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Osteogenic differentiation of mesenchymal stem cells by chondrocyte conditioned medium and mineralized scaffolds

연골세포 조정배지와 결정화된 지지체를 활용한 중간엽 줄기세포의 골 조직분화

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Abstract

Osteogenic differentiation of mesenchymal stem cells by chondrocyte conditioned medium and mineralized scaffolds

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During endochondral bone formation, regulative mechanisms via transient cartilage play pivotal roles in ossification stages. In order to recapitulate endochondral differentiation microenvironmental cues, C3H10T1/2 murine mesenchymal cells were expanded with chondrocyte conditioned medium (CM) and further differentiated with osteogenic medium (OM). Priming of C3H10T1/2 cells via CM expansion resulted in enhanced expression level of chondrogenic markers such as aggregcan, type II, X collagen and Sox9 rather than osteogenic genes. Furthermore, CM expansion resulted in reduced expression levels of osteogenic genes such as ALP, type I collagen, osteocalcin, and Runx2. However, C3H10T1/2 cells showed enhanced osteogenic differentiation as indicated by increased ALP
(Alkaline phosphatase) and Alizarin Red S stainings upon osteogenic factor exposure followed by chondrocyte-conditioned medium. In addition, CM expanded C3H10T1/2 mesenchymal cells were seeded onto mineralized scaffolds fabricated with polydopamine and SBF implanted in critical-sized calvarial defect mouse models. After 8 weeks of implantation, mouse skulls were collected, and bone tissue regeneration were evaluated by micro-CT imaging and Masson’s trichrome (MTC) staining. In accordance with in vitro analysis, the scaffold seeded CM expanded C3H10T1/2 resulted in high level of bone regeneration compared to the scaffolds seeded GM expanded C3H10T1/2 cells. In conclusion, chondrocytes-conditioned factors in along with mineralized micro-environment can simulate the bone forming environment of mesenchymal cells.

Keywords: biomimetic substrate, osteogenic differentiation, conditioned medium, stimulating body fluids, scaffolds, biomaterials, in vivo

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Contents

Abstract ............................................................................................................. 1

Contents ........................................................................................................... 3

List of tables and figures ................................................................................. 4

Chapter 1. Introduction ................................................................................... 5

Chapter 2. Osteogenic differentiation of mesenchymal stem cells by chondrocyte
conditioned medium and mineralized scaffolds with simulated body fluid

  2.1. Introduction ............................................................................................ 14

  2.2. Materials and methods ......................................................................... 16

  2.3. Results .................................................................................................... 22

  2.4. Discussion .............................................................................................. 36

Chapter 3. Osteogenic differentiation of mesenchymal stem cells by hydroxyapatite and
whitlockite scaffolds

  3.1. Introduction ............................................................................................ 40

  3.2. Materials and methods ......................................................................... 41

  3.3. Results .................................................................................................... 44

  3.4. Discussion .............................................................................................. 48

Chapter 4. Conclusion and future direction ..................................................... 49

Reference .......................................................................................................... 50

요약 (국문초록) ............................................................................................. 56
List of figures and tables

Table 1. Primer sequences used in real-time PCR analysis ........................................ 19
Fig. 1.1. EdU staining of C3H10T1/2 in GM and CM .............................................. 22
Fig. 1.2. The ratio of EdU positive cells per total cells and doubling time ................. 23
Fig. 1.3 Real-time PCR analysis of osteogenic markers in CM ................................. 24
Fig. 1.4. Real-time PCR analysis of chondrogenic markers in CM ............................ 24
Fig. 2.1. Alkaline Phosphatase staining and Alizarin Red S staining ......................... 25
Fig. 2.2. Real-time PCR analysis of osteogenic markers in osteogenic medium .......... 26
Fig. 3.1. Surface mineralization of silicon wafer .................................................. 27
Table 2. Surface elemental analysis of silicon wafer by EDX .................................... 27
Fig. 3.2. Water contact angle measurement of silicon wafer .................................. 28
Fig. 4.1. PLLA/PLGA scaffold after surface coating .............................................. 29
Fig. 4.2. SEM images of PLLA/PLGA scaffolds after surface coating ...................... 29
Fig. 4.3. The ratio of attached cells per seeded cells on the scaffolds ...................... 30
Fig. 4.4. Real-time PCR analysis on PLLA/PLGA scaffolds in GM ......................... 31
Fig. 4.5. Real-time PCR analysis of GM expanded cells on the scaffolds ................. 32
Fig. 4.6. Real-time PCR analysis of CM expanded cells on the scaffolds ................. 33
Fig. 5.1. Micro-CT images of calvarial defect models ........................................... 34
Fig. 5.2. The ratio of regeneration area per defect area ........................................ 35
Fig. 5.3. Masson’s trichrome staining of calvarial defect models ............................ 35
Fig. 6.1. Imaged of fabricated HAp and WH scaffolds ........................................ 45
Fig. 6.2. Live/dead and EdU staining of HAp and WH scaffolds .............................. 45
Fig. 6.3. F-actin staining and SEM image of HAp and WH scaffolds ....................... 46
Fig. 6.4. Real time PCR analysis of HAp and WH scaffolds .................................. 47
Chapter 1. Introduction

Current treatment for bone tissue regeneration

Bone is most hard tissue and takes several vital roles in the human body. One of the significant functional roles of the bone is to support the body structure. This tissue has relatively high level of compressive strength of 170 MPa compared other tissues in the body and protects internal organs from external shock and assists mechanical movement by the collaboration with muscle, tendon, ligament and cartilage tissues (1). In the view of synthesis and regulation of body homeostasis, bone tissue plays critical roles in human body. Hematopoietic stem cells reside in bone marrow, and they provide a reservoir for all types of blood cells such as red and white blood cells. In addition, bone tissue regulates ion concentrations by storing minerals, which become bone structure and release mineral ions into the fluids in the body. Bone tissue is composed of structural proteins, minerals, and various types of cells. Mainly type I collagen and hydroxyapatite, family of calcium phosphate, make the structure of bone tissue and bone cells induce the process of bone remodeling of living bone tissue. In bone remodeling, osteoclasts remove bone matrix and osteoblasts forms bone matrix at the same region. After remodeling cycle is completed, osteocytes appear in bone matrix and maintain the structure. The bone homeostasis is important because imbalance of bone remodeling can induce bone diseases such as osteoporosis, which is caused by higher resorption rate than the formation rate, and osteopetrosis, which is caused by the opposite situation.

Although bone has a high level of mechanical strength, the fracture in bone tissue often occurs by external shock with unexpected accidents. Like many tissues in our body,
bone has a natural ability to repair minor injuries resulting from small fractures. However, millions of patients worldwide suffer from bone disease or critical-sized bone fractures, which require autologous grafts, allografts, or synthetic grafts (2-4). Currently, the gold standard is autologous grafts harvested from a donor patient. These implants have qualified histocompatibility and no immune rejection. However, there are crucial limitations such as additional surgery at the donor site of the patient and limitation in the area of about 20cm$^2$ (5). In order to overcome the limitation of autologous grafts, allografts have been utilized in a clinical way, but they also have the limitations of the aspects of safety such as hazard of immune rejections and infections as well as economic issues.

**Cells for bone tissue regeneration**

Tissue engineering strategies for critical-sized bone fracture presents a new avenue for developing bone grafts. There are three main components of bone tissue engineering, stem cells, scaffolds and soluble factors. In tissue engineering, many kinds of stem cells have been utilized to fabricate targeted tissue. The primary characteristics of stem cells are self-renewal and differentiation. All kinds of stem cells have these two characteristics but have different capacity of differentiation according to types of stem cells. Pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the ability can differentiate into all kinds of cells in the body. However, ESCs have ethical issues, and it is impossible to obtain ESCs from adult human. In addition, iPSC has the limitation related with safety issues, which are caused by the application of viral vectors and potential risk to form a teratoma in the body. To avoid these problems, mesenchymal stem cells (MSCs) are applied in the various field of tissue engineering. MSCs are most widely used stem cells in laboratories, and they have multi-potency to differentiate into chondrocytes,
osteoblasts, adipocytes, fibroblasts and other cell types which present in mesenchymal origin tissues. Clear advantage of MSCs is that they can be derived from many adult tissues, such as bone marrow, adipose tissue, umbilical cord, and amniotic fluids. In addition, autologous MSCs have histocompatibility and no immune rejection, which can stimulate healing rate and efficiency. From these reasons, many strategies are being developed to utilize MSCs along with functional scaffolds and soluble factors in bone tissue engineering.

**Scaffolds for bone tissue engineering**

In our body, cells interact with their surrounding microenvironments (cell niche) by controlling the synthesis of extracellular matrix (ECM). Scaffold engineering may be utilized to recreate cellular microenvironment to stimulate ECM synthesis. In bone regeneration, porous structure and mineralization are key factors for the scaffold fabrication to mimic the condition of natural bone tissue. As the one of strategies for inducing pores in the scaffold, salt-leaching technique has been widely used in bone tissue engineering. Recent efforts towards developing bone graft substitutes focus biomimetic 3D scaffold design, which should mimic the nanostructure and properties of natural bone. Previous studies have demonstrated that stem cells cultured on mineralized surfaces showed high osteogenic potential (6-8). Since bone consists of a porous composite of hydroxyapatite and collagen, scaffolds should be porous composites with ceramic and polymer phases. Furthermore, scaffolds should be biodegradable and provide a temporary matrix for cells to attach, proliferate, and deposit extracellular matrix (ECM). In this spirit, biodegradable scaffolds such as poly(l-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA) have demonstrated success in bone tissue engineering applications (9-11).
In addition, recent advancements in scaffold fabrications have resulted in electrospun nanofibers for bone tissue engineering. Electrospinning system is composed of four main apparatus (syringe pump, spinneret, high-voltage-supply and grounded collector) and several minor components (tubing, syringe and wire). A syringe pump makes a steady flow of polymeric solution into a blunt-end spinneret. Following a high-voltage supply impressing the spinneret, a Tayler cone is formed at the tip of spinneret by electrostatic pulling force. A liquid polymer jet heads to a grounded collector covered with an aluminium foil for the final nanofiber collection. There are lots of operation parameters that can affect characteristics of nanofiber: the concentration of polymeric solution, flow rate controlled by syringe pump, distance between spinneret and collector, and types of collector. These are easily controllable parameters in nanofiber fabrication processes. Electrospun nanofiber has the enormous potential because it can be fabricated with the specific characteristics. When chosen with appropriate starting biomaterials, nanofiber can be synthesized with desirable mechanical properties. For example, poly(L-lactic-co-glycolide) (PLGA) nanofiber has larger stiffness compared to poly(caprolactone) (PCL) nanofiber (12). Another remarkable nature of nanofiber is a high-surface-area-to-volume ratio which enables electrospun nanofiber to contain relatively large amount of chemicals. Therefore, Kenawy et al. could make poly(ethylene-co-vinyl acetate) (PEVA) and poly(lactic acid) (PLA) nanofiber by co-electrospinning and incorporate tetracycline into fabricated PEVA/PLA nanofiber. The main advantage of this strategy is a controllable release profile of target chemicals to the maximum (13). From these advantages, composite nanofibers with hydrogels and minerals have been reported to induce osteogenic differentiation of MSCs. Haider et al. fabricated electrospun poly(lactic-co-glycolide) (PLGA) nanofiber incorporated with hydroxyapatite and confirmed enhanced cellular adhesion, proliferation and osteogenesis along with increased calcium ion concentration on the nanofiber matrix (11). For organizing injectable system, Baylan et al.
used polycaprolactone (PCL) nanofiber and collagen hydrogel for bone regeneration. MC3TR-E1 cells on this composite scaffold showed promoted proliferation, phenotype expression and formation of mineralized matrix (14). In addition, Meng et al. introduced a novel methods for *in vivo* osteogenesis with mechanical stimulation by integrating magnetic nanoparticles onto nanofiber matrix with exposure of magnetic field. They made electrospun poly(lactic acid) (PLA) nanofiber incorporated with super-paramagnetic γ-FeO nanoparticles (MNP) and hydroxyapatite nanoparticles. With static magnetic field, the scaffolds implanted in bone defect model showed enhanced bone regeneration compared to the scaffolds without magnetic stimulations, confirming potential application with external magnetic field that can be helpful bone healing and forming process (15).

As another widely used types of scaffolds in the field of tissue engineering, hydrogel scaffolds can obtain a large amount of water in their structure and also contain water-soluble molecules. For the application of bone tissue engineering, previous researches had been tried to fabricate osteo-inductive composite hydrogels with minerals and biomolecules. Flausse et al. applied powder of nacre which was mainly composed with calcium phosphate into polyethylene glycol (PEG) hydrogel for osteogenic differentiation of human bone marrow mesenchymal stem cells (16). Yu et al. reported the composite scaffold with micro-channeled calcium phosphate scaffold and collagen hydrogel and this hydrogel-calcium phosphate scaffold enhanced expression level of genes and proteins related with osteogenic differentiation of MSCs (17). Furthermore, Gandavarapu et al. fabricated PEG hydrogel with phosphate groups and immersed them into serum containing medium for increased proteins absorption into the hydrogel (18). Other groups focused on the incorporation of hydrogels and bone morphogenetic protein (BMP)-2 which mainly regulate osteogenic differentiation via BMP signaling pathway. Hulsart-Billstrom et al. and Kisiel et al. designed hyaluronic acid based hydrogel incorporated with BMP-2 and controlled the release
of the growth factor (19, 20). To promote the effect of BMP-2 onto the cell, Kim et al. induced Cbfa1 (also called by Runx2) overexpressing osteogenic cells from human embryonic stem cells (ESCs) and seeded these cells into BMP-2 containing poly(N-isopropylacrylamide-co-acrylic acid) (p(NiPAAm-co-AAc)) hydrogel having thermosensitivity (21). In addition, injectable systems with osteo-inductive hydrogels were fabricated to use of clinical way without surgical incisions. Kwon et al. made in situ-forming hydrogel by using polyethylene glycol (PEG) and polycaprolactone (PCL) and seeded human turbinate originated MSCs into the hydrogel. From the result of subcutaneous implantation without any incision, they proclaimed enhanced osteogenesis when hydrogels incorporated with human MSCs and osteogenic factors (22). Liao et al. showed p(NiPAAm) based thermoresponsive hydrogel copolymerized with hyaluronic acid as injectable system and confirmed osteogenic differentiation and ectopic bone formation of the hydrogel seeded with canine bone marrow-derived MSCs (23). Besides the use of synthetic polymers, natural polymers such as chitosan and collagen were also applied to organize the injectable system. Sun et al., reported thermo-sensitive injectable hydrogel composed with chitosan, collagen and β-glycerophosphate presented enhanced osteogenic differentiation in vitro and in vivo seeded with canine bone marrow-derived MSCs (24). Currently, biomaterials elasticities are being considered as a novel way for promoting osteogenic differentiation. S. Tan et al. utilized transglutaminase cross-linked gelatin (TG-Gels) hydrogel to control the degree of crosslinking. In their study, it was confirmed stiffer TG-Gels showed enhanced osteogenic differentiation in vivo and in vitro (25).

On the other hand, surface mineralization is also beneficial because the major component of natural bone tissue is calcium phosphates and other minerals. Widely used method to induce mineralization on the scaffolds is the incubation in simulated body fluid (SBF), which has similar ion concentration of human blood plasma. A surface modification
on porous scaffolds via mussel-inspired immobilization strategy may provide an efficient method to deposit mineralized matrix for osteomimetic microenvironment. Repeated unit of 3,4-dihydroxy-L-phenylalanine (L-DOPA; dopamine (DA) precursor) attributes a strong adhesion of mussel to virtually any material surfaces. DA contains a catechol functional group that promotes polymerization in alkaline condition via oxidative conversion of catechol to quinone, and polymerized DA, polydopamine (pDA) allow covalent incorporation of various bioactive molecules. Recently, pDA assisted surface immobilization of osteoinductive peptides onto the porous scaffolds has shown to enhance bone regeneration by adipose-derived stem cells (26). In this study, we report that mineralized surface modification on porous PLGA/PLLA scaffolds was achieved via pDA assisted coating of stimulating body fluids (SBF). Stimulated body fluids (SBF) with gelatin in the presence of pDA resulted in osteomimetic scaffold fabrication.

Furthermore, there are special phase of calcium phosphate in natural bone tissue such as hydroxyapatite and whitlockite as well as beta-tricalcium phosphate (β-TCP). Particularly, hydroxyapatite had been confirmed osteo-inductive property. Most important advantage of the application of hydroxyapatite is their similar chemical composition and crystalline structure to native bone tissue, and it has good integration into implanted native bone structure (27). Recently, Jang et al. reported optimized condition for synthesis of whitlockite, which as magnesium composition in their crystal structure. They also showed that synthesized whitlockite represented comparable biocompatibility and the expression level of osteogenic markers to hydroxyapatite by culturing human osteoblast cells on the scaffolds (28). In this study, bioactivity and osteo-inductivity were confirmed by culturing C3H10T1/2 cells, one of mouse mesenchymal cell, on the hydroxyapatite and whitlockite scaffolds with heat treatment of 700℃ as the further observation.
Soluble factors for bone tissue engineering

As the last part of bone tissue engineering, soluble factors can stimulate osteogenesis of stem cells incorporated with functionalized scaffolds. Most useful growth factors in bone tissue engineering are bone morphogenetic protein (BMP) family which regulate osteogenic differentiation of MSCs via transforming growth factor-β (TGF-β), BMP and SMAD pathway. BMPs were originally discovered in bone matrix by Reddi et al. and confirmed their capability for chondrogenesis and osteogenesis by previous researches (29-31). In addition, almost all kinds of BMPs except for BMP-3 in the members of TGF- β family have osteogenic activity while TGF- β and activins and inhibins do not have this activity (32). Another major signaling pathway affecting osteogenesis is Wnt signaling. Wnt signaling acts in bone remodeling process in dynamic states of living bone tissue and has been targeted to cure the disease related disorder of bone homeostasis (33). Besides of them, insulin-like growth factor (IGF) (34), vascular endothelial growth factor (VEGF) (35) and alternative small molecules such as melatonin, purmorphamine and resveratrol were reported as osteo-inducing soluble factors (36).

In addition, cellular component is also necessary for generation and establishment of extracellular matrix in the new tissues (37-39). Mesenchymal stem cells have been shown to be capable of giving rise to several different cell types including myoblasts, adipocytes, fibroblast, chondrocytes, and osteoblasts (40, 41). In skeletal development, soluble factors provide instructive signals that specify aspects of stem cell commitment and differentiation. In particular, during endochondral ossification, cartilage development lead to subsequent downstream alternation of bone formation and Gerstenfeld and colleagues have shown that chondrocyte-secreted factors induce selective osteogenic differentiation of mesenchymal stem cells (42). It may be hypothesized, then, that the local soluble microenvironment
created during transient cartilage formation may regulate downstream mesenchymal stem cell recruitment and bone formation. Although individual morphogenetic factors have been shown to promote tissue-specific differentiation of mesenchymal stem cells, it is critical to understand the complex interrelationship of chondrocyte-secreted factors for \textit{in vivo} commitment (43-45).
Chapter 2. Osteogenic differentiation of mesenchymal stem cells by chondrocyte conditioned medium and mineralized scaffolds with simulated body fluid

2.1. Introduction

Tissue engineering strategies for critical-sized bone fracture presents a new avenue for developing bone grafts. The basis of tissue engineering is to induce tissue regeneration with cell culturing onto three-dimensional (3D) scaffolds (46). Recent efforts towards developing bone graft substitutes focus biomimetic 3D scaffold design, which should mimic the nanostructure and properties of natural bone. Furthermore, scaffolds should be biodegradable and provide a temporary matrix for cells to attach, proliferate, and deposit extracellular matrix (ECM). In this spirit, biodegradable scaffolds such as poly(l-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA) have demonstrated success in bone tissue engineering applications (9-11).

A surface modification on porous scaffolds via mussel-inspired immobilization strategy may provide an efficient method to deposit mineralized matrix for osteomimetic microenvironment. Recently, pDA assisted surface immobilization of osteoinductive peptides onto the porous scaffolds has shown to enhance bone regeneration by adipose-derived stem cells (26). In this study, we report that mineralized surface modification on porous PLGA/PLLA scaffolds was achieved via pDA assisted coating of stimulating body fluids (SBF). Stimulated body fluids (SBF) with gelatin in the presence of pDA resulted in osteomimetic scaffold fabrication.

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In this chapter, we report pDA-mediated fabrication of mineralized surface modification onto biodegradable PLGA/PLLA scaffolds for enhancing osteogenic potential of C3H10T1/2 murine MSCs. Furthermore, we utilized chondrocyte-conditioned medium to prime the C3H10T1/2 murine MSCs for osteogenic fate. Osteogenic commitment of primed C3H10T1/2 murine MSCs was examined on mineralized scaffolds in vitro. Finally, mineralized PLGA/PLLA scaffolds seeded with primed C3H10T1/2 murine MSCs were transplanted in a mouse model of critical size calvarial defect model, and bone regeneration in the defects was evaluated by microcomputed tomograph (μ-CT) and histological analysis 8 weeks after implantation. Primed C3H10T1/2 murine MSCs in mineralized microenvironment significantly enhanced osteogenic differentiation both in vitro and in vivo, leading to bone regeneration in the defects.
2.2. Materials and Methods

Cell culture and differentiation

C3H10T1/2 cells were obtained from Korean Cell Line Bank (KCLB) of Seoul National University Hospital (SNUH), Korea. C3H10T1/2 cells were plated at $5 \times 10^5$ cells per 150mm-diameter culture plate (SPL lifescience) and maintained with DMEM (GIBCO) supplemented with 10% Fetal Bovine Serum (FBS, GIBCO) and 1% penicillin/streptomycin (Pen/Strep) (GIBCO). Bovine chondrocytes were isolated as previously described (47). Briefly, cartilage tissues were collected and chopped into small pieces. Chopped tissues were digested with 0.2% type II collagenase (Worthington Biochemical Corporation, USA) solution for 16 hours at 37°C. Isolated bovine chondrocytes filtered through 40μm mesh and washed with phosphate buffered saline (PBS). For chondrocyte-conditioned medium (CM), confluent primary bovine chondrocytes in 150mm-diameter culture plate were incubated with 15mL of DMEM with 1% Pen/Strep for 24 hours. Chondrocyte CM was then filtered through 0.2 μm syringe filter and further supplemented with 10% FBS. C3H10T1/2 cells were expanded with FBS supplemented CM for 14 days for priming. For osteogenic differentiation, cells were cultured in DMEM supplemented with 10% FBS, 1% Pen/strep, 50mg/mL L-ascorbic acid (Sigma), 10mM glycerol-2-phosphate (Sigma) and 100nM dexamethasone (Sigma) for 14 days. Differentiation medium was changed every other day.

Scaffold fabrication and characterization

Porous poly(L-lactic acid) (PLLA)/poly(lactic-co-glycolide) (PLGA) scaffolds were fabricated as previously described (48). Briefly, an equal amount PLLA and PLGA were...
dissolved in chloroform (Daejung chemical) to make 5% w/v solution. 300mg of Sodium chloride (NaCl) (Merck) powder above 300μm of diameter was put into the mold, and 250 L of PLLA/PLGA solution was added to make 7mm by 2mm disc-shaped scaffolds. Scaffolds were thoroughly dried and immersed in 500mL of distilled water for 3 days to leach out NaCl residue in the polymer scaffold. Distilled waters were exchanged at least 6 times. For the surface modification, simulated body fluid (SBF) containing 58.43g NaCl, 2.77g CaCl₂ and 1.39g NaH₂PO₄·H₂O in 1L deionized water was prepared. The scaffolds were then immersed in SBF solution (SBF group_ or SBF containing 2mg/mL dopamine hydrochloride (pDA, Sigma) and 0.1% gelatin (PDSBF group) for 4 days each. Mineralized scaffolds were sterilized under UV (3.1mW/cm²) for 1 hour prior to cell seeding. Control scaffold without any modification was utilized as control.

**Surface property measurement of mineralized substrates**

Atomic Force Microscopy (AFM) analysis was performed with NX10 (Park systems) by analyzing 10μm by 10μm area. Scaffold mineralization conditions were applied on silicon wafers. In addition, water contact angle measurements were performed with AM-413MT (Dino-Lite) by dropping a water droplet on mineralized silicon wafers. Furthermore, energy dispersive X-ray spectroscopy (EDX) was performed to verify the composition of the element on the surface at 20kV and 10μA (JSM-6701F, JEOL).

**Scanning electron microscope analysis**

Cell-seeded scaffolds were fixed with 4% paraformaldehyde (Polysciences) for 1 hour, serially dehydrated with ethanol (Daejung chemical), and exchanged with hexamethyldisilazane (HMDS) (Daejung chemical) for 1 hours. Samples were visualized

**Cell attachment and proliferation**

Cells were seeded at $5 \times 10^5$ cells per scaffold. Quant-iT™ PicoGreen dsDNA Assay Kit (P11496, Invitrogen) was used to evaluate cell attachment after 3 hours of seeding. Papain solution was prepared by adding 1.58mg cysteine and 25mg papain type III (Worthington) in PBE buffer. The samples were digested in papain solution at 60°C for 16 hours, and DNA contents were measured with PicoGreen solution diluted 200 times with TE buffer at by Infinite M200pro (TECAN) at 485nm (excitation) and 535 (emission). To assess cell proliferation, Click-iT EdU Flow Cytometry Assay Kits (Invitrogen) were used according to manufacturer’s instruction. In brief, C3H10T1/2 cells were seeded on glass bottom dish (SPL lifescience) and incubated with 0.1% 5-methynyl-2’-deoxyuridine (EdU) for 3 hours. Click-iT reaction cocktail were added on the dish for 30min and washed with distilled water. To label total cell nucleus, 4’,6-diamidino-2-phenylindole (DAPI) was added after EdU staining.

**Alkaline phosphatase and Alizarin Red S stainings**

After 14 day's culture, cell were fixed with a fixative solution (40μL citrate stock solution (85-4C, Sigma), 1.96mL distilled water and 3mL acetone) for 1 min and washed with distilled water and stained for alkaline phosphatase (ALP) (Sigma) as previously described (49, 50). In brief, diazonium salt solution was prepared by dissolving 0.24mg of Fast Blue RR Salt in 1mL of distilled water and adding 40μL of Naphthol AS-MX Phosphate solution. Cells were stained with diazonium salt solution for 1 hour and washed twice with
distilled water. For Alizarin Red S (ARS) (Sigma) staining, 20mg of ARS was dissolved in 1mL of distilled water and pH was adjusted to 4.1~4.2 with ammonium hydroxide. Fixed cells were stained with ARS solution for 20 min and washed with distilled water twice.

**Real time-PCR analysis**

Total RNA was collected by TRIzol method, and reverse transcribed into cDNA using TOPscript™ Reverse Transcriptase kit (Enzymomics) as manufacturer’s instruction. Real-time PCR were performed with TOPreal™ qPCR 2x PreMIX (Enzymomics) and the ABI StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA). cDNA samples were analyzed for the genes of interest, and GAPDH was used as a reference gene. The expression analysis was then evaluated as $-2^{\Delta\Delta C_t}$ as previously described (51). Samples were run in triplicates, and PCR primers are listed in table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (3'-5')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Osteogenic markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>GAAGTCCGTGGGCATCGT</td>
<td>CAGTGCGGTTCCAGACATAG</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>GGGCGAGTGGCTGTGCTTT</td>
<td>GGGACCCATGAGGACTGAA</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>CACACCTAGCAGACACCATG</td>
<td>CCGTAGATGCTTTGTTAGGC</td>
</tr>
<tr>
<td>Runx2</td>
<td>GGACGAGGGCAAGATTTCA</td>
<td>TGGTGCAAGAGTTGAGCAG</td>
</tr>
<tr>
<td><strong>Chondrogenic markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggrecan</td>
<td>GAAATGACAACCCCAAGCAC</td>
<td>TCTCCGCTGTTCAGTCCT</td>
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<tr>
<td>Type II collagen</td>
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<td>GCGACTTACGGGCACTCCT</td>
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<tr>
<td>Type X collagen</td>
<td>ATGCCGTGTCGTCCTTTTT</td>
<td>TCTGGAAGCTCCATGATTG</td>
</tr>
<tr>
<td>Sox9</td>
<td>CTTCTCCGCTTCCCGGCCA</td>
<td>GATCGAATCTGTCACCAGAC</td>
</tr>
</tbody>
</table>

**Table 1.** Primer sequences of osteogenic and chondrogenic markers used in real Time PCR analysis
**In vivo calvarial defect model**

All animal studies were approved by Institute of Laboratory Animal Resources Seoul National University. 6-week-old BALB/c mouse were used for critical size calvarial defect. Before the transplantation, cells were seeded onto PDSBF scaffolds and cultured osteogenic medium for 7 days. Mice were anesthetized with 30mg/kg of Zoletil and 10mg/kg of Rompun and small incision was made on their head. Two of 4mm-diameter defect were induced by using hand drilling machine, STRONG 106 (SAESHIN), on both sides of mouse skull and 4mm diameter scaffolds were implanted in defect site. After 8 weeks of transplantation, mouse skulls were collected and analyzed for bone formation.

**Micro-computed tomography (μ-CT) analysis**

Collected skulls were fixed with 4% PFA and micro-CT images were taken by Skyscan1172 (Bruker). Operation source voltage was 59kV and source current was 167μA. Each image was stacked by CTvox program (Bruker) and regeneration area of samples was evaluated by ImageJ.

**Histological analysis**

For histological analysis, tissues were decalcified with 14% EDTA for 4 days, serially dehydrated with ethanol, and embedded in paraffin for overnight. Paraffin-embedded tissues were sectioned into 5μm thickness by RM2145 (Leica) and mounted on glass slides. To perform Masson’s trichrome staining, the slides were deparaffinized with xylene, rehydrated with ethanol, immersed in Bouin’s solution (Sigma) at room temperature overnight. Pretreated slides were washed in running tap water and stained Weigert’s Iron Hematoxylin Solution for 5 min and Trichrome Stain solution for 5 min. After staining
process, the slides were placed in 0.5% acetic acid for 1 min to differentiate the tissue section and observed by phase-contrast microscope (CKX41, Olympus).

**Statistical analysis**

All data are represented as mean ± standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA single factor) with * $p < 0.05$, ** $p < 0.01$
2.3 Results

**CM enhanced proliferation of C3H10T1/2**

To investigate the effects of chondrocytes-secreted factors on stem cells, conditioned medium (CM) was collected from bovine chondrocytes and utilized to expand the C3H10T1/2 murine mesenchymal stem cells after supplementing CM with 10% v/v FBS. In CM, C3H10T1/2 showed enhanced cell proliferation compared to the cells in growth medium (GM). CM treatment for 4 hours resulted in two-fold increase in s-phase cells, as indicated by EdU positive cells, compare to cells in GM (Fig 1.1.). In addition, population doubling (PD) time for CM expanded cells were 35.65 hours while GM expansion resulted in PD time of 28.42 hours, indicating 22.86% faster PD time in CM medium compared to GM medium (Fig 1.2.).

![Fig 1.1. EdU staining of C3H10T1/2 in growth medium (GM) and chondrocyte conditioned medium (CM), scale bar = 300μm](image)
CM regulated gene expression of C3H10T1/2

CM expansion decreased the expression levels of osteogenic genes and enhanced the genes related to chondrogenic differentiation in C3H10T1/2 cells. CM expansion of C3H10T1/2 cells resulted in decreased levels Type I collagen gene and Runx2 gene expression, where the levels of gene expressions decreased 80.34 % and 88.39%, respectively after 8 days of CM expansion. However, ALP gene and osteocalcin gene expression levels remained unchanged via CM expansion (Fig 1.3.). During the course of CM expansion, the chondrocyte-secreted factors resulted in enhanced levels of aggrecan and type X collagen gene expression levels. Aggrecan gene increased 6.207 fold and type X collagen gene increased 7.731 folds after 8 days of CM expansion. However, Sox9 gene and type II collagen gene expressions remain unchanged via CM expansion, suggesting that perhaps 3D micromass or high density culture is necessary for the activation of these genes. (Fig 1.4.)
Fig 1.3. Real time PCR analysis of osteogenic markers in CM at day 4 (D4) and day 8 (D8) compared with non-priming (D0). * P<0.05, ** P<0.01

Fig 1.4. Real time PCR analysis of chondrogenic markers in CM at day 4 (D4) and day 8 (D8) compared with non-priming (D0). * P<0.05, ** P<0.01
**Osteogenesis of C3H10T1/2 in the differentiation medium**

Contrary to our hypothesis, CM expansion resulted in reduced osteogenic cell related gene markers. Since osteogenic differentiation of MSCs require ascorbic-2-phosphate, β-glycerophosphate and dexamethasone in the differentiation media, we further exposed CM expanded C3H10T1/2 cells to osteogenic medium (OM) for additional 14 days. CM expanded C3H10T1/2 cells that were further differentiated in osteogenic medium (COM) showed higher ALP activity and mineral deposition compared to control C3H10T1/2 cells in OM (Fig 2.1.). In real-time PCR analysis, osteogenic markers such as ALP, type I collagen, osteocalcin and Runx2 showed relatively higher expression level in COM group. Gene expressions of COM group were 4.345 folds increased in Runx2, 4.814 folds increased in ALP, 5.103 folds increased in osteocalcin, and 13.712 folds increased in type I collagen compared to GM expanded cells in growth medium (GM) group. In addition, COM group indicated 1.391 folds increased in ALP, 1.551 folds increased in osteocalcin, 1.571 folds increased in Runx2 and 3.671 folds increased in type I collagen compared to GM expanded cells in osteogenic medium (OM) group (Fig 2.2.). This suggests that CM expansion primed C3H10T1/2 cells and further exposure to OM resulted in an enhanced osteogenic commitment of C3H10T1/2 cells.
Fig 2.1. Alkaline Phosphatase (ALP) staining and Alizarin Red S (ARS) staining of C3H10T1/2 in growth medium (GM), C3H10T1/2 cells in osteogenic medium (OM) and CM expanded C3H10T1/2 in osteogenic medium (COM) at day 14, scale bar = 500μm

Fig 2.2. Real time PCR analysis of osteogenic markers of GM, OM and COM at day 14 (B). * P<0.05, ** P<0.01
Surface mineralization on a silicon wafer by SEM and AFM imaging

For successful bone tissue engineering, 3D mineralized microenvironment may be necessary. Previous reports have utilized simulated body fluid (SBF) for mineralization of scaffolds. We further utilized SBF along with pDA to facilitate the mineral deposition. Prior to the fabrication of mineralized 3D scaffolds, efficient mineral deposition conditions were investigated on silicon wafers. AFM analysis of uncoated silicon wafers showed a smooth surface. SBF alone was sufficient to allow mineral deposition on silicon wafers. However, mixture of SBF and 2mg/mL polydopamine (pDA) in 0.1% gelatin solution (SBF/pDA/Gel) resulted in enhance mineral deposition. In SEM and AFM analysis, surface mineralization was observed on the silicon wafers immersed in SBF and SBF/pDA/Gel. Particularly hundreds micrometer-sized crystals were formed on SBF/pDA/Gel silicon wafer, supporting that the incorporation of pDA and gelatin have enhanced the mineral deposition (Fig 3.1.). In addition, elemental composition of the surface was confirmed by Energy Dispersive X-ray spectroscopy (EDX). Calcium, phosphorus, sodium and chloride were detected on the surface of SBF/pDA/Gel silicon wafer but not detected on SBF silicon wafer at the same magnification of EDX (Table 2).
Fig 3.1. Surface mineralization of silicon wafer in 10mM Tris-HCl buffer (Control), simulated body fluid (SBF) and the mixture of SBF, 2mg/mL polydopamine, and 0.1% gelatin (SBF/pDA/Gel) visualized by SEM and AFM, scale bar = 5μm

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Table 2. Surface elemental analysis of silicon wafer in Control, SBF and SBF/Gel/pDA solution by EDX
**Surface mineralization on a silicon wafer by water contact angle measurement**

The water contact angle measurement demonstrated that the hydrophilicity of the mineralized silicon wafers changed over time (Figure 3.2.). Silicone surface treatment with SBF/pDA/Gel resulted in hydrophilic surface after 4 days while SBF treated or control silicon wafer surface remained relatively hydrophobic.

![Day 2 Day 4](image)

<table>
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**Fig 3.2.** Water contact angle measurement of silicon wafer in Control, SBF and SBF/pDA/Gel solution at day 2 and 4.

**Surface mineralization on 3D porous PLLA/PLGA scaffold**

To examine the effect of 3D mineralized microenvironment on CM expanded C3H10T1/2 cells, porous mineralized scaffolds were fabricated with average pore size of 300
30 μm. Scaffolds were also immersed in SBF or SBF/pDA/gelatin solution for 4 days to initiate mineralization. SBF/pDA/gelatin condition resulted in darkening of the scaffolds due to surface adhesion via pDA (Fig 4.1.). SEM analysis of scaffolds demonstrated that SBF solution induced mineral crystal deposition porous scaffolds. Furthermore, average densities of mineral crystals were higher with SBF/pDA/Gel scaffold (Fig 4.2.). Cells were attached well on all scaffold systems, and there were no significant difference in seeding efficiencies (Fig 4.3.).

![Fig 4.1. PLLA/PLGA scaffold after coating in 10mM Tris-HCl buffer (Control), simulated body fluid (SBF) and the mixture of SBF, 2mg/mL polydopamine and 0.1% gelatin (SBF/pDA/Gel) for 4 days, scale bar = 2mm](image1)

![Fig 4.2. SEM images of PLLA/PLGA scaffolds after coating in Control, SBF and SBF/pDA/Gel solution](image2)
RT-PCR analysis of C3H10T1/2 on mineralized scaffold in growth medium

To identify the effect of mineralized microenvironment, GM expanded C3H10T1/2 cells were cultured on control scaffolds, SBF scaffolds, or SBF/pDA/Gel scaffolds in growth medium for 12 days. ALP gene expression of C3H10T1/2 cells on SBF/pDA/Gel scaffolds showed 4.006 folds increase compared to cells in control scaffolds and 1.881 folds increase compared to the cells on SBF scaffolds. Similarly, type I collagen gene expression on SBF/pDA/Gel scaffold showed 3.825 folds increase compared to control scaffolds and 2.683 folds increase compared to cells on SBF scaffolds (Fig 4.4.).
We further plated GM expanded or CM expanded C3H10T1/2 cells on mineralized scaffolds and cultured in osteogenic medium for 12 days. In real-time PCR analysis, GM expanded cells on SBF/pDA/Gel scaffold (SBF/pDA/Gel group) showed 9.546 folds
increased in ALP and 9.882 folds increased in type I collagen expression compared to control PLLA/PLGA scaffold (Control group). In addition, this group presented 2.272 folds increased in ALP, and 3.174 folds increased in type I collagen expression compared to SBF scaffold (SBF group) (Fig 4.5.). Similar tendencies were observed with CM expanded cells but degree of expression levels were distinct. SBF/pDA/Gel group showed 21.38 folds increased in Runx2, 44.10 folds increased in ALP and 57.65 folds increased in type I collagen expression compared to Control group. Furthermore, this group indicated 13.83 folds increased in type I collagen, 14.59 folds increased in ALP and 16.75 folds increased in Runx2 expression compared to SBF group (Fig 4.6.).

Fig 4.5. Real time PCR analysis of osteogenic markers of C3H10T1/2 in osteogenic medium at day 14 on PLLA/PLGA scaffolds after coating in Control, SBF and SBF/pDA/Gel solution. * P<0.05, ** P<0.01
In vivo implantation with critical-sized calvarial defect model

To evaluate the effect of mineralized scaffold and chondrocyte secreted factors in vivo, critical sized mouse calvarial defect model was used. Prior to the implantation, 4mm diameter SBF/pDA/Gel scaffolds were seeded with CM expanded or GM expanded cells and cultured in osteogenic medium for 7 days. After 8 weeks transplantation, mouse skulls were

Fig 4.6. Real time PCR analysis of osteogenic markers of CM expanded C3H10T1/2 in osteogenic medium at day 14 on PLLA/PLGA scaffolds after coating in Control, SBF and SBF/pDA/Gel solution. * P<0.05, ** P<0.01
harvested and evaluated the degree of bone regeneration by μ-CT and histological analysis. Regeneration area of defect site was increased along with mineralized scaffold. Scaffold group, which was implanted SBF/pDA/Gel scaffold without cells showed 2.06 folds increase compared to the sham group without any treatment. In addition, C3H10T1/2 cells supported the regeneration of bone tissue. SBF/pDA/Gel scaffold seeded with GM expanded cells (GM group) showed 1.408 folds increased regeneration area compared to acellular scaffold control. Furthermore, SBF/pDA/Gel scaffold seeded with CM expanded cells (CM group) showed 1.929 folds increased bone regeneration area compared to acellular control when defect regeneration area was measured (Fig 5.1. and Fig 5.2.). In the result of Masson’s trichrome staining, regeneration of bone tissue started from the boundary region of defect in Scaffold and OM group, while there was no regeneration in Sham group without any treatment. However in CM group, bone regeneration sites were observed at the top of the scaffold as well as neighboring region to boundary bone tissue (Fig 5.3.).

**Fig 5.1.** Micro CT images of calvarial defect models after 8 weeks of implantation with no scaffold (Sham), SBF/pDA/Gel scaffold (Scaffold), SBF/pDA/Gel scaffold seeded C3H10T1/2 without CM expansion (GM) and SBF/pDA/Gel scaffold seeded CM expanded C3H10T1/2 (CM), scale bar = 1mm
**Fig 5.2.** The ratio of regeneration area per defect area of calvarial defect models implanted with no scaffold (Sham), SBF/pDA/Gel scaffold (Scaffold), SBF/pDA/Gel scaffold seeded C3H10T1/2 without CM expansion (GM) and SBF/pDA/Gel scaffold seeded CM expanded C3H10T1/2 (CM). * P<0.05 ** P<0.01

**Fig 5.3.** Masson’s trichrome staining of calvarial defect models implanted with no scaffold (Sham), SBF/pDA/Gel scaffold (Scaffold), SBF/pDA/Gel scaffold seeded C3H10T1/2 without CM expansion (GM) and SBF/pDA/Gel scaffold seeded CM expanded C3H10T1/2 (CM), scale bar = 200μm
2.4. Discussion

Recreation of microenvironments that mimic a targeted native tissue may provide an optimized differentiation conditions for stem cells in bone tissue engineering. In this study, we made an effort develop a novel differentiation system to promote osteogenic differentiation via exposing the cells with chondrocyte-secreted factors and mineralized microenvironment. During endochondral ossification process, mesenchymal stem cells are exposed to paracrine factors of chondrocyte and primary ossification center appear as a starting point of bone formation (52, 53). Furthermore, previous reports have demonstrated that chondrocyte secreted factors may induce both osteogenic and chondrogenic differentiations of mesenchymal cells and human embryonic-derived mesenchymal cells, respectively (42, 54, 55). Chondrocyte secreted factors were collected as conditioned medium (CM) from bovine chondrocytes in serum-free condition and further supplemented with 10% FBS for proper nutrient conditions (56-58). Our initial observation with chondrocyte secreted factors is that it facilitated the cellular proliferation. Furthermore, C3H10T1/2 cells, one of murine mesenchymal stem cells (MSCs), expansion with CM activated chondrocyte-specific genes such as aggrecan and type X collagen even in the low-density culture. However, contrary to our hypothesis and previously reported results, expansion of MSCs with CM reduced the expression level of osteogenic genes such as type I collagen and Runx2. Even though CM alone inhibited osteogenic related gene expression, we further investigated the osteogenic response of CM expanded cell when they were exposed to osteogenic differentiation medium containing L-ascorbic acid, β-glycerophosphate and dexamethasone. Significant increase in ALP activity, mineral deposition and the expression level of osteogenic markers were observed when CM expanded cells were further exposed to OM. This study is not the first to suggest that chondrocyte-
conditioned factors can facilitate the bone differentiation; however, this suggests that CM indirectly effects the commitment of stem cells via priming them for differentiation. Interestingly, in order to clearly observe the priming effects of CM on MSCs, around 6 days of expansions were required. Our gene expression analysis showed that CM expansion of MSCs activated BMP2R gene and BMP2 gene expressions after 4 days (Supplementary Figure 4).

Mineralized microenvironments enhanced osteogenic differentiation of stem cells (6, 8, 9). Recent study has suggested that calcium phosphate (CaP) rich microenvironment can facilitate the osteogenesis of mesenchymal stem cells via extracellular phosphate uptake through phosphate transporter, which results increased adenosine, an ATP metabolite, that acts as an autocrine/paracrine signaling molecules through A2b adenosine receptor (59). In order to provide a 3D environment for bone regeneration, we have fabricated porous PLLA/PLGA scaffolds, and further modified the surface of the scaffolds with simulated body fluids for CaP rich microenvironment. SBF have been widely used for surface mineralization (60, 61). Surface mineralizations were induced on via immersing porous scaffolds in SBF solution. However, in order to enrich the SBF on porous scaffolds, we additionally added dopamine and gelatin into the SBF solution. Incorporation of pDA and gelatin strongly enhanced the mineral deposition. Polydopamine is originated from mussel adhesion protein which can attach well onto various types of surface. During this coating process, adjacent molecules such as growth factors and peptides in the solution can absorb simultaneously onto the surface of scaffolds during the process of polymerization of dopamine hydrochloride at basic condition, resulting in enhanced surface characterization (26, 62).

From the results of this study, mineral enriched microenvironment via SBF/pDA/Gel
showed osteogenic response of C3H10T1/2 cells even in growth medium. GM expanded C3H10T1/2 cells showed enhanced osteogenic differentiation on SBF/pDA/Gel scaffold, which indicated the level of surface mineralization might affect osteogenesis of MSCs by inducing high concentration of phosphate ion at microenvironment of the cells and activated phosphate-ATP-adenosine metabolic signaling as previously described (59). Similar tendencies were observed in the result of osteogenic differentiation of GM or CM expanded MSCs on mineralized scaffolds in osteogenic medium for 12 days. Interestingly, CM expanded cells showed more significant difference between on non-mineralized and mineralized scaffold compared to the same condition of GM expanded cells. From this result, it is hypothesized that secreting factors in chondrocyte conditioned medium induced the change of osteogenic signaling pathway of MSCs, which promoted cells more sensitive onto the mineralized microenvironment.

Furthermore, we applied mineralized scaffolds into mouse calvarial defect model to evaluate the capability of bone tissue formation in vivo. Mineralized SBF/pDA/Gel scaffolds with or without cells were implanted into mouse calvarial defect model and harvested after 8 weeks implantation. After 8 week's implantation, mineralized scaffolds with SBF/pDA/Gel coating supported bone regeneration of defect site along with itself, and MSCs loaded mineralized scaffolds showed enhanced bone formation. Particularly the scaffold seeded CM expanded C3H10T1/2 presented bone formation at whole region of the scaffold in Masson’s trichrome staining. From this result, it is hypothesized that CM expanded C3H10T1/2 cells in the scaffold directly induced high level of bone tissue formation in vivo condition and mineralized scaffold.

Vascularization is essential in coordinating the phenotypic transformation from cartilage to bone during endochondral ossification process. Vascularizations during
endochondral ossification step allow transport of essential ions and mineral to ossification area, leading to complete bone formation. To promote vascularization into the scaffolds, incorporations with human vascular endothelial cells (HUVECs) and angiogenic growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) will be novel approaches to increase the capability of bone formation of the mineralized scaffold (63, 64). In future study, strategies incorporating controlled release VEGF or PDGF from scaffolds and co-culture-based cell seeding for comprehensive recapitulation of bone formation.

Our study substantiates the concept of mimicking endochondral ossification stages, first with chondrocyte secreted factors followed by mineralized microenvironment, can promote efficient bone tissues in a clinically relevant model. Specifically, the bone regenerate produced by the CM expanded cells on mineralized scaffolds showed denser bone formation as examined by μCT and histological analysis in critical size defect model. Furthermore, screening of functional molecules in CM with proteomics approach may help to find a new candidate molecule for bone healing and to identify unknown signaling pathway related with osteogenic differentiation.
Chapter 3. Osteogenic differentiation of mesenchymal stem cells by hydroxyapatite and whitlockite scaffolds

3.1. Introduction

In our bone tissue, there is particular phase of calcium phosphate in natural bone tissue such as hydroxyapatite and whitlockite as well as beta-tricalcium phosphate (β-TCP). Particularly, hydroxyapatite had been confirmed osteo-inductive property. Most significant advantage of the use of hydroxyapatite is their similar chemical composition and crystalline structure to native bone tissue, and it has good integration into implanted native bone structure (27). Recently, Jang et al. reported optimized condition for synthesis of whitlockite, which as magnesium balance in their crystal structure. They also showed that synthesized whitlockite represented comparable biocompatibility and the expression level of osteogenic markers to hydroxyapatite by culturing human osteoblast cells on the scaffolds (28). In this study, bioactivity and osteo-inductivity were confirmed by culturing C3H10T1/2 cells, one of mouse mesenchymal cell, on the hydroxyapatite and whitlockite scaffolds with heat treatment of 700°C as the further observation. In this experiment, biocompatibility and osteo-inductive capacity of whitlockite were compared with hydroxyapatite and the potential uses for bone tissue engineering was evaluated.
3.2 Materials and methods

Fabrication of HAp and WH scaffolds

Hydroxyapatite (HAp) and whitlockite (WH) powders were synthesized followed by previously described (28). For the synthesis of hydroxyapatite powder, phosphoric acid was added into aqueous calcium phosphate solution with stirring, and the precipitates were collected by freeze drying after 9 hour reaction. For the synthesis of whitlockite powder, calcium phosphate and magnesium hydroxide were mixed, and phosphoric acid were added into the mixture after heat process at 60°C to 90°C. Solution was incubated for more than 9 hours with stirring and the precipitates were collected by filtering and frozen drying. For the fabrication of cylindrical pellet scaffold, hydroxyapatite and whitlockite powders were pressed into a mold with 1cm diameter at 700°C for 2 hours. After fabrication processes, scaffolds were sterilized by autoclaving at 121°C for 15 min and UV curing for 1 hours.

Cell culture and differentiation

C3H10T1/2 cells were plated at $2 \times 10^6$ cells per 1cm-diameter hydroxyapatite and whitlockite scaffolds and differentiated with DMEM supplemented with 10% FBS, 1% Pen/strep, 50mg/mL L-ascorbic acid (Sigma), 10mM glycerol-2-phosphate (Sigma) and 100nM dexamethasone (Sigma) for 14 days. Cell expansion media were changed every other day.

Cell viability and proliferation test
For viability test, samples were stained with Live/Dead viability kit (Invitrogen) after 24 hours of cell seeding. Growth medium supplemented with 2μL calcein AM (component A) and 1μL ethidium homodimer-1 (component B) was added into C3H10T1/2 cells and incubated for 30 min for live/dead detection. Cells were fixed with 4% PFA and samples were imaged with confocal microscope (Zeiss LSM 700). To assess cell proliferation, Click-iT EdU Flow Cytometry Assay Kits (Invitrogen) were used according to manufacturer’s instruction. In brief, C3H10T1/2 cells on HAp and WH scaffolds were incubated with 0.1% 5-methynyl-2’-deoxyuridine (EdU) for 3 hours. Click-iT reaction cocktail were added on the scaffolds for 30min and washed with distilled water. To label total cell nucleus, 4’,6-diamidino-2-phenylindole (DAPI) was added after EdU staining.

**Scanning electron microscope analysis**

Cell-seeded scaffolds were fixed with 4% paraformaldehyde (Polysciences) for 1 hour, serially dehydrated with ethanol (Daejung chemical), and exchanged with hexamethyldisilazane (HMDS) (Daejung chemical) for 1 hours. Samples were visualized with Field Emission Scanning Electron Microscope (FE-SEM) (JSM-6701F, JEOL) after platinum coating.

**F-actin phalloidin staining**

C3H10T1/2 cells were seeded at 2 × 10⁶ cells per HAp and WH scaffold. After 24 hours, cells were fixed with 4% paraformaldehyde (Polysciences) and permeabilized with 0.1% Triton-X (sigma) for 15 min and immersed into 40 times diluted Alexa Fluor 594 Phalloidin (intivtrogen) in PBS (Gibco) for 1 hour. To label total cell nucleus, 4’,6-diamidino-2-
phenylindole (DAPI) was added after EdU staining. Confocal imaging was performed by LSM 700 series (Zeiss)

**Real-time PCR analysis**

Total RNA was collected by TRIzol method, and reverse transcribed into cDNA using TOPscript™ Reverse Transcriptase kit (Enzymomics) as manufacturer’s instruction. Real-time PCR were performed with TOPreal™ qPCR 2x PreMIX (Enzymomics) and the ABI StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA). cDNA samples were analyzed for the genes of interest, and GAPDH was used as a reference gene. The expression analysis was then evaluated as \(-2^{ΔΔCt}\) as previously described (51). Samples were run in triplicates, and PCR primers are listed in table 1.
3.3 Results

**Cell viability on HAp and WH scaffold**

We first examined the viability of the hydroxyapatite and whitlockite scaffolds. C3H10T1/2 cells were seeded onto both scaffolds and showed excellent biocompatibility as most of the seeded cells were live at day 1 (Fig 6.2.). However, WH scaffolds showed enhanced proliferation compared to Hap scaffolds. Cells on WH scaffold resulted in more S-phase nucleus cells (1.638 fold increase) compared to cells on HAp scaffold (Fig 6.2.). We further examined the cellular attachment on Hap and WH scaffolds. In order to account for the initial-stage of cellular attachment onto the scaffolds, cells were seeded onto the scaffolds and after 3 hours of seeding, samples were collected for total DNA content within the scaffolds. HAp scaffolds showed enhanced cellular attachment compared to WH scaffolds. HAp scaffolds resulted in 89% increased cell number compared to WH scaffolds (Fig 6.2.). We next examined the cellular morphology on the Hap and WH scaffolds. Morphological analysis was examined via fixing the cells and looking them under the SEM after 24 hours of seeding. Cellular spreading and morphology were indistinguishable between HAp and WH scaffolds. (Fig 6.3).
**Fig 6.1.** Image of fabricated hydroxyapatite and whitlockite scaffold, 

scale bar = 3mm

**Fig 6.2.** Live/dead assay and EdU staining images on hydroxyapatite and whitlockite scaffolds at day 1. Green is live cell and red is dead cell (Viability). 

Blue is nucleus of total cell and green is nucleus of S-phase cell (Proliferation), 

scale bar = 50μm
For real-time PCR analysis, C3H10T1/2 cells were seeded on tissue culture plate, HAp and WH scaffolds in osteogenic medium. After 14 days culture, both of HAp and WH scaffolds showed up to 150 times increased gene expression level of osteogenic markers compared to the result in tissue culture plate without the condition of calcium phosphate. In gene expression of ALP, type X collagen, osteocalcin, Runx2 and osterix, no significant differences were observed between the results of HAp and WH scaffolds (Fig 6.5)

*Fig 6.3. F-actin staining and SEM image on hydroxyapatite and whitlockite scaffolds.*

Blue is nucleus of total cell and red is F-actin staining, scale bar = 100μm

**RT-PCR analysis on HAp and WH scaffold**
**Fig 6.4.** Real time PCR analysis of C3H10T1/2 on tissue culture plate (ML), hydroxyapatite (HAp) and whitlockite (WH) in osteogenic medium after 14 days culture.
3.4 Discussion

Hydroxyapatite has been utilized in many years in bone tissue engineering scaffolds due to their osteo-inductive properties and mechanical properties. Even though hydroxyapatite resides in natural bone as one of the primary form of ECM, several decades old journals have reported whitlockite as another mineral composition within our native bone. Jang et al has resynthesized the exact composition of whitlockite and has shown that it may be utilized as an alternative functional mineral composition for bone tissue engineering (28). In this second part of the thesis, we have extended the whitlockite fabrication and examined the biological properties of whitlockite by molding it into disc-shaped scaffolds and further creating into porous composite-scaffolds with biodegradable polymers. Furthermore, we also have created hydroxyapatite scaffolds for comparison. In the results of this chapter, both of hydroxyapatite and whitlockite scaffolds showed a similar level of biocompatibility and enhanced osteogenic differentiation of C3H10T1/2. However, cells on whitlockite scaffolds showed enhanced proliferation rate while presenting decreased seeding efficiency. From this result, it is hypothesized that magnesium form on whitlockite scaffolds may affect cellular behavior and further observation about releasing profile of magnesium ion into the culture medium will be needed.
Chapter 4. Conclusion and future direction

In this thesis, we first provided the evidence that CM expansion can enhance osteogenic differentiation of mesenchymal stem cells both of in vivo and in vitro. In addition, via pDA coating strategy, we created highly mineralized scaffolds for bone tissue engineering. From the first part of the thesis, it may be concluded that the incorporation of autologous mesenchymal stem cells and chondrocyte-conditioned factors might be useful for the treatment of bone disease. Even though it was beyond the scope of the current thesis, identification of CM factors (proteins or soluble factors responsible for osteo-priming) and their downstream signaling targets will be needed in order to utilize CM factor in clinical setting. Furthermore, screening of CM factors via proteomics approach may help to find a new candidate for bone healing and to identify unknown signaling pathway related with osteogenic differentiation.

In the second part of the thesis, we have compared hydroxyapatite and whitlockite-based scaffolds for osteogenic differentiation of MSCs. By plating murine MSCs onto whitlockite composite scaffolds, enhanced osteo-inductivity of whitlockite was confirmed. In addition, to apply in bone tissue engineering, composite and porous scaffolds containing whitlockite and biodegradable polymers are currently being synthesized and fabricated. Whitlockite-based composite scaffolds are being currently investigated for their bone-inducing abilities in vivo. Successful application of whitlockite-based composite scaffolds for in vivo bone regeneration may provide alternative to hydroxyapatite for bone regeneration.
Reference

poly(lactic acid), and a blend. J Controlled Release.81:57-64. 2002.


41. Bashir J, Sherman A, Lee H, Kaplan L, Hare JM. Mesenchymal stem cell therapies in the
42. Gerstenfeld LC, Cruceta J, Shea CM, Sampath K, Barnes GL, Einhorn TA. Chondrocytes
provide morphogenic signals that selectively induce osteogenic differentiation of mesenchymal
stem cells. Journal of bone and mineral research : the official journal of the American Society for
43. Lin GL, Hankenson KD. Integration of BMP, Wnt, and notch signaling pathways in
44. Lamplot JD, Qin J, Nan G, Wang J, Liu X, Yin L, et al. BMP9 signaling in stem cell
osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. Experimental and
therapeutic medicine.7:625-9. 2014.
Springerplus.3:80. 2014.
47. Daniel JC, Pauli BU, Kuettner KE. Synthesis of cartilage matrix by mammalian
48. Levenberg S, Huang NF, Lavik E, Rogers AB, Itskovitz-Eldor J, Langer R. Differentiation of
human embryonic stem cells on three-dimensional polymer scaffolds. Proc Natl Acad Sci U S
49. Kaplow LS. A histochemical procedure for localizing and evaluating leukocyte alkaline
50. Ackerman GA. Substituted naphthol AS phosphate derivatives for the localization of
51. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
52. Asami G, Dock W. Experimental Studies on Heteroplastic Bone Formation. The Journal of
53. Brighton CT, Sugioka Y, Hunt RM. Cytoplasmic Structures of Epiphyseal Plate Chondrocytes -
Quantitative Evaluation Using Electron Micrographs of Rat Costochondral Junctions with Special
Reference to Fate of Hypertrophic Cells. Journal of Bone and Joint Surgery-American Volume.A
54. Hwang NS, Varghese S, Puleo C, Zhang Z, Elisseeff J. Morphogenetic signals from
chondrocytes promote chondrogenic and osteogenic differentiation of mesenchymal stem cells. J
Conditioned medium as a strategy for human stem cells chondrogenic differentiation. J Tissue Eng


요약 (국문초록)

연골세포 조정배지와 결정화된 지지체를 활용한 중간엽 줄기세포의 골 조직분화

발생단계의 연골내골화 과정에서 물령뼈조직이 연계된 일련의 조절 기작은 중요한 역할을 수행한다. 위와 같은 미세 환경을 조성하기 위해 본 연구에서는 쥐의 중간엽 세포인 C3H10T1/2 세포주를 연골세포 조정배지에서 배양한 뒤 골 분화 배지를 처리하여 골 조직분화를 유도하였다. 연골세포 조정배지에서의 배양을 시행했을 때 C3H10T1/2 세포주는 향상된 아그리간, 2형 및 10형 콜라겐, Sox9 등의 연골분화 관련 유전자의 발현 정도를 보였지만 ALP, 1형 콜라겐, 오스테오칼신, Runx2 등의 골분화 관련 유전자의 발현에 있어서는 오히려 감소된 발현 정도를 보였다. 그러나 이후 시행된 골 분화 배지를 이용한 배양에서는 연골세포 조정배지에서 배양되었던 C3H10T1/2 세포가 해당 단계를 거쳐지 않은 세포에 비해 향상된 ALP 및 Alizarin red S 염색 결과를 나타내었고 높은 골분화 관련 유전자의 발현 정도를 나타내었다. 결정화된 지지체에서의 세포의 골분화 정도를 확인하기 위해 본 실험에서는 종합접착 유래 폴리도포만과 의사체액을 이용하여 지지체의 결정화를 유도하였고 C3H10T1/2 세포주를 적용한 뒤 생쥐 두개골 결손 모델에 적용하여 생체 내에서의 골 조직 형성을 확인하였다. 마이크로 CT 기법과 Masson’s trichrome 염색법을 통해 생체 내에서 연골세포 조정배지에서 배양된 C3H10T1/2 세포주가 적용된 지지체가 그렇지 않은 지지체보다 더 향상된 골 조직 형성을 수행했으며 실험실 단계의 실험 결과와 유사성을 확보하였다. 이를 통해 본 연구에서는 연골세포 조정배지와 결정화된 지지체가 중간엽 줄기세포의 골 조직 형성에 기여
한다는 것을 확인하였다.

주요어: 생체 모방, 골 분화, 조정배지, 의사체액, 지지체, 생체 재료, 생체내

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