EXPERIMENTAL CELL RESEARCH 317 (2011) 221–233

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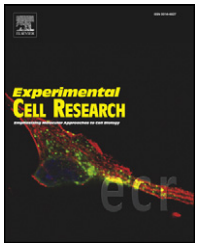

Research Article

Community effect triggers terminal differentiation of myogenic cells derived from muscle satellite cells by quenching Smad signaling

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ARTICLE INFORMATION

Article Chronology:
Received 18 April 2010
Revised version received
16 September 2010
Accepted 13 October 2010
Available online 20 October 2010

Keywords:
Community effect
BMP
Smad
ALK
Myogenesis
Osteogenesis

ABSTRACT

A high concentration of bone morphogenetic proteins (BMPs) stimulates myogenic progenitor cells to undergo heterotopic osteogenic differentiation. However, the physiological role of the Smad signaling pathway during terminal muscle differentiation has not been resolved. We report here that Smad1/5/8 was phosphorylated and activated in undifferentiated growing mouse myogenic progenitor Ric10 cells without exposure to any exogenous BMPs. The amount of phosphorylated Smad1/5/8 was severely reduced during precocious myogenic differentiation under the high cell density culture condition even in growth medium supplemented with a high concentration of serum. Inhibition of the Smad signaling pathway by dorsomorphin, an inhibitor of Smad activation, or noggin, a specific antagonist of BMP, induced precocious terminal differentiation of myogenic progenitor cells in a cell density-dependent fashion even in growth medium. In addition, Smad1/5/8 was transiently activated in proliferating myogenic progenitor cells during muscle regeneration in rats. The present results indicate that the Smad signaling pathway is involved in a critical switch between growth and differentiation of myogenic progenitor cells both in vitro and in vivo. Furthermore, precocious cell density-dependent myogenic differentiation suggests that a community effect triggers the terminal muscle differentiation of myogenic cells by quenching the Smad signaling.

Introduction

Signaling molecules have both permissive and repressive effects on gene expression in the myotomes during embryonic myogenesis. Skeletal muscle formation in the paraxial mesoderm is controlled by a number of signaling molecules emanating from neighboring tissues. Sonic hedgehog (Shh) and Wnt promote myogenesis [1]. In contrast, bone morphogenetic protein 4 (BMP4) inhibits premature differen-

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Abbreviations: BMP, bone morphogenetic protein; ALK, activin-like kinase; MyHC, myosin heavy chain; BPV, bupivacaine hydrochloride
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doi:10.1016/j.yexcr.2010.10.011

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