Lineage specific differentiation of myoblast derived induced pluripotent stem cells

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Abstract

Induced pluripotent stem (iPS) cells, reprogrammed from somatic cells with defined factors such as Oct4, Sox2, cMyc and Klf4, hold the potential to produce unlimited numbers of autologous cells to treat and model a variety of muscular dystrophies. However, the derivation of myogenic precursors from iPS cells remains elusive, and current differentiation protocols rely on multi-stage fluorescent cell sorting or the use of transgenes. Reprogrammed somatic cells exhibit epigenetic memory in the form of DNA methylation patterns and gene expression profiles characteristic of their tissue of origin. Here we show that myoblast (Mb) derived iPS cells maintain low level expression of myogenic markers, including MyoD, providing evidence that myogenic genes are not fully silenced in MB-iPSCs during the reprogramming process. In addition, Mb-iPS cells display preferential myogenic differentiation *in vitro* and *in vivo* compared to fibroblast (Fb) derived iPS cells. Exploiting this epigenetic memory, we establish a simple method for the derivation of myogenic progenitor cells from iPS cells, a critical step towards efficient cell therapy of Duchenne muscular dystrophy (DMD).

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Introduction

Duchenne muscular dystrophy (DMD) is an x-linked muscle-wasting disease affecting approximately 1 in 3,500 males.[1] DMD patients inherit or acquire non-sense mutations in the dystrophin gene, resulting in the absence of dystrophin in skeletal, cardiac, and smooth muscle.[2,3] Involved in mechano-transduction, dystrophin is a functional anchor protein that stabilizes muscle fibers by connecting them to the basal lamina.[4] In muscle lacking dystrophin, successive rounds of degeneration and regeneration lead to necrosis and exhaustion of the stem cell compartment, as well as the deposition of calcium and adipose in lieu of myofibers.[5,6] Patients suffer from a loss of ambulation and difficulty breathing in early life, generally dying in their mid to late twenties due to respiratory or cardiac failure.[7,8]

The dystrophin gene is the largest in the human genome, encompassing over 2,500 kilobases.[4] Its massive size and systemic expression provide considerable barriers to the development of successful treatment strategies. Current therapies include the administration of morpholinos or other small molecules for exon skipping, and drugs capable of producing read through translation of premature stop codons.[9-16] Although partially successful in restoring dystrophin expression, these treatments have failed to demonstrate functional benefits.

Recently, cell therapy has gained momentum as an alternative DMD treatment, and a wide range of cell types have been used with varying degrees of success. These include myoblasts, satellite cells, muscle derived stem cells, multi-potent adult progenitor cells, mesangioblasts, hematopoietic stem cells, $CD133^+$ cells, side population cells, and

pericytes.[17-32] Notably, purified satellite cells, the stem cells of skeletal muscle, are able to partially reconstitute the host satellite cell pool, and it is tempting to speculate that donor-derived cells could provide a continual source of dystrophin as they activate, divide, and fuse with myofibers. Regrettably, satellite cells lose their ability to self-renew and contribute to muscle fibers when expanded *ex-vivo*, making it impossible to obtain a sufficient number for clinical application.[31] In addition, donor cell survival is limited due to the hosts' immune system, and transplantation of even HLA-matched myoblasts requires immunosuppression.[26,27,33-35]

Embryonic stem cells differentiate into all three germ layers and undergo unlimited expansion *in-vitro*.[36] However, their therapeutic use may be limited due to legal restrictions, immunogenicity, availability, and biological impediments to the generation of new cell lines.[37,38] The recent development of induced pluripotent stem (iPS) cells—reprogrammed from somatic cells by defined factors such as Oct4, Sox2, cMyc and Klf4—may circumvent these issues.[39,40] iPS cells resemble embryonic stem cells in their gene expression, self-renewal, and multi-potency, and hold tremendous therapeutic promise due to their immunological compatibility, permitting the generation of an unlimited number of transplantable patient-specific cells.[41]

Clinical application of iPS cells will require safe, reproducible, and cost-effective methods for the production of relevant cell types. However, the derivation of myogenic precursors from iPS cells remains elusive, and current differentiation protocols rely on multi-stage fluorescent cell sorting or the use of transgenes, raising concerns over cost

and safety, respectively.[42-49] In addition, these protocols utilize lengthy differentiation periods, which last from three to eight weeks in duration.

A failure to recreate myogenic signaling pathways may be partially responsible for the lack of skeletal muscle progenitors in embryoid bodies. Skeletal muscles of the trunk and limbs are derived from somites, segments of paraxial mesoderm that flank the neural tube.[50] As somites mature, the dermomyotome, or dorsal portion of the somite, remains epithelial, housing myogenic progenitors that express the paired box transcription factors Pax3 and Pax7.[51,52] These $Pax3^{+}$/$Pax7^{+}$ cells migrate to the satellite cell position beneath the basal lamina of the muscle fiber where they later assume responsibility for the remarkable regenerative capabilities of adult skeletal muscle.[53-56]

Tissues adjacent to the somite, including the notochord, neural tube, and dorsal ectoderm, regulate skeletal myogenesis by secreting signaling molecules, notably Wnts, Sonic Hedgehog, and Bone Morphogenic Protein 4 (BMP4, Fig. 1).[50] These signals activate transcription factors involved in the specification of the skeletal muscle lineage, including Pax3, Meox1, and Gli2.[57-59] Further commitment is controlled by MyoD, myogenin, Myf5, and MRF4, the so-called myogenic regulatory factors (MRFs), a group of basic helix-loop-helix (bHLH) transcription factors that operate as a master switch, activating a suite of downstream myogenic genes.[60-63]

BMP4 is a member of the transforming growth factor beta (TGF-β) super family, and binds to type I and II BMP4 trans-membrane receptors, initiating the formation of a receptor complex.[64] This leads to the activation of the type I receptor kinase, resulting in the phosphorylation of receptor-regulated Smad (R-Smad) proteins. Activated R-Smads

migrate to the nucleus and regulate the transcription of target genes, including inhibitors of DNA differentiation/binding (Id) proteins.[65-67] Id proteins contain a HLH dimerization domain, but lack a DNA-binding domain. By sequestering E-proteins, including MyoD and myogenin, they negatively regulate bHLH transcription factors required for myogenesis.[68] Inhibition of BMP4 is required for the onset of skeletal muscle development, and its suppression occurs through noggin, an agonist secreted from the somites and notochord.[69-76] BMP4 also inhibits myogenesis in immortalized cell lines *in-vitro*, driving cells towards an osteogenic fate.[47,77]

Methyltransferase enzymes alter gene expression through the covalent transfer of methyl groups to pyrimidine rings.[78-80] Highly methylated regions of the genome are generally dormant, while the absence of DNA methylation is a prerequisite for active transcription.[81,82] Therefore, DNA methylation has been described as an epigenetic mechanism of gene regulation, a heritable change in gene function independent of changes in DNA sequence. During development, the genome undergoes substantial *de novo* methylation, but this process is reversible, and transcription factor based reprogramming resets the methylome, leading to massive changes in gene expression.[82-86] The result is a partial ablation of cell specificity and the creation of an embryonic methylation signature, mimicking the process that takes place during fertilization of an oocyte and the subsequent generation of a zygote.[87-89]

Human and murine iPS cells can be generated from a variety of somatic cell types, including peripheral blood cells, keratinocytes, hepatocytes, fibroblasts, pancreatic cells, lymphocytes, and neural stem cells.[41] However, recent work demonstrates that this

process is imperfect, and reprogrammed cells retain residual methylation patterns characteristic of their tissue of origin.[90-93] Although ES and iPS cells are similar, it is now clear that they possess distinct differences in gene expression and functionality.[94-96] These differences result in a variety of phenotypic characteristics, including retention of disease-specific traits, persistence of donor cell gene expression, and chromosomal instability. Importantly, somatic cell reprogramming favors subsequent differentiation along donor cell lineages, while restricting alternative cell fates.[85,97-101]

Recently, our laboratory was the first to reprogram myoblasts, transit amplifying cells able to proliferate and contribute to muscle regeneration, into iPS cells.[102] These cells differentiate into all three germ layers, express pluripotency markers, and undergo germ line transmission. The epigenetic memory displayed by other reprogrammed somatic cell types led us to ask whether myoblast-derived iPS (Mb-iPS) cells are fully reprogrammed, and if they hold an advantage over fibroblast-derived iPS (Fb-iPS) cells in the production of myogenic progenitors. Here we demonstrate that Mb-iPS cells maintain low level expression of myogenic markers, including MyoD, and display preferential myogenic differentiation *in vitro* and *in vivo* compared to Fb-iPS cells. Exploiting this epigenetic memory, we establish a simple, transgene-free method for the derivation of myogenic progenitors from iPS cells by means of BMP4-mediated differentiation. This process is further enhanced through the over expression of MyoD, a potent regulator of skeletal muscle myogenesis.

Materials and Methods

Mice

Myf5-nLacZ mice were kindly provided by Shahragim Tajbaksh. Doxycycline (Dox) inducible *MyoD* (*iMyoD*) transgenic mice were kindly provided by Dr. Michael Kyba. . *iMyoD* ES cells were generated by a cassette exchange method (Fig. 2; Iacovino, 2009). *Myf5-nlacZ/Rosa26-rtTA/iMyoD* mice were established by crossing *Myf5-nLacZ* and *Rosa26-rtTA/iMyoD* mice. Animals were housed in an SPF environment and monitored by the Research Animal Resources (RAR) of the University of Minnesota. All protocols were approved by the Animal Care and Use Committee (IACUC, Code Number: 1003A79635) of the University of Minnesota. Care was taken to minimize the number of animals used, as well as any pain and suffering.

Myoblast Isolation and Culture

Skeletal muscles from the hind limbs of 1 to 2 month old adult mice were dissected, rinsed briefly in phosphate buffered saline and minced using surgical scissors. The isolated tissue was digested in a solution of 1.5 U/ml collagenase D, 2.4 U/ml dispase II, and 2.5 mM $CaCl_2$ for 20 minutes at 37° C (Roche Applied Science, Basel, Switzerland, www.roch-applied-science.com) then triturated again and incubated for an additional 20 minutes. After digestion, 10 ml of Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, www.invitrogen.com) supplemented with 2% fetal bovine serum (FBS, Fisher Scientific, Pittsburgh, PA, www.fishersci.com) was added and the digested tissue was spun down at 800 rpm for 30 seconds. The supernatant was

transferred to a fresh tube and the wash/spin/transfer cycle was repeated an additional time. Cells were collected by centrifugation at 2000 rpm for 5 minutes at 4° C and re-suspended in fresh medium before undergoing magnetic activated cell sorting (MACS). MACS was performed using MiniMACS and MidiMACS cell sorting starter kits (Miltenyi Biotec, Cologne, Germany, www.miltenyibiotec.com). Cells underwent negative selection using phycoerythrin (PE)-labeled CD45, PE-labeled CD31, and PE-labeled Sca-1 antibodies (all from BD biosciences San Jose, CA, www.bdbiosciences.com) and anti-PE micro beads (Miltenyi Biotec). After clearance of the $CD45^-$, $CD31^-$, $Sca\text{-}1^-$ cell fraction, the remaining cells underwent positive selection using an Integrin α7 antibody (MBL International, Woburn, MA, www.mblintl.com) and anti-IgG1 micro beads (Miltenyi Biotec) to enrich for satellite cells. Sorted satellite cells were maintained on collagen coated dishes in myoblast growth medium consisting of HAM's F-10 (Invitrogen) supplemented with 20% FBS (Fisher Scientific), 50 μg/ml penicillin/streptomycin (Invitrogen), and 5 ng/ml basic fibroblast growth factor (R&D systems, Minneapolis, MN, www.rndsystems.com). Cells were enzymatically passaged every 4 to 5 days with 0.25% trypsin-EDTA (Invitrogen) onto new plates. To ensure that muscle cells retained physiological characteristics, all experiments were performed using cells passaged 2-4 times.

Preparation of Murine Embryonic Fibroblasts and Tail Tip Fibroblasts

For murine embryonic fibroblast (MEF) isolation, uteri isolated from 13.5 day pregnant mice were washed with phosphate buffered saline. The head and visceral organs

were removed and discarded, and the bodies were washed in fresh PBS, minced using surgical scissors, and transferred into 3 ml of a 0.1 mM trypsin/1 mM EDTA solution per embryo. After incubating the embryos at 37° C for 20 minutes, an additional 3 ml per embryo of 0.1 mM trypsin/1 mM EDTA solution was added, followed by an additional 20-minute incubation at 37° C. After trypsinization, an equal volume of Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 10% FBS was added and triturated to aid in tissue digestion. After incubation of the tissue at room temperature for 5 minutes, the supernatant was transferred to a new tube. Cells were collected by centrifugation at 200g for 5 minutes at 4° C and resuspended in fresh medium. Cells were cultured on 100 mm dishes at 37° C with 5% CO_2 and 5% O_2. MEFs were used within four passages to avoid replicative senescence. To prepare MEF feeders, cells were washed with 5 ml of PBS per 100 mm plate and incubated with 4 ml of 10 mg/ml mitomycin-c (Sigma-Aldrich, St. Louis, MO, www.sigmaaldrich.com) for three hours. Cells were then washed three times with 10 mL of PBS, trypsinized, and plated at a concentration of 5 x 10^4 cells per cm^2. For the isolation of tail tip fibroblasts (TTFs), the tails from adult mice were peeled and minced into 1 mm pieces, placed on culture dishes, and incubated in DMEM supplemented with 10% FBS and 50 μg/ml penicillin/streptomycin for 5 days. Cells migrating out of the tail pieces were transferred to new plates and cultured on 100 mm dishes at 37° C with 5% CO_2 and 5% O_2.

Generation and Culture of iPSCs

The *pMX* based retroviral vectors encoding the mouse cDNAs of *Oct4*, *Sox2*, *cMyc*, and *Klf4* (Addgene, Cambridge, MA, www.addgene.com) were kindly provided by Dr. Toshio Kitamura. The *pMX* based retroviral vectors were separately co-transfected by packaging defective helper plasmids into PlatE cells using Lipofectamine 2000 Reagent (Invitrogen). Forty eight hours after transfection, virus supernatants were harvested. Low passage myoblasts (1-2 passages) or tail tip fibroblasts (2-4 passages) were seeded at a density of 2-5 x 10^5 cells per 35 mm plate 24-48 hours prior to viral infection. Myoblasts and fibroblasts were incubated with the supernatants of the retroviral vectors containing the four factors (Oct4, Sox2, cMyc, and Klf4) with 10 μg/ml of polybrene (Millipore, Bedford, MA, www.millipore.com) for four hours. The medium was then replaced with myoblast growth medium and the cells were cultured for 12 hours before undergoing an additional round of infection. The infected cells were cultured for two days and re-plated onto MEF feeder cells on tissue culture treated dishes (BD biosciences) coated with 0.1% gelatin (Sigma Aldrich) in embryonic stem (ES) cell medium comprised of knockout DMEM (Invitrogen) supplemented with 20% knockout serum replacement (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma Aldrich), 0.1 mM nonessential amino acids (Invitrogen), 50 μg/ml penicillin/streptomycin, and 5,000 U/ml leukemia inhibitory factor (Millipore). One to four weeks after sub-culture, ES cell-like colonies were sub-cloned onto MEF feeder cells. iPS cells were maintained on MEF feeder cells in ES medium and enzymatically passaged every 2 to 3 days with 0.25% trypsin-EDTA onto a fresh feeder layer.

Immunostaining & X-Gal Staining

For β-D-galactoside staining, differentiated iPSCs or muscle tissues were fixed with 4% paraformaldehyde and immediately washed three times in a solution of 0.1 M sodium phosphate buffer (pH 7.2), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% nonidet-P40. Samples were then transferred to a lacZ staining solution comprised of 0.1 M sodium phosphate buffer (pH 7.2), 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.01% sodium deoxycholate, and 0.02% nonidet-P40 and incubated overnight at 37° C. For immunostaining, cells were fixed with 2% paraformaldehyde, washed twice with 0.01% Triton-X 100 in PBS, and permeabilized in 0.2% Triton-X 100 in PBS for 10 minutes. Permeabilized cells were then blocked in 1% bovine serum albumin (BSA) for 30 minutes and incubated with primary antibodies diluted 1:200 in blocking solution for 2 hours. The following primary antibodies were used: anti-Nanog (Bethyl Laboratories, Montgomery, TX, www.bethyl.com), anti-Oct4 (Santa Cruz, Santa Cruz, CA, www.scbt.com), anti-stage specific embryonic antigen-1 (SSEA-1, Developmental Studies Hybridoma Bank, Iowa City, IA, dshb.biology.uiowa.edu), anti-MyoD (BD Pharmingen, San Diego, CA, www.bdbiosciences.com), and anti-Pax7 (Developmental Studies Hybridoma Bank). After incubation with primary antibodies, the cells were washed four times with 0.01% Triton-X 100 in PBS and incubated with secondary antibodies diluted 1:1000 in blocking solution for 2 hours. The following secondary antibodies were used: Alexa-488-conjugated anti-mouse IgG, Alexa 594-conjugated anti-mouse IgM, and Alexa 594-conjugated anti-rabbit IgG (all from Invitrogen). After

incubation with secondary antibodies, cells were washed four times with 0.01% Triton-X 100 in PBS and nuclei were counter stained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) for 20 minutes. Fluorescence images were captured by a DP-1 digital camera attached to an IX80 inverted fluorescence microscope with x20 and x40 LUCPlanFLN objectives and a BX51 fluorescence microscope with x20 and x40 UPlanFLN objectives (all from Olympus America, Center Valley, PA, www.olympusamerica.com). Photoshop CS2 (Adobe Systems, San Jose, CA, www.adobe.com) was used for image processing.

RT-PCR & Microarray Analysis

Total RNA was isolated from cells by TRIZOL (Invitrogen). Purified total RNA was reverse transcribed (Roche Applied Science: Transcriptor First Strand cDNA Synthesis Kit) and 25-35 cycles were performed (Eppendorf Thermal Cycler, Hauppage, NY, www.eppendorf.com) using the gene-specific primer pairs listed in Table 2. Optimal PCR cycles for each pair were determined by several different amplifications of the PCR products.

Mouse WG-6 v2.0 chip arrays (Illumina, San Diego, CA, www.illumina.com) containing ≈45,000 known mouse genes and expressed sequence tags were used for gene expression based lineage mapping. Data analysis of Illumina microarrays was performed using GeneSpring 3.2.2 software (Agilent, Santa Clara, CA, http://www.home.agilent.com).

Embryoid Body Formation

Undifferentiated iPSCs were cultured in one of two ways. For hanging drop experiments, cells were seeded on the lids of 100 mm petri dishes (BD Biosciences) at a density of 800, 2,000, or 4,000 cells per 20 µl of differentiation medium consisting of DMEM (Invitrogen) supplemented with 50 µg/ml penicillin/streptomycin (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma Aldrich), 5% horse serum (Invitrogen), and 10% FBS (Fisher Scientific). The embryoid bodies (EBs) were transferred to ultra-low attachment six-well plates (Corning Life Sciences, Corning, NY, www.corning.com) and cultured for an additional three days in the presence or absence of 50 ng/ml retinoic acid (Sigma Aldrich) or 15 ng/ml BMP4 (Sigma Aldrich). For suspension culture experiments, EBs were formed by plating cells directly into ultra-low attachment six-well plates in differentiation medium for three days followed by the addition of retinoic acid or BMP4 for another three day period. Following suspension culture, embryoid bodes were plated in differentiation medium onto 35 mm plates coated with 0.1% gelatin, and medium was replenished every other day. For myoblast co-culture experiments, equal numbers of myoblasts and iPSCs were seeded in hanging drops for three days and then plated onto 35 mm 0.1% gelatin coated plates in differentiation medium. For MyoD over expression experiments, differentiation medium was supplemented with 10 µg/ml of doxycycline during hanging drop formation.

Teratoma Formation

1 x 10^6 iPSCs cultured on MEF feeder cells were subcuntaneously injected into 2 to 3 month old *Nod:Scid* immunodeficient mice. Three weeks after injection, teratomas were fixed with 4% paraformaldehyde and then stained with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase as described above.

Cell Counts

All counts were performed using three independently derived myoblast iPS cell lines and three independently derived tail tip fibroblast iPS cell lines. Three replicates were used per cell line and one count was performed per replicate (n = 9). Images of stained plates or slides were captured randomly using the DP-1 digital camera and IX80/BX51 inverted fluorescence microscopes with the x20 and x40 LUCPlanFLN objectives described above. Cells were counted manually after a six by six grid was placed over the images using Photoshop CS2. Percentages of positive cells were calculated using the following formula: % positive cells = positive nuclei/total cell nuclei x 100.

Statistics

All experiments were performed using three independently derived myoblast and three independently derived tail tip fibroblast iPS cell lines. Three experimental replicates were performed per cell line and one count was performed per replicate (n = 9). All data are expressed as ± the standard error of the mean (SEM). Statistical significance was

analyzed by a two-tailed Student's *t* test, one-way analysis of variance (ANOVA), and two-way ANOVA. Values denote mean ± SEM. Asterisks and double asterisks denote experimental pairs where differences between the compared values were statistically significant ($p < 0.05$) and ($p < 0.01$) respectively.

Results

Generation of Myoblast and Tail Tip Fibroblast Derived Induced Pluripotent Stem Cells

The directed differentiation of stem cells is time consuming and often fails to produce the cell type of interest. Therefore, we established a simple system to streamline the detection of myogenic progenitors following iPS differentiation *in-vitro* and *in-vivo*. To this end, we generated several iPS clones from the myoblasts and tail tip fibroblasts of *Myf5-nlacZ* mice (Fig. 3). *Myf5-nlacZ* mice carry a lacZ reporter gene inserted at the ATG start codon of the Myf5 gene. Thus, lacZ expression should recapitulate endogenous Myf5 expression which marks myoblasts, satellite cells, and differentiating myotubes. The dissociated skeletal muscle of *Myf5-nlacZ* mice was subjected to magnetic activated cell sorting (MACS) to isolate $CD31^-/Sca\text{-}1^-/CD45^-/\alpha\text{-}Integrin\text{-}7^+$ myoblasts. Immunostaining of isolated myoblasts with anti-Pax7, anti-MyoD, and anti-desmin antibodies clearly showed that more than 99% of isolated cells were $Pax7^+$, $MyoD^+$, and $desmin^+$ myoblasts (Fig. 4). Myoblasts and tail tip fibroblasts then underwent retroviral infection with vectors carrying the Oct4, Sox2, c-Myc, and Klf-4 genes. ES cell-like colonies emerged within 7 to 28 days after infection, and sub-clones were established through the transfer of colonies to fresh feeders. Both myoblast iPS cells (Mb-iPS cells) and tail tip fibroblast iPS cells (Fb-iPS cells) expressed markers of pluripotency, including nanog, Sox2, and SSEA-1 (Fig. 5). Following the differentiation of *Myf5-nlacZ* Mb-iPS and Fb-iPS cells, X-gal staining was able to detect the presence of $Myf\text{-}5\text{-}nlacZ^+$ myogenic progenitor cells (Fig. 6).

Phenotypic differences exist in the gene expression profiles and differentiation ability of iPS cell lines due to technical difficulties in the reprogramming process.[94,95,103,104] Therefore, all experiments in this study utilized three independently established Mb-iPS and Fb-iPS clones to eliminate the possibility that outcomes were the result of clonal variation. In addition, recent evidence indicates that serial passage of iPS cells diminishes epigenetic memory.[99,105] Therefore, iPS cells used in each experiment did not exceed 3-4 passages in culture.

Embryoid Body Design and Cell Number Affect the Myogenic Differentiation of iPS Cells

Embryoid bodies (EBs) are cell aggregates that partially reproduce the three-dimensional structure of the developing embryo and allow pluripotent stem cells to differentiate into all three germ layers.[106,107] Recently, it was shown that EB size affects the production of differentiated cell types due to cell to cell interactions.[108-110] Initially, we sought to optimize EB conditions to enhance the myogenic potential of iPS cells.

The standard methods of EB formation are suspension culture and hanging drop.[111,112] During suspension culture, stem cells self-aggregate in low attachment culture dishes. In contrast, the formation of hanging drops on the surface of inverted lids allows control of EB cell number and size. To determine if the mechanism of EB generation affects the myogenic differentiation of iPS cells, Mb-iPS or Fb-iPS cells were incubated in suspension culture or hanging drops for three days, plated onto gelatin-coated dishes, and assessed for the presence of Myf5-nlacZ^{+} myogenic progenitors after two weeks of

differentiation (Fig. 7). At day seven, hanging drop culture led to a four-fold increase in the number of Myf5-nlacZ$^+$ cells differentiated from Mb-iPS cells (0.24 ± 0.049% hanging drop, 0.057 ± 0.023% suspension culture). After two weeks of differentiation, hanging drop culture doubled the number of differentiated Myf5-nlacZ$^+$ Mb-iPS cells (hanging drop 0.28 ± 0.026%, suspension culture 0.13% ± 0.023) and increased the number of differentiated Myf5-nLacZ$^+$ Fb-iPS cells four-fold (hanging drop 0.21 ± 0.024%, suspension culture 0.053 ± 0.020%). This represented a significant increase in the number of Myf5-nlacZ$^+$ cells produced by hanging drop culture compared to suspension culture for both Mb-iPS and Fb-iPS cells (n = 9, $p < 0.05$). Taken together, these results suggest that hanging drop formation of EBs enhances the myogenic differentiation of iPS cells.

To determine whether EB size influences the myogenic differentiation of iPS cells, Mb-iPS and Fb-iPS cells were seeded in hanging drops at various cell densities and examined for the presence of Myf5-nlacZ$^+$ cells after two weeks of differentiation (Figs. 3 & 8). Increasing the number of cells per hanging drop from 8 x 10^2 to 2 x 10^3 resulted in a two-fold increase in the number of Myf5-nlacZ$^+$ Fb-iPS cells (8 x 10^2 cells 0.12 ± 0.012%, 2 x 10^3 cells 0.27 ± 0.032%). An additional increase from 2 x 10^3 to 4 x 10^3 cells resulted in fewer Myf5-nlacZ$^+$ cells after two weeks of differentiation (2 x 10^3 cells 0.27 ± 0.032%, 4 x 10^3 cells 0.19 ± 0.0058%). The differences between all three groups were significant (n =9, $p < 0.05$). Taken together, these results indicate that hanging drop cell number affects the myogenic potential of iPS cells, and divergence from optimal cell densities may result in a reduction of myogenic progenitors during iPS cell differentiation.

MB-iPS Cells Exhibit Lineage Specific Differentiation *In-Vitro*

Hepatocyte, pericyte, cardiomyocyte, B cell, granulocyte, and beta cell derived iPS cells have previously been shown to display preferential lineage specific differentiation *in-vitro*.[85,97-101] To assess whether Mb-iPS cells preferentially differentiate into their tissue of origin, Mb-iPS and Fb-iPS cells were seeded in hanging drops and examined for Myf5-nlacZ$^+$ cells after two weeks of culture (Figs. 6 & 9). In agreement with previous findings, Mb-iPS cells produced more Myf5-nlacZ$^+$ progenitors than Fb-iPS cells at days seven and fourteen of differentiation (D7 Mb-iPS 0.26 ± 0.015%, D7 Fb-iPS 0.13 ± 0.0033%, D14 Mb-iPS 0.31 ± 0.023%, D14 Fb-iPS 0.15 ± 0.018%). This represented a significant increase in the number of Myf5-nlacZ$^+$ cells produced by Mb-iPS cells compared to Fb-iPS cells ($n = 9$, $p < 0.05$). These results suggest that Mb-iPS cells display lineage specific differentiation *in-vitro*, producing significantly more myogenic progenitors than Fb-iPS cells.

Retinoic Acid Does Not Significantly Enhance Skeletal Myogenesis of iPS Cells

Retinoic acid (RA) influences a variety of development processes and has been shown to increase skeletal myogenesis of teratocarinomas and C2 mouse myoblasts.[116-118] Recently, RA was shown to increase myogenesis in differentiating cultures of P19 and mouse ES cells.[47] To determine if RA enhances the myogenic differentiation of iPS cells, Mb-iPS and Fb-iPS cells were seeded in hanging drops in the presence of 50 ng/mL of RA for three days and examined for the presence of Myf5-nlacZ$^+$ cells after two weeks of culture (Fig. 6 & 10). Retinoic acid treatment led to a slight increase in the number of

MB-iPS and TTF-iPS Myf5-nlacZ$^+$ cells after two weeks of differentiation (MB-iPS RA$^+$ 0.27 ± 0.060%, MB-iPS RA$^-$ 0.19 ± 0.0072%, TTF-iPS RA$^+$ 0.33 ± 0.12%, TTF-iPS RA$^-$ 0.17 ± 0.069%). However, this increase was not significant (n = 9, $p > 0.05$). In addition, MB-iPS cells failed to produce more Myf5-nlacZ$^+$ cells than TTF-iPS cells following RA treatment (MB-iPS RA$^+$ 0.27 ± 0.060%, TTF-iPS RA$^+$ 0.33 ± 0.12%). Taken together, these results indicate that RA treatment does not significantly enhance the myogenic differentiation of iPS cells.

Myoblast Co-culture Does Not Enhance Myogenic Differentiation of iPS Cells

Myoblast co-culture has been shown to induce myogenic differentiation of a variety of cell types.[119-122] To determine if myoblast co-culture induces myogenic differentiation of iPS cells, equal numbers of myoblasts and Mb-iPS or Fb-iPS cells were seeded in hanging drops and assessed for the presence of Myf5-nlacZ$^+$ cells after two weeks of differentiation on gelatin coated culture dishes (Fig. 11). Surprisingly, myoblast co-culture resulted in fewer Myf5-nlacZ$^+$ cells at day seven for both Mb-iPS cells (0.12 ± 0.018% myoblast$^+$, 0.26 ± 0.015 myoblast$^-$) and Fb-iPS cells (0.11 ± 0.012% myoblast$^+$, 0.13 ± 0.0033 myoblast$^-$). Myoblast co-culture also produced fewer Myf5-nlacZ$^+$ cells for both Mb-iPS cells (0.21 ± 0.015% myoblast$^+$, 0.31 ± 0.023 myoblast$^-$) and Fb-iPS cells (0.14 ± 0.014% myoblast$^+$, 0.15 ± 0.018 myoblast$^-$) at day fourteen. However, these differences were not significant. Taken together, these results suggest that myoblast co-culture does not enhance myogenesis of differentiating iPS cells.

Bone Morphogenic Protein 4 (BMP4) Treatment Enhances the Myogenic Differentiation of iPS Cells

Although Mb-iPS cells produced twice as many Myf5-nlacZ$^+$ cells as Fb-iPS cells, the total number of Myf5-nlacZ$^+$ cells produced by Mb-iPS and Fb-iPS cells remained extremely low after two weeks of differentiation. Therefore, we attempted to direct iPS cell differentiation through the use of developmentally relevant cytokines involved in myogenesis.

Ectopic expression of BMP4, a key regulator of skeletal muscle myogenesis, was recently shown to increase the number of satellite cells and myoblasts in the developing chick embryo.[113] In addition, treatment with BMP4 followed by its early removal was shown to enhance myogenesis in differentiating mouse ES cells.[48] To determine whether BMP4 enhances the myogenic differentiation of iPS cells, Mb-iPS and Fb-iPS cells were seeded in hanging drops in the presence of 10 ng/mL of BMP4 for three days and examined for the presence of Myf5-nlacZ$^+$ cells after two weeks of culture (Figs. 6 & 12). In the absence of BMP4, Mb-iPS cells produced significantly more Myf5-nlacZ$^+$ cells than Fb-iPS cells after two weeks of differentiation, supporting our previous results (Mb-iPS 0.42 ± 0.14%, Fb-iPS 0.25 ± 0.091%, n = 9, $p < 0.05$). However, BMP4-mediated differentiation produced twice the number of Myf5-nlacZ$^+$ cells for both Mb-iPS (BMP4$^+$ 0.92 ± 0.037%, BMP4$^-$ 0.42 ± 0.14%) and Fb-iPS cells (BMP4$^+$ 0.56 ± 0.073%, BMP4$^-$ 0.25 ± 0.091%), and this increase was statistically significant (n = 9, $p < 0.05$). In addition, BMP4-treated Mb-iPS cells produced significantly more Myf5-nlacZ$^+$ cells than

BMP4-treated Fb-iPS cells (Mb-iPS 0.92 ± 0.037%, Fb-iPS 0.56 ± 0.073%, n = 9, $p < 0.05$), confirming their lineage specific differentiation.

To confirm the presence of myogenic progenitors following BMP4-mediated iPS cell differentiation, Mb-iPS and Fb-iPS cells were seeded in hanging drops for three days in the presence of BMP4, plated on gelatin coated dishes, and immunostained with anti-Pax7, anti-MyoD, and anti-Myogenin antibodies after two weeks of differentiation (Figs. 13 & 14). Pax7 is a marker of early myogenic progenitor cells, while MyoD and myogenin are markers of myoblasts and more committed skeletal muscle cells. Mb-iPS cells produced significantly more $Pax7^+$, $MyoD^+$, and $Myogenin^+$ cells than Fb-iPS cells, confirming our previous results (Mb-iPS $Pax7^+$ 3.44 ± 0.96%, Mb-iPS $MyoD^+$ 3.85 ± 1.1%, Mb-iPS $Myogenin^+$ 5.9 ± 1.4%, Fb-iPS $Pax7^+$ 0.41 ± 0.076%, Fb-iPS $MyoD^+$ 0.93 ± 0.44%, Fb-iPS $Myogenin^+$ 0.55 ± 0.29%, n = 9, $p < 0.05$). Taken together, these results suggest that BMP4 treatment enhances the myogenic differentiation of iPS cells, and demonstrate that Mb-iPS cells display lineage specific differentiation *in-vitro*, producing myogenic progenitors more efficiently than Fb-iPS cells.

Ectopic Expression of MyoD Enhances iPS Cell Skeletal Myogenesis

Although BMP4-mediated differentiation resulted in a two-fold increase in the number of Myf5-nlacZ^+ cells, Myf5-nlacZ^+ cells only constituted about 1% of the total cell population after two weeks of differentiation (Mb-iPS 0.92 ± 0.037%, Fb-iPS 0.56 ± 0.073%). Therefore, we attempted to enhance the myogenic differentiation potential of iPS cells by ectopically expressing MyoD, a potent regulator of skeletal muscle

myogenesis, able to convert a variety of somatic cell types to a muscle-like state.[114] To this end, we crossed *Myf5-nlacZ* mice with *Rosa26-rtTA/iMyoD* mice to generate *Myf5-nlacZ/Rosa26-rtTA/iMyoD* mice, referred to henceforth as *Myf5-nlacZ/iMyoD* mice. *Rosa26-rtTA/iMyoD* mice carry a reverse tetracycline trans-activator (rtTA) gene inserted into the Rosa26 locus and a tetracycline response element (TRE) controlled MyoD gene. Administration of doxycycline induces MyoD expression in skeletal muscle and X-gal staining can detect the presence of Myf-5-nlacZ^{+} myogenic progenitor cells following differentiation of *Myf5-nlacZ/iMyoD* iPS cells (Fig. 6). CD31^{-}/Sca-1^{-}/CD45^{-}/α-Integrin-7^{+} myoblasts and tail tip fibroblasts were isolated from *Myf5-nlacZ/iMyoD* mice and underwent retroviral infection with vectors carrying the Oct4, Sox2, c-Myc, and Klf-4 genes. However, *Myf5-nlacZ/iMyoD* cells displayed remarkable resistance to reprogramming (Table 1).

Previously, our laboratory demonstrated that suppression of MyoD, expressed at relatively high levels in myoblasts, is required for iPS cell reprogramming, and that myoblasts display lower reprogramming efficiencies than fibroblasts.[102] In accordance with our previous data, only a single ESC-like colony was obtained following *Myf5-nlacZ/iMyoD* fibroblast infection, and *Myf5-nlacZ/iMyoD* myoblasts failed to produce any ES cell-like colonies, despite repeated reprogramming efforts. Basal expression levels, or "leakiness," remain the predominant problem for tetracycline inducible mammalian expression systems and may account for the inefficiency in the generation of *Myf5-nlacZ/iMyoD* iPS cells, as *Myf5-nlacZ/iMyoD* fibroblasts retained basal expression of MyoD (Fig. 15).

To determine whether ectopic expression of MyoD enhances iPS skeletal muscle myogenesis, *Myf5-nlacZ/iMyoD* iPS cells were seeded in hanging drops for three days in the presence or absence of doxycycline, and examined for Myf5-nlacZ^{+} cells after eighteen days of differentiation (Figs. 6 & 16). Doxycycline administration led to an increase in the number of Myf5-nlacZ^{+} cells after only six days of differentiation (dox^{+} 11.7 ± 0.0455, dox^{-} 2.04 ± 0.00602). After eighteen days of differentiation, nearly half of doxycyline induced cells expressed Myf5 (41.2 ± 0.0752). This represented a significant increase in the number of Myf5-nlacZ^{+} cells compared to control cells (dox^{+} 41.2 ± 0.0752, dox^{-} 9.76 ± 0.0268, n = 9, $p < 0.05$). Surprisingly, un-induced *Myf5-nlacZ/iMyoD* Fb-iPS cells produced a large number of Myf5-nlacZ^{+} progenitors after two weeks of differentiation (*Myf5-nlacZ/ iMyoD* Fb-iPS 2.56 ± 0.0212%, Myf-5-nlacZ Mb-iPS 0.28 ± 0.026%, *Myf-5-nlacZ Fb-iPS* 0.21 ± 0.024%, n = 9, $p < 0.05$, see Fig. 9). However, this may be due to the "leakiness" associated with the mammalian expression system utilized, as *Myf5-nlacZ/ iMyoD Fb-iPS* cells retained MyoD gene expression in the absence of doxycycline (Fig. 15).

MB-iPS Cells Exhibit Lineage Specific Differentiation *In-Vivo*

In order to evaluate the myogenic potential of Mb-iPS and Fb-iPS cells *in-vivo*, 1 x 10^{6} Mb-iPS or Fb-iPS cells were subcuntaneously injected into 2 to 3 month old *Nod:Scid* immunodeficient mice. Teratomas were removed three weeks later and examined for the presence of Myf5-nlacZ^{+} cells (Fig. 17). Teratoma size did not appear different based on gross visual inspection. Teratomas generated by Mb-iPS cells

displayed more Myf5-nlacZ^{+} cells than teratomas generated by Fb-iPS cells (Mb-iPS 577.3 ± 28.3 cells, Fb-iPS 864 ± 18.9 cells). However, this difference was not significant (n = 3, $p > 0.05$). This result suggests that Mb-iPS cells display lineage specific differentiation *in-vivo,* producing myogenic progenitors more efficiently than Fb-iPS cells.

MB-iPS Cells Retain Expression of Myogenic Genes

Induced pluripotent stem cells derived from a variety of cell types retain donor-cell gene expression profiles.[94-96] To determine whether Mb-iPS cells retained expression of myogenic genes, the cDNAs from three Mb-iPS and Fb-iPS cell lines were isolated and subjected to RT-PCR (Fig. 18). Both Mb-iPS and Fb-iPS cells expressed multiple markers of pluripotency, characteristic of embryonic stem cells, including Oct4, Sox2, KLF4, nanog, Eras, Esg-1, and FGF4. However, Mb-iPS cells retained gene expression of key myogenic regulatory factors, including MyoD, Myf5, myogenin, Mash4, Pax3, and Pax7, as well as the cell adhesion molecule and satellite cell marker M-cadherin, while Fb-iPS cells did not. These results indicate that Mb-iPS cells possess a gene expression profile consistent with an ES cell-like state, but retain donor-cell gene expression of key myogenic factors.

To directly compare the gene expression of Mb-iPS and TTF-iPS cell lines with myoblasts and embryonic stem cells as a reference, global gene expression analyses with microarray chips were performed (Fig. 19). As expected, iPS cells derived from myoblasts were globally similar to embryonic stem cells while remaining distinct from

parental myoblast cells, as shown by hierarchical cluster analyses. However, the expression profile for Mb-iPS cells was distinct from that of TTF-iPS cells, indicating a unique expression profile indicative of an intermediate state of reprogramming and retention of an epigenetic memory.

Discussion

Somatic cell reprogramming is an imperfect process, and reprogrammed cells retain methylation patterns that influence gene expression and functionality. Hepatocyte, pericyte, cardiomyocyte, B cell, granulocyte, and beta cell derived iPS cells have previously been shown to retain donor-cell gene expression and display preferential lineage specific differentiation.[85,97-101] In accordance with these studies, our results indicate that Mb-iPS cells express key myogenic transcription factors including Pax7, MyoD, and myogenin, and in the presence of BMP4, display enhanced myogenic differentiation *in-vitro* and *in-vivo* compared to Fb-iPS cells (Fig. 20).

The efficiency of direct iPS and ES skeletal muscle differentiation is poor, presumably due to the lack of dermomyotomal cells within EBs. By harnessing the epigenetic memory of Mb-iPS cells, we have generated a simple protocol for the derivation of myogenic progenitors, which is further enhanced by ectopic expression of MyoD. This protocol is reproducible across several Mb-iPS cell lines, excluding errors due to clonal variation. Importantly, after only fourteen days, a significant proportion of differentiating Mb-iPS cells expressed Pax7, Myf5, MyoD, and Myogenin, key regulators of skeletal muscle myogenesis.

Recently, RA was shown to increase myogenesis in differentiating cultures of P19 and mouse ES cells, while over-expression of BMP4 led to inhibition of P19 cell myogenesis.[47] However, we were unable to reproduce these results, and RA treatment did not lead to a significant difference in the number of skeletal muscle progenitors derived

from either Mb-iPS or Fb-iPS cells. Although previous studies have led to the conclusion that BMP4 is a repressor of skeletal muscle differentiation, it was recently reported that ectopic expression of BMP4 in developing chick embryos led to an increase in the number of fetal muscle fibers and satellite cells.[113] In addition, BMP4 increases proliferation of $Pax3^{+}$ cells, and its early removal from differentiating mouse ES cells was recently shown to increase the production of myogenic progenitors in embryoid bodies.[48,75] Developmental myogenesis is a complex spatiotemporal process, requiring a delicate balance between proliferation and differentiation, and the timing of growth factor administration, as well as the use of iPS cells in place of ES cells, may account for the BMP4-mediated myogenesis seen here.[72,123,124] Differentiating myoblasts have been shown to produce numerous factors involved in myogenesis, including several proteins involved in the down regulation of BMP-mediated myogenic pathways, and the myoblast secretome may account for the slight decrease in the number of Myf5-nlacZ^{+} cells observed during co-culture experiments. [129]

Of importance to the present study are recent findings which indicate myoblast-derived iPS cells do not display biased differentiation potential.[125] However, lab specific effects and reprogramming methods have been shown to result in phenotypic differences between iPS cell lines.[94,95,103,104] The secondary reprogramming system used by Tan et al. to generate iPS cells was more efficient than the retroviral delivery mechanism utilized here.[126] As such, it may have resulted in more complete reprogramming, and the subsequent loss of epigenetic memory exhibited by Mb-iPS cells in the current study. In addition, their analysis was restricted to *in-vivo* MHC expression in teratomas after only

14-21 days. As myosin heavy chain (MHC) is a terminal marker of muscle differentiation expressed at later stages of *in-vitro* iPS skeletal muscle differentiation, it is possible that the detection of earlier markers of myogenesis seen here were overlooked. Moreover, our findings indicate that a large number of Myf5-nlacZ^{+} myogenic precursors are present during *in-vitro* differentiation of Mb-iPS cells, as early as seven days after hanging drop formation.

Recently, transplantation of iPS-derived myogenic precursors was shown to result in functional improvement of the muscular dystrophy phenotype in *mdx* mice following the ectopic expression of Pax7.[44-46] The protocols established here are suitable for the purification of myogenic pre-cursors using fluorescent activated cell sorting, and the ability of Mb-iPS derived skeletal muscle progenitors to contribute to muscle fibers and improve muscle function will require further analysis. Although our protocol for myogenic BMP4-mediated differentiation omitted the use of transgenes, Mb-iPS cells were derived using retroviral transmission of the four Yamanaka factors, and alternative reprogramming methods, such as the use of small inhibitory micro RNAs or the transduction of recombinant proteins, will be required for the completely transgene-free derivation of myogenic progenitors for clinical applications.[127,128] Myoblasts are readily available from patient populations via routine muscle biopsies. Therefore, human Mb-iPS cells may provide a means to the production of large numbers of myogenic progenitors for efficient DMD cell therapy.

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Appendix 1: Tables

Cell Type	*Time to Colony Formation (Days)*	*# iPSC Colonies*	*Efficiency (Colonies/Cell Seeded)*
TTF-Myf5-LZ	13	32	0.016%
MB-Myf5-LZ	18	14	0.0070%
TTF-iMyoD/Myf5-LZ	24	1	0.0005%
MB-iMyoD/Myf5-LZ	No data	0	0%

Table 1. Efficiency and time to colony formation for induced pluripotent stem cells derived from myoblasts (MB) and tail tip fibroblasts (TTF). Myoblasts required longer reprogramming times and produced fewer iPS colonies than fibroblasts.

Gene	*Forward Primer*	*Reverse Primer*	*BP*
MyoD	CACGACTGCTTTCTTCACCACTCC	CGCAGGTCTGGTGAGTCGAAACAC	298
Myf5	TGCCATCCGCTACATTGAGAG	CCGGGGTAGCAGGCTGTGAGTTG	353
Myogenin	CCCTATTTCTACCAGGAGCCCCAC	GCGCAGGATCTCCACTTTAGGCAG	348
Mash4	CTACAAAACCGGTGAAAGGAAACG	GAAACGATGGACACCAGAAGCTAC	324
Pax3	GCCAGGGCCGAGTCAACCAG	GATCCGCCTCCTCCTCTTCTCCTT	414
Pax7	GCTTGGTGGGGTCTTCATCAACGG	CTGAGCACTCGGCTAATCGAACTC	357
M-Cadherin	CCACAAACGCCTCCCCTACCCACTT	TCGTCGATGCTGAAGAACTCAGGGC	447
Oct-4	TCTTTCCACCAGGCCCCCGGCTC	TGCGGGGCGGACATGGGGAGATCC	224
Sox2	TAGAGCTAGACTCCGGGCGAGAAACC	TTGCCTTAAACAAGACCACGAAA	297
KLF4	GCGAACTCACACAGGCGAGAAACC	TCGCTTCCTCTTCCTCCGACACA	711
Nanog	CTCAGCACCAGTGGAGTATCCCAG	ATGCGCATGGCTTTCCCTAGTGGC	343
Eras	CCAGGGACAACGGAGGCTACATTC	CTTGCTTGATTCGGCCACAGCCTC	387
Esg-1	CGTGGGTGAAAGTTCCTGAAGACC	CTCGATACACTGGCCTAGCTCCAG	317
FGF4	CGTGGTGAGCATCTTCGGAGTGG	CCTTCTTGGTCCGCCCGTTCTTA	197
β-actin	CACCCTGTGCTGCTCACCGAGGCC	ACCGCTCGTTGCCAATAGTGATGA	463

Table 2. Primer pairs used for RT-PCR.

Appendix 2: Figures

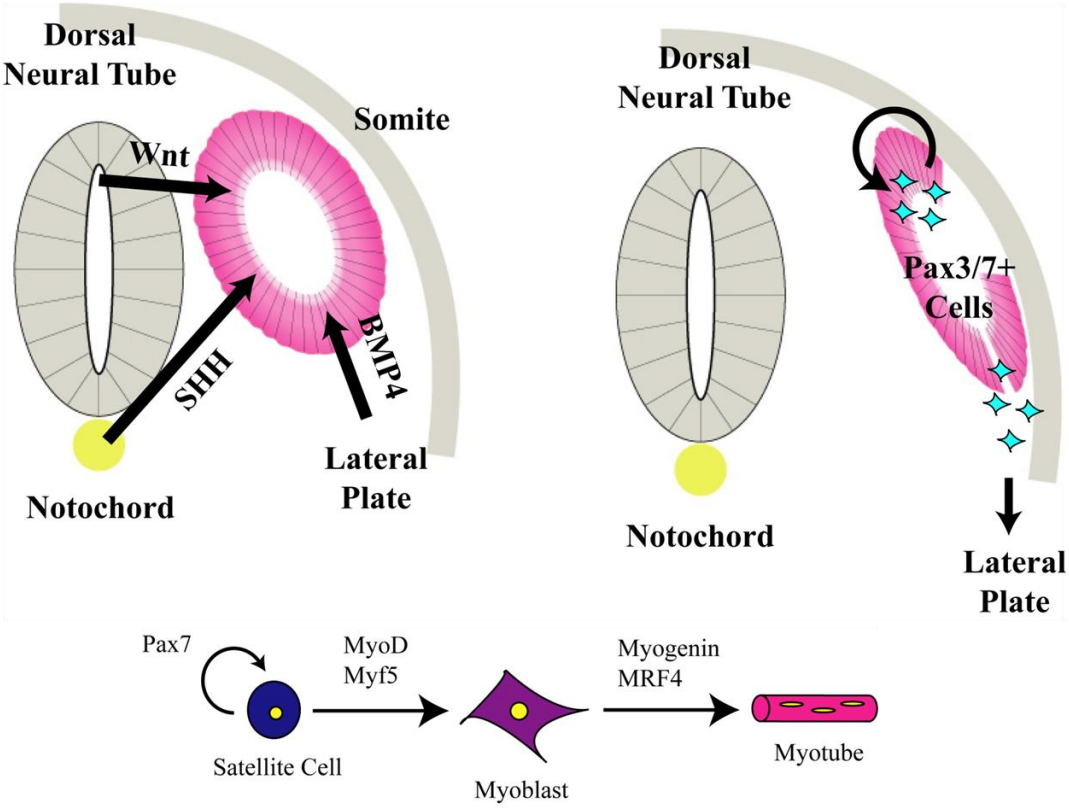

Figure 1. During embryogenesis, gradients of signaling molecules direct the development of satellite cells, in particular Wnt from the dorsal neural tube, sonic hedge hog (SHH) from the notochord, and BMP4 from the lateral plate. Pax3/7^{+} satellite cells infiltrate the somite to seed the muscles of the trunk and migrate outwards to seed the muscles of the limb.

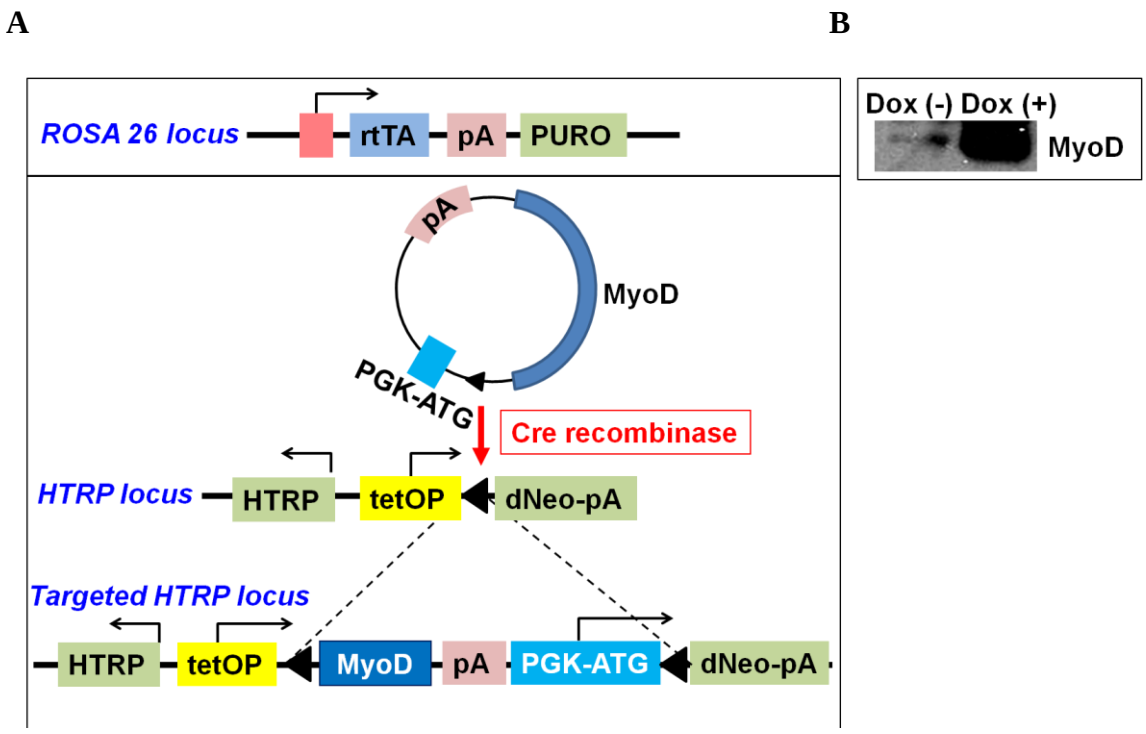

Figure 2. Strategy for creating Doxycycline (Dox)-inducible *MyoD* (*iMyoD*) mice. (A) Cre-loxP recombination in embryonic stem cells (ESCs). *Rosa26-rtTA* mice were used as a source of transactivator. (B) Administration of doxycycline led to an increase in the expression of MyoD in skeletal muscle.

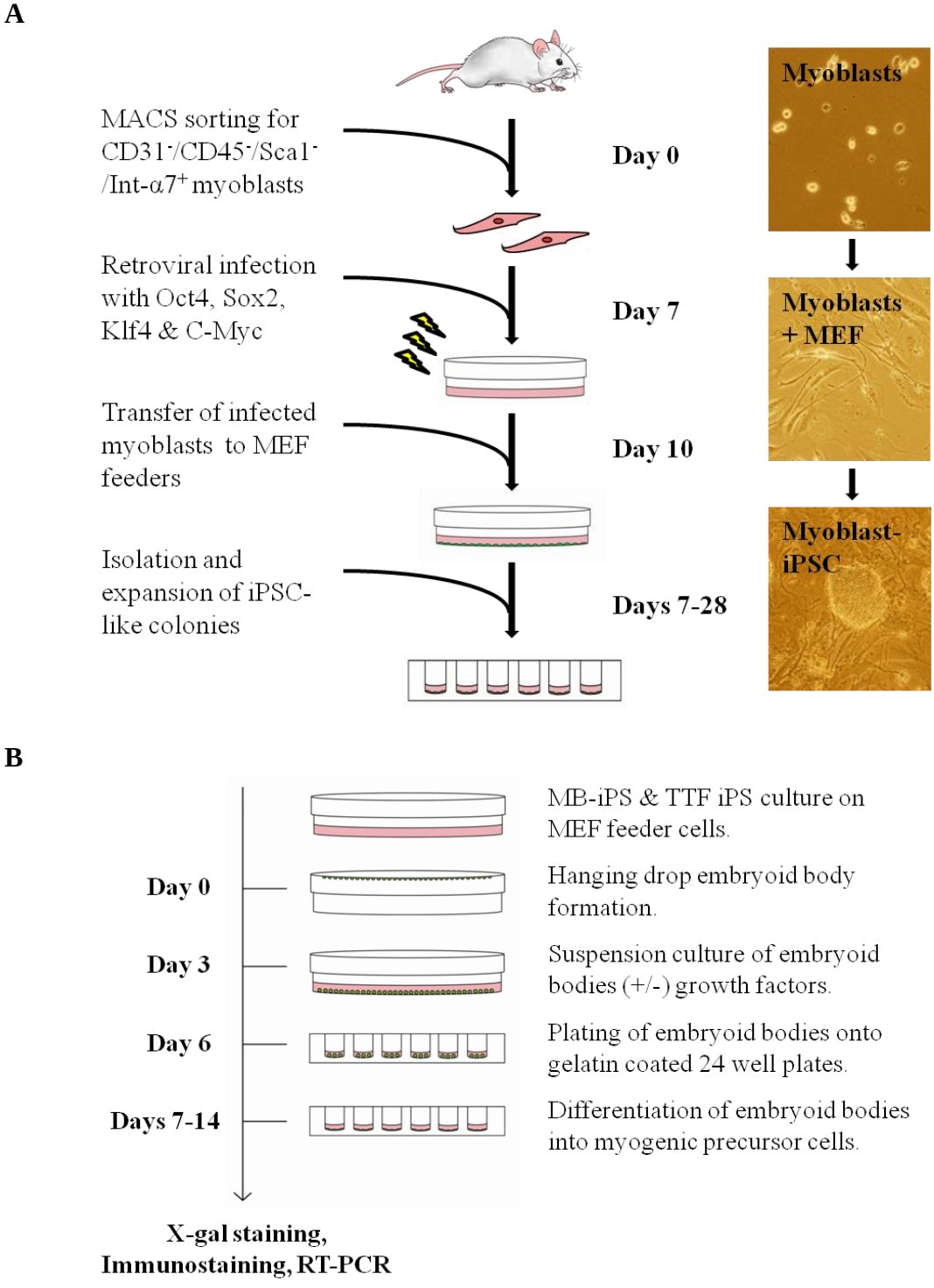

Figure 3. Protocols for the derivation of myoblast (MB) and tail tip fibroblast (TTF) derived iPS cells (A) and their directed differentiation into myogenic progenitors (B).

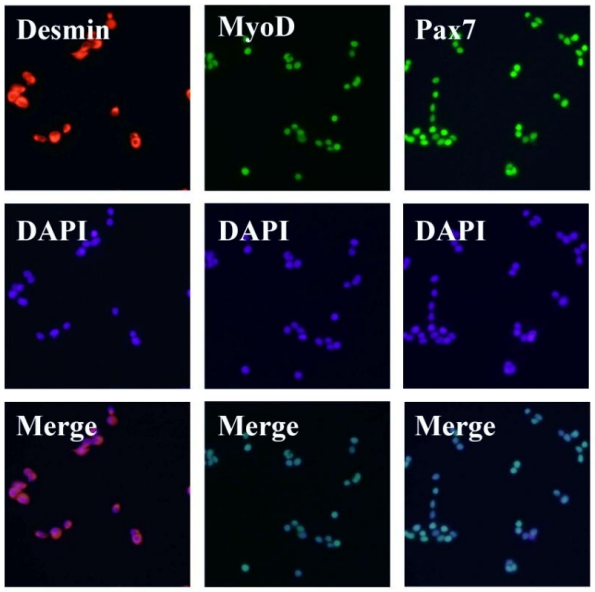

Figure 4. Myf5-nlacZ myoblasts were isolated using MACS sorting for $CD31^-/CD45^-/Sca1^-/Integrin\text{-}\alpha7^+$ cells and stained for desmin, myoD, and pax7 to ensure that induced pluripotent stem cells were derived from myoblasts. 99.8% of cells were $desmin^+$, $myoD^+$, $pax7^+$ myoblasts.

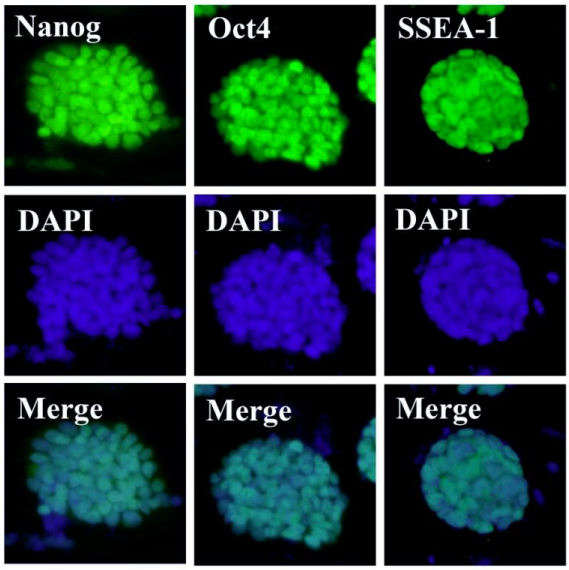

Figure 5. Following reprogramming and cloning, induced pluripotent stem cell colonies were stained for markers of pluripotency including nanog, Oct4, and SSEA-1 to ensure reversion to an embryonic-like state. All colonies examined were positive for nanog, Oct4, and SSEA-1

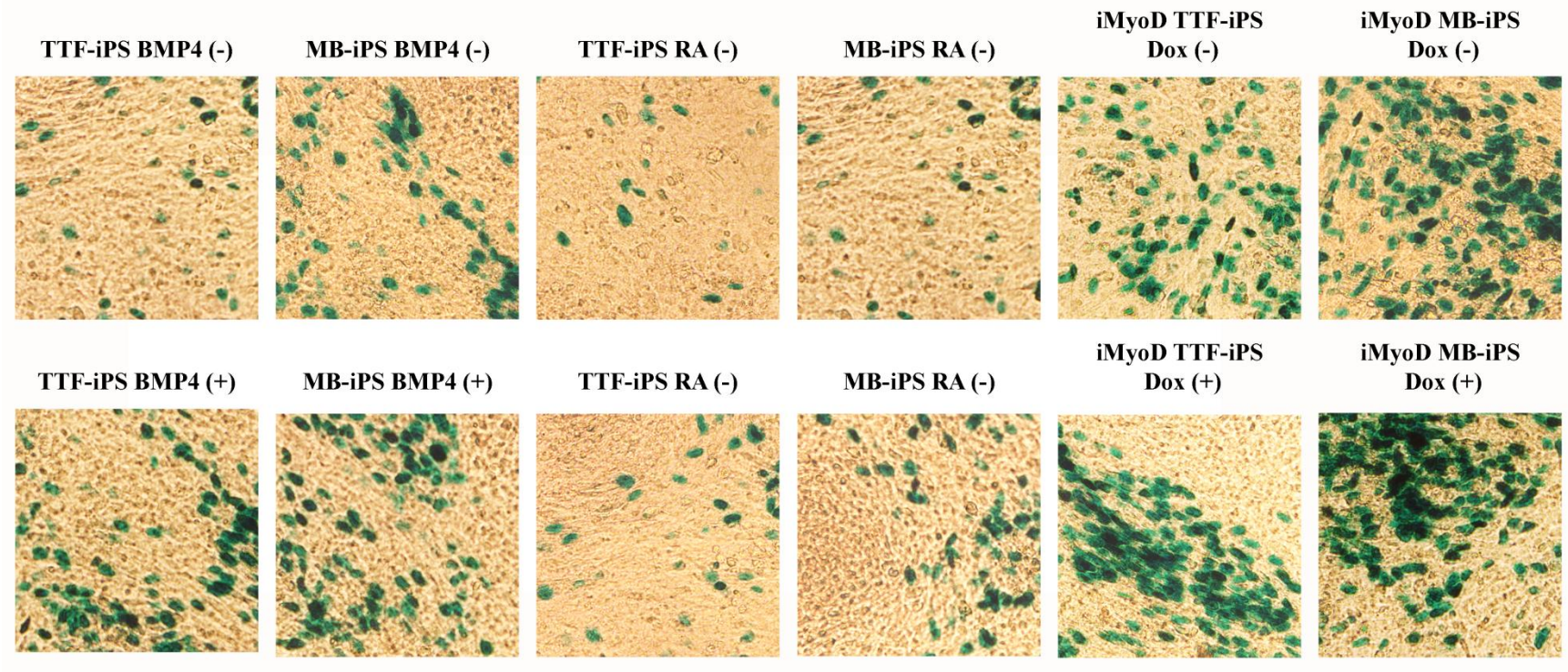

Figure 6. Representative images of X-gal staining for Myf5-nlacZ^{+} cells following differentiation of tail tip fibroblast (TTF) and myoblast (MB) derived iPS cells in the presence or absence of BMP4, retinoic acid (RA), or doxycycline (Dox).

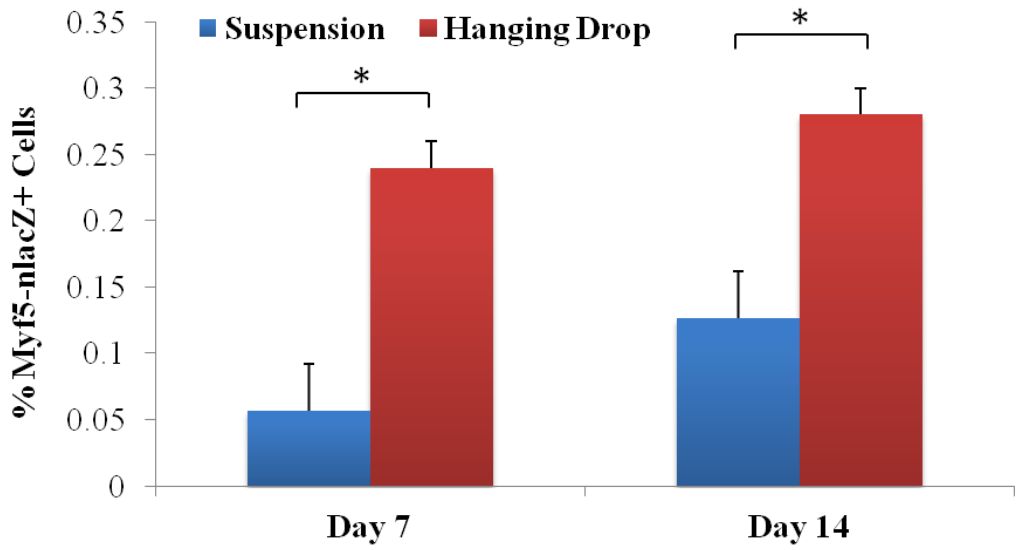

Figure 7. Comparison of hanging drop and suspension culture methods for the directed differentiation of induced pluripotent stem (iPS) cells. Hanging drop culture allows for control of embryoid body size and shape and significantly increased the number of Myf5-nlacZ^{+} myogenic progenitors at days 7 and 14 of differentiation.

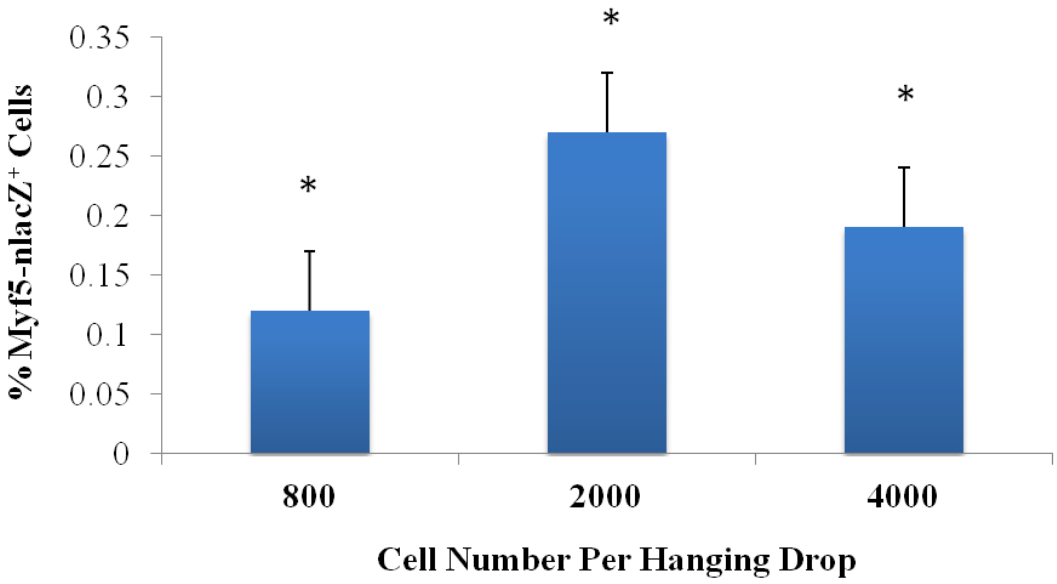

Figure 8. Cell number per hanging drop affects the myogenic differentiation of tail tip fibroblast (TTF) derived iPS cells after two weeks of differentiation. Hanging drops were seeded at varying cell densities and examined for the presence of lacZ^{+} cells after 14 days of differentiation.

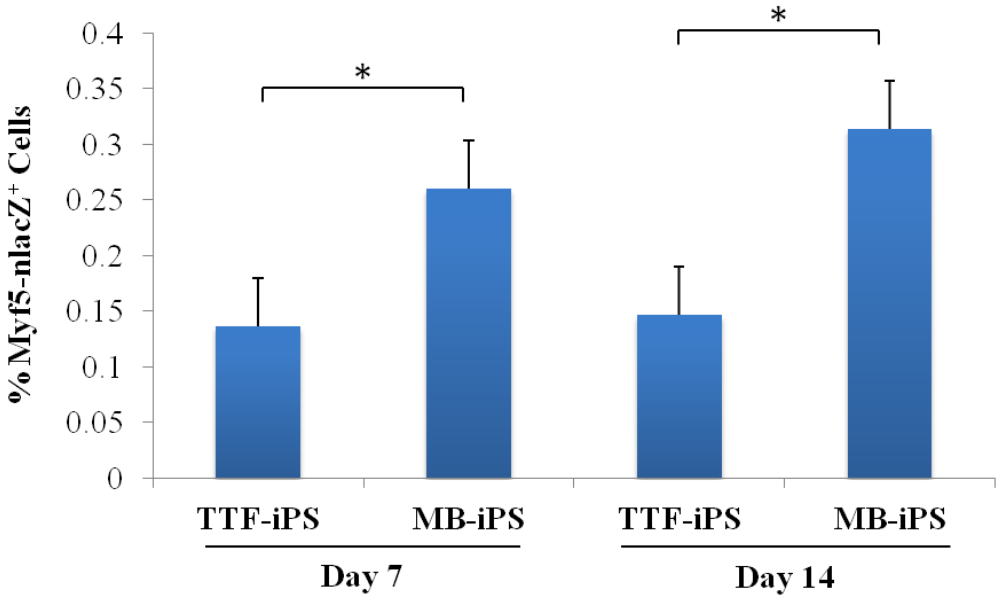

Figure 9. Comparison of the myogenic potential of tail tip fibroblast (TTF) and myoblast (MB) derived iPS cells. MB-iPS cells produced significantly more Myf5-nlacZ^{+} cells than TTF-iPS cells at both days 7 and 14 of differentiation.

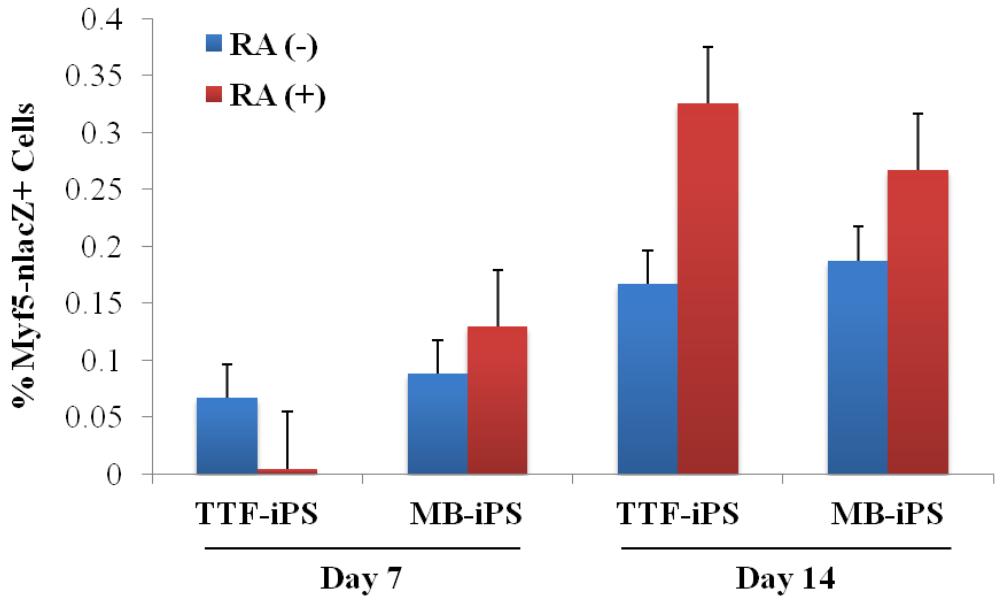

Figure 10. Retinoic acid-mediated differentiation of tail tip fibroblast (TTF) and myoblast (MB) derived iPS cells. Treatment with retinoic acid did not result in an increase in the number of MB-iPS or TTF-iPS Myf5-nlacZ^{+} cells.

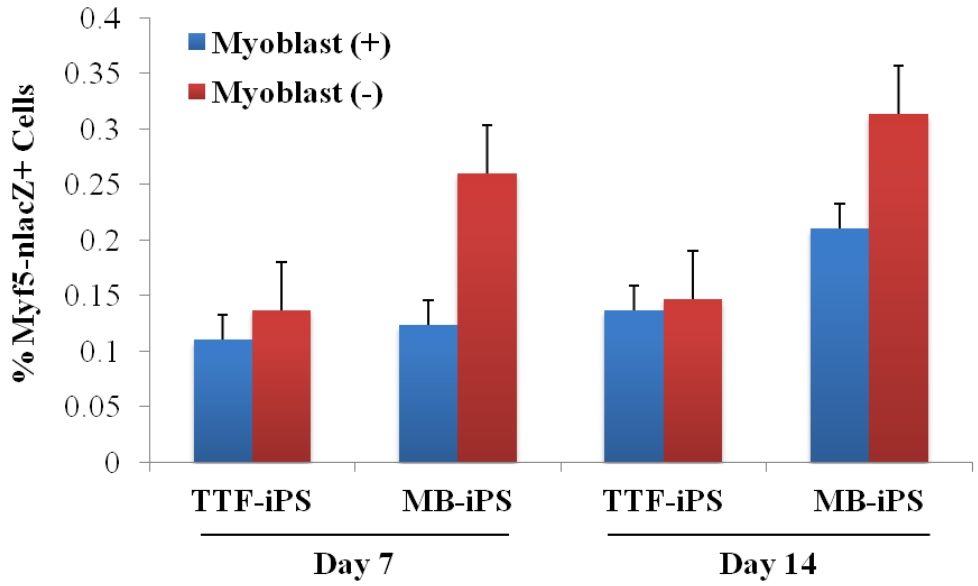

Figure 11. Co-culture of myoblast (MB) and tail tip fibroblast (TTF) derived iPS cells with myoblasts. Co-culture did not result in a significant difference in the number of Myf5-nlacZ^{+} myogenic progenitors.

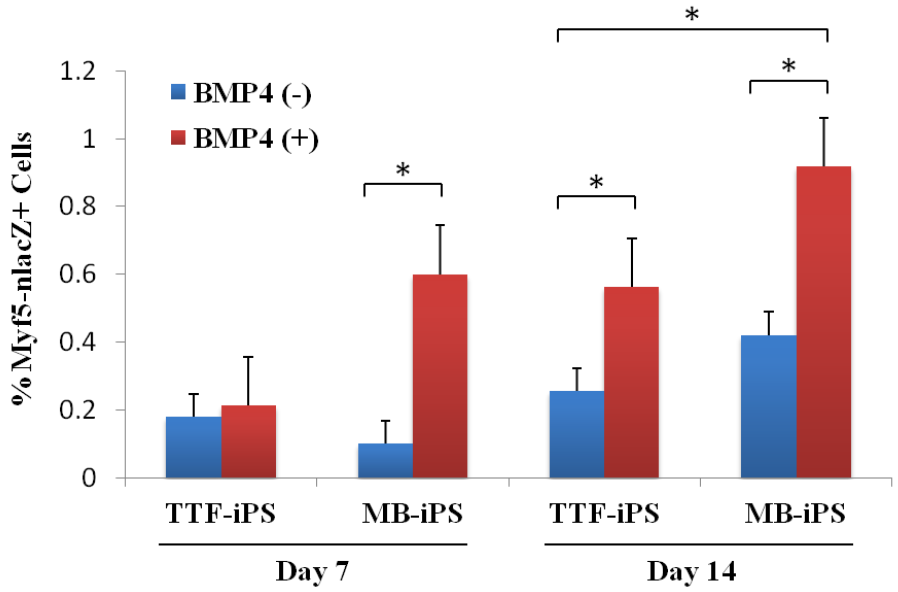

Figure 12. BMP4-mediated differentiation of tail tip fibroblast (TTF) and myoblast (MB) derived iPS cells. BMP4 treatment led to a significant increase in the number of lacZ^{+} cells at day 7 for MB-iPS cells and at day 14 for TTF-iPS and MB-iPS cells. MB-iPS cells also produced significantly more Myf5-nlacZ^{+} cells than TTF-iPS cells following treatment with BMP4.

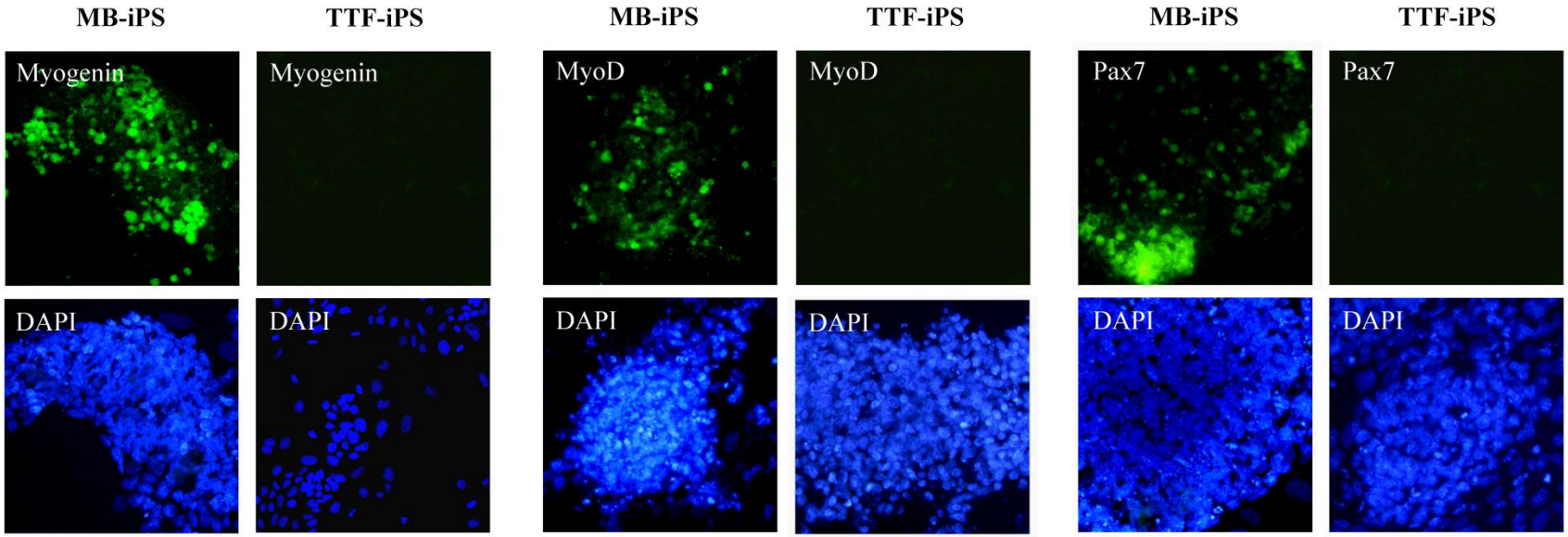

Figure 13. Immunostaining of tail tip fibroblast (TTF) and myoblast (MB) derived iPS cells for Pax7, MyoD, and Myogenin markers of satellite cells, myoblasts, and terminally differentiated myotubes respectively. MB- iPS cells produced more Myogenin^{+}, Pax7^{+}, and MyoD^{+} cells than TTF-iPS cells following their directed differentiation with BMP4.

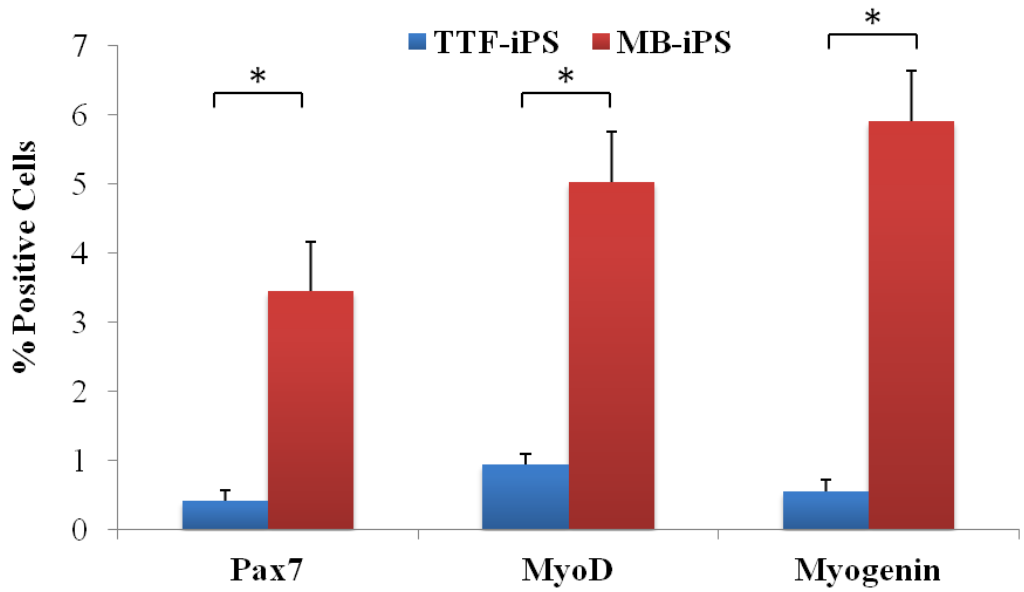

Figure 14. Quantification of Pax7^{+}. MyoD^{+}, and Myogenin^{+} cells following BMP4-mediated differentiation of tail tip fibroblast (TTF) and myoblast (MB) derived iPS cells.

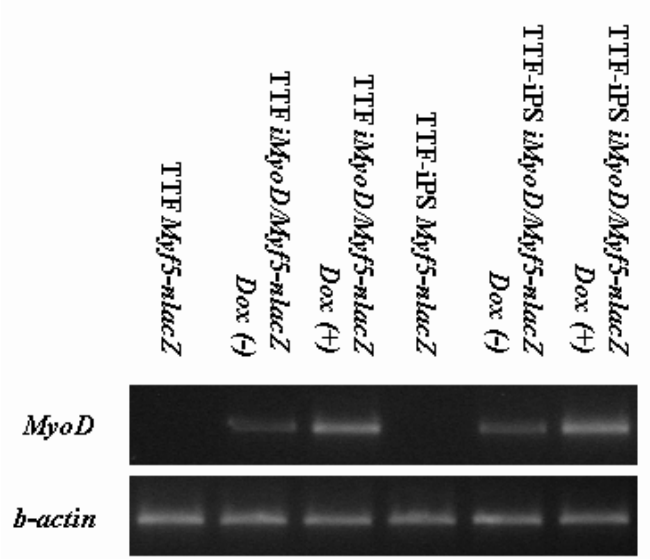

Figure 15. RT-PCR for tail tip fibroblast (TTF) and myoblast (MB) derived iPS cells from *Myf5-nlacZ* and *iMyoD/Myf5-nlacZ* transgenic mice. TTF-iPS cells from *iMyoD/Myf5-nlacZ* mice, as well as their parental fibroblasts, expressed MyoD even in the absence of doxycycline, which may account for the fibroblasts' resistance to reprogramming and the TTF-iPS cells increased myogenic potential during differentiation.

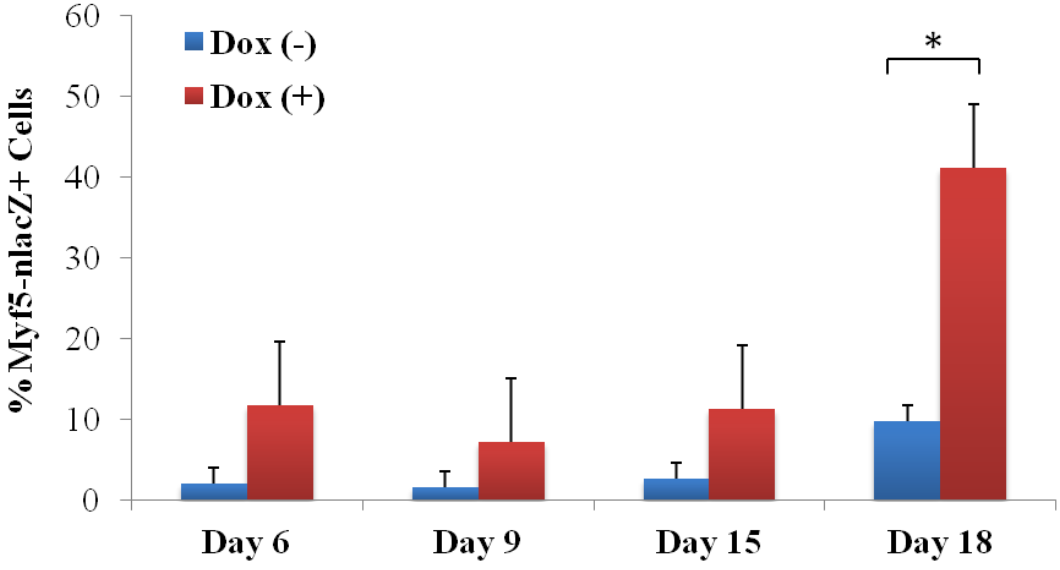

Figure 16. Differentiation of Fb-iPS cells carrying a tetracycline-inducible MyoD expression cassette. Doxycycline administration led to the ectopic expression of MyoD and a significant increase in the number of $Myf5^{+}$ cells at day 18.

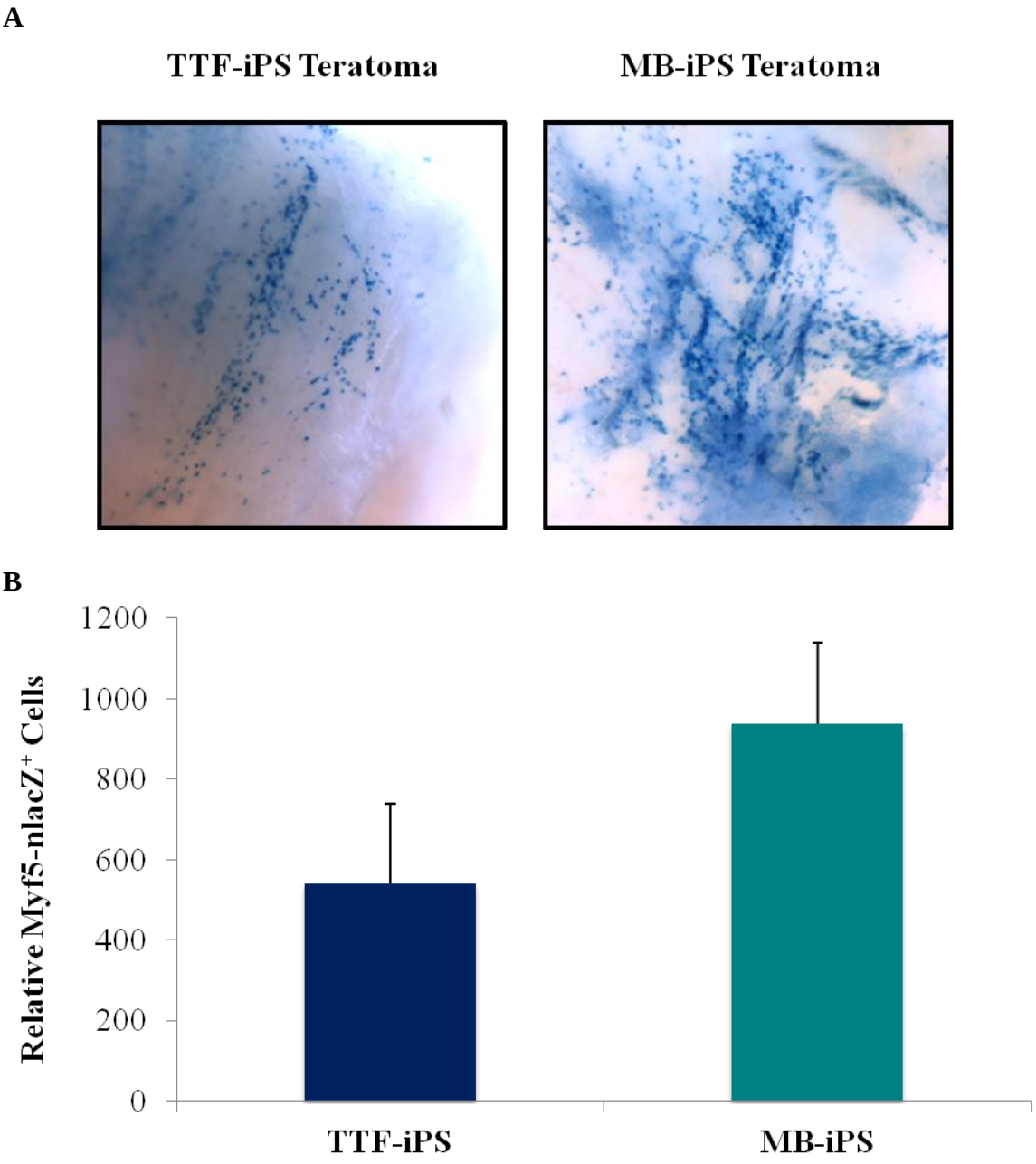

Figure 17. *In-vivo* differentiation of tail tip fibroblast (TTF) and myoblast (MB) derived iPS cells in teratomas. (A) X-gal staining of Myf5-nlacZ^{+} cells in teratomas. (B) Quantification of the number of Myf5-nlacZ^{+} cells in MB-iPS and TTF-iPS derived teratomas.

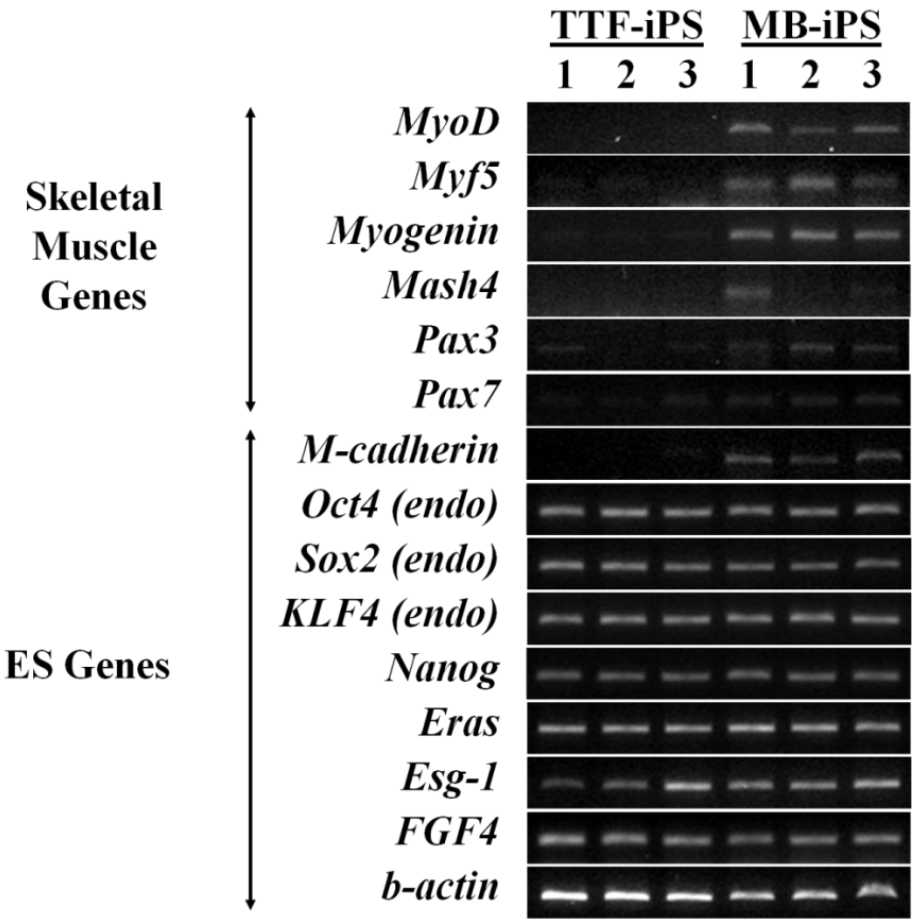

Figure 18. RT-PCR for myoblast (MB) and tail tip fibroblast (TTF) derived iPS cells prior to differentiation. MB-iPS cells retained expression of myogenic regulatory factors, including myoD, Myf5, and Myogenin but also expressed markers of pluripotency including Oct4, Sox2, Klf4, and Nanog.

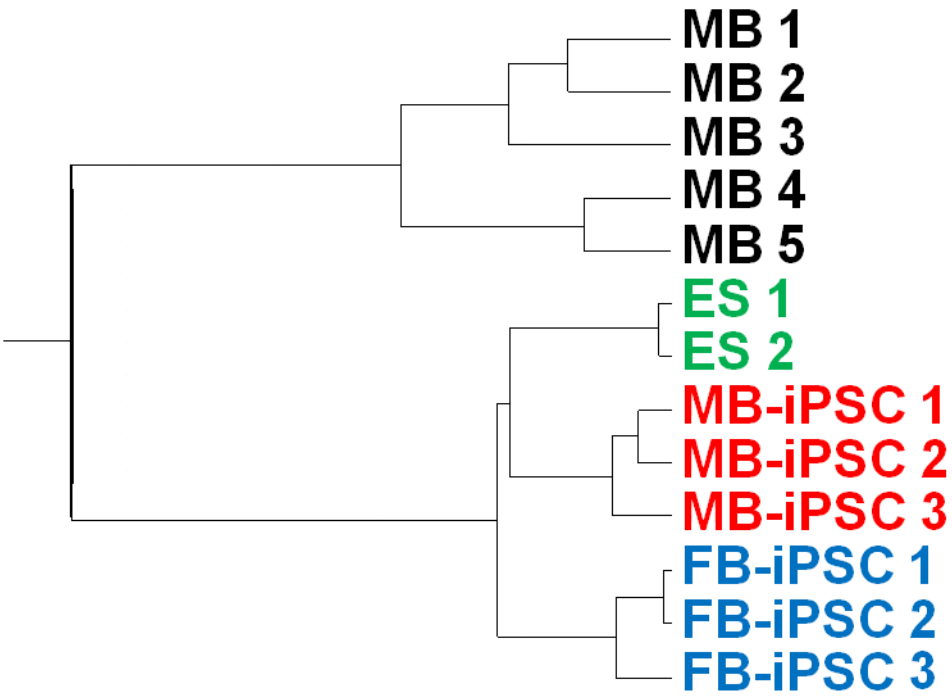

Figure 19. Dendrogram of hierarchical gene expression clustering for myoblasts (MB), embryonic stem (ES) cells, tail tip fibroblast (TTF) derived iPS cells, and myoblast derived iPS cells. MB-iPS cell gene expression was distinct from the gene expression of both ES cells and TTF-iPS cells, which may account for their enhanced myogenic potential.

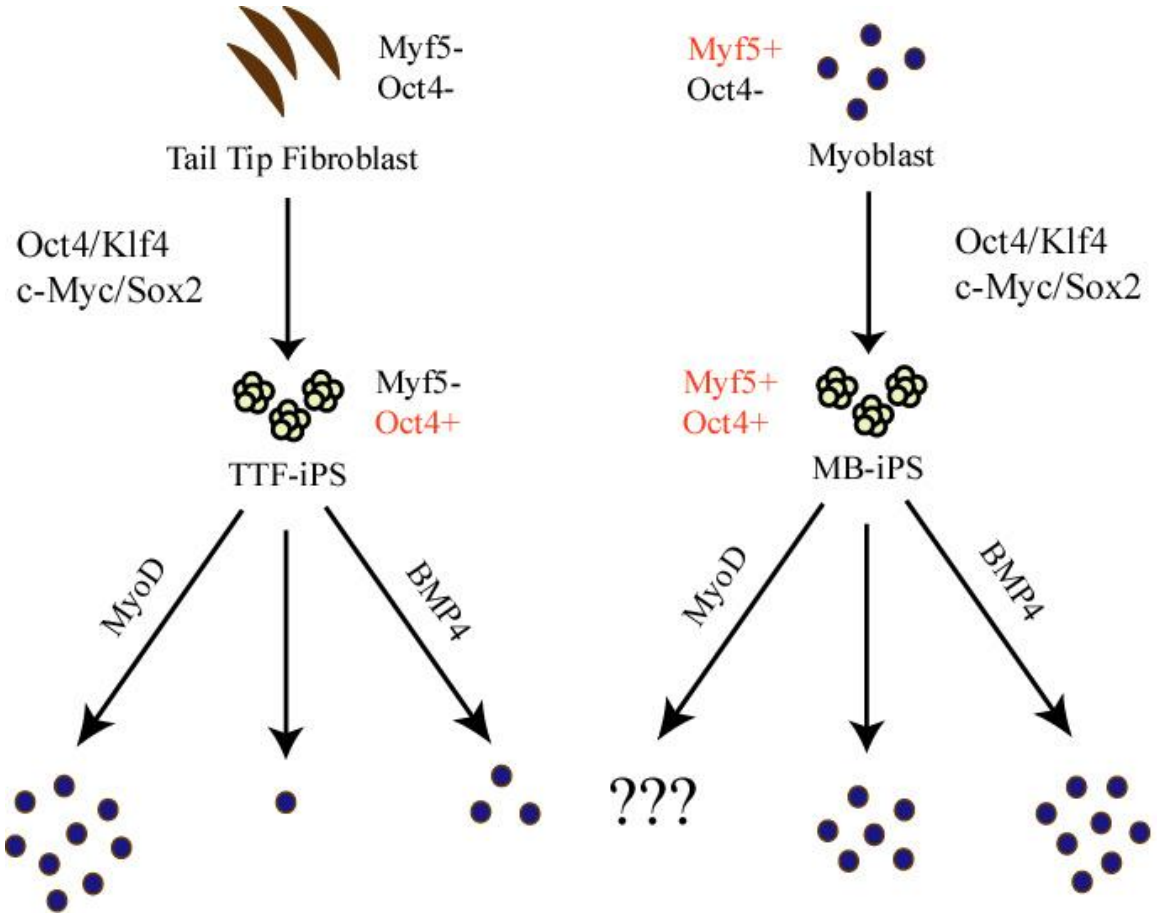

Figure 20. Graphical abstract of myoblast derived induced pluripotent stem cell lineage specific differentiation. Reprogramming of both tail tip fibroblasts and myoblasts leads to expression of pluripotency genes. MB-iPS cells but not TTF-iPS cells retain expression of myogenic regulatory proteins including Myf5 and display higher efficiencies when differentiating into myogenic progenitors. This myogenic differentiation is enhanced through administration of BMP4 or the ectopic expression of MyoD.