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Abstract of Thesis

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Title	A Novel 3D Culture Model for Bone Regeneration with Controlled Morphology (骨形態を再現しうる新しい三次元培養モデル)

OBJECTIVE: While bone tissue engineering has been successfully achieved, accomplishing proper integration of regenerated bone into the well-organized host bone requires better morphological control. In this study, a 3D culture model was developed by leveraging the effects of an osteoblast (OB) layer to regulate bone regeneration by highly purified osteoprogenitors (HipOPs) with tailored morphology.

MATERIALS AND METHODS

1. Isolation of HipOPs from BMSC

Bone marrow stem cells (BMSCs) were collected from male C57BL/6 mice and plated in culture medium (CM). Three days later, culture dishes were washed three times with phosphate-buffered saline to exclude non-adherent cells. Adherent cells were maintained in CM to reach 80% confluence and harvested to eliminate hematopoietic cells by negative sorting with magnetic beads (Miltenyi Biotec). After the purification, HipOPs were collected as unlabeled cells for further experiments.

2. Osteoblast differentiation of HipOPs on collagen gel

To generate an OB monolayer, HipOPs were first seeded into a 24-well plate (2.5×10^5 cells/well), which was previously coated with collagen gel (250 μL/well). Osteoinduction was initiated by replacing the medium with an osteoinductive medium (OM). On days 1, 2, 3, 6, and 9, alkaline phosphatase (ALP) and von Kossa staining, as well as RT-qPCR analysis, were performed to assess extracellular matrix formation and to detect signaling molecules secreted by OBs during osteogenic differentiation, respectively.

3. Coculture of HipOPs and OBs in 3D culture

To evaluate cell interactions in the 3D culture system, cylindrical gelatin sponges (5 mm in diameter, 3 mm thick) were prepared to seed HipOPs (10^6 cells/sponge) and then transferred onto the OB layers on collagen gel in a 96-well plate. After 48 h in CM, the cell-seeded sponges were bisected transversely to examine HipOP responses to signal concentration gradients. RNA was collected separately from the upper and lower halves of the sponges in both the HipOP/none group (no OB layer) and the HipOP/OB group (coculture with OBs) for gene expression profiling via RNA sequencing. Additionally, total RNA was extracted from intact sponges in each group to analyze osteogenic gene expression by RT-qPCR. On day 7, cell-seeded sponges were collected to assess proliferation via MTT assay and to visualize osteogenic differentiation through ALP staining.

4. *In vivo* transplantation

For transplantation, collagen solution (50 μL) was loaded into the bottom of a perforated poly-lactidecoglycolide (PLGA) tubular scaffold (5 mm diameter × 5 mm height), followed by osteogenic induction of HipOPs. Once a confluent OB monolayer was confirmed on the collagen gel, HipOP-embedded sponges were added to complete the 3D model for the HipOP/OB group. For the HipOP/none group, the OB layer was omitted. The cell-scaffold constructs were maintained in CM for 48 h before subcutaneous transplantation into immunodeficient mice (Crlj:CD1-Foxn1nu mice). Samples were collected eight weeks after transplantation for further analysis. All animal experiments were conducted in accordance with the guidelines of the Animal Experiments Committee of Osaka University Graduate School of Dentistry (R-01-001-0).

RESULTS AND DISCUSSION

1. Osteoblast differentiation of HipOPs on collagen gel

Compared to BMSCs, HipOPs exhibited robust osteogenic differentiation, with ALP activity detectable from day 1 and calcium deposition starting from day 3. Both ALP staining and mineralized areas expanded progressively with time, reaching their peak at day 9. In the RT-qPCR analysis of the HipOP group, *Wnt1* and *Wnt3a* were not expressed during the first nine days of differentiation. However, other Wnt family members showed rapid up-regulation, peaking at day 3 (*Wnt5a* and *Wnt7b*) or day 6 (*Wnt5b* and *Wnt10b*) and then gradually decreasing. Similarly, the growth factors *BMP2* and *VEGFA* exhibited a gene expression pattern comparable to that of *Wnt5a* and *Wnt7b*. These findings suggested that mature OBs, which demonstrated positive von Kossa staining after three days in OM, were most effective in producing biochemicals that could positively influence the osteogenic differentiation of HipOPs. Since the majority of the selected genes, including *Wnt5a*, *Wnt7b*, *BMP2*, and *VEGFA*, showed their most significant upregulation between days 2 and 3 before being downregulated (one-way ANOVA and Tukey's HSD test), the OB layer at day 2 was selected as the optimal stage for coculture with HipOPs in the 3D model.

2. Effects of the OB layer on HipOPs in the 3D culture model

After 48 h of coculture, the HipOP population exhibited distinct gene expression profiles in RNA sequencing analysis with significantly enriched Gene Ontology terms related to bone formation, response to stimuli, cell communication, and regulation of vascular development in the lower half of the sponge contacting the OB layer. In contrast, the upper half of the HipOP/OB group displayed intermediate expression levels for these biological processes, while both halves of the HipOP/none group showed low expression levels. RT-qPCR analysis further demonstrated significant upregulation of osteogenic differentiation markers, including *ALP*, *Col-I*, *BSP*, and *OCN*, in HipOPs after 48 h of coculture with the OB layer (Student's *t*-test, $p < 0.05$). By day 7, ALP staining confirmed robust osteogenic differentiation in the HipOP/OB group, with stronger staining intensity observed in the part closer to the OB layer. In addition, MTT assay results showed a significantly higher cell number in the lower half of the sponge in the HipOP/OB group compared to the upper half (Student's *t*-test, $p < 0.001$). In contrast, the HipOP/none group showed minimal ALP staining with a higher proliferation rate in both halves of the sponge. These findings suggest that the OB layer provided signals that promoted osteogenic differentiation rather than proliferation after coculture and simultaneously stimulated them to migrate to the bottom part where the OBs were located.

3. *In vivo* bone formation capacity of the 3D culture model

After eight weeks of transplantation, the average bone volume in the HipOP/none group was significantly smaller than that in the HipOP/OB group (Student's *t*-test, $p < 0.05$). The results from micro-CT analysis and Masson's trichrome staining showed a continuous bone-like tissue formation with density gradually decreasing from the bottom where the HipOP-seeded sponge contacted the OB layer. This pattern demonstrated the concentration-dependent responses and chemotactic migration as effects of the OB layer on HipOPs. In contrast, the HipOP/none group showed two distinct ossification clusters in the lower and upper areas, which was attributed to passive cell condensation and improved nutrient exchange in these regions. HE staining results confirmed the histology of the bone tissue in the HipOP/OB samples, showing osteocytes embedded in the bone matrix and osteoblasts lining the inner bone surface. Furthermore, bone marrow tissue, including adipocytes, hematopoietic cells, blood vessels, and sinusoids, was identified in the bone cavity. Immunohistochemical staining for CD31 revealed that the HipOP/OB group had a highly developed vascular network throughout the lower half of the sample. Conversely, the HipOP/none group showed minimal vascularization.

CONCLUSIONS

Our proposed 3D culture model effectively utilized OB-derived signaling molecules to direct cell migration and osteogenic differentiation of HipOPs, achieving bone organ regeneration with controlled morphology, including cortical bone, spongy bone, and bone marrow tissue.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

本研究は，骨芽細胞層上で間葉系幹細胞集団（HipOPs）を培養することで，形態を再現した骨組織を再生しうるのではないかという仮説のもと，新規 3 次元培養法を開発したものである．

その結果，新規 3 次元培養法の骨芽細胞層から生じるシグナル伝達分子は HipOP に作用し，皮質骨や海綿骨の形成を誘導することがわかった．また，骨芽細胞層から離れた領域には骨髄組織を形成し，本法を用いることで骨・骨髄構造を再現した組織を再生しうることが明らかとなった．

以上の研究成果は，骨形態を再現した骨再生の実現につながるものであり，本研究は博士（歯学）の学位論文として価値のあるものと認める．