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Specific Serum-free Conditions can Differentiate Mouse Embryonic Stem Cells into Osteochondrogenic and Myogenic Progenitors.

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1. Introduction

Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells have great potentials for cell-based therapies based on their abundant potentials of self renewal and differentiation into all cell lineages (1,2). A serum-free ES cell differentiation system has an advantage for clinical applications because it can efficiently induce a specific cell lineage, and can avoid the risk of viral or prion infection by biomaterials. This study was initiated to examine how to efficiently induce paraxial mesodermal progenitor cells from ES cells in serum-free cultures. BMP4 acted as a key factor to promote the primitive streak-type mesoderm in both mouse development and ES cell differentiation in culture. Many lateral mesodermal derivatives such as hematopoietic cells, endothelial cells and cardiomyocytes, and intermediate mesoderm derivatives such as renal progenitors have been induced by BMP4 stimulation. However, differentiation of paraxial mesodermal cells from ES cells in serum-free culture has remained elusive. In this study, we developed a simple culture system with BMP4 and lithium chloride (LiCl) in serum-free conditions to promote two types of paraxial mesodermal progenitors, osteochondrogenic progenitors, and myogenic progenitors, which were identified using the paraxial mesodermal marker PDGFR-α.

2. Materials and methods

2.1 Cell culture and in vitro ES cell differentiation

CCE ES cells and ES cells expressing the *LacZ* gene (CCE/nLacZ) were kindly gifted by Dr. Nishikawa.

For serum-free culture of ES differentiation, type IV collagen (Nitta Gelatin), serum-free culture medium, SF-O3 (Sanko Junyaku), 0.2% Bovine serum albumin (BSA), 2-mercaptoethanol, and recombinant human BMP4 (R&D systems) were used. For myogenic mesodermal progenitor cell differentiation, initial induction by BMP4 was the same as osteo-chondrogenic progenitors. Three days after BMP4 treatment, the medium was changed entirely to SF-O3 (Sanko Junyaku) supplemented with 2.5mM LiCl, and cells were cultured in the medium for four days. For further myocyte induction, the cells which were sorted as

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described below were re-cultured on a collagen type I-coated 24-well dish (Iwaki) in SF-O3 with 2ng/ml IGF-1 (R&D systems), 10ng/ml HGF (R&D systems) and 2ng/ml bFGF (R&D systems). Three days after re-culture, the medium was changed entirely to SF-O3 with 2ng/ml IGF-1. Four days after the medium was changed, the medium was changed again to SF-O3 with 2ng/ml IGF-1 and 10ng/ml HGF, and the cells were cultured for seven days.

2.2 Antibodies, cell staining, FACS analyses and cell sorting

Rat monoclonal antibodies (MoAbs), APA5 (anti-PDGFR-α), AVAS12(anti-VEGFR2) and ECCD2 (anti-E-cadherin) were kindly gifted by Dr. Nishikawa. Phycoerythrin-conjugated streptavidin (BD Pharmingen) was used to detect the biotinylated-APA5 antibody. ECCD2 and AVAS12 were directly conjugated by a standard method using allophycocyanin (APC).
Cultured cells were harvested and collected in 0.05% trypsin-EDTA (GIBCO). Single-cell suspensions were stained as previously described (3) and analyzed or sorted by FACSCalibur or FACSVantage-HG (Becton Dickinson).

2.3 Transplantation of ESC-derived mesodermal progenitors into immunodeficient mice

We carried out mouse experiments according to protocols approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine. The PDGFR-α^+ and PDGFR-α^-ECD$^+$ populations were purified and collected by FACS (> 5×10^5 cells). Cells were resuspended at a density of 2.5×10^4 cells/μl in αMEM. For intra-muscular transplantation, a quadriceps femoris muscle of a KSN nude mouse was injured by direct cramping with a diethyl ether anesthesia. Twenty micro liters of collected cell suspension were directly injected into the injured quadriceps of each mouse. For intra-bone marrow transplantation, a hole in the tibial bone was bored through to the bone marrow at the knee joint using a 21G needle with the anesthesia diethyl ether, and twenty micro liters of collected cell suspension were directly injected into bone marrow.

3. Results

3.1 In vivo muscle regeneration by paraxial mesodermal progenitors derived from murine ES cells.

First, we simply seeded ES cells onto a 10-cm dish coated with type IV collagen in αMEM supplemented with 10% fetal calf serum. Four days later, the cells were harvested and stained with anti-PDGFR-α and anti-VEGFR-2 antibodies. PDGFR-α + cells were sorted by FACS. Cell suspensions were directly injected into the injured quadriceps femoris muscle of a KSN nude mouse. The majority of PDGFR-α mesodermal progenitors were located in the interstitial zone of muscles, especially in the area adjacent to the myofibers (Fig. 1).
Since the ES cells have a *LacZ* marker, we stained with Pax7 and CD34 antibodies, and found LacZ positive cells have these satellite cell markers. Satellite cells were isolated from the KSN nude mouse that was injected with the PDGFR-α^+ cells in the quadriceps femoris muscle. Many LacZ$^+$ cells were observed in the culture, and some of them exhibited fiber formation like other host satellite cell-derived myofibers (Fig. 1) (3).

3.2 Serum-free culture to induce mesodermal progenitors

We attempted to differentiate ES cells into mesodermal lineage cells in serum-free medium. For paraxial mesoderm differentiation, 2×10^5 ES cells were plated on a 10-cm dish coated

with type IV collagen, and were differentiated in a serum-free culture medium, which was comprised of SF-O3 supplemented with BSA, 0.1mM 2-mercaptoethanol, and 1 ng/ml BMP4. PDGFR-α+ cells emerged after four days of differentiation, and reached a peak on day five, with almost half of the cells becoming PDGFR-α+. The morphology of the cell aggregates changed from ES cell-like round colonies to cobblestone monolayers. The expression of T, Msgn, Tbx6 and Pax3, which play an important role in mesodermal development, was detected. We conclude that the addition of BMP4 to SF-O3 medium permits efficient induction of paraxial mesodermal progenitor cells from mouse ES cells.

3.3 BMP4-induced paraxial mesodermal progenitor cells have osteogenic and chondrogenic potentials *in vivo.*

We investigated the *in vivo* tissue differentiation potentials of paraxial mesodermal progenitor cells. At 4 to 6 days of LacZ-positive ES cell culture in serum-free medium with BMP, cells were stained with anti-PDGFR-α and anti-ECD antibodies. The PDGFR-α+ and PDGFR-α-ECD+ populations were sorted by FACS and were directly injected into bone marrow of the tibia of a KSN nude mouse. Twenty-eight days later, β-galactosidase staining was performed to detect engrafted cells. LacZ positive cells, which have light blue nuclear staining, were observed in the trabecular bone. ES cell-derived PDGFR-α+ cells could differentiate to osteocytes *in vivo*. These PDGFR-α+ and PDGFR-α-ECD+ populations were also directly injected into injured quadriceps femoris muscle. The engrafted tissues were then analyzed four weeks after transplantation. Although we could not detect muscle cells derived from Lac Z ES cells, we were surprised to detect ectopic cartilage in engrafted skeletal muscle. The ectopic cartilage was derived from engrafted cells as confirmed by the expression of LacZ by fluorescent immunohistochemistry (Fig. 2).

3.4 Serum-free induction of myogenic progenitor cells by ES

In order to differentiate ES cells to myogenic progenitor cells, we exposed cells to BMP for the first three days and then replaced it with LiCl for four days. The PDGFR-α+ population strongly expressed the dermomyotome markers Pax3 and Pax7, and the myogenic regulatory genes Myf-5 and Myo-D. Next, we asked whether this procedure could induce PDGFR-α+ cells to form mature myofibers in vitro. In mouse skeletal muscle regeneration, many growth factors such as insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), or basic fibroblast growth factor (bFGF) activate proliferation of Myf5+/MyoD+ myoblasts. Although IGF-1 could promote myogenin expression in this culture independently, adding both HGF and bFGF for three days enhanced myogenin expression and stimulated MRF4 expression. Myogenin expression was confirmed by immunohistochemistry. Some spindle-shape cells with mono- or multi-nuclear myogenin staining were observed. Further treatment with IGF-1 and HGF promoted mature skeletal muscle cells which expressed skeletal muscle actin (Fig.3).

4. Discussion

4.1 In vivo regeneration of muscle, cartilage and bone by ES-derived paraxial mesoderm cells

Here we described ES cell differentiation into paraxial mesoderm and paraxial mesoderm derived- tissues in vivo. First, we succeeded in regenerating muscle by direct injection of differentiated ES cells into injured muscle. In this case, we injected PDGFRα + cells, which

were differentiated from ES cells by two-dimensional culture on Type IV collagen with FCS (Fig. 1). These simple methods produced progenitors of muscle satellite cells. Because muscle satellite cells are tissue stem cells, the transplanted cells may continuously replicate in transplanted tissues. Next, we tried to differentiate ES cells to muscle cells using serum-free medium. Because BMP has been shown to be essential to mesoderm formation (4), we cultured ES cells in serum-free medium with BMP4, and transplanted PDGFRα + cells into damaged muscle. We expected to get myogenic progenitor cells by this method. However, we detected ectopic cartilage tissues in transplanted, injured muscle. These results indicate that BMP4 induces progenitors of cartilage cells. However, further experimentation revealed that BMP-4-induced progenitors also differentiated into osteogenic cells when sorted PDGFRα cells were injected into the bone marrow cavity (Fig. 2). These results indicate that, *in vivo,* BMP4-induced mesodermal progenitor cells differentiate into different cell types, depending on the surrounding tissue.

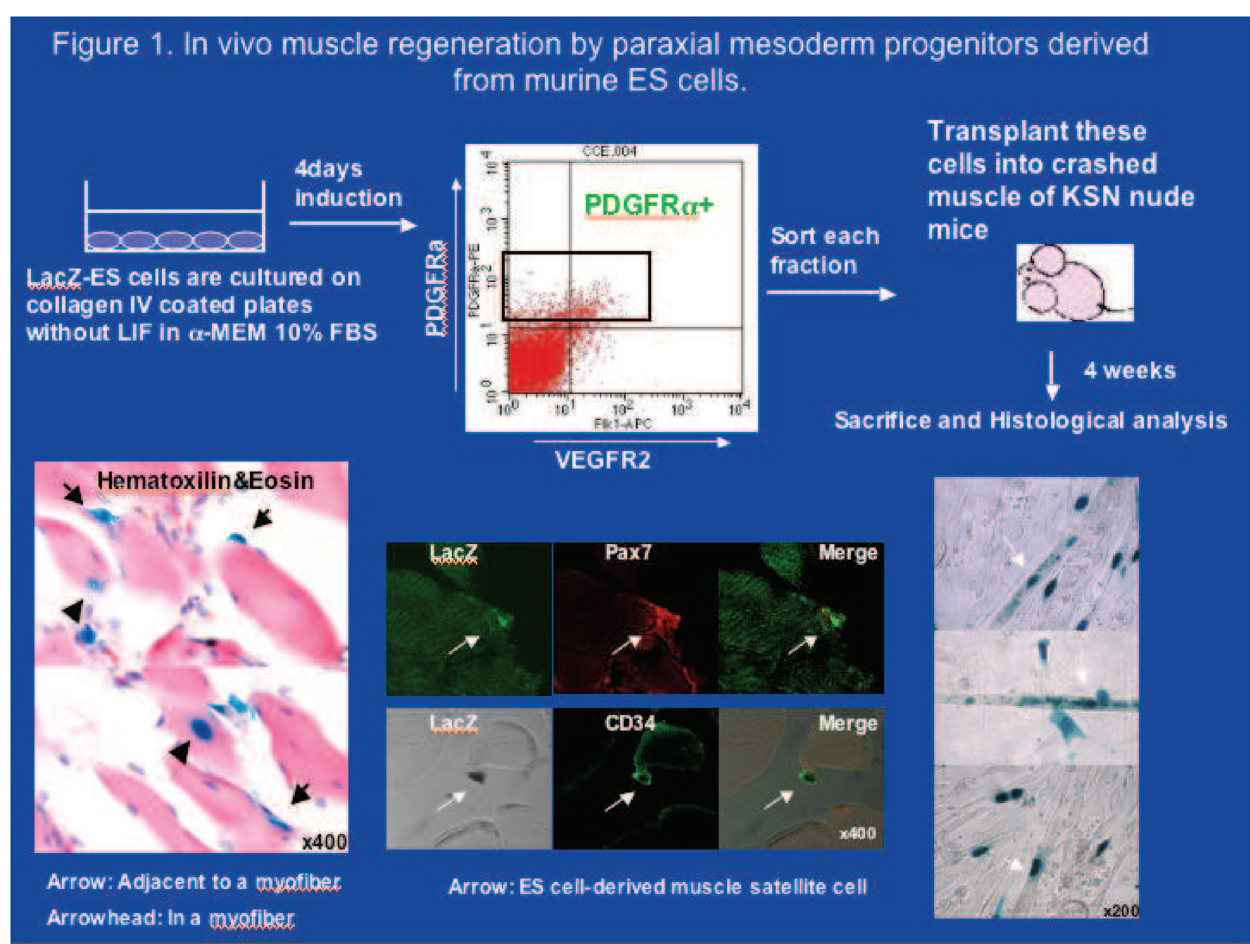

Fig. 1. ES cells were cultured on type IV collagen-coated dishes with 10%FCS. PDGFR-α + cells were sorted by FACS. Cell suspensions were directly injected into the injured quadriceps femoris muscle of a KSN nude mouse.

4.2 Serum-free induction of myogenic progenitor cells from ES cells

Early removal of BMP-4 followed by LiCl treatment promotes the differentiation of PMPs to myogenic progenitors. In mouse embryogenesis, the establishment of the myotome from the

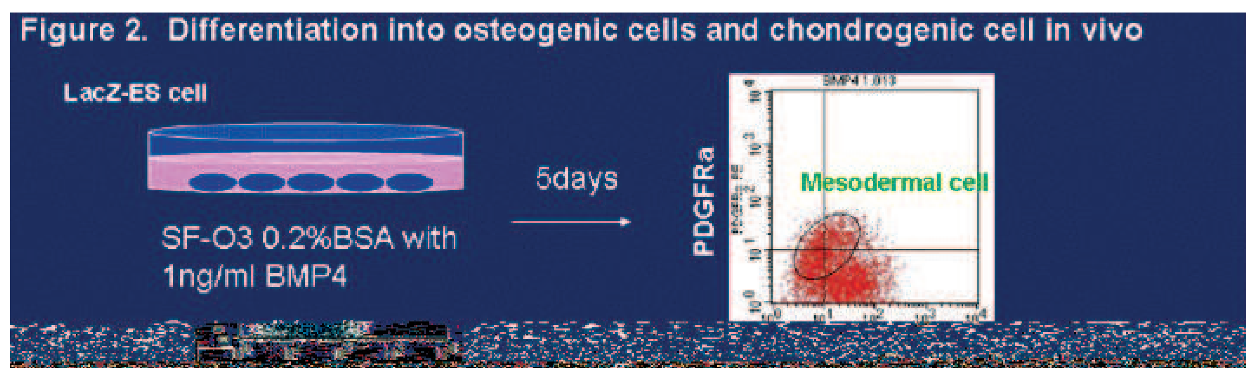

Fig. 2. BMP4-induced paraxial mesodermal progenitor cells can differentiate into chondrogenic and osteogenic cells in vivo. ES cells were plated on a 10 cm dish coated with type IV collagen and differentiated in a serum-free culture medium supplemented with 1ng/mlBMP4. At day 5 of culture, PDGFR-$\alpha^{+\sim low}$ and ECD^{low} populations were sorted (Fraction 1) and injected into bone marrow of tibia of KSN nude mouse.

dermomyotome is stimulated by Wnt signaling and by the expression of Noggin, which inhibits the BMP signaling pathway (5). Our culture system mimics these developmental events by removing BMP-4 after the induction of paraxial mesodermal progenitors and adding LiCl which inhibits GSK-3 and causes translocation of cytoplasmic β-catenin to the nucleus (6.).

In this study, we successfully induced the efficient differentiation of mouse ES cells towards myogenic cell types using chemically defined conditions. Also, simple differentiation of ES cells by BMP4 induced bone and cartilage tissues *in vivo*.

5. References

[1] Nishikawa S, Jakt LM, Era T. Embryonic stem-cell culture as a tool for developmental cell biology. Nature reviews. 2007;8:502-507.

[2] Yamanaka S. A Fresh Look at iPS Cells 2009; 137:13-17.

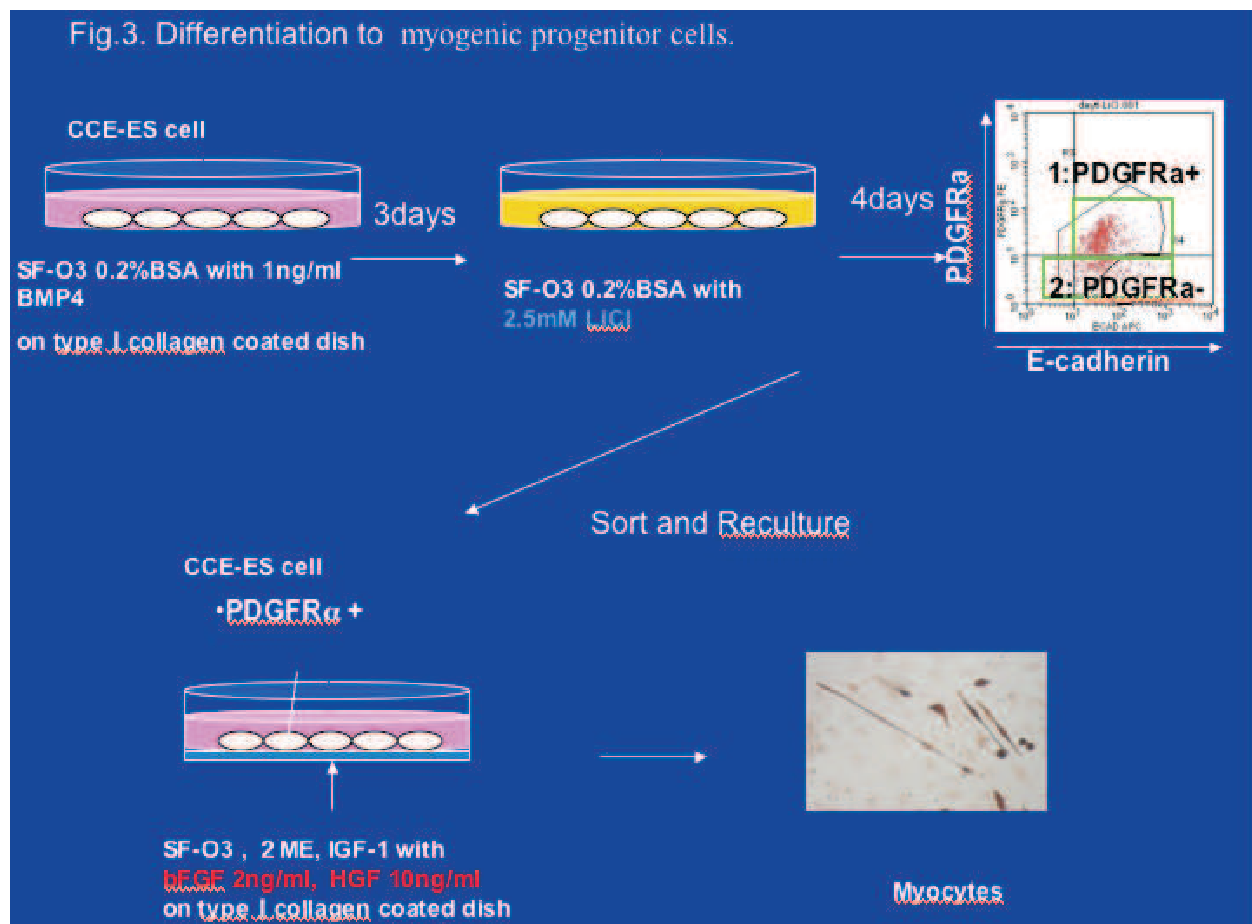

Fig. 3. Myogenic progenitor cells are induced from ES cells by transient exposure to BMP4 and subsequent LiCl treatment in chemically defined media. After sorting, fraction 1 was recultured as described.

[3] Sakurai H, Okawa Y, Inami Y, Nishio N, Isobe K. Paraxial mesodermal progenitors derived from mouse embryonic stem cells contribute to muscle regeneration via differentiation into muscle satellite cells. Stem Cells. 2008 Jul;26(7):1865-73.

[4] Winnier G, Blessing M, Labosky PA and Hogan BL. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev. 1995 9: 2105-2116.

[5] Parker, M.H., Seale, P. and Rudnicki, M.A. Looking back to the embryo: defining transcriptional networks in adult myogenesis. Nat. Rev. Genet. 4 (2003) 497-507.

[6] Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takada, S. and Kikuchi, A. Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability. J Biol Chem 274 (1999) 10681-4.

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This book discusses key aspects of MEMS technology areas, organized in twenty-seven chapters that present the latest research developments in micro electronic and mechanical systems. The book addresses a wide range of fundamental and practical issues related to MEMS, advanced metal-oxide-semiconductor (MOS) and complementary MOS (CMOS) devices, SoC technology, integrated circuit testing and verification, and other important topics in the field. Several chapters cover state-of-the-art microfabrication techniques and materials as enabling technologies for the microsystems. Reliability issues concerning both electronic and mechanical aspects of these devices and systems are also addressed in various chapters.

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