tiation of the paraxial mesoderm. In addition, the negative action of BMP4 is counteracted by a specific BMP antagonist, noggin [1].

In contrast to embryonic skeletal muscle formation, the contribution of the signaling molecules to regulation of myogenesis during postnatal growth and repair of skeletal muscles remains to be determined. Skeletal muscle stem cells of adult muscle are known as muscle satellite cells that were characterized as a different class of myogenic cells from embryonic and fetal myoblasts [2]. Nonetheless, the signaling molecules that control embryonic and fetal myogenesis are likely to play a role in the regulation of growth and differentiation of muscle satellite cells and their descendant progenitor cells. Shh and Wnt have been reported to promote the proliferation of postnatal myogenic cells derived from muscle satellite cells [3–6]. It has been well established that BMPs induce heterotopic osteogenic terminal differentiation in myogenic cells derived from satellite cells [7–9]. Forced expression of constitutively active forms of BMP type I receptors and those of activin-like kinases (ALK) or receptor-regulated Smads (Smad1/5/8) induces ectopic osteogenesis in myogenic cells [10–13]. These results strongly suggest that the BMP–ALK–Smad signaling pathway renders the myogenic cell fate osteogenic. However, the concentration of exogenous BMPs required to induce osteogenesis in more than a half of myogenic cells is 100 ng/ml or higher [8,9,14,15] (Supplementary Fig. S1; Hashimoto, unpublished data). Recently, gene expression analyses of human skeletal muscle demonstrate that BMP4 is involved in the regulation of myogenic progenitor proliferation in human fetal skeletal muscle [16]. Co-Smad, Smad4, is involved in the inhibition of myogenesis but not the induction of osteogenesis, both of which are triggered by BMPs [15]. Therefore, these studies imply that the Smad signaling pathway plays a distinct role independently of heterotopic osteogenesis.

In vitro culture systems of myogenic cells have greatly contributed to elucidation of the molecular mechanisms underlying myogenic terminal differentiation of muscle satellite cells. Mouse and rat myoblast cell lines such as C2C12 [17], Ric10 [18], and L6 [19] from muscle satellite cells in postnatal muscle have been established and represent excellent cell culture models to analyze the proliferation and differentiation of myogenic progenitor cells. Terminal muscle differentiation of myoblastic cells is usually induced by reduction of the serum concentration in the culture medium. A number of studies have provided mechanistic insights into myogenesis using this differentiation-inducing condition because terminal muscle differentiation is synchronously induced in cells cultured in the serum-reduced differentiation medium. Nevertheless, we should keep in mind that the reduction of serum and/or growth factors would never be triggering mechanisms of terminal muscle differentiation in vivo. Another option to induce myogenesis of cultured myoblasts is high cell density culture. A number of muscle cell biologists may have had the common experience of myogenic cells beginning to undergo terminal muscle differentiation even in growth medium supplemented with a high concentration of serum when cultures become confluent. The spontaneous differentiation occurs asynchronously and focally in myoblast cultures under that growth condition. Thus, cell density-dependent and growth factor-independent induction of spontaneous myogenesis in vitro might be a unique model to investigate the initiation of myogenesis in regenerating muscles, which may contain large amounts of growth factors and cytokines. Mechanistic insights into spontaneous myogenesis may provide a new hypothesis concerning the molecular switch between growth and differentiation of myogenic cells during muscle regeneration.

In this study, we focused on the physiological role of the Smad signaling pathway in the switch between growth and differentiation of myogenic progenitor cells. Smad1/5/8 was activated in undifferentiated mouse myogenic Ric10 cells under growth conditions without the stimulus of exogenous BMPs. We show here that the community effect of myogenic cells quenches the Smad signaling pathway and triggers terminal muscle differentiation.

Materials and methods

Cell culture

The mouse myogenic cell line Ric10 was established from muscle satellite cells of the normal gastrocnemius muscle of an adult female ICR mouse [9,18,20]. Ric10 cells were plated on dishes coated with type I collagen (Sumilon, Tokyo, Japan) and cultured at 37 °C under 10% CO_2 in primary myocyte growth medium (pmGM) consisting of Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (FBS), 2% Ultroser G (Biosepra, Cedex-Saint-Christophe, France), and glucose (4.5 mg/ml) [9,14,21,22]. For induction of myogenic differentiation, the cells were plated and cultured for 24 h in pmGM, and then the medium was changed to primary myocyte differentiation medium (pmDM) consisting of the chemically defined medium TIS [23,24] supplemented with 2% FBS.

For micromass culture, dissociated single cells were cultured in pmGM at a density of 5×10^4 or 1×10^5 cells per 100-μl spot in a 35-mm dish or 5×10^2–2.5×10^4 cells per 50-μl spot in a well of a 24-well plate. After incubation for at least 2 h, pmGM was carefully added to each dish or well.

For inhibition of the Smad signaling pathway, Ric10 cells were cultured in medium supplemented with dorsomorphin (Calbiochem, Darmstadt, Germany) or recombinant mouse noggin and Fc of human IgG_1 chimeric protein (R&D Systems, Minneapolis, MN).

Promoter assay

Ric10 cells (2×10^4 per well in 12-well plates) were transfected with 0.75 μg of plasmids in the presence of 4.5 μl of FuGENE6 transfection reagent (Roche Diagnostic, Mannheim, Germany) as described [20,23–25]. A reporter plasmid MGN-luc was constructed by subcloning a 1.4 kb fragment of mouse myogenin promoter [26] (kindly provided by Y. Nabeshima) into pGL2 (Promega, Madison, WI). The transcriptional activity of Smad1/5/8 was determined using a BMP/Smad-dependent specific enhancer-containing reporter plasmid BRE-luc [27] (kindly provided by K. Miyazono). FLAG-tagged mouse Smad6 cDNA (kindly provided by K. Watanabe) and ALK2(KR) cDNA encoding a dominant negative form of human ALK2 (kindly provided by T. Imamura) were subcloned into pcDNA3 (Invitrogen, San Diego, CA). An expression plasmid for *Renilla* luciferase, pRL-TK (Promega) was co-transfected for normalization of transfection efficiency. A dual luciferase assay using a dual luciferase assay system was done essentially according to the manufacturer's instructions (Promega).

Induction of muscle regeneration by bupivacaine

Gastrocnemius muscles of Sprague–Dawley rats were injected with 500 μl of 0.5% bupivacaine hydrochloride (Marcain; Astellas Pharma, Tokyo, Japan) [28]. Three to four days after injection, the

gastrocnemius muscles were removed and quickly frozen in isopentane cooled with liquid nitrogen and processed for preparation of cryosections as described [29]. Muscle specimens were sectioned at a thickness of 8 μm with a cryostat.

Immunofluorescence analysis

The frozen sections and cultured cells were fixed with 4% paraformaldehyde for 10 min at room temperature or on ice, respectively, and then incubated with primary antibodies. Primary antibodies included mouse monoclonal antibodies to mouse MyoD (1:10, Novocastra, Newcastle, UK), chicken sarcomeric myosin heavy chain (MHyC) (MF20, undiluted culture supernatant) [30], rat myogenin [31] (ascites, 1:1000, Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit polyclonal antibodies to human phosphorylated Smad1/5/8 (pSmad) (1:100, Cell Signaling, Denver, CO), rat myogenin (1:200) [25], or mouse Id1 (1:100, affinity purified antibodies against β-galactosidase-mouse Id1 fusion protein) (Hashimoto, unpublished). Secondary antibodies included biotinylated or Cy3-labeled antibodies to mouse or rabbit immunoglobulin G (Jackson ImmunoResearch Laboratory, Bar Harbor, ME). The biotinylated antibodies were detected with streptavidin-conjugated Alexa488 (Jackson ImmunoResearch Laboratory). Cell nuclei were stained with 2,4-diamidino-2-phenylindole dihydrochloride n-hydrate (DAPI) (Sigma, St. Louis, MO). Samples were visualized using an upright microscope (model BX50; Olympus, Tokyo, Japan) and an inverted microscope (model IX71; Olympus) and a CCD camera (DP70; Olympus). Images were post processed using Adobe Photoshop (Adobe Systems, San Jose, CA). The signal intensity of pSmad in the nucleus was quantified using Image J software (NIH, Bethesda, MA). The signal density of pSmad in each sample was estimated from [signal intensity/nuclear area].

Immunoblotting

Sample preparation and immunoblot analysis were performed as described [24,25,32]. Immune complexes were detected by colorimetry with a BCIP/NBT detection kit (Nacalai, Kyoto, Japan) or an ECL kit (GE Healthcare, Piscataway, NJ). Primary antibodies included antibodies to MyHC, myogenin, Smad1 (Abcam, Cambridge, MA), pSmad, Id1 (Santa Cruz Biotech., Santa Cruz, CA) and flotilin-1 (Santa Cruz Biotech). Secondary antibodies included alkaline phosphatase (DAKO, Carpinteria, CA)- or horseradish peroxidase (GE Healthcare)-labeled antibodies to mouse or rabbit immunoglobulin G. The PVDF membranes (Fluoro Trans W; Pall, Port Washington, NY) or X-ray films (Hyperfilm ECL; GE Healthcare) were scanned, and the signal intensity of each band was quantified using Image J software.

Results

Smad1/5/8 are phosphorylated in undifferentiated myogenic cells under growth conditions

To understand the physiological role of BMP–ALK–Smad signaling pathway during the growth and differentiation of postnatal myogenic cells, we detected phosphorylated Smad1/5/8 in the mouse myogenic progenitor cell line Ric10 with or without BMP2 stimulation. Smad1, Smad5, and Smad8 are functionally activated through their phosphorylation in a BMP–ALK–Smad axis-dependent manner [33]. The amount of Smad1, a major Smad protein in Ric10 cells, remained constant even under BMP stimulation (Fig. 1A, upper panel). Exogenous BMP2 activated the Smad signaling pathway: although the amount of phosphorylated Smad1/5/8 in BMP-stimulated Ric10 cells was not more than 1.5 times that in unstimulated cells (Fig. 1A), exogenous BMP2 multiplied the transcriptional activity of Smad1/5/8 up to more than 10 times that in unstimulated cells (Fig. 1Ba). However, Smad1/5/8 was also phosphorylated in growing Ric10 cells without exposure to any exogenous ligand (Fig. 1A, left lane). Phosphorylated Smad1/5/8 was localized in nuclei of unstimulated, growing Ric10 cells (Fig. 1C). In addition, ALK2(KR), the dominant negative form of ALK2, significantly reduced the transcriptional activity of Smad1/5/8 in unstimulated cells (Fig. 1Bb). Exogenous BMP2 induced glycosylphosphatidylinositol-anchored alkaline phosphatase (ALP), an early marker of osteogenic differentiation through the activation of Smad signaling pathway in mouse myogenic cells (Supplementary Fig. S1) [9,14]. In contrast, the basal level activation of Smad1/5/8 didn't induce ALP expression in unstimulated mouse myogenic cells (data not shown) [9,14]. The results suggest that the Smad signaling pathway plays a physiological role that is independent of osteogenic differentiation in unstimulated, undifferentiated growing Ric10 cells.

Myogenic terminal differentiation-inducing conditions reduce the amount of phosphorylated Smad1/5/8

Myogenic terminal differentiation of cultured myogenic cells depends highly on both the serum concentration in the medium and the cell density in the culture. Despite the low cell density, myogenic progenitor cells actually undergo myogenic differentiation in differentiation medium supplemented with a low concentration of serum. However, myogenic cells under the high cell density culture condition give rise to myotubes faster than those under the low cell density

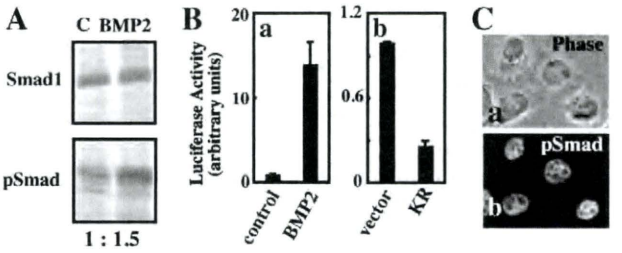


Fig. 1 – Phosphorylation of Smad1/5/8 in undifferentiated growing myogenic cells. (A) Total protein (20 mg) was prepared from Ric10 cells that had been cultured in pmGM supplemented with (lane BMP2) or without (lane C) BMP2 (100 ng/ml) for 24 h. Smad1 and phosphorylated Smad1/5/8 (pSmad) were detected on immunoblots. Relative amounts of pSmad are shown below the lower panel. (B) Ric10 cells were transfected with BRE-luc, Renilla luciferase-expression plasmid pRL-tk, and pcDNA3.1 (a and vector in b) or an expression plasmid for dominant negative ALK2 (KR). Then the cells were cultured in pmDM for 24 h and harvested for dual luciferase assay according to the manufacturer's instructions (Hashimoto and Ogashiwa, 1997). In (a), the cells were stimulated with BMP2 (100 ng/ml) or without (control) for 24 h. (C) Unstimulated, growing Ric10 cells showed nuclear localization of pSmad. The images were obtained by phase contrast (a) and epifluorescent (b) microscopy. Scale bar: 10 μm.

culture condition. To reveal the optimal culture condition for myogenic differentiation, different numbers of Ric10 cells were cultured for 24 h in the chemically defined differentiation medium TIS supplemented with different concentrations of serum. Actually, myogenic differentiation of Ric10 cells was exclusively induced under the culture condition with low serum and high cell density in an early period of culture (Fig. 2A). Then we determined the activation level of the Smad signaling pathway in Ric10 cells under different culture conditions. Smad1/5/8 was phosphorylated in low cell density culture (Fig. 2B, lower panel). In contrast, the amount of phosphorylated Smad1/5/8 severely declined in the high cell density culture. The serum concentration in the medium also affected the phosphorylation level of Smad1/5/8 both in low and high cell density cultures. In addition, a low cell density in the culture and a high serum concentration in the medium synergistically activated the Smad signaling pathway (Fig. 2C). However, a low cell density in the culture more potently activated the Smad signaling pathway than a high serum concentration in the medium.

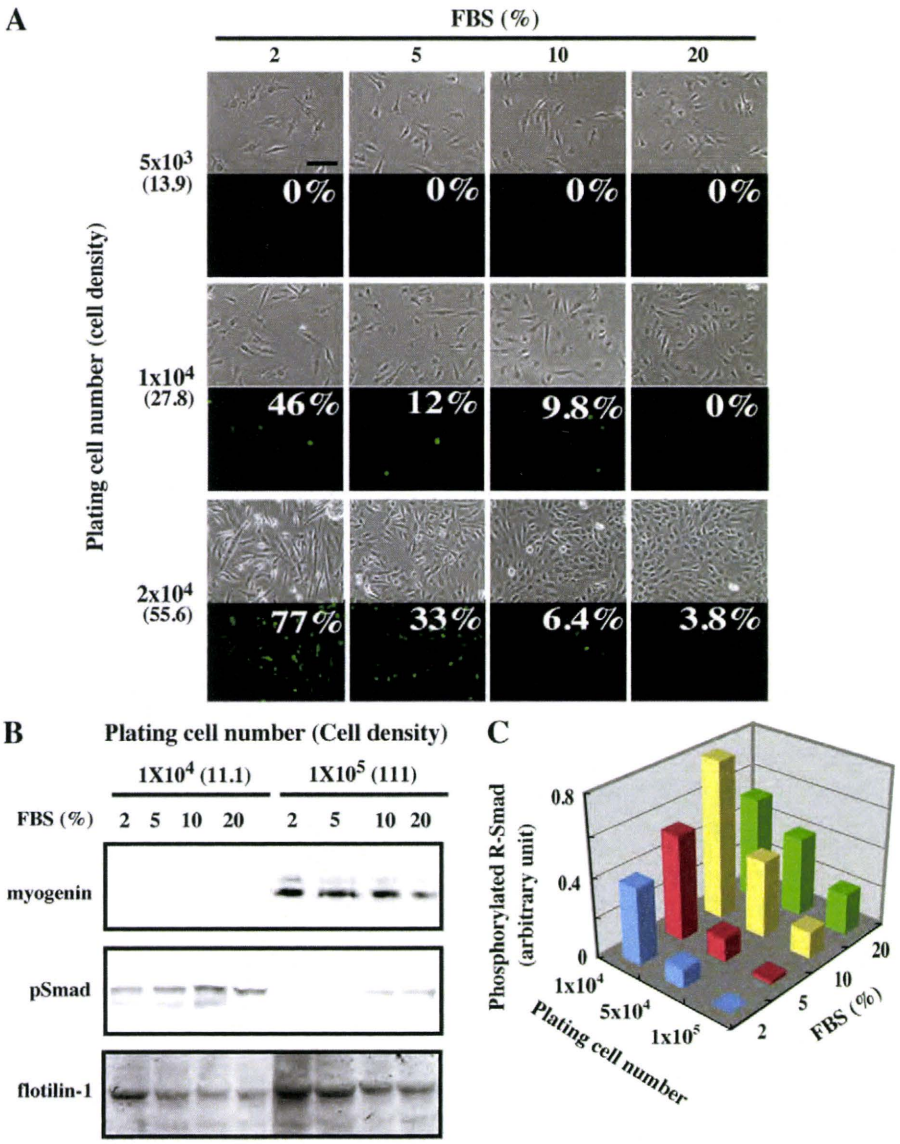


Fig. 2 – Down-regulation of Smad signaling pathway under myogenic differentiation-inducing conditions. (A) Ric10 cells were plated at a density of 5×10^3, 1×10^4, and 2×10^4 cells in a well of a 12-well culture plate and then cultured for 24 h in medium supplemented with different concentrations of fetal bovine serum (FBS). Cell density at seeding (cells per mm^2) is shown in parentheses left of the panels. Myogenin-positive nuclei were detected with a specific antibody and appeared as green dots. The percentage of myogenin-positive nuclei is shown inside the panels. Phase contrast and immunofluorescence images of the identical fields are shown as pairs. Scale bar: 100 μm. (B and C) Different numbers of Ric10 cells were plated in 35-mm dishes and cultured for 24 h in medium supplemented with different concentrations of FBS. Cell density at seeding (cells per mm^2) is shown in parentheses in (B). Myogenin, pSmad, and flotilin-1 were detected in 20 mg of total protein by immunoblotting analysis. Flotilin-1 was used as a loading control. Amounts of phosphorylated Smad1/5/8 on immunoblots were quantified and are represented as a three-dimensional graph (C).

The myogenic differentiation-specific transcription factor myogenin was expressed exclusively in the high cell density cultures of Ric10 cells, in which phosphorylation of Smad1/5/8 was down-regulated (Fig. 2B, upper panel). It should be noted that Ric10 cells undergo myogenic differentiation during prolonged culture (more than 48 h), even under the low cell density culture condition (Fig. 3A) [18]. Therefore, myogenic terminal differentiation was enhanced in the high cell density culture associated with inactivation of the Smad signaling pathway. The results suggest that the Smad signaling pathway is activated in undifferentiated proliferating cells and inactivated during the myogenic differentiation induced under the high cell density culture condition. The results imply the involvement of the Smad signaling pathway in a critical switch between growth and differentiation of myogenic cells.

Quenching of Smad signaling pathway is rate-limiting for myogenic differentiation induced by serum reduction

To determine whether the Smad signaling pathway is involved in the regulation of myogenic differentiation triggered by serum reduction, Ric10 cells were seeded at low (22 cells per mm^2) or high (111 cells per mm^2) cell density and then induced to differentiate in differentiation medium pmDM containing 2% serum for up to 48 h. Ric10 cells seeded at high cell density gave rise to myotubes after 24 h under the serum-reduced culture condition whereas those under the low cell density culture condition began to fuse after 36 h (Fig. 3A). Expression of myogenin and a muscle differentiation marker MyHC was induced by serum reduction faster in the high cell density culture than the low cell density culture (Fig. 3B). In contrast to the muscle-specific proteins, the amount of a downstream factor of the Smad signaling pathway, Id1, declined faster in the high cell density culture than the low cell density culture. Serum reduction decreased the amount of phosphorylated Smad1/5/8 in both low and high cell density cultures. However, the phosphorylated Smad1/5/8 still remained under the serum-reduced, low cell density culture condition (Fig. 3B).

The previously mentioned results imply that quenching of the Smad signaling pathway is rate-limiting for myogenic differentiation induced by serum reduction. To explore this possibility, Ric10 cells were treated with the Smad signaling pathway inhibitor dorsomorphin [34]. Ric10 cells were seeded at low cell density (18 cells per mm^2) and then induced to differentiate in pmDM for up to 48 h. More than 90% of Ric10 cells underwent myogenic differentiation under this condition (Fig. 3A) [18]. Dorsomorphin further reduced the amount of remaining

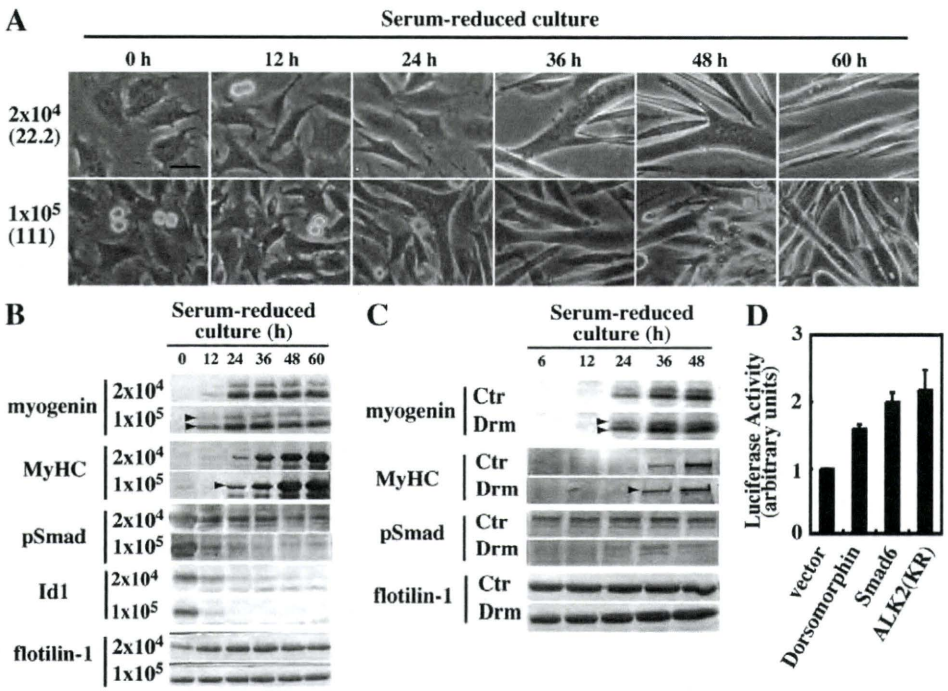


Fig. 3 – Activation levels of Smad signaling pathway during serum-reduced, low cell density culture. (A and B) Ric10 cells (2×10^4 cells per 35-mm dish; 22 cells per mm^2 for low cell density culture or 1×10^5 per 35-mm dish; 111 cells per mm^2 for high cell density culture) were plated and cultured in pmDM for up to 60 h. Images in (A) were obtained by phase contrast microscopy. Scale bar: 50 μm. Total cell lysates (20 μg of proteins) were subjected to immunoblotting analyses for MyHC, myogenin, pSmad, Id1, and flotilin-1 (B). Flotilin-1 was used as a loading control. Plating cell number is shown at the left of panels. Cell density at seeding (cells per mm^2) is shown in parentheses in (B). Arrowheads indicate the enhanced expression of MyHC and myogenin. (C) Ric10 cells (1×10^5 per 100-mm dish; 18 cells per mm^2) were cultured in pmDM with dorsomorphin (3 μM) or without (Control) for the indicated periods (h) shown at the top of panels. Total cell lysates (20 μg of proteins) were subjected to immunoblotting analyses for MyHC, myogenin, pSmad, and flotilin-1. Flotilin-1 was used as a loading control. Arrowheads indicate the enhanced expression of MyHC and myogenin in dorsomorphin-treated culture. (D) Ric10 cells were transfected with MGN-luc, pRL-tk, and pcDNA3.1 (vector and Dorsomorphin) or an expression plasmid for Smad6 or dominant negative ALK2 (ALK2(KR)). Then the cells were cultured in pmDM for 24 h and harvested for dual luciferase assay.

phosphorylated Smad1/5/8 (Fig. 3C). Expression of myogenin and MyHC was enhanced in Ric10 cells treated with dorsomorphin at 24 and 36 h of culture in pmDM. Taken together with the results, lowering the level of phosphorylated Smad may enhance myogenic differentiation triggered by serum reduction.

To determine whether the myogenic differentiation is negatively regulated through the activation of Smad1/5/8 under the serum-reduced culture condition, we determined the promoter activity of myogenin in Ric10 cells when the function of Smad1/5/8 was directly inhibited at distinct steps of the cellular signaling pathway: dorsomorphin inhibits phosphorylation of Smad1/5/8. Smad6, an inhibitory Smad, interrupts the interaction between Smad1/5/8 and Smad4 or BMP type I receptors [35,36]. ALK2(KR), the dominant negative form of ALK2, suppresses the activation of the BMP-receptor ALK2 [12]. Activity of the myogenin promoter in Ric10 cells during 24 h of differentiation culture was significantly enhanced by administration of dorsomorphin or expression of Smad6 or ALK2 (KR) (Fig. 3D). The results suggest that the quenching of Smad signaling pathway plays a role in the rate-determining step of terminal muscle differentiation triggered by serum reduction.

Concentration of phosphorylated Smad1/5/8 declines during myogenic differentiation in a cell density-dependent fashion

High cell density culture is another option for induction of myogenic differentiation in vitro. Ric10 cells were cultured in micromass to undergo myogenic differentiation independently of the serum concentration in the medium. When 5×10^4 or 1×10^5 Ric10 cells were seeded in a 100-μl spot, cells gave rise to a circular mass, the diameter of which was 6–8 mm (Fig. 4A). The cell density within a micromass declined along a central–peripheral axis. Myogenic terminal differentiation was induced exclusively in the central region of a cell mass during the 48 h after seeding, even when cells were cultured in growth medium pmGM containing 20% serum and supplementary growth factors (Fig. 4B). In the central region of the culture, the extremely high cell density was associated with a loss of phosphorylated Smad1/5/8 and the induction of myogenin expression even in pmGM (Fig. 4C). Myogenin expression was induced in a fraction of the nuclei in which the amount of phosphorylated Smad1/5/8 was severely reduced (Supplementary Fig. S2). In contrast, the cells retained phosphorylated Smad1/5/8 and did not express myogenin in the peripheral region with relatively low cell density. The results indicate that the Smad signaling pathway was inactivated under the high cell density culture condition, and also suggest that quenching of the Smad signaling pathway induces precocious myogenic differentiation even in the presence of growth factors.

Id1 gene is one of the target genes of the Smad signaling pathway [27]. Its gene product, Id1, inhibits transcription of the MyoD family and suppresses terminal myogenic differentiation [37]. Id1 expression was inhibited in Ric10 cells exclusively in the central region even in pmGM (Fig. 4D), although Id1 is normally expressed in a serum-dependent manner (Fig. 3B) [37]. Similar expression patterns of phosphorylated Smad1/5/8 and Id1 imply that inactivation of the Smad signaling pathway may induce myogenin expression and myogenesis through suppression of Id1 expression.

Furthermore, differentiation-inducing culture for 24 h in pmDM following 24 h of culture in pmGM triggered the robust

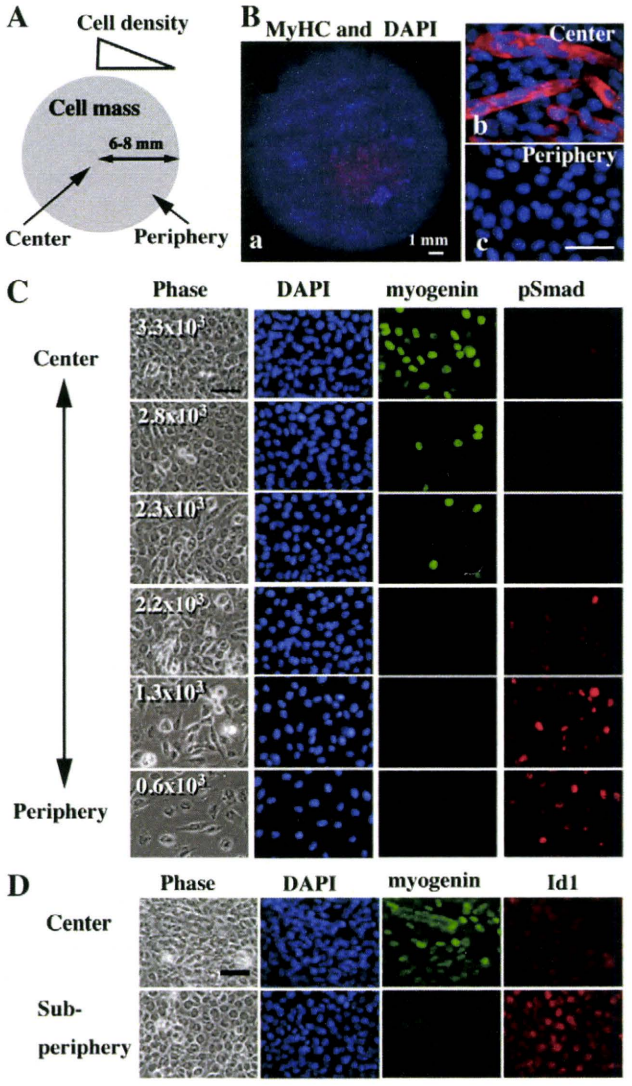


Fig. 4 – Down-regulation of phosphorylated Smad and Id1 during myogenesis triggered in high cell density culture. (A and B) For a micromass culture, 1×10^5 Ric10 cells were plated in a 100-μl spot and cultured in pmGM for up to 48 h. A diagram of micromass culture shows that cell density is reduced along with the center–periphery axis (A). MyHC was detected with a specific antibody (red) and nuclei were stained with DAPI (blue) in (B). Pictures show whole cell mass (a) and regions of center (b) or periphery (c). Scale bars, 1 mm (a) and 50 μm (b and c). (C and D) Ric10 cells (5×10^4 cells per 100-μl spot) were cultured in micromass for 48 h in pmGM. The cells were subjected to immunostaining with anti-myogenin and anti-pSmad or anti-Id1 antibodies. Nuclei were stained with DAPI. The numbers in phase contrast images in (C) represent the cell density of the indicated field (cells per mm^2). Images in each row were obtained from the same field. Scale bars: 50 μm.

expression of myogenin from the center to the sub-periphery of the cell mass (Fig. 5A). Ric10 cells gave rise to myotubes in the sub-peripheral region as well as the central region of the micromass. Serum reduction caused a rapid fall in the amount of phosphorylated Smad1/5/8 in micromass cultures (Fig. 5A). Id1 was down-

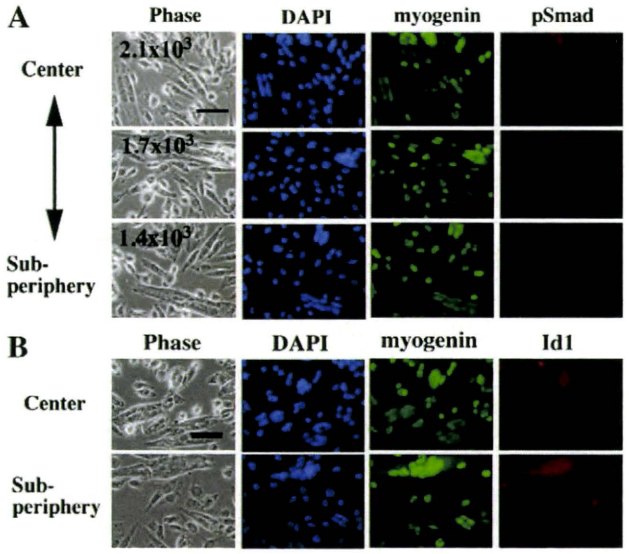


Fig. 5 – Synergistic induction of robust myogenesis by high cell density and serum reduction. (A and B) Ric10 cells (5×10^4 cells per 100 μl of spot) were cultured in micromasses in pmGM for 24 h and then further cultured in pmDM for up to 24 h. The cells were subjected to immunostaining with anti-myogenin, anti-pSmad, and anti-Id1 antibodies. Nuclei were stained with DAPI. The numbers in the phase contrast images in (A) represent cell density of the indicated field (cells per mm^2). Images in each row show the same field. Scale bars: 50 μm.

regulated even in the peripheral region of the micromass in pmDM (Fig. 5B). It should be noted that the cell density in the peripheral region of the micromass ($4–6\times10^2$ cells per mm^2) was still much higher than that of the low cell density cultures shown in Figs. 2 and 3 (less than 100 cells per mm^2). The results indicate that high cell density and serum reduction synergistically induced robust myogenic differentiation, possibly through quenching the Smad signaling pathway.

Dorsomorphin enhances myogenic differentiation in a cell density-dependent fashion under growth condition

To determine whether the Smad signaling pathway is involved in the regulation of myogenic differentiation induced in the high cell density culture, Ric10 cells were cultured in micromass under the growth condition and treated with dorsomorphin for 24 h after seeding. Myotube formation was markedly enhanced by dorsomorphin in a dose-dependent fashion (Fig. 6A). Expressions of MyHC and myogenin were also enhanced by dorsomorphin in a similar manner (Fig. 6B). Myotubes were robustly formed in both dorsomorphin-treated and untreated cultures when the cells were cultured for the prolonged period (48–60 h). The result indicates that dorsomorphin induced myogenic differentiation precociously.

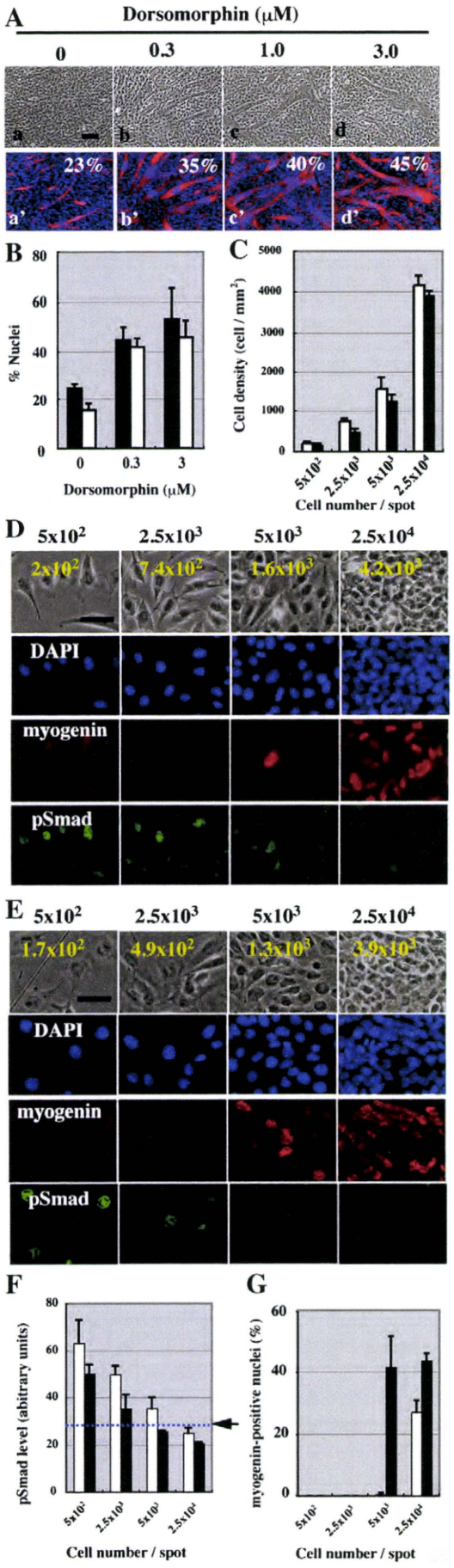
In the next series of experiments, various numbers of Ric10 cells were cultured in micromass to understand whether cell density affects the enhancement of myogenesis by dorsomorphin. The highest concentration of cells in the central region of the cell mass increased along with the number of plated cells (Fig. 6C). Phosphorylation of Smad1/5/8 was suppressed in high cell density cultures (Fig. 6D). Dorsomorphin also inhibited phosphorylation of Smad1/5/8 and markedly enhanced myogenic differentiation in high cell density culture whereas its effects were obscure in relatively lower cell density culture (Fig. 6E). Dorsomorphin continuously but limitedly decreased the level of phosphorylated Smad1/5/8 per nucleus (Fig. 6F). Dorsomorphin and high cell density synergistically down-regulated phosphorylation of Smad1/5/8. When the concentration of phosphorylated Smad1/5/8 fell below the putative threshold that is represented by an arrow in Fig. 6F, expression of myogenin was induced (Fig. 6G). Dorsomorphin-treatment also resulted in down-regulation of Id1 and up-regulation of MyoD (Supplementary Fig. S3). These results imply that down-regulation of the Smad signaling pathway induces myogenic differentiation even in pmGM under the high cell density culture condition. Similar results were obtained from another mouse myogenic cell line, COM3, that was re-cloned from C2C12 cells (Supplementary Figs. S4–6) [23].

BMP antagonist noggin triggers myogenic terminal differentiation in a cell density-dependent fashion under growth condition

To determine whether precocious cell density-dependent, serum concentration-independent myogenic differentiation is suppressed by ligand BMP, Ric10 cells cultured in micromass were exposed to a BMP antagonist, noggin. The expression of MyHC and myogenin were induced in the center of cell mass exclusively (Fig. 7A). Recombinant noggin (5 mg/ml) enhanced myotube formation and the expression of MyHC and myogenin in the region with the relatively lower cell density (Fig. 7B). However, noggin failed to induce MyHC expression in Ric10 cells in the peripheral region where cell density was lower than 1000 cells per mm^2 (Fig. 7C). Consistent with the results in myogenesis assay, noggin decreased the level of phosphorylated Smad1/5/8 (Fig. 7D). The results suggest that ligand-dependent activation of the Smad signaling pathway contributes to the suppression of precocious differentiation of myogenic cells.

Smad signaling pathway is transiently activated in myogenic progenitor cells during muscle regeneration in vivo

To examine the involvement of the Smad signaling pathway in postnatal muscle regeneration, the phosphorylation of Smad1/5/8 was determined in regenerating muscle. Muscle regeneration was induced by injection of BPV into the gastrocnemius muscle of rats. During the early phase of muscle regeneration, myogenic progenitor cells derived from muscle satellite cells express MyoD but not myogenin in BPV-injected rat muscle. Then, the cells are induced to express both MyoD and myogenin, and they give rise to myofibers [38] (Umeda and Hashimoto, unpublished observation). We detected phosphorylated Smad1/5/8 in more than 50% of nuclei of MyoD-expressing myogenic cells in regenerating muscle on days 3 and 4 after BPV injection (Fig. 8Aa–d). A fraction of the myogenin-positive myogenic cells also showed phosphorylated Smad1/5/8 in their nuclei (Fig. 8Ae–h), but the percentage of phosphorylated Smad1/5/8-positive cells in the myogenin-positive mononucleated cells was less than that in the number of MyoD-positive mononucleated cells (39% vs. 65%). The results indicate that the Smad signaling pathway is first activated and then inactivated in myogenic progenitor cells during postnatal muscle regeneration. Therefore, the Smad signaling pathway may be involved in the regulation of growth and



differentiation of postnatal myogenic cells in vivo as well as in vitro.

Discussion

Smad signaling pathway determines cell fates of myogenic cells

In the present study, we provide evidence for a novel role of the BMP–ALK–Smad axis in the switch between growth and differentiation of myogenic cells during postnatal muscle growth and repair. Autonomous cell expression of BMP receptors and downstream Smad proteins in postnatal myogenic cells implies a physiological function of the BMP–ALK–Smad axis during postnatal muscle differentiation. Actually, growing undifferentiated myogenic cells show significant activation of the Smad signaling pathway without administration of any exogenous BMPs. Four independent methods of blocking the BMP–ALK–Smad axis demonstrated that this physiological Smad signaling suppresses precocious differentiation of myogenic cells and keeps their undifferentiated state (Fig. 8B): inhibition of BMP function by noggin, inhibition of ALK2 activation by dominant negative ALK2, inhibition of the interaction between Smad1/5/8 and Smad 4 or ALK2 by an inhibitory Smad, and inhibition of phosphorylation of

Fig. 6 – Induction of precocious myogenic differentiation by dorsomorphin. (A and B) Ric10 cells (5×10^4 cells per 100-μl spot) were cultured in micromass in pmGM for 24 h and then further cultured in pmGM with (Ab–d) or without (Aa) dorsomorphin for up to 24 h. Each medium contained 0.03% DMSO. The cells were subjected to immunostaining with anti-MyHC alone (A) or anti-myogenin and anti-MyHC antibodies (B). Nuclei were stained with DAPI. Images of central regions of micromasses with the highest cell density were obtained by phase contrast (Aa–d) and epifluorescent microscopy (Aa′–d′). The percentages of nuclei in MyHC-positive cells in the total number of nuclei are shown in the lower panels (Aa′–d′). The percentages of nuclei in myogenin- and MyHC-positive cells in the total number of nuclei were calculated, and averages and standard deviations of three independent cultures are shown as solid and open bars, respectively in (B). (C–G) The indicated numbers of Ric10 cells per 50-μl spot were cultured in micromass in pmGM for 24 h and then further cultured in pmGM with 3 μM dorsomorphin (E and solid bars in C, F and G) or without (D and open bars in C, F and G) for up to 24 h. The cells were subjected to immunostaining with anti-myogenin and anti-pSmad antibodies. Nuclei were stained with DAPI. Images of the central regions of micromasses with the highest cell density were obtained by phase contrast (top panels) and epifluorescent microscopy (lower panels) in (D and E). Cell density (cells per mm²) at the end of culture was calculated and is shown as averages and standard deviations of three independent cultures (C), or represented as numbers in the top panels (D and E). The relative concentration of pSmad in nuclei (F) and the percentages of myogenin-positive nuclei in the total number of nuclei (G) were calculated, and averages and standard deviations of three independent cultures are shown. Scale bars: 10 μm.

Smad1/5/8 by a protein kinase inhibitor dorsomorphin. The correlation between the levels of phosphorylated nuclear Smad1/5/8 and myogenin indicates a threshold of the Smad signal intensity that is sufficient to maintain myogenic cells in an undifferentiated state (Fig. 6F). When the level of Smad signaling is below the threshold, myogenic progenitor cells begin to undergo terminal myogenic differentiation (Fig. 8B).

Our previous study suggests that the exposure to high concentrations of BMP2 causes extraordinary activation of the Smad signaling pathway resulting in induction of osteogenesis in myogenic cells [9]. Hyper-activated Smad1/5/8 irreversibly prevents myogenesis, whereas spontaneously activated Smad1/5/8 suppresses precocious myogenic differentiation reversibly without exposure to exogenous BMPs. Then, hyperactivation of the Smad signaling pathway results in expression of Smad target genes that are not induced during myogenesis but are required for osteogenesis (Fig. 8B).

Recently, we found that high concentrations of BMPs induce ectopic osteogenesis of Ric10 in a cell density-dependent fashion (Supplementary Fig. S7). The results suggest a continuum in the effect of BMPs between the inhibition of myogenic differentiation and transdifferentiation into an osteogenic cell fate. In addition, low concentrations of BMP2 induced osteogenesis in Ric10 cells at low cell density (Supplementary Figs. S7B and C). Taken together with the Supplementary results, the magnitude of Smad signaling might play a critical role in generation of different fates from myogenic progenitor cells (Fig. 8B). In addition, we have found that the exogenous BMP-induced osteogenesis is facilitated by a co-signal (Yanagisawa and Hashimoto, unpublished). Therefore, cellular context and co-signals may determine whether a given BMP stimulus induces which cell fates. From this point of view, it is very interesting that the migrating Ric10 cells at the margin of a cell mass were refractory when exposed to high concentrations of BMP2 (Supplementary Figs. S7D and E).

Quenching of Smad signaling is rate-limiting for myogenic differentiation

The present study indicates that quenching of the Smad signaling pathway triggers myogenic differentiation under the high cell density culture condition. Serum reduction also lowered the phosphorylation level of the Smad signaling pathway. However, high cell density was more potent for inactivating the Smad signaling pathway than low serum concentration in the medium. The present study shows that the Smad signaling pathway is also rate-limiting for myogenic differentiation induced by serum reduction. However, the enhancement of myogenic differentiation by dorsomorphin was quite limited under the serum-reduced, low cell density culture condition. Thus, it is likely that distinct

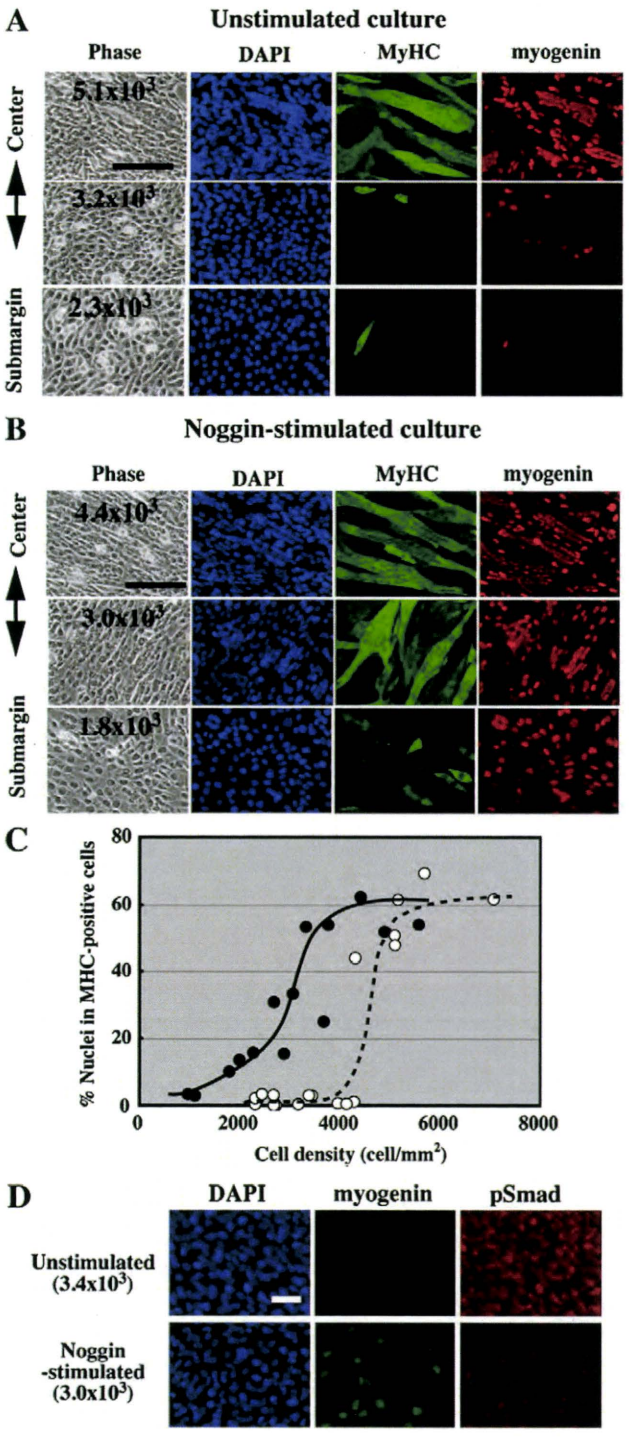


Fig. 7 – Induction of precocious myogenic differentiation by BMP antagonist noggin. (A, B and C) Ric10 cells (5×10^4 cells per 100-μl spot) were cultured in micromasses in pmGM for 24 h and then further cultured in pmGM with (B) or without (A) noggin (5 mg/ml) for up to 24 h. The cells were subjected to immunostaining with anti-myogenin and anti-MyHC antibodies. Nuclei were stained with DAPI. Images of various regions were obtained by phase contrast and epifluorescent microscopy. Numbers in the left-hand panels in A and B represent cell density (cell per mm^2). (C) Cell density and the percentages of nuclei in MyHC-positive cells in the total number of nuclei were calculated in cultures stimulated with (solid circles) or without (open circles) noggin. (D) Ric10 cells (5×10^4 cells per 100-μl spot) were cultured in micromasses in pmGM for 24 h and then further cultured in pmGM with (lower panels) or without (upper panels) noggin (5 mg/ml) for up to 24 h. The cells were subjected to immunostaining with anti-myogenin and anti-phosphorylated Smad1/5/8 antibodies. Nuclei were stained with DAPI. Images were obtained by epifluorescent microscopy. The numbers in parentheses at the left of panels represent cell density (cell per mm^2) at the end of culture. Scale bars: 50 μm.

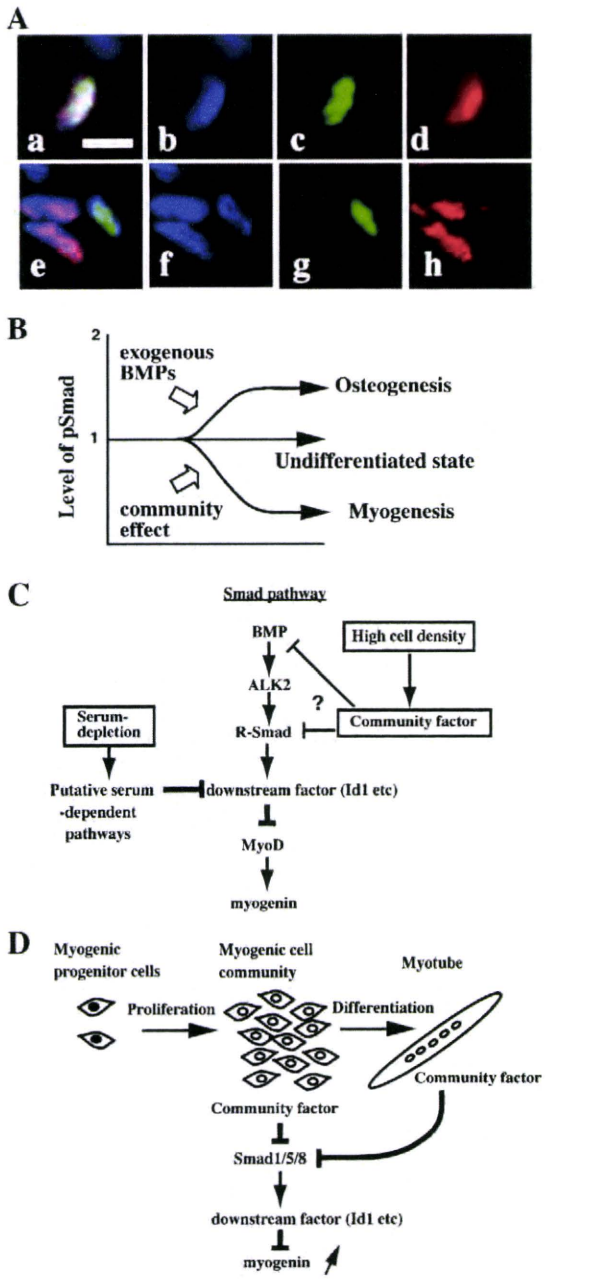


Fig. 8 – Role of Smad signaling pathway in switch between growth and differentiation of postnatal myogenic cells. (A) BPV was injected into the gastrocnemius muscle of rats. Cryosections were prepared from the muscles removed on day 3 (a–d) or 4 (e–h) after BPV injection, and stained with anti-MyoD (c), anti-myogenin (g), anti-phosphorylated Smad1/5/8 (pSmad) (d and h) and DAPI (b and f), respectively. (b, c, and d and f, g, and h) were merged in (a) and (e), respectively. Scale bar: 10 μm. (B) Crucial role of phosphorylated Smad1/5/8 (pSmad) in generation of different fates from myogenic progenitor cells. (C) Hypothetical role of down-regulation of Smad signaling pathway during myogenic differentiation induced in vitro. High cell density and serum reduction synergistically induce myogenic differentiation although they down-regulate downstream factors including Id1 through distinct pathways. (D) Community effect on myogenesis during postnatal muscle growth and repair. See detailed explanation of B, C and D in the "Discussion".

signaling pathways also play a role in the rate-determining step for myogenic differentiation under the serum-reduced, low cell density culture condition. The basal levels of the Smad signaling may induce expression of multiple target genes in undifferentiated, growing myogenic cells. *Id1* is a well-known downstream target gene of the Smad signaling pathway (Fig. 8C). *Id1* encodes an inhibitor protein of the MyoD family and is down-regulated during myogenic differentiation induced by serum reduction [37,39,40]. Previous studies have shown several factors that down-regulate Id1 protein independently of the effects of the BMP–ALK–Smad axis on serum reduction: interferon-inducible protein p204 and micro RNA miR-206 repress Id1 protein and promote myogenic differentiation [39,40]. In addition, lowering the level of p204 inhibits myogenic differentiation in serum-reduced culture [40]. In contrast, quenching of the Smad signaling pathway alone seems critical and rate-limiting for myogenic differentiation and induces myogenic differentiation under the high cell density culture condition even in the high serum-containing culture. Therefore, the two myogenic differentiation-inducing conditions, high cell density and serum reduction, may induce myogenic differentiation in different ways (Fig. 8C): suppression of the Smad signaling pathway alone is rate-limiting for myogenesis or both the Smad signaling pathway and the other signaling pathways play a role in the rate-determining step for myogenesis. It is conceivable that the Smad signaling pathway plays a role in the rate-limiting step for postnatal myogenesis in vivo. From this point of view, it is noteworthy that the Smad signaling pathway is actually down-regulated during BPV-induced muscle regeneration.

Community effect triggers terminal differentiation of postnatal myogenic cells

The present study suggests that muscle satellite cell-dependent myogenesis in postnatal mice depends on a "community effect," which means the expression of a differentiation potential when a certain cell density is exceeded [41], and also provides evidence that quenching the Smad signaling pathway in postnatal myogenic cells is required for the community effect. Skeletal muscle formation in amphibian embryos provides a paradigm of the community effect [42]. Dissociation of muscle progenitor cells reduces their differentiation, whereas the reaggregated cells differentiate [43]. In mouse embryos, muscle differentiation also depends on a community effect [44]. Previous studies on embryonic myogenesis and the present study on postnatal myogenesis both suggest that the developmental timing of a community effect is important as a critical switching mechanism between growth and differentiation of myogenic progenitor cells during embryonic and postnatal muscle growth, repair, and differentiation in mice (Fig. 8D).

Both signals from adjacent tissues and a community effect are necessary for the formation of skeletal muscle in embryos [45]. Fibroblast growth factors (FGFs) are candidates for community factors in *Xenopus* embryos [41]. During postnatal muscle growth and repair in mice and humans, muscle satellite cells and their descendant progenitor cells express and release a number of trophic factors that are candidates for community factors, including growth factors and cytokines such as BMPs, myostatin, FGFs, hepatocyte growth factor, insulin-like growth factors, interleukin-6, leukemia inhibitory factor, and tumor necrotic

factor α [21,46–52]. In embryos, BMP4 is released from the adjacent neural tube and lateral plate mesoderm, and inhibits MyoD and Myf5 gene expression [53,54]. However, the role of BMP signaling in a community effect during skeletal muscle formation in embryos remains to be determined. In contrast, the present study shows that a community effect quenches the Smad signaling pathway in postnatal myogenic cells. Thus, BMP antagonists are possible candidates for community factors. The list of BMP antagonists includes noggin, chordin, gremlin, follistatin, Cerberus, sclerostin, and their related and family proteins [55]. Careful description of the spatiotemporal expression patterns of these antagonists during myogenesis induced by high cell density may provide a hypothetical and mechanistic view of the community effect. However, the activity of the BMP–ALK–Smad axis could be also modulated by Notch signaling [56] or Src tyrosine kinase [57]. Therefore, we should observe whether a simple community factor story can explain a community effect on the terminal differentiation of postnatal myogenic cells.

The origin of ligands that stimulate the Smad signaling pathway in growing myogenic progenitor cells remains puzzling. We cannot exclude the possibility that the FBS in the medium contains an adequate amount of BMP to support the activation of Smad1/5/8, but putative BMP derived from FBS is unlikely to contribute to the activation of Smad1/5/8 in growing myogenic cells because serum reduction does not significantly affect the level of phosphorylated Smad1/5/8 except in higher density culture. In addition, the Smad signaling pathway is inactivated exclusively in the central region of a micromass, even in pmGM supplemented with 20% FBS. Therefore, BMP4 produced by myogenic progenitor cells themselves [21] is a possible candidate for ligands that stimulate their own Smad signaling pathway.

Community effect guarantees myogenic cell fusion following expression of muscle-specific genes

Skeletal muscle terminal differentiation of muscle satellite cells is composed of a highly ordered series of steps that includes activation of quiescent satellite cells, proliferation of descendent progenitor cells, expression of muscle-specific genes, and cell fusion to give rise to syncytia. Cell fusion is the last step of terminal muscle differentiation and is a multi-cellular event, whereas the other steps are uni-cellular responses. To differentiate into myotubes, a differentiating myogenic cell requires direct contact with its fusion partner cell. If a single myogenic progenitor cell is cultured without contact with other cells under the serum-reduced condition, it will undergo the myogenic differentiation process up to the expression of muscle-specific genes but be unable to form myotubes. Therefore, neighboring myogenic cells, including progenitor cells and myofibers, are required for terminal differentiation of myogenic progenitor cells. The community effect induces myogenic differentiation when a certain cell density is exceeded and guarantees myogenic cell contact relevant for syncytium formation. The community effect also provides a probable explanation of how non-synchronized and local myogenic differentiation is induced in culture when myogenic cells are distributed unevenly throughout a culture dish. In addition, high-level expression of noggin in myotubes (Hashimoto, unpublished observation) raises the possibility of differentiated cell-induced differentiation: terminally differentiated cells enhance the community effect and induce differentiation of neighboring undifferentiated myogenic cells (Fig. 8D).

Concluding remarks

We have shown a novel physiological role of the Smad signaling pathway in a switch between growth and differentiation of postnatal myogenic cells. Further studies identifying factors that quench the Smad signaling pathway will provide mechanistic insight into a community effect on postnatal myogenesis.

Supplementary materials related to this article can be found online at doi:10.1016/j.yexcr.2010.10.011.

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ORIGINAL ARTICLE

Plasma sex hormone levels and mortality in disabled older men and women

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Aim: To investigate the relationship between circulating sex hormone levels and subsequent mortality in disabled elderly.

Methods: This prospective observational study was comprised of 214 elderly subjects aged 70–96 years (117 men and 97 women; mean ± standard deviation age, 83 ± 7 years), receiving services at long-term care facilities in Nagano, Japan. All-cause mortality by baseline plasma sex hormone levels was measured.

Results: After excluding deaths during the first 6 months, 27 deaths in men and 28 deaths in women occurred during a mean follow up of 32 months and 45 months (up to 52 months), respectively. Mortality rates differed significantly between high and low testosterone tertiles in men, but did not differ significantly between middle and low tertiles. Compared with subjects in the middle and high tertiles, men with testosterone levels in the low tertile (<300 ng/dL) were more likely to die, independent of age, nutritional status, functional status and chronic disease (hazard ratio [HR] = 3.27, 95% confidence interval [CI] = 1.24–12.91). In contrast, the low dehydroepiandrosterone sulfate (DHEA-S) tertile was associated with higher mortality risk in women (multivariate adjusted HR = 4.42, 95% CI = 1.51–12.90). Exclusion of deaths during the first year and cancer deaths had minimal effects on these results. DHEA-S level in men and testosterone and estradiol levels in women were not related to mortality.

Conclusion: Low testosterone in men and low DHEA-S in women receiving care at facilities are associated with increased mortality risk, independent of other risk factors and pre-existing health conditions. **Geriatr Gerontol Int 2010; 10: ••–••.**

Keywords: dehydroepiandrosterone, disabled elderly, mortality risk, testosterone.

Introduction

Japan has the longest life expectancy at birth in the world for both men and women, although women live 8 years longer than men on average.[1,2] One explanation for this phenomenon is that estradiol production during the premenopausal years partially protects women from cardiovascular disease (CVD). In contrast, there has been a suspicion that testosterone itself is harmful; however, recent studies support the hypothesis that testosterone may be beneficial to survival in aging men.[3–8]

It is well established that endogenous androgens decline with advancing age in men.[9] Because testosterone has important physiological effects on muscle, bone, brain, erythropoietin and the vascular system, decreased testosterone levels could contribute to age-associated symptoms and diseases in older men, such as decreased muscle mass and strength,[10] impaired physical performance,[11,12] osteoporosis[13] and fractures,[12,14]

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depressed mood,[15] cognitive impairment,[16,17] anemia[18,19]and frailty.[20] In our previous study in which older persons receiving day-care services or admitted to a facility were investigated, higher plasma testosterone levels were associated with better activities of daily living (ADL), cognitive function and vitality in men.[21] On the other hand, several epidemiological studies have demonstrated that a decline in testosterone level was associated with mortality risk in community-dwelling middle-aged or older men.[3–8] In cause-specific analyses, some studies have shown that a low testosterone level was associated with an increased risk of death due to CVD.[4,5] However, the above-mentioned studies were performed in community samples of Caucasian men, and this issue remains to be clarified in frail or disabled older men.

The majority of dehydroepiandrosterone (DHEA), an endogenous steroid precursor to testosterone and estrogen, exists as the sulfated form (DHEA-S) in the circulation, and DHEA and DHEA-S are the most abundant adrenal sex steroid hormones, with concentrations reported to be more than 100-fold higher than those of testosterone and estradiol,[22] suggesting an important physiological role of DHEA(-S). Their circulating levels also peak in young adults and decline with age in both men and women. Although the role of androgens in older women's health is not fully understood, postmenopausal women with intact ovaries continue to produce androgens, DHEA and testosterone, while their production of estradiol is minimal.[23] In our previous study,[21] in older women, higher DHEA and DHEA-S levels were related to better ADL, while estradiol and testosterone levels showed no relations. Other reports have shown a correlation between DHEA level and cognitive function,[24] depression,[25] osteoporosis[26] and frailty in older women.[27] Several studies that examined the association between DHEA-S and mortality in women have shown mixed results,[28–32] and mostly found no relation; however, both low and high levels of DHEA-S at baseline[28] and some trajectory patterns such as a steep decline or extreme variability[32] have been reported to correlate with increased mortality.

These lines of evidence suggest that endogenous androgens, including testosterone and DHEA(-S), may play a role in physical and mental function as well as longevity in older individuals. We hypothesized that low plasma androgen levels could be a mortality risk factor even in elderly with disability who are receiving facility services.

Methods

Study population

In this longitudinal observational study, 218 consecutive persons aged 70 years or older (121 men aged 70–96 years and 97 women aged 70–95 years; mean ± standard deviation [SD] age, 83 ± 6 and 83 ± 5 years, respectively) who attended health service facilities for the elderly (facilities that provide nursing care and rehabilitation services to elderly people with disability, *Mahoroba-no-Sato*) located in Nagano Prefecture, Japan were enrolled. The participants were in a chronic stable condition and receiving services under Long-term Care Insurance, which is provided by the Japanese Government, either under admission or as day care. The principal exclusion criteria were malnutrition (serum albumin <3.5 mg/dL or body mass index [BMI] <16 kg/m^2), extremely low ADL status (Barthel Index[33] <50), malignancy, acute inflammation (fever, white blood cell count >10 000/μL, or other signs of infection within 4 weeks before enrollment), severe anemia (blood hemoglobin <10.0 g/dL) and overt endocrine disease because these conditions may affect both plasma sex hormone levels and mortality. Deaths that occurred during the first 6 months of follow up (four men and no women) were also excluded to minimize the influence of comorbidity on both sex hormone levels and mortality; therefore, the remaining 214 persons were analyzed in this study. The institutional review board of *Mahoroba-no-Sato* approved the study protocol, and all participants and/or their family members gave written informed consent.

Hormone measurements

Blood samples were obtained from the participants in the morning after an overnight fast, and plasma hormone levels in addition to blood cell counts and blood chemical parameters were determined by a commercial laboratory (Health Sciences Research Institute, Yokohama, Japan). Testosterone and estradiol were assayed using chemiluminescence immunoassays with minimum detection limits of 7 ng/dL (0.2 nmol/L) and 4 pg/mL (14.7 pmol/L), respectively. DHEA-S was assayed using a sensitive radioimmunoassay with a minimum detection limit of 2.0 μg/dL (0.05 μmol/L). The intra-assay coefficients of variation for these measurements were less than 5%.

Functional and anthropometric measurements

Trained nurses and physical therapists visited the participants at the health facilities and performed comprehensive geriatric assessments. Basic ADL was assessed by Barthel Index,[33] cognitive function by Hasegawa Dementia Scale – Revised (HDS-R, 30-point scale),[34] mood by the Geriatric Depression Scale (GDS, 15 items),[35] and ADL-related vitality by Vitality Index (10-point scale).[36] BMI was calculated

as weight in kilograms divided by the square of height in meters.

Comorbidity

Diseases were ascertained by experienced physicians according to pre-established criteria that combine information from self-reported physician diagnoses, medical records, current medication, clinical examinations and blood tests. Diseases included in the current analysis were hypertension, heart disease (including any of angina pectoris, myocardial infarction, congestive heart failure and arrhythmia), stroke, diabetes mellitus, osteoarthropathy (arthritis, rheumatism, osteoporosis and history of fractures), lung disease (including bronchial asthma and chronic obstructive pulmonary disease) and other chronic diseases (chronic kidney disease, gastrointestinal disease, Parkinson's disease and psychological disorders). We also obtained data on anti-androgenic treatment and intake of glucocorticoids, opiates and hormone supplements that could affect plasma hormone levels, but no subject was taking any of these.

Follow up

The subjects were followed up in 2002–2009, for a period of up to 52 months (mean ± SD, 32 ± 13 [34] months in men and 45 ± 11 [49] months in women). Time and causes of death of deceased persons were ascertained using medical records and death certificates. All deaths were registered with International Classification of Diseases, 10th version (ICD-10) codes,[37] based on the information from death certificates. We categorized deaths into the following four specific causes: (i) diseases of the circulatory system (I00–I99) including heart disease and cerebrovascular disease; (ii) diseases of the respiratory system (J00-J99); (iii) neoplasms (C00-D48); and (iv) other causes. Subjects who were alive were confirmed by checking appointment records of the facilities. Survival of 16 subjects whose records were not available was ascertained by the phone interview of each subject. Causes of death were determined for all the subjects without any missing cases.

Statistical analysis

Differences between testosterone tertiles in men and between DHEA-S tertiles in women were analyzed using ANOVA for continuous variables and χ^2-test for categorical variables. Survival was analyzed using Kaplan–Meier plots and log-rank tests. Hazard ratios (HR) for mortality were analyzed using Cox proportional hazards regression. Significance tests were two-sided, with an α-level of 0.05. Data were analyzed using SPSS statistical software.

Results

Characteristics of study subjects

Over the follow-up period, 27 men and 28 women died, yielding a mortality rate of 86.5/1000 person-years at risk in men; and 69.9/1000 person-years at risk in women. Of those, 13 deaths were due to diseases of the circulatory system (eight to ischemic and other heart disease and five to cerebrovascular disease), 10 to diseases of the respiratory system and four to cancer in men; while 14 deaths were due to diseases of the circulatory system (nine to ischemic and other forms of heart disease and four to cerebrovascular disease), eight to diseases of the respiratory system, five to cancer and two to other causes in women. Men who died were significantly older, had lower serum albumin and cholesterol, lower ADL and cognitive status, higher prevalence of heart disease, and lower testosterone level than survivors; whereas in women, subjects who died were older, had lower hemoglobin, higher prevalence of heart disease and lower plasma DHEA-S level than survivors (data not shown).

Table 1 shows the baseline characteristics of the male subjects by tertile of plasma testosterone. A significant difference was observed in serum albumin and hemoglobin levels, ADL and cognitive status among tertiles of testosterone in men. Table 2 shows the baseline characteristics of the female subjects by tertile of plasma DHEA-S. A significant difference was found in age and ADL status among DHEA-S tertiles in women, while other variables did not differ between the tertile groups.

Mortality and plasma sex hormone levels in men

As shown in Figure 1(a), Kaplan–Meier survival analysis by tertile of plasma testosterone level revealed that testosterone level was associated with mortality in men. After adjusting for age, Cox proportional hazards models showed that there was an inverse relation between testosterone level and mortality. Mortality rate differed significantly between the high and low testosterone tertiles, but not significantly between the middle and low tertiles: tertile 3 (high), reference; tertile 2 (middle), HR = 2.51 (95% confidence interval [CI] = 0.66–9.50); and tertile 1 (low), HR = 6.63 (95% CI = 1.92–23.21). Accordingly, we investigated the increased mortality in tertile 1 versus tertiles 2–3 (Table 3). Compared with subjects within tertiles 2–3,

Table 1 Association between potential confounding variables and testosterone tertiles in men

Characteristic	Testosterone tertiles T1 <10.4 nmol/L (<300 ng/dL), $n = 39$	T2 10.4–16.3 nmol/L (300–470 ng/dL), $n = 40$	T3 >16.3 nmol/L (>470 ng/dL), $n = 38$	*P*-value
Age, years	83 ± 7	83 ± 6	81 ± 6	0.11
Nutritional parameters				
Body mass index, kg/m^2	21.3 ± 3.4	22.8 ± 3.8	21.7 ± 3.0	0.21
Hemoglobin, g/dL	12.7 ± 1.9	13.8 ± 1.3	14.0 ± 1.7	<0.01
Albumin, g/dL	4.0 ± 0.3	4.1 ± 0.2	4.2 ± 0.3	<0.01
Total cholesterol, mg/dL	173 ± 38	195 ± 36	176 ± 28	0.05
Prevalent diseases, *n* (%)				
Hypertension	17 (44)	16 (40)	12 (32)	0.53
Heart disease	10 (26)	5 (13)	7 (18)	0.32
Stroke	12 (31)	15 (38)	8 (21)	0.34
Diabetes mellitus	8 (21)	5 (13)	8 (21)	0.31
Osteoarthropathy	8 (21)	9 (23)	7 (18)	0.94
Lung disease	2 (5)	3 (8)	3 (8)	0.52
Other chronic diseases	17 (44)	19 (48)	18 (47)	0.95
Functional parameters				
Barthel Index	79 ± 12	82 ± 11	87 ± 13	0.04
HDS-R	18 ± 7	19 ± 6	22 ± 5	0.02
Vitality Index	9.2 ± 1.1	9.3 ± 0.9	9.5 ± 0.9	0.46
GDS	5.0 ± 3.1	5.6 ± 3.7	5.6 ± 2.9	0.66
Sex hormone levels				
Testosterone, nmol/L	7.6 ± 2.5	13.3 ± 1.6	20.9 ± 3.9	<0.01
(ng/dL)	(219 ± 73)	(382 ± 43)	(602 ± 112)	
DHEA-S, μmol/L	1.7 ± 1.1	1.8 ± 1.6	1.7 ± 1.2	0.94
(μg/dL)	(64 ± 42)	(69 ± 57)	(63 ± 45)	

Values are shown as mean (standard deviation). Differences between the groups were analyzed using ANOVA for continuous variables and χ^2-test for categorical variables. DHEA-S, dehydroepiandrosterone sulfate; GDS, Geriatric Depression Scale; HDS-R, Hasegawa Dementia Scale – Revised.

a testosterone level within tertile 1 was associated with approximately fourfold higher mortality risk. Adjustment for age, nutritional parameters (BMI, albumin, hemoglobin, total cholesterol) and functional parameters (Barthel Index, HDS-R, Vitality Index, GDS), and prevalent diseases showed no major influence on the result. In order to examine how follow-up time and cancer impacted on the results, assuming that the subjects may have had subclinical cancer or a fatal illness at baseline, we performed further analyses excluding deaths that occurred in the first 12 months ($n = 9$) and deaths from cancer ($n = 4$). However, the significant associations remained after these exclusions (Table 3). On the other hand, DHEA-S level was not associated with mortality when DHEA-S was entered as tertiles (data not shown).

Although the statistical power was not strong enough, we studied the risk for cause-specific mortality by tertiles of testosterone level in men. Neither deaths from diseases of the circulatory system nor those from non-circulatory causes showed a significant association with testosterone tertiles (tertile 1 vs tertile 2–3, HR = 3.18, 95% CI = 1.87–11.6, $P = 0.17$; HR = 3.46, 95% CI = 0.29–7.29, $P = 0.64$, respectively).

Mortality and plasma sex hormone levels in women

As shown in Figure 1(b), a low DHEA-S level was associated with higher mortality by Kaplan–Meier survival analysis. Age-adjusted Cox proportional hazards models revealed that the association was not significant when each tertile of DHEA-S was entered as a continuous variable; however, a significant association was observed when tertile 1 was compared with tertiles 2–3 (Table 3). The association remained significant after excluding deaths that occurred in the first 12 months ($n = 2$) and deaths from cancer ($n = 5$). Moreover, further adjustment had no major influence on the result. In women, testosterone and estradiol levels were not associated with mortality when they were entered as tertiles (data not shown).

In cause-specific mortality analysis, compared with tertiles 2–3, the low tertile of DHEA-S level was associated with higher risk of death from diseases of the

Table 2 Association between potential confounding variables and DHEA-S tertiles in women

Characteristic	DHEA-S tertiles T1 <1.17 μmol/L (<43 μg/dL), $n = 33$	T2 1.17–1.49 μmol/L (43–55 μg/dL), $n = 32$	T3 >1.49 μnmol/L (>55 μg/dL), $n = 32$	*P*-value
Age, years	83 ± 6	82 ± 6	80 ± 6	0.08
Nutritional parameters				
Body mass index, kg/m^2	22.3 ± 2.7	22.5 ± 3.2	23.7 ± 2.7	0.31
Hemoglobin, g/dL	12.6 ± 1.4	12.6 ± 1.2	13.1 ± 1.1	0.16
Albumin, g/dL	4.1 ± 0.3	4.2 ± 0.3	4.3 ± 0.2	0.18
Total cholesterol, mg/dL	205 ± 30	204 ± 35	205 ± 35	0.99
Prevalent diseases, *n* (%)				
Hypertension	10 (30)	14 (44)	15 (47)	0.47
Heart disease	4 (12)	7 (22)	8 (25)	0.46
Stroke	5 (15)	4 (13)	6 (19)	0.79
Diabetes mellitus	5 (15)	4 (13)	5 (16)	0.90
Osteoarthropathy	8 (24)	11 (34)	13 (40)	0.47
Lung disease	3 (9)	2 (6)	2 (6)	0.56
Other chronic diseases	17 (52)	19 (59)	18 (56)	0.90
Functional parameters				
Barthel Index	90 ± 7	93 ± 8	95 ± 8	0.04
HDS-R	23 ± 6	22 ± 7	25 ± 5	0.39
Vitality Index	9.2 ± 1.4	9.1 ± 2.2	8.8 ± 2.9	0.35
GDS	6.8 ± 2.6	5.9 ± 3.4	6.9 ± 3.3	0.16
Sex hormone levels				
DHEA-S, μmol/L	0.8 ± 0.2	1.3 ± 0.1	2.0 ± 0.3	<0.01
(μg/dL)	30 ± 7	49 ± 4	73 ± 12	
Testosterone, nmol/L	1.2 ± 0.6	1.2 ± 0.6	1.3 ± 0.5	0.81
(ng/dL)	35 ± 17	36 ± 17	37 ± 13	
Estradiol, pmol/L	56 ± 32	57 ± 37	67 ± 46	0.41
(pg/mL)	15.3 ± 8.6	15.5 ± 10.2	18.3 ± 12.5	

Values are shown as mean (standard deviation). Differences between the groups were analyzed using ANOVA for continuous variables and χ^2-test for categorical variables. DHEA-S, dehydroepiandrosterone sulfate; GDS, Geriatric Depression Scale; HDS-R, Hasegawa Dementia Scale – Revised.

circulatory system (HR = 13.1, 95% CI = 2.39–72.3, $P < 0.01$), while there was no association with deaths from non-circulatory causes (HR = 0.93, 95% CI = 0.86–1.02, $P = 0.14$).

Discussion

In this small prospective study of Japanese elderly who were receiving care in facilities, a low testosterone level was associated with mortality in men independent of multiple risk factors and pre-existing health conditions. In addition, a low DHEA-S level in older women was related to increased mortality. In contrast, DHEA-S level in men and testosterone and estradiol levels in women were not related to mortality.

Recent prospective cohort studies in Western countries have yielded inconsistent findings about the use of a low total testosterone level as a predictor of all-cause and cardiovascular mortality in middle-aged to older men.[4,5,38,39] In the two studies that found no significant prediction of mortality,[38,39] the populations were younger (mean or median ages were in the early 50s), testosterone levels were higher and mortality rates were lower (11.6 and 15.4/1000 person-years, respectively) compared to those in studies that found positive results. In the present study, although the sample size was small, the subjects were frail and older than those in any previously reported studies, with a relatively small age range and higher mortality rate. Therefore, the relation between testosterone level and mortality might have been easier to detect in our study than in other studies with healthy middle-aged and older men.

There could be several mechanisms by which endogenous testosterone affects mortality in men. Although the number of subjects was too small to perform cause-specific analysis in the present study, other studies have reported that a low testosterone level predicted increased risk of death due to CVD.[4,5] Further, in addition to the relation to muscle strength, physical performance and ADL,[10–12,21] some but not all reports have

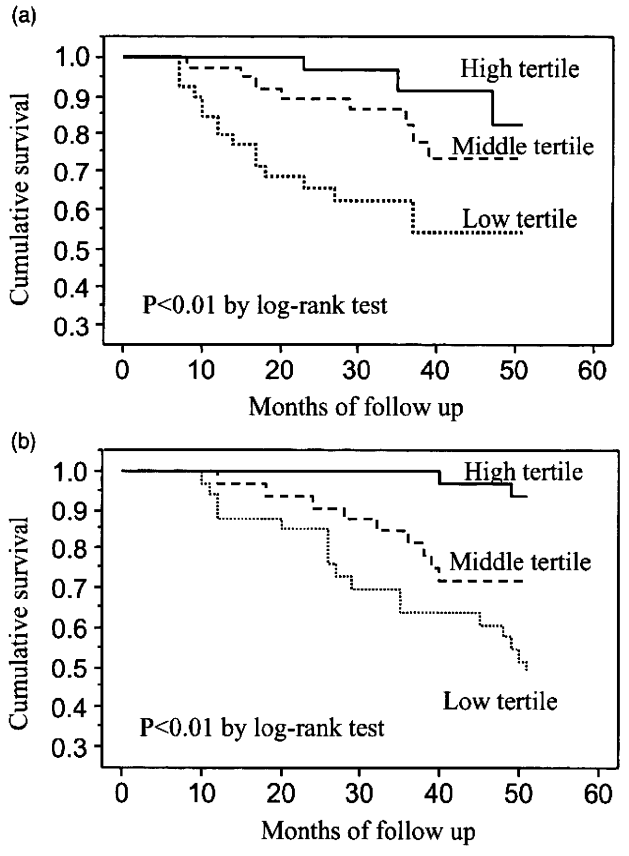

Figure 1 (a) Survival curves by tertile group of plasma testosterone level in men. (b) Survival curves by tertile group of plasma dehydroepiandrosterone sulfate level in women.

demonstrated an association between low testosterone level in older men and risk of a fall or fracture and frailty.[12–14,20] It is noteworthy that in the 10 men who died of respiratory infection, four had a history of a fall and fracture, which resulted in worse disability. Accordingly, a low testosterone level may contribute to frailty, which influences men's susceptibility to illness and falls and the capability to recover from disease or fractures, and thereby affects mortality.

Other than aging, systemic illness can result in decreased testosterone levels; therefore, low testosterone levels in older men could be attributable to acute and chronic diseases,[40] and the possible reverse causality should be considered. To evaluate this possibility, we excluded the first 12 months of observation and still found that in 12–52 months of observation, men in the low testosterone tertile had a greater risk of mortality from all causes than those in higher tertiles. We carefully excluded subjects with critical diseases and conditions at baseline, although our subjects were old with multiple chronic diseases, and it is difficult to exclude the possibility that men with subclinical critical conditions might have been included. Moreover, at baseline, there was a significant difference in functional status (ADL and cognition) and nutritional parameters (serum albumin and hemoglobin levels) between testosterone tertiles, as reported previously;[21] thus, our results need to be confirmed in a cohort with no difference in these factors between testosterone groups to exclude the influence of these biases on mortality. Also, it needs to be explored whether low testosterone in older men plays a pathogenic role, such as affecting the immune system, developing physical frailty and depression, or simply serves as a marker for biological vulnerability and poor prognosis. Long-term studies also need to test whether testosterone treatment should yield clinically significant improvements in mortality in appropriately selected older men, with consistent symptoms and signs and unequivocally low serum testosterone levels.

Low DHEA-S has been associated with increased all-cause and cardiovascular mortality in older men;[26,27,41] however, no association was found in the present study. Because DHEA(-S) is an inactive prohormone and we and others have found an association between testosterone and mortality,[3–8] it is suggested that testosterone could be a stronger predictor of mortality in older men.

On the other hand, a low DHEA-S level in older women was associated with a poor prognosis after adjusting for multiple factors related to mortality. Other previous reports showed an inconsistent relationship between DHEA-S level and mortality in older women,[29–31] possibly due to differences in the cohorts including age, DHEA-S level, heterogeneity of health status and mortality rate, and the method of statistical analysis used to demonstrate the relationship, regression models with linear/non-linear assumption.

Previous studies support a potential physiological role of DHEA-S, which could contribute to reduced mortality, an anti-inflammatory action and immune regulatory activity.[42] However, there are still many unanswered questions regarding DHEA's role in aging, and there is insufficient evidence to support DHEA replacement for increasing longevity in older women. It also needs to be explored whether the DHEA-S level contributes to mortality or is merely a biomarker of the underlying health condition of older women.

Our study has some limitations. First, the sample size was too small to reach a clear conclusion with strong statistical power, thus limiting the precision of the estimates, which is reflected in the broad range of HR for mortality. Second, the results are based on single measurements of sex hormones, which do not allow assessment of changes in levels over time; therefore, they may overestimate or underestimate the association between hormone levels and mortality. Third, we did not measure estradiol levels in men, although it would have been helpful to see whether the effects of testosterone on mortality are mediated by testosterone itself or by aromatization to estradiol in older men. Finally, active forms of testosterone such as bioavailable and

Table 3 Hazard ratios for low tertile 1 vs tertiles 2–3 of plasma sex hormone levels for all-cause mortality in men and women

	Unadjusted	Model 1	Model 2
Men ($n = 117$)			
HR of low testosterone for mortality	3.83 (1.74–8.40)**	3.71 (1.54–8.04)**	3.27 (1.24–12.91)*
Excluding first-year deaths ($n = 108$)	3.81 (1.53–6.93)**	3.49 (1.14–7.39)**	3.08 (1.11–13.62)*
Excluding deaths from cancer ($n = 113$)	4.18 (1.77–9.86)**	4.03 (1.70–9.58)**	5.02 (1.51–15.41)*
Women (N = 97)			
HR of low DHEA-S for mortality	3.77 (1.77–8.07)**	3.86 (1.79–8.32)**	4.42 (1.51–12.90)*
Excluding first-year deaths ($n = 95$)	3.38 (1.55–7.37)**	3.43 (1.56–9.54)**	3.58 (1.12–11.46)*
Excluding deaths from cancer ($n = 92$)	3.82 (1.69–8.60)**	3.55 (1.54–8.19)**	3.92 (1.28–11.98)*

*$P < 0.05$; **$P < 0.01$ vs reference group (tertile 2–3). Values are expressed as HR (95% CI). Model 1, adjusted for age; Model 2, adjusted for age, nutritional parameters, functional parameters and prevalent disease. DHEA-S, dehydroepiandrosterone sulfate; HR, hazards ratio.

calculated free testosterone were not measured, because a direct assay of bioavailable testosterone or an assay of sex hormone binding globulin, which is necessary for free testosterone calculation, is not available in Japan. However, because most of the above-mentioned previous reports have shown an association of total testosterone with mortality, the fundamental findings might not have differed if active forms of testosterone had been analyzed.

In conclusion, a low testosterone level in men and a low DHEA-S level in women are associated with increased mortality risk, independent of multiple risk factors and several pre-existing health conditions in disabled elderly. To our knowledge, the present study is the first that showed testosterone as a predictor of mortality in Asian men. Also, this is the first study that investigated frail or disabled older persons receiving care at facilities. Our results imply the clinical importance of measuring plasma androgen levels even in disabled elderly to estimate their prognosis.

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