not been reported.

Proliferating neural cell conversions

The problems associated with direct conversion into a post-mitotic cell type may be mitigated with the generation of a dividing cell, which has also been achieved within the neural lineage with the creation of induced neural stem cells and also induced oligodendrocyte precursor cells. Han et al. (2012) demonstrate that induced neural stem cells (iNSCs) can be generated through the forced expression of Sox2, Klf4, Myc, Brn4, and Tcf3. These cells display the morphological, gene expression, and functional properties of NSCs, but they are created with constitutive retroviruses, which leads to varying levels of exogenous factor expression in the final cells (Han et al., 2012). Also, the generation of iNSCs is a slow and inefficient process resulting in just a couple of iNSC lines, making it unclear how reproducible this technique is. In addition, Southern blot analysis was not employed to ensure that the cell lines arose independently. The

inefficiency of their conversion may be due to the presence of rare neural progenitors residing in the MEF starting population. Although brain cells and internal organs were removed for the creation of MEFs, the neural tissue of the spinal cord was not removed, and the transformation of neural progenitors originating there cannot be excluded.

Wernig and colleagues were able to generate neural stem-like cells from embryonic fibroblasts that had been manually dissected away from the spinal and brain neural cells (Lujan et al., 2012). They found that overexpression of three transcription factors—Sox2, Brn2, and FoxG1—leads to the creation of cells that resemble NSCs by marker expression and differentiation capacity (Lujan et al., 2012). However, it should be noted that these cells differentiate spontaneously upon exogenous factor withdrawal and therefore have not activated the endogenous NSC transcriptional circuitry necessary for maintaining themselves.

Another proliferating cell type that has been generated *in vitro* through transcription factor overexpression in fibroblasts is induced oligodendrocyte precursor cells (iOPCs). Through the forced expression of Sox10 and Olig2, and combinations of additional factors for three weeks, two groups were able to create transient precursor cells that are restricted to forming either both astrocytes and oligodendrocytes (Yang et al., 2013) or just oligodendrocytes (Najm et al., 2013). The generation of oligodendrocyte-like cells may have therapeutic implications, as evidenced by their efficacy in rescuing the hypomyelineation phenotype of the shiverer mutant mouse upon transplantation (Yang et al., 2013; Najm et al., 2013; Chernoff, 1981). However, it remains to be determined how potent these factors are in generating bona fide iOPCs,

since the exogenous factors are expressed throughout the generation, differentiation, and terminal maturation of the iOPCs. Moreover, Wernig and colleagues maintained induction of the transduced factors after transplantation into the shiverer mouse by adding the inducing agent to the drinking water (Yang et al., 2013), so it is unclear if the correction of the myelination defect would occur without continuous transgene expression.

Direct conversion into a proliferating cell type is more compelling than into a post-mitotic cell. Having numerous cell divisions would allow the induced cell to modify and reset its epigenome to ensure that the gene expression and other changes that have occurred can be maintained over time. Furthermore, multiple cell divisions after transgene withdrawal may result in a more accurate representation of the true nature of the induced cell because the results of functional and other assays would not be obscured by a large amount of exogenous factor expression.

However, the proliferative nature of the induced cell also presents some added complications for analyzing direct conversion experiments. For one, since the cells are cultured long-term after factor induction, a rare somatic cell—like a somatic stem cell or endogenous version of the target cell—that contaminates the starting cell population could conceivably have enough divisions to grow out and be observed as a “converted” cell, thus making it difficult to determine the true extent of conversion. A second issue arising from the numerous cell divisions required to induce and maintain the target cell is that the cell may transiently go through a pluripotent intermediate step. Although the

correct target cell would be generated, it would not be through “direct conversion,” but rather through reprogramming to pluripotency and subsequent differentiation.

How similar are converted cells and their endogenous counterparts?

Cells created through direct conversion share many features with their *in vivo*, natural counterparts; however, it remains to be seen if the two are functionally and molecularly equivalent. Virtually all the studies claiming “direct conversion” characterize the induced cells in the presence of exogenous factors. This makes it difficult to determine if the observed phenotypes are intrinsic to the induced cell or are artifacts of transgene overexpression. For instance, gene expression analysis in the presence of exogenous factors makes it unclear if the endogenous core transcriptional circuitry governing cell type-specific gene expression has been fully activated. Furthermore, the overexpression of transduced factors complicates the interpretation of functional assays because it is not clear how much of the cells’ perceived functionality is contributed by the exogenous factors. Without comprehensive epigenetic analysis, it is unknown if the unique, cell type-specific epigenetic marks that maintain proper gene expression have been established and are maintained independently of exogenous transgene overexpression.

Therapeutic implications of direct conversion

The direct conversion technology may have a tremendous effect on cell-based therapies because it can potentially be used to create cell types that are otherwise

difficult to generate. There are currently two strategies for applying direct conversion—converting cells *in vivo*, or converting cells *in vitro* and then transplanting them *in vivo*.

There have been a few reports that indicate that cells can be generated *in vivo* for cell therapy. One example is the conversion of pancreatic exocrine cells into β-cells through the forced expression of Ngn3, Pdx1, and MafA (Zhou et al., 2008), which may be of interest for the treatment of diabetes. Another clinically relevant example of *in vivo* conversion is the generation of cardiomyocytes from cardiac fibroblasts by overexpressing Gata4, Mef2c, and Tbx5 in the heart (Qian et al., 2012). However, factor delivery remains a technical impediment for this technique, since vector integration into the patient genome can have detrimental effects for the patient (Hacein-Bey-Abina et al., 2003). Also, some conversions, such as the induction of β-cells from exocrine cells, only occur with certain starting cell types (Zhou et al., 2008), so it is unclear how the factors will be introduced specifically to the relevant cells. When these technical issues have been solved, this may be a valuable tool for treating patients with certain disorders with their own cells.

Transplantation can provide a better therapeutic option for genetic disorders because causative mutations can be corrected *in vitro*, and healthy cells can be introduced to the patient. This strategy has been shown to be effective in treating dysmyelination and Parkinsonian symptoms in mice (Yang et al., 2013, Najm et al., 2013, Kim et al., 2012). However, since the cells created thus far have not been shown to function independently of exogenous factor expression, it remains to be determined if they can be generated without introducing transgenes into the genome.

Reprogramming cells to iPS and then re-differentiating them may be a more suitable option for therapy, since iPS cells can be generated without modifying the genome, they can be checked for their karyotype or for other mutations, and disease-relevant mutations can be corrected in iPS cells (Hanna et al., 2007).

Biological implications of direct conversion

The discovery that cells can be reprogrammed to pluripotency had an enormous impact on the understanding of epigenetic plasticity in differentiated cells, and elucidating the interconversion of differentiated cell types would further refine our understanding. Although pluripotent cells are readily induced and stabilized *in vitro*, it is not known if other cell types can be stably induced and maintained in a similar manner. A convincing demonstration of direct conversion would show that the pluripotent state is not uniquely attainable and that the epigenome of other cell types can also be stabilized *in vitro* from somatic sources. Furthermore, factors capable of inducing direct conversion may hint at the key regulators of that somatic cell type, just as Oct4, Sox2, Klf4, and Nanog are crucial for pluripotent cells. Additionally, true direct conversion would provide another system to analyze the transcription factors, chromatin modifiers, and other genes involved with repressing a differentiated cell's chromatin and establishing an unrelated cell type.

It is unclear how much novel information is gained from the current direct conversion experiments. Weintraub and colleagues' discovery that the forced expression of *Myod* can give myoblast characteristics to a distantly related cell type was

a novel discovery because at the time it was not known how cells undergo cell type-specific gene expression changes or what factors may be responsible for initiating a differentiation process. Now, however, we know all of the genes in the genome, as well as their expression patterns in many cell types. Current direct conversion experiments are generally performed by choosing factors thought to be important for the target cell type (based on genetic, gene expression, biochemical, etc. analyses), overexpressing these factors in an unrelated cell type, and then monitoring the cells in the presence of the exogenous factors for phenotypes that the factors were thought to be critical for. If, however, the transduced cells were analyzed in the absence of the exogenous factors, the results may reveal which factors are the most important critical regulators for establishing and/or maintaining cell identity. As outlined in Table 1 and Table 2, few (if any) of the currently published studies have completely characterized the functional properties of converted cells in the absence of exogenous factors, so it is unclear what the biological implications of these studies are.

The work presented in this thesis attempts to address some of the shortcomings of the previous direct conversion experiments. Self-maintaining neural stem cells have been generated from mouse embryonic fibroblast cultures by overexpressing factors shown to be important for this cell type. These cells have been characterized in the absence of exogenous factor induction and were shown to differentiate and be transcriptionally and epigenetically similar to endogenous embryonic cortical neural stem cells. Finally, a “secondary” system has been created for reproducibly generating neural stem cells without additional factor transduction.

Table 1. Somatic Cell Conversion Experiments (Non-Neural Target Cell type)

Donor (Starting) Cell Type	Target (Ending) Cell Type	Exogenous Reprogramming Factors	Transduction Method	Exogenous Factor Independence	Transcriptional Analysis	Epigenetic Analysis	Reference
Fibroblasts	Myoblast-like cells	MyoD	Stable transfection	Dependent	Gene-specific	None	Davis et al. 1987; Weintraub et al., 1989
Fibroblasts	Melanocyte-like cells	MITF	Stable transfection	Not determined	Gene-specific	None	Tachibana et al., 1996
Monocyte-neutrophil progenitors	Erythroid-, eosinophil-, and basophil-like cells	Gata1	Inducible retrovirus	Dependent	Gene-specific	None	Heyworth et al. 2004
B-cells	Macrophage-like cells	C/EBPα ± PU.1	Constitutive retrovirus	Not determined	Gene-specific	None	Xie et al., 2004
T-cells	Macrophage-like cells	C/EBPα ± PU.1	Constitutive retrovirus	Not determined	Gene-specific	None	Laiosa et al., 2006
Fibroblasts	Macrophage-like cells	C/EBPα ± PU.1	Constitutive retrovirus	Dependent	Genome-wide	None	Feng et al., 2008
Pancreatic exocrine cells	β-cells-like cells	Ngn3, Pdx1, and MafA	Consitutive adenovirus	Not determined	Genome-wide	None	Zhou et al., 2008
Fibroblasts	Brown fat-like cells	C/EBPβ and PRDM16	Constitutive retrovirus	Not determined	Genome-wide	None	Kajimura et al., 2009
Fibroblasts	Hepatocyte-like cells	Hnf4α and FoxA1/FoxA2/ FoxA3	Constitutive retrovirus	Not determined	Genome-wide	None	Sekiya and Suzuki, 2010
Fibroblasts	Hepatocyte-like cells	Gata4, Hnf1α, and FoxA3	Constitutive lentivirus	Not determined	Genome-wide	None	Huang et al., 2011
Fibroblasts	Sertoli-like cells	Gata4, Nr5a2, Wt1, Dmrt1, and Sox9	Inducible lentivirus	Not determined*	Genome-wide	None	Buganim et al., 2012
Fibroblasts	Cardiomyocyte-like cells	Gata4, Mef2c, and Tbx5	Constitutive retrovirus/ inducible lentivirus	Not determined*	Gene-specific	Gene-specific	Ieda et al., 2010

*No functional data post exogenous factor withdrawal

Table 2. Somatic Cell Conversion Experiments (Neural Target Cell Type)

Donor (Starting) Cell Type	Target (Ending) Cell Type	Exogenous Reprogramming Factors	Transduction Method	Exogenous Factor Independence	Transcriptional Analysis	Epigenetic Analysis	Reference
Fibroblasts	Neuron-like cells	Brn2, Ascl1, and Mytl1	Inducible lentivirus	Not determined*	None	None	Vierbuchen et al., 2010
Hepatocytes	Neuron-like cells	Brn2, Ascl1, and Mytl1	Inducible lentivirus	Not determined*	Genome-wide	None	Marro et al., 2011
Fibroblasts (Human)	Neuron-like cells	Brn2, Ascl1, Mytl1, and NeuroD1	Inducible lentivirus	Not determined**	Gene-specific/ single-cell	None	Pang et al., 2011
Fibroblasts	Motor neuron-like cells	Hb9, Isl1, Lhx3, Ngn2, Brn2, Ascl1, Mytl1	Constitutive retrovirus	Not determined	Genome-wide analysis	None	Son et al., 2011
Fibroblasts (Human)	Dopaminergic neuron-like cells	Ascl1, Brn2, Myt1l, Lmx1a, FoxA2	Constitutive and inducible lentiviruses	Not determined	None	None	Pfisterer et al, 2011
Fibroblasts	Dopaminergic neuron-like cells	Ascl1, Pitx3, Nurr1, FoxA2, Lmx1a, and En1	Inducible lentivirus	Not determined*	Gene-specific	None	Kim et al., 2011
Fibroblasts	Dopaminergic neuron-like cells	Ascl1, Nurr1, and Lmx1a	Inducible lentivirus	Independent	Genome-wide	Gene-specific	Caiazzo et al., 2011
Fibroblasts	Neural stem-like cells	Sox2, Klf4, Myc, and Brn4 (± Tcf3)	Constitutive retrovirus	Not determined	Genome-wide	Gene-specific	Han et al., 2012
Fibroblasts	Neural stem-like cells	Sox2, Brn2, and FoxG1	Inducible lentivirus	Dependent	Gene-specific	None	Lujan et al., 2012
Fibroblasts	Oligodendrocyte precursor-like cells	Sox10, Olig2, and Zfp536	Inducible lentivirus	Not determined	Genome-wide	None	Yang et al., 2013
Fibroblasts	Oligodendrocyte precursor-like cells	Sox10, Olig2, Olig1, Nkx2.2, Nkx6.2, ST10, Gm98, Myt1l	Inducible lentivirus	Not determined--function *in vivo* without induction	Genome-wide	None	Najm et al., 2013

*No functional data post exogenous factor withdrawal
**Limited functional data post exogenous factor withdrawal

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Chapter 2

Generation of transgene-independent neural stem cells from fibroblasts by defined factors and growth conditions

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Author Contributions
JPC and RJ conceived the project and wrote the chapter. JPC designed experiments and conducted all experiments unless otherwise listed. ACD performed ChIP and microarray experiments. ZPF analyzed ChIP and microarray experiments. YL performed *in utero* injections.

SUMMARY

The overexpression of four transcription factors Oct4, Sox2, Klf4, and c-Myc reprograms a somatic nucleus to one that is transcriptionally and epigenetically indistinguishable from an embryonic stem (ES) cell. However, it is still unclear if transcription factors can completely convert the nucleus of a differentiated cell into that of a distantly related cell type with complete transcriptional and epigenetic reprogramming maintained in the absence of exogenous factor expression. To test this idea, we generated doxycycline (dox)-inducible vectors encoding neural stem cell-expressed factors. We found that stable, self-maintaining NSC-like cells could be induced under defined growth conditions after transduction of factors including Brn2, Hes1, Hes3, Klf4, Myc, Notch, PLAGL1, Rfx4, and dominant-negative REST. These cells were characterized in the absence of exogenous factor induction and were shown to be transcriptionally, epigenetically, and functionally similar to endogenous embryonic cortical NSCs. Additionally, a cellular system was created for reproducible generation of dox-independent iNSCs without additional factor transduction. Our results show that a transcriptionally and epigenetically reprogrammed somatic nucleus can be stabilized *in vitro* providing a tool to study the mechanism of somatic cell conversion.

INTRODUCTION

Factor-mediated reprogramming, the process by which overexpression of a defined set of transcription factors converts one cell type into another, has important implications for regenerative medicine and demonstrates the power that transcription factors have as cell fate determinants (Jaenisch and Young, 2008). This has been shown for pluripotent stem cells, where three transcription factors (Oct4, Sox2, and Klf4) are sufficient to induce any cell type to become an induced pluripotent stem (iPS) cell (Takahashi and Yamanaka, 2006; Okita et al., 2007; Wernig et al., 2007; Nakagawa et al., 2008; Wernig et al., 2008a). iPS cells are transcriptionally, epigenetically, and functionally indistinguishable from embryonic stem (ES) cells (Okita et al., 2007; Wernig et al., 2007; Boland et al., 2009; Zhao et al., 2009). The overwhelming biological and medical interest in iPS cells driven attempts to generate other cell types through forced expression of defined factors.

However, pluripotency may represent a unique cellular state that is more attainable *in vitro* than a somatic one (Hanna et al., 2010). This has been suggested by cell fusion experiments, which show that ES cells express dominant *trans*-acting factors that can induce a somatic nucleus to become pluripotent upon cellular fusion (Tada et al., 2001). Also, pluripotent cells express a set of well-characterized transcription factors that are known to regulate each other, thereby forming an endogenous autoregulatory gene circuitry (Boyer et al., 2005) that may maintain the ES cell state once it is activated. Pluripotency may be further stabilized *in vitro* by its unique

epigenetic environment, which includes the regulators responsible for silencing retroviruses specifically in pluripotent cells (Wolf and Goff, 2007; Jahner et al., 1982) as well as a destabilized nucleosome remodeling and histone deacetylation (NuRD) complex (J. Hanna, unpublished data). Furthermore, the growth advantage that pluripotent cells have over somatic cells and their dome-like morphology may aid the detection of rare reprogramming events.

Recently, a number of cell types have been generated by the forced expression of key transcription factors, including cells resembling blood cells (Heyworth et al., 2004; Xie et al., 2006), brown fat cells (Kajimura et al., 2009), hepatocytes (Sekiya and Suzuki, 2010; Huang et al., 2011), sertoli cells (Buganim et al., 2012a), and various cells of the neural lineage (Veirbuchen et al., 2010; Kim et al., 2011; Son et al., 2011; Han et al., 2012; Lujan et al., 2012; Najm et al., 2013; Yang et al., 2013). However, it is unclear how similar these cells are to their endogenous counterparts because they were generated either with constitutive or with inducible vectors, but were functionally and molecularly characterized in the presence of the inducing agent. Furthermore, an extensive epigenetic analysis has not been performed to determine if the chromatin has been reset to allow for cell type-specific gene expression to persist in the absence of the exogenous factors. Cell type conversion requires analysis of the resulting cells in the absence of exogenous factors to assess the intrinsic qualities of the cell (Wager and Weissman, 2004; Graf and Enver, 2009; Hanna et al., 2010).

The induction of stable iPS cells requires multiple cell divisions (Hanna et al., 2009). Reasoning that defined-factor-mediated induction of a somatic cell type may

also require multiple cell divisions to establish a stable epigenetic state, we sought to generate neural stem cells (NSCs)—the multipotent precursors of the developing mammalian nervous system. NSCs are readily grown *in vitro* and can be generated either by explanting them directly from the developing or adult mouse brain (Doetsch et al., 1999) or by deriving them *in vitro* by differentiating ES cells in a defined medium containing insulin, transferrin, selenium, and fibronectin (ITSFn) (Okabe et al., 1996). The *in vitro* derived cells share the features of their *in vivo* counterparts, including the ability to self-renew indefinitely in the presence of EGF and FGF, as well as the ability to differentiate into both neurons and glia upon growth factor withdrawal (Okabe et al., 1996).

Several factors are known to have important roles in NSC biology and/or are preferentially expressed in NSCs. For instance, Notch and its effectors are critical for NSC maintenance (Imayoshi et al., 2010), as are Sox9 (Scott et al., 2010) and the redundant SoxB1 transcription factors Sox1, Sox2, and Sox3 (Bylund et al., 2003; Graham et al., 2003). POU-domain proteins like Brn1 and Brn2 are known to bind collaboratively with Sox proteins and enhance their induction of target genes (Dailey and Basilico, 2001), and the Rfx and NF-I binding motifs were found at Sox2/Brn2 co-bound sites (Lodato et al., 2013), which suggests that these factors may also have prominent NSC regulatory roles as well. Many genes like Plagl1, Plzf, Lhx2, and Zic1 are highly expressed in rosette-stage NSCs, an early NSC type with broad differentiation potential (Elkabetz et al., 2008; Elkabetz and Studer, 2008).

In the present study, we generated self-maintaining neural stem cells from mouse embryonic fibroblast cultures by transducing the cells with a pool of defined factors known to be important for neural stem cells and then growing the cells in defined growth conditions. These induced NSCs (iNSCs) can differentiate *in vitro* and *in vivo* and have similar genome-wide gene expression patterns and enhancer usage as primary NSCs isolated from developing mouse brains. Finally, we demonstrate the reproducibility of this process by establishing a 'secondary' system in which iNSCs can be generated from embryo chimeras containing cells with clonal iNSC proviral integrations that can be induced without the need for additional factor transduction. These findings demonstrate that a non-pluripotent somatic cell can be reproducibly induced that maintains itself in the absence of exogenous factor overexpression with a transcriptionally and epigenetically reprogrammed nucleus.

RESULTS

Defined growth conditions promote the induction of self-maintaining NSCs by defined factors

A library of neural stem cell (NSC)-expressed transcription factors and chromatin-modifying factors thought to be important for NSCs or with NSC-enriched gene expression was created for overexpression in fibroblasts (Table S1) (Bylund et al., 2003; Elkabetz et al., 2008; Imayoshi et al., 2010; Scott et al., 2010). In addition to NSC factors, a dominant-negative version of the RE1-silencing transcription factor (DN-REST) was included to inhibit the endogenous Rest complex, which silences neural

genes in non-neural cell types (Ballas and Mandel, 2005; Chong et al., 1995). The library also included the neuronal induction factors Ascl1, Myt1l, and mir124a (Ambasudhan et al., 2011; Yoo et al., 2011; Vierbuchen et al., 2010) as well c-Myc, which acts as an amplifier of gene expression and may serve a general role during reprogramming (Lin et al., 2012; Nie et al., 2012; Bugamin et al., 2013). All transgenes were controlled by the doxycycline (dox)-inducible Tet-On promoter to regulate exogenous factor expression.

Fibroblasts from the Sox2-GFP reporter mouse, in which one allele of the endogenous *Sox2* gene was replaced by GFP, were used to monitor for NSC properties in transduced cells (Ellis et al., 2004). In the mouse embryo, Sox2 is highly expressed in the developing neural tissues (Ellis et al., 2004; Avilion et al., 2003; Zappone et al., 2000), but also found in other somatic stem cell compartments (Arnold et al., 2011). The Sox2 expressing neural tissue was removed by dissection, and the rare GFP+ cells that remain have been shown to not have neural characteristics (Lujan et al., 2012).

Initially, the strategy to generate NSC-like cells was to transduce the pool of factors into MEFs and then culture the cells in NSC medium that contained dox (Figure S1A). GFP analysis showed a weakly-positive population of Sox2-GFP+ cells that increased over time after factor induction (Figure S1B). These cells could be grown *in vitro* as adherent cultures or as neurosphere-like floating cultures (Figures S1C and S1D), and FACS-sorted cells stained for the NSC marker Nestin (Figure S1E).

Figure S1. Generation of transgene-dependent NSC-like cells with limited NSC characteristics

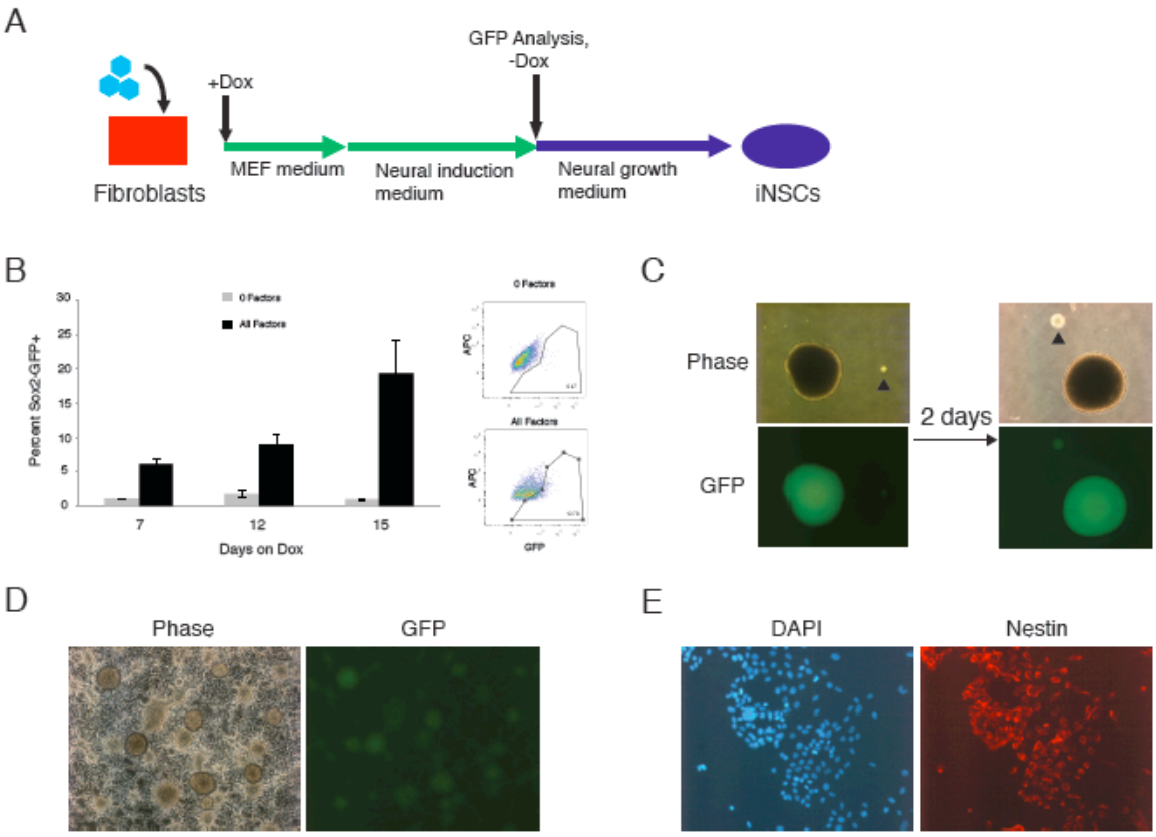

Figure S1. Generation of transgene-dependent NSC-like cells with limited NSC characteristics

(A) Experimental strategy for inducing NSCs from fibroblasts. After library transduction, cells were grown with dox for 3-4 weeks (4 days in MEF medium and then in neural induction medium), then GFP was analyzed, and cells were replated without dox. (B) Left, time course of Sox2-GFP expression in transduced and untransduced MEFs. Right, representative FACS plots. The APC channel was used to control for autofluorescence. (C) Sox2-GFP+ cells could grow as spheres and proliferate over time. Note that the sphere marked with an arrowhead expanded over a 2 day time frame. Images were taken using the same microscope objective. (D) Morphology and Sox2-GFP fluorescence of transduced cells growing as adherent spheres and monolayer culture. (E) Immunostaining for Nestin in adherent cells sorted for Sox2-GFP+ expression.

However, these Sox2-GFP+ cells remained dependent on the exogenous transgenes for survival, as they did not survive dox withdrawal (data not shown).

To screen for conditions that could produce NSC-like cells able to be maintained independent of dox, we varied both the exogenous factors and the culture conditions. First, MEFs from E12.5 embryos were used, since these cells were from an earlier embryonic period than the typical (E14.5) MEFs and therefore may be more amenable to generating self-maintaining NSCs *in vitro* by defined factors. Additionally, dox was withdrawn, and NSC-like cells were selected for during a period of growth in ITSFn, a medium that is normally used during ES cell differentiation to NSCs to select for Nestin-expressing cells (Okabe et al., 1996). Finally, specific factors or groups of factors were omitted from the viral pools used to transduce MEFs to assess whether certain factors were detrimental for dox-independent NSC formation (Figure 1A).

GFP expression was measured at two time points: before ITSFn selection while the cells were on dox, and again three weeks after dox withdrawal (Figure 1A). We found robust activation of the Sox2-GFP reporter with most factor combinations in the presence of dox. No Sox2-GFP induction was seen in the uninfected control cultures and when both Notch (NICD) and its effector Hes1 were omitted (Figure 1B), supporting the finding that Notch signaling is important for the maintenance of NSCs (Kageyama et al., 2009; Imayoshi et al., 2010). Many of the factor combinations that induced robust Sox2-GFP expression in the presence of dox had reduced or no GFP expression following dox withdrawal. In fact, only one factor combination—the pool in which Olig1,

Figure 1. Defined growth conditions promote the induction of NSC-like cells from fibroblasts

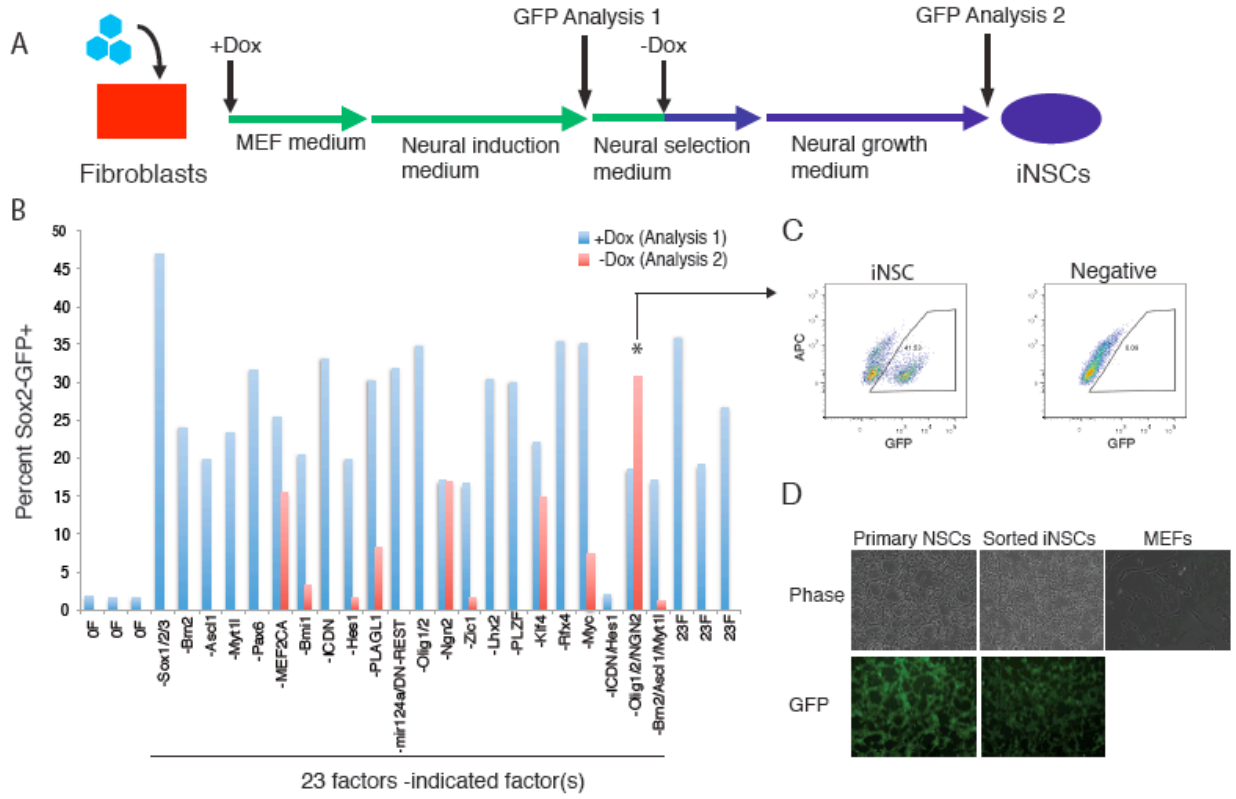

Figure 1. Defined growth conditions promote the induction of NSC-like cells from fibroblasts

(A) Experimental strategy for inducing and selecting transgene-independent NSC-like cells from transduced E12.5 MEFs. Dox was withdrawn on day 4 of a 10 day growth period in a neural selection medium (ITSFn). See methods section for experimental details. (B) Sox2-GFP fluorescence measured at two time points—before (blue bars, GFP Analysis 1) and after (red bars, GFP Analysis 2) dox withdrawal. The * denotes transduction that generated dox-independent NSC-like cells, also called iNSCs. (C) FACS plots showing the Sox2-GFP expression of dox-independent cells (iNSCs). A Sox2-GFP-negative culture from the same experiment is shown for reference. The APC channel was used to control for autofluorescence. (D) Morphology and Sox2-GFP fluorescence of iNSCs sorted for Sox2-GFP+ cells (Sorted iNSCs), as well as primary cortical NSCs from Sox2-GFP mouse (Primary NSCs), and MEFs. (E) Immunostaining of sorted iNSCs for NSC markers Nestin, Sox2, and Sox1. (F) Quantitative PCR comparing the expression of fibroblast genes Col5a2 and Thy1 and NSC genes Sox1, Nestin, and Brn1 in ES cells, MEFs, E14.5 derived neurospheres (E14.5 NS), and the iNSCs. Values were normalized to Gapdh expression for each cell type.

Olig2, and Ngn2 were omitted—showed an increase in the Sox2-GFP+ fraction 3 weeks after dox withdrawal (Figure 1B). The GFP expression of these cells was distinct from the Sox2-GFP-negative population (Figure 1C), and it was much stronger than the weak GFP expression of the dox-dependent Sox2-GFP+ cells (comparing Figure S1A and Figure 1C). In pools containing Olig1, Olig2, and Ngn2, the cultures were overgrown by GFP-negative cells after dox withdrawal, which may have masked the generation of Sox2-GFP+ cells (data not shown).

The dox-independent Sox2-GFP+ cells were isolated by FACS and could be expanded in the presence of EGF and FGF with a morphology and GFP expression similar to primary cortical NSCs isolated from Sox2-GFP+ embryos (Figure 1D). The sorted cells had the same growth factor dependence as primary NSCs in that they proliferated rapidly in the presence of EGF/FGF or EGF alone, but experienced slower proliferation or cell death upon EGF withdrawal from EGF/FGF cultures (Figure S2 and S3) (Conti et al., 2005; Pollard et al., 2006). The sorted cells stained for the NSC-expressed intermediate filament Nestin, as well as the endogenous transcription factors Sox1 and Sox2 (Figure 1E). Importantly, quantitative PCR (qPCR) analysis showed that these cells expressed the neural genes *Nestin*, *Sox1*, and *Brn1*, but did not express the fibroblast genes *Col5a2* or *Thy1* (Figure 1F).

Shortly after growth factor withdrawal, the Sox2-GFP+ sorted cells began to express doublecortin (Dcx) (Figure 2A), which is normally expressed in differentiating NSCs (Francis et al., 1999). The cells differentiated into neuronal and glial cells,

Figure S2. iNSC growth factor dependence

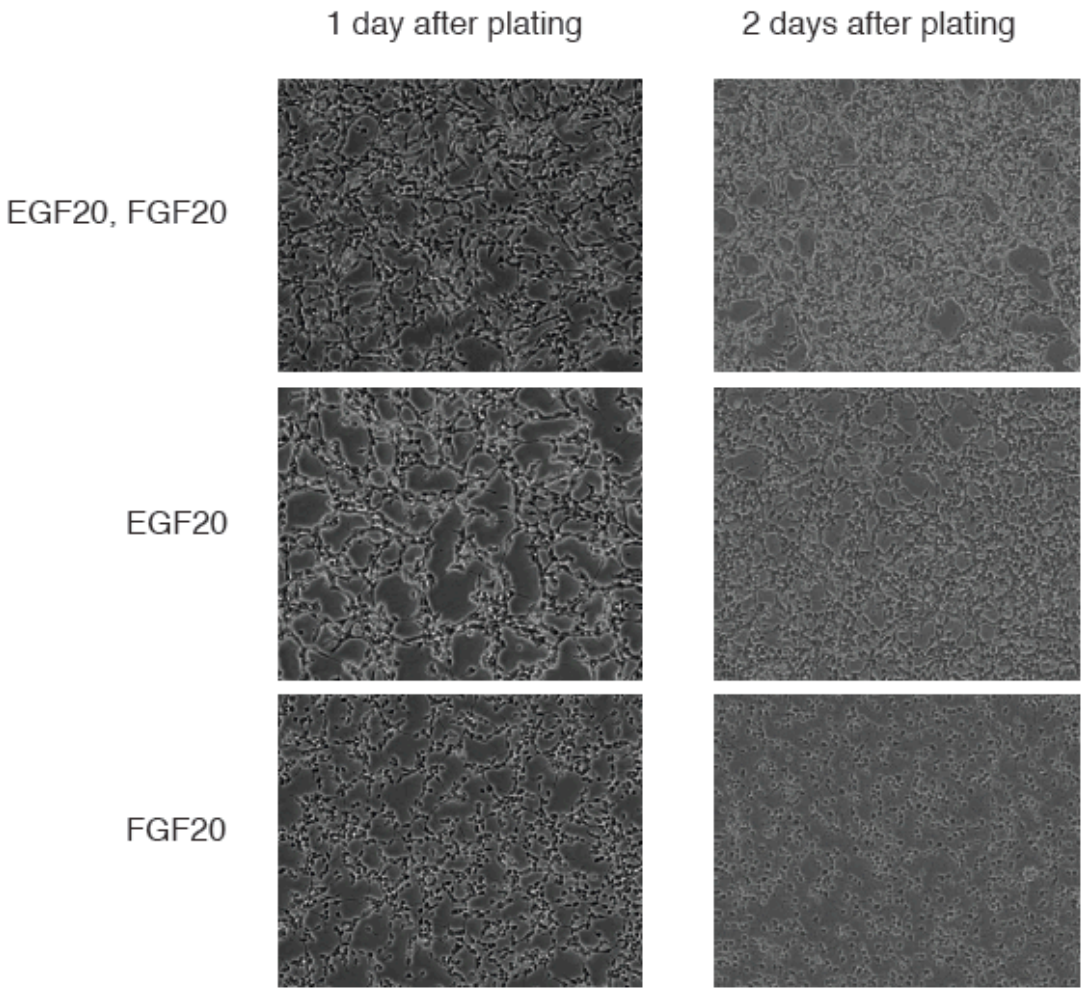

Figure S2. iNSC growth factor dependence

Morphological analysis of iNSCs 24 and 48 hours after being plated with the indicated growth factors. iNSCs display a similar growth dependence as that reported for ES cell-derived NSCs (Conti et al., 2005; Pollard et al., 2006). EGF20 is 20 ng/ml EGF and FGF20 is 20 ng/ml FGF.

Figure S3. iNSC morphological changes upon differentiation

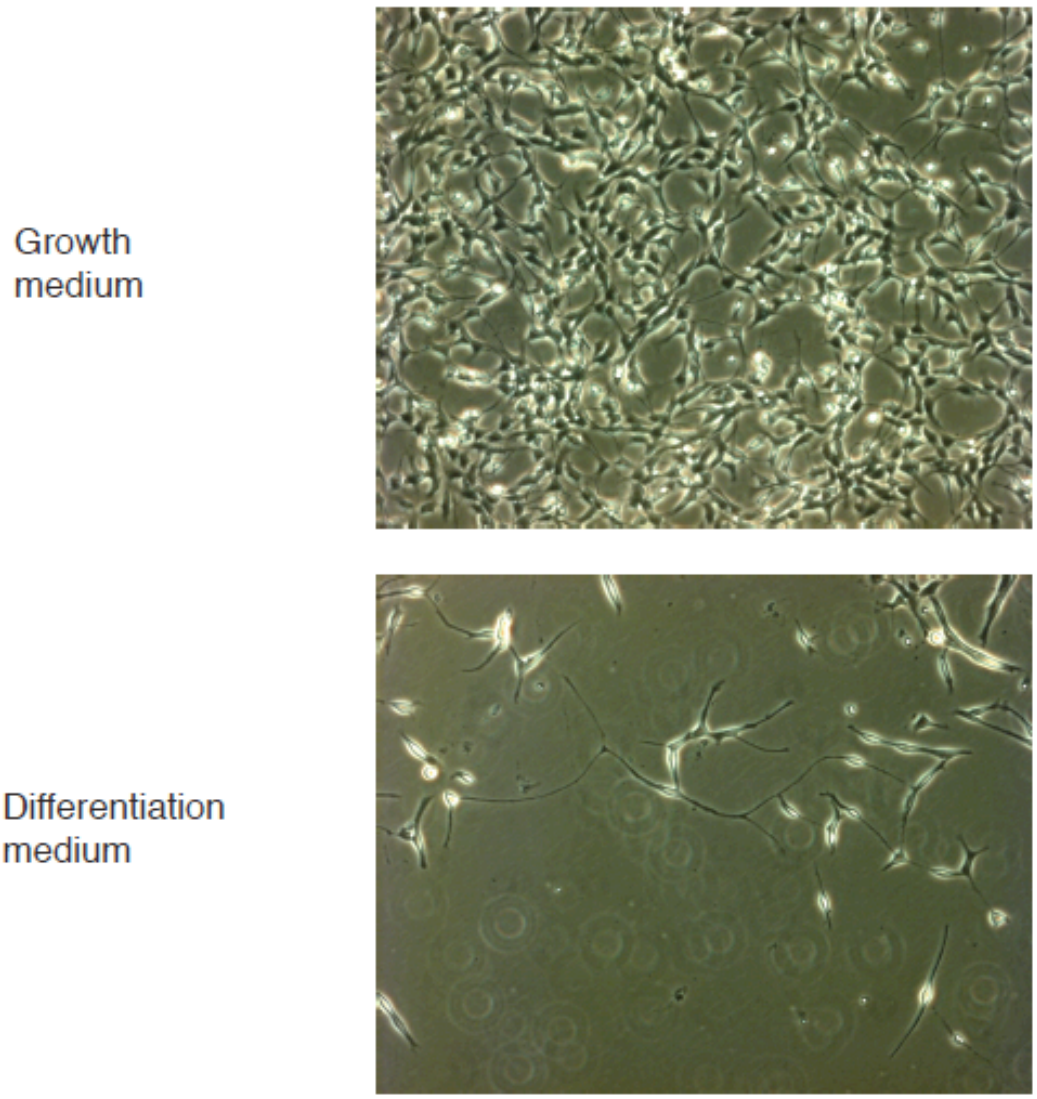

Figure S3. iNSC morphological changes upon differentiation

Morphological analysis of iNSCs cultured for 4 days in either growth medium (containing EGF and FGF) or a differentiation medium (containing forskolin, FGF, and PDGF) (Glaser et al. 2007).

which immunostained for β-III tubulin (TuJ1) and glial fibrillary acidic protein (GFAP), respectively (Figure 2B). When allowed to spontaneously differentiate in the absence of growth factors, differentiation was skewed toward astrocytes (Figure S4), as has been reported for NSCs grown *in vitro* (Mikkelsen et al., 2008; Conti et al., 2005).

The ability of these induced cells to integrate, migrate, and differentiate *in vivo* was assessed by labeling them with a constitutive tdTomato lentivirus (FUW-tdTomato) and injecting them into the ventricles of developing mouse brains. Analysis one month after birth revealed that the injected cells that had integrated into the endogenous NSC niche of the subventricular zone stained for endogenous Dcx. (Figure 2C). Cells that migrated to the cortex could differentiate *in vivo* and stained for the glial marker GFAP or the mature neuron marker NeuN (Figure 2C).

Since these cells grew like NSCs, expressed the transcripts and proteins associated with NSCs, and could differentiate into neurons and glia both *in vitro* and *in vivo*, they were designated as “induced neural stem cells”, or iNSCs.

iNSC induction from E14.5 MEFs

We wanted to determine if a similar culture protocol could be used to generate iNSCs from later stage MEFs. To that end, fibroblasts were isolated from E14.5 embryos after removal of the neural tissue. The three factors that were detrimental

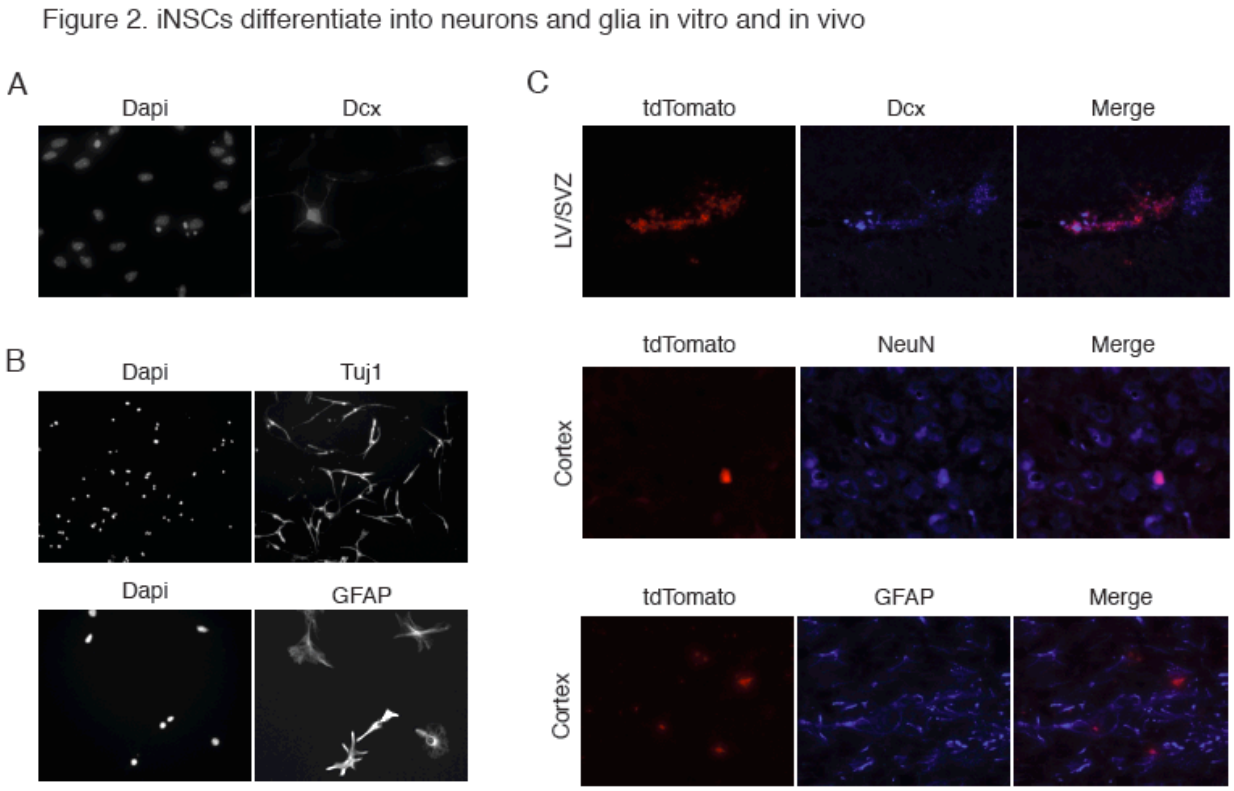

Figure 2. iNSCs differentiate into neurons and glia *in vitro* and *in vivo*

(A) Immunostaining for the early differentiation marker doublecortin (Dcx) shortly after growth factor withdrawal. (B) Immunostaining for neuronal marker β-III tubulin (TuJ1) in cells differentiated with forskolin (top), and immunostaining for glial marker glial fibrillary acidic protein (GFAP) in cells differentiated with 1% serum (bottom). (C) iNSCs labeled with FUW-tdTomato were transplanted into the ventricles of E14.5 mouse brains and immunostained for differentiation markers one month after birth. iNSCs and endogenous cells in the lateral ventricle/ subventricular zone (LV/SVZ) were immunostained for Dcx (top), and cells in the cortex were immunostained for differentiation markers GFAP (middle) and neuron-specific neuronal nuclei (NeuN) (bottom).

Figure S4: Co-generation of neurons and glia

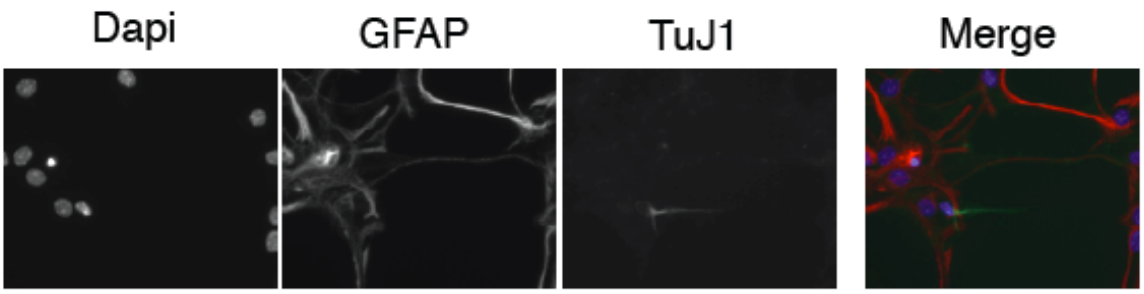

Figure S4. Co-generation of neurons and glia

GFAP (red) and TuJ1 (green) immunostaining in cells differentiated by growth factor withdrawal. Dapi (blue) is shown for reference.

for iNSC formation (Olig1, Olig2, and Ngn2) were omitted from future transductions; however, the Notch effectors Hes3 and Hes5 as well as Sox9 were included in the new library of factors.

Parallel transductions of this new pool of factors into MEFs resulted in multiple iNSC lines with morphologies and Sox2-GFP expression similar to primary NSCs (Figure 3A and Figure 1D). RT-PCR analysis revealed that these iNSC lines expressed the endogenous NSC genes Sox1, Sox3, Pax6, Blbp, and Brn1 (Figure 3B). When measured quantitatively by qPCR, the transcript expression in iNSC cell lines was similar to primary NSCs and distinct from MEFs—iNSCs expressed NSC gene Sox1, Sox2, and Pax6, but not fibroblast-specific gene Col5a2 (Figure 3C). Importantly, upon growth factor withdrawal, these cell lines differentiated and stained for TuJ1 and GFAP (Figure 3D).

iNSCs can be generated with 7 or 8 factors

To determine which factors were responsible for generating iNSC lines, we developed a PCR strategy to detect the viral transgenes that had integrated in the genome of characterized iNSC lines. We used primers that either spanned introns or, for single-exon genes, amplified between the TetO lentiviral promoter and the gene of interest. PCR analysis revealed that many different lenti-proviruses had integrated into the genome of the iNSC lines, ranging in number from 13 different factors in iNSC5 to

Figure 3. Characterization of multiple iNSC lines induced from E14.5 MEFs

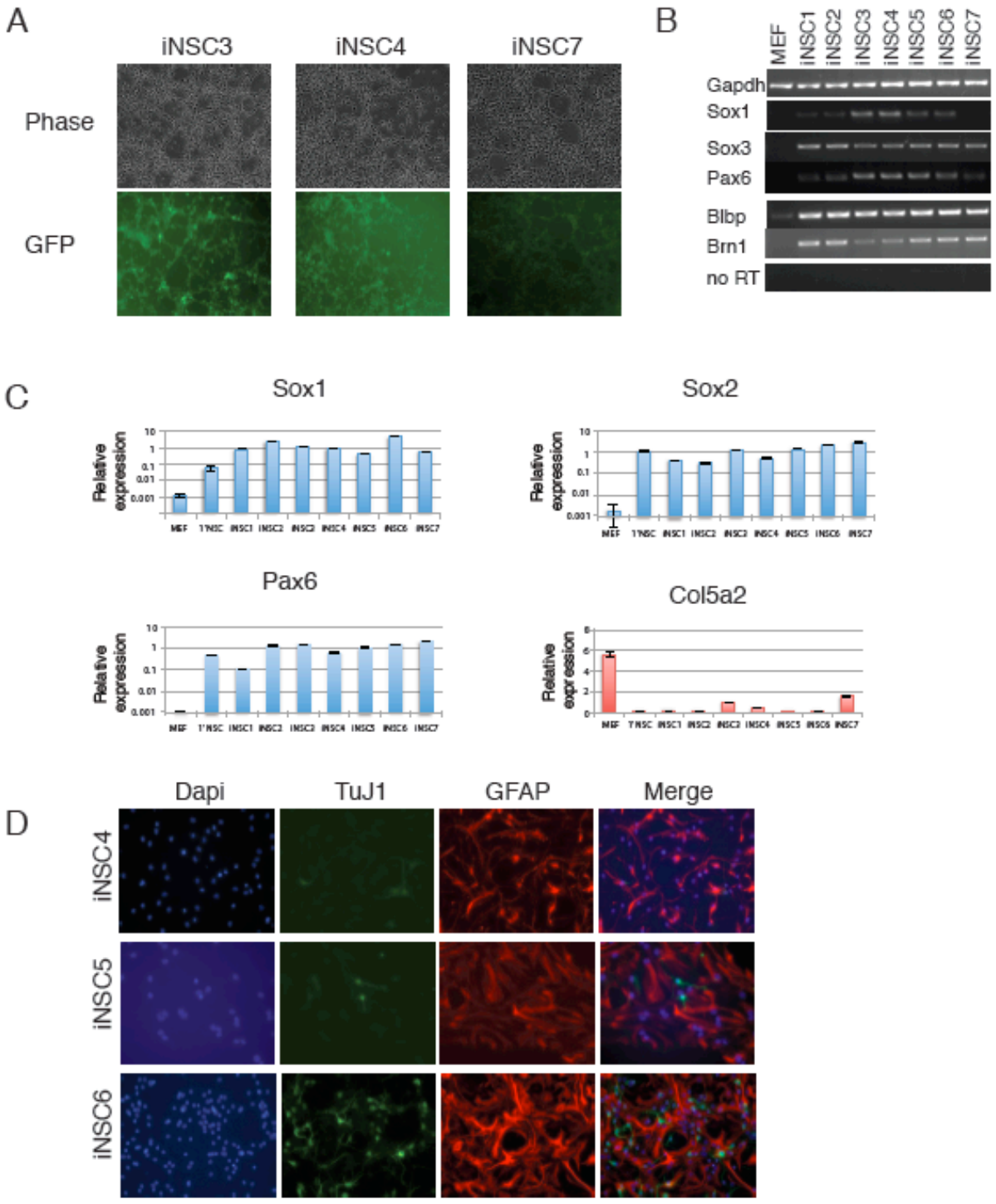

Figure 3. Characterization of multiple iNSC lines induced from E14.5 MEFs

(A) Morphology and GFP fluorescence of iNSCs generated from transduced E14.5 MEFs. Compare to MEFs shown in Figure 1D. (B) Non-quantitative (RT)-PCR analysis of NSC transcript expression in iNSC lines and uninfected MEFs. (C) Quantitative PCR (qPCR) analysis of indicated NSC and MEF transcripts in MEFs, primary cortical NSCs (1' NSC), and iNSC lines. Expression values were normalized to Gapdh expression for each cell type. (D) Immunostaining of differentiation markers GFAP and TuJ1 after growth factor withdrawal.

21 in iNSC2 (Figure 4A). The high number of integrated factors in these lines may reflect a requirement for many different factors to impart stable NSC-like characteristics on MEFs, or it may be due to heterogenous cultures made up of multiple cell lines.

To narrow down the factors, we focused on iNSC5 because it had the fewest number of integrated factors, suggesting that some combination of these factors was sufficient for generating iNSCs. We therefore infected E14.5 MEFs with the 13 factors present in iNSC5—Sox2, Hes1, Hes3, Brn2, Klf4, Rfx4, Zic1, DN-REST, NICD, Lhx2, PLAGL1, Myc, and Bmi1—or with the 13 factors plus FoxG1, which was recently reported to be important for creating dox-dependent iNSCs (Lujan et al., 2012). A time course was performed with dox induction periods ranging from 3 to 30 days to determine the duration of exogenous factor induction sufficient for iNSC formation (Figure 4B). iNSCs were generated from both lentiviral pools (13 factors and 14 factors), but only after 30 days on dox (Figure 4B). The resulting cells lines (iNSC-13F and iNSC-14F) had morphologies and GFP expression patterns similar to those of the previously characterized iNSC lines and primary NSCs (Figure 4C and 4D). After 3 weeks of culture in NSC growth medium, these populations formed stable cell lines and homogenously expressed Sox2-GFP in more than 90% of cells (Figure 4D). Additionally, the cells expressed NSC marker genes *Pou3f3* (Brn1) and *Sox2* by qPCR, but did not express the MEF genes *Col5a2* or *Thy1* (Figure 4E). Like previous iNSC lines and primary NSCs, the cells were competent for neuronal and glial differentiation

Figure 4. Transgene-independent iNSCs can be induced by 7 or 8 factors

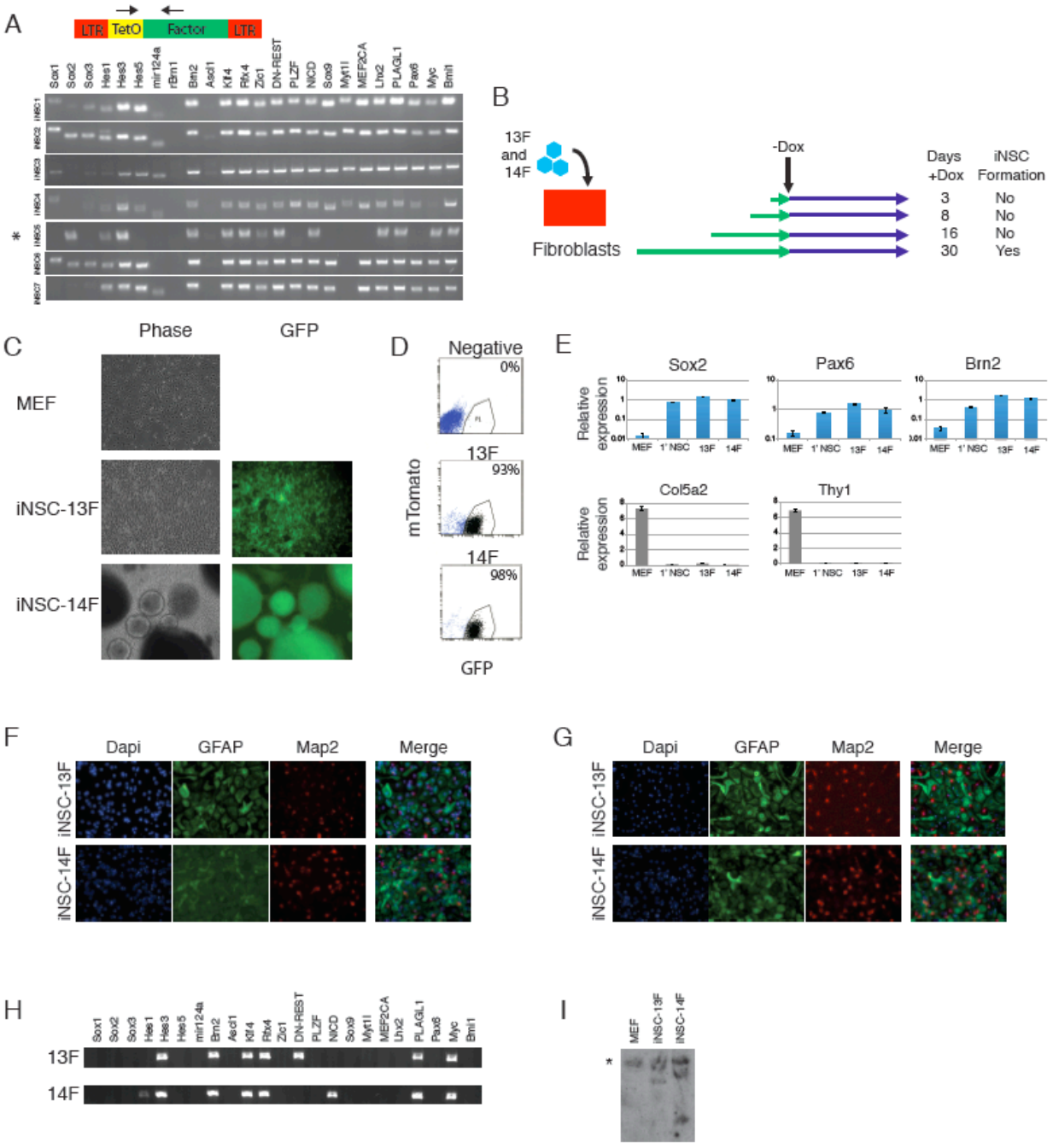

Figure 4. Transgene-independent iNSCs can be induced by 7 or 8 factors

(A) Schematic showing the PCR strategy to detect transduced factors in the genomic DNA of iNSC lines (top). Primer sets used either included an universal primer recognizing the TetO promoter and a factor-specific reverse primer, or two factor-specific primers separated by at least one intron in the endogenous gene. Bottom, PCR genotyping results for the transduced factors detected in iNSC lines. The * denotes line iNSC5, which had the fewest number of transduced factors. (B) Time course with varying durations of dox induction. MEFs were transduced with the 13 factors present in iNSC5 (13F) or with the 13 factors plus FoxG1 (14F) and grown in the presence of dox for the indicated lengths of time. iNSCs were not generated at time points earlier than 30 days. (C-D) Morphology and GFP expression of iNSC-13F (13F) and iNSC-14F (14F). The mTomato channel was used to control for autofluorescence. (E) qPCR analysis of NSC and MEF transcript expression in iNSC-13F, iNSC-14F, MEFs, and primary cortical NSCs (1' NSC). (F-G) Immunostaining for differentiation markers GFAP and TuJ1 (F) and GFAP and Map2 (G) in iNSC-13F and iNSC-14F cultures after growth factor withdrawal. (H) PCR analysis to detect factors integrated in iNSC-13F and iNSC-14F genomic DNA. (I) Southern blot analysis of uninfected MEFs, iNSC-13F, iNSC-14F using a Klf4 probe to determine the independence of these two lines. The * indicates endogenous gene.

upon growth factor withdrawal (Figure 4F).

We next determined which transgenes had integrated into iNSC-13F and iNSC-14F. We found that 7 factors had integrated into iNSC-13F and 8 factors into iNSC-14F (Figure 4G). Interestingly, these lines shared 6 factors in common: Brn2, Hes3, Klf4, Myc, PLAGL1, and Rfx4, with the additional factors being DN-REST in iNSC-13F and NICD and Hes1 in iNSC-14F (Figure 4G). Furthermore, we confirmed the absence of FoxG1 integration as well as independence of the cell lines by Southern blot analysis of FoxG1 and Klf4, respectively (Figure 4I and data not shown).

Genomic reprogramming of iNSCs

We next compared the genome-wide gene expression patterns of iNSC-13F and iNSC-14F, the starting MEF population, and primary cortical NSCs derived from the same genetic background (Sox2-GFP+, rtTA+/+). Microarray analysis revealed that iNSCs expressed many genes that are commonly transcribed in NSCs but have silenced MEF-specific marker genes (Figure 5A). When analyzed for genes differentially expressed between published MEF and ES-derived neural precursor cell (NPC) datasets (Mikkelsen et al., 2008), iNSCs showed a global gene expression pattern similar to the NPCs and primary NSCs (Figure 5B). Moreover, when the gene expression pattern of iNSCs was compared to that of diverse tissue types, such as ES cells, ES-derived NPCs, mature neural cell lineages, and MEFs, hierarchical clustering showed that iNSCs clustered with primary NSCs and ES-derived NPCs and were distinct from fibroblasts and the other cell types (Figure 5C).

Figure 5. Genome-wide gene-expression analysis of iNSCs

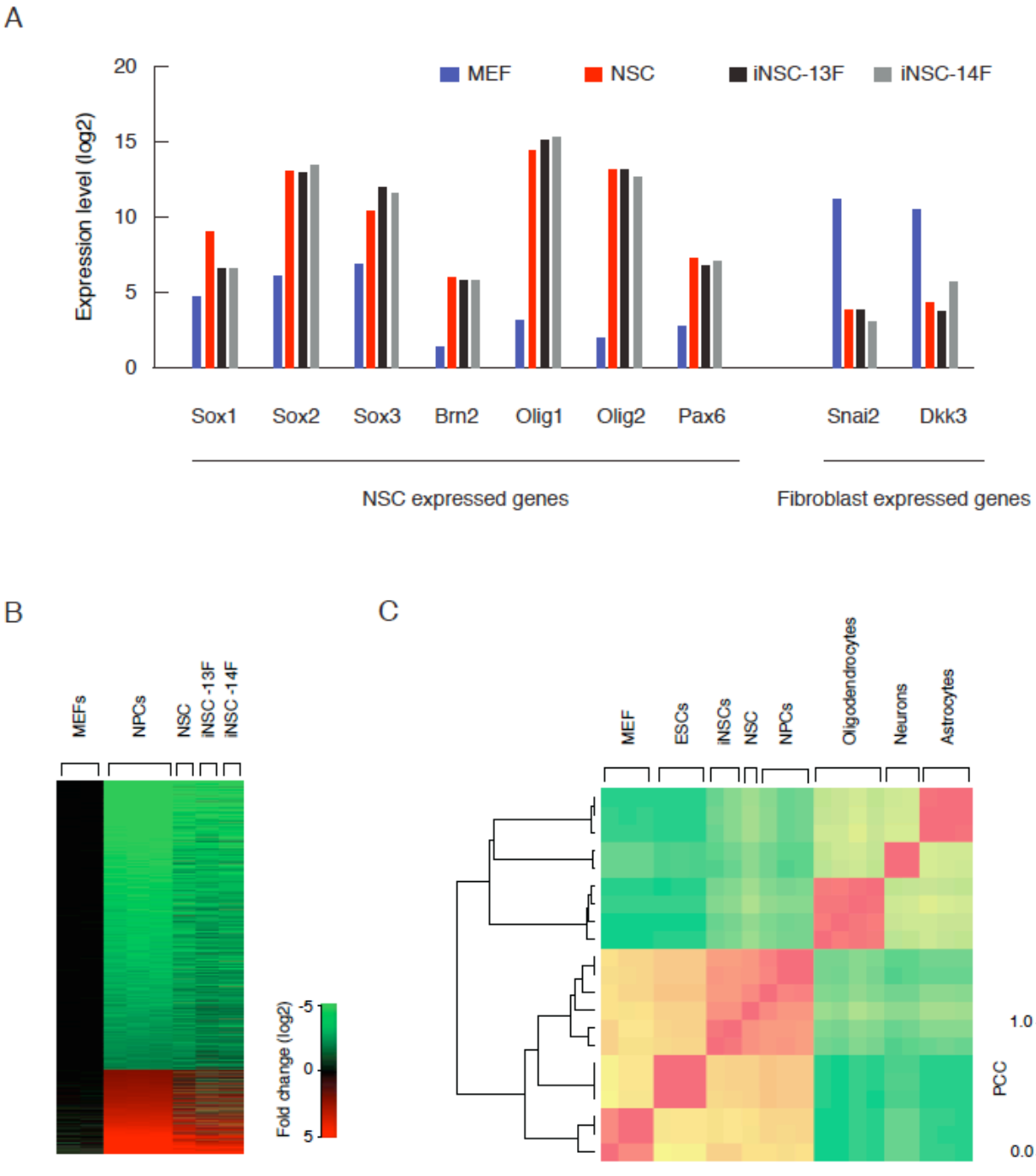

Figure 5. Genome-wide expression analysis of iNSCs

(A) Microarray analysis was performed on the untransduced MEF starting population (MEF), primary cortical NSCs (NSC), iNSC-13F, and iNSC-14F. Shown is the normalized expression values at NSC expressed genes and fibroblast expressed genes. (B) Differentially expressed genes were determine by analysis of published ES-derived NPC (NPC) and MEF datasets (See methods). The MEF expression level was set to 0. NSC is primary NSC from the same genetic background as iNSC-13F and iNSC-14F. (C) Expression profiles of iNSC-13F and iNSC-14F were compared to ES cells, ES-derived NPCs, mature neural cell lineages, and MEFs. The datasets were analyzed by hierarchical clustering and Pearson correlation analysis.

Enhancers are chromatin sites where transcription factors, chromatin remodelers, histone modifiers, and mediators of the transcriptional machinery bind to control the cell type-specific expression of nearby genes (Buecker and Wysocka, 2012; Calo and Wysocka, 2013). Active enhancers, or enhancers that are associated with active genes, are marked by histone H3 lysine K27 acetylation (H3K27ac), which displays a unique cell type-specific profile (Creyghton et al., 2010; Rada-Iglesias et al., 2011). The dynamic nature of enhancers and their cell type-specificity make them stringent markers of cell identity. To determine if iNSCs reprogrammed their epigenome and had enhancer usage similar to primary NSCs, we analyzed H3K27ac genome-wide by chromatin immunoprecipitation, followed by massively parallel DNA sequencing (ChIP-seq). iNSC-13F had a similar H3K27ac profile as primary NSCs at key NSC loci like *Olig1*/*Olig2* and was not marked at a fibroblast-expressed collagen locus (Figures 6A). Genome-wide, iNSC-13F had an active enhancer profile that was similar to primary NSCs and distinct from the starting population of MEFs (Figure 6B). Although the vast majority of the iNSC-13F genome was reprogrammed to the NSC-like state, there were rare sites that displayed a MEF-like H3K27ac pattern (Figure S5), suggesting that epigenetic memory of the MEF starting population may persist in these iNSCs.

Overall, these genome-wide analyses indicated that iNSCs had transcriptionally and epigenetically reprogrammed their nucleus to a state that was highly similar to primary NSCs, although a few rare genes may indicate residual epigenetic memory from their non-neural origin.

Figure 6. Genome-wide active enhancer profile of iNSCs

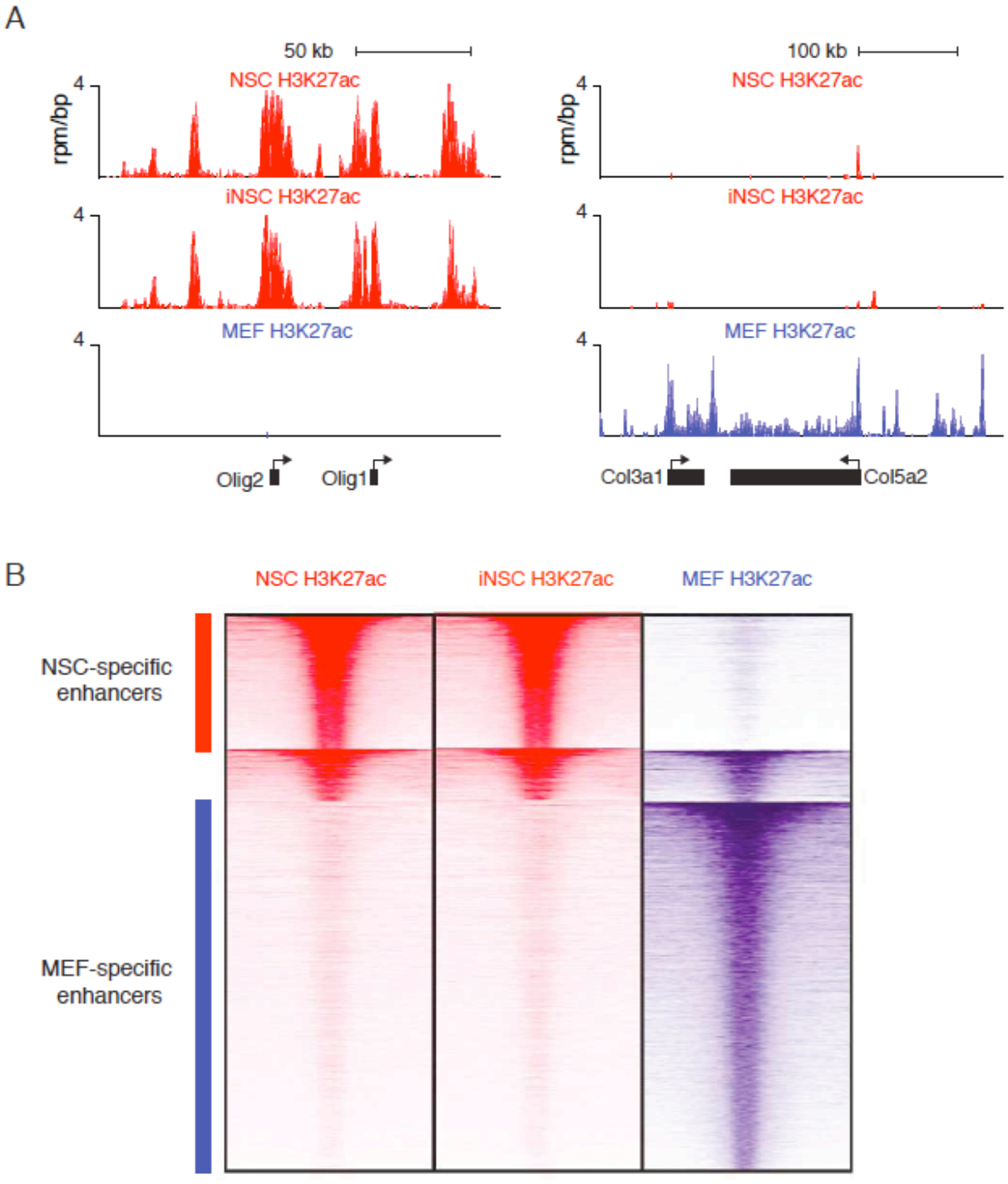

Figure 6. Genome-wide profiling of iNSC active enhancer usage

(A) H3K27ac ChIP-seq profiling in primary NSCs (NSC), iNSC-13F (iNSC), or uninfected MEF (MEF). Shown are the H3K27AC maps at the neural expressed *Olig1/Olig2* locus and at the MEF enriched *Col3a1/Col5a2* locus. (B) Heatmap representation of active enhancers in the genome NSC-specific enhancers are shown on top and MEF-specific enhancers are shown on the bottom. Enhancers were defined as regions with H3K27ac enrichment at least 5kb from the transcriptional start site.

Figure S5. iNSC genes displaying MEF epigenetic enhancer profile

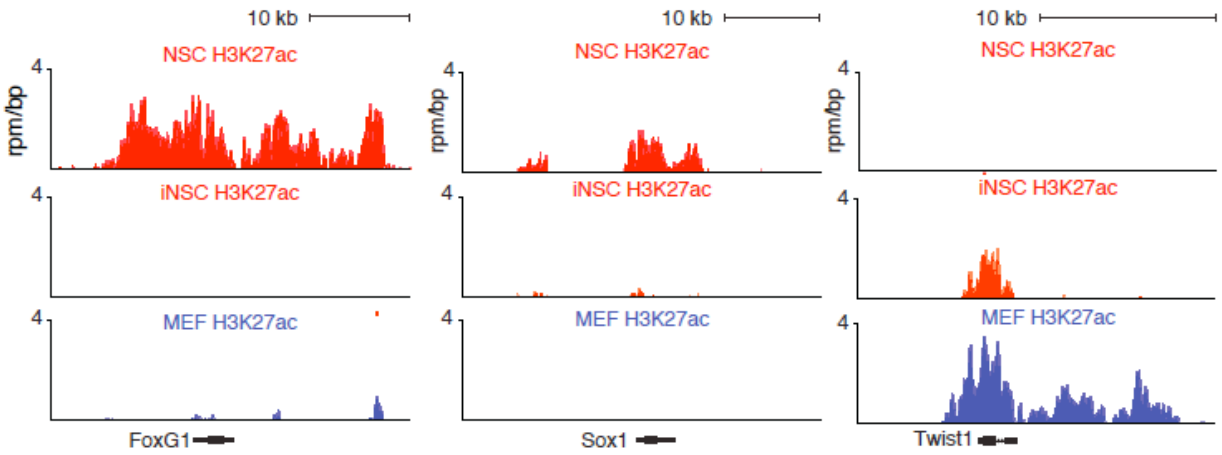

Figure S5. iNSC genes with MEF epigenetic enhancer profile
H3K27ac profiles for 3 gene that display the MEF enhancer marks: FoxG1 (left), Sox1 (middle), and Twist1 (right).

A genetically homogenous system reproducibly converts MEFs to iNSCs

We next wanted to test the reproducibility of these factors for generating iNSCs. To avoid the inherent complications from factor transduction—such as variation in transduction efficiency, heterogenous integration patterns, etc.—we developed a 'secondary' iNSC reprogramming system based on a similar technology for mouse iPS reprogramming because it does not require additional factor transduction (Wernig et al., 2008b). First, we generated iPS cells from iNSCs that were transcriptionally, epigenetically, and functionally similar to primary NSCs (Figure 7A). Since the integrated neural induction factors were dox-inducible, we transduced iPS-reprogramming factors with Moloney retroviral vectors, which are permanently silenced in pluripotent cells (Jahner et al., 1982) and would not interfere with secondary iNSC formation. The iPS cells were pluripotent and formed differentiated teratomas with cells of all three germ layers (Figure 7B). Furthermore, when injected into developing blastocysts, the cells were able to contribute to chimeras, as shown by the patchy expression of the Sox2-GFP reporter in the brain and the spinal cord (Figure 7C).

To test the ability of these cells to reprogram into iNSCs, MEFs were isolated from E14.5 chimeras after removing the neural tissue and selecting with puromycin. These fibroblasts were genetically homogenous and contained the transduced neural factors in the exact stochiometry and proviral integration sites that were sufficient to produce the original ("primary") iNSC line. Upon dox addition, the cells proliferated more rapidly and underwent morphological changes that were not observed in

Figure 7. Genetically homogenous system for reproducible iNSC formation

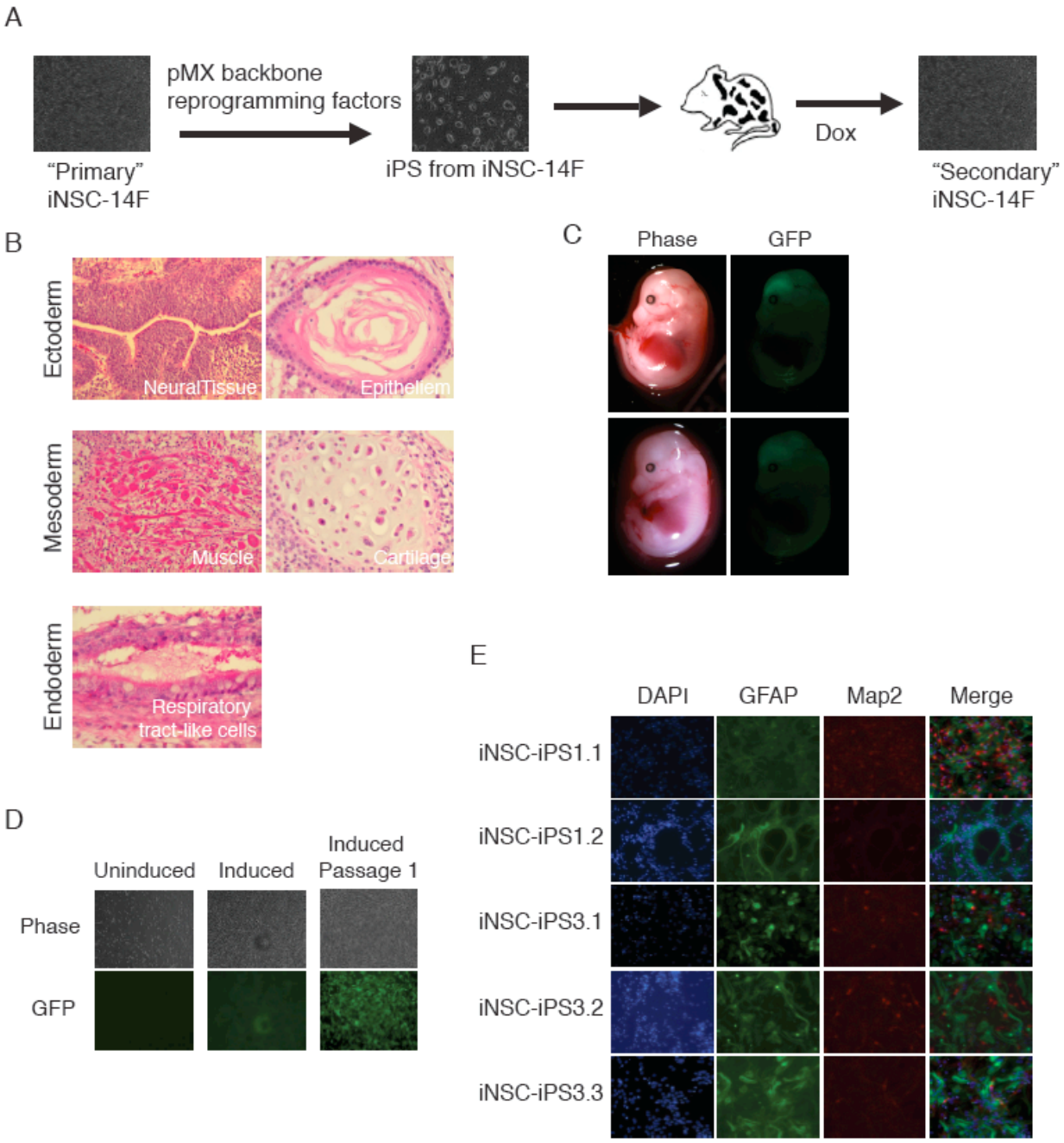

Figure 7. Genetically homogenous system for reproducible iNSC formation

(A) Experiental strategy. iNSC clone iNSC-14F is reprogrammed to pluripotency using retroviral vectors, which are transcriptionally silenced in pluripotent cells. Stable iPS cells are then used to generate a chimera. The cells of this chimera can be isolated and contain the exact proviral pattern as iNSC-14F. The NSC transgenes can then be activated by induction of doxycycline (Dox), thus iNSCs can be reprogrammed without further factor transduction. (B) Teratoma analysis confirming pluripotency of iPS clones. (C) Chimera analysis of E14.5 embryos. The GFP fluorescence comes from the Sox2-GFP reporter of the iNSC-14F cells. (D) iNSC formation in cells induced by doxycycline (dox). Morphology and Sox2-GFP expression in uninduced (left) and induced (middle) secondary MEFs 2 weeks after dox withdrawal. Morphology and GFP expression of induced culture after one passage (right). (E) Multiple secondary iNSC lines were generated from 2 different iPS clones, iPS1 and iPS3. Shown is immunostaining for GFAP and Map2 in 5 independent iNSC lines 6 days after growth factor withdrawal.

uninduced cells. Using the same protocol as before, Sox2-GFP+ "secondary" iNSC lines could be generated, and after one passage these cells had similar morphology, growth properties, and Sox2-GFP expression patterns as embryonic NSCs and the original iNSC line (Figure 7D). The two iPS clones tested were both able to generate multiple independent secondary iNSC lines that differentiated into neurons and glia upon growth factor withdrawal (Figure 7E). Thus, the secondary system showed that 8 transcription factors are sufficient to generate iNSCs from embryonic fibroblasts.

DISCUSSION

Reprogramming somatic cells to pluripotency by overexpressing defined factors is a well-established phenomenon; however, it is unknown whether a non-pluripotent cell type can be induced from somatic cells in a similar manner, or whether a reprogrammed non-pluripotent nucleus can be maintained in the absence of transgenic factor overexpression (Hanna et al., 2010; Jaenisch and Young, 2008). Here we have shown that the forced expression of defined factors can generate neural stem-like cells (iNSCs) from MEF cultures. Importantly, when analyzed in the absence of exogenous factor overexpression, these cells exhibit many features of endogenous NSCs including morphology, growth properties, and capability to differentiate into neurons and glia, as well as genome-wide transcriptional and epigenetic patterns.

The use of dox-inducible lentiviral vectors for factor induction (Brambrink, 2008) allows iNSCs to be maintained in the absence of exogenous factor overexpression.

This is important because naturally occurring cell states are stable, and a cell that has been truly converted would be equally stable and would not require exogenous perturbations to maintain its properties. Furthermore, expressed transgenes may contribute to the reprogrammed cells' phenotypes and mask their inherent properties, thus making it difficult to determine the cells' true identity, or whether the endogenous circuitry is fully activated.

The stable iNSCs generated in this study show a transcriptionally and epigenetically reprogrammed genome. Our genome-wide epigenetic analysis shows that iNSCs maintain NSC-specific active enhancers in the absence of exogenous factor induction. This indicates that they have activated the endogenous regulatory circuitry responsible for maintaining the NSC cell state. To our knowledge, this stringent assessment of cell type-specific epigenetic identity has not been reported for "induced" somatic cells. Furthermore, the activation and stabilization of a somatic cell's endogenous regulatory network have not been conclusively shown prior to these experiments. In the previous studies, the maintenance of the iNSC state was dependent on continuous transgene expression from either constitutive or inducible vectors (Han et al., 2012, Ring et al., 2012; Lujan et al., 2011; Najm et al., 2013; Ying et al., 2013).

Despite being induced by slightly different sets of factors, the dox-independent iNSC lines generated in this study are highly similar to one another. For instance, the cell lines have similar qPCR gene expression patterns, morphology, and bipotent differentiation potentials. This may be due to the highly redundant sets of transcription

factors responsible for each cell line—all cell lines share a core set of 6 transcription factors: Brn2, Hes3, Klf4, Myc, PLAGL1, and Rfx4—that may promote the induction of these characteristics. Alternatively, the defined growth conditions may select for this specific type of NSC.

Although the minimal factors necessary for iNSC formation have not been determined, the secondary system has shown that 8 factors—the 6 core factors plus Hes1 and Notch (NICD)—are efficient in reproducibly inducing iNSC formation. The finding that a core set of 6 transcription factors is present in all iNSC lines suggests that these factors are important for transgene-independent iNSC induction. Most of the core factors are not well-studied in NSCs so their roles during this reprogramming process are obscure, but they may represent key genes for understanding NSC biology. For instance, recently it was shown that forced expression of Brn2 in ES cells functionally recruits Sox2 to certain NSC enhancers (Lodato et al., 2013). Although Sox2 has not been transduced in most iNSC lines, Brn2 may serve a similar recruitment role with one of the other exogenous factors, such as Rfx4, which also binds at active NSC enhancers (Creyghton et al., 2010). Hes3 is a Notch effector gene and has been shown to be important for NSC maintenance (Imayoshi et al., 2010). It remains to be determined if there is a functional difference between Hes1, Hes3, and Hes5 that explains why Hes3 is transduced in all iNSC lines and the other genes are not, since they have previously been shown to be functionally redundant (Imayoshi et al., 2010). PLAGL1 function has not been studied in NSCs, but it was found to have the highest level of differential expression between rosette NSCs and EGF/FGF responsive NSCs

(Elkabetz, et al., 2008). Myc and Klf4 are both expressed in NSCs, but their role in iNSC formation may be a more general reprogramming effect, since they are also important for iPS formation.

The secondary system for iPS cells has provided a wealth of information about the iPS reprogramming process, as well as the molecular players important for reprogramming *in vitro* and *in vivo* (Wernig et al., 2008c; Hanna et al., 2009; Buganim et al., 2012b; Mansour et al., 2012). The secondary reprogramming system for iNSC formation may be a similarly useful tool for studying the NSC-like state. The system will allow for many unresolved issues of iNSC formation to be addressed, such as the efficiency of reprogramming and the duration of factor induction required for full epigenetic reprogramming. It also provides an unlimited supply of different cell types that are genetically competent for iNSC formation that can be used to dissect the molecular determinants of this reprogramming process, screen for genes or chemicals that accelerate this iNSC formation, and find factors that facilitate complete epigenetic reprogramming eliminating epigenetic memory.

The results presented in this study demonstrate that embryonic fibroblasts can be epigenetically reprogrammed into neural stem-like cells. However, whether adult cells can be similarly converted is currently not known. Many adult cell types are more difficult to transduce than MEFs, but they are efficiently reprogrammed to iPS cells using the iPS-secondary system (Hanna et al., 2008; Wernig et al., 2008b). Our iNSC-secondary system provides an optimal tool for studying adult cell conversion. It would be interesting to determine if iNSCs can be induced from adult cells and if forced

expression of factors can epigenetically convert, or “transdifferentiate,” cells derived from other germ layers such as endoderm cells.

EXPERIMENTAL PROCEDURES

Mouse embryonic fibroblast (MEF) isolation

MEFs were isolated from E14.5 embryos that contained two alleles of the M2 reverse tetracycline trans-activator (M2rtTA) in the constitutively active *Rosa26* locus (Rosa26-rtTA +/+) (Beard et al., 2006). The embryos also harbored one allele of Sox2-GFP in which the endogenous *Sox2* gene was replaced by a sequence encoding eGFP (Ellis et al., 2004). After removing the head, vertebral column, and internal organs, MEFs were dissociated in 0.25% trypsin (Sigma) for 10 minutes, split onto two 15-cm plates, and grown in MEF medium [DMEM supplemented with 10% FBS (Hyclone), penicillin and streptomycin (100 μg/ml) (Life Technologies), L-glutamine (2mM) (Life Technologies), and nonessential amino acids (Life Technologies)] until confluent and then frozen. MEFs passaged one to two times were used for transduction experiments. MEFs in the secondary iNSC reprogramming experiments were isolated from E14.5 chimeras using the same protocol as above except that chimeria cells were selected in medium containing puromycin for 4-6 days.

Lentiviral cloning and infections

To create lentiviral factors, genes of interest were amplified by PCR either from cDNA libraries or from cDNA expression vectors using primers flanked by EcoRI restriction sites (MfeI was used instead of EcoRI for genes that contained internal EcoRI restriction sites). The PCR products were cloned into the TOPO-TA cloning vector (Life

Technologies) following the manufacturer's instructions. The genes were then excised from the TOPO-TA vector and ligated into the EcoRI site of the FUW-TetO lentiviral backbone in which transgene expression is controlled by the tetracycline-reponsive operator sequence and a minimal CMV promoter (TetO) (Beard et al., 2006).

Lentivirus was generated in 6-well plates by co-transfecting 293T cells with 2.5 μg of lentiviral vector, 0.625 μg of pMD2.G, and 1.875 μg psPAX2 (packaging vectors from Addgene) using Fugene 6 (Promega) according to the manufacturer's instructions. MEF medium was replaced 16-24 hours after transfection. Viral supernatants were harvested 48 and 72 hours after transfection and filtered through a 0.45 μm filter. MEFs were transduced by adding a 1:1 mixture of viral supernatants and MEF medium to the cells in the presence of 8 μg/ml polybrene (Sigma). After 24 hours, the MEF medium was replaced and on the following day doxycycline (Sigma) was added (2 ug/ml).

Neural cell culture

Primary neural stem cells and established iNSCs were cultured in N2 medium (Okabe et al., 1996) [DMEM/F-12 medium containing insulin (5 μg/ml) (Sigma), transferrin (100 μg/ml) (Sigma), sodium selenite (30 nM) (Sigma), progesterone (20 nM) (Sigma), putrescine (100 nM) (Sigma), and penicillin and streptomycin (100 μg/ml) (Life Technologies)] supplemented with 20 ng/ml epidermal growth factor (EGF) (R & D systems), 20 ng/ml basic fibroblast growth factor (bFGF) (Sigma), and laminin (1 μg/ml) (Life Technologies). Medium was replenished every 24-48 hours, and cells were passaged every 2-3 days. Typically, cells were differentiated by EGF withdrawal for 2

days followed by complete growth factor withdrawal for 4-6 additional days. Directed differentiations were performed by withdrawing EGF and bFGF and supplementing N2 medium with 5 μM forskolin (Stemgent) for neuronal differentiation or with 1% FBS for glial differentiation.

Primary neurosphere and cortical NSC derivation

Neurospheres were isolated from the subventricular zone of E12.5 embryos essentially as described previously (Doetsch et al., 1999). Briefly, subventricular zone tissue was collected in Pipes buffer [20 mM Pipes, 25 mM glucose, 0.12 M NaCl, and 0.5 mM KCl] and digested with activated papain (Sigma). After 1 hr at 37°, cells were triturated, centrifuged, and resuspended in the presence of ovomucoid (Sigma) and DNase (Qiagen). Cells were purified by Percoll gradient centrifugation and plated on non-adherent plastic dishes in N2 medium supplemented with 20 ng/ml EGF and 20 ng/ml bFGF.

For Cortical NSCs, the forebrain cortex of E12.5 embryos was collected in Hank's buffered saline solution (HBSS). Tissue was dissociated by tritruation and then incubated in HBSS for 10 minutes at room temperature. Cells were collected by centrifugation and plated in N2 containing EGF (20 ng/ml), FGF (20 ng/ml), and laminin (1 ug/ml).

iNSC reprogramming

For the generation of iNSCs, transduced MEFs were grown for 4 days in MEF

medium supplemented with 2 μg/ml doxycycline (Sigma) before being switched to neural induction medium [N2 medium plus 10ng/ml EGF, 10ng/ml FGF (Sigma), 1 μg/ml laminin, and 2 μg/ml doxycycline]. The cells were grown in neural induction medium for 2-3 weeks before the addition of neural selection medium, which is also called ITSFn [DMEM/F-12 medium containing insulin (25 μg/ml), transferrin (50 μg/ml), sodium selenite (30 nM), fibronectin (5 μg/ml) (Sigma), and penicillin and streptomycin (100 μg/ml)] (Okabe et al., 1996). The selection medium was supplemented with doxycycline (2 μg/ml) for the first 4 days, and on day 10 the cultures were dissociated by incubating them with 0.25% trypsin (Sigma) for 5-10 minutes. The trypsin was quenched with 10% serum (Hyclone), and the cells were collected by centrifugation and washed once with DMEM/F-12. The cells were then replated onto plates coated with polyornithine (15 μg/ml) (Sigma) for 24 hours and then laminin (1 μg/ml) for an additional 24 hours and then grown in neural expansion medium [N2 supplemented with 20 ng/ml EGF, 20 ng/ml bFGF, and 1 μg/ml laminin]. Medium was replenished every other day for 2-3 weeks until NSC-like foci became visible, after which cells were fed daily to promote the growth of NSC-like cells. Sox2-GFP+ cells were sorted directly into polyornithine and laminin coated plates using the BD FACSAria IIU cell sorter (BD Biosciences).

RT-PCR

RNA was purified using the Trizol reagent (Life Technologies) according to the manufacturer's instructions, treated with DNase (Qiagen), and reverse transcribed using Superscript III (Life Technologies). Quantitative PCR was performed using SYBR-

Green master mix (Life Technologies) and analyzed on the ABI 7900 machine. Non-quantitative and quantitative PCR primer sequences are listed in Tables S3 and S4).

Immunostaining

For NSC and iNSC immunostaining, cells were washed twice with HBS, fixed in 4% paraformaldehyde for 20 minutes at room temperature, and then washed twice in PBS containing magnesium and calcium ions (PBS+). Cells were blocked by incubating them in PBS+ containing 5% normal donkey serum (Jackson Immunoresearch) and 0.3% Triton X-100 (Sigma) for 60 minutes at room temperature. Antibodies were diluted in a solution of PBS+ with 1% BSA (Sigma) and 0.3% Triton X-100. Primary antibodies were incubated overnight at 4°, and secondary antibodies were incubated for 60 minutes at room temperature in the dark. Cells were washed 3 times with PBS+ after the primary antibody incubation and twice after secondary antibody incubation. For staining nuclei, cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) for 5-10 minutes and subsequently washed with PBS+. The following antibodies were used: mouse anti-Nestin (Developmental Studies Hybridoma Bank (DSHB), 1:500), rabbit anti-Sox1 (Cell Signaling, 1:500), goat anti-Sox2 (R&D systems, 1:500), goat anti-Doublecortin C-18 (Santa Cruz, 1:500), rabbit anti-GFAP (DAKO, 1:1000), and mouse anti-Tuj1 (Covance, 1:1000).

Transplantation assay

Transplantation assays were performed essentially as described (Wernig et al., 2008c; Brustle et al., 1997). Briefly, iNSCs were dissociated in 0.25% trypsin (Sigma) for 3 minutes and quenched with 10% serum (Hyclone) in DMEMF-12. Cells were collected and washed twice with DMEM/F-12. Cells were resuspended at a concentration of 100,000 cells/μl in N2 medium. 1 μl of cells was injected into the lateral ventricle of E14.5 stage Balb/c embryos. Cell integration was measured one month after birth by performing a 4% paraformaldhyde perfusion followed by overnight postfix in preparation for floating section analysis. Sections with iNSC-specific FUW-tdTomato expression were analyzed with the following antibodies: goat anti-Doublecortin C-18 (Santa Cruz, 1:500), mouse anti-NeuN (Millipore, 1:100), and rabbit anti-GFAP (DAKO, 1:1000).

iPS reprogramming

The pMXs Moloney viruses Oct4, Klf4, Myc, Nanog, and Sall4 were purchased from Addgene (Takahashi and Yamanaka, 2006). pMXs-Esrrb was generated by digesting TetO-Esrrb (Buganim et al., 2012) with EcoRI and then ligating it into the EcoRI site of pMXs. To reprogram iNSCs to pluripotentcy, equal amounts of pCLeco and either Oct4, Klf4, Myc, Nanog, Sall4, or Esrrb lentiviral plasmids were co-transfected into 293T cells using the Fugene 6 transfection reagent (Promega). One day after transfection, the 293T culture medium was exchanged for N2 supplemented with 20 ng/ml EGF, 20 ng/ml bFGF, 1 μg/ml laminin, and 5 μg/ml fibronectin. Viral supernatants were collected 48 and 72 hours after transfection and pooled for infection.

The infected cells were grown in NSC medium for 4 days and then grown in ES medium [DMEM supplemented with 10% FBS (Hyclone), penicillin and streptomycin (100 μg/ml) (Life Technologies), L-glutamine (2mM) (Life Technologies), nonessential amino acids (Life Technologies), 0.1 mM β-mercaptoethanol, and leukemia inhibitory factor (Lif)]. When pre-iPS colonies began to emerge, the culture was switched to serum-free 2i/Lif medium [1:1 mixture of DMEM/F-12 (Life Technologies) and Neurobasal (Life Technologies) base mediums plus N2 supplement (Life Technologies), B27 supplement (Life Technologies), recombinant human LIF, 2 mM L-glutamine (Life Technologies), 1% nonessential amino acids (Life Technologies), 0.1 mM β-mercaptoethanol (Sigma), penicillin and streptomycin (100 μg/ml) (Life Technologies), 5 μg/mL BSA (Sigma), 1 μM PD0325901 (Stemgent), and 3 μM CHIR99021 (Stemgent)] (Hanna et al., 2010). iPS colonies were manually picked and after 1 passage in 2i/lif medium, they were cultured in ES medium.

Teratomas and blastocyst injections

For teratoma analysis, cells were dissociated in 0.25% trypsin and then collected in ES medium. 5 x 10^5 cells were injected subcutaneously into both flanks of recipient immunocomprimised SCID mice (Brambrink et al., 2008). Tumors approximately 1 cm in diameter were harvested 3-4 weeks after injection for paraffin sectioning and stained with hematoxylin and eosin. Blastocyst injections were performed as described (Wernig et al., 2008b) except that E14.5 embryos were extracted from pregnant females 12 days after blastocyst injection for the isolation of MEFs.

[Computational Methods Provided by ZFP]

Identifying ChIP-Seq enriched regions

The MACS version 1.4.1 (Model-based analysis of ChIP-Seq) (Zhang et al., 2008) peak finding algorithm was used to identify regions of ChIP-Seq enrichment over background. A p-value threshold of enrichment of 1 X 10^{-9} was used for all data sets.

Defining active enhancers

Active enhancers were defined as regions of enrichment for H3K27ac outside of promoters (greater than 5 kb away from any transcriptional start site (TSS)). H3K27ac is a histone modification associated with active enhancers (Creyghton et al., 2010; Rada-Iglesias et al., 2011).

ChIP-Seq density heatmaps and composite ChIP-Seq density profiles

In order to display ChIP-Seq levels at enhancers, a heatmap representation was used. In the heatmap representation, each row represented the ±5 kb centered on the center of H3K27ac enriched region. Each 50 bp bin in each row was shaded based on intensity of ChIP-Seq occupancy using Java Treeview (http://jtreeview.sourceforge.net/).

Microarray analysis

All expression profiles including the previous published expression datasets were processed together to generate Affymetrix MAS5-normalized probe set values. We

processed all CEL files by using the probe definition ("mouse4302 cdf") and the standard MAS5 normalization technique within the affy package in R to get probe set expression values.

The expression profiles were compared and clustered by hierarchical clustering using average linkage. The distance matrix was calculated using Pearson correlation coefficients of the top 50 percent of probe sets with the largest coefficients of variation across expression profiles.

The differentially expressed probe sets between the published datasets of mouse embryonic fibroblasts (MEFs) and mouse neural precursor cells (mNPCs) were determined using a linear model within the limma package in R. The empirical Bayes approach was used to estimate variances. The differentially expressed probe sets were required to have absolute value of log2-fold change greater than 2 and FDR-adjusted p-value less than 0.01.

Previously published gene expression datasets

Two previously published datasets using the Affymetrix Mouse Genome 430 2.0 Array (platform ID GPL1261) microarray platform were obtained from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) database (Supplementary Table 5). For Mikkelsen et al. (2007), data were obtained from the GEO database accession GSE8024. For Cahoy et al. (2008), data were obtained from the GEO database accession GSE15148.

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Supplementary Table 1. Defined factors screened for iNSC formation in MEFs

Gene Name	Species	Genbank Accession
Ascl1	Mouse	NM_008553
Bmi1	Mouse	NM_007552
Brn2	Mouse	NM_008899
DN-REST (P73 REST)	Human	NM_005612 (REST)
Hes1	Mouse	NM_008235
Hes3	Mouse	NM_008237
Hes5	Mouse	NM_010419
Klf4	Mouse	NM_010637
Lhx2	Mouse	NM_010710
MEF2CA	Human	NM_002397 (MEF2C)
mir124a	Human	NR_029668
c-Myc	Mouse	NM_010849
Myt1l	Mouse	NM_001093775
Ngn2	Mouse	NM_009718
NOTCH- intracellular domain (ICD)	Human	NM_017617 (NOTCH1)
Olig1	Mouse	NM_016968
Olig2	Mouse	NM_016967
PLAGL1	Human	NM_002656
PLZF	Human	NM_006006
Rfx4	Mouse	NM_001024918
Sox1	Mouse	NM_009233
Sox2	Mouse	NM_011443
SOX3	Human	NM_005634
Sox9	Mouse	NM_011448
Zic1	Mouse	NM_009573

Supplementary Table 2. Primers for PCR genotyping factor integrations

Gene Name	Forward Primer	Reverse Primer
Ascl1	ATCCACGCTGTTTTGACCTC	GTTTGCAGCGCATCAGTTC
Bmi1	GTGTACGGTGGGAGGCCTAT	TGCAACTTCTCCTCGGTCTT
Brn2	GTGTACGGTGGGAGGCCTAT	CACCCTGCTGTACCACCAC
DN-REST	GAATCTGGCTCTTCCACTGC	GAGGTTTAGGCCCATTGTGA
Hes1	ATCCACGCTGTTTTGACCTC	GTCACCTCGTTCATGCACTC
Hes3	GTGTACGGTGGGAGGCCTAT	AGGCAAGGGTTGAGAACAGA
Hes5	GTGTACGGTGGGAGGCCTAT	CAGGAGTAGCCCTCGCTGTA
Klf4	GTGTACGGTGGGAGGCCTAT	CTAGGTCCAGGAGGTCGTTG
Lhx2	GCGAATACCCAGCACACTTT	TAAAAGGTTGCGCCTGAACT
MEF2CA	CTTATGAGCTGAGCGTGCTG	GTGAGCCAGTGGCAATAGGT
mir124a	ATCCACGCTGTTTTGACCTC	AATCAAGGTCCGCTGTGAAC
c-Myc	GTGTACGGTGGGAGGCCTAT	ACCGCAACATAGGATGGAGA
Myt1l	GTGTACGGTGGGAGGCCTAT	CCCCTTGCTCATCATTGTCT
Ngn2	ATCCACGCTGTTTTGACCTC	GTCTTCTTGATGCGCTGCAC
NOTCH ICD	CATGGACGACAACCAGAATG	CATGTTGTCCTGGATGTTGG
Olig1	ATCCACGCTGTTTTGACCTC	GTGGCAATCTTGGAGAGCTT
Olig2	ATCCACGCTGTTTTGACCTC	GGGCTCAGTCATCTGCTTCT
PLAGL1	CAAGTGTGTGCAGCCTGACT	ATCTCTGGGCACAGAACTGG
PLZF	GTGTACGGTGGGAGGCCTAT	CTGGATGGTCTCCAGCATCT
Rfx4	GTTACTGGAGGAACCCGACA	GAATATGCCACCGTCTGCTT
Sox1	GTGTACGGTGGGAGGCCTAT	GTCCTTCTTGAGCAGCGTCT
Sox2	GTGTACGGTGGGAGGCCTAT	CTCCGGGAAGCGTGTACTTA
SOX3	GTGTACGGTGGGAGGCCTAT	CTGCGTTCGCACTACTCTTG
Sox9	AGGAAGCTGGCAGACCAGTA	CCCTCTCGCTTCAGATCAAC
Zic1	TCTGCTTCTGGGAGGAGTGT	CTGTTGTGGGAGACACGATG

Supplementary Table 3. Primers used for RT-PCR

Gene	Forward Primer	Reverse Primer	Size
Gapdh	AGGCCGGTGCTGAGTATGTC	TGCCTGCTTCACCACCTTCT	297
Sox1	AGGGCCCAAGAGTAAGGAAA	TGGGATAAGACCTGGGTGAG	443
Sox3	CGTAACTGTCGGGGTTTTGT	CACGCACACCTGGCTATAAA	371
Pax6	GGTCACAGCGGAGTGAATCAGC	AGCCAGGTTGCGAAGAACTCTG	267
Blbp	GAAGGTGGCAAAGTGGTGAT	GGGACTCCAGGAAACCAAGT	307
Brn1	TCTATGGCAACGTGTTCTCG	CGTCATGCGTTTTTCCTTTT	347

Supplementary Table 4. Primers used for qPCR

Gene	Forward Primer	Reverse Primer
Col5a2	TAGAGGAAGAAAGGGACAAAAAGG	GTTACAACAGGCACTAATCCTGGTT
Thy1	CGAATCCCATGAGCTCCAAT	CCAGCTTGTCTCTATACACACTGATA
Sox1	TGTAATCCGGGTGTTCCTTC	AACCCCAAGATGCACAACTC
Nestin	GCGGTGCGTGACTACCAG	CAGCTGCTGCACCTCTAAGC
Brn1	TCAACAGCCACGACCCTC	GGTGAAACCCAGCTTGATGC
Sox2	ACTTTTGTCCGAGACCGAGA	CTCCGGGAAGCGTGTACTTA
Pax6	GAAGGAGGGGGAGAGAACAC	CTCCAGAGCCTCAATCTGCT

Supplementary Table 5. Previously published gene expression datasets used for this study

Cell Type	GEO ID	Name	Reference
Neural precursor cells	GSM198065	ES derived NPC Replicate 1	Mikkelsen et al., 2007
Neural precursor cells	GSM198066	ES derived NPC Replicate 2	Mikkelsen et al., 2007
Neural precursor cells	GSM198067	ES derived NPC Replicate 3	Mikkelsen et al., 2007
Mouse Embryonic Stem Cells	GSM198062	Mouse Embryonic Stem Cells Replicate 1	Mikkelsen et al., 2007
Mouse Embryonic Stem Cells	GSM198063	Mouse Embryonic Stem Cells Replicate 2	Mikkelsen et al., 2007
Mouse Embryonic Stem Cells	GSM198064	Mouse Embryonic Stem Cells Replicate 3	Mikkelsen et al., 2007
Mouse embryonic fibroblasts	GSM198070	Mouse embryonic fibroblasts Replicate 1	Mikkelsen et al., 2007
Mouse embryonic fibroblasts	GSM198072	Mouse embryonic fibroblasts Replicate 2	Mikkelsen et al., 2007
Oligodendrocytes	GSM241889	Oligodendrocytes	Cahoy et al., 2008
Oligodendrocytes	GSM241891	Oligodendrocytes	Cahoy et al., 2008
Oligodendrocytes	GSM241892	Oligodendrocytes	Cahoy et al., 2008
Oligodendrocytes	GSM241917	Oligodendrocytes	Cahoy et al., 2008
Astrocytes	GSM241912	Astrocytes	Cahoy et al., 2008
Astrocytes	GSM241914	Astrocytes	Cahoy et al., 2008
Astrocytes	GSM241926	Astrocytes	Cahoy et al., 2008
Neurons	GSM241904	Neurons	Cahoy et al., 2008
Neurons	GSM241896	Neurons	Cahoy et al., 2008

Chapter 3

Conclusions and Perspectives

Conclusions

The discovery that any cell can be reprogrammed to pluripotency revolutionized the way we thought about development: it went from being a rigidly firm, unidirectional process to one that can be reversed to pluripotency through the concerted action of 3 or 4 transcription factors. However, pluripotency may represent a special cellular state that is uniquely capable of being captured *in vitro* because it has a transcriptional circuitry that induces and regulates itself, as well as chromatin regulators that are not expressed in somatic cells. The work presented in this thesis has shown that it is possible to generate stable epigenetically reprogrammed somatic cells using transcription factors.

As mentioned in the introduction, previous studies on the induction of neural stem-like cells using transcription factors were inconclusive in demonstrating that the resulting cell was truly converted. First, in those studies transgene expression was not turned off, so it is not possible to conclude that the maintenance of the iNSC state was independent of the continuous expression of inducing factors. Second, minimal or no epigenetic analysis was performed to indicate that the epigenome was reprogrammed to the patterns of the endogenous cell. Third, iNSC formation in these studies was rare and not quantitated, making it difficult to assess reprogramming efficiency.

We have demonstrated here that it is indeed possible to generate a stably reprogrammed, transgene-independent NSC epigenetic state. This was accomplished using the dox-inducible transgene expression and then maintaining and characterizing the cells after dox withdrawal. Our finding that many Sox2-GFP+ cells are induced in the presence of dox, but few—if any—maintain their GFP expression after dox removal

highlights the importance of cell analysis without transgene induction because the exogenous factors can have a dramatic effect on the cell's properties.

We performed genome-wide H3K27ac profiling and found that the enhancer usage in iNSCs is nearly identical to that of NSCs and distinct from MEFs. This suggests that iNSCs have an epigenetically reprogrammed nucleus. However, the extent of reprogramming is unclear, since a few genes maintained the pattern characteristic of MEFs. It would be interesting to compare these genes to determine if they are regulated by a common factor that is necessary for complete epigenetic reprogramming.

We have shown that iNSC formation is reproducible by generating a 'secondary' iNSC reprogramming system that allows for iNSC formation without the need for additional viral transduction. Both of the iPS clones used to make fibroblasts were able to generate multiple independent secondary iNSC lines.

The establishment of dox-independent iNSC clones was greatly aided by two factors: 1) the selection medium and 2) the proliferative nature of the NSCs. The medium used for selecting iNSCs is also used during *in vitro*-derivation of NSC from ES cells and was empirically found to promote the preferential growth of NSCs (Okabe et al., 1996). Similar to iPS reprogramming, when dox is withdrawn from the reprogramming cells, much of the culture dies and only a few clones survive. Since iNSCs proliferate, the rare clones with proper neural properties can grow out and be isolated either by manual picking or FACS. The dox withdrawal and selection medium may act cooperatively to ensure that only fully reprogrammed iNSCs survive.

Unresolved issues

One unresolved issue from this study is the minimum group of factors necessary to generate stable iNSCs. The identity of these factors may hint at the critical regulators of NSC biology, since these factors are able to induce and stabilize the NSC phenotype. It is interesting, however, that 3 Notch family members (Notch-ICD, Hes1, and Hes3) are among the 9 factors found in the iNSCs (iNSC-13F and iNSC-14F). This supports the finding that Notch signaling is important for NSC maintenance (Kageyama et al., 2009; Imayoshi et al., 2010). The reproducibility of reprogramming using the secondary system will allow for optimizing growth conditions for efficient iNSC formation, which could aid the screening of factors necessary for generating iNSCs.

A second unresolved issue is whether iNSCs are *de novo* induced rather than selected from a pre-existing cell such as a neural crest-derived cell present in the starting population. Since cells are cultured long-term for iNSC reprogramming, a rare somatic cell—like a somatic progenitor cell—could be selected by the culture conditions and be scored as a "reprogrammed" cell. Generating iNSCs from an adult starting population would resolve these issues. The most stringent criteria for reprogramming would be a genetic marker such as the rearrangement of the TCR or Ig locus in T- or B-cells.

A third unresolved issue is whether iNSC formation transiently passes through a pluripotent state during the numerous cell divisions required for the process. This is a concern because the ITSFn medium used for iNSC selection is also used to differentiate ES cells to NSCs *in vitro*. However, no ES-specific factors are used for iNSC

reprogramming, so it is unclear how pluripotency would be achieved. One way to determine if iNSC formation passes through pluripotency is to make iNSCs in a genetic background that includes both a Cre driven by the endogenous Oct4 promoter and a lox-stop-lox GFP allele. If the endogenous Oct4 gene is activated, Cre will genetically mark the cells and GFP will be expressed.

A fourth unresolved issue is the extent of transgene independence. The dox-inducible (Tet-On) system used for inducing exogenous factor expression is known to allow for low levels of residual transgene expression, which may alter the cells' molecular characteristics (Soldner et al., 2009). However, since the residual expression does not interfere with the differentiation of iNSCs or their reprogramming to pluripotency, it is unlikely that the basal expression of the neural factors is enough to maintain NSC characteristics. One way to stringently demonstrate transgene-independence is to create iNSCs using excisable vectors, like lentiviruses with LoxP sites in the LTRs. Then the exogenous factors can be physically removed from the genome after Cre-mediated recombination, and the molecular features of the iNSCs can be assessed in the absence of any viral reprogramming factors.

Outlook: towards transdifferentiation

In the most strict definition, transdifferentiation is the conversion of one somatic cell state directly into a distinctly different somatic cell state without passing through a pluripotent intermediate (Wagers and Weissman, 2004; Graf and Enver, 2009; Hanna et al., 2010). Although iNSCs have been generated from MEFs, this study does not fulfill

these criteria for two reasons. First, the starting cell is a heterogenous mix of embryonic cells. Although the CNS tissue is removed, there is no way to retrospectively determine the origin of the starting cell. Second, we cannot rule out the possibility that the conversion event may have gone through a pluripotent intermediate. Thus, using the secondary system described in this thesis, the most stringent criteria for transdifferentiation would include:

1. Excluding a pluripotent intermediate step by deleting Oct4 in the somatic cell. The secondary cells come from a background with both alleles of Oct4 floxed (*Pou5f1*$^{flox/flox}$), such that the endogenous Oct4 allele is knocked out upon Cre administration and therefore pluripotency cannot be achieved (Nichols et al., 1998).

2. The use of a genetic marker to retrospectively identify the donor cell. This could be accomplished by reprogramming B- or T-cells carrying VDJ or TCR rearrangements, respectively. Alternatively, the iPS cells could be targeted with a cell type-specific Cre, like Albumin-Cre (marking hepatocytes) or Mx1-Cre (marking blood cells), which would genetically alter the Oct4 alleles specifically in the cell types that express the gene.

In summary, factor-mediated reprogramming demonstrates that forced expression of transcription factors can induce pluripotency on a differentiated cell. The work in this thesis shows that the effect of transcription factors is not simply restricted to inducing pluripotency, but can also induce and stabilize somatic cell types. Furthermore, the tools and techniques presented here may be applicable to future investigations aiming to accomplish cross-lineage transdifferentiation.

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