博士論文（要約）

Development of three-dimensional and xeno-free culture system for differentiating pluripotent stem cells into osteoblasts

(多能性幹細胞を骨芽細胞へ分化させる 3 次元・ゼノフリー培養システムの開発)

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1. Introduction

The number of patients with bone defects due to diseases, tumors, and trauma has increased gradually in recent years. According to the American Academy of Orthopedic Surgeons, approximately 2.2 million of musculoskeletal procedures involving bone grafting are done annually worldwide[1]. Although autologous bone transplantation is the gold standard, donor site morbidity and the limitation of available volume lead to an important need for developing fully-functional artificial bones. Scaffolds with MSC-derived osteogenic cells are currently commercially available (trinity evolution, osteocell, Map3, BIO4/Stryker, etc.); however, there is insufficient evidence for the real therapeutic effect of these engineered grafts.

Pluripotent stem cells are defined by two characteristics: self-renewal and pluripotency. Embryonic stem cells (ESCs), which exist only at the earliest stages of development, and induced pluripotent stem cells (iPSCs), which have been reprogramed from somatic cells, can give rise to any cell type of the embryonic proper. Upon osteogenic differentiation, human iPSC-derived osteoblasts (hiPSCs-OB) have shown higher proliferation rate as well as improved osteogenic features in vitro than those derived from MSCs[2, 3]. Therefore, there is a growing interest for developing effective systems to achieve osteoblast specification of PSCs[4,5,6]. However, none of strategies developed so far is a fully xeno-free, which is crucial for safe clinical translation. Based on this need, the present study aims to establish an effective protocol for osteoblast differentiation from hiPSCs by using small molecule inducers under fully defined, xeno-free, and 3D conditions.

2. Experimental

2.1. Reagents

The following small molecules were used for manipulation of specific signaling pathways. PD0325901 for inhibiting mitogen-activated protein kinase kinase, CHIR99021 (CHIR) for activating canonical Wnt signaling. Smoothened agonist (SAG) and cyclopamine (Cyc) for activating and repressing hedgehog (Hh) signaling, respectively. BMP-dependent osteogenic molecule TH was also used[7].

2.2 Mouse pluripotent stem cell (mPSCs) culture

Mouse PSCs including mESCs and miPSCs were maintained on gelatin-coated culture dishes (2D PSCs) under the 2iLIF culture conditions described previously[8]. An atelocollagen porous scaffold (Mighty sponge, KOKEN CO., LTD) was used for 3D PSC cultures under the same culture conditions. Subsequent osteoblast differentiation was carried out as previously reported[4].

2.3. Human induced pluripotent stem cell (iPSC) culture

Three independently derived human iPSC lines maintained in Essential 8™ medium (Thermo Fisher Scientific) on vitronectin-coated dishes were used for developing the osteoblast induction

method using small molecule inducers. For 3D cultures, porous scaffolds fabricated from human collagen I-based recombinant peptide (Cellnest™, Fujifilm) were used.

3. Results and discussion

3.1. Establishment of a three-dimensional (3D) culture system for osteogenic differentiation of pluripotent stem cells

To elucidate the relevant features of the 3D culture of PSCs, I cultured mESCs and miPSCs with atelocollagen scaffolds and compared them with those cultured in conventional gelatin-coated plates in terms of pluripotency maintenance and differentiation under defined conditions.

3D mESCs strongly expressed pluripotency markers (*Nanog*, *Oct4*, *Sox2*) at comparable levels to 2D mESCs. However, 3D mESCs maintained expressions of all pluripotency markers tested in the absence of 2iLIF, whereas 2D mESCs showed downregulation of those genes. Thus, 3D culture can maintain mESCs in an undifferentiated state with the self-renewal capacity even in the absence of exogenous stimulation.

Next, I evaluated the differentiation potential of 3D mESCs. Gene expression patterns in the osteogenic 3D culture, in comparison with those in the osteogenic 2D culture, showed similar trend. Pluripotency markers were progressively downregulated while the osteogenic markers were gradually upregulated throughout the osteoblast induction. In contrast, *Dmp1* and *Sost* (osteocyte markers) were highly expressed in the osteogenic 3D culture on days 19 and 26, but not in the 2D culture, compared to day 0 (Figure 1A). Immunohistochemistry of the cell-scaffold complex showed nuclear localization of SP7 in most of the cells, and superficial cell clusters expressing DMP-1 (Figure 1B). The von Kossa staining with nuclear fast red staining revealed cells embedded in the calcified ECM within the scaffold, and SEM examination highlighted calcium nodule-like structures covering the ECM (Figure 1C).

Thus, mESCs successfully underwent small molecule-mediated, terminal differentiation into mature osteoblasts and osteocytes in the 3-D culture. Unlike the 2-D culture, the osteogenic 3-D culture further induced maturation of osteoblasts into osteocytes. Also, the present 3-D culture platform successfully worked in miPSCs.

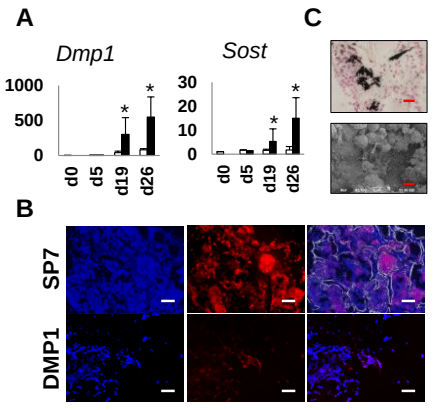

Figure 1. (A) mRNA expression determined by RT-qPCR analysis in the osteocyte markers 2-D (white bars) or 3-D (black bars) cultures of mESCs. The data are expressed as the mean ± SDs from six independent experiments. *p < 0.001. (B) SP7 and DMP1 protein expression in the osteogenic 3-D culture of mESCs. Nuclei were stained with DAPI. Scale bars, 100 μm. (C) Representative picture of von Kossa staining with nuclear fast red (upper panel), and SEM (lower panel). Scale bars, 50 μm and 5 μm, respectively.

3.2. Development of a fully-defined, xeno-free protocol for osteogenic differentiation of human induced pluripotent stem cells (iPSCs)

Although the strategy used above leads the differentiation and maturation of mouse PSCs into osteoblasts even in 3-D cultures, it fails to produce terminal osteoblast commitment and maturation in hiPSCs. Since sustained activation of the Hh signaling has been shown to inhibit osteoblast commitment in human mesenchymal stem cells[10], treatment with SAG was restricted just for the specification to osteoblast precursors upon mesoderm induction (phase I). In contrast, the removal of canonical Wnt signaling in early osteoblast progenitors was reported to inhibit the terminal osteoblast differentiation[11]. Therefore, a subsequent treatment with CHIR was considered for the optimization of the present strategy (phase II), followed by 9 extra days of culture without any inducer (phase III). Figure 2A shows the improved strategy.

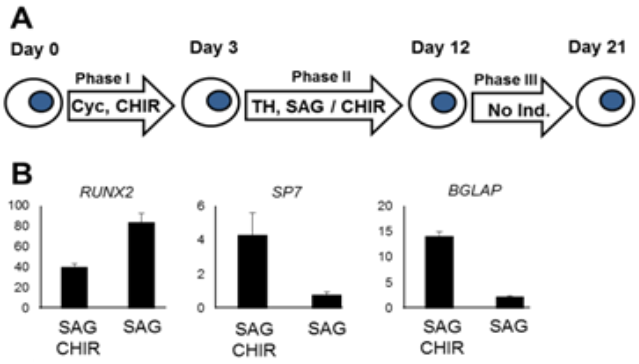

Figure 2. (A) A schematic showing the developed strategy for inducing osteogenic differentiation of hiPSCs. (B) mRNA expression determined by RT-qPCR analysis of the osteogenic markers. The data are expressed as the mean ± SDs from four independent experiments.

The hiPSCs maintained under a feeder-free condition were subjected to the developed protocol. The contribution of the Wnt/β-catenin signaling activation by using CHIR to the middle stage of osteoblast differentiation (from day 10 to day 12) was verified by mRNA expression analysis; stage specific treatment with SAG and CHIR (SAG, CHIR) exerted stronger effects on osteoblast differentiation than sustained SAG treatment (SAG) as indicated by the upregulation of osteoblast markers at day 21 (Figure 3B). In addition, this improved chemically defined xeno-free strategy allowed the successful differentiation of three independent hiPSC lines into functional osteoblasts.

3.2. Development of a fully-defined, xeno-free protocol for osteogenic differentiation of human induced pluripotent stem cells (iPSCs)

To investigate whether the optimized xeno-free strategy can be applied to differentiate hiPSCs into osteoblasts under 3D culture conditions, hiPSC-derived mesodermal cells (Phase I) were seeded in the 3D scaffolds.

By day 21 the cells in the 3D culture highly expressed the osteoblast markers at comparable levels than those from the 2D culture. Importantly, the resulting cell population

formed calcified structures in vitro and exhibited a sustained expression of *BGLAP*, a bona fide marker of mature osteoblasts. When transplanted in critical-sized calvarial defects in mice, hiPSC-derived osteoblasts induced accelerated bone repair as indicated by MicroCT scans and histological analysis. In addition, the presence of human nuclear-positive cells in the regenerated sites suggested that the hiPSC-derived osteoblasts exerted their positive effect on bone repair by engraftment with the host tissue as well as via self-assembled bone-like tissue formation (Figure 3).

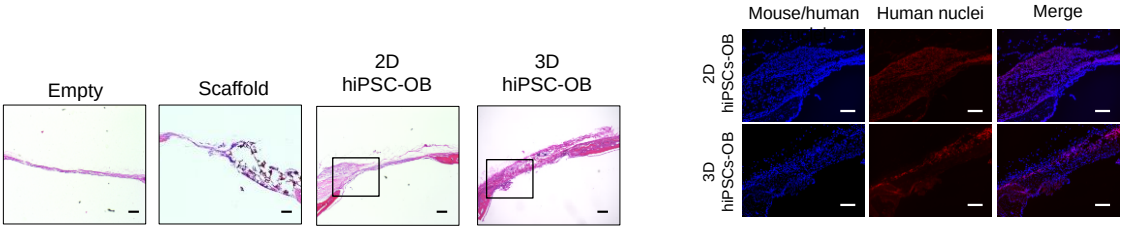

Figure 3. Representative H&E staining (left) and immunostaining with human nuclear antigen (right) in calvarias at 4 weeks post-transplantation.

4. Conclusion

A 3D, fully defined, and xeno-free strategy to direct human iPSCs toward osteoblasts was established for bone regeneration. The proposed small-molecule mediated protocol produces functional hiPSC-derived osteoblast-like cells both in vitro and in vivo. The xeno-free condition, the short treatment period, and the stability of the inducers in the developed protocol together provide a new step toward the clinical applications of hiPSCs to skeletal regenerative medicine, and it may have also significant advantages for drug screening and basic research.

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