Direct Reprogramming of Mouse Embryonic Fibroblasts to Oligodendrocyte Progenitor
Cells Using Various Transcription Factors

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INTRODUCTION

Spinal Cord Injury

The field of stem cell research has created an opportunity to use cell replacement therapies for a number of diseases and injuries. One example is spinal cord injury. Spinal cord injury is a prevalent medical issue that has few treatment options. The Centers for Disease Control and Prevention predict that about 200,000 people in the United States are living with spinal cord injury (SCI). Annually, 12,000-20,000 people become new patients. Most of these cases occur in young adults (18-35 years of age) who are mostly male. Of all SCIs, 46% are due to motor vehicle accidents. Other causes of SCI include acts of violence (i.e. gunshot wounds), sports injuries, and falls. SCI results in neurologic impairments in the central nervous system which includes paraplegia, tetraplegia, and respiratory, urinary, and gastrointestinal problems. Psychological side effects also occur including lifetime recurrence of depression and anxiety. For patients of SCI, the average medical cost annually is $15,000-$30,000 [49].

Two types of SCI exist based on the severity of the injury. The severity is determined clinically by "completeness" based on neurological examination [43]. According to the Sacral Sparring definition, patients with a complete SCI have a total loss of motor and sensory function below the site of injury, or in the sacral segments [43]. Incomplete SCI includes some ability to convey messages to the brain as they retain minimal motor and sensory function below the site of injury in the sacral segments [43]. Both types are life-altering and lead to a life of treatment and rehabilitation.

In cases of spinal cord injury, there are two stages: the initial injury (e.g. crushed vertebrae from a car accident), and a secondary injury which involves an excessive release of neurotransmitters and cytokines to the injury site. During secondary injury other mechanisms occur that worsen the functional impairment of the spinal cord including shifts in electrolytes, lipid peroxidation, accumulation of excitatory amino acids, and an overabundance of free radicals [54]. There are a number of vascular changes that occur including loss of auto regulation, neurogenic shock, hemorrhage, and reduction of blood flow. Electrolyte changes occur including increased intracellular calcium, extracellular potassium, and sodium permeability. Other changes are biochemical, like increases in neurotransmitter accumulation, free-radical production, and lipid peroxidation that lead to neuronal death [46].

Oligodendrocytes

Oligodendrocytes are a glial cell lineage in the central nervous system that generate myelin membranes rich in lipids. Oligodendrocytes arise from oligodendrocyte progenitor cells (OPCs). PDGFR alpha positive cells are believed to be the source of OPCs in the spinal cord. Previous experiments have shown that PDGFR alpha positive cells in the optic nerve and spinal cord co-label with OPC markers such as NG2 or Olig1/2. PDGFR alpha positive cells all differentiated into oligodendrocytes when cultured in defined medium [13]. During mid-neurogenesis OPCs arise from neuroepithelial precursor cells [50]. This occurs in mice around E15 and mature into adult oligodendrocytes by E20/P1 [52] (Figure1). OPCs disperse from the ventral to the dorsal section of the spinal cord around E12.5 in the mouse in response to sonic hedgehog signaling from the notochord and the floor plate and are dispersed throughout the cord around E14.5 (Figure 2) [41] [36][38]. Once cells reach the white matter, they become multi-processed and may differentiate into immature oligodendrocytes [29]. After the initial migration, dorsal OPCs arise independent of sonic hedgehog signaling [36]. OPCSs are able to migrate several millimeters during development into the parenchyma where they differentiate into mature oligodendrocytes that can myelinate numerous axons [36].
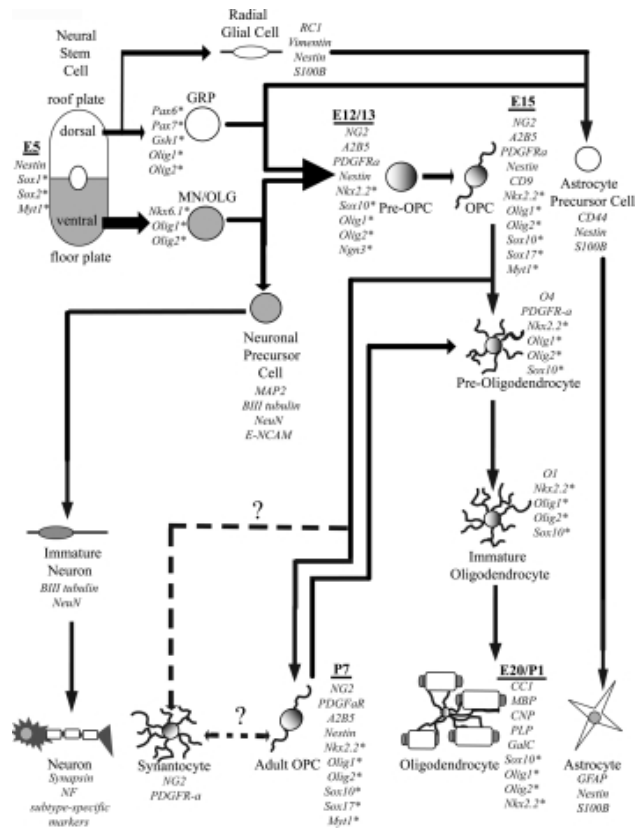
Figure 1. Oligodendrocyte Differentiation with markers for each stage.

Oligodendrocytes arise from neural stem cells in the ventral part of the floor plate and mature into oligodendrocytes after migration into white matter [52].
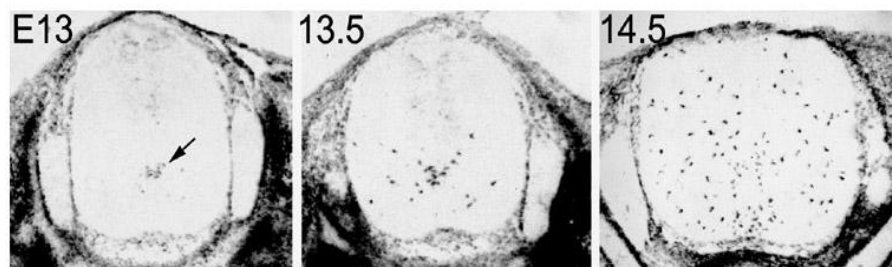
Figure 2. Oligodendrocyte progenitor cells in the mouse spinal cord.

PDGFRα+ oligodendrocyte progenitor cells appear in the ventral VZ on E13 (arrow), then they proliferate and migrate away from the midline. Within a day-and-a-half (E14.5) they spread through most of the cord [39].

Oligodendrocytes role in spinal cord injury

      Neurons and oligodendrocytes are susceptible to the secondary injury of SCI. Previous studies in rats and monkeys have shown a pattern of expression of apoptosis after SCI shows secondary cellular degeneration of oligodendrocytes within the fiber tracts in response to the degeneration of damaged axons at the injury site [5]. During secondary injury, most oligodendrocytes are lost from the epicenter of the injury to surrounding tissue, due to apoptosis and necrosis [22]. Loss of oligodendrocytes is important because they function as myelin-forming cells that provide a myelin sheath for axonal conductivity. With damaged axonal conductivity, propagation of axon potentials is blocked and the recovery of neural function following SCI is limited [54]. After injury, endogenous oligodendrocyte progenitor cells migrate under the control of Sonic Hedgehog protein and BMP4 [28]. Oligodendrocyte progenitor cells respond to injury with proliferation, migration, and eventual differentiation into myelinating mature oligodendrocytes [8]. Unfortunately, the spontaneous remyelination that occurs after migration is not sufficient for effective repair [1].

      Current treatments for SCI include surgery to relieve compression and remove debris, medications for pain control, and long-term rehabilitation to strengthen muscle. There is no current treatment that addresses the issue of demyelination, one reason for loss of neurological function. Some neural function after SCI only requires about 5-10% myelinated axons at the lesion site [54]. Therefore, experimental procedures that may increase myelination, providing some improved neurological function, are at a high demand. Cell therapy in combination with immediate injury treatment may be the most beneficial therapy for spinal cord injury by reducing further damage and replacing important cell types in the spinal cord.

Experimental Treatment of Spinal Cord Injury

      There are two main experimental options to overcome the problem of demylinated axons. The first involves preventing axonal demyelination with the addition of neurotrophic factors such as BDNF or LIF. Koda et al. (2002) suggest that BDNF suppresses delayed apoptosis of oligodendrocytes after SCI if injected immediately or three days after injury, as seen in a reduced number of TUNEL-positive cells in a rat model [21]. Previous studies have shown that LIF can support oligodendrocyte survival by promoting differentiation and survival of oligodendrocyte progenitor cells. In rat models LIF-mediated reduction of oligodendrocyte apoptosis decreased the amount of axonal demyelination [7]. Another study in 2004 showed that injection of

minocycline, a form of tetracycline, reduced the numbers of apoptotic oligodendrocytes in nerve fiber tracts near the site of injury, suggesting minocycline has a neuroprotective effect [44]. Unfortunately, if not treated immediately after spinal cord injury, prevention of axonal demyelination is not sufficient to have a significant effect.

The second experimental approach involves enhancing axonal remyelination through cell transplantation. Originally, Schwann cells seemed to be a potential source for therapy because they can be autologous to the patient and showed improvement in thoracic SCI models. Schwann cells are myelin-forming cells in the peripheral nervous system and participate in remyelination; however, they do not have efficient integration into the central nervous system due to astrocyte inhibition [12]. Schwann cells may also differentiate into two other cell types that are non-myelinating [18].

Another source of transplantable cells is neural precursors which are multipotent stem cells that may differentiate into the three neural cell types: neurons, astrocytes, and oligodendrocytes. Work by Mothe and fellow researchers found that neural stem/progenitor cells that were transplanted as neurospheres, produced cells that primarily expressed an oligodendrocytic phenotype. 73% of grafted cells differentiated into oligodendrocytes when transplanted into the white matter [31]. Bambakidis and Miller (2004) transplanted oligodendrocyte precursor cells into lesion sites of rats with SCI. The rats had significant locomotor improvement and the electrophysiological results were comparable to positive control animals up to 28 days post-transplantation. The cells survived, integrated, and proliferated [3].

Oligodendrocyte progenitor cells from multiple sources

To generate high numbers of OPCs, techniques using embryonic stem cells as well as induced pluripotent stem cells were studied. In 2005, Keirstead and colleagues showed that hESC-derived oligodendrocyte progenitor cells could remyelinate axons and promote improvement of motor function in adult rats with SCI when they were transplanted 7 days post injury. Transplanted cells that were injected 10 months after injury were still able to survive and distribute around the site of injury, however there was no locomotor improvement [16]. From this study, the authors proposed that there is a window of opportunity in treatment.

Initial iPSC studies looked at the potential of iPS-derived oligodendrocytes from mouse cells. A group in 2011 generated mature oligodendrocytes that myelinated axons *in vitro* and *in vivo,* when transplanted into the corpus callosum of a mouse model of demyelination [6]. Also in 2011, a group was able to establish oligodendrocyte progenitor cells from human induced

pluripotent stem cells that came from adult somatic cell lines [33]. However, the differentiation efficiency was low (less than 0.01%) [33]. Another experiment in 2013, also used human IPS cell lines to obtain OPCs identified with Olig2, PDGFRα, NKX2.2, and Sox10. The OPCs were neonatally engrafted and myelinated shiverer mice brains which increased survival compared with untreated controls [51]. Although these are significant findings and hold great potential for therapies, there are a few problems with using hESC or IPSC-derived OPCs. Firstly, there are the ethical concerns surrounding the use of embryonic stem cells. Secondly, both hESC and IPSC have a potential to cause teratomas due to some cells undifferentiating from a pluripotent state. Finally, hESCs are not autologous to the patient. Cell transplantation of allogenic cells may require immune suppression in the patient, causing a number of other complications and unwanted side effects. IPSC methods may take around 10 months to establish autologous OPCs from patient somatic cells; therefore, this strategy would require further refinement.

Direct Reprogramming

An alternative and upcoming strategy to using iPSCs or ESCs is direct reprogramming. Direct reprogramming is defined as the generation of a desired cell type by over expression of lineage-specific transcription factors [14]. Often, the reprogramming involves crossing of the germ layers; for example, reprogramming fibroblasts to neurons [10]. One of the benefits of using direct reprogramming is that it may take less time than IPSC because it skips the stage needed to return cells to an undifferentiated state (Figure 3). Direct reprogramming can be dated back to the 1960's when John Gurdon reprogrammed a nucleus of a differentiated frog cell to a totipotent state when the nucleus was transferred into an enucleated egg. In the 1980's the Weintraub team reprogrammed fibroblasts into skeletal muscle cells using MyoD. Another experiment by Zhou included reprogramming pancreatic exocrine cells to beta cells with three transcription factors: Ngn3, Pdx1, and Mofa. These experiments represent the two types of direct reprogramming: somatic cell-specific factor mediated direct reprogramming, which targets a specific somatic cell using somatic-cell specific transcription factors or pluripotent cell-specific factor mediated, which uses the induced pluripotent stem cell factors introduced by Yamanaka [19].
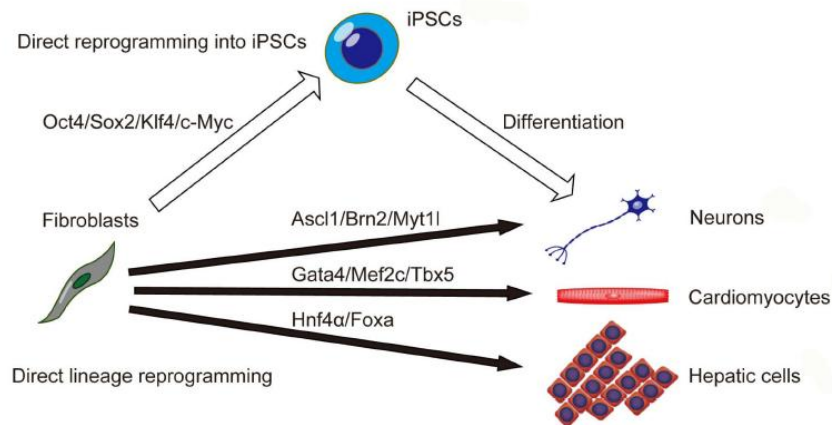
Figure 3. Generation of cell types by two techniques. Desired cell types may be obtained by either direct reprogramming or reprogramming with the iPSC stage. [14]

Somatic cell-specific transcription factor mediated direct reprogramming has been used to develop a variety of cell types (Figure 4). This includes neural stem cells (NSCs) or neural progenitor cells. The Han group reprogrammed mouse fibroblasts to induced neural stem cells using a host of factors including Brn4, Pou3f4, Sox2, Klf4, and C-Myc. Thier also induced neural stem cells with three or four factors that included Sox2, Klf4, C-Myc, and with or without Oct4 [48]. Ring's group established NSCs that were self-renewable, multipotent, and could be differentiated into a neural lineage with just one factor, Sox2 [40]. Treatments for spinal cord injury would benefit most from a more lineage-specific progenitor cell due to the goal being to replace mostly oligodendrocytes. Therefore, the ultimate experiment would produce oligodendrocyte precursor cells that could survive after transplantation, and myelinate axons in the injured area. In 2011, Marius Wernig's group was able to convert mouse fibroblasts to self-renewing tripotent neural precursor cells using three factors out of an original 11 candidate genes. After adding and deleting factors, Wernig suggested that FoxG1, Brn2, and Sox2 were able to induce neural precursor cells that differentiate into neurons and glial cells [25]. FoxG1 and Sox2 were able to produce neurons and some glial cells. With the addition of Brn2, the neurons were immature and astrocytes and oligodendrocyte populations increased [25]. Therefore, Wernig suggested that FoxG1 and Brn2 alone can induce a more pure oligodendrocyte population. Wernig's group followed up this experiment in 2013, where a set of three factors (Sox10, Olig2, and Zfp536) reprogrammed mouse and rat fibroblasts to what they termed induced oligodendrocyte progenitor cells that gave rise to mature oligodendrocytes [56]. Another group

repeated this work using mouse fibroblasts and another set of three different transcription factors: Nkx6.2, Sox10, and Olig2 [32].
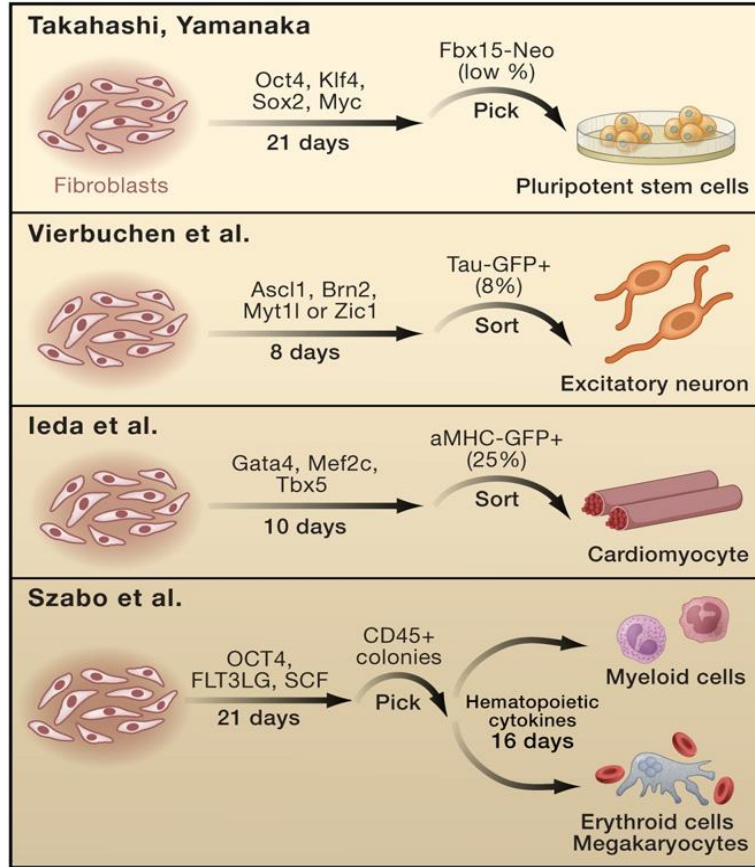
Figure 4. Direct reprogramming of various cell types. Pluripotent and somatic cell specific direct reprogramming can be used to create a variety of cell types across different lineages [4].

Olig2Cre-ER System

As previously mentioned, there are a variety of transcription factors that are used to designate stages of oligodendrocyte development. These include Olig1, Olig2, and Sox10 [18]. Olig2 is a basic helix-loop-helix factor that is induced by sonic hedgehog in the ventral neural tube and is important for motor neuron and oligodendrocyte development [45]. Olig2 starts expressing in the pMN domain of the mouse spinal cord around E8.5 and is expressed in adulthood by oligodendrytic cells. Early Olig2+ cells generate motor neurons, but after E12.5, they only produce glial cells [26]. Therefore, Olig2 is used as a cell marker for identification of

oligodendrocyte lineage cells. Figure 4 shows Olig2 expression in the mouse spinal cord [23]. In 2002, the Olig2 Cre-ER mouse strain was created. Exon 2 of the Olig2 locus was replaced with a tamoxifen-inducible Cre recombinase (Cre-ER^{TM}) (Figure 5). Mutant mice were backcrossed with C57BL/6N mice. We decided to use this system because it provides live fluorescence imaging. When tamoxifen is added and once there is expression of Olig2, the Cre is activated, enters the nucleus, and recombines the targeted vector to express the reporter gene. Reporter gene expression stays on for the duration of the experiment making any positive cells identifiable even if the Olig2 expression in the cell declines or fades.
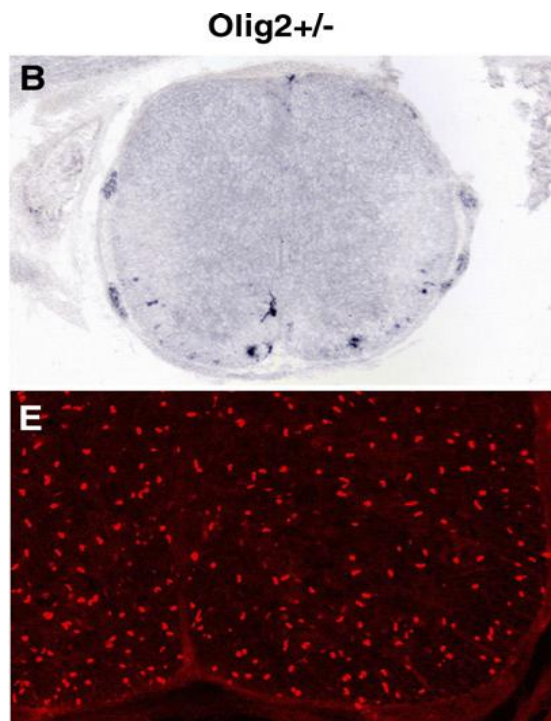

Figure 5. Olig2 expression in mouse spinal cord. E16.5 mouse spinal cords show expression of Olig2 [23].
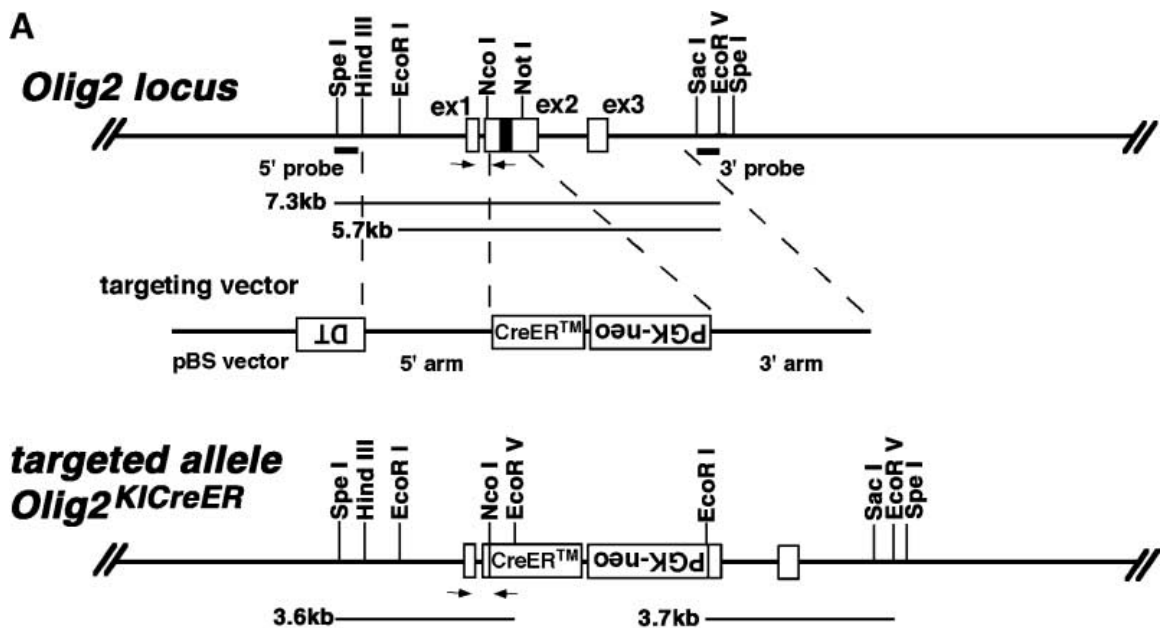
Figure 6. The targeted mutation of the Olig2 locus to create Olig2Cre-ER mice. Exon 2 of the Olig2 Locus was targeted for a mutation to replace with Cre-ER[TM]. [45]

       With previous experimentation considered, the goal of this project was to use the tool of direct reprogramming to efficiently reprogram mouse embryonic fibroblasts using a simple retroviral method into oligodendrocyte progenitor cells using the two factors Wernig's group proposed would result in a more pure population of OPCs: FoxG1 and Brn2. We also attempted to expand the Sox2 protocol first reported by Ring's group by differentiating the neural precursor cells to an oligodendrocyte progenitor lineage. We hypothesize that oligodendrocyte progenitor cells resulting from direct reprogramming would be consistent with the hallmarks of oligodendrocyte progenitor cells (i.e. express OPC-specific genes) and would be able to express myelin basic protein (MBP) *in vitro*. The goal is to have a stable population of oligodendrocyte progenitor cells that will engraft, survive, and myelinate axons in an *in vivo* mouse or rat model.

Methods

Mouse embryonic fibroblasts taken from an Olig2Cre-ER knock-in mouse were used for a direct reprogramming protocol used to produce oligodendrocyte progenitor cells. Different methods of virus production were tested to find the most efficient method. Protocols for reprogramming were taken from previous publications with minor alterations [25][40].Transfection efficiency was assessed along with morphological changes and positive fluorescence of OPC markers for positive identification of any OPCs produced.

Genotyping

First, to establish a colony of transgenic mice that could express Cre upon tamoxifen addition, Olig2Cre-ER knock-in mice from a strain created by Dr. Takebayashi were ordered from Riken. An Olig2Cre-ER female mouse was mated with a BL6 male to establish a colony. Genotyping was performed to verify transgene expression. Tail tips were taken from all offspring. DNA extraction was performed using the Promega Wizard® Genomic DNA Purification Kit protocol. Riken genotyping PCR protocol (Table 1) was used for each DNA sample with three primers: Common sense, Olig2KlCre-Er, and Olig2 (Table 2). Gel electrophoresis was performed to assess a positive or negative expression (Figure 6). All transgene negative offspring were sacrificed while positive mice were kept for breeding with a reporter mouse for fibroblast extraction.

Step	Temp (°C)	Time (sec)	Notes
1	95	900	
2	94	30	
3	60	90	
4	72	90	30 Cycle Repeat
5	72	600	

Table 1. PCR protocol for genotyping of Olig2Cre-ER mice. The PCR protocol was slightly altered changing the temperature of 63 to 60 due to initial inefficient results.

Primer	Sequence	Concentration
Common Sense	TCGAGAGCTTAGATCATCC	10pmol/μL
Olig2^{KICreER}	AGCATTGCTGTCACTTGGT	10pmol/μL
Olig2	CACCGCCGCCCAGTTTGTCC	10pmol/μL

Table 2. Primer sequences and concentration for genotyping PCR. 0.5μL per sample were used.

Figure 7. Resulting gel from PCR genotyping. Double bands designate a transgenic mouse (ex. Lane #4, + control lane #8).

Olig2Cre-ER mice were crossed with a reporter mouse line

To establish a transgenic mouse line that would express GFP in recombinant mice, an Olig2Cre-ER mouse was mated with a reporter mouse. Transgenic mice contained the Olig2Cre-ER locus as well as a homozygous double-fluorescent Cre reporter strain mT/mG (Jackson Laboratory, Stock No. 007576) that identified Cre recombination by the replacement of tdTomato with EGFP expression upon addition of tamoxifen (Figure 7).
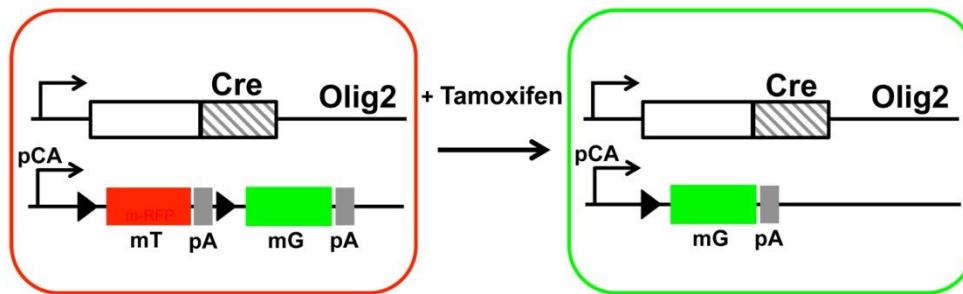
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Figure 8. Olig2Cre-ER Reporter Mouse Transgene Construct. The expression of Cre is identified by the replacement of tdTomato with EGFP expression with the addition of tamoxifen in transgenic mice.[11]

Testing the Olig2Cre-ER transgenic system in endogenous tissues

To test the Olig2Cre-ER system in our mice, a pregnant Olig2Cre-ER transgenic female mouse was sacrificed and E16 embryos were obtained. One embryo was transgenic based on tail-tip genotyping as described above. The brain and section of spinal cord were dissected from the embryo and fixed in formalin. Tissues were then placed in sucrose. The next day, the tissues were washed in PBS and put into cryomold molds in OCT in preparation for sectioning. The brain was cross-sectioned and the spinal cord was sectioned longitudinally.

Fibroblast Extraction

Next, fibroblasts were extracted from a transgenic mouse to establish a source of cells for multiple experiments. A pregnant Olig2Cre-ER female mouse was sacrificed at Day 15 and 10 embryos were removed. Yolk sacs were used for genotyping. Each embryo was dissected, discarding the spinal cord and head region. Limbs were dissected and a scalpel was used to create smaller pieces of tissue. Fibroblasts were grown on gelatin-coated flasks from passage 0 to passage 5 and multiple vials were frozen down in 1mL cryovials once cells were confluent on a T175 flask.

Cell Culture and Maintenance

Mouse embryonic fibroblasts, balbc fibroblasts, and plat-e cells were all maintained in MEF basal media with media changes and passaging when cells came to about 80% confluency. MEF basal media consists of 450 mL HiGlucose DMEM, 50mL FBS, 5mL L-Glutamine, and 5mL Anti-Anti. With every passage, some cells were frozen down in media and freezing media which consists of 90% FBS and 10% DMSO.

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Plasmid Preparation

Before virus production, plasmids for each gene were prepared. The Origene Powerprep HP Plasmid Maxi kit with pre-filters protocol was used to prepare purified DNA plasmids for FoxG1, Brn2, Sox2, and GFP. Each plasmid was obtained by first pelleting cultures 100mL overnight. Cells were then suspended in 20mL suspension buffer containing RNase A. Following suspension, cells were lysed with 10mL cell lysis solution and incubated at room temperature for 5 minutes. 10mL of neutralization buffer was added and mixed. After neutralization, the cell lysate was placed in an equilibrated prefilter/column. After flow stops, flow-through was discarded and the column was washed with 10mL wash buffer. The prefilter was then removed and the column was washed with 50mL wash buffer. The plasmid DNA was then eluted by adding 15mL of low salt elution buffer. DNA was precipitated by adding 10.5mL isopropanol. The solution was mixed and centrifuged at 15,000 x g at 4 degrees Celsius for 30 minutes. The pellet was washed with 5mL of 70% ethanol and centrifuged at the same speed for 5 more minutes. The ethanol wash was pipetted off and was air dried for 10 minutes. The resulting pellet was dissolved in 500µL TE buffer and stored at -20 degrees Celsius.

Retrovirus Production:

For all retrovirus production, the Plat-E cell line, a retrovirus packaging line, was used. Plat-E cells contain an EF1α promoter and yield a stable retroviral structure protein expression of gag, pol, and ecotropic env [30]. Plat-E cells were transfected with transfection reagents and DNA and retroviral supernatant was collected on various days for MEF infection.

X-treme Gene Method

The first retrovirus production method attempted was the X-treme gene method from Roche Applied Sciences. Plat-E cells were thawed, passaged, and 2 million cells were plated on gelatin-coated 10cm dishes. 9µg of plasmid DNA was added to 27µL of X-treme Gene transfection reagent in 300µL serum-free DMEM. The solution was added drop wise to each 10cm dish. P2 Olig2Cre-ER fibroblasts were plated onto 2 gelatin-coated T-25 flasks in MEF media. On day 1, the supernatant was collected and filtered. MEF media was replaced on each 10cm dish. On day 2, the secondary virus was collected and filtered. 4mL of the primary collection was added to 4mL of the secondary collection and 8µL of 8µg/mL polybrene was

added. 4mL of virus was added to each T-25 flask with fibroblasts. On day 3, virus was replaced with 4mL of fresh virus supernatant. On day 4, media was changed on each T-25 flask.

Calcium Phosphate Method

Due to low efficiency with the X-treme gene method, calcium phosphate was used in the next set of retrovirus production experiments. $CaCl_2$ transfection method was used to produce a retrovirus using the plasmid DNA of FoxG1, Brn2, Sox2, and GFP. Plat-E cells were thawed and plated onto 2 gelatin-coated T-25 flasks. The following day, Plat-E's were passaged to a T-175 flask. On day 3, Plat-Es were passaged to 2 million cells per 10cm dish (gelatin-coated) in 8mL of MEF media. On day 4, 2x Hepes-Buffered Saline (HBS) was made and titrated to a pH of 6.95. 5mL conical tubes were labeled for each plasmid and 1mL of DPEC water was added to each. 112.5µL of 10x $CaCl_2$ was added to the water and mixed by hand. 35µg of each DNA plasmid was added to the appropriate tube and mixed by hand. 1.125mL of 2x HBS was added drop wise from about 3 inches above the solution and was mixed by pipetting up and down 5 times. 1.125mL of the final solution was added drop wise to each 10cm dish and placed in a 3% CO_2 incubator for 24 hours. On day 5 the media was removed from each 10cm dish and replaced with DMEM Hi-Glucose + 2% FBS. GFP transfection was observed under fluorescence to show transfection efficiency. Passage 2 Olig2Cre-ER fibroblasts were plated at 125,000 cells per well of a poly-ornithine coated 6-well plate. The first retrovirus collection occurred on day 6. Supernatant was collected from the 10cm dishes using a 60mL syringe and luerlock 0.22µm filter. Supernatant was replaced with fresh 2% FBS media. 24 hours later the supernatant was removed from each 10cm plate and 8µg/mL polybrene was added. Supernatant was added to the fibroblasts drop wise and incubated.

PEI Method

A third method was attempted and resulted in the highest transfection efficiency. This retrovirus production method uses the PEI reagent. The PEI reagent may be ordered from Polysciences (cat # 23966-2). A stock solution is made by dissolving PEI in endotoxin-free dH_2O, heated to around 80℃. After cooling to room temperature, the solution is neutralized to a pH of 7.0, filtered using a 0.22µM filter, and stored at -20℃. Plat-E cells were thawed, plated on a T175 flask and left for 3 days in MEF media. Cells were then passaged and 2 million cells were plated on to gelatin-coated 10cm dishes. The virus was made with DMEM with no serum, 9 µg of the

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plasmid DNA and 27 µL of the PEI reagent. To assess virus efficiency, GFP plasmid was transfected into Plat-E cells, infected into MEFS and were assessed by immunofluorescence.

FoxG1/Brn2 Reprogramming Protocol

   A protocol taken from Lujan et al. that was published in 2012 was used with minor changes (Figure 8). The reprogramming protocol began 24 hours after transfection. Media was replaced with N3 medium which consisted of 100 mL DMEM F12, 25µg/mL insulin, 50µg/mL transferrin, 30nM sodium selenite, 20nM progesterone, 100nM peutriscine, 10ng/mL FGF2, and 10ng/mL EGF. Cells were left for 24 days with media changes every three days. 100µM tamoxifen (1µL/mL) was administered to each well during the first media change and at each subsequent media change. On day 24, media was changed to OPC differentiation media consisting of 10ng/mL FGF2, 10ng/mL PDGF, and 10nM forskolin. After five days media was changed to OPC differentiation media consisting of 200nM absorbic acid and 30ng/mL T3 and left for five more days. It was previously published that PDGF and bFGF maintain oligodendrocyte progenitor cells that will not differentiate spontaneously into oligodendrocytes [26]. After immunofluorescence analysis, cells were fixed and stained for Olig2 and Nkx2.2, Tuj1 and GFAP, and Pax6 and Nestin.
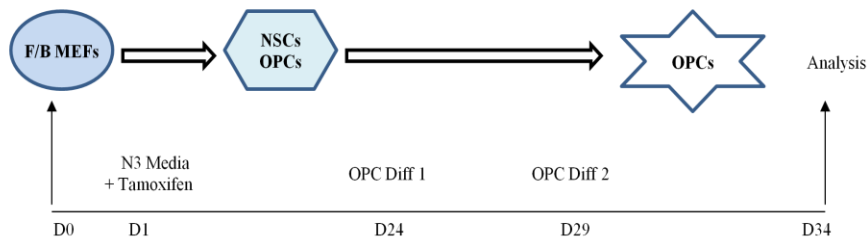

Figure 9. Protocol for reprogramming. After transfection with FoxG1 and Brn2, cells underwent a 34 day protocol with media changes every 3 days.

Direct Reprogramming with Sox2

Following the Ring et al protocol, MEFs were used for direct reprogramming using the single factor, Sox2 (Figure 9). MEFs were plated onto 10cm dishes for retroviral transfection. One day after transfection cells were passaged and plated onto 6-well plates coated with poly-ornithine. Media was changed to NSC-BM+++ which consisted of DMEM F12, B27 Supplement, N2 Supplement, EGF, and bFGF. Cells were left for 10 days with media changes every other day. On day 11, cells were collected with accutase and plated onto uncoated low attachment 6-well plates. Cells were cultured for 5-7 days and spheres were collected and plated onto poly-ornithine coated plates. A total of three rounds of neurosphere and monolayer expansion were performed. After 31 total days with passages to monolayer cultures every 3-5 days, cells could be characterized.
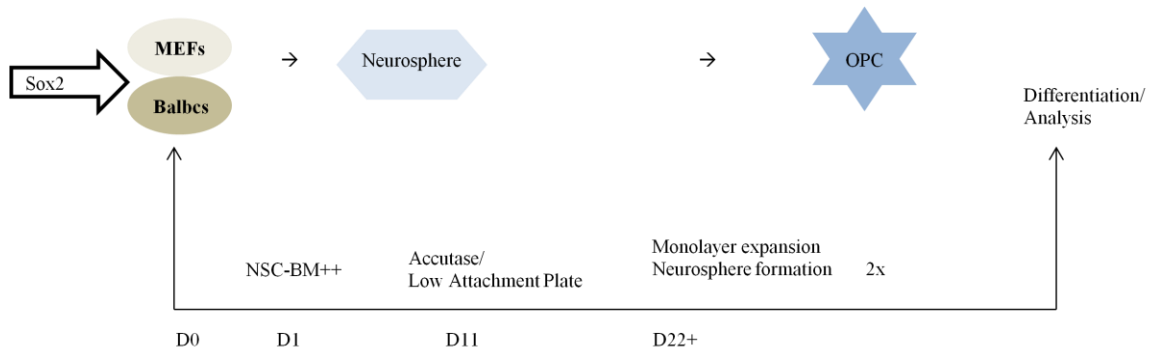

Figure 10. Protocol for reprogramming. Sox2 transfected MEFs and Balbcs underwent an approximate 30 day protocol with 3 rounds of neurosphere formation and monolayer expansion.

Immunohistochemistry

To assess transgene expression in transfected fibroblasts, a sample of cells was used for antibody staining. Cells were fixed in 1mL 10% formalin for 15 minutes then washed for 2 minutes in 2mL PBS. Cells were washed in PBS 2 more times for 5 minutes. Cells were then blocked in 0.5mL donkey serum and 9.5mL PBS-T for 30 minutes. Block was removed and 1000μL of the diluted primary antibody was added to the well. Primary antibodies were ordered from Abcam and were diluted 1:1000 in block (Table 3). Cells were left in 4°C overnight. The next day, cells were washed 3 times with PBS-T. The secondary antibody, diluted 1:500, was

added and left for 1 hour at room temperature. Cells were then washed with PBS-T and 1mL

PBS-T was left on each well for microscopy verification.

Antibody	Animal	Concentration	Dilution	Company/Catalog#
FoxG1	Goat	0.5mg/mL	1:1000	Abcam: ab3394
Brn2	Rabbit	0.5mg/mL	1:1000	Abcam: ab94977
Sox2	Mouse		1:1000	R&D: MAB2018

Table 3. Primary Antibodies used for immunohistochemistry. Secondary antibodies used were all Alexafluor 488 diluted 1:500.

Results

Olig2 Cre-ER system shows GFP and Olig2 Expression in the brain and spinal cord
Spinal cord and brain slices showed Cre GFP+ expression of endogenous oligodendrocytes of E16 mice (Figure10). Brain also showed Olig2 antibody expression (Figure11).

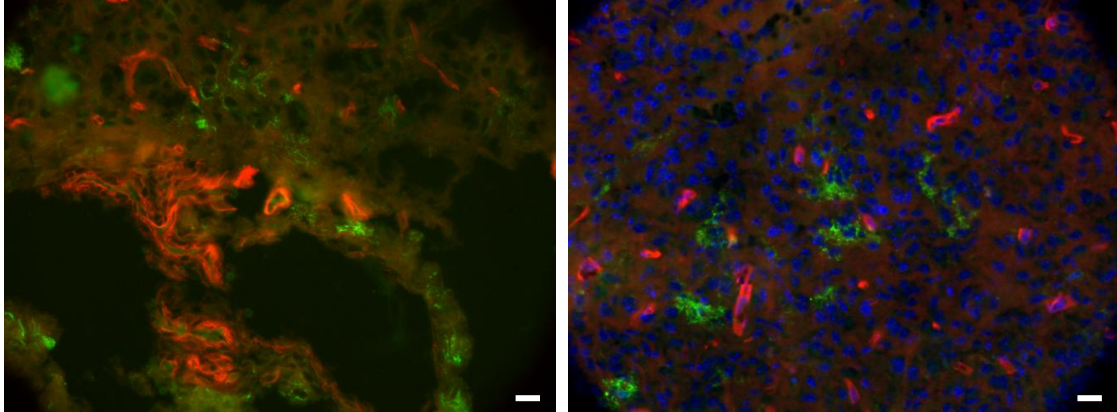**Figure 11. Spinal cord and brain sections showing GFP+ oligodendrocytes.** E16 transgenic mouse shows GFP expression after tamoxifen administration. Images = 40x Scale bar = 50µM
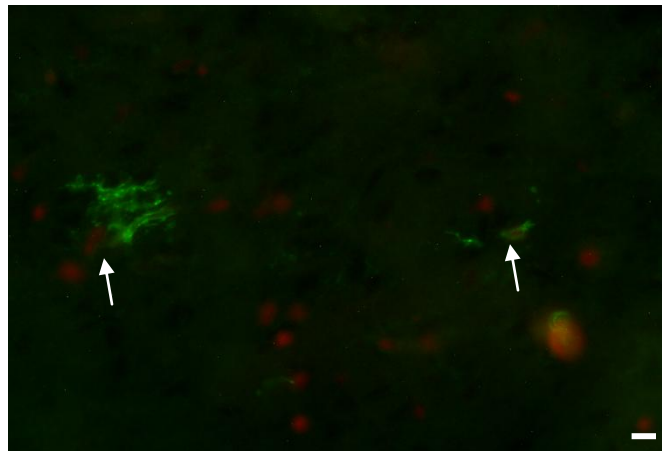

Figure 12. Brain section showing Olig2 expression near GFP+ oligodendrocytes. Olig2 antibody expression (Alexafluor 647) is consistent with GFP+ oligodendrocytes in an E16 transgenic mouse (arrows). Scale bar = 50µM

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PEI was the most effective retrovirus production protocol

After obtaining or making the DNA plasmids, three virus production methods were used to create viruses that efficiently transfected plat-e cells and mouse embryonic fibroblasts (MEFs). The X-Treme Gene protocol produced almost no transfection of plat-e cells based on immunofluorescence (data not shown). The calcium phosphate method produced GFP positive plat-e cells; however, the transfection efficiency of MEFs was only about 30% for each gene resulting in a double transfection efficiency of FoxG1 and Brn2 at about 15% (Figure 12). The third protocol attempted involved the use of the transfection reagent, polyethylenimine (PEI). The PEI method resulted in 90-100% GFP transfection of plat-e cells and MEFs in two different experiment attempts, proving to be the most efficient method for this protocol (Figure 12).
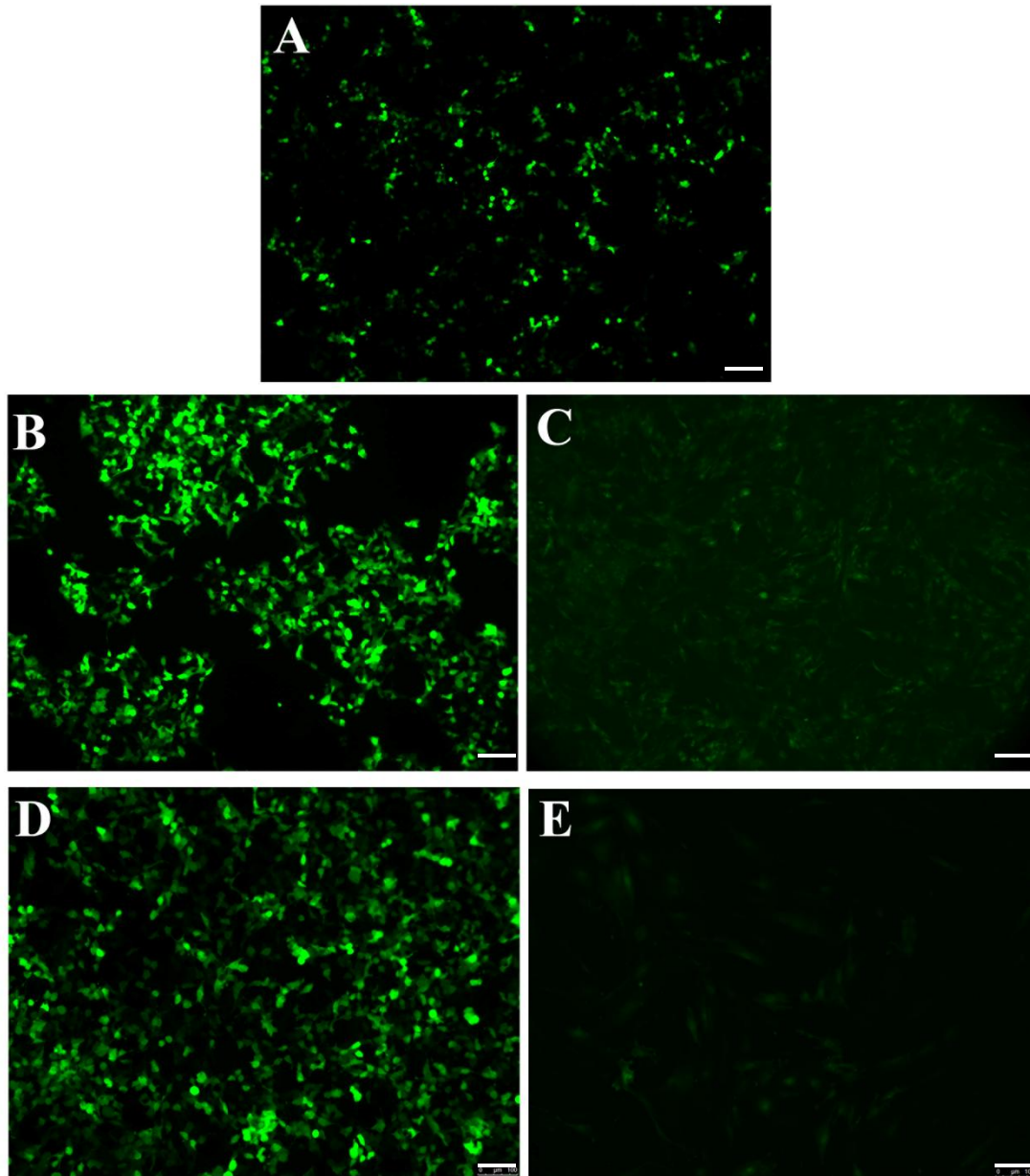
Figure 13. GFP Transfection using CaCl₂ and PEI protocols. A.) CaCl₂ protocol produces low GFP positive plat-E cells. **B-E.)** Two transfections using the PEI protocol. PEI produces a high number of GFP positive plat-e cells and high transfection in MEFs. Scale bar = 50 µm

Mouse Embryonic Fibroblasts without viruses do not produce GFP+ cells or change morphology

Controls of mouse embryonic fibroblasts without virus transfection were used to assess the possibility of any GFP+ cells or change in morphology due to cytokines or components of the medium. MEFs were plated following the same protocol as the virus-transfected cells and were

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maintained in the same medium over the 34 day protocol. Microscopy showed no GFP+ fluorescence at day 1 or day 21 and cells maintained the original red fluorescence (Figure 13). MEFs were also given tamoxifen to ensure the Olig2Cre-ER system would not produce GFP+ cells without FoxG1 and Brn2 transfection. There were not GFP+ cells after tamoxifen administration (Figure 14).
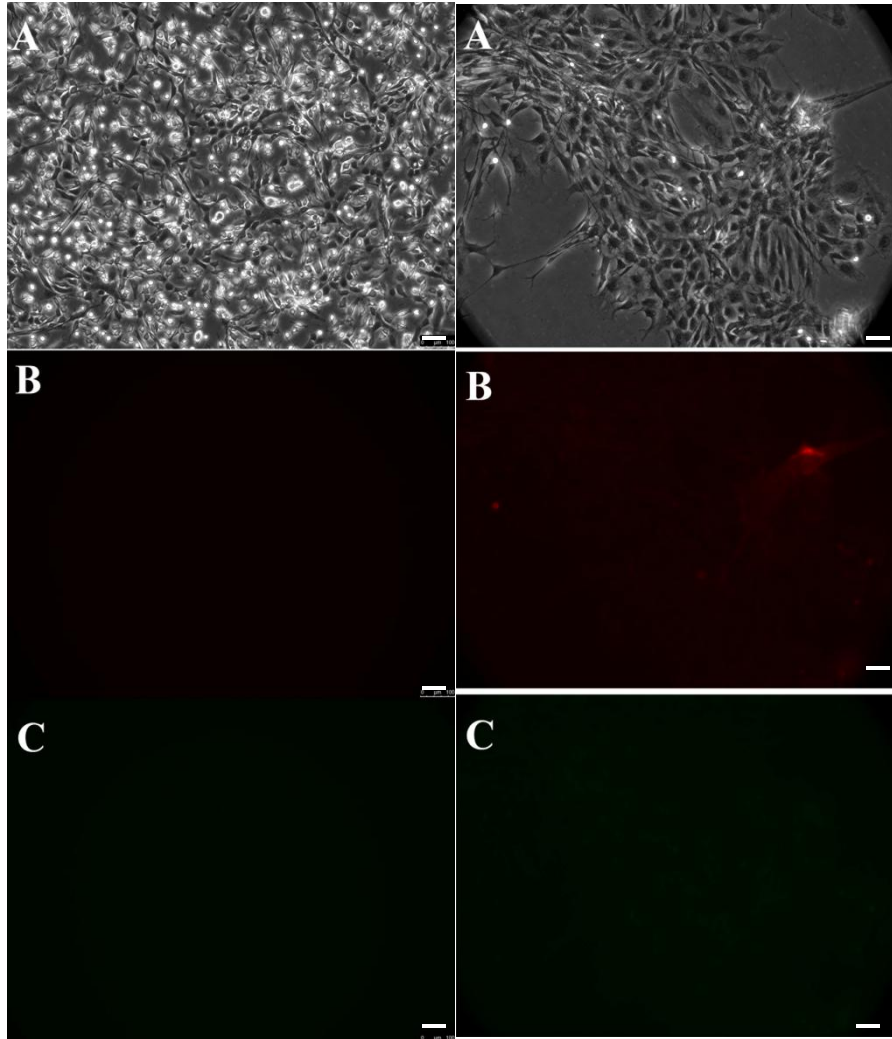

Figure 14. MEFs without virus transfection are not Olig2+, maintain tdTomato fluorescence, and do not change morphology in N3 medium. Left A.) Day 1 phase picture shows normal MEF morphology. **B.)** MEFs maintain endogenous tdTomato fluorescence. **C.)** MEFs do not show any GFP+ cells. **Right A-C.)** Day 21 MEFs phase, tdTomato, and GFP microscopy images. Scale bar = 100μm.
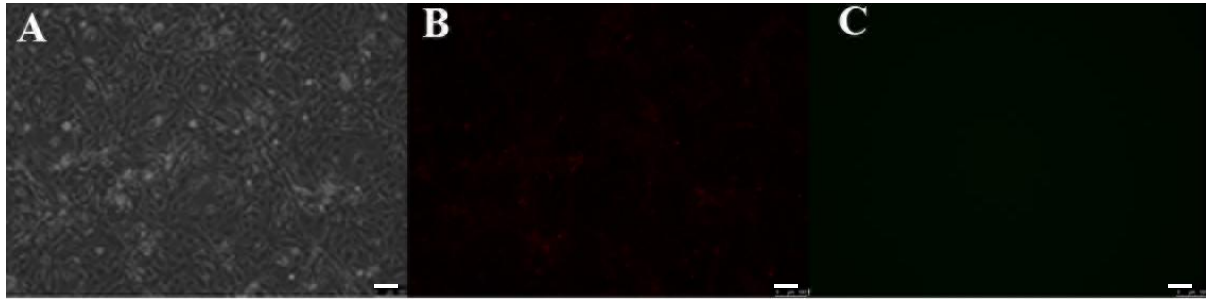
Figure 15. MEFs without virus transfection with tamoxifen do not produce GFP+ Olig2 cells. A.) Phase picture of MEFs. **B.)** tdTomato positive MEFs. **C.)** No GFP+ cells. Scale Bar = 100μM

PEI protocol retroviruses efficiently transduce cells

After an appropriate retrovirus production protocol was established, the reprogramming protocol previously published was attempted to convert mouse embryonic fibroblasts to induced neural progenitor cells and then differentiated into oligodendrocyte progenitor cells. MEFs transfected with the FoxG1 and Brn2 retroviruses were plated onto poly-ornithine coated 6-well plates and maintained in N3 Medium. A sample of cells was plated onto gelatin-coated plates and primary antibodies were added for FoxG1 and Brn2. Secondary antibodies were added and assessed with immunofluorescent microscopy. Alexafluor 488+ FoxG1/Brn2 positive cells were counted as well as DAPI stained cells and transfection efficiency was determined comparing the number of positive cells versus total cells. Four fields of view were used for counting resulting in 181/249 Brn2 cells and 91/124 FoxG1 cells. Both FoxG1 and Brn2 retroviral transfection resulted in about 73% positive cells (Figure 15).
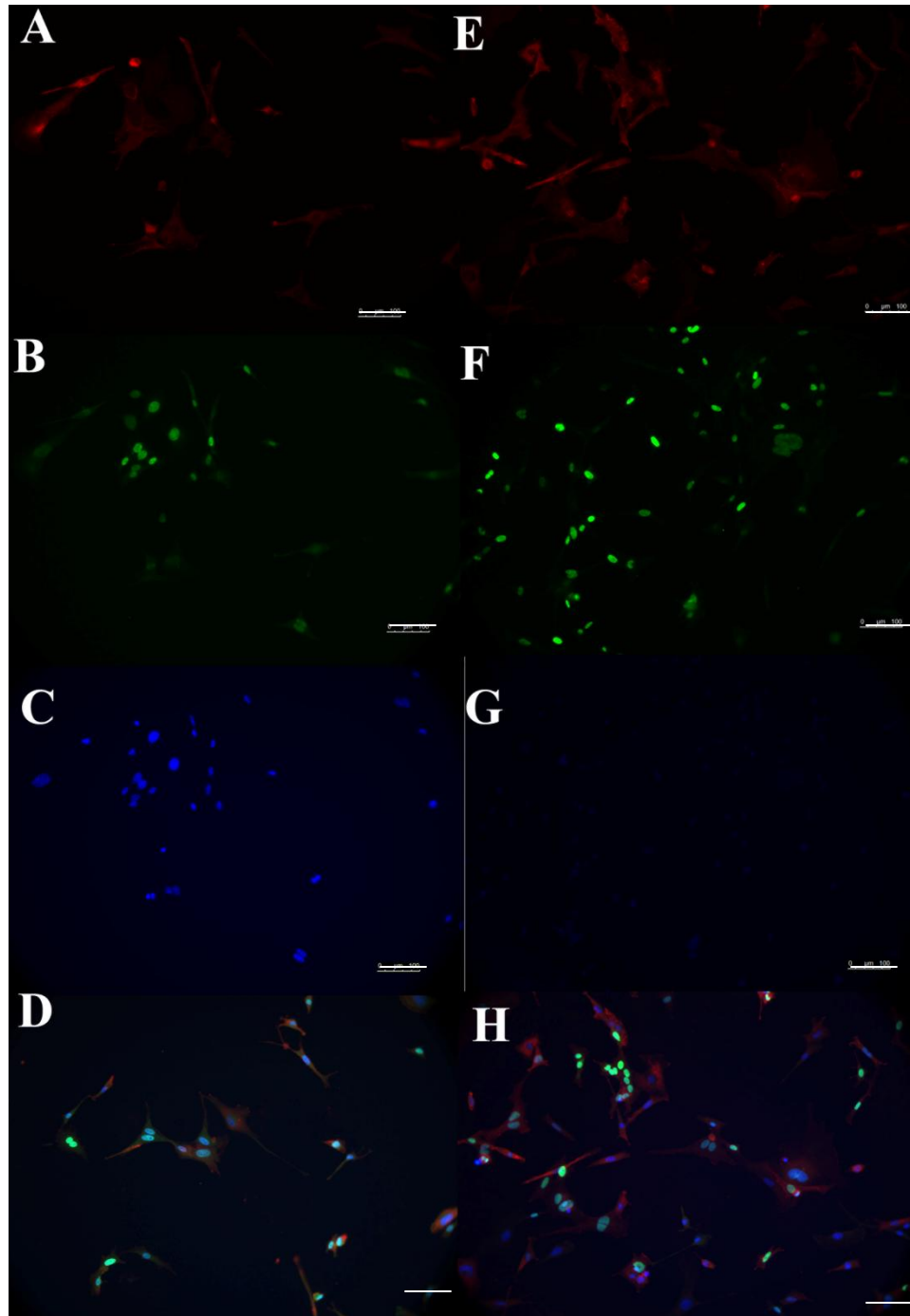
Figure 16. FoxG1 (Left) and Brn2 (Right) expression transfected MEFs. A &E.) tdTomato positive MEFs after transfection. **B&F.)** Alexafluor 488+ cells for FOXG1/Brn2. **C&G.)** DAPI Staining. **D&H.)** Merge images. Scale = 100μM

FoxG1 and Brn2 transfected cells show changes in morphology

      Previous studies reported that mouse embryonic fibroblasts after transfection showed morphological changes beginning at day 4 and significant morphological changes at day 7 where cells showed an elongated shape. Colony formation was first observed at day 13 [25] (Figure 16). We saw similar morphological changes beginning at day 3 with colonies forming at day 14 (Figure 17).
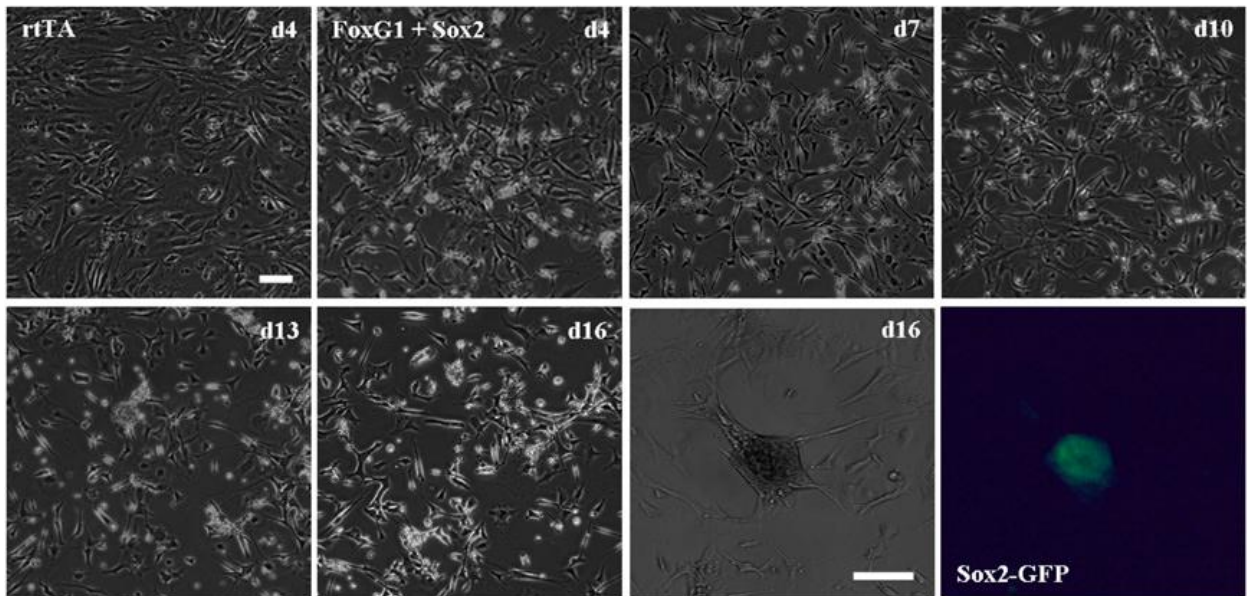

Figure 17. Time-lapse images of FoxG1 + Sox2 infected cells [24]. MEFs show morphological changes starting as early as day 4 with elongation at day 7. Colonies began forming at days 13-16.
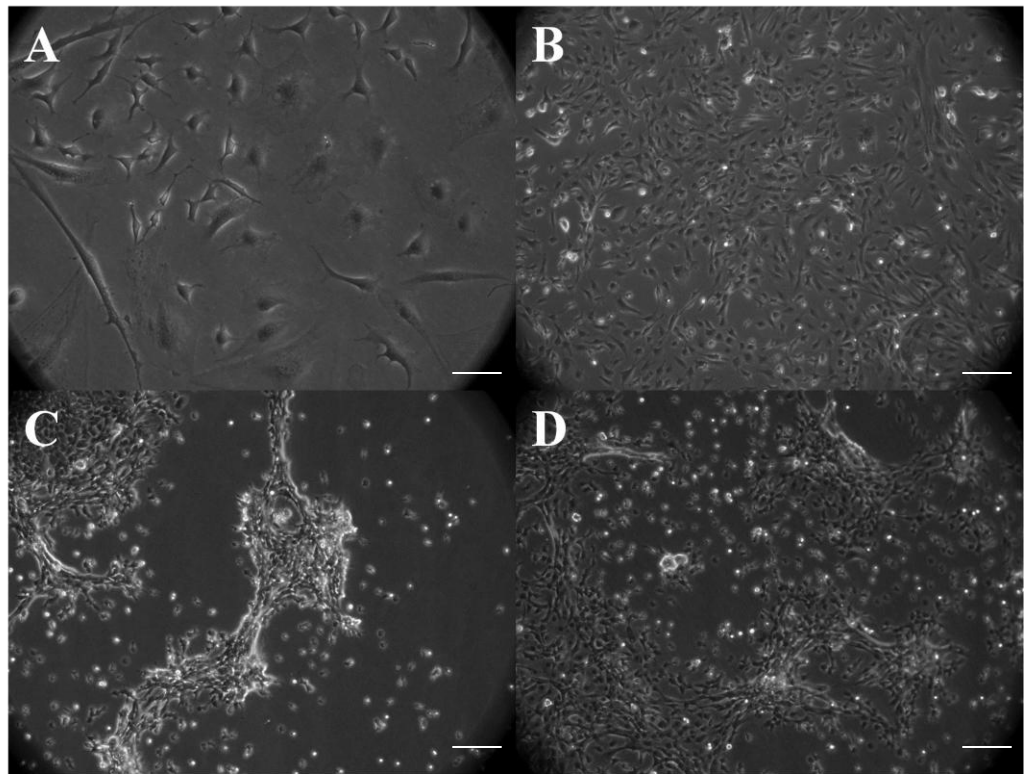
Figure 18. Morphological changes of transfected MEFs over 18 days. A.) Day 3 MEFs show normal fibroblast morphology with some elongated structures. **B.)** Day 9 MEFs begin to form elongated cells and have areas of clumping. **C-D.)** MEFs form colonies. Scale bar = 100μm

No GFP+ cells were identified over the 24 day protocol. After day 24, N3 medium was replaced with OPC differentiation medium as proposed in the established protocol [25].Cells were confluent the first day of OPC differentiation (day 24); however, cell colonies broke up and significant cell death occurred from day 24 to day 34 (Figure 18). There were no GFP+ cells determined by fluorescent microscopy (Figure 19). Cells were stained for multiple factors including Olig2, Nkx2.2, Tuj1, Nestin, Pax6, and GFAP. Cell staining was negative.

Figure 19. Morphological changes during OPC differentiation. A-B.) Phase contract images of transfected MEFs at Day 1 (Left) and Day 10 (Right) of OPC differentiation. **C-D.)** tdTomato positive MEFs. **E-F.)** There are no GFP+ cells during or after OPC differentiation. Images = 10x
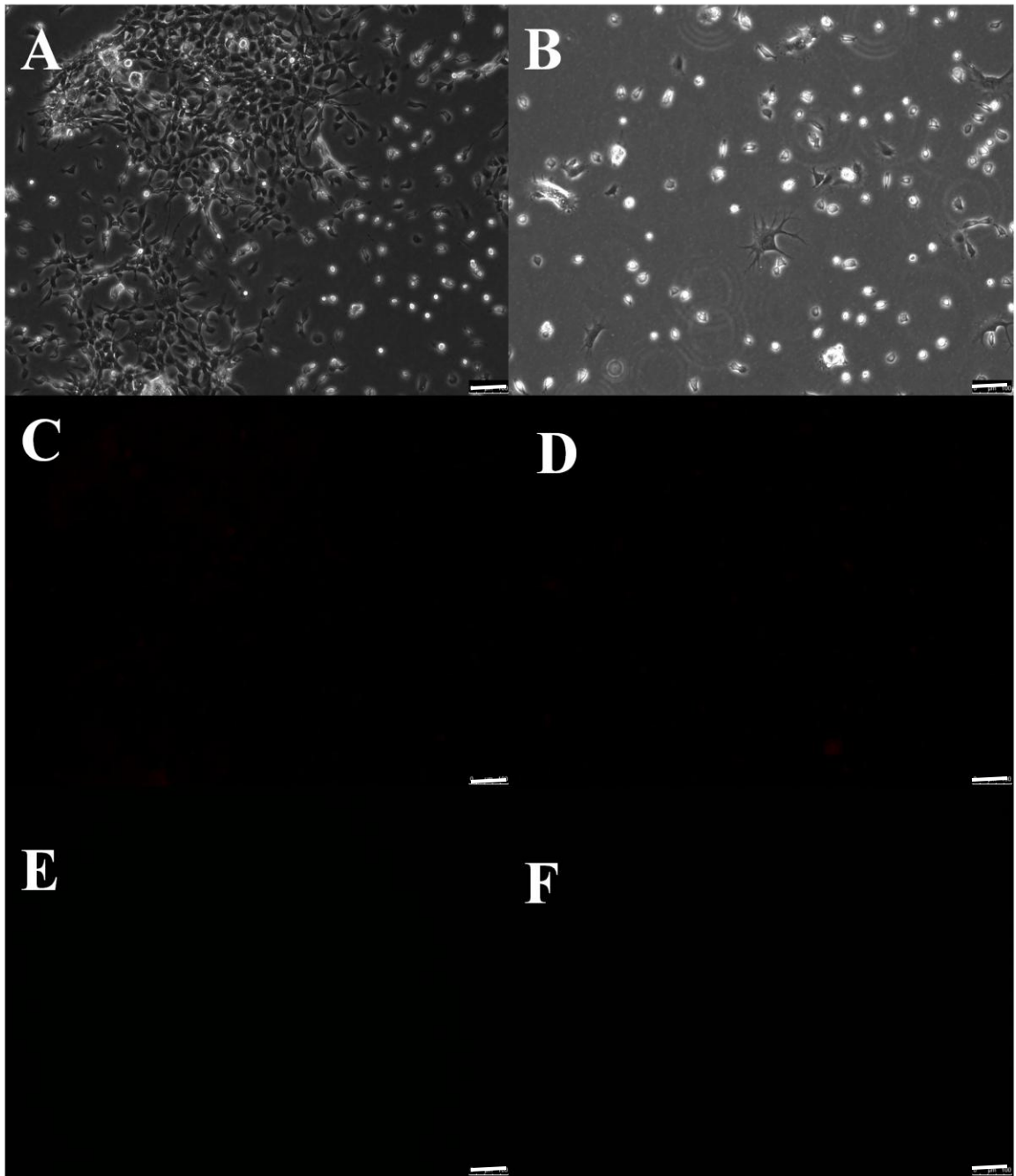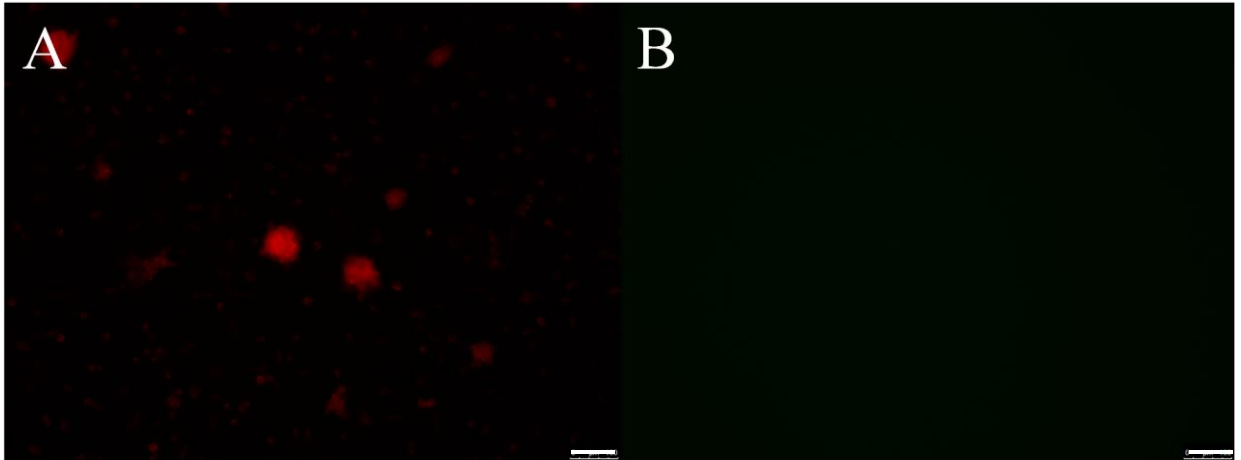
Scale bar = 100μm

Figure 20. Fluorescent microscopy of day 24 MEFs. A.) MEFs are still positive for endogenous tdTomato. **B.)** There are no GFP+ cells at the first day of OPC differentiation. Scale = 100μm

Sox2 transfected cells change morphology but are easily contaminated

Sox 2 transfected MEFs were done in parallel with the FoxG1/Brn2 protocol using a protocol previously published [40]. Cells were transfected on day 0 and left with media changes until day 11 in which the first neurosphere stage began. Cell morphology changes dramatically over the first 11 days and neurospheres were formed after placement onto uncoated low attachment 6-well plates. However, at day 20 it was noticed that cells that were not yet given tamoxifen fluoresced green (Figure 20). The GFP+ cells were replated for the monolayer stage and assessed on day 22. Although cells could still form a monolayer and undergo expansion, they fluoresced green and had lost the endogenous red fluorescence (Figure 21). We believe this is due to contamination of neural stem cells in the NSC basal medium that was used. The contaminant cells took over the expansion and all Olig2Cre-ER fibroblasts died. Other experiments performed resulted in green cells as well. Further experimentation done with new media resulted in expanding cells that endogenously fluoresced red and no green; however, this experiment could not be completed and images were only obtained for day 2 and day 11 (Figure 22).

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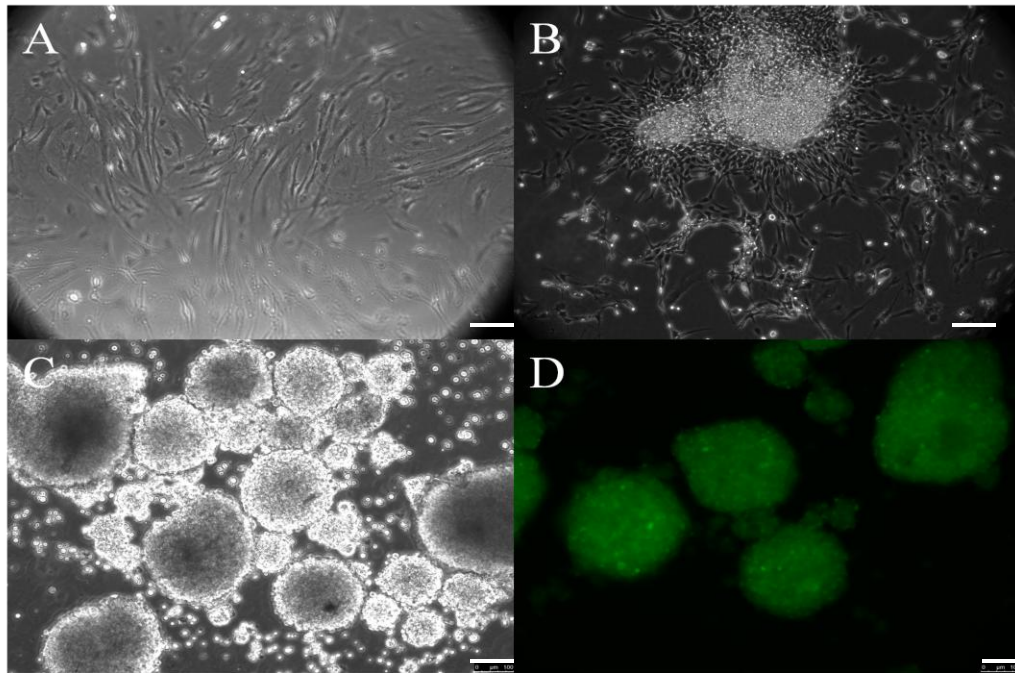
Figure 21. Sox2 transfected MEFs change morphology and fluoresce green without tamoxifen. A.) Sox2 MEFs on day 1. **B.)** Sox2 MEFs on day 8, form colonies. **C.)** Sox2 Neurospheres on day 18. **D.)** Sox2 MEFs exhibit green fluorescence on day 20 despite no addition of tamoxifen. Scale = 100 μm
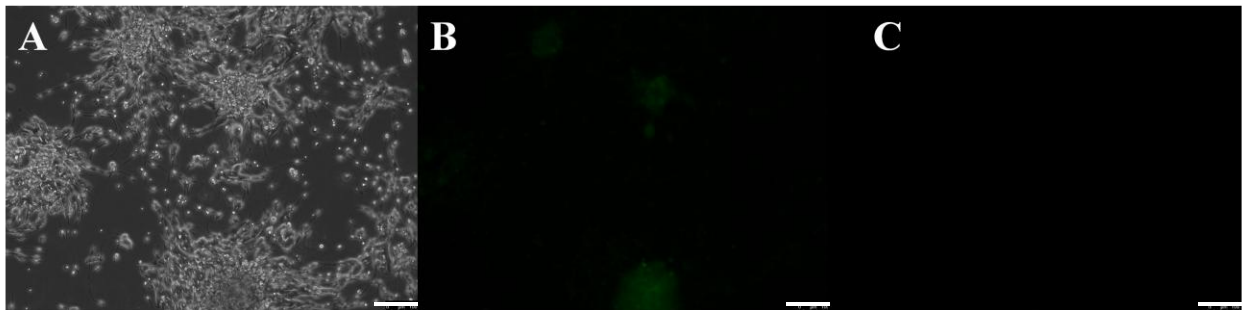
Figure 22. Sox2 MEFs replated for monolayer expansion. A.) Sox2 MEFs during monolayer expansion on day 22. **B.)** Sox2 MEFs exhibit green fluorescence without tamoxifen. **C.)** Sox2 MEFs on day 22 show no endogenous red fluorescence. Scale = 100 μm
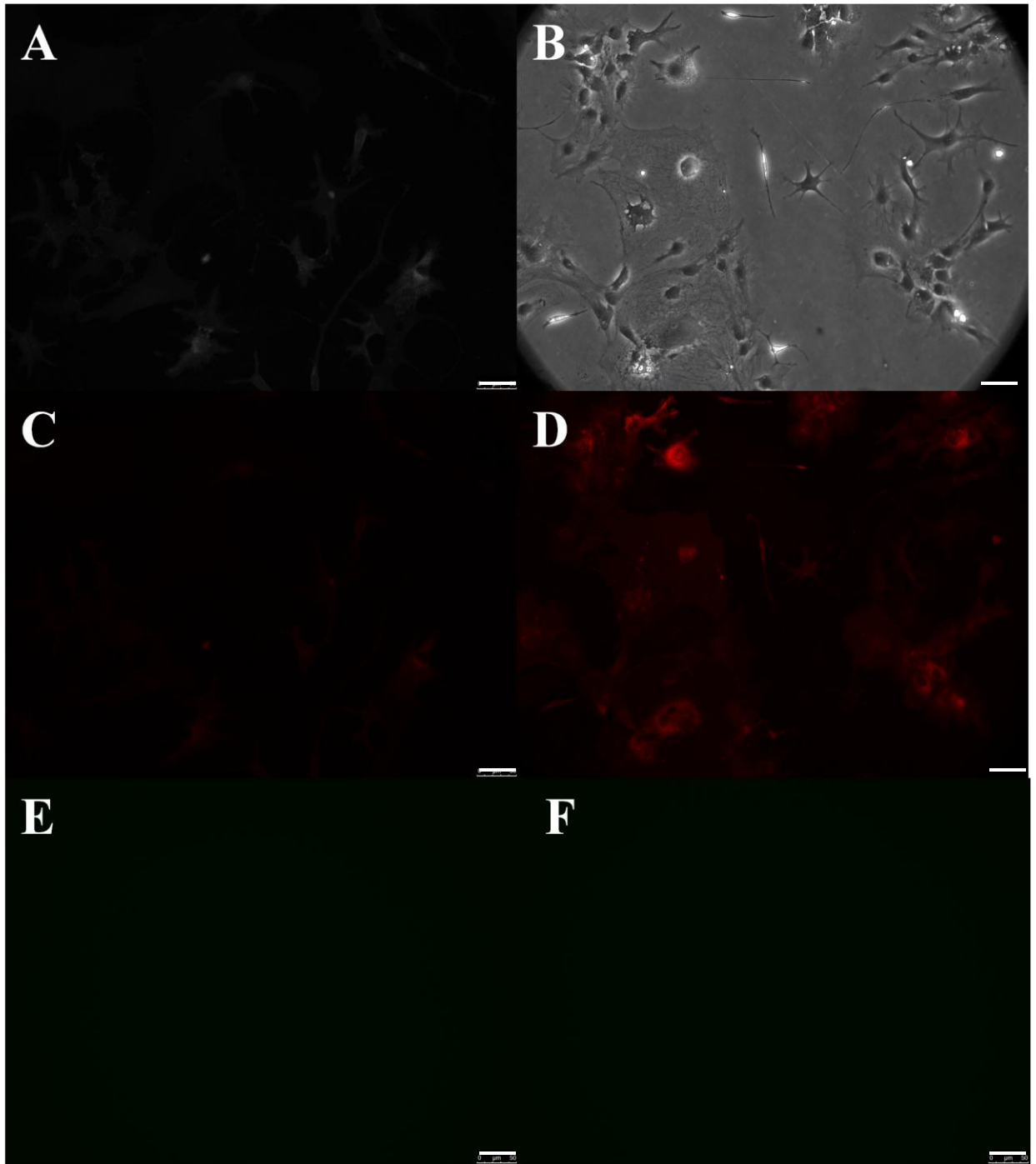
Figure 23. Sox2 MEFs have morphological changes with no contamination. A-B.) Sox2
MEFs phase images on day 2(Left) and day 11(Right). **C-D.)** Endogenous tdTomato fluorescence
expression of Sox2 MEFs. **E-F.)** Sox2 MEFs do not express GFP. Scale bar = 100μm

A total of 9 experimental runs were attempted, each with varying virus type and transfection methods (Table 4). As shown in the table, some experiments were not carried to completion for the full 30-34 day protocol duration. Others were completed with no GFP+ cells at the end of the 24 days or after OPC differentiation. Transfection efficiencies did increase as the PEI transfection method was practiced using retrovirus transfection.

Experiment Number	Virus Type	Transfection Method	Transfection Efficiency	Results
1	Lentivirus	X-Treme Gene	N/A	No GFP+ with doxycycline
2	Retrovirus	X-Treme Gene	N/A	No GFP + with tamoxifen (D24)
3	Retrovirus	$CaCl_2$	N/A	Contaminated
4	Retrovirus	X-Treme Gene	<10%	No GFP+ (D34)
5	Retrovirus	$CaCl_2$	~34%	No GFP+
6	Retrovirus	PEI	<15%	No GFP+ (D22)
7	Retrovirus	PEI	~80%	Lost after D11
8	Retrovirus	PEI	~73%	Lost after D11
9	Retrovirus	PEI	N/A	Death after tamoxifen

Table 4. Description of all experimental runs. Throughout experiments the virus type and transfection methods were altered to obtain high efficiency.

DISCUSSION

Direct reprogramming has become a new strategy for development of a specific cell type that may be useful for cell transplantation. Past studies have demonstrated the reprogramming potential of a variety of cell types including skeletal muscle, cardiomyocytes, pancreatic beta cells and neural cells [13]. Further research has shown that progenitor cells are the most useful cell type for transplantation because they can differentiate into a specific-cell type, lessening the chances of unwanted cell types taking over the injured or diseased area. Taking into consideration past studies from Wernig's group as well as others, we hypothesized that direct reprogramming is a useful technique to generate oligodendrocyte progenitor cells with a future potential of being used for spinal cord injury therapies.

The protocols used were previously published and resulted in two different cell stages. FoxG1 and Brn2 cells resulting from the protocol were described as bipotent neural precursor cells [24] while cells from the Sox2 protocol resulted in neural progenitor cells, both of which we hoped to expand with differentiation into OPCs [38]. Studies occurring after these two protocols show a variation in the stages of OPC formation and the reprogramming factors chosen [54][46][31]. We chose to use the least number of reprogramming factors necessary in hopes to obtain a more simple protocol; however, many limiting factors still existed. For example, three retrovirus production methods were tried before efficient reprogramming numbers were obtained. This may be due to the individual methods, or in the case with FoxG1 and Brn2, it may be due to each virus being made separately and then transfected into the cells. This limits the chances that each cell will receive both genes. It took subsequent experiments to obtain a high enough efficiency that would allow for the possibility of reprogramming. In the original paper as well as others, a lentivirus with transcriptional control using a TetO promoter was used, which varies from our protocol. Wernig's publication in 2013 states that induced OPC generation is still at a low efficiency and therefore increasing the viral titer of the reprogramming factors is necessary. The group also recommends that further experimentation to understand the appropriate stoichiometry and expression levels of the factors are needed [54].

Other technical issues decreased chances of effective reprogramming including cell proliferation. Specifically for the Sox2 transfection, balbc fibroblasts often died upon viral transfection. The mouse embryonic fibroblasts originally harvested and frozen away were difficult to thaw and grow efficiently. These technical issues limited the number of viable cells for viral transfection that could survive and differentiate. FoxG1/Brn2 transfected cells in one

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trial were proliferating and had morphological changes until the OPC differentiation media was added. After day 24 there was prolific cell death, suggesting that something in the differentiation media was not appropriate to maintain cells. Another technical issue is the addition of tamoxifen at appropriate concentrations. Late experiments using newly-made tamoxifen in the same concentration resulted in cell death around day 12, suggesting the ratio was not correct. These technical issues greatly limited the potential of a successful experiment.

Future experimentation should take into consideration any technical difficulties such as cell viability, tamoxifen ratio, media components, etc. Therefore, any difficulties due to these issues may be resolved before reprogramming experiments are attempted. It would be beneficial to try to produce a double construct vector for the FoxG1 and Brn2 genes so that each transfected cell would receive both genes, ultimately increasing the transfection efficiency; or utilize a lentiviral system previously published by other groups. Another alternative is to try a variety of transcription factors other than FoxG1 and Brn2. Although it is easier using one or two transcription factors, it may be more efficient using another combination. Many groups have published reprogramming reports of neural stem cells and OPCs using a variety of combinations suggesting that it is possible to use more than two factors.

Finally, the ultimate purpose of these experiments was to generate autologous OPCs that would myelinate axons *in vivo*. If further experimentation produces reprogrammed cells it will be necessary to test the cells in a mouse or rat model of demyelination such as a shiverer mouse or rat with a spinal cord injury. For any future clinical application, the cells must migrate and myelinate *in vivo* for an extended period of time. Once the mouse system is well understood, direct reprogramming of OPCs will need to be extended with the use of human somatic cells as the starting cell population. Other areas of experimentation will need to include culturing techniques that are serum-free and are done in consideration with good manufacturing practice guidelines so that the cells are appropriate for transplant into humans. Although other techniques exist that produce OPCs, direct reprogramming holds the potential of producing a faster method for obtaining OPCs for not only autologous transplants to improve function after SCI but also patient-specific models of disease and models for drug screening or understanding of OPC development.

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