DIRECT REPROGRAMMING OF FIBROBLASTS INTO MUSCLE OR NEURAL LINEAGES BY USING SINGLE TRANSCRIPTION FACTOR WITH OR WITHOUT MYOD TRANSACTIVATION DOMAIN

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Abstract

The generation of induced pluripotent stem cells (iPSCs) from somatic cells has opened new doors for regenerative medicine by overcoming the ethical concerns surrounding embryonic stem (ES) cell research. However, iPSC technology presents several safety concerns, such as the potential risk of tumor formation, that have caused apprehension for use of iPSCs in humans. One such approach is "direct reprogramming" which can bypass the iPSC or pluripotent stage and obtain tissue-specific cell types from somatic cells. In this study, we examined whether an important transcription factor involved in myogenesis (Pax3) or neurogenesis (NeuroD1) alone can directly reprogram the mouse embryonic fibroblasts (MEFs) into myogenic or neurogenic lineages, respectively. In addition, we created fusion transcription factors (Pax3 or NeuroD1) with the potent MyoD transactivation domain (MDA) that could facilitate radical acceleration of reprogramming into the desired cell type through chromatin modification compared to wild-type factors. Here, we showed that Pax3 can reprogram MEFs towards a myogenic lineage and that MDA-Pax3 further enhances this myogenic reprogramming event. In addition, ectopic expression of NeuroD1 and MDA-NeuroD1 is able to induce neurogenic genes in MEFs, suggesting the partial neurogenic conversion of MEFs. Furthermore, we also showed that the ectopic expression of NeuroD1 but not MDA-NeuroD1 in myoblasts could suppress myogenic differentiation. These data suggest that single gene transduction such as Pax3 or NeuroD1 will become a feasible therapeutic approach for neuro- and muscle degenerative diseases, respectively.

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1. INTRODUCTION

Over the years, the nuclear transfer studies by John Gurdon and experiments by Yamanaka and his colleagues demonstrated that the differentiated state of somatic cells is not irreversibly locked[1,2]. Yamanaka and his colleagues discovered that transduction into fibroblasts using retroviral expression vectors encoding for transcription factors highly enriched in embryonic stem (ES) cells (Sox2, Oct4, c-Myc and Klf4), now often referred to as the "Yamanaka factors", led to the cells, known as iPSCs (induced pluripotent stem cells) to acquire pluripotent-like phenotypes [3,4]. The use of iPSCs for regenerative medicine can eliminate ethical concerns surrounding ES cell research. However, iPSC technology presents several safety concerns such as the potential risk of tumor formation (due to the presence of oncogenes in the reprogramming cocktail) and the genetic and epigenetic aberrations they carry have caused apprehension for use of iPSCs in humans[5,6]. To overcome these difficulties, researchers are seeking alternative reprogramming method that could translate into clinical application. One such approach is "direct reprogramming", which can bypass the iPSC or pluripotent stage (see Fig.1).

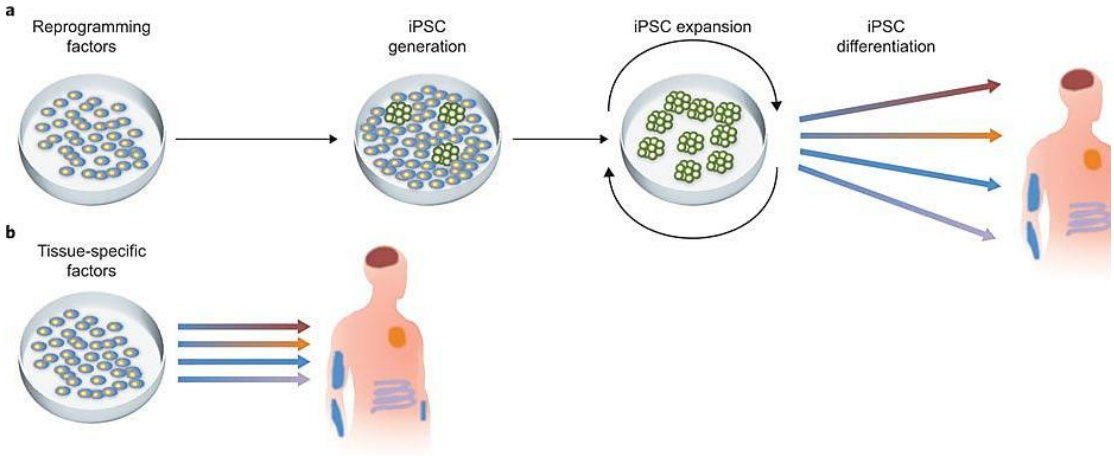

Figure 1. Reprogramming strategies allowing for generation of specific cell type.
(**a**) Generation of iPSCs allows for an unlimited supply of pluripotent cells, which further can be differentiated into specific cell types. (**b**) Direct lineage conversion allows for the

generation of specific cell types defined by the transcription factor method of reprogramming, bypassing the pluripotent state[6].

Direct lineage conversion or direct reprogramming is the process by which one cell type can be converted into another in the absence of any intermediate states[1]. The proof-of-principle for direct lineage conversion was demonstrated by Davis and his colleagues, when they successfully converted fibroblasts into myoblasts through ectopic expression of a single transcription factor MyoD, establishing the possibility of initiating the direct fate switch without returning to a pluripotent state[7]. Since then, many groups have been successful in using the transcription factor based direct reprogramming to obtain different cell lineages by ectopic expression of a combination of transcription factors (Fig. 2)[8].

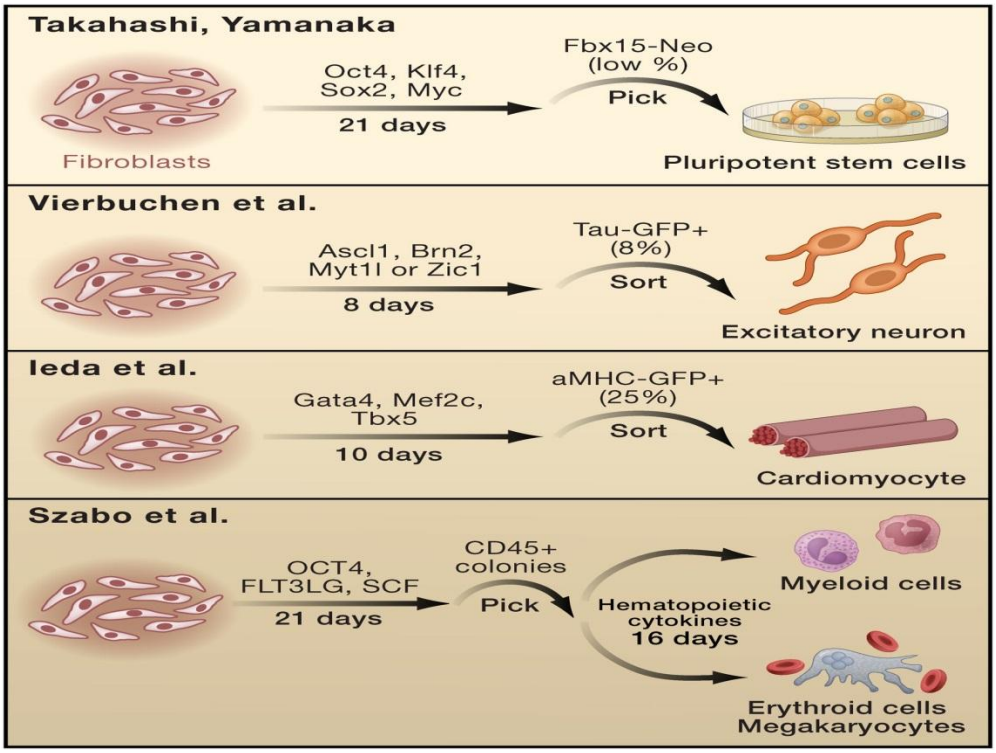

Figure 2. Schematic showing the use of direct reprogramming by ectopic expression of transcription factors[8]

Overexpression of transcription factors has been known to "open" the epigenetic state that is favorable for the generation of desired cell types by changes in the extracellular signals, without reversion to pluripotency[6,9].The phenotypic changes observed in the case

of direct conversion are a matter of hours or days, in contrast to days or weeks when using iPSCs to generate a cell lineage of interest[10-13].The other advantage of direct conversion is that it generates a unipotent post-mitotic population of cells which in theory can reduce the risk of teratoma formation after transplantation, in contrast to iPSC derived cells which may contain undifferentiated or multipotent cells[14,15]. One of the major weaknesses of iPSCs, which may be overcome by direct reprogramming, is the age of the desired tissue type[8]. The current protocols generate iPSC derived cells that may not correspond to the particular age of human tissue development. This is an important challenge in modeling human diseases such as Alzheimer's or Parkinson's disease that affect at later stages of human life[16].

The potential advantage of direct reprogramming is the overall time required to obtain the desired cell type and simplicity of the differentiation conditions. On the other hand, disease modeling using iPSCs takes time from generation stage to differentiating pluripotent stem cells[8]. Another benefit of direct reprogramming is the minimum steps required to change the epigenetic status of cell that can be reset or rearranged. In contrast, iPSC generation requires the complete change in epigenetic status to revert back to pluripotent stage.

Finally, one promising strategy which can prove advantageous is that reprogramming can be applied *in situ* for therapeutic purposes. The proof-of-principle concept was given by Zhou et al. in 2008, when they ectopically expressed transcription factors to convert exocrine cells to beta-cell like lineages in the pancreas of mice[8,17]. The *in vivo* direct reprogramming method was also used by Qian et al. in 2012 where they converted murine cardiac fibroblasts into functional cardiomyocytes, owing to the therapeutic potential of this approach[18]. However, for the direct reprogramming to be applied *in situ*, stable infection and delivery methods of the reprogramming factors have to be developed for *in vivo* direct cell conversion. Some of the major differences in reprogramming strategies are summarized in Table 1.

Reprogramming strategy	SCNT	iPSC	Direct lineage conversion
Time frame	Hours–days	Weeks–months	Hours–days
Efficiencies	Moderate	Very low	High
Differentiation potential	Pluripotent	Pluripotent	Unipotent
Requirement for cell proliferation	None	Yes	None
Risk for teratoma	None	High	Low
Mechanisms	De-differentiation	De-differentiation	Trans-differentiation

Table 1. Comparison of the different reprogramming strategies. SCNT, somatic cell nuclear transfer [1]

In addition to direct *in vivo* conversion, direct reprogramming has other applications such as :

- Disease modeling – to study the characteristics of a particular disease *in vitro*.
- Drug screening – to test new drugs and study their effect on disease phenotype.
- Toxicity tests – to study the side effects of newly developed drugs (Fig. 3)[19].

The method of direct reprograming can be applicable to tissues which are non-regenerative such as nervous and cardiac tissue. As the direct reprogramming method bypasses pluripotent state, samples from patients can be collected in huge numbers at once and be studied in parallel for genome wide disease modelling[19].

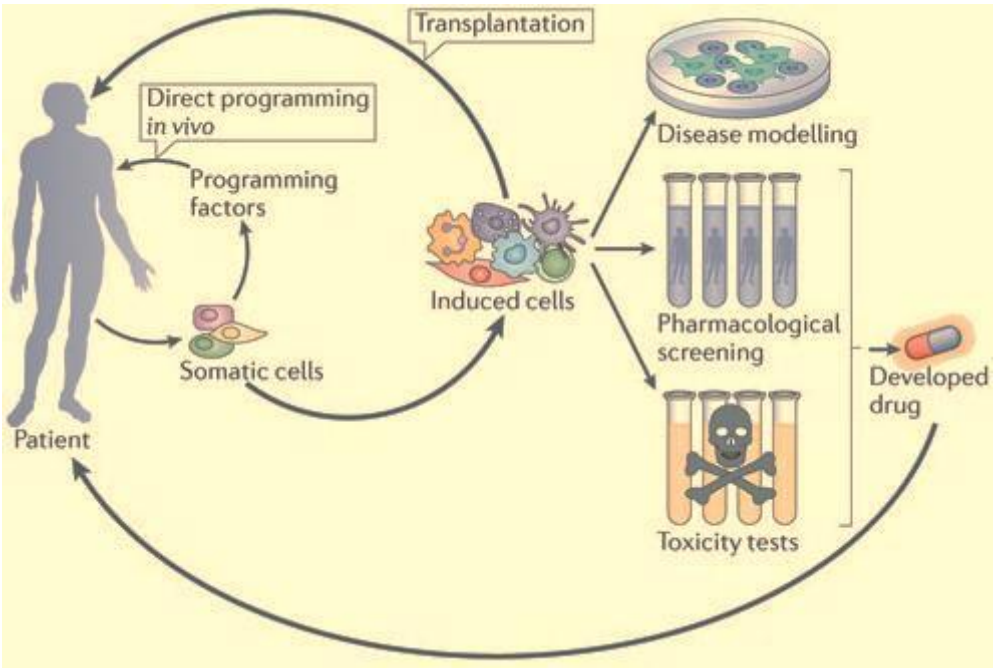

Figure 3. Schematic showing the biomedical application of direct cell fate conversion[19]

As with any therapeutic stem technology, the need to improve the strategies for facilitating adult lineage reprogramming remains to be one of the near-term objectives. One such strategy is the use of chemicals such as HDAC (histone deacetylases) inhibitors that loosen the chromatin structure enabling easy access for reprogramming factors, thereby enhancing cellular reprogramming[20,21]. The cells are thought to be most vulnerable to epigenetic change during uncoiling of DNA wrapped around nucleosome, to provide an open chromatin network for the reprogramming factors. Direct cell fate conversion may be stimulated by cell proliferation[21,22]. Although inhibitors that loosen chromatin can be used, naturally occurring chromatin modifiers such as transactivation domains found in DNA binding transcription factors, which act as powerful gene activators by initiating the program of cell differentiation, have been used as a potent transcriptional activation (transactivation) model in gene reporter assays and yeast two hybrid assays[23].

One such potent transactivation domain (TAD) is MyoD TAD, one of the domains of MyoD that mediates transcriptional activation of genes in repressive chromatin during myogenesis[24]. MyoD belongs to the myogenic basic helix-loop-helix (bHLH) transcription factors that play essential roles in myogenic specification, differentiation and maintenance during muscle development and regeneration[25]. MyoD is a classic example of a master control gene for cell differentiation in the sense that transduction of this gene alone is sufficient to activate the whole genetic program of muscle differentiation in non-muscle cells[26,27]. MyoD, like any other DNA-binding transcription factor, contains two important domains: a DNA-binding domain (DBD) and a transcriptional activation domain (TAD). The DBD is structurally conserved in the basic domain of the bHLH(basic helix-loop-helix) motif (Fig. 4A) that directs the transcription factor to the target gene by recognizing a specific DNA sequence termed "E-box"

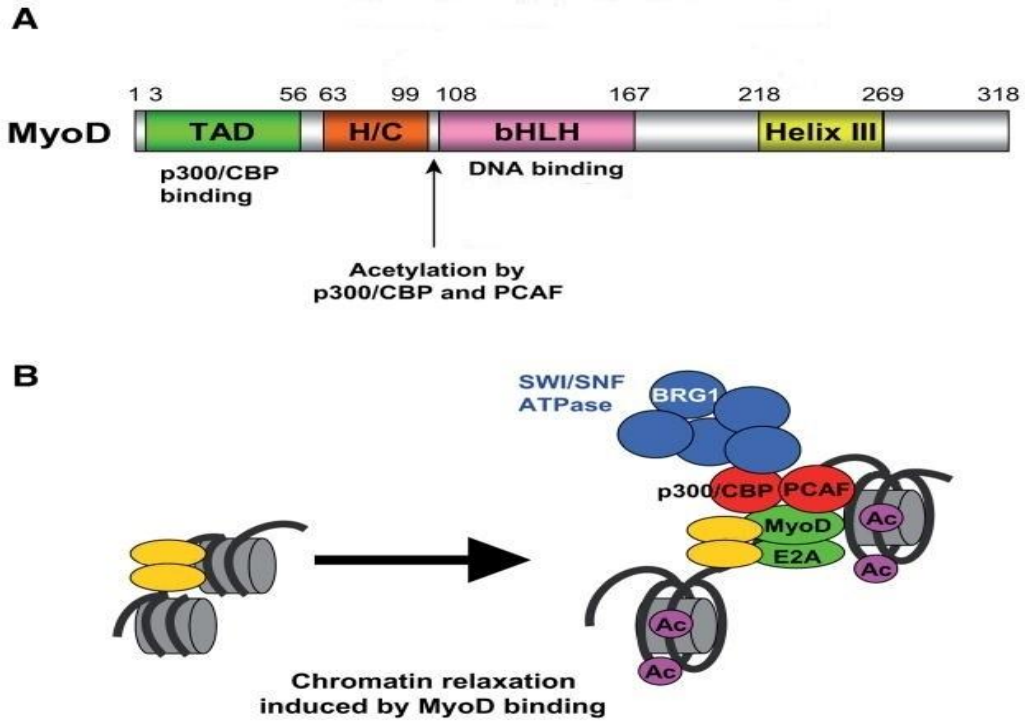

Figure 4. **MyoD Transactivation domain and its interaction with chromatin regulators**.**(a)** MyoD structure with MyoD TAD(green) and DBD (pink) with amino acid positions.**(b)** Proposed mechanism of interaction of MyoD TAD with chromatin modifiers. Adopted and modified from (Hirai *et al*. *Int J Dev Biol*. 2010)[23].

(CANNTG motif). Once recruited to the right target genes, the TAD serves as a scaffold to recruit and assemble additional transcription factors and chromatin remodeling proteins to initiate transcription[23].

MyoD TAD is at the amino terminal of MyoD (Fig. 4A), which contains 12 acidic amino acids out of a total of 54 amino acids, belonging to the classic acidic TAD groups[23].The MyoD TAD is known to interact with two histone acetyltransferases(HATs) namely P300/CBP complexes[28]. p300/CBP are recruited to the target promoters, which then acetylate histones H3 and H4 leading to a more open chromatin structure . The HATs neutralize the positive charge of lysine present on the histones, thereby reducing the affinity between the histones and the negatively charged DNA that is wrapping around the histones. Subsequently, a nucleosome remodeling complex namely the BRG1-containing SWI/SNF complex is also recruited to the promoters which remodels nucleosomes and stabilizes the DNA binding of MyoD as shown in Fig. 4B [23].

The fusion of MyoD TAD, to heterologous transcription factors has been shown to increase the efficiency of direct reprogramming by effectively remodeling patterns of DNA methylation, chromatin accessibility, and histone modifications[29-31]. Kikyo *et.al* showed that the efficiency of iPS generation can be increased by creation of a fusion gene between *Oct4* and MyoD TAD termed "M_3O". Transduction of M_3O along with three other Yamanaka factors *Sox2*, *Klf4*, and *c-Myc* into fibroblasts significantly increased the efficiency of making mouse and human iPSCs more than 50-fold in comparison to normal Yamanaka factors (OSKM)[30].The same group fused the MyoD TAD to Mef2c, Gata4, Hand2, or Tbx5 and transduced these genes in various combinations into mouse fibroblasts to induce cardiomyocytes. It was observed that the transduction of the MyoD TAD- Mef2c with the wild-types of the other three genes produced much larger beating clusters of cardiomyocyte-like cells faster than compared to wild-type gene

combinations, with an efficiency of 3.5% and was more than 15-fold greater than the wild-type genes combination[29].

With the advantages of direct reprogramming and the potency of MyoD TAD, we hypothesized that fusion of MyoD TAD with transcription factors related to muscle and neuronal lineage can facilitate direct reprogramming of fibroblasts into respective lineage cell types through increased transcriptional activity and chromatin remodeling.

The first step was to find suitable transcription factors related to muscle and neuronal cell lineages that play important roles in embryonic myogenesis and neurogenesis. The overall reprogramming can be improved by reducing the number of reprogramming factors used, as shown by Ieda *et al.* and Vierbuchen *et al* [32,33]. In this study, we set out to find such master transcription factors that play an important role in maintenance of myogenic or neural precursor cells.

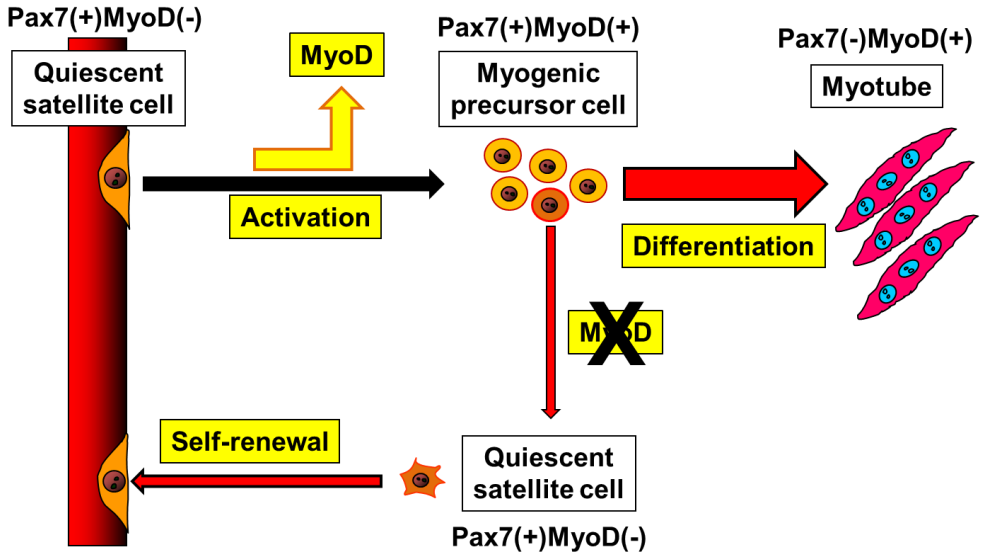

Figure 5. The model for satellite cell self-renewal regulated by MyoD. MyoD is not expressed in quiescent satellite cells [Pax7(+)MyoD(-)]. MyoD is expressed in myogenic precursor cells or myoblasts [Pax7(+)MyoD(+)] which undergo muscle differentiation to form multinucleated myotube [Pax7(-)MyoD(+)]. A minor population of myogenic precursor cells go back to quiescent satellite cells [Pax7(+)MyoD(-)] as a self-renewal.

In muscle, although ectopic expression of MyoD alone in fibroblasts can give rise to myoblasts followed by myotubes, it is rather difficult to obtain muscle precursor cells

or satellite cells (stem cells of muscle)[34]. Satellite cells are a small population of myogenic stem cells located beneath the basal lamina of skeletal muscle fibers. They are characterized by the expression of Pax7 but are negative for MyoD (Fig. 5)[35,36]. Satellite cells are responsible for muscle repair, wherein these muscle stem cells are normally mitotically quiescent. Following injury, satellite cells are activated and initiate MyoD expression and proliferation to produce their progeny, myogenic precursor cells or myoblasts, which mediate the regeneration of muscle[37]. After several rounds of cell proliferation, myoblasts cease expression of Pax7, exit the cell cycle and fuse to each other to create multinucleated myotubes, which are immature myofibers. Some minor populations of myoblasts cease MyoD expression and go back to satellite cells as a self-renewal process for maintenance of the muscle stem cell pool. Use of satellite cells with self-renewal is required for the efficient therapy of muscle diseases for the long term maintenance of donor-derived cells in the recipient muscle. The paired-box transcription factor 3, (Pax3) is a powerful myogenic regulatory factor during embryonic-myogenesis. The expression of Pax3 is involved in determining somatic cell fate, resulting in the formation of the first specified group of cells, termed the myotome, acting upstream of the muscle-specific program[38-40]. Ectopic expression of Pax3 at the early stage of differentiation enhances mesoderm formation and increases the muscle potential of Pax3-induced ES cells[40]. Pax3-expressing cells are known to possess migratory capacity, and therefore can enhance skeletal muscle regenerative capacity by migrating to the site of injury. Taking into account the crucial role of Pax3 in muscle formation, we chose Pax3 as a candidate master factor for the development of myogenic progenitor cells or satellite cells in our study.

In neurogenesis, bHLH transcription factor NeuroD1 is known to be involved in cell fate determination and differentiation during development similar to the myogenic bHLH factor MyoD that plays a key role in myogenesis. The ectopic expression of NeuroD in *Xenopus* ectodermal cells can convert them to neurons, indicating the crucial role of NeuroD in neurogenesis [41]. NeuroD1 regulates neurogenic cell differentiation fates, such as granule cells in the cerebellum, early retinal ganglion cells and inner ear sensory neurons. Given the importance of NeuroD1 in early neuronal development and

its requirement for terminal differentiation, we examined NeuroD1 as a candidate master factor for neuronal lineage development.

In this study, we examined whether a single transcription factor involved in myogenesis (Pax3) or neurogenesis (NeuroD1) alone can directly reprogram the mouse embryonic fibroblasts (MEFs) into myogenic or neurogenic lineages respectively. In addition, we created fusion transcription factors (Pax3 or NeuroD1) with the potent MyoD transactivation domain (MyoD TAD or MDA hereafter) that could facilitate radical acceleration of reprogramming into the desired cell type through chromatin modification compared to wild-type factors.

2. MATERIALS AND METHODS

Myoblast isolation and culture

Pax3 is known to prevent terminal differentiation in myoblasts. And also NeuroD1 is a bHLH(basic helix-loop-helix) transcription factor that shares homology with MyoD, a muscle bHLH transcription factor. We therefore cultured myoblasts to study the comparative effects of myogenic and neurogenic programs, when myoblasts are overexpressed with wild type Pax3, NeuroD1 and fusion transcription factors namely MDA-Pax3 and MDA-NeuroD1.

Myf5$^{+/nLacZ}$ mice in which *β-galactosidase* gene is inserted in *Myf5*-locus were kindly provided by Shahragim Tajbaksh. Skeletal muscles were isolated from the hind limbs of 1 to 2 month old *Myf5*$^{+/nLacZ}$ mice, rinsed briefly in phosphate buffered saline (PBS) before being minced with surgical scissors. The minced muscle tissue was digested with a solution of collagenase type B and dispase II (Roche Applied Science) for 20 minutes at 37°C. The tissue was minced again and incubated for an additional 20 minutes. After the incubation, 10 ml of Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 2% fetal bovine serum (FBS, Fisher Scientific) was used to rinse the digested tissue. The tissue and medium was divided between two tubes and spun down at 800 rpm for 30 seconds. The supernatant was transferred to a fresh tube and spun down once more for a second wash. The cells were then collected by centrifugation at 2000 rpm for 5 minutes at 4°C. The cells were resuspended in fresh medium before undergoing magnetic activated cell sorting (MACS). MiniMAC and MidiMAC kits (Miltenyi Biotec) were used to sort cells. Negative selection was performed using phycoerythrin (PE)-labeled CD31, PE-labeled CD45, PE-labeled Sca-1 antibodies (BD biosciences), and anti-PE micro beads (Miltenvi Biotec). After negative selection, the cells underwent positive selection using Integrin α7 antibodies (MBL International) as well as anti-IgG1 micro beads (Miltenyi Biotec). Myoblasts were cultured on dishes coated with collagen (BD Biosciences; Franklin Lakes, NJ) in HAM's F10 (Invitrogen) supplemented with 20% FBS, Fibroblast growth factor (2.5ng/ml) (FGF, R&D systems), and penicillin/streptomycin (50 µg/ml; Invitrogen). Cells were incubated at 37°C in 100% humidity and with 5% CO_2 and 5% O_2, passaged at ~80% confluence. Differentiation medium, DMEM supplemented with 5% horse serum (Invitrogen), and

penicillin/streptomycin (50 µg/ml), was used to induce myogenic differentiation. The Institutional Animal Care and Use Committee (IACUC) for the University of Minnesota approved all experimental protocols. Care was taken to minimize the number of animals used, as well as any pain and suffering.

Murine embryonic fibroblast (MEF) isolation and culture

The myogenic factor Myf5 is one of the earliest markers of myogenesis. Thus, expression of lacZ recapitulates the endogenous Myf5 mRNA expression in MEFs isolated from *Myf5*$^{+/nLacz}$ mice and can be a marker for myogenic conversion.

For *Myf5*$^{+/nLacZ}$ MEF isolation, uteri isolated from 13.5 day pregnant *Myf5*$^{+/nLacZ}$ mice were washed with PBS. 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 DMEM 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 collagen-coated 100 mm dishes in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37°C with 5% CO_2 and 5% O_2. MEFs were used within four passages to avoid replicative senescence. For genotyping *Myf5*$^{+/nLacZ}$ allele, PCR was performed by Thermal Cycler (Eppendorf Thermal Cycler, Hauppauge, NY) with primers (5′-TGAAGGATGGACATGACGGAC-3′) and (5′-GGGCGATCGGTGCGGGCCTCTTCGC-3′) as previously reported (Verma et.al, 2011)[42].

cDNA amplification by PCR

To obtain cDNAs for MyoD TAD (MDA), Pax3, and NeuroD1 with appropriate restriction sites, the reaction mixture containing AccuPrime™ Pfx SuperMix (Invitrogen), template plasmid vectors containing mouse MyoD, or cDNA from mouse embryos (E11.5 day), and forward and reverse primers were mixed in PCR tubes. The PCR cycle parameters used were as follows: 95°C for 5 minutes, 40 cycles of: 95°C for 15 seconds,55–65°C for 30 seconds, 68°C for 1 minute by Thermal Cycler (Eppendorf Thermal Cycler, Hauppage, NY). The reaction was maintained at 4°C after cycling. Resulting products were separated electrophoretically in 2% agarose gel and visualized by ethidium bromide staining.

Target	Forward Primer	Reverse Primer
MyoD TAD	CGAATTCCATGGAGCTTCTATCGCCGCCACTCC	GAATTCCCGTGCTCCTCCGGTTTCAGGAGGGC
Pax3	CCCTCGAGATGACCACGCTGGCCGGCGCTGTG	GGGCGGCCGCCCTAGAACGTCCAAAGGCTTACTTTG
NeuroD1	CCCTCGAGATGACCAAATCATACAGCGAGAGC	GGGCGGCCGCCTAATCGTGAAAGATGGCATTAAG

Table 2. List of primers and their target gene used for PCR amplification

Retroviral vector construction

The Myc epitope tag was obtained from pCS2+MT vector containing 6x Myc tags. The full length MyoD TAD, termed "MDA", fragment was PCR amplified using the primer pairs listed in Table 2 with a vector carrying MyoD cDNA as a template. Frist, to create retroviral expression vectors, the 6x Myc tag (MT) fragment was cloned into BamH1/Not1 sites of the pMX-Puromycin (pMX-Puro) plasmid (CELL BIOLABS, Inc) containing a puromycin resistant gene expression cassette for drug selection to obtain pMX-MT-Puro vector. Next, MDA fragments (encoding position 1-60 amino acid of MyoD) were cloned into EcoR1 sites of the pMX-MT-Puro plasmid to obtain pMX-MT-MDA-Puro vectors.

Next, to construct the pMX-MT-Puro-transcription factor gene vectors and pMX-MT-MDA-Puro-transcription factor gene vectors, the full length coding regions of Pax3 and NeuroD1 were amplified using the primers listed in Table 2 and subcloned into Xho1 and Not1 sites of the pMX-MT-Puro and pMX-MT-MDA-Puro vectors (Fig. 6).

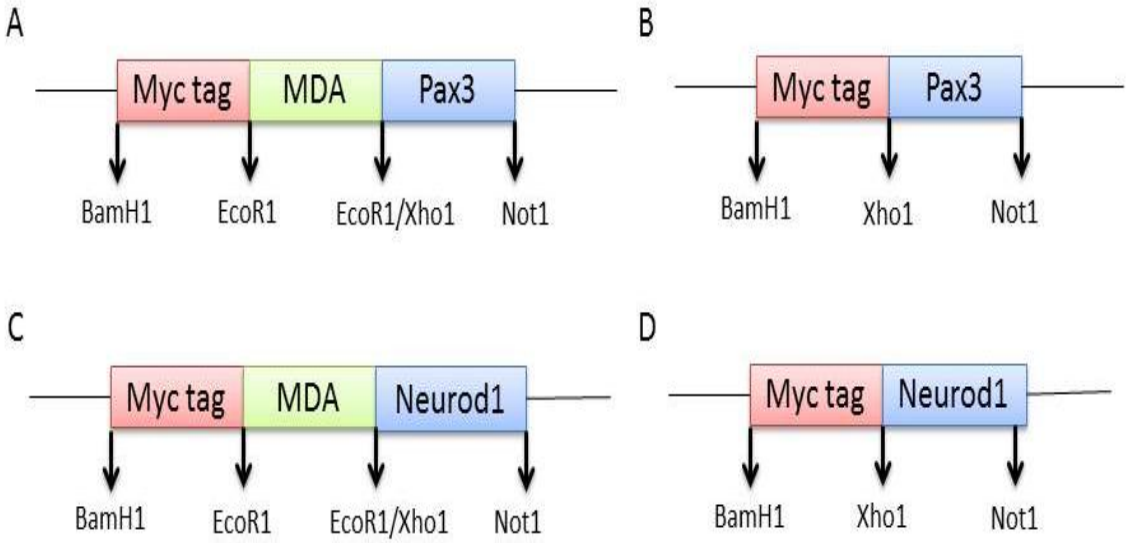

Figure 6. Fusion constructs with restriction map. A) & (B): The Pax3 with MDA and control vector without MDA. **(C) & (D)**: The NeuroD1 with MDA and control vector without MDA. The restriction sites indicated are on the pMX-Puromycin (pMX-Puro) backbone.

Retroviral vector production

Plat-E cells (CELL BIOLABS, Inc) were thawed and plated onto 10 cm culture dishes containing DMEM supplemented with 10% FBS and 100 µg/ml penicillin and 100 µg/ml streptomycin with total 8 ml of culture media for 2 days, or until cells became 70% confluent (Fig. 7). The Plat-E cells were then seeded onto new 10 cm culture dish without penicillin and streptomycin one day before transfection. Transfection of pMX constructs was started after 24 hours, when cells were 80% confluent. In a sterile 15 mL tube, 25 µg pMX plasmid DNA, 25 µl of warmed-up TransIT-2020 transfection reagent (Mirus Bio LLC), and 1 mL of Opti-MEM (Invitrogen) were added and pipetted gently. The mixture

was incubated at room temperature for 20 minutes to allow sufficient time for TransIT-2020:DNA complexes to form. The prepared TransIT-2020:DNA complexes were added drop-wise to different areas of 10 cm culture dish containing Plat-E cells containing 5 mL of culture medium (DMEM with 10% FBS and without antibiotics). The culture dish was incubated in a CO_2 incubator at 37°C for 24 hrs, after which media was replaced with 10mL fresh growth media. The supernatant containing the retroviral vectors was harvested into 2.0 ml cryotubes the following day and stored at -80°C.

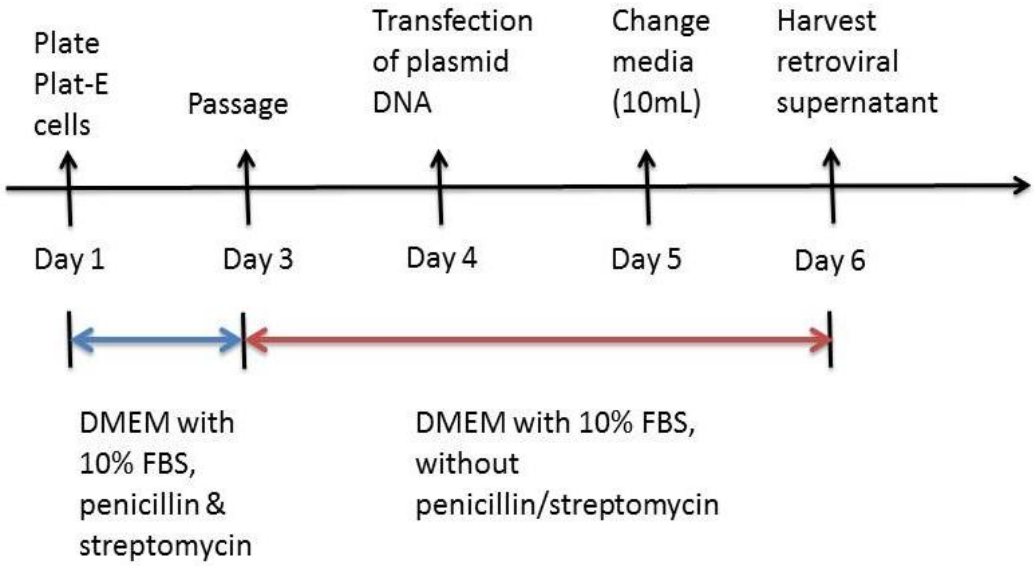

Figure 7. Timeline showing retroviral vector production from Plat-E cell culture before and after plasmid DNA transfection.

Retroviral infection

MEFs were seeded onto 10 cm culture dishes and allowed to expand. 5×10^4 *Myf5*$^{+/nLacZ}$ MEFs were seeded onto 3 cm culture dishes supplemented with DMEM, 10% FBS, and penicillin/streptomycin. 750 µl of retroviral viral supernatant was added to the fibroblasts with 10 µg/ml polybrene (Millipore) (Fig. 8). After 24 hrs, the virus supernatant was changed to fibroblast medium (DMEM, 10%FBS, penicillin/streptomycin) which was

replaced every other day thereafter. The cells were grown in fibroblast medium with 1 ug/ml puromycin (Invitrogen) selection for 72 hrs. Similar strategy was used for transfection of *Myf5*$^{+/nLacZ}$ myoblasts cultured in myoblast media (HAMs F10, 20%FBS, bFGF, penicillin/streptomycin). The puromycin resistant transformant cells were used for further analysis.

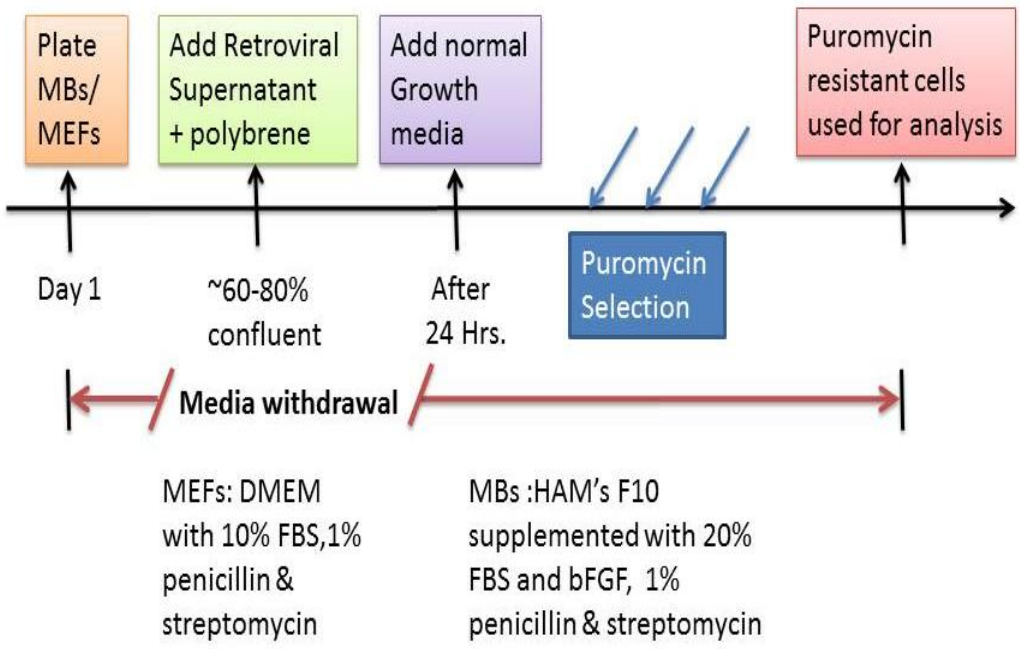

Figure 8. Timeline showing the retroviral infection of MEFs and MBs

Immuno Fluorescence staining

Cells in 3 cm dishes were fixed with 2% paraformaldehyde solution at room temperature for 5 minutes, followed by rinsing with 0.01% Triton X in PBS twice. Cells were permeabilized with 0.2% Triton in PBS for 5-7 minutes and blocked with 1% BSA in PBS for 30 minutes. A hydrophobic pen (Sigma Aldrich) was used before blocking to prevent loss of antibodies in subsequent steps. After blocking step, cells were incubated with 200 µl of primary antibodies diluted with blocking solution as shown in Table 3 and stored in a moist box overnight at 4°C. The following day, cells were rinse with 0.01% Triton X in PBS twice and incubated with secondary antibodies Alexa Fluor 488-labeled

anti-mouse IgG and anti-rabbit Alexa-594-labeled anti-rabbit IgG (diluted with blocking solution as shown in Table 3) for 1 hour at room temperature, followed by rinsing with 0.01% Triton X in PBS twice and costaining with DAPI dye for 20 minutes. A cover slip was placed on the cells with one drop of DAKO mounting media (DAKO) and was then ready to be viewed under the fluorescence microscope.

Table 3. Antibodies along with their dilutions used for immunostaining.

Primary Antibodies

Antibody	**Dilution**	**Manufacturer**
Anti-myc tag	1:50000	Abcam
Anti-sarcomeric myosin heavy chain (MHC) antibody (MF20)	1:20	Developmental Hybridoma Study Bank (DHSB)
Neural filament-M (NFM) (2H3)	1:20	Developmental Hybridoma Study Bank (DHSB)
MyoD (C-20)	1:1000	Santa Cruz Biotechnology
Pax7	1:200	R&D systems
Tuj1	1:1000	Covance
Phosphorylated histone H3	1:1000	Cell signaling technology

Secondary Antibodies

Antibody	**Dilution**	**Manufacturer**
Alexa Fluor 488–labeled anti-mouse IgG	1:1000	Invitrogen
Alexa Fluor 594–labeled anti-rabbit IgG	1:1000	Invitrogen

X-gal staining

Cells in 3 cm dishes were fixed with 2% PFA at room temperature for 5 minutes. Cells were rinsed thrice with 1 ml of X-gal buffer solution (0.1M Phosphate buffer pH7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40). The cells were then stained with 1 ml of X-gal staining solution [5 mM Ferrocyanide, 5 mM Ferricyanide, 0.5 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) prepared freshly overnight. The next day, cells were washed with 1 ml of X-gal buffer thrice, followed by costaining with DAPI dye for 20 minutes. The stained cells were stored at 4°C until characterization.

Imaging

Fluorescence images were captured by a DP-70 or DP-80 digital camera attached to an IX80 inverted fluorescence microscope with x4, x10, x20, and x40 LUCPlanFLN objectives and a BX51 fluorescence microscope with x4, x10, x20, and x40 UPlanFLN objectives (all from Olympus America, Center Valley, PA). The CellSens dimension 1.8 (Olympus; Center Valley, PA) imaging software was used for image capture. Photoshop CS3 (Adobe Systems; San Jose, CA) was used for image processing.

Quantitative real time PCR

Total RNAs were isolated from the cells by TRIZOL (Invitrogen) and quantitated by Nanodrop Spectrophotometer (Thermo Scientific). 1 μg RNAs were reverse transcribed by Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Amplified cDNA qualities were assessed by PCR using β-actin primers with 25 cycles (Eppendorf Thermal Cycler, Hauppage, NY), followed by running 2% agarose gel. Quantitative real-time PCR was performed by GoTaq qPCR Master Mix (Promega) with Mastercycler ep realplex (Eppendorf). cDNAs of 7-day differentiated mouse embryonic stem cells (ES day 7) and proliferating myoblasts were used for control. The amounts of transcripts were normalized to that of β-actin as an internal control. The expression level of ES day 7 was set as 1.0 then relative value of each sample was calculated. Primer sets used for PCR were summarized in Table 4.

Name	Forward primer	Reverse primer
Otx1	AGGCGCTGTTCGCAAAGA	CCTCCTCGCGCATGAAGA
TuJ1	GCGCATCAGCGTATACTACAA	TTCCAAGTCCACCAGAATGG
Vglut1	GTGCAATGACCAAGCACAAG	TAGTGCACCAGGGAGGCTAT
Brn2	CGCGCATTTGACAGTTACTT	GAGAGAGAGTGGGAGAAGCG
Ascl1	GAGAACGAGTTGGGGACG	CTTTGATCGTGCTTCGCA
Pax3	GCCAGGGCCCGAGTCAACCAG	TGTATCCCCTCACCAGAGGAT
NeuroD1	GCTCCAGGGTTATGAGATCGTCAC	CCTCGTCCTGAGAACTGAGACACTC
β-Actin	CACCCTGTGCTGCTCACCGAGGCC	ACCGCTCGTTGCCAATAGTGATGA

Table 4. Primer pairs used for Q-PCR analysis

Statistical analysis

All data are expressed as average percentage of 3 randomly chosen fields ± SEM. The SEM was calculated as STDEV/SQRT (sample size).All the data are expressed as percentage of antibody positive nuclei (or in some cases as lacZ positive) per DAPI positive nuclei.

3. RESULTS

Myogenic and neurogenic reprogramming in mouse embryonic fibroblasts by expression of a single transcription factor.

To examine whether transduction of a single transcription factor gene can directly reprogram mouse embryonic fibroblasts (MEFs) toward myogenic or neurogenic cell lineages, we created retroviral expression vectors with a puromycin selection cassette (pMX-Puro) carrying the Myc-tag (MT)-expressing Pax3, or NeuroD1 genes or these same MT-transcription factor genes fused with the strong MyoD transactivation domain (referred to as MDA hereafter) at their N-termini.

First, retroviral vectors carrying the MT-Pax3 (referred to as Pax3 hereafter) or MT-NeuroD1 (referred to as NeuroD1 hereafter) genes, either fused or not fused with MDA at their N-termini, MT-MDA-Pax3 (referred to as MDA-Pax3 hereafter) and MT-MDA-NeuroD1 (referred to as MDA-NeuroD1 hereafter), were infected into MEFs isolated from *Myf5*$^{+/nLacz}$ mice to examine the possibility of direct reprogramming toward myogenic or neurogenic cell lineages. To examine the expression of exogenous genes in infected MEFs, the MEFs were stained with an anti-MT antibody on day 6 after puromycin selection (Fig. 9A). Nearly 90% of the infected MEFs were anti-MT positive, indicating the successful retroviral gene transduction and efficient puromycin drug selection (Fig. 9B).

Next, we asked whether Pax3 or NeuroD1 ectopic expression can reprogram *Myf5*$^{+/nLacz}$ MEFs toward a myogenic cell lineage. The myogenic factor Myf5 defines the early onset of myogenesis in vertebrates during development that lead to the specification of muscle progenitor cells[43]. Expression of lacZ in MEFs isolated from *Myf5*$^{+/nLacz}$ mice can be a marker for myogenic conversion since the nuclear lacZ (nlacZ) reporter gene was knocked-in into the locus of one of the alleles of *Myf5*, and lacZ expression in the *Myf5*$^{+/nLacz}$ mice recapitulates the endogenous Myf5 mRNA expression. The retroviral vectors carrying Pax3, MDA-Pax3, NeuroD1, and MDA-NeuroD1 were infected into *Myf5*$^{+/nLacz}$ MEFs. After infection, MEFs were stained with X-gal by day 3 without puromycin selection and by day 6 after puromycin selection (Figs. 10A, 11A). By day 3, the Myf5-lacZ expression in MEFs infected with Pax3 was not significantly increased

compared to the control (Fig. 10B, 3.1% vs. 2.7 %). By contrast, the MDA-Pax3 infected MEFs showed a significant increase in Myf5-lacZ expression compared to the control (Fig. 10B, 17.5 % vs. 2.7%). We noticed that a few control MEFs already expressed Myf5-lacZ without any transduction (2.7%). This may have been due to the fact that, since the MEFs were prepared from whole embryos (lacking the head region), contamination with embryonic myogenic cells could have occurred. We cannot rule out the possibility that expression of Pax3 or MDA-Pax3 simply induced proliferation of such contaminated LacZ positive myogenic cells but not actually induced myogenic conversion in cultures. This possibility can be confirmed by clonal assay, whether it is the actual conversion or promoting proliferation of Pax3 or MDA-Pax3 infected MEFs. We also noticed that NeuroD1 but not MDA-NeuroD1 infected MEFs displayed reduced Myf5-lacZ expression, suggesting that expression of NeuroD1 but not MDA-NeuroD1 suppresses myogenic differentiation. At day 6 after transduction with puromycin selection, the Pax3-infected MEFs showed a marked increase in Myf5-lacZ expression (18.8%) compared to control MEFs (2.0%) (Fig. 11A, B). Strikingly, the MDA-Pax3-infected MEFs showed further increase Myf5-lacZ expression (45.7%). We noticed that infection of MDA-NeuroD1 to MEFs also induced Myf5-lacZ in MEFs (6.7%), suggesting that an addition of MDA could modify NeuroD1 target specificity capable of partial myogenic reprogramming. Alternatively, since it was reported that Myf5-lacZ and Myf5 mRNA, but not Myf5 protein, are expressed in the central nervous system (CNS) during embryogenesis[44], MDA-NeuroD1-mediated Myf5-lacZ expression may reflect the expression of Myf5 mRNA in the CNS. Taken together, these data indicate that Pax3 alone can reprogram MEFs toward a myogenic cell lineage. In addition, MDA-Pax3 can cause radical enhancement of Myf5-lacZ expression and efficiently initiate the myogenic program in MEFs.

To further assess the myogenic potential of Pax3 and MDA-Pax3 in MEFs, we co-stained the infected MEFs with two myogenic markers, Pax7 and MyoD, by day 6 after infection (Fig. 12A). Pax7 single positive cells represent embryonic myogenic progenitor cells or satellite cells. MyoD single positive cells represent terminally differentiating myocytes. Pax7 and MyoD double positive cells represent proliferating

myoblasts. As a result, the percentage of Pax7 single positive cells was significantly higher in Pax3 infected MEFs compared to control MEFs (18.8% vs. 0.7%, Fig. 12B). The MDA-Pax3-infected MEFs showed a further increase in the percentage of Pax7 single positive cells (49.8%). Similarly, the percentage of MyoD single positive cells and Pax7/MyoD double positive cells was also significantly higher in Pax3 infected MEFs compared to control MEFs. The MDA-Pax3-infected MEFs showed further increase in the percentage of MyoD single positive cells and Pax7/MyoD double positive cells. However, terminal differentiation markers such as sarcomeric myosin heavy chain (MHC) were not detected in any MEFs (control, Pax3-expressing, and MDA-Pax3-expressing) both under growth and differentiation conditions (data not shown), indicating that expression of Pax3 and MDA-Pax3 promote myogenic conversion but suppress terminal myogenic differentiation in MEF cultures. By contrast, expression of NeuroD1 or MDA-NeuroD1 in MEFs could not show any additional myogenic conversions compared to control MEFs. Thus, these data strongly indicate Pax3 alone or MDA-Pax3 is able to reprogram MEFs toward myogenic cell lineage, especially toward Pax7-expressing embryonic myogenic progenitor cells or satellite cells in addition to Pax7/MyoD double positive myoblasts.

Next, to examine whether NeuroD1 and/or MDA-NeuroD1 has the capability to initiate the neurogenic program when infected into MEFs, we stained for TuJ1 (neuron-specific class III β-tubulin), an early marker for neurogenesis, by day 9 after infection (Fig. 13A). The NeuroD1-infected MEFs showed a significant increase in TuJ1 expression (51.7%) compared to control MEFs (0%) (Fig. 13B). The MDA-NeuroD1-infected MEFs also induced TuJ1 (31.7%), but relatively lower to that of NeuroD1. Taken together, these data indicate that NeuroD1 alone is sufficient to induce TuJ1 expression in MEFs. However, an addition of MDA to NeuroD1 could not enhance this neurogenic induction activity. The q-PCR analysis of MEFs on day 3 after infection showed that the Pax3 and NeuroD1 levels (detecting both endogenous and exogenous expressions) were up-regulated in MEFs by ectopically expression with Pax3 with or without MDA, and NeuroD1 with or without MDA respectively (Fig. 14A,B). Ectopic expression of NeuroD1 showed up-regulation of early neuronal marker genes such as

Tuj1, *Brn2 (Brain-2)/Pou2f3* and *OTX1*, and a mature neuronal marker gene *V-glut1*, while MDA-NeuroD1 infected MEFs further enhanced the expression levels of these genes (Fig. 14C, D, F, G). The expression levels of early neurogenic factor *Ascl1* (*Achaete scute complex like-1*) was undetected in MEFs infected with NeuroD1 or MDA-NeuroD1, suggesting that *Ascl1* acts upstream of NeuroD1 in the hierarchy of neurogenesis (Fig. 14E). Taken together these data indicate NeuroD1 and MDA-NeuroD1 are able to induce neurogenic genes in MEFs, suggesting the partial neurogenic conversion of MEFs by NeuroD1.

Effects of myogenic and neurogenic induction in myoblasts by expression of a single transcription factor.

We showed that Pax3 and MDA-Pax3 have a strong potential to reprogram MEFs toward a myogenic cell lineage, especially to Pax7(+)MyoD(-) myogenic progenitor cells or satellite cells. In addition, ectopic expression of NeuroD1 and MDA-NeuroD1 are able to induce a neurogenic marker in MEFs, suggesting the partial neurogenic conversion of MEFs by NeuroD1. The myogenic regulatory factor Pax3, which is expressed in myogenic progenitor cells or satellite cells, plays an important role in myogenic specification and migration during early embryogenesis[45]. In adult myoblasts, it was reported that Pax3 and Pax7 promote and regulate cell proliferation and prevent terminal differentiation[46]. By contrast, NeuroD1 was reported to be expressed in post-mitotic neurogenic cells during embryogenesis, and expression of NeuroD1 suppresses neuronal cell proliferation through up-regulation of cell cycle inhibitor p21[47] .

Therefore, we first compared cell proliferation between the empty control, Pax3, MDA-Pax3, NeuroD1, and MDA-NeuroD1-infected primary myoblasts isolated from adult mouse muscle. By day 6 after infection, the cell proliferation was assessed by anti-phosphohistone H3 (pHisH3) antibody which marks the mitotic-phase of cells (Fig. 15A). Myoblasts infected with the MDA-Pax3 vector showed significantly increased pHisH3 levels compared to that of the control vector (Fig. 15B, 7.1% vs. 3.0%). The MDA-NeuroD1 vector-infected myoblasts also showed slightly increased pHisH3 levels compared to that of the control vector (Fig. 15B, 3.9% vs. 3.0%), while NeuroD1 vector-

infected myoblasts showed slightly decreased pHisH3 levels compared to that of the control vector (Fig. 15B, 2.0% vs. 3.0%). Taken together, these data indicate that MDA-Pax3 acts as a strong mitogen and increases cell proliferation of myoblasts. In addition, while NeuroD1 was shown to be a weak suppressor for cell proliferation, MDA-NeuroD1 was shown to be a weak promoter for cell proliferation.

Next, we compared myogenic differentiation between empty control, Pax3, and MDA-Pax3-infected primary myoblasts. In addition, we examined whether ectopic expression of NeuroD1 and MDA-NeuroD1 have an ability to suppress myogenic differentiation in myoblasts. Myoblasts were stained for sarcomeric myosin heavy chain (MHC), a terminal differentiation marker for muscle, under growth conditions by 6 days after infection and under differentiation conditions (low serum for 72 h) following the 6 days growth medium (Figs. 16A, 17A). Under growth conditions, a few control myoblasts underwent spontaneous terminal differentiation (3.0%). Under differentiation conditions, more than half of control myoblasts (56.2%) underwent MHC-expressing terminal differentiation. The expression of Pax3 increased spontaneous differentiation of myoblasts (6.9%) under growth conditions, while slightly suppressing terminal differentiation under differentiation conditions (44.5%). By contrast, MDA-Pax3-vector-infected myoblasts showed significantly reduced MHC expression (0.1%) under growth conditions, while slightly increased terminal differentiation (66.0%) under differentiation conditions. The MDA-Pax3-expressing myoblast culture also displayed formation of mature elongated myotubes under differentiation conditions (Fig. 17A). These data indicate that MDA-Pax3 stimulates mitotic activity in myoblasts and suppresses spontaneous myogenic differentiation under growth conditions. However, terminal differentiation rate was increased in MDA-Pax3-expressing myoblasts under differentiation conditions. This may have occurred, because the cell density for MDA-Pax3-expressing myoblasts became nearly confluent, which promoted terminal differentiation under differentiation conditions. Interestingly, ectopic expression of NeuroD1 almost completely suppressed myogenic differentiation in both growth conditions (0%) and differentiation conditions (4.3%), indicating that NeuroD1 possesses strong anti-myogenic effects. By contrast, the MDA-NeuroD1-vector-infected myoblasts

did not inhibit spontaneous myogenic differentiation under growth conditions (5.0%) and even significantly enhanced terminal differentiation under differentiation conditions (89.0%). In addition, formation of mature elongated myotubes also increased in MDA-NeuroD1-expressing myoblast cultures under differentiation conditions (Fig. 17A). This may have also occurred, because the cell density for MDA-NeuroD1-expressing myoblasts became nearly confluent, which promoted terminal differentiation under differentiation conditions. Alternatively, an addition of MDA fragment to NeuroD1 may be able to modify NeuroD1 target specificity which in turn promotes myogenic terminal differentiation in myoblast cultures.

We showed that ectopic expression of NeuroD1 and MDA-NeuroD1 is able to induce a neurogenic marker in MEFs, suggesting the partial neurogenic conversion potential for both transcription factors. To perform a comparative study and examine the effect of myogenic and neurogenic programs in the retrovirally infected myoblasts, the myoblasts were co-stained for 140 k dalton neurofilament medium (NFM), a neuronal marker, and for MyoD by day 6 after infection (Fig. 18A). The control myoblasts showed that almost all cells expressed MyoD, and among them, around 10% of cells expressed both MyoD and NFM, consistent with the previous work in which a proliferating C2C12 myoblast cell line expressed NFM in culture[48,49]. In the myoblasts infected with Pax3 and MDA-Pax3 vectors, the levels of MyoD were comparable to that of control myoblasts (Fig. 18A, B), indicating that there is no significant effect on MyoD expression by ectopic expression of Pax3 or MDA-Pax3. While Pax3-expressing myoblasts display reduced numbers of NFM expressing cells, the number of MDA-Pax3-expressing myoblasts that were NFM-positive cells did not change. In contrast, the myoblasts infected with NeuroD1 and MDA-NeuroD1 showed a significant decrease in MyoD levels (Fig. 18A, B, 47.4% for NeuroD1, 19.6% for MDA-NeuroD1, 83.4 % for control). These data confirmed that NeuroD1 possesses an anti-myogenic ability. However, MDA-NeuroD1 could promote MHC-positive myogenic terminal differentiation but suppressed MyoD expression in myoblast cultures. These data suggest that MDA-NeuroD1 has partial myogenic differentiation induction ability, possibly through binding to E-boxes located in muscle-specific gene regulatory regions such as sarcomeric MHC gene while

preventing MyoD expression. Interestingly, while NeuroD1-expressing myoblasts show reduced numbers of NFM-positive myoblasts, the MDA-NeuroD1-infected myoblasts showed significantly increased numbers of NFM-positive myoblasts compared to that of control myoblasts (Fig. 18A, B, 47.3% vs. 11.7%). Taken together these data indicate that both NeuroD1 and MDA-NeuroD1 down-regulate MyoD expression, but only MDA-NeuroD1 possesses neurogenic induction activity in myoblast cultures.

DISCUSSION

The first important finding in this study was that ectopic Pax3 expression is sufficient to directly reprogram MEFs toward a myogenic cell lineage in the absence of any inducing signals: *Myf5*$^{+/nLacZ}$ MEFs were induced to express the myogenic markers, Myf5-lacZ, Pax7 and MyoD when Pax3 was ectopically expressed. Similar myogenic reprogramming activity has been already reported when the myogenic master transcription factor family, consisting of MyoD, Myf5, Myogenin and MRF, was force expressed in non-muscle cells including fibroblasts and in differentiated melanoma, neuroblastoma, liver, and adipocyte lines[26].During embryonic myogenesis, Pax3 expression is initiated in paraxial mesoderm followed by the dermomyotome before any myogenic master transcription factors appear[50,51]. Pax3-expressing dermomyotomal cells are the precursor cell source for myogenic cells in the myotome where expression of myogenic master transcription factors is initiated. Previous studies also demonstrated that ectopic Pax3 expression in chicken embryonic neural tubes, paraxial mesoderm and lateral plate mesoderm activates MyoD and Myf-5 expression and induces the myogenic program in these non-muscle embryonic tissues[39].Therefore, Pax3 is also considered as a myogenic master regulatory factor up-stream of the MyoD family during embryogenesis. In addition, recent studies have shown that myogenic cells expressing Myf5 and MyoD can be efficiently obtained from ES cells when Pax3 or Pax7 is ectopically expressed[52]. Therefore, embryonic tissues and cells have a potential to undergo conversion to myogenic cell lineages when Pax3 is over-expressed. However, to our knowledge, this study is the first showing that ectopic expression of Pax3 can directly reprogram cultured MEFs toward a myogenic cell lineage.

We also created Pax3 fusion protein with a potent MyoD transactivation domain (MDA) to examine whether MDA-Pax3 can more efficiently induce the myogenic program in MEFs. Interestingly, the MDA-Pax3 expression further enhanced Pax3-mediated myogenic reprogramming in MEFs. In addition, mitotic effects of Pax3 which have been reported were also significantly enhanced by adding the MDA fragment to

Pax3. These results strongly suggest that Pax3 or MDA-Pax3 gene-mediated myogenic conversion may be a useful therapeutic approach for stem cell transplantation for muscle diseases such as Duchenne muscular dystrophy (DMD). In addition, the system in which expression of Pax3 or MDA-Pax3 can directly reprogram MEFs toward myogenic cells allows us to understand hierarchical molecular mechanisms of early myogenesis by characterization of Pax3 function in this system. Such characterization can be done by searching for Pax3 down-stream genes using RNA-seq and ChIP-seq.

The second important finding in this study was that ectopic NeuroD1 expression is sufficient to directly reprogram MEFs toward a neurogenic cell lineage in the absence of any inducing signals: ectopic expression of NeuroD1 in MEFs induces neurogenic markers, Tuj1 and mRNAs for Tuj1, Brn2, Otx1 and v-GluT1. Previous data demonstrated that ectopic expression of NeuroD1 and other neurogenic members of the bHLH family such as NeuroD2, Neurogenin2 (Ngn2) and mouse ASCL1 (MASH1) are able to initiate the neurogenic program in embryonic ectodermal cells, retinal pigment epithelial (RPE) cells, P19 embryonal carcinoma cells[41,53-55]. Recent studies demonstrated the direct reprogramming of fibroblasts including MEFs using several different cocktail combinations of neurogenic transcription factors[12,33]. Vierbuchen et al. first demonstrated that the expression cocktail (ABM) of Achaete-scute complex-like 1 (Ascl1), Brain-2 (Brn2a)/Pou2f3, and Myelin transcription factor-like 1 (Myt1l), together efficiently converts mouse embryonic and postnatal fibroblasts into functional neurons *in vitro* [33]. Thereafter, expression cocktail of ABM + NeuroD1, cocktail of Ascl1, Nurr1/Nr4a2 and Lmx1a, cocktail of miR-124, Brn2/Pou3f2 and Myt1l, cocktail of miR-9/9*, miR-124, Ascl1, Myt1l and NeuroD2, cocktail of Mash1, Ngn2, Sox2, Nurr1, and Pitx3, and cocktail of Pax6, Ngn2, Hes1, Id1, Ascl1, Brn2/Pou3f2, c-Myc, and Klf4 together convert, murine and human fetal and postnatal fibroblasts, murine hepatocytes, and murine sertoli cells into neurons including dopaminergic neurons[56-62]. Similarly, expression cocktail of cocktail of ABM, Lhx3, Hb9, Isl1, and Ngn2, together converts MEFs, murine and human postnatal fibroblasts into functional motor neurons[63]. Kim et al. reported that among different combinations of 11 neurogenic transcription factors, an expression cocktail of Ascl1, Pitx3, Lmx1a, Nurr1, Foxa2 and EN1 showed the highest

neurogenic reprogramming activity in murine postnatal fibroblasts[64]. More recently, expression of the five factors Rfx4, Id4, FoxG1, Lhx2, and Sox2, or Brn4/Pou3f4, Sox2, Klf4, c-Myc, and E47/Tcf in MEFs is sufficient to induce multipotent neural precursor cells[65,66]. However, studies have failed to show that a single transcription factor can efficiently reprogram fibroblasts into a neuronal cell lineage.

In this study, we also attempted to address whether NeuroD1 fused to MyoD transactivation domain (TAD) will have efficient neurogenic activity when expressed in MEFs. However, our experiments revealed that MDA-NeuroD1 expression does not enhance NeuroD1-mediated neurogenic reprogramming in MEFs. By contrast, expression of MDA-NeuroD1 partially initiates the myogenic program in MEFs and enhances myogenic terminal differentiation in primary myoblasts, while expression of MDA-NeuroD1 also up-regulates NFM expression in myoblasts. Conversely, expression of NeuroD1 suppresses myogenic differentiation but does not induce neurogenic genes in myoblasts. These data suggest that NeuroD1 possesses an ability to convert fibroblasts into a neurogenic cell lineage. However, MDA-NeuroD1 possesses both neurogenic and myogenic reprogramming abilities. One possibility is that myogenic activity of MDA-NeuroD1 may be due to its enhanced transcriptional activity because of the MDA fragment fusion. Recent work demonstrated that MyoD and NeuroD2 bind a shared CAGCTG E box motif and specific E box motifs (CAGGTG for MyoD and CAGATG for NeuroD2)[67]. Since NeuroD1 possesses the highest homology to NeuroD2, hyper-activated NeuroD1 as a result of adding the MDA fragment may be able to remodel epigenetic status through binding at shared sites and possibly at MyoD-specific binding sites as well, which may lead to partial activation of myogenic genes. The other possibility is that MyoD TAD (MDA) fragment itself contains myogenic activity since it possesses a powerful chromatin remodeling activity[24] . Previous work demonstrated that Oct4 fusing with this MyoD TAD has a significantly higher reprogramming activity for iPS cell generation from MEFs and human fibroblasts when combined with other Yamanaka factors[30] . In addition, a cardiac transcription factor GATA4 fusing with this MyoD TAD has a significantly higher cardiomyocyte inducing activity in MEFs when combined with other cardiogenic transcription factors[29] . However, it remains unknown

whether these fusion proteins have myogenic abilities. Developmentally, NeuroD1 does not only regulate neurogenesis but also functions as a regulatory switch for endocrine pancreatic development, and is an essential transcription factor for differentiation of pancreatic islet cells including insulin- and glucagon-producing cells. NeuroD1 gene knockout mice display pancreatic islet morphogenesis defects and induces diabetes[68]. In humans, two mutations in NeuroD1 have been identified: These heterozygous NeuroD1 mutations are associated with type 2 diabetes[69]. Therefore, it is important to study the down-stream gene targets of NeuroD1 and MDA-NeuroD1 that activate the neurogenic, myogenic and pancreatic program through RNA seq and ChIP-seq studies.

In this study, we also demonstrate that Pax3 or MDA-Pax3 expression in MEFs does not only induces myogenic cells but also efficiently induce Pax7(+)MyoD(-) myogenic progenitor-like cells or satellite cell-like cells. These Pax7(+)MyoD(-) cells may undergo differentiation to Pax7(+)MyoD(+) myoblasts followed by becoming Pax7(-)MyoD(+) terminally differentiated myocytes. Therefore, use of Pax7(+)MyoD(-) myogenic progenitor-like cells or satellite cell-like cells may have an advantage for stem cell therapy. Therapeutic stem cell transplantation has been widely explored for regenerative medicine in the last decade. Transplantation of primary myoblasts into DMD model animals and DMD patients has shown that normal myoblasts can participate in regeneration, resulting in dystrophin expression in the host muscle[70] . However, severe limitations exists that hinder practical application in patients, including failed satellite cell self-renewal. By contrast, freshly isolated satellite cells are capable of self-renewal when transplanted into regenerating muscle, and such self-renewal activity is quickly lost when satellite cells are cultured[71] . Recently, we demonstrated that myoblasts isolated from mice lacking the MyoD gene (*MyoD*$^{-/-}$) displayed increased engraftment, survival, and satellite cell contribution compared to wild-type myoblasts following transplantation into regenerating muscle[46,72]. Interestingly, these *MyoD*$^{-/-}$ myoblasts up-regulate Pax3 expression through down-regulation of Pax3-targetting microRNAs. Recently, the Perlingeiro group demonstrated that expression of Pax3 or Pax7 promotes myogenic induction in mouse and human ES and iPS cells[40,73] . These Pax3/7-induced myogenic progenitor cells display efficient engraftment capability in regenerating muscle.

Importantly, these induced myogenic progenitor cells possess self-renewal activity in transplanted muscle, indicating the advantage of use of Pax3/7-induced myogenic cells for stem cell therapy.

We also showed that expression of NeuroD1 alone could convert MEFs to neurogenic cell lineage. However, our results only showed the up-regulation of some neurogenic markers in MEFs, including early neurogenic markers (Brn2/Pou2f3, Otx1 and Tuj1) and mature neurogenic marker (v-GluT1). Therefore, to confirm true neurogenic conversion of MEFs, it is necessary to show their morphological and functional properties as neuronal cells. In this regard, the culture conditions which we used for this study are not optimal for neurons to grow and differentiate. Indeed, the neurogenic cells induced from MEFs by NeuroD1 lack typical neuronal morphology. Therefore, neuronal cell permissive culture conditions should be used to allow the immature neuronal cells to develop into structurally and functionally differentiated neurons. In addition, it is also intriguing whether the NeuroD1-induced neurogenic cells are capable of contribution to regenerating neurons *in vivo* after cell transplantation into brain.

Utilizing myogenic progenitor cells or neurons directly converted from postnatal fibroblasts as a means to expand patient-specific cells and reprogram them to transplantable myogenic or neurogenic progenitor cells can potentially overcome the problems of immune rejection of cell based therapy. Application of the direct reprogramming of fibroblasts is collectively expected to generate an effective cell therapy for neuro-degenerative diseases including Alzheimer's disease and Parkinson’s disease and muscle degenerative diseases including DMD. In this regard, recent works have shown that transplanted human fibroblasts and astrocytes are able to convert into neurons when a reprogramming gene cocktail (ABM) are expressed after transplantation[74]. In addition, endogenous mouse astrocytes residing in the striatum can be directly reprogrammed into neurons *in vivo* when transgene for ABM is activated in mice. Similar direct *in vivo* conversion has already been successful in other organs such as the pancreas and heart[17,18]. Taken together, these published data provide a proof of concept that *in vivo* direct reprogramming is a viable therapeutic strategy for many regenerative diseases. In

the future, single gene transduction or activation of a single master reprogramming gene by small molecules will become a feasible therapeutic approach for regenerative medicine.

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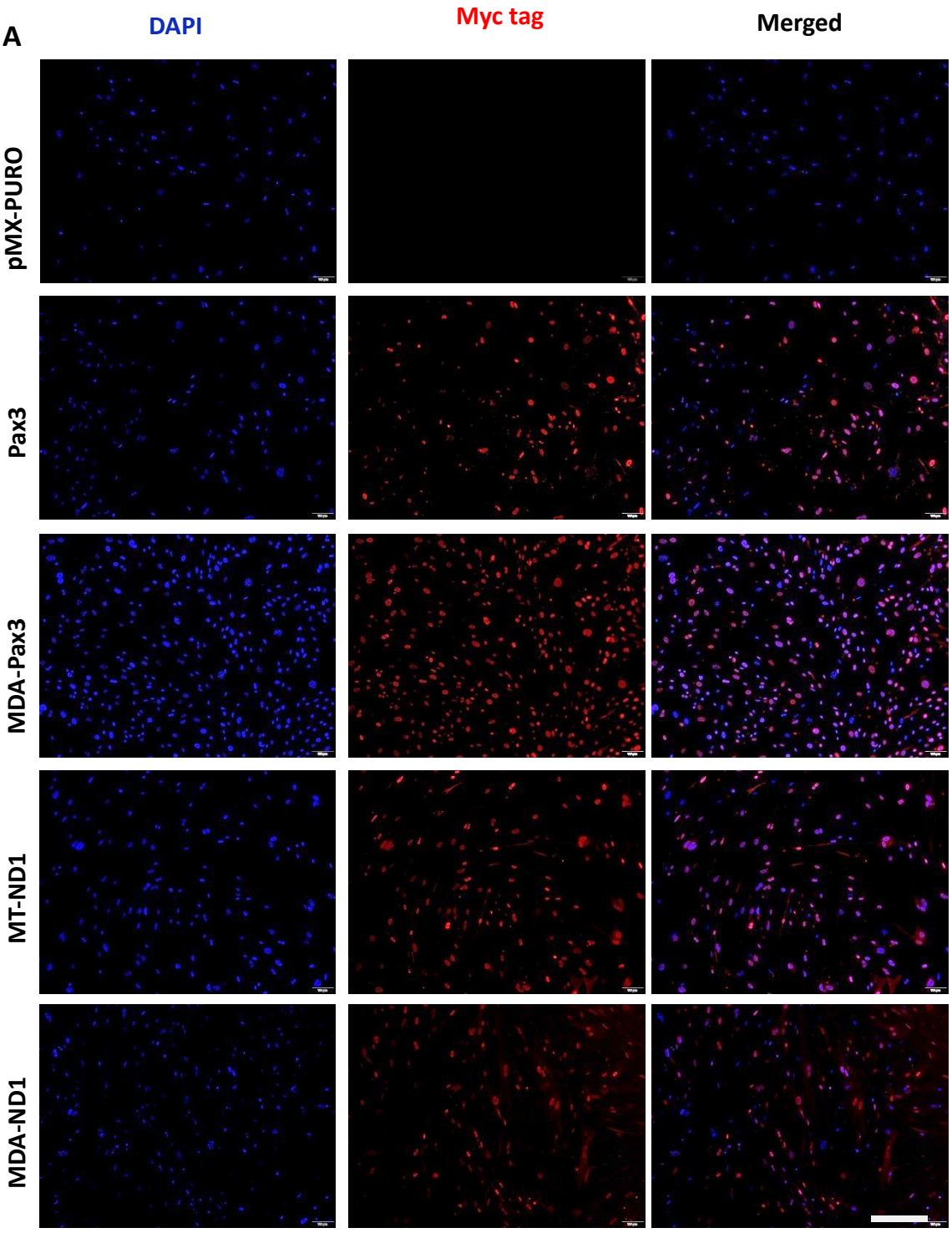
APPENDIX

Figure 9.



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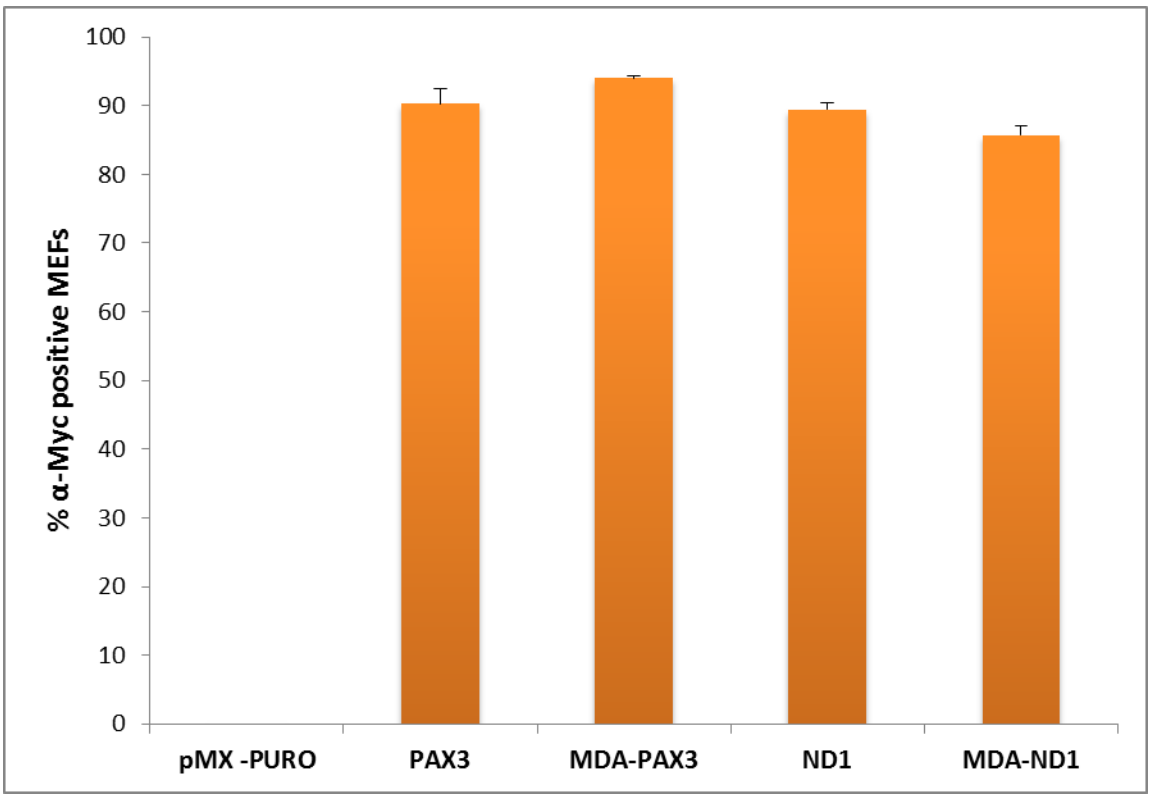
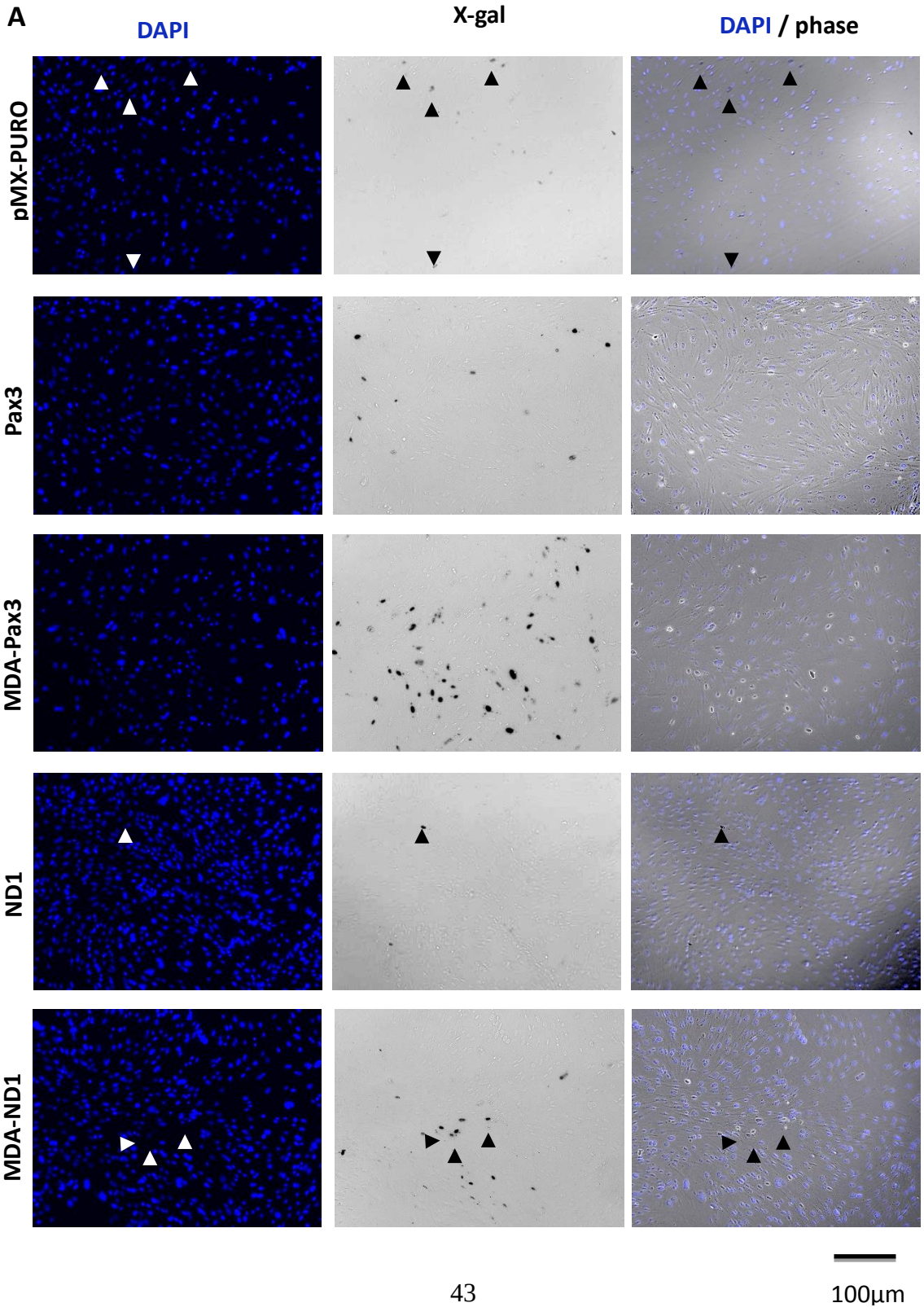


Figure 9. The retrovirally infected MEFs selected in presence of puromycin constitutively expressing MT fusion proteins. A) MEFs retrovirally infected with control pMX-Puro, pMX-MT-Pax3-Puro (Pax3) or pMX-MT-NeuroD1-Puro (ND1) with or without MDA were stained for Myc-tag (MT) (red) by 6 days after infection. DAPI (blue) was used to stain the all nuclei. Scale bar = 100µm. **B)** The percentage of nuclear numbers in MT-positive cells per DAPI positives after infection with retroviral vectors.All data are expressed as average percentage of 3 randomly chosen fields ± SEM. Abbreviations: MT, Myc tag; ND1, NeuroD1; MDA, MyoD transactivation domain.

Figure 10.


A
DAPI
X-gal
DAPI / phase
pMX-PURO
Pax3
MDA-Pax3
ND1
MDA-ND1
100µm

B

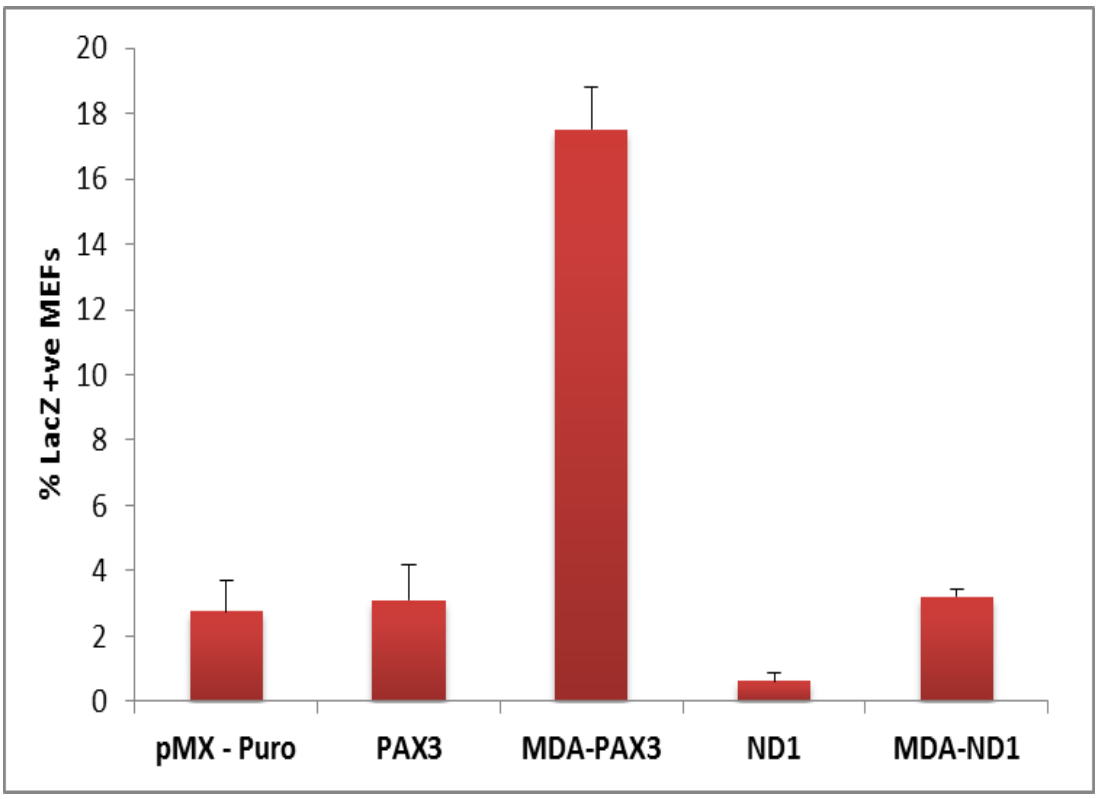
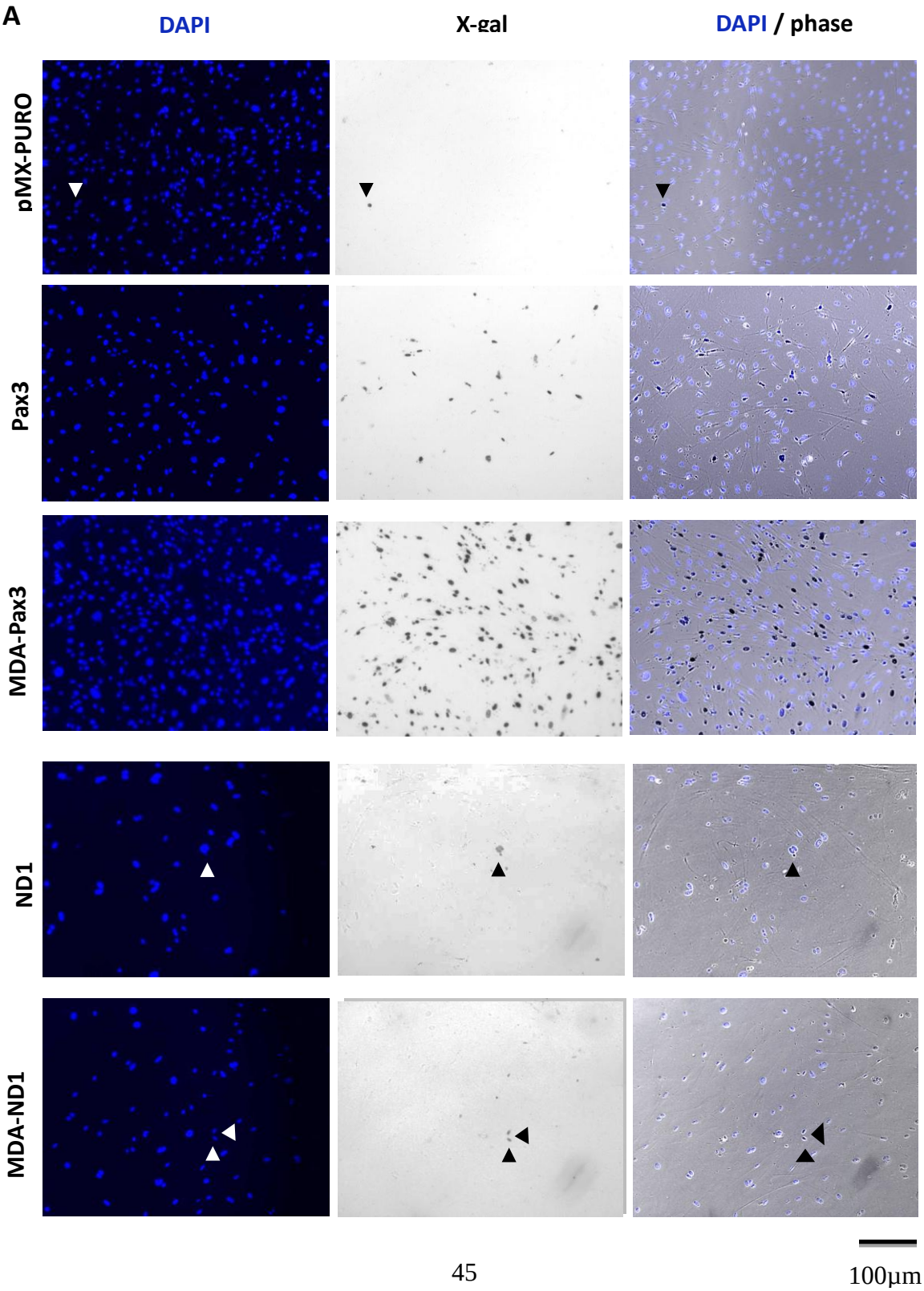


Figure 10. Myf5-lacZ expression in MEFs isolated from *Myf5*$^{+/nLacZ}$ on day 3 without puromycin selection , after retroviral infection with control and Pax3/NeuroD1 with or without MDA. A) MEFs retrovirally infected with control pMX-Puro, pMX-MT-Pax3-Puro (Pax3) or pMX-MT-NeuroD1-Puro (ND1) with or without MDA were stained with X-gal (green) by 3 days after infection. DAPI (blue) was used to stain the all nuclei. Scale bar = 100µm **B)** The percentage of X-gal-positive nucleai per DAPI positive nuclei after infection with retroviral vectors. All data are expressed as average percentage of 3 randomly chosen fields ± SEM.. Abbreviations: ND1, NeuroD1; MDA, MyoD transactivation domain.

Figure 11.


A
DAPI
X-gal
DAPI / phase
pMX-PURO
Pax3
MDA-Pax3
ND1
MDA-ND1
100μm

B

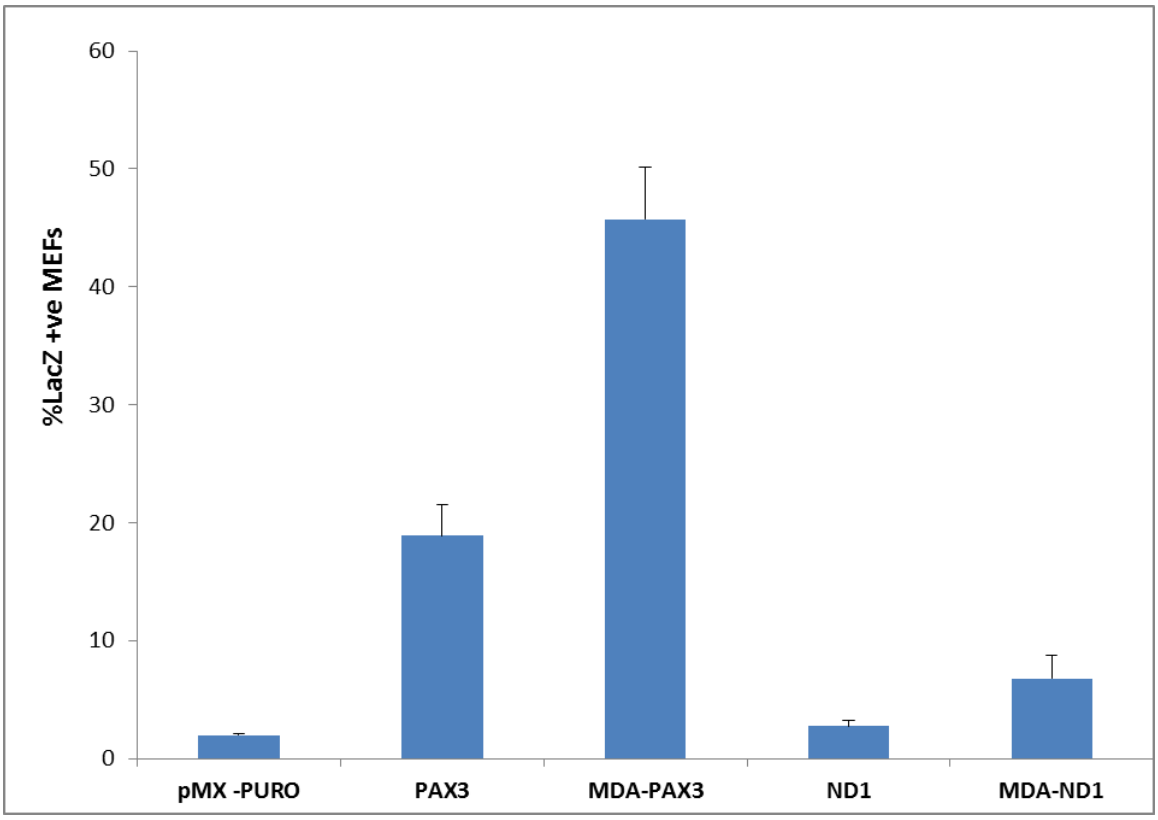
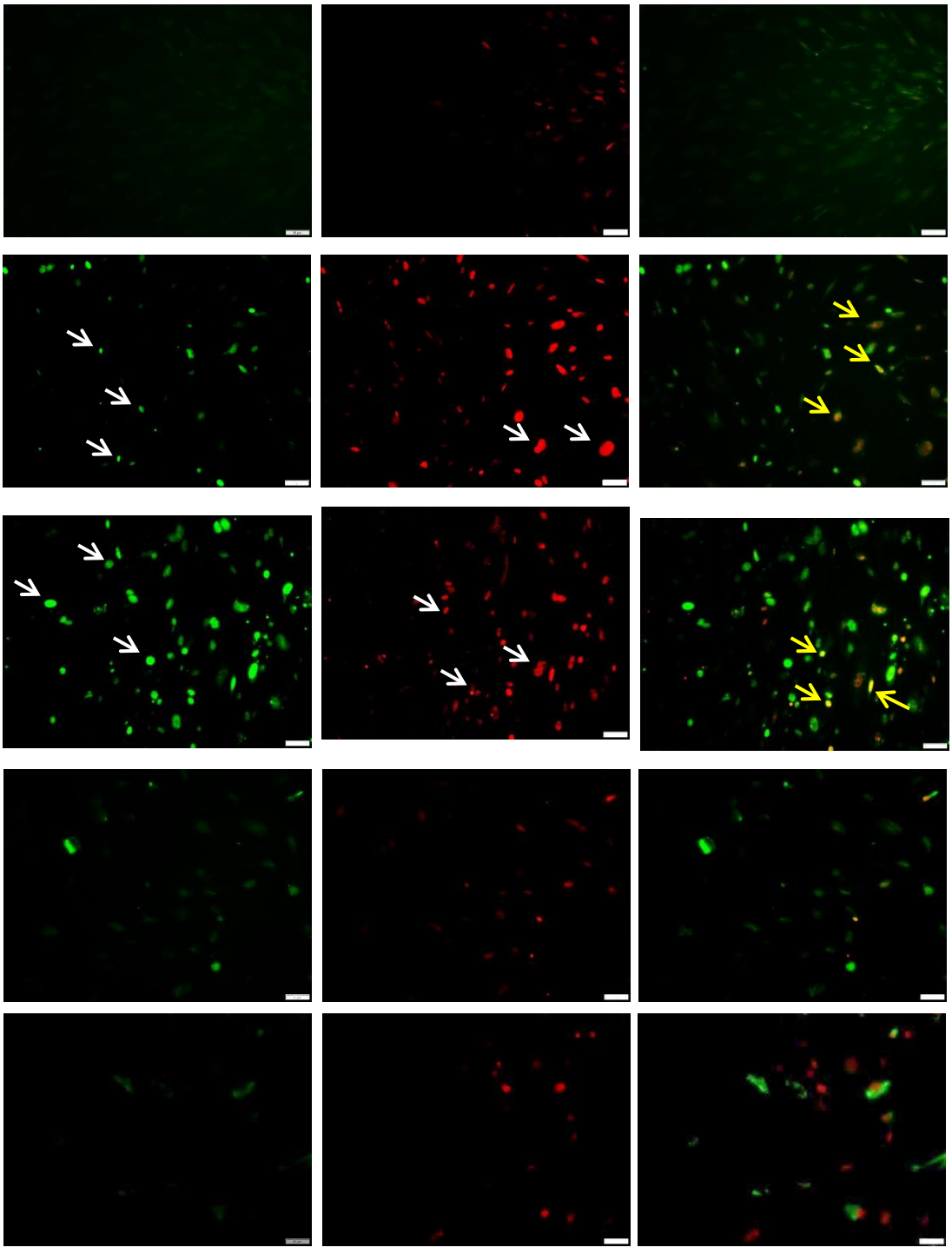


Figure 11. Myf5-lacZ expression in MEFs isolated from *Myf5*$^{+/nLacZ}$ on day 6 with puromycin selection after retroviral infection with control and Pax3/NeuroD1 with or without MDA. A) MEFs retrovirally infected with control pMX-Puro, pMX-MT-Pax3-Puro (Pax3), or pMX-MT-NeuroD1-Puro (ND1) with or without MDA were stained with X-gal (green, arrowheads) by 6 days after infection with puromycin selection. DAPI (blue) was used to stain the all nuclei. Scale bar = 100μm **B)** The percentage of X-gal-positive nuclei per DAPI positive nuclei after infection with retroviral vectors All data are expressed as average percentage of 3 randomly chosen fields ± SEM.. Abbreviations: MT, Myc tag; MDA, MyoD transactivation domain; ND1, NeuroD1.

Figure 12.

A



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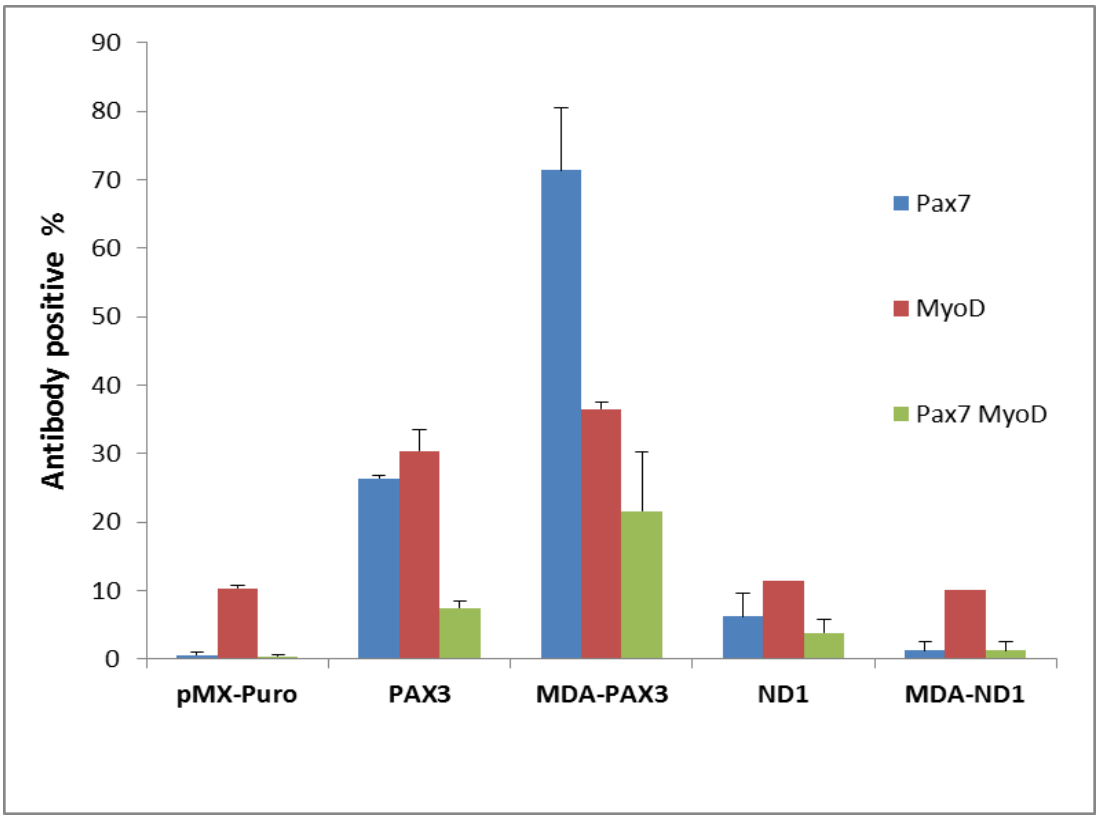
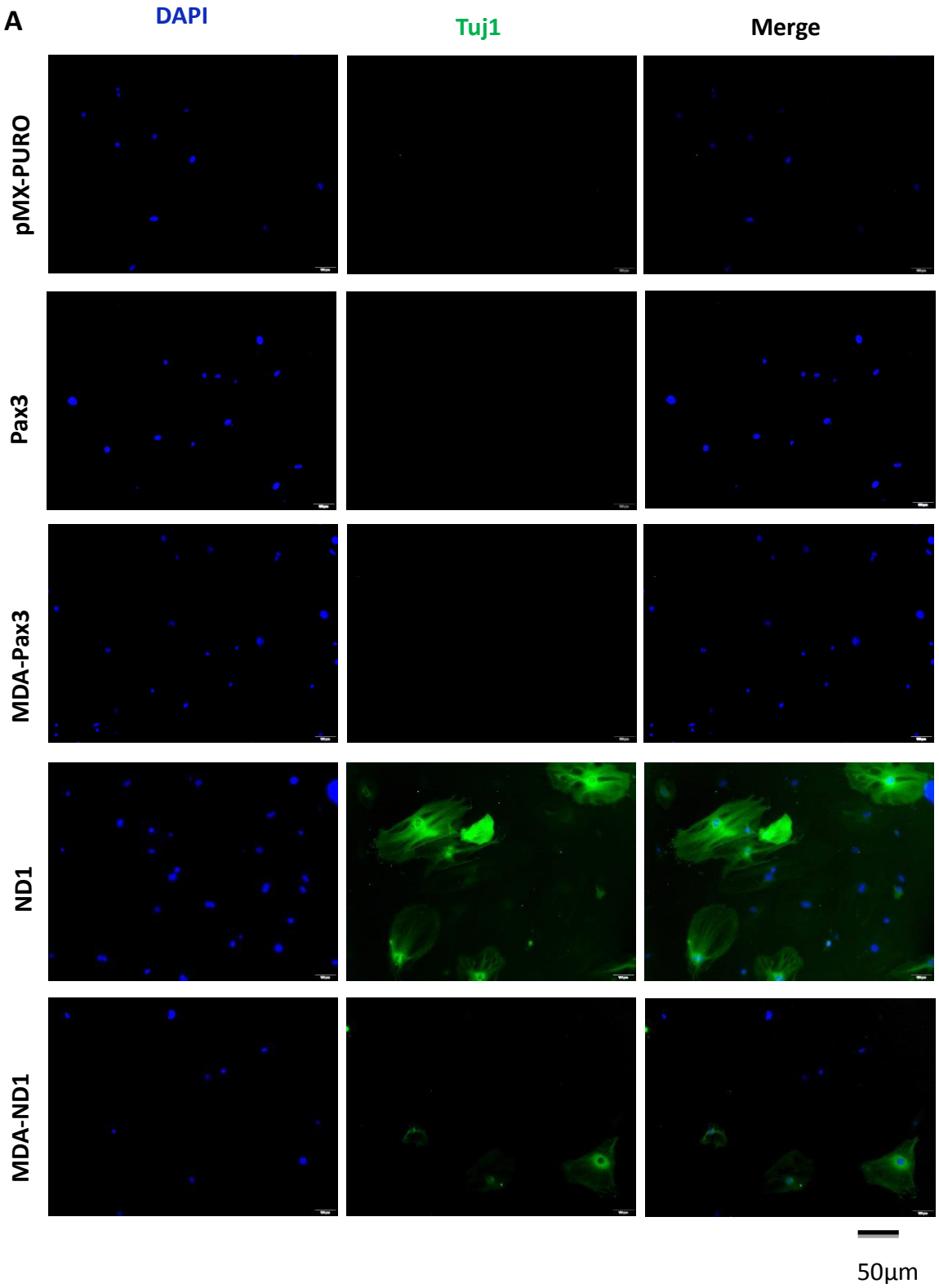


Figure 12. Pax3 is sufficient to induce myogenic markers in MEFs.

A) MEFs retrovirally infected with control pMX-Puro, pMX-MT-Pax3-Puro (Pax3), or pMX-MT-NeuroD1-Puro (ND1) with or without MDA were stained for Pax7 (green) and MyoD (red) by 6 days after infection with puromycin selection. DAPI (blue) was used to stain the all nuclei. White arrows indicate single antibody positives [Pax7(+)MyoD(-) or Pax7(-) MyoD(+)] and yellow arrows indicate double antibody positives [Pax7(+)MyoD(+)].Scale bar = 100µm.**B)** The percentage of antibody-positive nuclei per DAPI positive nuclei after infection with retroviral vectors. All data are expressed as average percentage of 3 randomly chosen fields ± SEM. Abbreviations: MT, Myc tag; MDA, MyoD transactivation domain; ND1, NeuroD1.

Figure 13.



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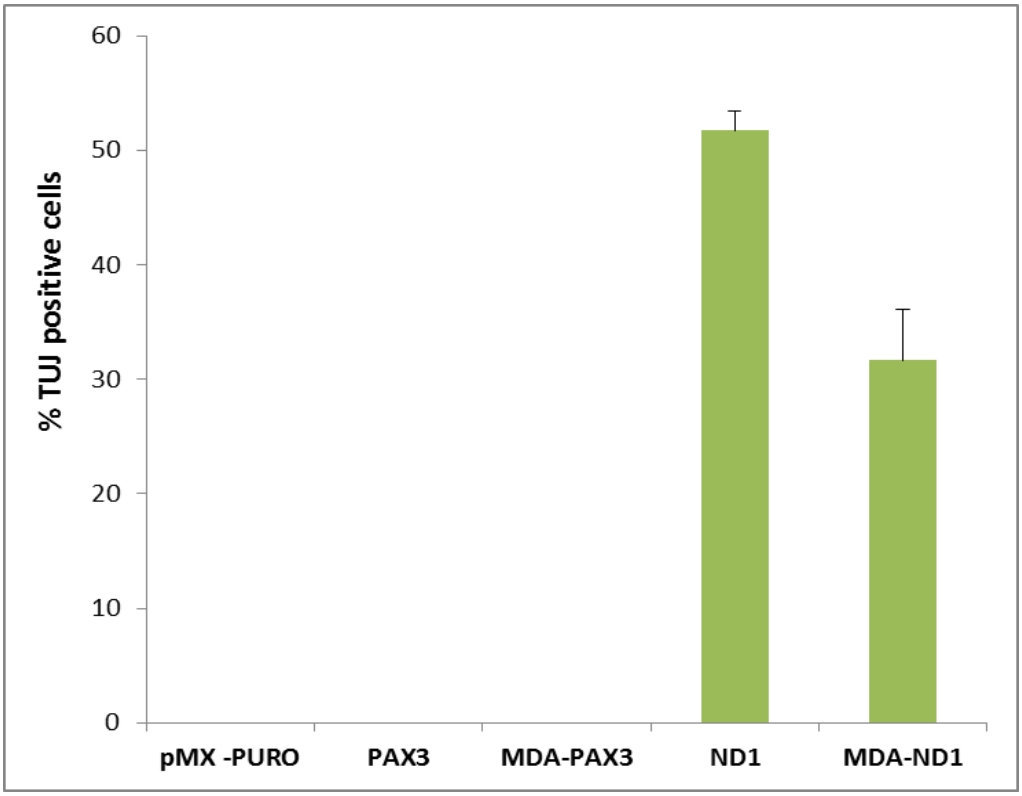


Figure 13. NeuroD1 is sufficient to induce early neuronal marker Tuj1 in MEFs.

A) MEFs retrovirally infected with control pMX-Puro, pMX-MT-Pax3-Puro (Pax3) or pMX-MT-NeuroD1-Puro (ND1) with or without MDA were stained for Tuj1 (green) by 9 days after infection with puromycin selection. DAPI (blue) was used to stain the all nuclei. . Scale bar = 50µm **B)** The percentage of TuJ1-positive nuclei per DAPI positive nuclei after infection with retroviral vectors. All data are expressed as average percentage of 3 randomly chosen fields ± SEM. Abbreviations: MT, Myc tag; MDA, MyoD transactivation domain; ND1, NeuroD1.

Figure 14.

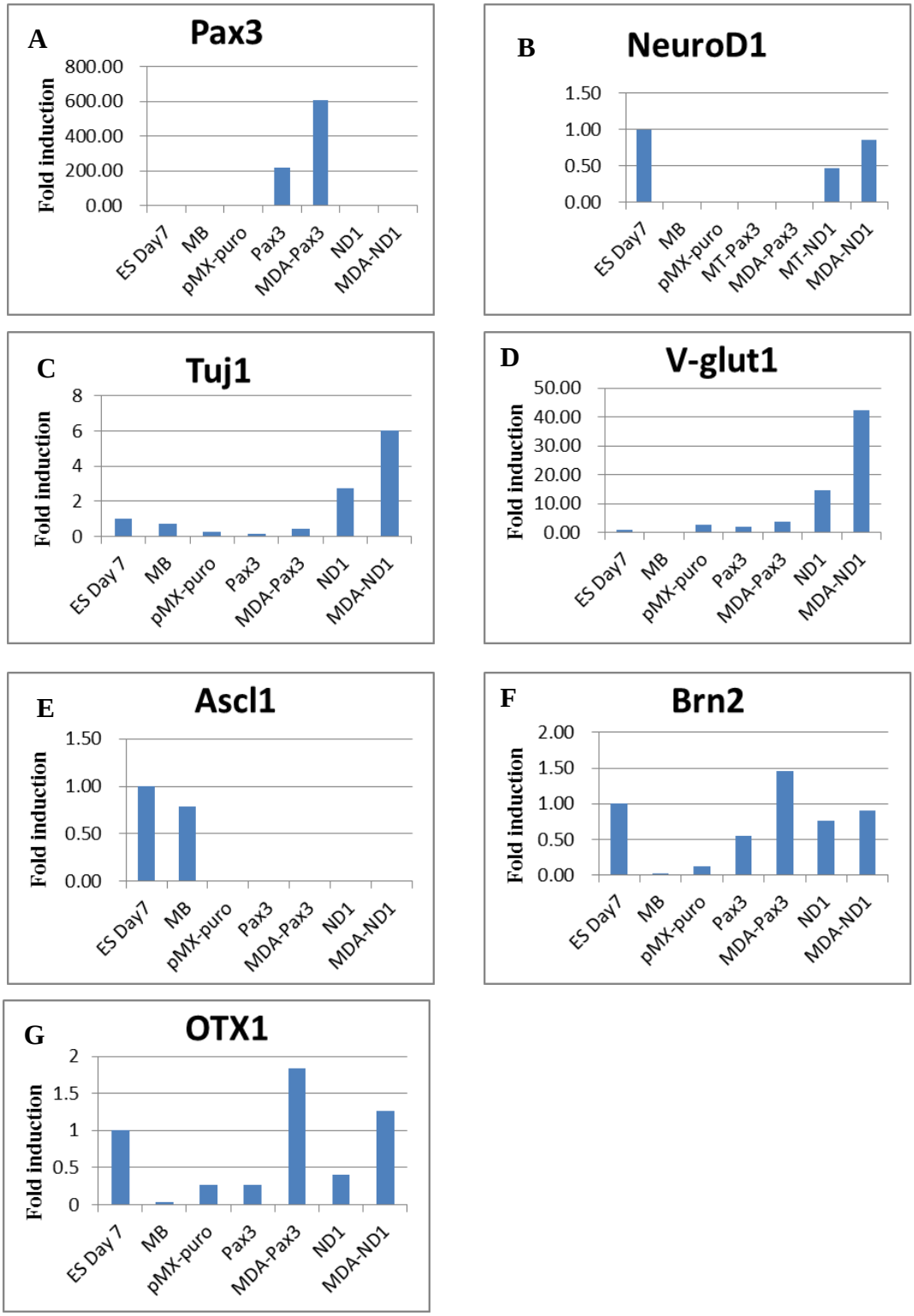
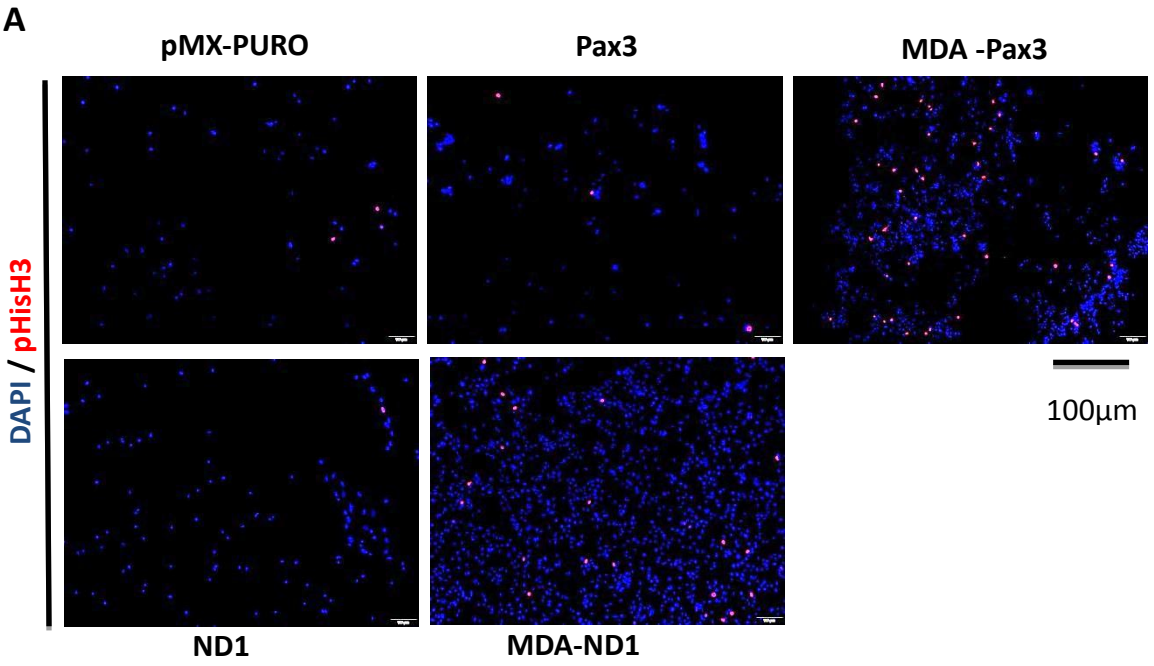

A
Pax3
Fold induction
800.00
600.00
400.00
200.00
0.00
ES Day7
MB
pMX-puro
Pax3
MDA-Pax3
ND1
MDA-ND1
B
NeuroD1
1.50
1.00
0.50
0.00
ES Day7
MB
pMX-puro
MT-Pax3
MDA-Pax3
MT-ND1
MDA-ND1
C
Tuj1
8
6
4
2
0
D
V-glut1
50.00
40.00
30.00
20.00
10.00
0.00
E
Ascl1
F
Brn2
2.00
1.50
1.00
0.50
0.00
G
OTX1
2
1.5
1
0.5
0

Figure 14. Q-PCR analysis of neurogenic genes in MEFs on day 3 after retroviral infection with control and Pax3/NeuroD1 with or without MDA.

(A-G) q-PCR analysis of the expression levels of various neurogenic genes in MEFs retrovirally infected with control pMX-puro, pMX-MT-Pax3-Puro or pMX-MT-NeuroD1-Puro with or without MDA. cDNAs of 7-days differentiated ES cells (ES day 7) and proliferating myoblasts were used for control. The amounts of transcripts were normalized to that of β-actin as an internal control. The expression level of ES day 7 or myoblasts was set as 1.0, and then relative value of each sample was calculated. Genes: Pax3-Paired box transcription factor 3, TuJ1- Neuron-specific class III β -tubulin,V-glut1- Vesicular glutamate transporter 1, Ascl1- Achaete-scute complex homolog 1, Brn2- POU class 3 homeobox 2, OTX1- orthodenticle homeobox 1. Abbreviations: MDA-MyoD transactivation domain



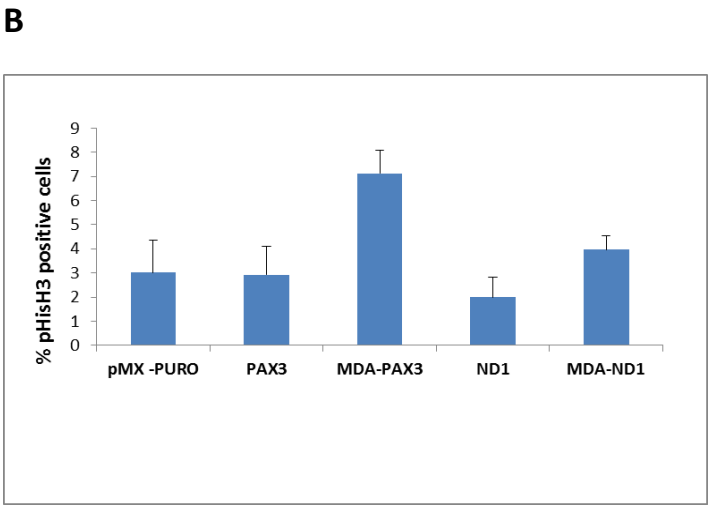
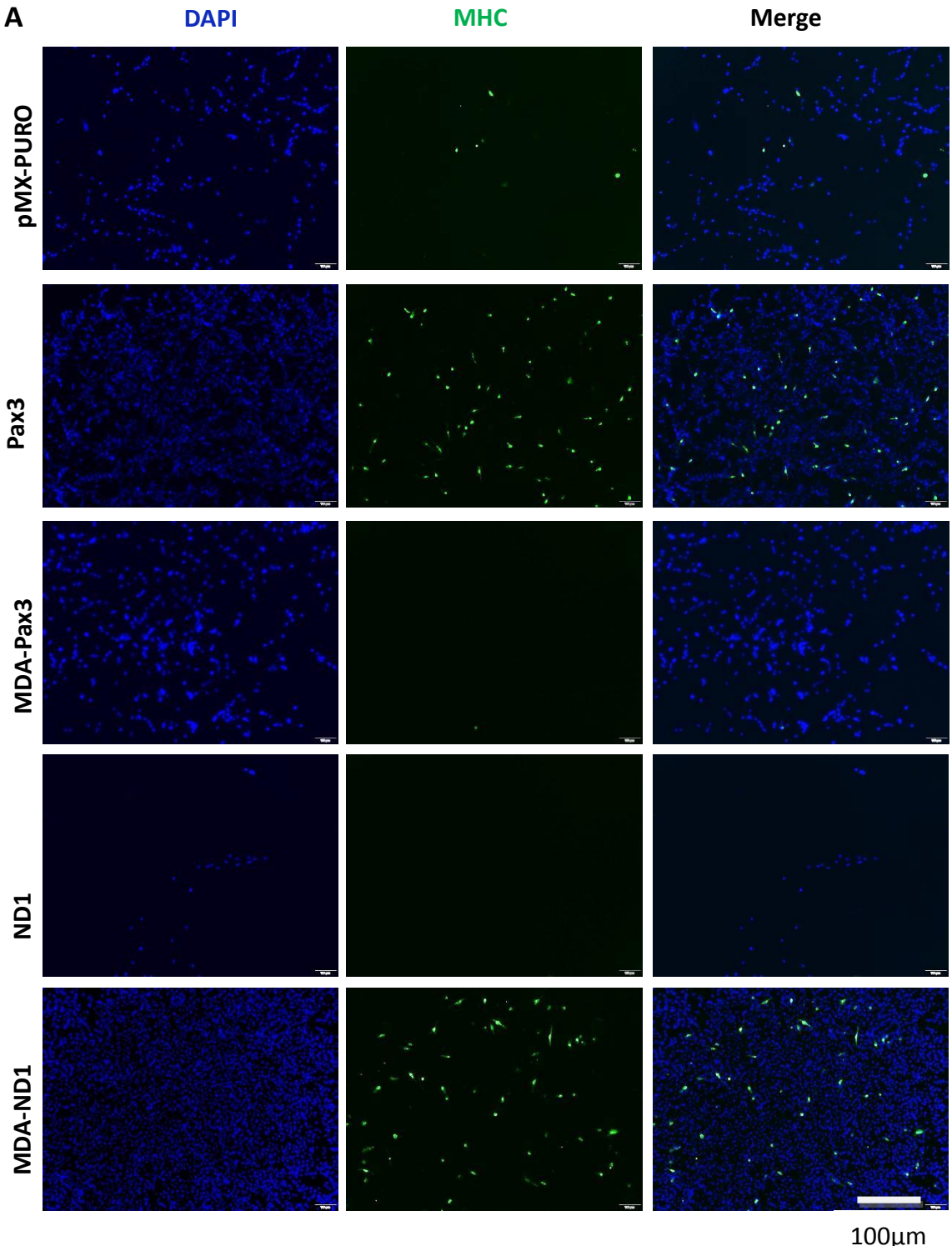


Figure 15. Myoblast proliferation assay by anti-phosphohistone H3 antibody staining after retroviral infection with control and Pax3/NeuroD1 with or without MDA . A) Myoblasts retrovirally infected with control pMX-Puro, pMX-MT-Pax3-Puro (Pax3) or pMX-MT-NeuroD1-Puro (ND1) with or without MDA were stained for phospho-histone H3 (pHisH3) (green) by 6 days after infection. DAPI (blue) was used to stain the all nuclei.Scale bar = 100µm **B)** The percentage of pHisH3-postive cells per DAPI positive nuclei after infection with retroviral vectors. All data are expressed as average percentage of 3 randomly chosen fields ± SEM. Abbreviations: MT, Myc tag; MDA, MyoD transactivation domain; ND1, NeuroD1.

Figure 16.



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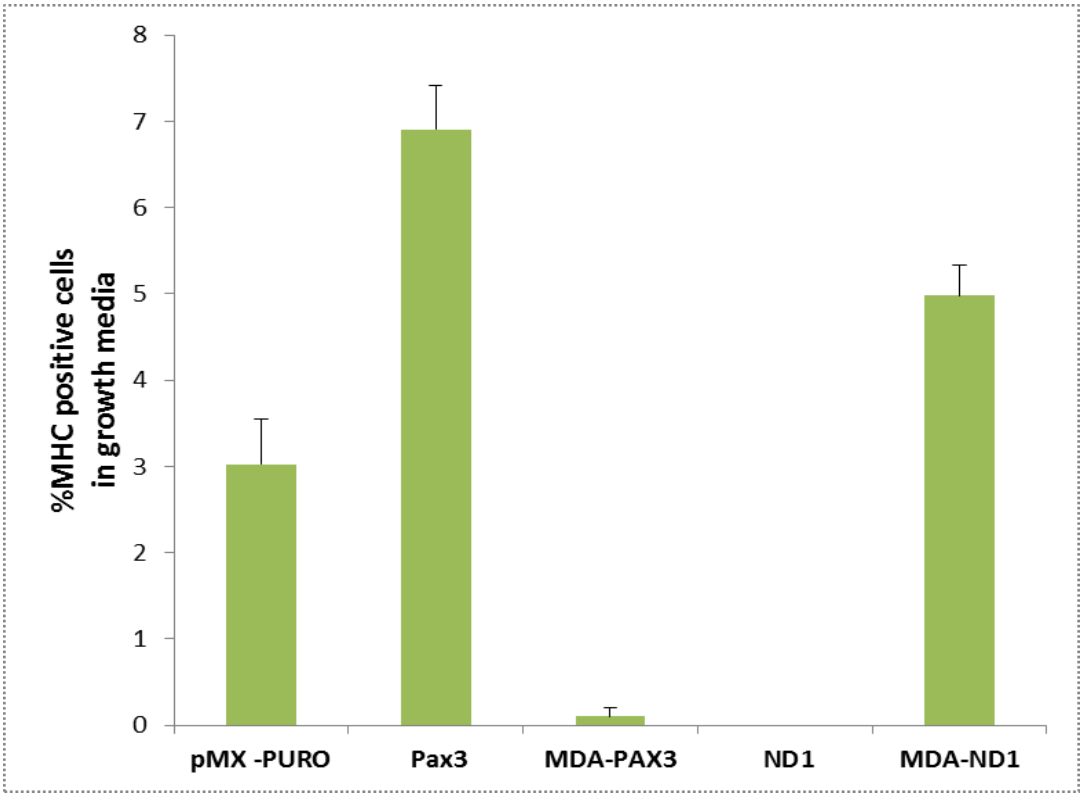
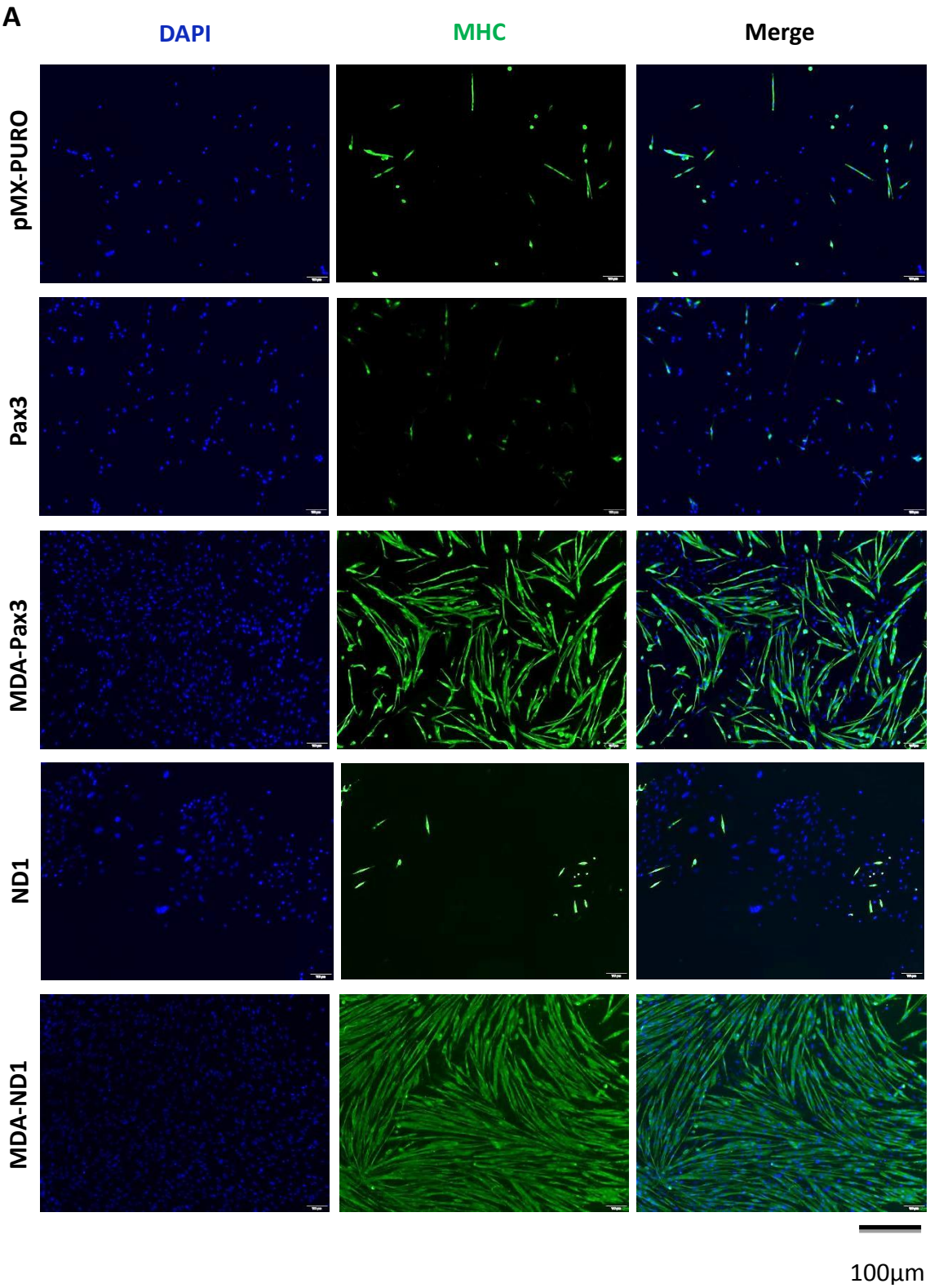


Figure 16. Myosin heavy chain (MHC) expression in myoblasts retrovirally infected with control and Pax3/NeuroD1 with or without MDA under growth conditions.

A) Myoblasts retrovirally infected with control, pMX-Puro, pMX-MT-Pax3-Puro (Pax3), or pMX-MT-NeuroD1-Puro (ND1) with or without MDA were stained for Myosin MHC (green) by 6 days after infection under growth conditions. DAPI (blue) was used to stain the all nuclei. Scale bar= 100µm **B)** The percentage cells expressing MHC per DAPI-positive nuclei after infection with retroviral vectors. All data are expressed as an average percentage of 3 randomly chosen fields ± SEM. Abbreviations: MT, Myc tag; MDA, MyoD transactivation domain; ND1, NeuroD1.

Figure 17.



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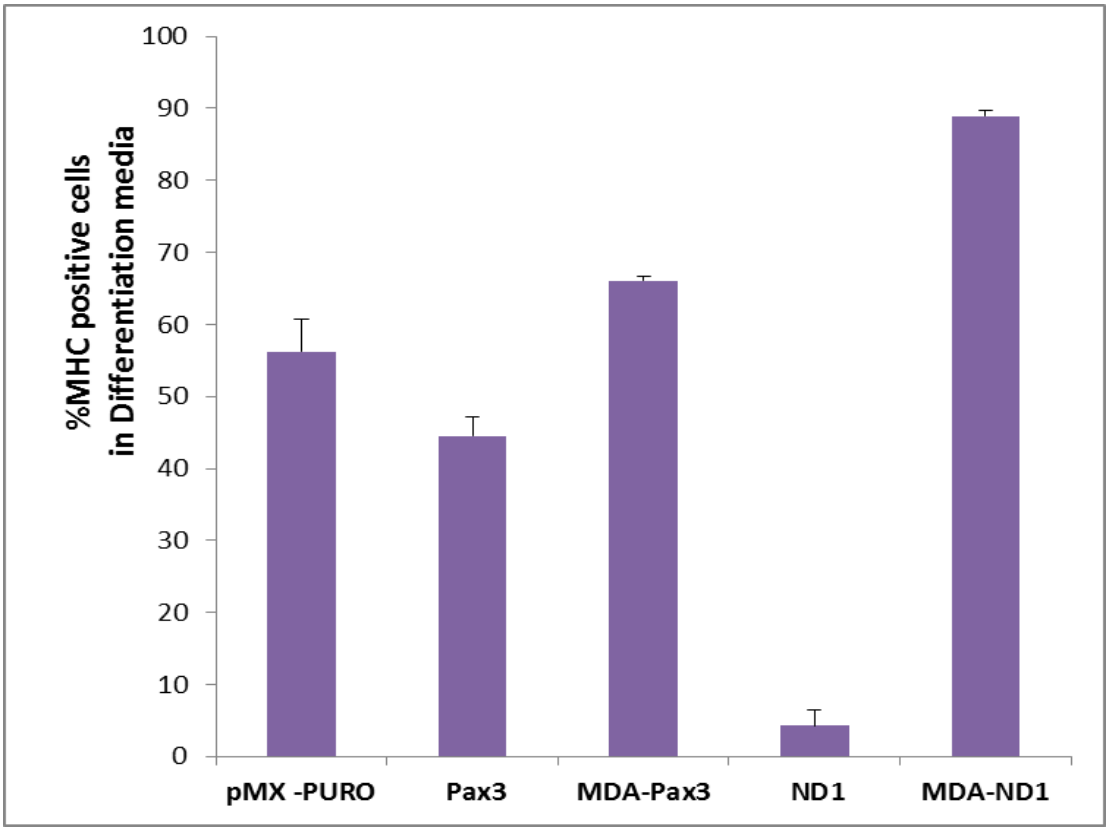
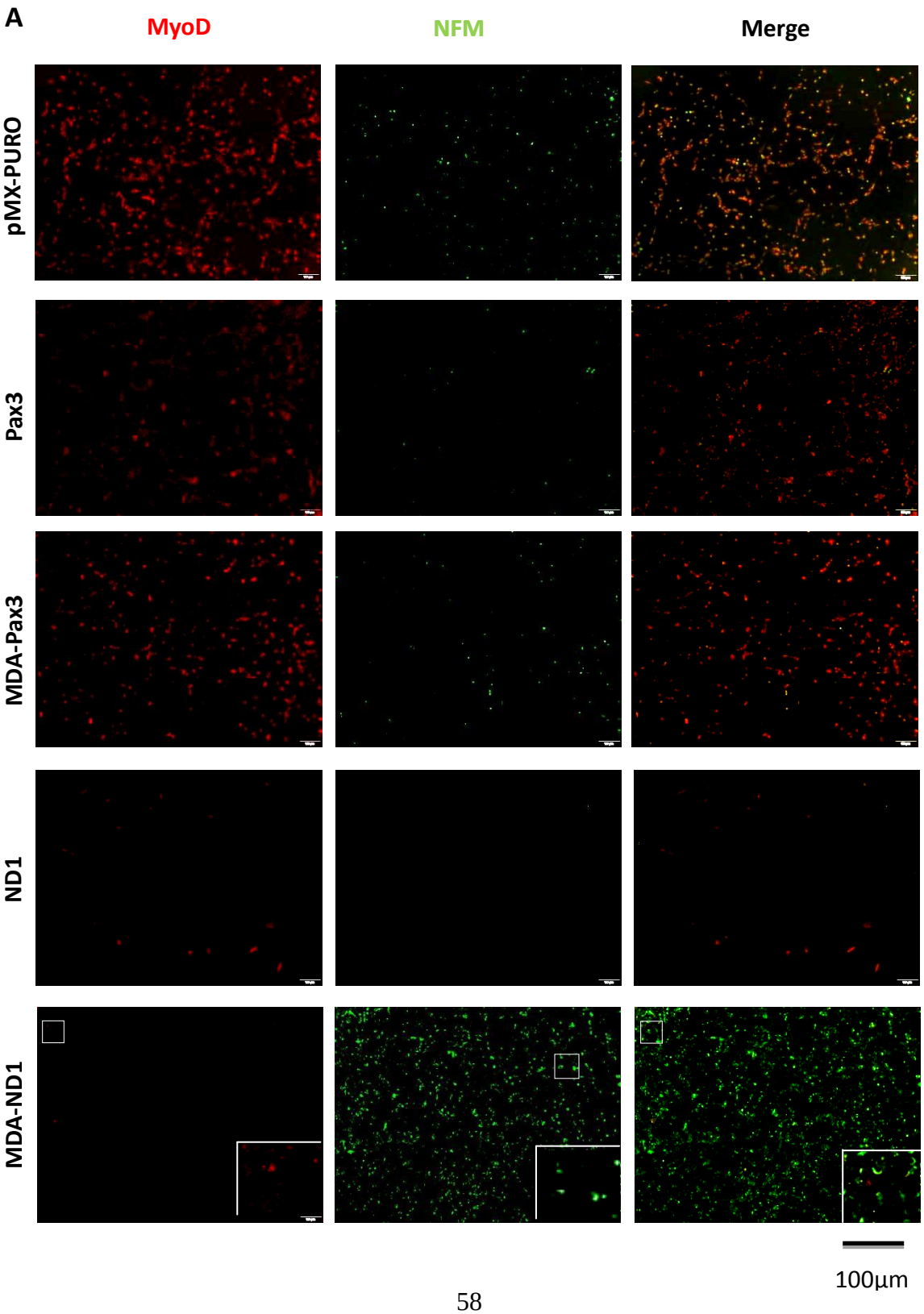


Figure 17. MHC expression in myoblasts retrovirally infected with control and Pax3/NeuroD1 with or without MDA under differentiation conditions. A) Myoblasts retrovirally infected with control pMX-Puro, pMX-MT-Pax3-Puro (Pax3), or pMX-MT-NeuroD1-Puro (ND1) with or without MDA were stained for Myosin MHC (green) by 6 days after infection followed by 3 additional days in differentiation medium. DAPI (blue) was used to stain the all nuclei. Scale bar = 100μm **B)** The percentage of MHC positive cells per DAPI positive nuclei after infection with retroviral vectors. All data are expressed as average percentage of 3 randomly chosen fields ± SEM. Abbreviations: MT, Myc tag; MDA, MyoD transactivation domain.

Figure 18.



B

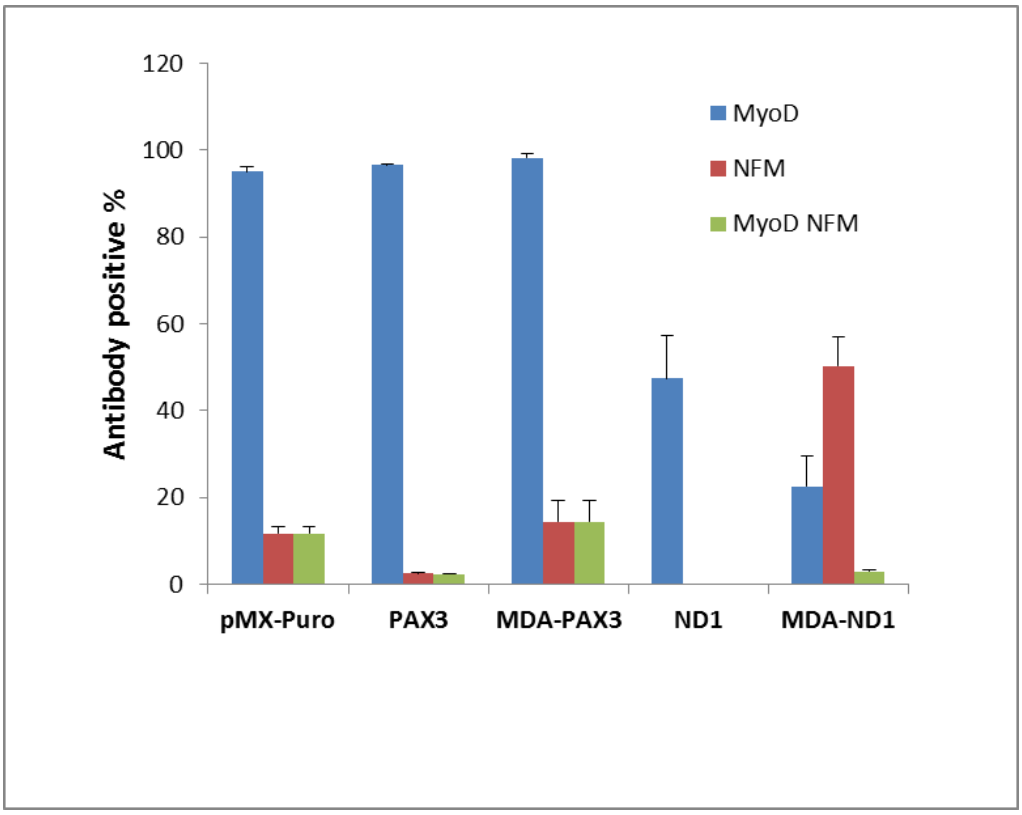


Figure 18. Up-regulation of Neurofilament-M (NFM) in myoblasts infected with MDA-NeuroD1. **A)** Myoblasts retrovirally infected with control pMX-Puro, pMX-MT-Pax3-Puro (Pax3) or pMX-MT-NeuroD1-Puro (ND1) with or without MDA were stained for Neurofilament-M (green) and MyoD (red) by 6 days after infection. DAPI (blue) was used to stain the all nuclei. Scale bar = 100µm. **B)** The percentage of cells expressing Neurofilament-M (NFM) per DAPI positive nuclei after infection with retroviral vectors. All data are expressed as average percentage of 3 randomly chosen fields ± SEM. Abbreviations: MT, Myc tag; MDA, MyoD transactivation domain; ND1, NeuroD1.