Efficient induction of osteogenic and chondrogenic progenitors and myogenic progenitors from mouse ES cells in chemically defined medium

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Abstract

Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells represent a renewable cellular resources for regenerative therapies. Here, we try to differentiate ES cells to paraxial mesodermal progenitors cells (PMPs), which give rise to osteogenic and chondrogenic progenitors and myogenic progenitors without fetal calf serum. Here, we demonstrate the methods for inducing paraxial mesodermal progenitors efficiently by treatment of BMP4 using PDGFR-a and ECD as markers for purifying them. The BMP4 induced PMPs exhibit fine potentials of differentiation to osto- and chondro-genic cells. Furthermore, early extraction of BMP4 and additional LiCl treatment promote the differentiation of myogenic progenitor cells. Further addition of IGF-1, bFGF and HGF induced the differentiation of PMPs to myocytes in vitro.

Introduction

Embryonic Stem (ES) cells and induced pluripotent stem cells (iPS) have great potentials for cell-based therapies because of their abundant potentials of self renewal and differentiation to all cell lineages. Recently we have demonstrated the process of injured muscle regeneration by the engrafted ES cell-derived mesodermal progenitors (1 and Fig.1). Serum-free ES cell differentiation system has an advantage for clinical application 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 induce efficiently the paraxial mesodermal progenitor cells from ES cells in serum-free culture. BMP4 acted a key factor to promote the primitive streak-type mesoderm in both mouse development and ES cell differentiation culture. Many lateral mesodermal derivatives such as hematopoietic cell, endothelial cell and cariomyocytes, and intermediate mesoderm derivatives such as renal progenitor

have been induced by BMP4 stimulation. However, differentiation of paraxial mesodermal cell from ES cell in serum-free culture has remained elusive. Here, we developed the simple culture systems with BMP4 and Lithium Chloride (LiCl) in serum-free condition to promote two types of paraxial mesodermal progenitors, osteo- and chondrogenic progenitors and myogenic progenitor, using paraxial mesoderm marker PDGFR-α and undifferentiated cell marker ECD.

Materials and Methods

Cell culture and in vitro ES cell differentiation

CCE ES cells and the ES cells expressing the LacZ gene (CCE/nLacZ) were kindly gifted by Dr. Nishikawa. CCE/nLacZ ES cells were maintained as described previously (1).

For paraxial mesoderm differentiation, $2x10^5$ ES cells were plated on a 10-cm dish coated with type IV collagen (Nitta Gelatin) and differentiated in a serum-free culture medium, SF-O3 (Sanko Junyaku) supplemented with 0.2% Bovine serum albumin (BSA), 0.1mM 2-mercaptoethanol and 1 ng/ml recombinant human BMP4 (R&D systems). Four to six days later, osteogenic and chondrogenic mesodermal progenitor cells were obtained as the PDGFR–α+~^{low}, ECD^{low} cells. For further osteocytes and chondrocytes induction, the PDGFR-α+~^{low}, ECD^{low} cells which were sorted as described below, were re-cultured as described previously (1).

For myogenic mesodermal progenitor cell differentiation, initial induction by BMP4 was the same as osteo- and chondro-genic progenitors. Three days after BMP4 treatment, the medium were changed entirely to SF-O3 (Sanko Junyaku) supplemented with 2.5mM LiCl and cells were cultured in the medium for four days. For further myocytes induction, the cells which were sorted as described below, were re-cultured on 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 were changed entirely to SF-O3 with 2ng/ml IGF-1. Four days after medium change, the medium were changed again to SF-O3 with 2ng/ml IGF-1 and 10ng/ml HGF and the cells were cultured for seven days.

Antibodies, cell staining, FACS analyses and cell sorting

Rat monoclonal antibodies (MoAbs), APA5(anti-PDGFR-α) and ECCD2(anti-ECD), were kindly gifted by Dr. Nishikawa. Phycoerythrin-conjugated streptavidin (BD Pharmingen) was used to detect biotinylated-APA5 antibody. ECCD2 MoAbs 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 (1) and analyzed or sorted by FACSCalibur or FACSVantage-HG (Becton Dickinson).

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 (> $5x10^5$ cells). Cells were

resuspended at a density of $2.5x10^4$ cells/_l in αMEM. For intra-muscular transplantation, a quadriceps femoris muscle of a KSN nude mouse was injured by direct cramping with the anesthesia diethyl ether. Twenty micro liters of collected cell suspension were directly injected into the injured quadriceps of each mouse. For intra-bone marrow transplantation, a tibial bone was holed through bone marrow by 21G needle from knee joint with the anesthesia diethyl ether, and twenty micro liters of collected cell suspension were directly injected into bone marrow.

Results and discussion

We plated the ES cells at a density of 2×10^5 cells per 10 cm dish. PDGFR-α^+ cells emerged after four days of differentiation reaching 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, were expressed. We conclude that addition of BMP4 to SF-O3 medium permits efficient induction of paraxial mesodermal progenitor cells (PMPc) from mouse ES cells.

Next, we investigated the in vitro and in vivo osteogenic and chondrogenic potentials of PMPc induced by BMP4 (Fig.1). Four to six days later, the PDGFR-$\alpha^{+\sim low}$ ECD^{low} cells were sorted and were re-cultured as described previously (1). The PDGFR-$\alpha^{+\sim low}$ ECD^{low} population differentiated into osteocytes producing an Alizarin red positive calcium matrix at 21 days after osteocytic induction culture. The CCE/nLacZ ES cell-derived PDGFR-$\alpha^{+\sim low}$ ECD^{low} population was grafted into tibial bone marrow of immunodeficient mice. Twenty-eight days later, β-galactosidase staining was performed to detect engrafted cells. LacZ positive cells which have light blue nuclear staining are observed in trabecular bone (Fig. 2). The CCE/nLacZ ES cell-derived PDGFR-$\alpha^{+\sim low}$ ECD^{low} population was directly transplanted into injured quadriceps femoris muscles of immunodeficient mice. Twenty-eight days later, β-galactosidase staining was performed to detect engrafted cells. Ectopic cartilage and bone were detected in engrafted muscle tissues (Fig. 2). The ectopic cartilage was derived from engrafted cells as confirmed by the expression of LacZ by fluorescent Immunohistochemistry .

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 dermomyotome markers, Pax3 and Pax7, and 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) activated 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) .

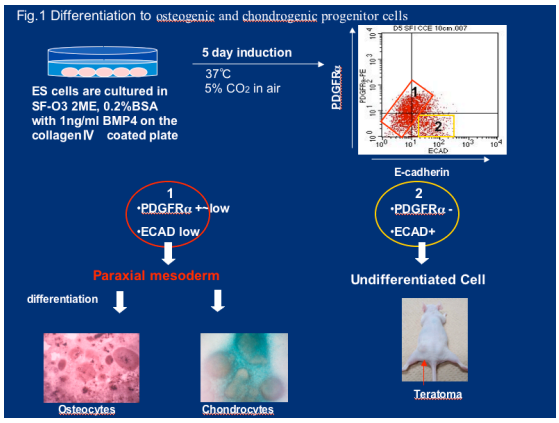

Figure 1. Osteogenic and chondrogenic potentials of BMP4-induced paraxial mesodermal progenitor cells in vitro. ES cells were plated on a 10 cm dish coated with type IV collagen and differentiated in a serum-free culture medium. PDGFR-$\alpha^{+\sim low}$ ECD^{low} population were sorted (Fraction 1) and were recultured in osteocytic induction culture. They differentiated into osteocytes producing an Alizarin red positive calcium matrix (at 28 day culture). They were also recultured in chondrocytic induction culture and gave rise to Alcian blue positive chondrocytes (at 21 days).

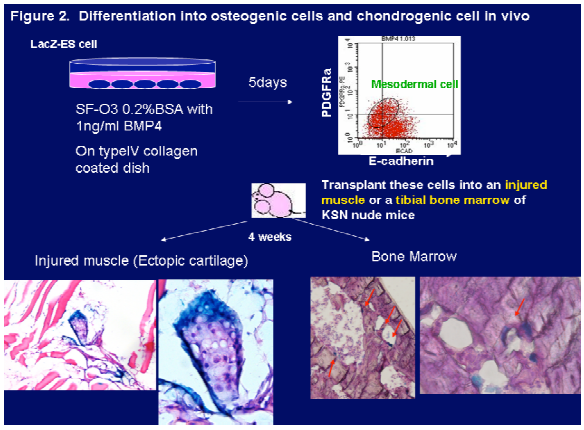

Figure 2. BMP4-induced paraxial mesodermal progenitor cells can differentiate into chondrogenic and osteogenic cells in vivo. LacZ positive (light blue) cells are derived from ES cells.

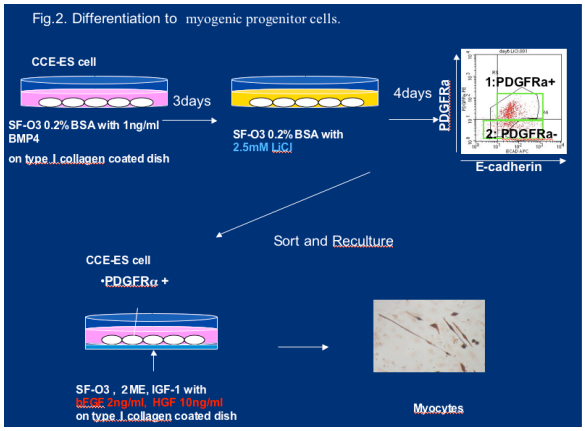

Figure 3. Myogenic progenitor cells are induced from ES cells by transient exposure to BMP4 and subsequent LiCl treatment in chemically defined media. After sorting, fraction 1 was recultured as descrived in Materials and Methods.

References

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