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Lack of Myostatin Reduces MyoD Induced Myogenic Potential of Primary Muscle Fibroblasts

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ABSTRACT

Conversion of skin fibroblasts into myoblasts by transducing the cells with myogenic master regulator MyoD has been in practice for more than two decades. The purpose of such conversion is due to scarcity of muscle biopsies during muscle wasting, hence conversion of fibroblasts to myogenic lineage from various genetic backgrounds offers a great alternative for cell therapies. Here, we have investigated if eliminating Myostatin, a potent negative regulator of myogenesis, could improve the myogenic conversion of fibroblasts. In the present study, we have isolated primary muscle fibroblasts from the skeletal muscles of wild-type (WT) and *myostatin* null (*Mstn*$^{-/-}$) mice and transduced the muscle fibroblasts with MyoD using adenoviral, lentiviral transduction, and electroporation methods. In contrast to what we predicted, it is only in WT muscle fibroblasts we detected significant ectopic expression of MyoD, and myogenic conversion. Muscle fibroblasts from *Mstn*$^{-/-}$ genotype failed to take up as much MyoD using the three methods and, therefore, failed to form myotubes. The aforesaid condition of greater MyoD uptake by WT muscle fibroblasts was attributed to the presence of adenoviral receptors, which facilitated adenoviral transduction. However, in *Mstn*$^{-/-}$ fibroblasts we detected negligible levels of adenovirus receptors. Moreover, we also detected significantly higher levels of MyoD antagonists, c-Fos, c-Jun, and cyclin D1 in *Mstn*$^{-/-}$ muscle fibroblasts. Taken together, our results demonstrate that lack of myostatin reduces myogenic potential of muscle fibroblasts by inhibiting MyoD function. J. Cell. Biochem. 115: 1908–1917, 2014.

KEY WORDS: FIBROBLASTS; MYOBLASTS; MyoD; MYOSTATIN; MYOGENESIS

Muscle biopsy as a starting material has been used not only for evaluating basic histopathological, and immunohistochemical changes associated with genetic muscle diseases but also for establishing myoblasts to identify defects in signaling pathways [Ikezawa et al., 1998]. Even though obtaining such muscle biopsies from adult patients pose minimal risk, procuring muscle biopsies from pediatric patients of skeletal muscle disorders is difficult [Wedel, 1992]. Therefore, uses of alternative source of scalable quantities of myoblasts are suggested to be useful. One common method used for obtaining a large number of muscle cells from non-muscle cells like skin fibroblasts or amniotic fluid cells is by using forced expression of MyoD [Roest et al., 1999].

MyoD is a member of the muscle specific basic helix loop helix (bHLH) family of transcription factors, which play a major role in the determination and differentiation of all skeletal muscle lineages [Tapscott and Weintraub, 1991; Arnold and Braun, 1996; Arnold and Winter, 1998]. MyoD can induce myogenic differentiation in most cell types derived from all the germ layers of the embryo [Gerhart

Abbreviations used: αSMA, alpha smooth muscle actin; bHLH, basic helix loop helix; CAR, Coxsackie and adenovirus receptor; CDK, cyclin dependent kinase; DMEM, Dulbecco's modified eagle's medium; PBS, phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; Mstn, Myostatin; Mstn$^{-/-}$, myostatin null; MPP1, muscle preplate1; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; TGF-β, transforming growth factor beta; WT, wild-type.

The authors declared that they have no conflicts of interest.

Sudheer Shenoy P and Bipasha Bose contributed equally to this work.

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et al., 2001]. Muscle specific genes can be activated in a variety of cell types by using forced expression of MyoD like skin fibroblasts, cardiac fibroblasts [Murry et al., 1996], C3H10T(1/2) mouse mesenchymal cells [Tapscott and Weintraub, 1991], P19 embryonal carcinoma cells [Skerjanc et al., 1994], melanoma, neuroblastoma, hepatocytes, and adipocytes [Weintraub et al., 1989]. Introduction of the *MyoD* gene into cells and further incubation of these cells under reduced horse serum culture conditions converts these myogenic cells into functional multinucleated myotubes. In addition to MyoD other myogenic determination factors, such as Myogenin/Myf4, Myf5, Myf6, and MRF4 can convert many different cell types into skeletal muscle in vitro [Thayer et al., 1989; Tapscott and Weintraub, 1991; Weintraub et al., 1991; Rudnicki et al., 1993; Olson and Klein, 1994]. Interestingly, such myogenic conversion with forced expression of MyoD has been only met with 50–60% efficiency [Weintraub et al., 1989].

We further reasoned that the presence of negative regulators or inhibitors of myogenesis could be governing low efficiency of myogenic conversion; therefore, lowering the levels of such negative regulators of myogenesis in non-myogenic cells might improve the efficacy of myogenic conversion. Hence we chose to investigate if inactivation of myostatin, a potent negative regulator of myogenesis, in fibroblasts would increase the efficacy of myogenic conversion upon MyoD overexpression. Myostatin (GDF-8) is a growth factor, of the TGF-β super family and excess levels of myostatin have been shown to inhibit not only myoblast proliferation but also differentiation [Thomas et al., 2000; Taylor et al., 2001]. In addition, myostatin expression is also detected in fibroblasts, and it has been shown that excess levels of myostatin increase fibroblast proliferation [Mendias et al., 2008]. In contrast to our hypothesis, our results here indicated that the myogenic potential of $Mstn^{-/-}$ fibroblast is, in fact, significantly less than that seen when MyoD was transduced into WT muscle fibroblasts. The molecular analysis suggested that this could be due to reduced transduction efficiency as we detected reduced levels of adenoviral receptors together with negligible ectopic expression of MyoD in $Mstn^{-/-}$ fibroblasts. We also propose that increased expression of MyoD antagonists, c-Fos, c-Jun, and cyclin D1 in $Mstn^{-/-}$ muscle fibroblasts could have contributed to the reduced myogenesis seen in $Mstn^{-/-}$ fibroblasts following transduction of MyoD.

MATERIALS AND METHODS

All experiments involving animals were approved by the Nanyang Technological University Institutional Animal Care and Use Committee (IACUC), Singapore (Approval Number: ARF SBS/NIE-A 0057).

CELL CULTURE AND DERIVATION OF MUSCLE FIBROBLASTS FROM WT AND $Mstn^{-/-}$ MICE (C57/BL/6J)

Isolation of muscle fibroblasts was carried out following the protocol described by Gharaibeh et al. [2008]. The rapidly adhering fraction (RAF) called muscle preplate1 (MPP1) containing muscle fibroblasts isolated both from WT and $Mstn^{-/-}$ mice (both 5 weeks old) were used for the current study. The cells were maintained in DMEM (high glucose Invitrogen) supplemented with 20% fetal bovine serum (FBS) (Hyclone), 10% horse serum (HS) (Invitrogen), 0.5% chick embryo extract (CEE US Biologicals), 1% penicillin and streptomycin (Invitrogen) and 0.1 mM β-mercaptoethanol (Sigma). Population doubling time was calculated as described previously [Sherley et al., 1995]. Cells from early passage were taken for all the studies.

ADENOVIRAL TRANSDUCTION

Adenoviral clone Ad-MyoD was a kind gift from Charles E Murry Department of Pathology University of Washington Seattle WA 98195 [Murry et al., 1996]. Starter cultures of Ad-MyoD were generated by transducing the original stock at 5.3×10^6 pfu/ml into one well of six well plate containing 50% confluent 293T cells for 48 h till the cytopathic effect was visible. Larger volumes of the virus were generated by transducing 1 ml of the starter culture into one T-175 flask of 50% confluent 293T cells till cytopathic effect became visible. The viral cultures were concentrated using Amicon filters (Millipore). The final viral concentrate contained 5×10^9 pfu/ml. Two hundred microliters of viral concentrate was used to infect 1×10^6 muscle fibroblasts growing on 10 cm dish isolated each from WT and $Mstn^{-/-}$ mice. Viral infection dose was kept constant at 1,000 pfu/cell. Twenty-four hours of virus infection were followed by switching the media to classical myogenic differentiation media-DMEM supplemented with 2% HS. The virus concentrate was tested for its ability to induce myotube formation up to 72 h.

LENTIVIRAL TRANSDUCTION

Lentiviral transduction was performed as previously described with modification [Kimura et al., 2008]. The pLenti-based expression vector (pLv-CMV-MyoD, plasmid 26808) containing MyoD gene of interest, Lentiviral plasmid (pLCAG-EGFP, Plasmid 14857) with green fluorescent protein, (GFP) and the components of the lentiviral packaging mix were purchased from Addgene. 293T cells were co-transduced with plasmids, in order to, prepare the lentiviral stocks as described earlier [Kimura et al., 2008]. The viral cultures were concentrated using Amicon filters (Millipore). On the day of transduction, 200 μl of lentiviral concentrate was gently mixed and added on to 1×10^6 fibroblasts derived from WT and $Mstn^{-/-}$ mice and incubated at 37°C for 24 h. The culture medium was replaced with fresh media DMEM (Gibco) supplemented with 2% HS after 24 h. The virus concentrate was tested for its ability to induce differentiation of fibroblasts to myoblasts and then to multinucleated myotubes up to 72 h. The expression of MyoD was determined after 24 h of transduction by FACS.

ELECTROPORATION

Electroporation was done performed as previously described [Pfeifer et al., 2002]. Electroporation was initiated by washing the cells with PBS, counted and resuspended in Gene Pulsar electroporation buffer (Bio-Rad, Hercules, CA) to a cell density of 5×10^6 cells/ml unless otherwise indicated, and mixed with nucleic acid. The following plasmids were used: 20 μg of Lentiviral plasmid (pLCAG-EGFP, Plasmid 14857) with green fluorescent protein (GFP) and 20 μg Lentiviral expression plasmid (pLv-CMV-MyoD, plasmid 26808) both purchased from Addgene were co-transfected into muscle fibroblasts derived from WT and $Mstn^{-/-}$ mice. Bio-Rad Gene Pulsar was set at 260 volts and 960 μF. Transfected cells were plated into

10 cm dishes (Nunc) for 24 h in DMEM supplemented with 10% FBS. The culture medium was replaced with fresh, media DMEM supplemented with 2% HS after 24 h. The efficiency of the MyoD was tested for its ability to induce differentiation of fibroblasts to myoblasts and then to multinucleated myotubes up to 72 h. pLCAG-EGFP alone was electroporated as a negative control (NC) and was used to assess electroporation efficiency by Flow Cytometry.

GENE EXPRESSION BY qRT-PCR

RNA isolation was done using the RNeasy Mini-kit (74104, Qiagen). cDNA synthesis was performed from 1 μg of RNA using iScript c-DNA synthesis kit (Bio-Rad). Real time quantitative PCR was performed with Eva Green SYBR green qPCR supermix-(Bio-Rad) in Eppendorf realplex/ABI 7000 (Eppendorf) equipment. Relative gene expression was calculated by the $2(-\Delta\Delta Ct)$ method compared to the respective untransduced cells (day 0), using GAPDH as housekeeping gene. ΔCt values >16 were considered not expressed (NE). The list of primers used can be found in the supplementary information Table SI. WT primary myoblasts and primary myoblasts differentiated into myotubes (myotube positive control) were taken as positive controls.

IMMUNOFLUORESCENCE

Immunofluorescence assessment of WT and *Mstn*$^{-/-}$ muscle fibroblast cells was performed before and after 72 h of transduction with adenovirus, lentivirus, and electroporation. Cells were grown in eight well permanox chamber slides (Nunc), fixed using freshly prepared 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 in PBS. The non-specific binding sites were blocked with 5% normal donkey serum in PBS for 1 h and incubated overnight at 4°C with primary antibodies (supplementary information Table SII for antibody details), followed by the addition of appropriate secondary antibody for 1 h, counterstained with DAPI. The slides were observed, and images were captured under Leica fluorescent microscope (Leica, Germany). The cells were assessed for the presence of myogenic markers-MyoD, myosin heavy chain 1 (MyHC1), alpha smooth muscle actin (αSMA), Vimentin, adenovirus and adenoviral receptors. Likewise, untransduced muscle fibroblasts cells from WT and *Mstn*$^{-/-}$ were also subjected to same immunofluorescence assessment as negative controls.

FLOW CYTOMETRY

Untransduced, adenovirus, lentivirus transduced, and electroporated muscle fibroblasts of 24 h time-point from WT and *Mstn*$^{-/-}$ respectively were washed with PBS and fixed with ice-cold 4% PFA in PBS. The cells were then permeabilized with 0.1% Triton X-100, incubated with primary antibody for 1 h followed by fluorescence conjugated secondary antibody for 20 min (supplementary information Table SII for antibody details). Flow cytometric analysis was performed on BD LSR-II flow cytometer (BD Biosciences, San Jose, CA) using BD FACSDiva software.

STATISTICAL ANALYSIS

Results are expressed as means ± standard errors. A one-way ANOVA test was used for statistical analysis. *P*-Value of $^{**}P<0.01$, $^{***}P<0.001$ was considered to be significant.

RESULTS

FIBROBLAST CULTURES FROM SKELETAL MUSCLE OF WT AND *Mstn*$^{-/-}$ EXHIBITED DIFFERENT GROWTH CHARACTERISTICS

We first assessed the primary muscle fibroblasts obtained as preplate1 cells from hind limb muscles of WT and *Mstn*$^{-/-}$ mice for their morphology, growth characteristics and expression of fibroblast as well as myogenic markers. The results indicated the difference in morphology of fibroblasts isolated from WT and *Mstn*$^{-/-}$ mice. Fibroblasts from WT exhibited more nuclear to cytoplasmic ratio in contrast to fibroblasts from *Mstn*$^{-/-}$ mice, which showed a flat morphology having more cytoplasmic to nuclear ratio (Fig. 1A,B). After the initial lag phase both the cell types demonstrated exponential cell growth and exhibited different population doubling times; 49 h for fibroblasts from WT and 75 h for fibroblasts from *Mstn*$^{-/-}$ mice (Fig. 1C). Cells from both the genotypes expressed significant amounts of fibroblast markers such as vimentin (Fig. 1D,F) and alpha smooth muscle actin (αSMA) (Fig. 1E,G) as determined by immunofluorescence. Furthermore, both WT and *Mstn*$^{-/-}$ fibroblasts did not express any detectable levels of major myogenic marker proteins including Pax7, MyoD, and Myf5 as determined by flow cytometric analysis (Fig. 1H,I). Lack of expression of MyoD in fibroblasts cultured from both the genotypes was also confirmed by immunofluorescence (Fig. 2A,B). Collectively these results confirmed the purity of fibroblasts isolated from WT and *Mstn*$^{-/-}$ genotypes.

WT PRIMARY MUSCLE FIBROBLASTS EXPRESSED SIGNIFICANTLY MORE MyoD AS COMPARED TO *Mstn*$^{-/-}$ MUSCLE FIBROBLASTS UPON MyoD GENE DELIVERY

In order to assess the expression of MyoD in fibroblasts as a result of gene delivery, MyoD transduced or electroporated cells were subjected to FACS and immunofluorescence analysis. Furthermore, qRT-PCR analysis was also performed to quantify *MyoD* levels. FACS analysis revealed that 70%, 83%, and 37% of WT fibroblasts were positive for MyoD expression when transduced with either MyoD over expressing adenovirus (Ad-MyoD) or lentivirus (Lenti-MyoD) or upon electroporation of MyoD over expressing plasmid (E-MyoD) respectively (Fig. 2C,E,G). However, the observed MyoD uptake in *Mstn*$^{-/-}$ fibroblasts was found to be 13% with adenoviral transduction, 42% with lentiviral transduction and 27% with electroporation (Fig. 2D,F,H). Furthermore, FACS data were confirmed by MyoD immunofluorescence of WT and *Mstn*$^{-/-}$ fibroblasts 24 h following either transduction or electroporation (images are displayed along with FACS data in Fig. 2). Approximately 70% and 83% of WT muscle fibroblasts expressed the *MyoD* Protein following transduction with either Ad-MyoD or Lenti-MyoD (Fig. 2). The increase in MyoD levels was confirmed through qRT-PCR analysis. While the transduction resulted in a dramatic increase in ectopic expression of *MyoD* in WT fibroblasts, (∼750 fold for Ad-MyoD; ∼850-fold for Lenti-MyoD and ∼350-fold for E-MyoD) MyoD expression was consistently lower in *Mstn*$^{-/-}$ fibroblasts despite transduction/electroporation of MyoD. The fold increase in MyoD expression in *Mstn*$^{-/-}$ transduced/electroporated fibroblasts was ∼8.50 for Ad-MyoD, ∼550-fold for Lenti-MyoD and ∼6.50-fold for E-MyoD.

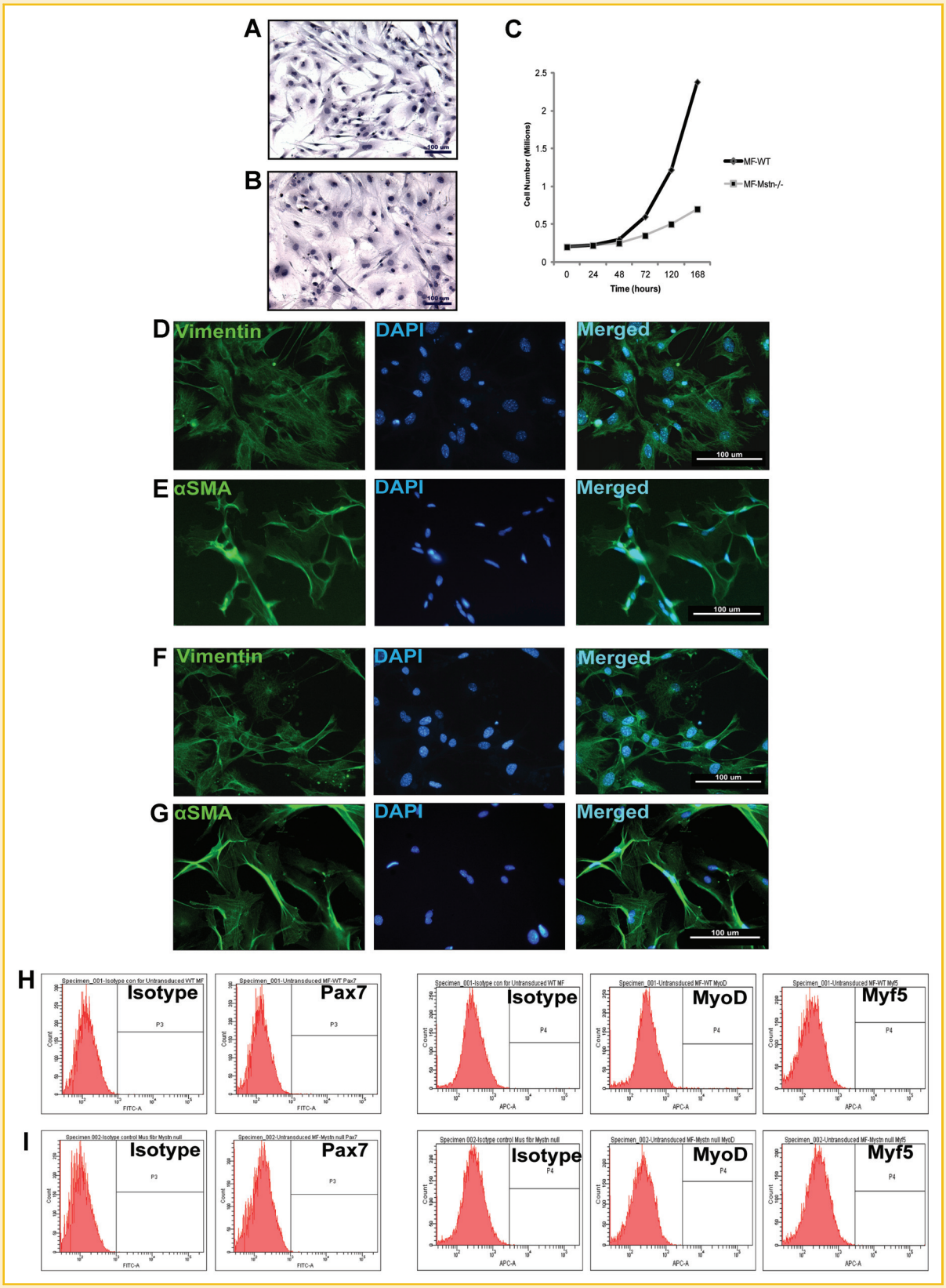

Fig. 1. Characterization of muscle fibroblasts isolated from WT and *Mstn*$^{-/-}$ mice. A: Phase contrast photomicrograph of muscle fibroblasts from WT mice. B: Phase contrast photomicrograph of muscle fibroblasts from *Mstn*$^{-/-}$ mice. C: Population doubling of muscle fibroblasts isolated from WT and *Mstn*$^{-/-}$ mice in culture (cultures derived from three different isolates from muscle). D,E: Immunofluorescence analysis of Vimentin and αSMA on muscle fibroblasts from WT mice. F,G: Immunofluorescence analysis of Vimentin and αSMA on muscle fibroblasts from *Mstn*$^{-/-}$ mice. H,I: FACS analysis of Pax7, MyoD, Myf5 on muscle fibroblasts from WT and *Mstn*$^{-/-}$ mice.

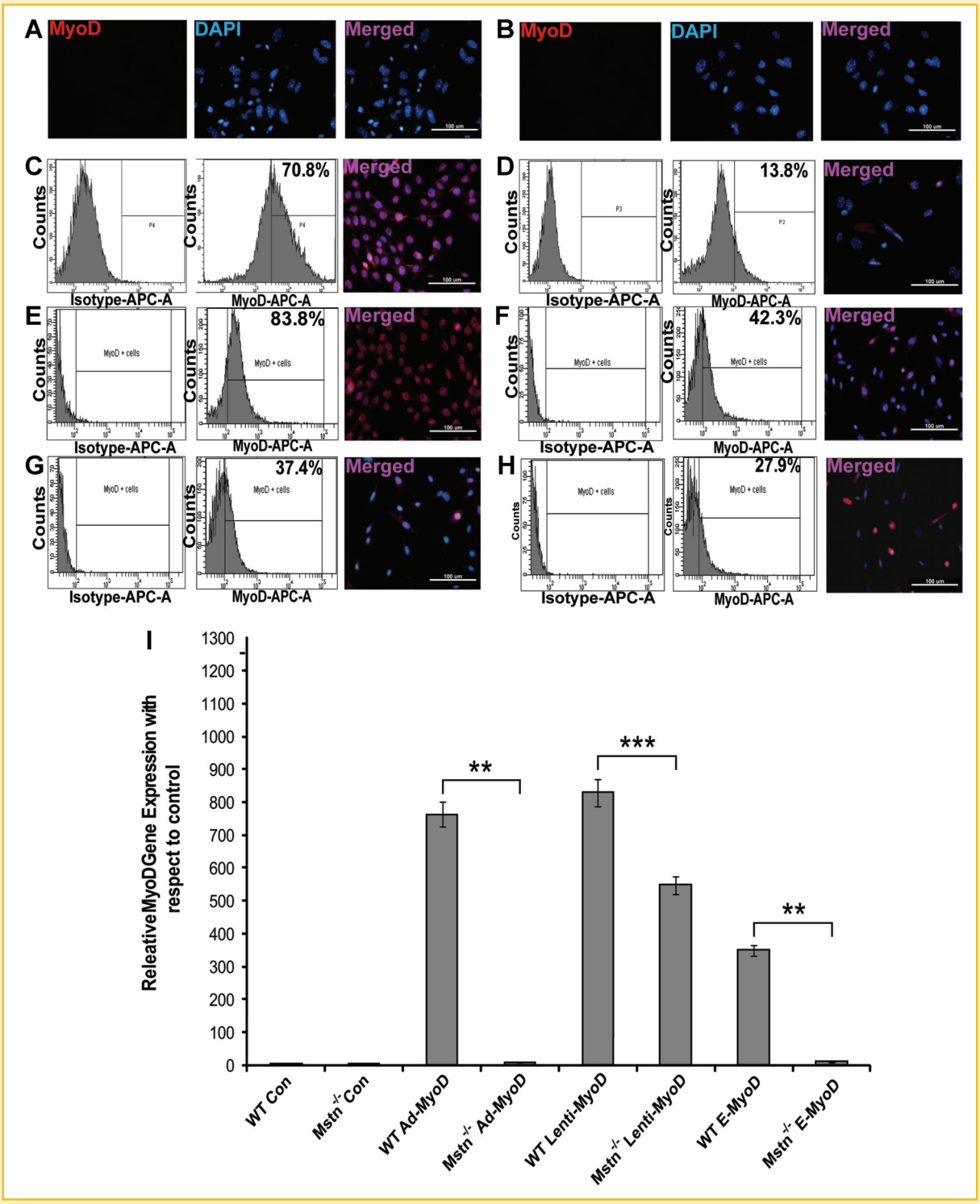

Fig. 2. Characterization of muscle fibroblasts isolated from WT and *Mstn*$^{-/-}$ mice before and after MyoD transduction. A,B: Immunofluorescence staining of MyoD on muscle fibroblasts from WT and *Mstn*$^{-/-}$ mice before (untransduced) MyoD transduction. C,D: FACS and immunofluorescence analysis on muscle fibroblasts from WT and *Mstn*$^{-/-}$ mice after 24 h of adenoviral transduction of MyoD. E,F: FACS and immunofluorescence analysis on muscle fibroblasts from WT and *Mstn*$^{-/-}$ mice after 24 h of lentiviral transduction of MyoD. G,H: FACS and immunofluorescence analysis on muscle fibroblasts from WT and *Mstn*$^{-/-}$ mice after 24 h of electroporation of MyoD. I: Gene expression levels (fold change) of MyoD in muscle fibroblasts isolated WT and *Mstn*$^{-/-}$ mice before and after 24 h MyoD transduction by adenovirus, lentivirus, and electroporation. Values are means of ±SD of three samples. $^{**}P < 0.01$, $^{***}P < 0.001$ versus untransduced control.

ONLY WT, BUT NOT *Mstn*$^{-/-}$ MUSCLE FIBROBLASTS, GAVE RISE TO FUNCTIONAL MYOTUBES IN VITRO FOLLOWING MyoD GENE DELIVERY

Assessment of in vitro myogenecity was carried out through the identification of multinucleated myotubes in *MyoD* transduced/electroporated muscle fibroblasts from both WT and *Mstn*$^{-/-}$ mice. MyoD transduced/electroporated cells were cultured in myogenic differentiation medium containing 2% horse serum for 72 h following transduction/electroporation. Using phase contrast microscopy, we could identify the presence of multinucleated myotubes in WT muscle fibroblasts transduced or electroporated with *MyoD* (Fig. 3A–C). Adenoviral transduction of MyoD resulted in moderate amount of cells differentiated into multinucleated myotubes (Fig. 3A). Lentiviral transduction of MyoD exhibited more fibroblasts differentiating into myotubes (Fig. 3B) and negligible amount of fibroblasts were differentiated to myotubes with electroporation of MyoD (Fig. 3C), respectively. However, no myotubes were observed in MyoD transduced/electroporated muscle fibroblasts from *Mstn*$^{-/-}$ mice in response to addition of differentiation medium (Fig. 3D–F). Consistent with these data, MyHC1 positive myotubes could be readily observed only in WT muscle fibroblasts following *MyoD* transduction/electroporation (Fig. 3).

Mstn$^{-/-}$ MUSCLE FIBROBLASTS DO NOT EXPRESS ADENOVIRAL RECEPTORS

We wanted to determine if the observed difference in MyoD expression in MyoD transduced/electroporated *Mstn*$^{-/-}$ fibroblasts was due to differences in the expression of adenoviral receptors (coxsackie and adenovirus receptor [CAR]) [Sengupta et al., 2011]. Immunofluorescence staining of Ad-MyoD transduced muscle fibroblasts from WT and *Mstn*$^{-/-}$ mice, using antibodies to specifically detect adenoviral receptors (CAR) and adenovirus confirmed that both adenoviral receptors and MyoD adenovirus were readily detected in Ad-MyoD transduced WT muscle fibroblasts (Fig. 4A). However, in contrast, Ad-MyoD transduced *Mstn*$^{-/-}$ muscle fibroblasts did not express either the CAR or adenovirus (Fig. 4B). Gene expression analysis also corroborated the immunofluorescence data, with expression of adenoviral receptors only detected in Ad-MyoD transduced WT muscle fibroblasts, but not in Ad-MyoD transduced *Mstn*$^{-/-}$ muscle fibroblasts (Fig. 4C).

ELEVATED EXPRESSION OF *c-Fos*, *c-Jun*, AND *Cyclin* D1 WAS OBSERVED IN *Mstn*$^{-/-}$ FIBROBLASTS

Since it has been previously shown that the *MyoD* gene promoter is repressed by *c-Fos* and *c-Jun* [Li et al., 1992], we next estimated the expressions for *c-Fos* and *c-Jun* in WT and *Mstn*$^{-/-}$ fibroblasts. Results revealed increased expression of *c-Fos* and *c-Jun* in *Mstn*$^{-/-}$ fibroblasts as compared to the WT fibroblasts (Fig. 4D). It has also been previously reported that high levels of G1/S cyclins, such as cyclin D1 can inhibit myogenesis [Rao et al., 1994; Zhang et al., 1999]. Thus, we next estimated the transcript levels of cyclin D1 and the cyclin dependent kinase inhibitor p21 in both WT and *Mstn*$^{-/-}$ muscle fibroblasts. Results revealed the presence of elevated *cyclin D1* and reduced *p21* expression in *Mstn*$^{-/-}$ muscle fibroblasts, as compared to the WT muscle fibroblasts (Fig. 4D), which is consistent with the impaired myogenesis in the *Mstn*$^{-/-}$ muscle fibroblasts.

DISCUSSION

Fibroblasts are easily available and can be expanded in large numbers, transduced and then differentiated into the myogenic lineage in vitro. Conversion of fibroblasts into myoblasts, via over expression of MyoD, has been previously demonstrated by various laboratories [Weintraub et al., 1989; Choi et al., 1990; Huard et al., 1998; Lattanzi et al., 1998]. However, the efficiency of myogenic conversion of muscle-derived fibroblasts is far less, as compared to the efficiency of myogenic conversion of fibroblasts from other tissues [Lattanzi et al., 1998]. Also, fibroblasts from dermal origin can be more myogenic when compared to fibroblasts of other origin (muscle, bone marrow) [Lattanzi et al., 1998]. In the current study, we compared the myogenic conversion efficiency of WT and *Mstn*$^{-/-}$ derived muscle fibroblasts upon over expression of MyoD. We speculated that the muscle fibroblasts obtained from *Mstn*$^{-/-}$ genotype might have a greater myogenic propensity, as compared to muscle fibroblasts obtained from WT genotype.

The main reason behind using three different gene delivery methods for over expression of MyoD (adenoviral, lentiviral, and electroporation) was to make sure there was significant ectopic over expression of MyoD in the fibroblasts. Indeed, our results suggest that viral transduction leads to high expression of MyoD in WT fibroblasts. Even though there was a large increase in MyoD expression, only a small percentage of MyoD expressing fibroblasts could be differentiated into myotubes in culture. From previous work, it is known that multiple copies of the *MyoD* gene together with high levels of MyoD protein are required for myogenic conversion of fibroblasts. Although we see high levels of MyoD expression in transduced/electroporated WT fibroblasts, we speculate that many of the cells do not express high enough levels of MyoD to induce myogenesis in fibroblasts [Murry et al., 1996]. In contrast to WT fibroblasts, only a very small percentage of *Mstn*$^{-/-}$ muscle fibroblasts expressed MyoD, therefore only a modest ectopic MyoD expression was noted. Consistent with reduced MyoD expression in *Mstn*$^{-/-}$ muscle fibroblasts, no myotubes were observed in MyoD transduced/electroporated fibroblasts isolated from *Mstn*$^{-/-}$ mice (Fig. 3).

The fusion of myoblasts and the generation of differentiated myotubes is reliant upon the action of as number of proteins including Myosin heavy chain 1. Therefore, high MyHC1 expression displayed by WT fibroblasts during differentiation may explain the myotube formation detected. However, since we failed to detect any MyHC1 expression in *Mstn*$^{-/-}$ muscle fibroblasts upon over expression of MyoD, we speculate that this could be a critical reason as to why we observed no morphological evidence of myotube formation in the absence of *Myostatin*.

Since we detected lower levels of MyoD expression in *Mstn*$^{-/-}$ fibroblasts, we searched for molecular mechanisms. Our results here clearly suggest that *Mstn*$^{-/-}$ fibroblasts express significantly reduced levels of the adenoviral receptor coxsackie and adenovirus receptors (CAR). Adenoviruses attach to host cells via cell surface

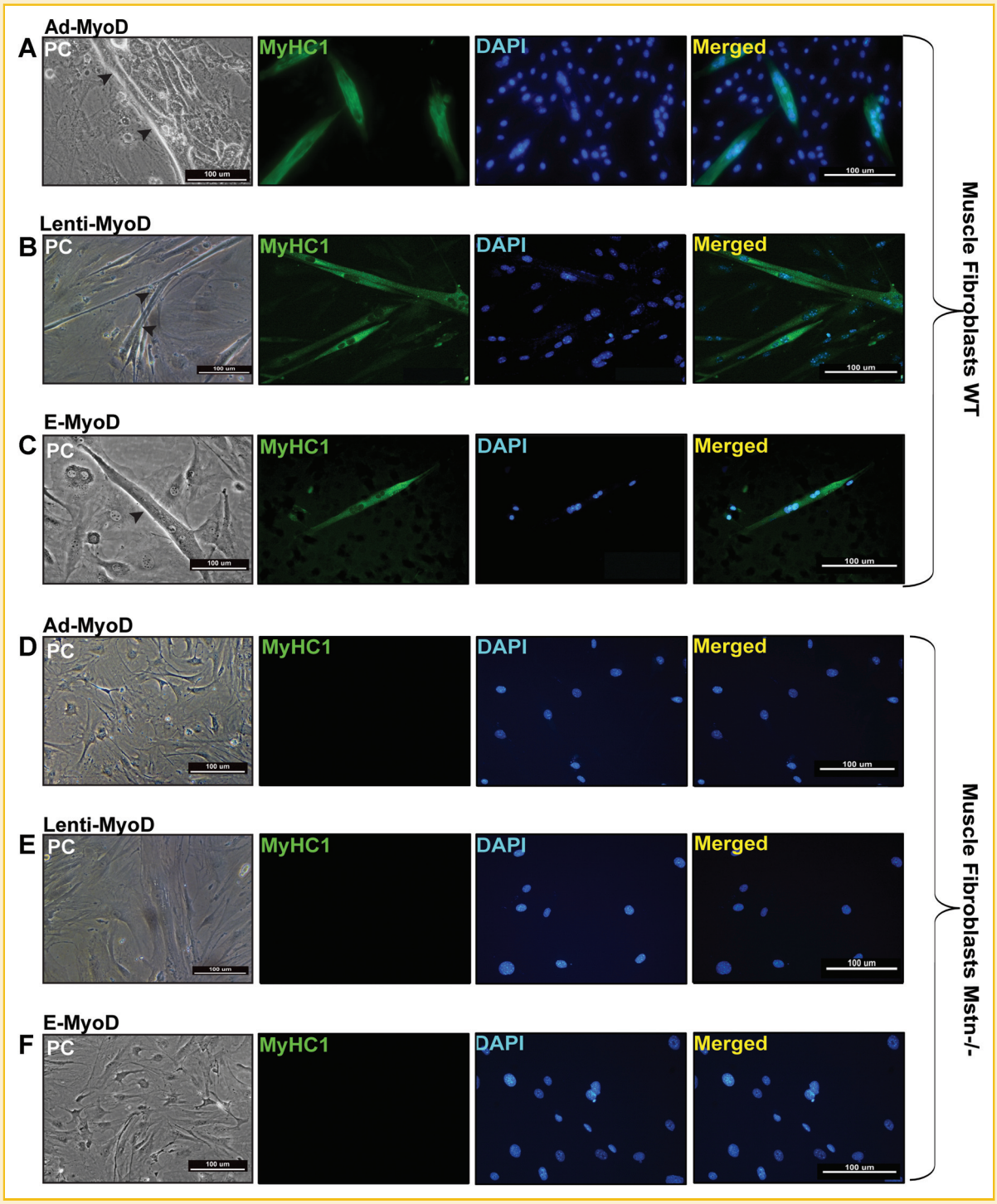

Fig. 3. Phase contrast (black arrow heads showing Myotubes) and immunofluorescence analysis photomicrographs of MyHC1, 72 h post MyoD transduction (2% HS induced differentiation) in WT and *Mstn*$^{-/-}$ mouse muscle fibroblasts. A–C: Seventy-two hours post adenoviral, post lentiviral and post electroporation of MyoD on muscle fibroblasts from WT mice. D–F: Seventy-two hours post adenoviral, post lentiviral and post electroporation of MyoD on muscle fibroblasts from *Mstn*$^{-/-}$ mice.

receptors known CAR, this attachment is facilitated by binding with integrins on the host cell surface [Bergelson et al., 1997, 1998]. Successful adenoviral-mediated delivery of MyoD in WT muscle fibroblasts was expected, since WT muscle fibroblasts displayed robust expression of adenoviral receptors on the cell surface as assessed by gene expression and immunofluorescence analysis. In stark contrast, we noted a complete absence of adenoviral receptors on the cell surface of muscle fibroblasts isolated from *Mstn*$^{-/-}$ mice (Fig. 4). The molecular mechanisms of how *Myostatin* regulates CAR receptor needs to be investigated further in future.

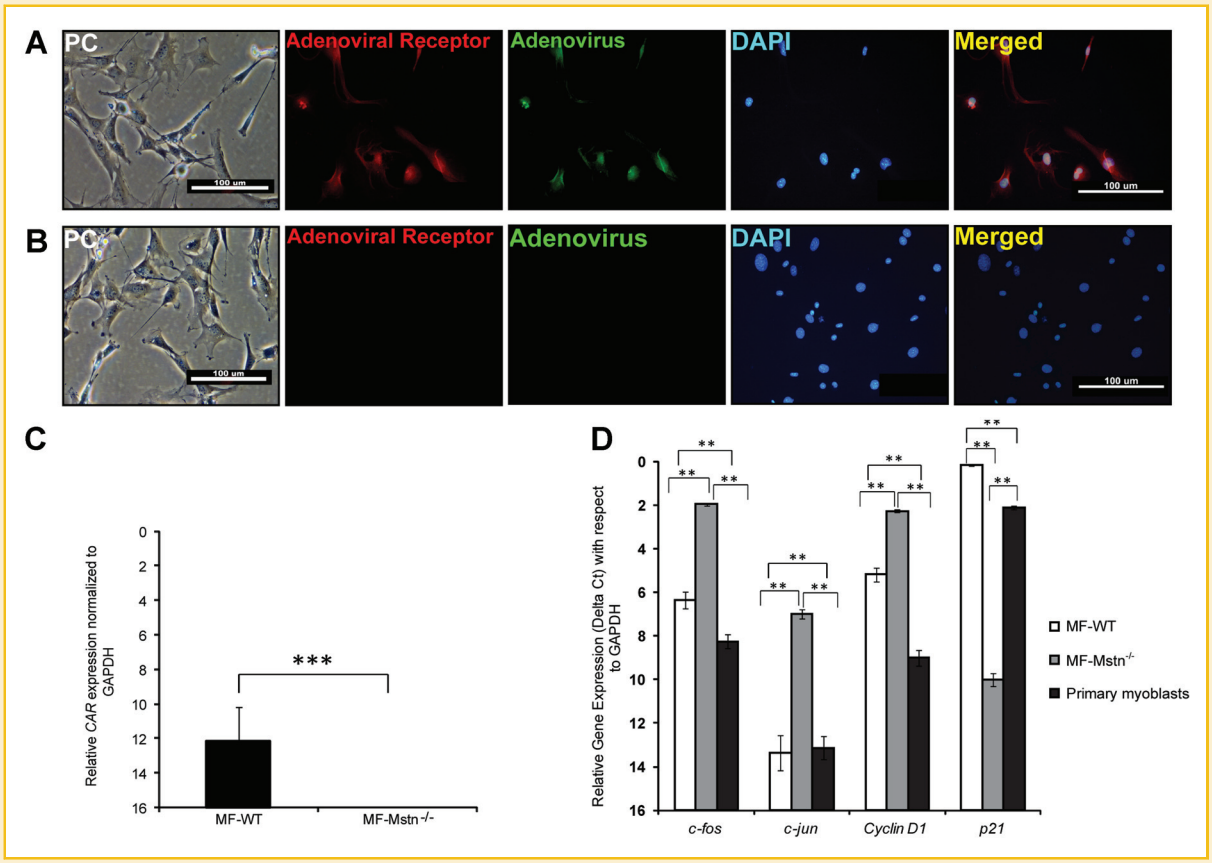

Fig. 4. Characterization of adenoviral receptors and adenovirus on untransduced and transduced WT and *Mstn*$^{-/-}$ mouse muscle fibroblasts. A: Phase contrast and immunofluorescence analysis photomicrographs of adenoviral receptors and adenovirus on WT muscle fibroblasts. B: Phase contrast and immunofluorescence analysis photomicrographs of adenoviral receptors and adenovirus on *Mstn*$^{-/-}$ muscle fibroblasts. C: Gene expression levels (Delta Ct) of Coxsackievirus and adenovirus receptor (CAR) in untransduced WT and *Mstn*$^{-/-}$ mouse muscle fibroblasts. Values are means of ±SD of three samples. ***$P < 0.001$ versus WT control. D: mRNA expression analysis of proto-oncogenes (c-Fos, c-Jun), cell cycle regulator (Cyclin D1) and cell cycle inhibitor (p21) in untransduced WT, Mstn$^{-/-}$ mouse muscle fibroblasts and primary myoblasts. Values are means of ±SD of three samples. **$P < 0.01$.

MyoD can induce differentiation of fibroblasts into the myogenic lineage in a wide variety of cells; however, some cells are resistant to conversion [Weintraub et al., 1989; Choi et al., 1990]. Serum and peptide growth factors, such as FGF and TGF-β, have been shown previously to suppress the activation of muscle specific genes [Florini et al., 1991]. Other molecules, which are known to inhibit MyoD, are c-Jun, v-Jun, c-Fos, v-Fos, and Jun B, which can mimic the inhibitory effects of growth factors on myogenesis. Furthermore, these molecules have been shown to silence the transcriptional activating capacity of the muscle specific activators myogenin and MyoD (repression by Fos and Jun is a specific property of myogenic HLH proteins) [Li et al., 1992; Sengupta et al., 2011]. In addition to reduced adenoviral receptor levels we further observed increased expression of MyoD antagonists in the absence of *Myostatin*. In the present study, our results revealed increased expression of *c-Fos* and *c-Jun* in *Mstn*$^{-/-}$ fibroblasts as compared to the WT fibroblasts. These data suggest that increased expression of MyoD antagonists may play a role in preventing myogenic conversion in *Mstn*$^{-/-}$ fibroblasts even in upon over expression of *MyoD* (Fig. 5). In addition to Fos and Jun, MyoD function is suppressed in proliferating cells by the formation of cyclin D1-cyclin dependent kinase (cdk) complex, which subsequently phosphorylates MyoD [Skapek et al., 1995]. The formation of MyoD–cdk4 complex in the nucleus specifically inhibits the functions of MyoD thereby inhibiting myogenesis [Zhang et al., 1999]. Our results here also demonstrated high levels of cyclin D1 transcripts in *Mstn*$^{-/-}$ fibroblasts, as compared to WT fibroblasts; highlighting a further mechanism through which myogenesis may be repressed in *Mstn*$^{-/-}$ fibroblasts, despite over expression of MyoD. The presence of the cyclin dependent kinase inhibitor p21, p16, and MEF2 has been shown to enhance myogenic conversion after MyoD transfection [Kaushal et al., 1994; Halevy et al., 1995; Molkentin et al., 1995]. Our results exhibits increased levels of p21 expression in WT muscle fibroblasts which confirms myogenic differentiation after MyoD transfection. Similarly the presence of low levels of p21 gene in *Mstn*$^{-/-}$ fibroblasts, also confirmed repressed myogenesis in *Mstn*$^{-/-}$ fibroblasts (Fig. 5).

Although, we clearly demonstrate that lack of Myostatin increases the expression of different MyoD inhibitors in muscle fibroblasts, the reason behind this phenomenon remains to be elucidated. However,

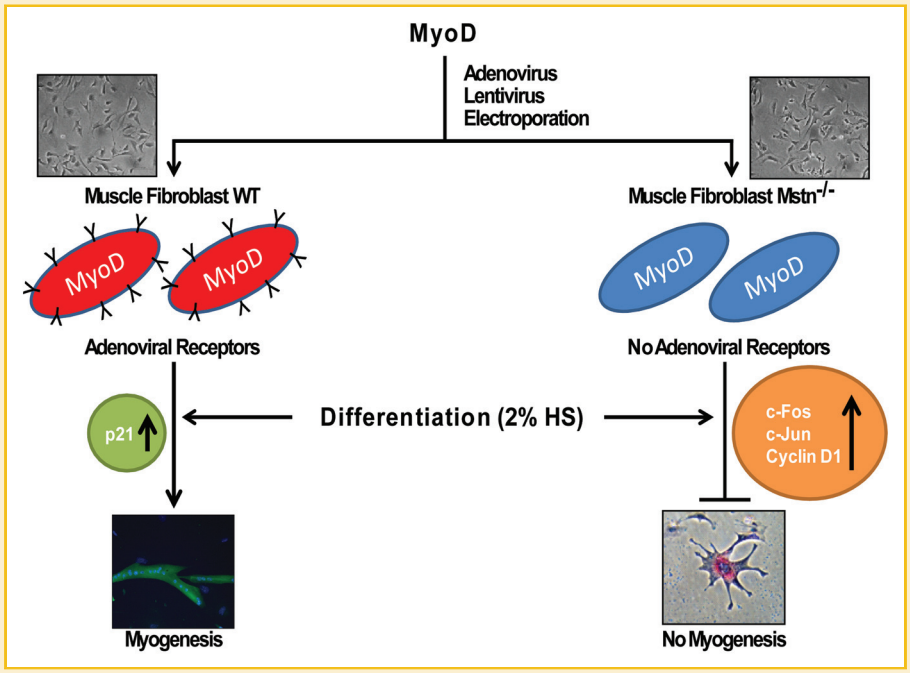

Fig. 5. Schematic representation of MyoD uptake by muscle fibroblasts from WT and *Mstn*$^{-/-}$ mice leading to myogenesis. Transduction of MyoD gene by three methods, that is, adenoviral, lentiviral, and electroporation in muscle fibroblasts isolated from WT and *Mstn*$^{-/-}$ mice. Myogenesis was observed only in muscle fibroblasts from WT genotype by all the three methods. In case of WT muscle fibroblasts, more MyoD is taken up due to the presence of adenoviral receptors and high expression of p21 when compared to muscle fibroblasts from *Mstn*$^{-/-}$. Furthermore, these WT muscle fibroblasts when kept in 2% HS differentiation media for 72 h readily undergo myogenesis and express MyHC1. The reason for muscle fibroblasts from *Mstn*$^{-/-}$ mice, which does not undergo myogenesis is because no sufficient MyoD taken up by the cells. Due to increased levels of proto-oncogenes (c-Fos, c-Jun), cell cycle regulator (Cyclin D1) leading to transcriptional repression of *MyoD* gene. Furthermore, these Mstn$^{-/-}$ muscle fibroblasts when kept in 2% HS differentiation media for 72 h do not undergo myogenesis.

we speculate that this is a possible adaptive mechanism to stop further conversion of non-muscle tissue to muscle tissue, to maintain a balance between non-contractile and contractile tissue. Further work is required to probe this hypothesis.

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