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REGULAR ARTICLE

Bidirectional induction toward paraxial mesodermal derivatives from mouse ES cells in chemically defined medium

Hidetoshi Sakurai [a,b,1], Yuta Inami [a], Yukie Tamamura [c], Toru Yoshikai [a], Atsuko Sehara-Fujisawa [d], Ken-Ichi Isobe [a,*]

[a] *Department of Immunology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8560, Japan*
[b] *Center for iPS Cell Research and Application, Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, 69 Konoe-cho, Yoshida, Sakyo-ku, Kyoto, 606-8501, Japan*
[c] *Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8560, Japan*
[d] *Department of Growth Regulation, Institute for Frontier Medical Sciences, Kyoto University, 53 Syogoin-kawaramachi, Sakyo-ku, Kyoto, 606-8507, Japan*

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Abstract Embryonic stem cells (ESCs) are a renewable cell source of tissue for regenerative therapies. The addition of bone morphogenetic protein 4 (BMP4) to serum-free ESC cultures can induce primitive streak-like mesodermal cells. In differentiated mouse ESCs, platelet-derived growth factor receptor-α (PDGFR-α) and E-cadherin (ECD) are useful markers to distinguish between paraxial mesodermal progenitor cells and undifferentiated and endodermal cells, respectively. Here, we demonstrate methods for BMP4-mediated induction of paraxial mesodermal progenitors using PDGFR-α and ECD as markers for purification and characterization. Serum-free monolayers of ESCs cultured with BMP4 could efficiently promote paraxial mesodermal differentiation akin to embryonic mesodermal development. BMP4 treatment alone induced paraxial mesodermal progenitors that could differentiate into osteochondrogenic cells *in vitro* and *in vivo*. Furthermore, early removal of BMP4 followed by lithium chloride (LiCl) promoted the differentiation to myogenic progenitor cells. These myogenic progenitors were able to differentiate further *in vitro* into mature skeletal muscle cells. Thus, we successfully induced the efficient bidirectional differentiation of mouse ESCs toward osteochondrogenic and myogenic cell types using chemically defined conditions.

Abbreviations: ESCs, embryonic stem cells; BMP4, bone morphogenetic protein 4; PDGFR-α, platelet-derived growth factor receptor-α; ECD, E-cadherin; PMPs, paraxial mesodermal progenitor cells; MPI, myogenic progenitor induction.

* Corresponding author. Fax: +81 52 744 2972.
E-mail address: kisobe@med.nagoya-u.ac.jp (K.-I. Isobe).
[1] Present address: Department of Growth Regulation, Institute for Frontier Medical Sciences, Kyoto University, 53 Syogoin-kawaramachi, Sakyo-ku, Kyoto, 606-8507, Japan.

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Introduction

Embryonic stem cells (ESCs), with their abundant potential of self-renewal and differentiation into all cell lineages, have been investigated both as an experimental tool for developmental biology and as a new source of cell-based therapy. Differentiation of ESCs under chemically defined conditions (i.e., by adding or removing specific factors) enables analysis of the molecular processes of early developmental events (Nishikawa et al., 2007) and is desirable for clinical applications because it eliminates the risk of viral or prion infection from biomaterials.

Specification of the mesoderm is established following gastrulation by a process initiated in the posterior region of the embryo resulting in the formation of the primitive streak. The primitive streak contains mesodermal cells that migrate laterally and diverge into three mesodermal lineages which are defined by their distances from the axis: paraxial, intermediate, and lateral. At approximately 6.5 days of mouse development, a gradient of bone morphogenetic protein (BMP) signaling is established along the proximal-distal axis of the embryo with restricted BMP4 expression in the proximal region and BMP antagonist expression in the distal region (McMahon et al., 1998); (Bachiller et al., 2000). Targeted disruption of BMP4 revealed that BMP4 plays a critical role in primitive streak formation (Winnier et al., 1995). The administration of recombinant BMP4 to mouse ESC culture induces expression of primitive streak and primitive streak-derived mesoderm-specific genes such as the T-box transcriptional factor Brachyury (T), Mixl1, Tbx6, and VEGFR2 (Johansson and Wiles, 1995; Ng et al., 2005; Era et al., 2008), and inhibits neuronal development (Kawasaki et al., 2000). Many lateral mesodermal derivatives have been induced by BMP4 stimulation including hematopoietic cells (Pick et al., 2007), endothelial cells (Park et al., 2004), cardiomyocytes (Honda et al., 2006), and intermediate mesodermal derivatives such as renal progenitors (Bruce et al., 2007). However, differentiation of paraxial mesodermal derivatives from ESCs in serum-free culture has remained elusive.

Previously, we demonstrated that platelet-derived growth factor receptor-α (PDGFR-α) expression was a marker for the paraxial mesodermal progenitors in differentiated mouse ESCs (Sakurai et al., 2006). The expression of PDGFR-α is found in paraxial mesoderm, somites, neural tube, and spinal chord during mouse embryogenesis (Kataoka et al., 1997; Schatteman et al., 1992). *In vitro* fate analyses of ESC-derived PDGFR-α^+ cells demonstrated their potential to differentiate into osteocytes, chondrocytes, and skeletal muscle cells, which are derivatives of somites (Sakurai et al., 2006; Nakayama et al., 2003). We have also demonstrated that PDGFR-α^+ cells can be subdivided into PDGFR-α^+/VEGFR2$^+$ and PDGFR-α^+/VEGFR2$^-$ cells (Sakurai et al., 2006). The PDGFR-α^+/VEGFR2$^-$ cells mainly consist of paraxial mesodermal progenitors, while the PDGFR-α^+/VEGFR2$^+$ cells have the bipotential to differentiate into both paraxial and lateral mesodermal progenitors. However, in paraxial mesoderm induction, the PDGFR-α^+/VEGFR2$^+$ cells can differentiate into paraxial mesodermal derivatives, as well as PDGFR-α^+/VEGFR2$^-$ cells. Indeed, in the previous study, we demonstrated that PDGFR-α^+ cells could differentiate into muscle satellite cells but not into vascular endothelial cells in intramuscular transplantation (Sakurai et al., 2008). Therefore, we handled the PDGFR-α^+/VEGFR-2$^-$ and the PDGFR-α^+/VEGFR-2$^+$ population as a single population of paraxial mesodermal progenitors. However, the percentage of PDGFR-α^+ cells in conventional serum-containing differentiation media is limited to no more than 40% of the differentiated cells (Sakurai et al., 2006).

Here, we report the development of serum-free culture systems supplemented with BMP4 or LiCl to efficiently differentiate osteochondrogenic and myogenic paraxial mesodermal progenitors. Our system was established without the use of gene manipulation to isolate/enrich the paraxial mesodermal progenitors. Instead we used the paraxial mesodermal marker PDGFR-α and the undifferentiated cell marker E-cadherin (ECD) for sorting and characterizing cell subpopulations. Our findings should facilitate the generation of bone, cartilage, and skeletal muscle from human pluripotent stem cells for future clinical applications.

Results

Paraxial mesodermal differentiation of ESCs in BMP4-supplemented serum-free culture

BMP4 promoted the expression of PDGFR-α in a dose-dependent manner during *in vitro* ESC differentiation (Fig. 1a). When ESCs were differentiated without BMP4 for 6 days, only 3% of the cells expressed PDGFR-α. Inclusion of low doses of BMP4 (0.1 or 0.3 ng/ml) had little impact on differentiation. However, moderate doses of BMP4 (1 to 10 ng/ml) induced up to half of the cells to express PDGFR-α (Fig. 1a). We used BMP4 for differentiation to mesoderm at 1 ng/ml in the following studies. Cell density inversely affected the expression of the PDGFR-α, with greater frequency of expression of PDGFR-α occurring below 2×10^5 cells per 10-cm dish (Fig. 1b). However, the extent of ESC proliferation over a 6-day period was the greatest when starting at 2×10^5 cells per 10-cm dish, increasing about 15-fold during differentiation (Fig. 1c). Total PDGFR-α^+ cell number was also the highest at the starting cell density of 2×10^5 per 10-cm dish, increasing about 5-fold to input cell number (Fig. 1c). In the higher starting cell density, viable harvested cell number was reduced, indicating that the proliferation of ESCs was inhibited in the higher cell density (Fig. 1c).

Based on these results, we plated the ESCs at a density of 2×10^5 cells per 10-cm dish in the following studies. PDGFR-α^+ cells emerged after 4 days of differentiation reaching a peak on Day 5, with almost half of the cells becoming PDGFR-α^+ (Fig. 1d, upper row). The morphology of the cell aggregates changed from ESC-like round colonies to cobblestone monolayers (Fig. 1d, lower row). Total PDGFR-α^+ cell number was increasing in a time-dependent manner for 7 days of differentiation and we obtained about 11-fold increase of PDGFR-α^+ cells over the number of input ESCs (Fig. 1e). Serum-free culture was superior to serum-containing culture on the production of PDGFR-α^+ cells after 5 days differentiation (Fig. S1b). However, differentiated ESCs began to die after 7 days in culture and the number of PDGFR-α^+ cells was greatly reduced (data not shown). Time-course gene expression profiling by RT-PCR revealed that the genes