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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-

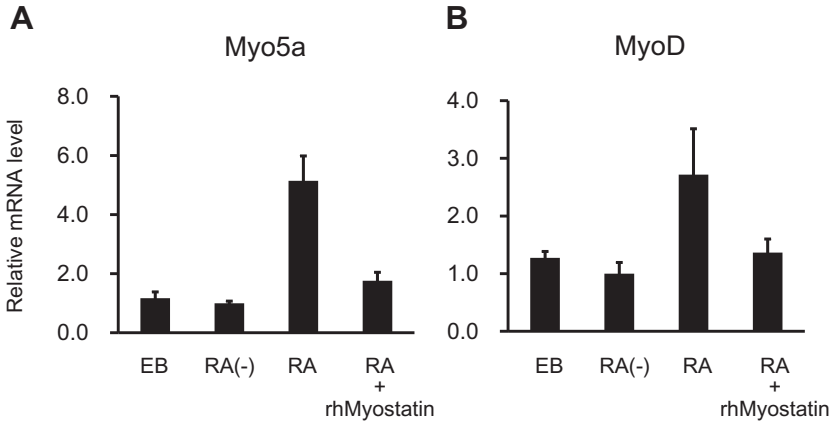

Fig. 1. Myostatin prevented induction of muscle-specific transcription factors in human iPS-derived cells that were stimulated to differentiate into myoblasts. EBs induced from human iPS cells were cultured for 10 days in the presence or absence of RA and rhMyostatin as described in the Section 2. RNA 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. $^*P < 0.05$.