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# Myostatin acts as an autocrine/paracrine negative regulator in myoblast differentiation from human induced pluripotent stem cells

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ABSTRACT

Myostatin, also known as growth differentiation factor (GDF-8), regulates proliferation of muscle satellite cells, and suppresses differentiation of myoblasts into myotubes via down-regulation of key myogenic differentiation factors including MyoD. Recent advances in stem cell biology have enabled generation of myoblasts from pluripotent stem cells, but it remains to be clarified whether myostatin is also involved in regulation of artificial differentiation of myoblasts from pluripotent stem cells. Here we show that the human induced pluripotent stem (iPS) cell-derived cells that were induced to differentiate into myoblasts expressed myostatin and its receptor during the differentiation. An addition of recombinant human myostatin (rhMyostatin) suppressed induction of MyoD and Myo5a, resulting in significant suppression of myoblast differentiation. The rhMyostatin treatment also inhibited proliferation of the cells at a later phase of differentiation. RNAi-mediated silencing of myostatin promoted differentiation of human iPS-derived embryoid body (EB) cells into myoblasts. These results strongly suggest that myostatin plays an important role in regulation of myoblast differentiation from iPS cells of human origin. The present findings also have significant implications for potential regenerative medicine for muscular diseases.



## 1. Introduction

During the prenatal development of skeletal muscle in mammals, the myoblasts derived from somitic dermomyotomes proliferate to increase their number, subsequently align and fuse into multi-nucleated myotubes, which no longer proliferate but further mature into contractile myofibers [1]. In the mature muscle tissue of postnatal mammals, myoblasts are differentiated from the satellite cells that are the resident stem cells located between the plasma membrane of the muscle fiber and the basement membrane in the skeletal muscle tissue of mammals [2,3]. The satellite cells are basically quiescent, but minimal proliferation of activated satellite cells is required for muscle repair, because subtle injuries of myofiber may routinely occur throughout life. Upon larger trauma of muscle, greater numbers of satellite cells are recruited, and the newly generated myoblasts subsequently undergo repeated cell division and either fuse into a pre-existing myofiber or form new myofibers. The development as well as regeneration processes of the muscle are highly orchestrated by the signals from the muscle niche and microvasculature, as well as by inflammatory responses. Some soluble factors play important regulatory roles, including the transforming growth factor (TGF)-beta, fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), insulin-like growth factor (IGF) and neuronal nitric oxide synthase (NOS) [2,3].

Among the soluble regulatory factors, myostatin, also known as growth differentiation factor 8 (GDF8), plays important roles in the control of muscle development and regeneration [4]. Myostatin belongs to the TGF superfamily and shares several features with other TGF superfamily members. The role of myostatin in skeletal muscle was discovered using the myostatin gene-deficient mice, which showed a drastic and widespread increase in muscle mass due to an increase in number (hyperplasia) and size (hypertrophy) of their muscle fibers [4]. Similar phenotypes were also demonstrated in natural mutations of myostatin genes in cattle [5,6], sheep [7], dogs [8] and humans [9]. In mice, myostatin activates canonical Smad3 signaling to maintain the satellite cells in a quiescent state, and inhibit myoblast proliferation as well, by up-regulating the cyclin-dependent kinase (Cdk) inhibitor p21 [10–12], down-regulating the level and activity of Cdk2 [11] and activating c-Jun N-terminal kinase (JNK) signaling pathway [13]. Myostatin also inhibits differentiation of myoblasts via down-regulation of MyoD [14] and activation of extracellular signal-regulated kinase 1/2 (Erk1/2) cascade [15].

*Abbreviations:* EB, embryoid body; ACVR2B, activin type 2B receptor.

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Recent advances in stem cell biology enabled generation of myoblasts from immature stem cells with pluripotent differentiation potential such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells [16]. The molecular mechanisms underlying the artificial differentiation of myoblasts from pluripotent stem cells may be different from those underlying the physiological development and regeneration of the myoblasts from satellite cells. The regulatory programs involved in myoblast differentiation from pluripotent stem cells have not been well understood, and it has not been reported whether myostatin is produced from, and/or acts on, the cells during the differentiation process.

In this context, we aimed at clarifying whether myostatin is involved in the regulation of myoblast differentiation from human iPS cells.

## 2. Materials and methods

### 2.1. iPS cells culture

Human iPS cells were established as described with some modifications [17]. Briefly, normal human epidermal keratinocytes (NHEK) (Kurabo, Osaka, Japan) were co-transfected with the following Epstein-Barr virus (EBV)-based episomal vectors [18]. pEF.oriP9.OKS.E contains the Oct3/4-2A-Klf4-2A-Sox2 fusion gene under the control of elongation factor (EF) gene promoter, EBV nuclear antigen (EBNA1) gene and EBV oriP sequence, while pEF.oriP9.MiL carries a polycistronic expression cassette composed of the EF promoter and c-Myc gene-internal ribosomal entry site (IRES) sequence-LIN28 gene, the EBNA1 gene and EBV oriP. pEF.oriP9. Large T contains SV40 large T antigen gene driven by the EF promoter, EBNA1 gene and EBV oriP. After electro-transfection using the Neon Electroporation System (Invitrogen, Eugene, OR), cells were cultured on the MSTO feeder cells in the Primate ES Cell Medium (Repro Cells, Yokohama, Japan) that was supplemented with 2 mM sodium valproate (VPA) for initial 10 days. Twenty-two days after transfection, iPS cell colonies were picked up. The clone hiPS1-2-8 was used in the following study.

### 2.2. Myoblast differentiation

hiPS1-2-8 cells were scraped off from the dish and resuspended in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Osaka, Japan) supplemented with 100 U/ml Penicillin (Nacalai Tesque), 100 μg/ml Streptomycin (Nacalai Tesque), and 10% fetal bovine serum (complete medium) (day-7). Cells were seeded into Lipidure (R)-CM (MPC polymer)-coated plates of 60 mm in diameter (NOF America Corporation, White Plains, NY) to allow formation of EB. After culturing in a humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C for 7 days, cells were harvested from the plates, resuspended in fresh complete medium in the presence or absence of 50 nM all-trans retinoic acid (RA) (Wako, Osaka, Japan) and cultured under adherent condition in 12 well-plates for 10 days. In some experiments, recombinant human myostatin protein (rhMyostatin) (Aviscera Bioscience Inc., Santa Clara, CA) was added to some wells at a final concentration of 8 μg/ml during the induction of EB to myoblasts (day 0–10).

### 2.3. RNA interference (RNAi)

Three short interfering RNA (siRNA) duplexes targeting human myostatin gene (siMyostatin), i.e., Hs-GDF8-1 (Cat No. SI00074627; Target sequence: 5′-CTGATGCTATCTCAACAATAA), Hs-GDF8-2 (Cat No. SI00074634; Target sequence: 5′-ACGGTACAAGGTATACTGGAA), Hs-GDF8-3 (Cat No. SI00074641; Target sequence: 5′-AGGAGTATGCTTTAAAGTCTA), and Hs-GDF8-4 (Cat No. SI00074648; Target sequence: 5′-CTCAGTAAACTTCGTCTGGAA), were purchased from Qiagen (Hilden, Germany). EBs were harvested from the culture and transfected with a mixture of these siRNA or control siRNA (Cat No. SIC-001) (Sigma Aldrich, St. Louis, MO) using Neon Electroporation System (Invitrogen). Briefly, EBs were resuspended in R. Buffer (Invitrogen) and mixed with a siRNA solution in such a manner that a 10 μL aliquot contained $10^5$ cells and 40 pmol of siRNA. After pulsation at 1400 V for 30 ms, cells were cultured in myogenic medium as described above.

### 2.4. Real time-RT-PCR

Total RNA was extracted from cells by the guanidinium acid phenol method using ISOGEN II RNA Extraction Reagent (Wako). One microgram of total RNA was reverse transcribed using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan), and cDNA was subjected to real-time PCR using the 7300 Real Time PCR System (Applied Biosystems, Carlsbad, CA). The matching primers and dye probe for the Myo5a (Hs00165309-m1), MyoD (Hs00159528-m1), activin type 2B receptor (ACVR2B)(Hs00609603-m1), myostatin (Hs00976237-m1), Myosin heavy chain (MHC) 3 (Hs00159463-m1) and beta-actin (Hs99999903-m1) were pur-

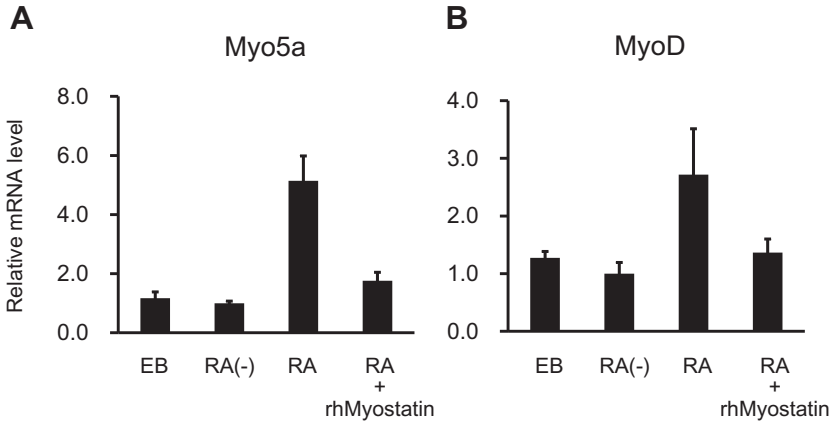


**Fig. 1.** Myostatin prevented induction of muscle-specific transcription factors in human iPS-derived cells that were stimulated to differentiate into myoblasts. EBs induced from human iPS cells were cultured for 10 days in the presence or absence of RA and rhMyostatin as described in the Section 2. RNA was extracted from these cells as well as non-stimulated EBs as a control, and subjected to real time-RT-PCR using primers/probes specific for Myo5a (A) and MyoD (B) genes. Relative mRNA levels (average ± SD) are shown. $^*P < 0.05$.

chased from Applied Biosystems. mRNA levels were quantified by RQ software (Applied Biosystems).

### 2.5. Immunohistochemistry

Cells were washed 3 times with PBS (-) and fixed with 4% PFA for 10 min. at room temperature. After washing 3 times with PBS (-), the cells were blocked with Blocking One Histo (Nakarai Tesque) at 4 °C overnight, followed by further washing with PBS (-). The cells were then incubated with anti-myosin heavy chain (MHC) (R&D Systems) (final concentration: 10 μg/ml) or anti-alpha actin (Bioworld Technology, Inc, Mineapolis, MN) (final concentration: 10 μg/ml) antibodies. As the secondary antibodies, Alexa 488-conjugated goat anti-mouse IgG (Invitrogen, Eugene, OR) (final concentration: 4 μg/ml) and Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen) (final concentration: 4 μg/ml) were used, respectively. After washing, cells were observed under fluorescent microscope.

### 2.6. Cell proliferation assay

Tetrazolium salt-based assay was performed as described previously [19]. Briefly, EBs derived from iPS cells were seeded in triplicates into 96 well plate at a density of 10,000 per well, and cultured under the adherent conditions as described above. On days 0, 2, 4, 6, 8 and 10, a solution of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST8) (Nacalai Tesque) was added into each well at 20 μL/well. After incubation for an additional 2 h, 100 μL of the supernatant was collected to measure the OD at 450 nm.

## 3. Results and discussion

To examine whether myostatin may influence differentiation of human myoblasts from iPS cells, iPS cells were first induced to differentiate into EBs, and subsequently cultured in the presence or absence of RA and rhMyostatin. Real time-RT-PCR analysis was

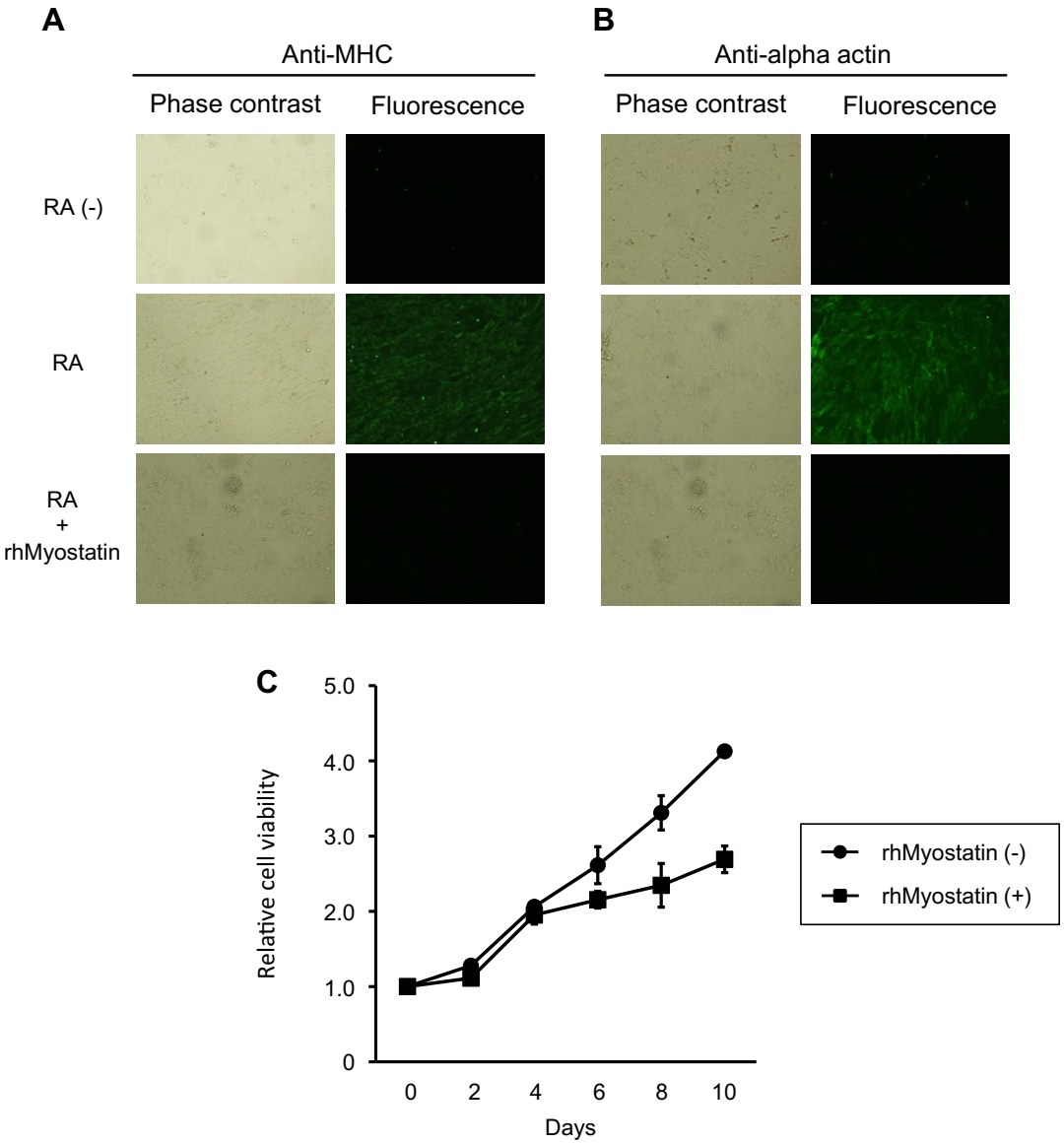


**Fig. 2.** Myostatin inhibited myogenesis from the human iPS-derived EBs. A and B, EBs induced from human iPS cells were cultured in the presence or absence of RA or rhMyostatin as in Fig. 1. Ten days later cells were fixed and immune-stained with anti-myosin heavy chain (MHC) (A) and anti-alpha-actin (B) antibodies as described in the Section 2. Shown are phase-contrast (left) and fluorescent (right) images of the cells. Original magnification was ×400. C, EBs induced from human iPS cells were cultured in myogeneic medium containing RA, with or without supplementation of rhMyostatin. On the indicated days, cell numbers were counted. Shown are average cell counts ± SD. $^{*}P < 0.05$.

performed to evaluate expression levels of mRNA for MyoD and Myo5a genes that play key roles in muscle differentiation. Upon RA-induced differentiation of EBs into myoblasts, the cells expressed MyoD and Myo5a mRNA (Fig. 1). In constrast, the addition of rhMyostatin resulted in significant reduction of the mRNA levels of the transcription factors, strongly suggesting that myostatin signal suppressed myogenic differentiation from EBs.

The effect of rhMyostatin on myoblast differentiation was also assessed by immunostaining using specific antibodies for myosin heavy chain (MHC) and alpha actin. As shown in Fig. 2A and B, both of the skeletal muscle-specific proteins were strongly expressed in the majority of the cells that were cultured with RA, whereas rhMyostatin drastically prevented the induction of these proteins. The findings were consistent with the phase-contrast microscopic observation of the cells, because the RA-treated cells showed typical morphological characteristics of myoblasts, whereas such phenotypic change was almost completely restored by the addition of rhMyostatin (Fig. 2A and B). These results strongly suggested that rhMyostatin potently suppressed myoblast differentiation from the EBs that had been derived from the human iPS cells.

Next, experiments were conducted to examine whether exogenous myostatin influenced proliferation of cells that were induced from iPS cells to differentiate into myoblasts. Tetrazolium salt-based assay indicated that an addition of rhMyostatin failed to affect cell growth on and before day 4, but significantly reduced proliferation of cells at later culture periods (Fig. 2C).

The negative regulatory role of myostatin on differentiation and proliferation of myoblasts strongly suggested that the developing myoblasts expressed functional myostatin receptor. Active myostatin mostly binds to, and mediates the signal through, the ACVR2B on the surface of the target cells. Real time-RT-PCR analysis was performed to evaluate the kinetic change of ACVR2B mRNA upon myoblast differentiation. As expected, mature myoblasts that appeared on day 10 expressed a high level of the myostatin receptor (Fig. 3A). Interestingly, the ACVR2B mRNA was moderately up-regulated in EB-derived cells 5 days after an addition of RA, suggesting that the immature EB-derived cells are susceptible to the myostatin signal before full maturation into myoblasts.

Myoblasts have been shown to produce myostatin that suppresses their growth in an autocrine/paracrine manner. Next, experiments were conducted to determine whether the myostatin was also induced during the myoblast differentiation. As shown in Fig. 3B, mRNA level for the myostatin gene was robustly elevated in EB-derived cells after differentiation (day 10), although the expression level was very low, if any, at an earlier phase of the culture (day 5). The results strongly suggest that the myostatin may contribute to an autocrine negative feedback regulation for human myogenesis in these culture systems.

To address the above mentioned hypothesis further, we attempted to block the myostatin production and examined its influence on myoblast differentiation. EB cells were transfected with a mixture of three siMyostatin duplexes, and after two days of culture under the myogenic conditions, myostatin mRNA was evaluated by real time-RT-PCR. We found that the siRNA transfection reduced the mRNA level to approximately 10% (data not shown). The EBs transfected with the siMyostatin were cultured in RA-containing medium for 10 days, and mRNA for MyoD and MHC genes was estimated. As shown in Fig. 4, the silencing of myostatin significantly elevated mRNA expression of these genes in the iPS-derived myoblasts, strongly suggesting that myostatin produced from developing myoblasts may actually suppress myogenesis.

Effects of myostatin on myogenesis have been clarified in detail in mice, particularly using genetic deficient mice [4,11] and the C2C12 myoblastoid cell line [10,12–15], demonstrating important regulatory roles of this TGF-beta family protein on satellite cells and myoblasts during muscle development and regeneration [4]. Inhibition of myostatin signal in adult mice also resulted in significant increase in muscle mass, suggesting potential feasibility of a myostatin blockage to regenerative medicine for muscular diseases [20]. Post-natal blockade of myostatin showed widespread effect on musculature in dystrophin-deficient mdx mice, a model for Duchenne muscular dystrophy [21].

However, involvement of myostatin in human myogenesis has not been fully elucidated. Recently, McFarlane et al. reported that human myostatin negatively regulates proliferation of human primary myoblasts by up-regulating p21, and suppresses their differentiation into myotubes via Notch signaling pathway [22]. To our knowledge the present study reports for the first time that myostatin is involved in artificial induction of human myoblasts from pluripotent stem cells in an autocrine/paracrine fashion.

Notably, ACVR2B was expressed at a moderate level on immature EB-derived cells before full maturation into myoblasts (day 5) (Fig. 3A), while expression of myostatin was evident at a later phase of differentiation (day 10) (Fig. 3B). The delay of myostation expression compared to its receptor's expression may suggest that the proliferation and differentiation of immature myoblasts are controlled by myostatin signal delivered from mature myoblasts. This is compatible with the effect of myostatin on cell proliferation, because rhMyostatin significantly suppressed cell growth on and after day 6 of culture (Fig. 2C). Such a paracrine feedback loop is

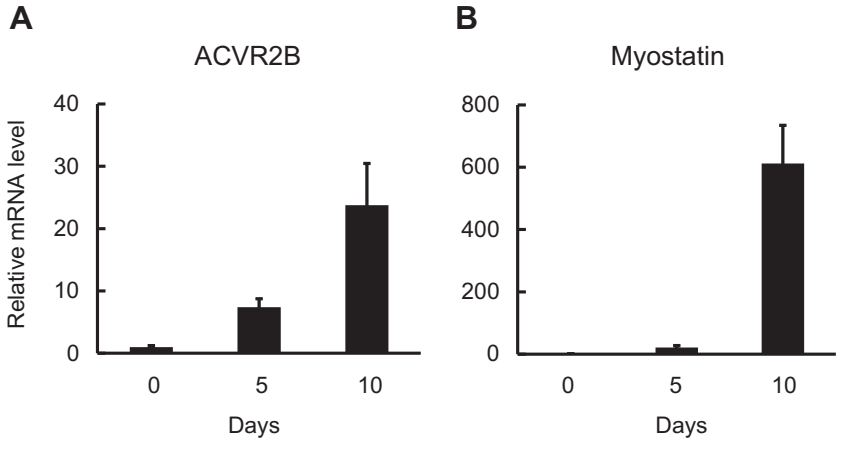


**Fig. 3.** iPS-derived cells expressed myostatin and its receptor after induced to differentiate into myoblasts. EBs induced from human iPS cells were cultured in myogeneic medium containing RA. On the indicated days, mRNA for ACVR2B (A) and myostatin (B) genes was evaluated by real time-RT-PCR. Relative mRNA levels (average ± SD) are shown. $^{*}P < 0.05$.

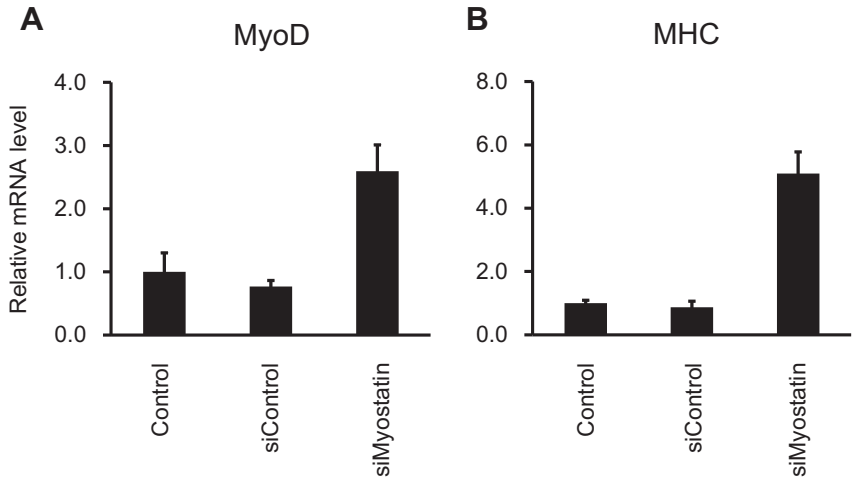


**Fig. 4.** Silencing of myostatin promoted myoblast differentiation from human iPS-derived EBs. EB cells were transfected with a mixture of three siMyostatin as described in the Section 2. The cells were cultured in the presence of RA for 10 days, and mRNA for MyoD and MHC was estimated by real time-RT-PCR. Relative mRNA levels (average ± SD) are plotted. $^{*}P < 0.05$.

considered as a regulatory mechanism to maintain muscle mass in physiological muscle development and regeneration [20], and the present study may suggest the presence of similar regulation in iPS-derived myogenesis. In this context, RNAi-mediated silencing of myostatin may facilitate myoblast generation probably by blocking this autocrine network (Fig. 4). This likelihood is also consistent with the present finding that an addition of rhMyostatin almost completely abrogated myogenesis (Figs. 1 and 2).

Pluripotent stem cells may differentiate into various cell types in culture, including ectodermal (neurons, epidermis, etc.), endodermal (intestinal mucosa etc.), and mesodermal (cardiomyocytes, chondrocytes, myoblasts, etc.) lineages dependent on culture conditions [17]. These technologies are not only of great significance in cell biology and basic medical science, but also have enormous impact on clinical medicine from the prospect of regenerative therapeutics. As for the skeletal muscle, generation of myoblasts may provide novel therapeutic modalities for muscular diseases, including Duchenne muscular dystrophy [21,23], cachexia caused by malignancies, adult immunodeficiency syndrome and heart failure [24], as well as sarcopenia associated with aging [25,26]. However, epigenetic mechanisms that underlie the control of the cells fate remain obscure, hindering establishment of technologies applicable to regenerative medicine for muscular diseases. Our present findings that myostatin acts as a negative regulator for myoblast induction from pluripotent stem cells may provide useful information for understanding regulatory mechanisms of the artificial differentiation of myoblasts, as well as for improving technologies that enable more effective production and engineering of the myoblasts feasible to therapeutic purposes in the future.

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