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FAM5C is a soluble osteoblast differentiation factor linking muscle to bone

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ABSTRACT

Muscle mass is related to higher bone mass and a reduction in fracture risk. However, the interactions between muscle tissues and bone metabolism are incompletely understood and there might be some humoral factors that are produced in muscle tissues and exhibit bone anabolic activity. We therefore investigated the role of FAM5C in osteoblast differentiation and the interactions between muscle and bone. A reduction of endogenous FAM5C by siRNA reduced the levels of osterix, alkaline phosphatase (ALP) and osteocalcin (OCN) mRNA as well as the levels of type 1 collagen and β-catenin in mouse osteoblastic MC3T3-E1 cells and mouse calvarial osteoblasts, although FAM5C overexpression significantly antagonized the levels of osterix, ALP and OCN mRNA induced by bone morphogenetic protein-2 in C2C12 cells. The conditioned medium from FAM5C-overexpressed and -suppressed C2C12 cells increased and decreased the levels of osterix, ALP and OCN mRNA in MC3T3-E1 cells, respectively. In conclusion, the present study is the first to show that FAM5C enhances osteoblast differentiation in differentiated osteoblasts, and that the effects of the conditioned medium from FAM5C-modulated myoblastic cells were positively correlated with the effects of FAM5C on osteoblast phenotype in osteoblasts. FAM5C might be an important humoral bone anabolic factor produced from muscle cells.

1. Introduction

Linkages between bone and cardiovascular systems, nervous systems, adipose tissues and pancreas have been suggested, and the relationships between bone metabolism and organ systems other than bone have recently been noted. Previous studies demonstrated that muscle mass is potently related to high bone mass and a decrease in fracture risk in postmenopausal women [1,2]. Recent animal studies showed that resection of a large muscle segment impairs tibial fracture healing and coverage with a muscle flap is superior to coverage with local skin in encouraging early return of strength at the site of an osteotomy [3,4], indicating that fractures that are covered with relatively intact muscle improve more rapidly than fractures associated with more severe damage. These findings suggest that there might be some interactions between muscle tissues and bone metabolism.

Proinflammatory cytokines, in particular tumor necrosis factor-α (TNF-α), at the site of fracture induced the differentiation of stromal cells present in muscle into osteoprogenitor cells, and promoted bone fracture healing [5]. In this previous study, muscle-derived mesenchymal cells were more effective as the source of cells that differentiate into osteoblastic cells than bone marrow-derived mesenchymal cells. These findings suggest that muscle tissues play some important roles through certain interactions between muscle tissues and bone metabolism. Muscle tissues can produce local growth factors, which have anabolic effects in bone tissues. For example, insulin-like growth factor-1 (IGF-1), IGF binding protein-5 (IGFBP-5) and fibroblast growth factor (FGF)-2 are secreted from muscle tissues [6–8]. We therefore speculated that there might be some humoral factors that are produced in muscle tissues and affect bone in an anabolic fashion.

The gene of FAM5C, family with sequence similarity 5, member C, is located on chromosome 1q31.1 and was originally identified in mouse brain as a gene that is induced by bone morphogenetic protein (BMP) and retinoic acid signaling [9]. FAM5C is related to several cellular functions, such as proliferation, migration and atherosclerosis [10]. FAM5C alleles are implicated in the risk of myocardial infarction [11]. These findings suggest that this protein is related to various cellular functions as well as pathological

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conditions, such as atherosclerosis and inflammation. However, the role of FAM5C in bone remains unknown. Therefore, in the present study, we investigated the role of FAM5C in osteoblast differentiation and the interactions between muscle and bone.

2. Materials and methods

2.1. Materials

MC3T3-E1 cells were provided by Dr. Kodama (Ohu Dental College, Koriyama, Japan). Mouse calvarial osteoblasts from 2- to 4-day-old ICR mice were obtained from Primary Cell Co. Ltd., Sapporo, Japan. Human (h) recombinant bone morphogenetic protein-2 (BMP-2), anti-β-actin and anti-troponin T antibodies were obtained from Sigma–Aldrich Corp. (St. Louis, MO). Anti-alkaline phosphatase (ALP) and anti-β-catenin antibodies, FAM5C siRNA(m) and control siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-type 1 collagen (Col1) antibody was from Calbiochem (Gilbertsville, PA). The coding region of mouse FAM5C was amplified by reverse transcription PCR of total RNA from C2C12 cells using 5′-TAAGTCCCCAGTGGAAGCATGATAT-3′ as the forward primer and 5′-GGTTGTGCTTGACATTTATGGTTAAC-3′ as the reverse primer. The cDNA was TA-cloned into the pCR2.1 vector (Invitrogen Life Technologies Inc., Grand Island, NY) according to the manufacturer's specifications. The FAM5C cDNA insert was cloned into the mammalian expression vector pcDNA3.1(−) (Invitrogen). All constructs were verified by restriction enzyme analysis and nucleotide sequencing.

2.2. Cell culture

Mouse osteoblastic MC3T3-E1 and mouse calvarial osteoblastic cells were cultured in α-MEM (containing 50 μg/ml ascorbic acid) with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Invitrogen). Mouse myoblastic C2C12 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The medium was changed twice a week.

2.3. Transient transfection

Each vector was transfected into MC3T3-E1 or C2C12 cells with Lipofectamine (Invitrogen), as previously described [12]. Six hours later, the cells were supplied with fresh α-MEM or DMEM containing 10% FBS. Forty-eight hours later, the transiently transfected cells were used for experiments.

2.4. Protein extraction and Western blot analysis

Cells were lysed with radioimmunoprecipitation buffer containing 0.5 mM phenylmethylsulfonylfluoride, complete protease inhibitor mixture (Roche Applied Science, Tokyo, Japan), 1% Triton X-100 and 1 mM sodium orthovanadate. Proteins were transferred in 25 mM Tris, 192 mM glycine and 20% methanol to polyvinylidene difluoride. Blots were blocked with 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 0.1% Tween and 20.3% dried milk powder. The membranes were immunoblotted with each primary antibody. The antigen–antibody complexes were visualized using the appropriate secondary antibodies (Sigma–Aldrich Corp.) and an enhanced chemiluminescence detection system, as recommended by the manufacturer (Amersham Biosciences, Arlington Heights, IL). The results depicted in each figure are representative of at least three independent cell preparations. Each experiment was repeated three times.

2.5. RNA extraction and real-time PCR

Total RNA was prepared from cells using Trizol reagent. cDNA was synthesized using the First-Standard Synthesis System™ for RT-PCR (GIBCO BRL). Specific mRNA was quantified by real-time PCR using a 7500 Real-time PCR system (Applied Biosystems, Rotkreuz, Switzerland) with SYBR Premix Ex TaqTM ∏ (Perfect Real Time) kits (TaKaRa) according to the manufacturer's standard protocol. The mRNA value for each gene was normalized relative to the mouse GAPDH mRNA levels in RNA samples. Primer sequences (forward and reverse) were as follows:

GAPDH, 5′-GTGTACATGGTTCCAGTATGAGTCC-3′ and 5′-AGTG AGTTGTCATATTTCTCGTGGT-3′; FAM5C, 5′-CAGACTGGGAGCG GACTAAG-3′ and 5′-TCAAAGTGCATGCTGTAGCC-3′; osteocalcin (OCN), 5′-CCTGAGTCTGACAAAGCCTTCA-3′ and 5′-GCCGGAG TCTGTTCACTACCTT-3′; Runx2, 5′-AAATGCCTCCGCTGTTATGAA-3′ and 5′-GCTCCGGCCCACAAATCT-3′; ALP, 5′-ATCTTTGGTCTGGCTC CCATG-3′ and 5′-TTTCCCGTTCACCGTCCAC-3′; Col1, 5′-ATGCCT GGTGAACGTGGT-3′ and 5′-AGGAGAGCCATCAGCACCT-3′; osterix, 5′-AGCGACCACTTGAGCAAACAT-3′ and 5′-GCGGCTGATTGGCTT CTTCT-3′; MyoD, 5′-GACGGCTCTCTCTGCTCCTT-3′ and 5′-AGTAG AGAAGTGTGCGTGCT-3′; myogenin, 5′-GCTGCCTAAAGTGGAGAT CCT-3′ and 5′-GCGCTGTGGGAGTTGCAT-3′.

2.6. Small interfering (si) RNA

Mouse FAM5C siRNA or control siRNA was transfected as recommended by the supplier (Santa Cruz Biotechnology) into cells seeded at 5×10^5 per well using LipofectamineTM RNAi MAX (Invitrogen).

2.7. Statistics

All experiments were repeated at least three times. Data are expressed as mean ± SEM. Statistical analysis was performed using ANOVA. A P-value < 0.05 was taken to indicate a significant difference.

3. Results

3.1. FAM5C expression during osteoblast and myotube differentiation

Myoblastic C2C12 cells differentiate into osteoblastic cells when stimulated with BMP-2. BMP-2 decreased the level of FAM5C mRNA with increasing time (Fig. 1A). MC3T3E1 cells in culture undergo osteoblastic differentiation with mineralization starting after 2–3 weeks. The levels of FAM5C mRNA increased for up to 3 weeks in MC3T3-E1 cells (data not shown). C2C12 cells differentiate into myotube cells when stimulated with horse serum. The level of FAM5C mRNA did not change during myotube differentiation (Fig. 1B).

3.2. Effect of a reduction in endogenous FAM5C on osteoblast phenotype

We examined whether a reduction in endogenous FAM5C would affect osteoblast phenotype in MC3T3-E1 cells. As shown in Fig. 2A, a reduction in endogenous FAM5C level by siRNA suppressed the levels of osterix, ALP and OCN mRNA in MC3T3-E1 cells (Fig. 2A). A reduction in endogenous FAM5C level by siRNA suppressed the levels of Col1 and β-catenin protein in MC3T3-E1 cells (Fig. 2B). Moreover, we examined the effects of a reduction in endogenous FAM5C level by siRNA on the osteoblast phenotypes in mouse primary calvarial osteoblast cultures. As shown in Fig. 2C, a reduction in endogenous FAM5C level by siRNA

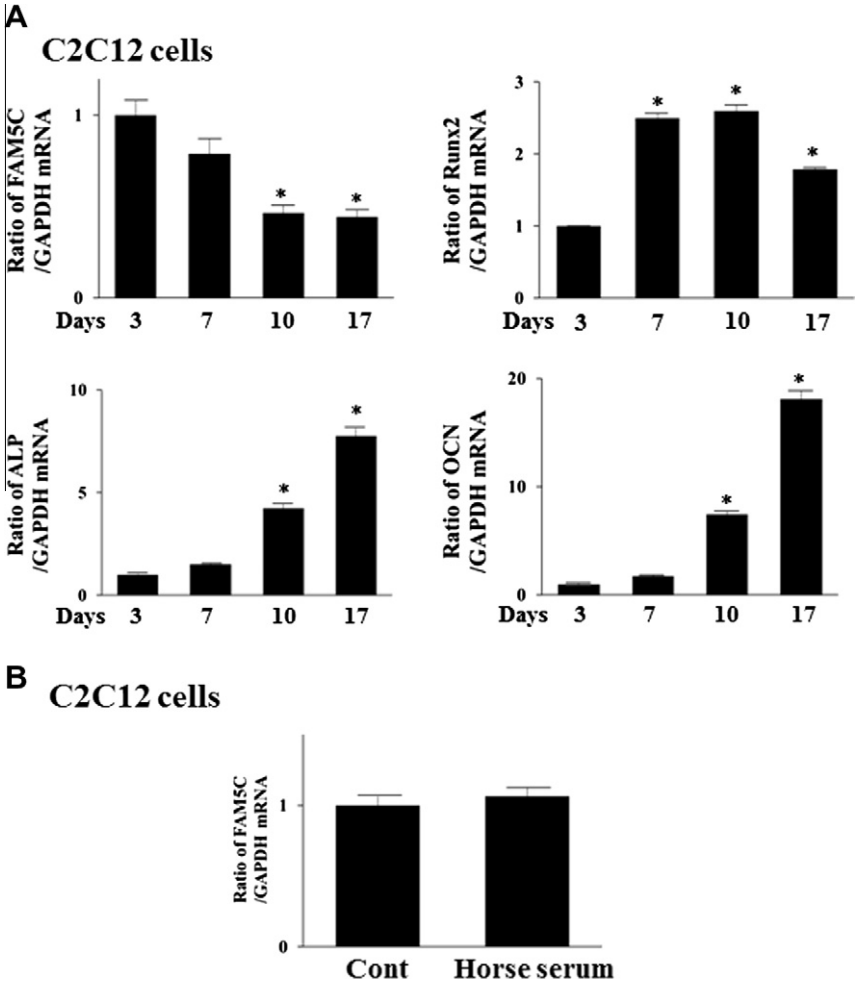

Fig. 1. Expression of FAM5C during osteoblast and myotube differentiation. (A) Total RNA was extracted from wild-type C2C12 cells treated with 300 ng/ml BMP-2 for 3, 7, 10 or 17 days and real-time RT-PCR for FAM5C, Runx2, ALP or OCN mRNA was performed. Data are expressed relative to the GAPDH mRNA value. $^{*}P < 0.01$ relative to C2C12 cells treated for 3 days. (B) Total RNA was extracted from wild-type C2C12 cells cultured with or without horse serum for 6 days and real-time RT-PCR for FAM5C mRNA was performed. Data are expressed relative to the GAPDH mRNA value.

suppressed the levels of osterix, ALP and OCN mRNA in mouse primary osteoblasts. It suppressed the levels of Col1 and β-catenin protein in mouse primary osteoblasts (Fig. 2D).

3.3. Effects of FAM5C on BMP-2-induced differentiation of myoblasts into osteoblasts

We examined the effects of FAM5C on BMP-2-induced differentiation of myoblasts into osteoblasts. Transient FAM5C overexpression significantly suppressed the levels of osterix, ALP and OCN mRNA induced by BMP-2 in C2C12 cells (Fig. 2E).

3.4. Effects of FAM5C on the differentiation of myoblasts into myotubes

Next, we examined the effect of FAM5C on the differentiation of mouse myoblasts into myotubes using FAM5C-overexpressed C2C12 cells. The levels of troponin T induced by horse serum were suppressed by FAM5C overexpression (Fig. 3A). Moreover, the levels of myogenin and MyoD mRNA were suppressed by FAM5C overexpression (Fig. 3B).

3.5. Role of myoblastic cell-derived FAM5C in osteoblasts

FAM5C overexpression and a reduction of endogenous FAM5C levels by siRNA increased and decreased the levels of FAM5C mRNA in C2C12 cells, respectively (Fig. 4A). We obtained the conditioned medium from each of FAM5C-overexpressed and -suppressed mouse myoblastic C2C12 cells. The conditioned medium from transiently FAM5C-overexpressed C2C12 cells enhanced the levels of osterix, ALP and OCN mRNA in MC3T3-E1 cells, compared with the conditioned medium from empty vector-transfected C2C12 cells (Fig. 4B). Moreover, the conditioned medium from C2C12 cells with reduced endogenous FAM5C levels by siRNA suppressed the levels of osterix, ALP and OCN mRNA in MC3T3-E1 cells, compared with the conditioned medium from control siRNA-transfected C2C12 cells (Fig. 4B). Moreover, in our preliminary study, FAM5C was detected in human serum by enzyme-linked immunosorbent assay (ELISA) Kit (Uscn Life Science Inc.) (data not shown).

4. Discussion

FAM5C is localized to the mitochondria and overexpression of this molecule leads to proliferation, migration and invasion of pituitary cells [10]. FAM5C mRNA expression in tongue squamous cell carcinoma-derived cell lines was decreased, compared with that in normal oral keratinocytes, suggesting that FAM5C might be a tumor suppressor gene [13]. Connelly et al. showed that FAM5C was expressed in proliferating aortic smooth muscle cells, and the FAM5C gene level correlated with the risk of myocardial

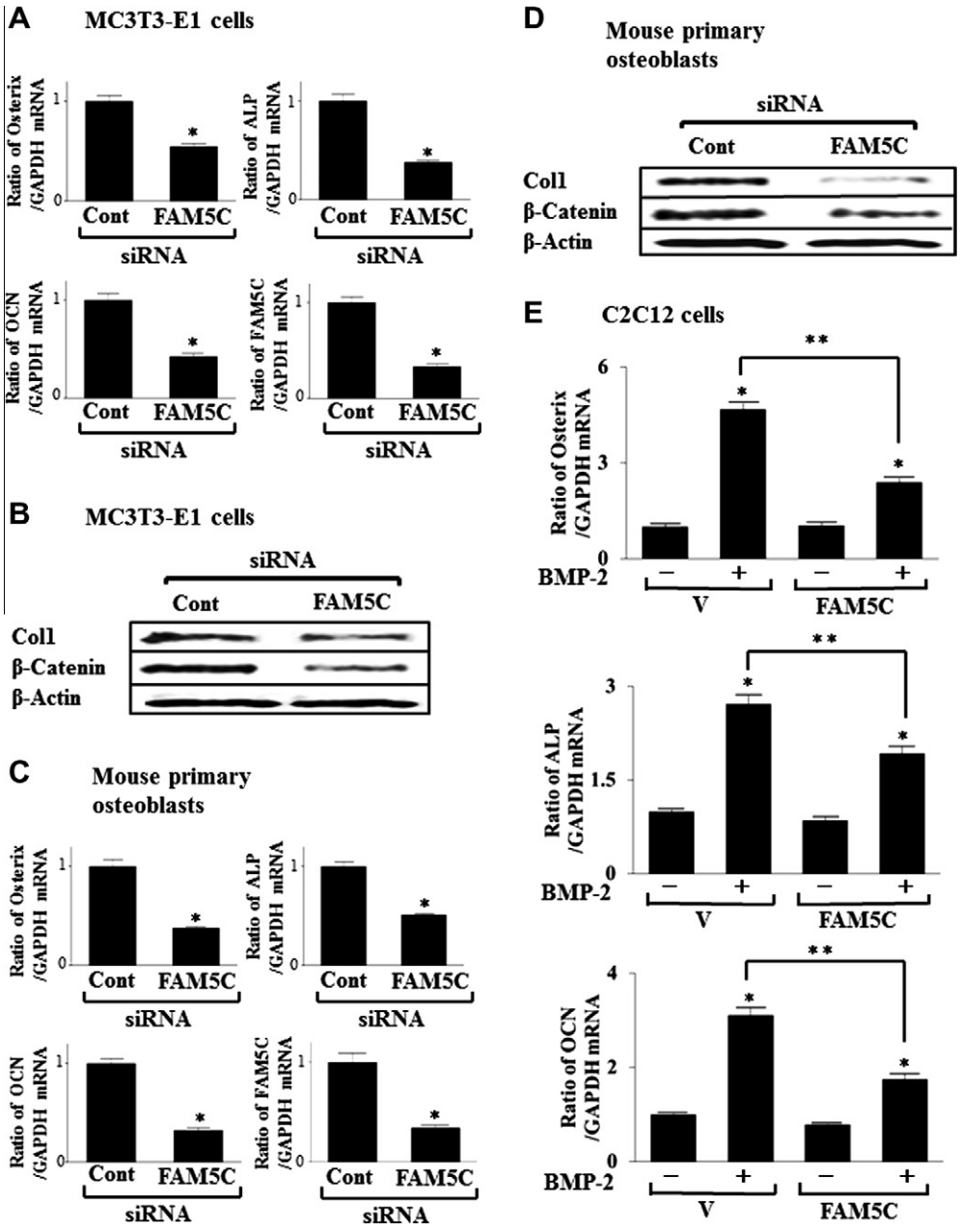

Fig. 2. Role of FAM5C in osteoblast phenotype. (A) Total RNA was extracted from MC3T3-E1 cells transfected with control siRNA or FAM5C siRNA, and real-time RT-PCR for osterix, ALP, OCN, FAM5C or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. $^{*}P < 0.01$ relative to control siRNA-transfected cells. (B) Control siRNA- or FAM5C siRNA-transfected MC3T3-E1 cells were analyzed by Western blot with anti-Col1, β-catenin and β-actin antibodies. (C) Total RNA was extracted from control siRNA- or FAM5C siRNA-transfected mouse calvarial osteoblasts, and real-time RT-PCR for osterix, ALP, OCN, FAM5C or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. $^{*}P < 0.01$ relative to control siRNA-transfected cells. (D) Control siRNA- or FAM5C siRNA-transfected mouse calvarial osteoblasts were analyzed by Western blot with anti-Col1, β-catenin and β-actin antibodies. (E) C2C12 cells transiently transfected with empty vector (V) or FAM5C were treated without (−) or with (+) 300 ng/ml BMP-2 for 72 h. Total RNA was extracted, and real-time RT-PCR for osterix, ALP, OCN or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. $^{*}P < 0.01$ relative to BMP-2-untreated, V-transfected cells. $^{**}P < 0.01$ relative to BMP-2-treated, V-transfected cells.

infarction [11]. Vasan et al. reported that FAM5C may be a candidate cardiac and vascular replication gene in a genome-wide association study [14]. These previous findings suggest that FAM5C has an important role in the proliferation and senescence of cells. In a recent study, FAM5C mRNA expression was significantly higher in aggressive periodontitis, and its expression correlated with the levels of several cytokines, such as interleukin (IL)-1β, IL-17A, IL-4 and receptor activator of nuclear factor κB ligand mRNA [15], suggesting that FAM5C contributes to aggressive periodontitis. Therefore, FAM5C may influence bone and muscle tissues. However, there have been no reports on the role of FAM5C in bone and muscle tissues.

The present study revealed that the levels of FAM5C mRNA decreased during the differentiation of myoblasts into osteoblasts, although its level did not change during the differentiation of myoblasts into myotubes. On the other hand, the levels of FAM5C mRNA increased during differentiation in mouse osteoblastic cell line. These findings suggest that FAM5C has some specific roles after cells have differentiated into osteoblasts. Our study showed that a reduction in endogenous FAM5C by siRNA suppresses the levels of osterix, ALP and OCN mRNA as well as the levels of β-catenin and Col1 in MC3T3-E1 cells and mouse primary osteoblasts. On the other hand, FAM5C overexpression significantly antagonized the levels of osterix, ALP and OCN mRNA induced by BMP-2 in

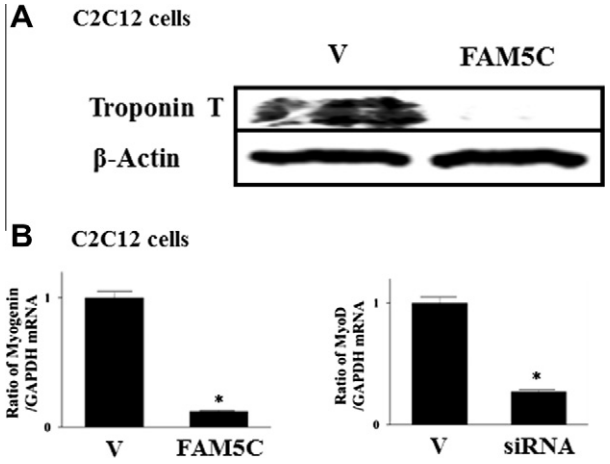

Fig. 3. Effect of FAM5C on myotube differentiation. (A) Total protein was extracted from empty vector (V)- or FAM5C-transfected C2C12 cells cultured with horse serum for 6 days, and Western blot analysis for troponin T and β-actin antibodies was performed. (B) Total mRNA was extracted from transient V- or FAM5C-transfected C2C12 cells cultured with horse serum for 6 days and real-time PCR for myogenin, MyoD or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. $^{*}P < 0.01$ relative to V-transfected C2C12 cells.

C2C12 cells. A previous study demonstrated that the osteoblast number increased at an early differentiation stage, but the osteoblast functions were impaired in osteoblast-specific Runx2-overexpressing transgenic mice [16], suggesting that Runx2 inhibits osteoblast differentiation of osteoblastic cells at a later differentiation stage, whereas it induces osteoblast differentiation in immature osteoblasts at an earlier differentiation stage. These findings suggest that FAM5C has an anabolic effect in differentiated osteoblasts, although it suppresses the differentiation of myoblasts into osteoblasts. Since this evidence suggests a relationship between FAM5C and cardiovascular systems, FAM5C might play some roles in the linkage of bone to cardiovascular systems. Further progress in this research is anticipated.

TGF-β inhibits an IGF-II-stimulated autocrine amplification cascade that is necessary for muscle differentiation in vitro [17]. Myostatin inhibits myoblast differentiation by binding to activin receptor typeIIB receptor in Smad-mediated and non-Smad pathways [18]. These findings suggest that muscle differentiation is regulated by hormones, growth factors and cytokines. In the present study, FAM5C inhibited the levels of troponin T as well as myogenin and MyoD mRNA, indicating that FAM5C suppresses the differentiation of myoblasts into myotubes.

Previous reports showed that muscle tissues secrete some cytokines and growth factors. Muscle tissues produce humoral factors that affect other organs. For example, IL-6 and IL-8 are produced by muscle fibers. IL-6 increases hepatic glucose production during exercise and lipolysis in adipose tissues, and IL-8 plays a role in exercise-induced angiogenesis [19]. Moreover, some factors produced in muscle tissues affect bone tissues. For example, IGF-1, IGFBP-5 and FGF-2 are known as osteogenic factors produced by muscle [6–8], although these factors affect various tissues in autocrine and paracrine manners as well as by acting as humoral factors. In the present study, conditioned medium from FAM5C-overexpressed and endogenous FAM5C-suppressed C2C12 cells

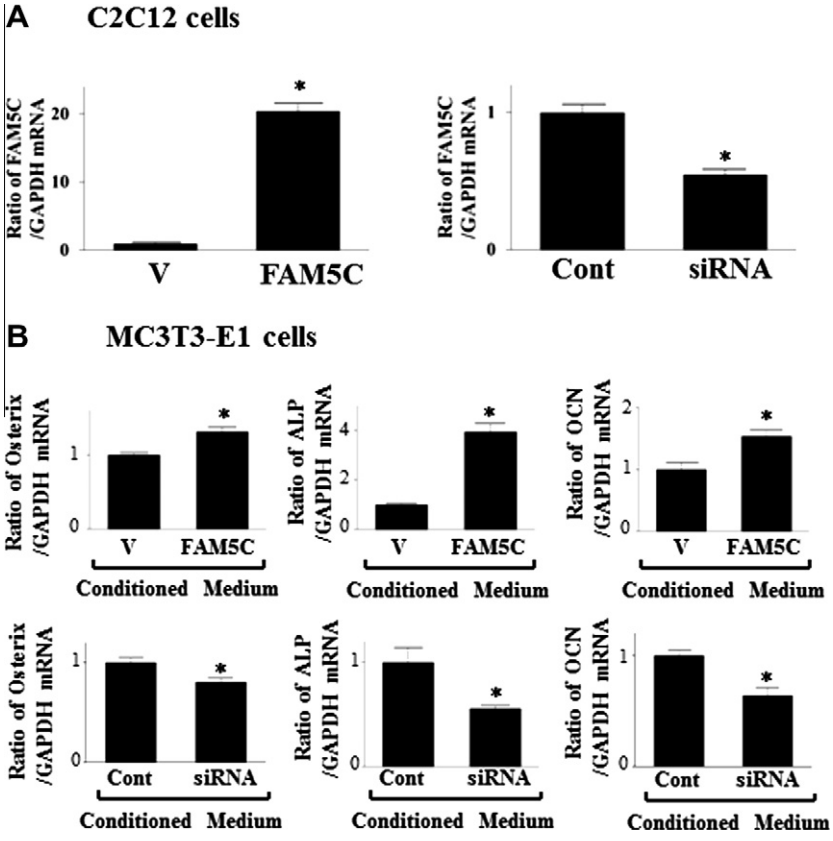

Fig. 4. Effects of conditioned medium from FAM5C-overexpressed or -suppressed myoblastic cells on osteoblast phenotype. (A) Total RNA was extracted from transient empty vector (V)- and FAM5C-transfected C2C12 cells and control siRNA- or FAM5c siRNA-transfected C2C12 cells, and real-time RT-PCR for FAM5C or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. $^{*}P < 0.01$ relative to V-transfected groups or control siRNA-transfected groups. (B) Conditioned medium was obtained from the cultures of V- and FAM5C-transfected C2C12 cells or the cultures of control siRNA- and FAM5c siRNA-transfected C2C12 cells for 24 h. Wild-type MC3T3-E1 cells were cultured with 20% of each conditioned medium for 24 h. Total RNA was extracted from these cells, and real-time RT-PCR for osterix, ALP, OCN or GAPDH mRNA was performed. Data are expressed relative to the GAPDH mRNA value. $^{*}P < 0.01$ relative to V-transfected groups or control siRNA-transfected groups.

increased and decreased the levels of osterix, ALP and OCN mRNA in mouse osteoblastic cells, respectively. These findings were consistent with the effect of a reduction in endogenous FAM5C on osteoblast differentiation. Moreover, FAM5C is measurable in human serum using ELISA. These findings suggest that FAM5C, produced in muscle tissues, exhibits bone anabolic activity in osteoblasts.

Osteoporosis and sarcopenia are increasingly problematic with the rapid aging of society. Although an increase in muscle mass is effective for the treatment of osteoporosis, it is difficult to maintain an active state for increasing and retaining muscle strength. However, the development of humoral bone anabolic factors, such as FAM5C, may provide some clues for target molecules for the treatment of osteoporosis.

In conclusion, the present study is the first to show that FAM5C enhances osteoblast differentiation in differentiated osteoblasts, and that the effects of the conditioned medium from FAM5C-modulated myoblastic cells on osteoblast differentiation were positively correlated with the effects of FAM5C on osteoblast phenotype in osteoblasts. FAM5C may be an important humoral bone anabolic factor produced by muscle cells. Further study will be necessary to clarify the precise mechanism of FAM5C actions in the interaction between muscle tissues and bone metabolism.

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