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Introduction

Mesenchymal stromal cells (MSCs) show great potential for therapeutic use in tissue engineering due to their immunoregulatory and paracrine effects as well as their ability to transdifferentiate (Parekkadan et al., 2010; Uccelli et al., 2008). However, injection of a dissociated single-cell suspension of MSCs to deliver cells to target tissues has the disadvantage of the cells disappearing from the system soon after transplantation (Matsuura et al., 2014). To overcome this obstacle, Okano and coworkers developed a cell sheet technique to improve the therapeutic potential of MSCs. These cell sheets could be detached intact from the culture dish at lower temperatures (Egami et al., 2014; Kim et al., 2005), enabling the retention of abundant endogenous extracellular matrix (ECM) and proteins on the cell surface. In addition, an intact cell sheet structure ensures homeostasis of the cellular microenvironment (Akahane et al., 2008; Egami et al., 2014; Guo et al., 2015; Inagaki et al., 2013; Ma et al., 2010; Nakamura et al., 2010;

Pirracco et al., 2011; Yang et al., 2007) while delivering growth factors and cytokines over a prolonged period of time to promote tissue repair (Matsuura et al., 2014).

The cell sheet technique has been applied to repair several diseased organs such as large lesions of the esophagus, impaired periodontal ligaments, and various thin-layered tissues, including the cornea (Nishida et al., 2004; Ohki et al., 2012). Cell sheet transplantation has been used to repair damage in three-dimensional thickened tissues such as the heart muscle (Sekine et al., 2011). Recently, functional recovery of the liver and pancreas was evaluated following cell sheet transplantation therapy (Ohashi et al., 2007; Saito et al., 2011). Osteogenically differentiated MSC sheets exhibit osteogenic potential *in vitro* (Akahane et al., 2008; Guo et al., 2015; Ma et al., 2010; Nakamura et al., 2010; Wei et al., 2012). In addition, direct application of osteogenic cell sheets to damaged bone promoted bone repair (Inagaki et al., 2013; Nakamura et al., 2010; Pirracco et al., 2011; Uchiyama et al., 2011), and osteogenic cell sheets implanted under subcutaneous tissues

demonstrated *in vivo* bone formation (Akahane et al., 2008; Ma et al., 2010). However, the optimal time needed to culture cell sheets for therapeutic applications as well as the requirement of osteogenic differentiation medium to culture MSCs for therapeutic use have not been elucidated. Therefore, in the present study I determined the culture time needed to ensure the highest osteoblastic activity of cells as well high levels of matrix formation. I also compared the osteogenic potential of osteogenic differentiated and undifferentiated cell sheets.

Materials and Methods

Isolation and culture of adipose tissue – derived (Ad)-MSCs

Canine Ad-MSCs were harvested according to the methods described in our previous paper (Ryu et al., 2009). In brief, adipose tissues were collected aseptically from the gluteal subcutaneous fat of 2-year-old beagle dogs (4 females). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-141210-1), Korea. Adipose tissue (approximately 1 g) was washed extensively with Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY, USA) and then minced with scissors. The minced tissue was digested with 1 mg/ml collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37 °C. The samples were then washed with DPBS and centrifuged at $980 \times g$ for 10 min. The resulting pellet of the stromal vascular fraction was resuspended by DPBS and filtered through a 100- μm nylon mesh,

followed by overnight incubation in low-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone) at 37 °C in a 5% CO₂ humidified atmosphere. After 24 h, the unattached cells and residual non-adherent red blood cells were removed by washing with DPBS. The medium was changed at 48-h intervals until the cells became confluent. The cells were subcultured after they reached 90% confluence and the cells at passage 3 were used for the experiments (Kang et al., 2013).

Cell seeding and harvesting

Cells were seeded in tissue culture polystyrene dishes and cultured in low-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (HyClone) at 37 °C in a 5% CO₂ humidified atmosphere (basal medium). After the cells reached 60–70% confluence, the basal medium was changed to a different medium based on the desired experimental conditions. The undifferentiated cells (UC) were cultured in basal medium until reaching 100% confluence. The MSCs for

undifferentiated cell sheets (U-CS) were cultured in basal medium supplemented with 50 µg/ml L-ascorbic acid 2-phosphate (Asc; Sigma-Aldrich). The MSCs for osteogenic differentiated cell sheets (O-CS) were cultured in high-glucose DMEM supplemented with 10% FBS, antibiotics, 10^{-7} M dexamethasone (Dex; Sigma-Aldrich), and 50 µg/ml Asc. Both the cells and cell sheets were harvested at 4, 7, 14, and 21 days of culture.

Morphological examination

ECM formation and morphological changes occurring in cells during culture were monitored under an inverted light microscope (model CKX41SF, Olympus, Tokyo, Japan) and images were captured with a digital camera (model E-330, Olympus, Tokyo, Japan).

Reverse transcription – polymerase chain reaction

Total RNA was extracted using a Hybrid-R RNA Extraction Kit (GeneAll, Seoul, Republic of Korea) and the RNA concentrations were

determined by measuring light absorbance at 260 nm using ImplenNanoPhotometer (model 1443, Implen GmbH, Munich, Germany). PrimeScript II First-strand cDNA Synthesis Kit (Takara, Otsu, Japan) was used to synthesize cDNA using 1000 ng of total RNA as template, and 1 μ l of cDNA was amplified by PCR using the T3000 Thermocycler (Whatman, Biometra, Biomedizinische Analytik GmbH, Goettingen, Germany). The PCR mixture (25 μ l) contained 12.5 μ l of PCR Premix (EmeraldAmpTM PCR Master Mix, Takara), 0.2 μ M forward primer and 0.2 μ M reverse primer (Standard Oligo, Bioneer, Daejeon, Korea), 1 μ l of cDNA, and 9.5 μ l of distilled water. The primer sequences used for transforming growth factor- β (*TGF- β*), bone morphogenetic protein-7 (*BMP7*), runt-related transcription factor-2 (*RUNX2*), alkaline phosphatase (*ALP*), collagen1 α -1 (*COL1 α -1*), and osteocalcin (*OCN*) are shown in Table 1. The mRNA levels for each gene were normalized to that of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The PCR conditions used were: denaturation at 94 °C (5 min), 27 to 37 cycles at 94 °C (1 min), annealing at 50–60 °C

(1 min), extension at 72 °C (1 min), and a final elongation at 72 °C. The PCR products were analyzed by electrophoresis on a 2% agarose gel (LE agarose, Dongin, Seoul, Republic of Korea). Redsafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Korea) was used to stain the 2% agarose gel. The longitudinal sections of the visualized gel were analyzed using ImageJ (version 1.37, National Institutes of Health, Bethesda, MD, USA). All transcripts obtained from cultured cells were compared to those obtained from 4-day cultures of UC.

Table 1. Specific primers for reverse-transcription polymerase chain reaction with amplicon sizes and optimal annealing temperatures.

Gene		Primer sequence (5'-3')	Amplicon size (base pair)	Ann. Temp. (°C)
<i>GAPDH</i>	Forward	CATTGCCCTCAATGACCACT	104	58
	Reverse	TCCTTGGAGGCCATGTAGAC		
<i>TGF-β</i>	Forward	CTCAGTGCCCACTGTTCCCTG	215	58
	Reverse	TCCGTGGAGCTGAAGCAGTA		
<i>BMP7</i>	Forward	TCGTGGAGCATGACAAAGAG	370	58
	Reverse	AACTTGGGGTTGATGCTCTG		
<i>RUNX2</i>	Forward	CGCATTCCTCATCCCAGTAT	768	58
	Reverse	GGCCACTGCTGAGGAATTT		
<i>ALP</i>	Forward	CCGAGACACAAGCACTCTCA	843	58
	Reverse	GCTGGCCATCTGTCATAGGT		
<i>COL1a-1</i>	Forward	GTAGACACCACCCTCAAGAG	119	58
	Reverse	TTCCAGTCGGAGTGGCACAT		
<i>OCN</i>	Forward	CTGCTCACAGACCCAGACAG	449	60
	Reverse	GATGACAAGGACCCCACT		

GAPDH: glyceraldehyde-3-phosphate dehydrogenase, *TGF-β*: transforming growth factor-β, *BMP7*: bone morphogenetic protein-7, *RUNX2*: runt-related transcription factor-2, *ALP*: alkaline phosphatase, *COL1a-1*: collagen1α-1, *OCN*: osteocalcin

Alkaline phosphatase activity measurement

ALP activity in cells and cell sheets cultured in 100 × 20-mm cell culture dishes was measured using an ALP activity colorimetric assay kit (Biovision, San Francisco, CA, USA). Cells were washed with DPBS, detached using a cell scraper, and sonicated in 300 μl of assay buffer. After sonication, the solution was centrifuged at 13,000 ×g for 3 min at 4 °C, and 80 μl of the supernatant was added to 50 μl of *p*-nitrophenyl phosphate substrate in a 96-well plate followed by incubation for 1 h at room temperature (22–25 °C) in the dark. Stop solution (20 μl) was added to the colored samples and the absorbance of each well was measured at 415 nm with a microplate reader (model 680, Bio-Rad, Hercules, CA, USA).

Detection of mineralization

In Alizarin Red S (ARS; Sigma-Aldrich) staining, cells and cell sheets cultured in 6-well plates were washed with DPBS twice and fixed in 4% paraformaldehyde (Wako, Tokyo, Japan) at room temperature for 10 min. The cells were then washed twice with distilled water, and 1 ml of 40 mM 2% ARS (pH 4.1–4.3) was added to each well. The plates were then incubated at room temperature for 20 min with gentle shaking. Any unincorporated dye was aspirated from the wells and the wells were washed four times with distilled water (Gregory et al., 2004).

The calcium content per well was measured using a calcium colorimetric assay kit (Biovision) based on the ortho-cresolphthalein complexone method. Cells and cell sheets cultured in 6-well plates were washed in DPBS twice and incubated in 0.5 ml of 10% formic acid (Sigma-Aldrich) and 0.5 ml of 0.2% IGEPAL CA-630 (Sigma-Aldrich) containing 1 mM $MgCl_2$ for 24 h at 4 °C with gentle shaking (Akahane et al., 2008). The chromogenic reagent (90 μ l) and calcium assay buffer (60 μ l) were added to 50 μ l of each extract and mixed well. Samples

were incubated for 10 min at room temperature and the optical density was measured at 540 nm using a microplate reader (Kang et al., 2013).

Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). The Kruskal–Wallis test was used to analyze differences among the groups. A post-hoc test and the Mann–Whitney U test were used to confirm the differences among groups. A P -value < 0.05 was considered to be statistically significant.

Discussion

The addition of 50 µg/ml of Asc to the culture medium stimulated the formation of cell sheets between 2 and 4 days of culture. Previous studies have shown that Asc supplementation to culture medium stimulated cell sheets to form multiple layers and secrete abundant ECM (Guo et al., 2015; Wei et al., 2012; Yu et al., 2014). Culturing cells in tissue culture polystyrene dishes preserves the structure of the ECM even after detaching cells using a cell scraper (Akahane et al., 2008; Guo et al., 2015; Inagaki et al., 2013; Ma et al., 2010; Nakamura et al., 2010). Similar to some previous reports, we observed that the edges of the cell sheets started to fold away from the cell culture dish after 7–10 days of culture (Guo et al., 2015). Further, the cells in O-CS started to aggregate at 10–12 days and only a partial matrix was observed by 21 days of culture. The cellular aggregation that occurs during osteogenic differentiation *in vitro* (Hakki et al., 2014; Vieira et al., 2010) could have contributed to the partial loss of the matrix observed in the present

study. Therefore, the O-CS sheets cannot be used for therapeutic application after 10–12 days of culture.

MSCs express many growth factors, including TGF- β (Uccelli et al., 2008). TGF- β 1 promotes the proliferation of MSCs and regulates the formation and degradation of the ECM (Chin et al., 2004; Phillips et al., 1992). TGF- β isoforms are stored in the ECM and interact with ECM proteins (Dallas et al., 2002). The significant increase in *TGF- β* expression observed in both U-CS and O-CS at 4 days of culture compared to UC was probably due to higher TGF- β secretion into the ECM from the MSCs.

BMP7 expression in O-CS was up-regulated after 4 days of culture, demonstrating that osteogenic medium facilitates osteogenic differentiation. In addition, the up-regulation of *BMP7* in U-CS at 7 days of culture could be attributed to TGF- β -driven osteogenic differentiation. The coordination of *RUNX2* and BMP/TGF- β signaling is critical for the early stages of bone formation. TGF- β and *BMP7* signaling induce osteoblast differentiation through several pathways

such as SMAD, MAPK, and Wnt, all of which converge at the *RUNX2* gene to facilitate mesenchymal precursor cell differentiation (Chen et al., 2012; Dallas et al., 2002; Komori et al., 2005; Tou et al., 2003). In the present study, *RUNX2* expression in both U-CS and O-CS was up-regulated at day 7, followed by up-regulation of *TGF- β* and *BMP7*. *RUNX2* regulates the commitment of mesenchymal cells to an osteoblast lineage and induces the expression of genes encoding bone matrix proteins such as COL1 α -1 and ALP (Komori et al., 2005; Komori et al., 2006; Lee et al., 2000). The ALP mRNA expression and activity at the protein level as well as *COL1 α -1* expression were up-regulated from 7 days of culture in O-CS.

The level of ALP activity increases during ECM maturation; the initiation of mineralization correlates with the peak in ALP activity, and mineralization correlates with a decrease in ALP activity (Chang et al., 2006; Chang et al., 2000; Lian et al., 1992; Mathews et al., 2012; Sabokbar et al., 1994). The peaks of ALP activity in U-CS and O-CS were observed at 14 and 7 days of culture, respectively. This suggests

that the osteogenic ECM maturation of O-CS was earlier than that of U-CS. MSCs cultured in osteogenic medium without Dex have been reported to exhibit higher ALP activity and to mineralize, although after a delay of 1 week relative to Dex-treated cultures (Hoemann et al., 2009). Dex in osteogenic medium could accelerate RUNX2-related transcriptional changes by facilitating DNA binding of RUNX2 (Komori et al., 2005). In the present study, the use of O-CS medium resulted in faster differentiation compared to the use of U-CS medium.

After ECM maturation, mineralization occurs by deposition of calcium and phosphate (Park et al., 2007). However, in the present study, the small amount of mineral deposition in both sheets was not sufficient to be detected by ARS staining. RUNX2 inhibits the expression of OCN during the maturation phase of osteoblasts (Komori et al., 2006). O-CS and U-CS, which progressed later than O-CS, might differentiate until the maturation phase, so that mineralization of neither sheet had yet occurred.

In the present study, ALP activity and the mRNA expression of *RUNX2*,

ALP, and *COL1a-1* indicated that U-CS and O-CS show osteogenic potential in the stage of ECM maturation. Considering the observed peak in ALP activity and morphological changes, the optimal times for application of O-CS and U-CS are between 7 and 10 days and after 14 days of culture, respectively.

References

- Akahane, M.; A. Nakamura, H.; Ohgushi, H.; Shigematsu, Y.; Dohi, Y.; Takakura, Y. Osteogenic matrix sheet-cell transplantation using osteoblastic cell sheet resulted in bone formation without scaffold at an ectopic site. *J. Tissue Eng. Regen. Med.* 2(4):196-201; 2008.
- Chang, P. L.; Blair, H. C.; Zhao, X. C.; Chien, Y.-W.; Chen, D.; Tilden, A. B.; Chang, Z. J.; Cao, X.; Faye-Petersen, O. M.; Hicks, P. Comparison of fetal and adult marrow stromal cells in osteogenesis with and without glucocorticoids. *Connect Tissue Res.* 47(2):67-76; 2006.
- Chang, Y. L.; Stanford, C. M.; Keller, J. C. Calcium and phosphate supplementation promotes bone cell mineralization: Implications for hydroxyapatite (HA)-enhanced bone formation. *J. Biomed. Mater. Res.* 52(2):270-278; 2000.
- Chen, G. Q.; Deng, C. X.; Li, Y. P. TGF-beta and BMP signaling in osteoblast differentiation and bone formation. *Int. J. Biol. Sci.* 8(2):272-288; 2012.
- Chin, D.; Boyle, G. M.; Parsons, P. G.; Coman, W. B. What is transforming growth factor-beta (TGF-beta)? *Br. J. Plast. Surg.* 57(3):215-221; 2004.

- Dallas, S. L.; Rosser, J. L.; Mundy, G. R.; Bonewald, L. F. Proteolysis of latent transforming growth factor-beta (TGF-beta)-binding protein-1 by osteoclasts - A cellular mechanism for release of TGF-beta from bone matrix. *J. Biol. Chem.* 277(24):21352-21360; 2002.
- Egami, M.; Haraguchi, Y.; Shimizu, T.; Yamato, Y.; Okano, T. Latest status of the clinical and industrial applications of cell sheet engineering and regenerative medicine. *Arch. Pharm. Res.* 37(1):96-106; 2014.
- Gregory, C. A.; Gunn, W. G.; Peister, A.; Prockop, D. J. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal. Biochem.* 329(1):77-84; 2004.
- Guo, P.; Zeng, J. J.; Zhou, N. A novel experimental study on the fabrication and biological characteristics of canine bone marrow mesenchymal stem cells sheet using vitamin C. *Scanning* 37(1):42-48; 2015.
- Hakki, S. S.; Bozkurt, B.; Hakki, E. E.; Kayis, S. A.; Turac, G.; Yilmaz, I.; Karaoz, E. Bone morphogenetic protein-2, -6, and -7 differently regulate osteogenic differentiation of human periodontal ligament stem cells. *J. Biomed. Mater. Res. B Appl. Biomater.* 102(1):119-130; 2014.

- Hoemann, C. D.; El-Gabalawy, H.; McKee, M. D. In vitro osteogenesis assays: Influence of the primary cell source on alkaline phosphatase activity and mineralization. *Pathol. Biol(Paris)*. 57(4):318-323; 2009.
- Inagaki, Y.; Uematsu, K.; Akahane, M.; Morita, Y.; Ogawa, M.; Ueha, T.; Shimizu, T.; Kura, T.; Kawate, K.; Tanaka, T. Osteogenic matrix cell sheet transplantation enhances early tendon graft to bone tunnel healing in rabbits. *Biomed. Res. Int.* 2013:842192; 2013.
- Kang, B.-J.; Kim, Y.; Lee, S. H.; Kim, W. H.; Woo, H.-M.; Kweon, O.-K. Collagen I gel promotes homogenous osteogenic differentiation of adipose tissue-derived mesenchymal stem cells in serum-derived albumin scaffold. *J. Biomater. Sci. Polym. Ed.* 24(10):1233-1243; 2013.
- Kim, Y. S.; Lim, J. Y.; Donahue, H. J.; Lowe, T. L. Thermoresponsive terpolymeric films applicable for osteoblastic cell growth and noninvasive cell sheet harvesting. *Tissue Eng.* 11(1-2):30-40; 2005.
- Komori, T. Regulation of skeletal development by the runx family of transcription factors. *J. Cell. Biochem.* 95(3):445-453; 2005.
- Komori, T. Regulation of osteoblast differentiation by transcription factors. *J. Cell. Biochem.* 99(5):1233-1239; 2006.

- Lee, K. S.; Kim, H. J.; Li, Q. L.; Chi, X. Z.; Ueta, C.; Komori, T.; Wozney, J. M.; Kim, E. G.; Choi, J. Y.; Ryoo, H. M.; Bae, S. C. Runx2 is a common target of transforming growth factor beta 1 and bone morphogenetic protein 2, and cooperation between runx2 and smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol. Cell. Biol.* 20(23):8783-8792; 2000.
- Lian, J. B.; G. S. Stein. Concepts of osteoblast growth and differentiation: Basis for modulation of bone cell development and tissue formation. *Crit. Rev. Oral Biol. Med.* 3(3):269-305; 1992.
- Ma, D.; Ren, L.; Liu, Y.; Chen, F.; Zhang, J.; Xue, Z.; Mao, T. Engineering scaffold-free bone tissue using bone marrow stromal cell sheets. *J. Orthop. Res.* 28(5):697-702; 2010.
- Mathews, S.; Bhonde, R.; Gupta, P. K.; Totey, S. Extracellular matrix protein mediated regulation of the osteoblast differentiation of bone marrow derived human mesenchymal stem cells. *Differentiation* 84(2):185-192; 2012.
- Matsuura, K.; Utoh, R.; Nagase, K.; Okano, T. Cell sheet approach for tissue engineering and regenerative medicine. *J. Control. Release.* 190:228-239; 2014.

- Nakamura, A.; Akahane, M.; Shigematsu, H.; Tadokoro, M.; Morita, Y.; Ohgushi, H.; Dohi, Y.; Imamura, T.; Tanaka, Y. Cell sheet transplantation of cultured mesenchymal stem cells enhances bone formation in a rat nonunion model. *Bone* 46(2):418-424; 2010.
- Nishida, K.; M. Yamato, M.; Hayashida, Y.; Watanabe, K.; Yamamoto, K.; Adachi, E.; Nagai, S.; Kikuchi, A.; Maeda, N.; Watanabe, H.; Okano, T.; Tano, Y. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N. Engl. J. Med.* 351(12):1187-1196; 2004.
- Ohashi, K.; Yokoyama, T.; Yamato, M.; Kuge, H.; Kanehiro, H.; Tsutsumi, M.; Amanuma, T.; Iwata, H.; Yang, J.; Okano, T.; Nakajima, Y. Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. *Nat. Med.* 13(7):880-885; 2007.
- Ohki, T.; Yamato, M.; Ota, M.; Takagi, R.; Murakami, D.; Kondo, M.; Sasaki, R.; Namiki, H.; Okano, T.; Yamamoto, M. Prevention of esophageal stricture after endoscopic submucosal dissection using tissue-engineered cell sheets. *Gastroenterology* 143(3):582-U556; 2012.
- Parekkadan, B.; Milwid, J. M. Mesenchymal stem cells as therapeutics. *Annu. Rev. Biomed. Eng.* 12:87-117; 2010.

- Park, B.-W.; Hah, Y.-S.; Kim, D. R.; Kim, J.-R.; Byun, J.-H. Osteogenic phenotypes and mineralization of cultured human periosteal-derived cells. *Arch. Oral. Biol.* 52(10):983-989; 2007.
- Phillips, C. L.; Tajima, S.; Pinnell, S. R. Ascorbic-acid and transforming growth factor-beta-1 increase collagen biosynthesis via different mechanisms: Coordinate regulation of pro-alpha-1(I) and pro-alpha-1(III) collagens. *Arch. Biochem. Biophys.* 295(2):397-403; 1992.
- Pirracò, R. P.; Obokata, H.; Iwata, T.; Marques, A. P.; Tsuneda, S.; Yamato, M.; Reis, R. L.; Okano, T. Development of osteogenic cell sheets for bone tissue engineering applications. *Tissue Eng. Part A.* 17(11-12):1507-1515; 2011.
- Ryu, H.-H.; Lim, J.-H.; Byeon, Y.-E.; Park, J.-R.; Seo, M.-S.; Lee, Y.-W.; Kim, W. H.; Kang, K.-S.; Kweon, O.-K. Functional recovery and neural differentiation after transplantation of allogenic adipose-derived stem cells in a canine model of acute spinal cord injury. *J. Vet. Sci.* 10(4):273-284; 2009.
- Sabokbar, A.; Millett, P. J.; Myer, B.; Rushton, N. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. *Bone Miner.* 27(1):57-67; 1994.
- Saito, T.; Ohashi, K.; Utoh, R.; Shimizu, H.; Ise, K.; Suzuki, H.; Yamato, M.; Okano, T.; Gotoh, M. Reversal of diabetes by the creation of

- neo-islet tissues into a subcutaneous site using islet cell sheets. *Transplantation* 92(11):1231-1236; 2011.
- Sekine, H.; Shimizu, T.; Dobashi, I.; Matsuura, K.; Hagiwara, N.; Takahashi, M.; Kobayashi, E.; Yamato, M.; Okano, T. Cardiac cell sheet transplantation improves damaged heart function via superior cell survival in comparison with dissociated cell injection. *Tissue Eng. Part A.* 17(23-24):2973-2980; 2011.
- Tou, L.; Quibria, N.; Alexander, J. M. Transcriptional regulation of the human *runx2/Cbfa1* gene promoter by bone morphogenetic protein-7. *Mol. Cell. Endocrinol.* 205(1-2):121-129; 2003.
- Uccelli, A.; Moretta, L.; Pistoia, V. Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.* 8(9):726-736; 2008.
- Uchiyama, H.; Yamato, M.; Sasaki, R.; Sekine, H.; Yang, J.; Ogiuchi, H.; Ando, T.; Okano, T. In vivo 3D analysis with micro-computed tomography of rat calvaria bone regeneration using periosteal cell sheets fabricated on temperature-responsive culture dishes. *J. Tissue Eng. Regen. Med.* 5(6):483-490; 2011.
- Vieira, N. M.; Brandalise, V.; Zucconi, E.; Secco, M.; Strauss, B. E.; Zatz, M. Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. *Cell Transplant.* 19(3):279-289; 2010.

- Wei, F. L.; Qu, C. Y.; Song, T. L.; Ding, G.; Fan, Z. P.; Liu, D. Y.; Liu, Y.; Zhang, C. M.; Shi, S. T.; Wang, S. L. Vitamin C treatment promotes mesenchymal stem cell sheet formation and tissue regeneration by elevating telomerase activity. *J. Cell. Physiol.* 227(9):3216–3224; 2012.
- Yang, J.; Yamato, M.; Shimizu, T.; Sekine, H.; Ohashi, K.; Kanzaki, M.; Ohki, T.; Nishida, K.; Okano, T. Reconstruction of functional tissues with cell sheet engineering. *Biomaterials* 28(34):5033–5043; 2007.
- Yu, J. S.; Tu, Y. K.; Tang, Y. B.; Cheng, N. C. Sternness and transdifferentiation of adipose-derived stem cells using L-ascorbic acid 2-phosphate-induced cell sheet formation. *Biomaterials* 35(11):3516–3526; 2014.

국문초록

세포시트는 자가 유래 중간엽 줄기세포를 이용해서 손쉽게 만들 수 있으며, 지지체를 이용하지 않고 골재생을 유도할 수 있다. 본 연구는 중간엽 줄기세포를 이용해 만든 세포시트들의 골분화 능력을 평가하였다. 개의 지방세포 유래의 중간엽 줄기세포로 미분화셀시트(Undifferentiated cell sheets)와 골분화셀시트(Osteogenic differentiated cell sheets)를 만들었다. 미분화세포(Undifferentiated cells)는 대조군으로 사용되었다. 골분화의 평가는 배양 후 4, 7, 14, 21일에 알칼리인포스파타제(alkaline phosphatase activity; ALP activity)의 활성 정도와 역전사 중합효소 연쇄반응을 이용한 골분화와 관련된 유전자가 발현되는 정도로 평가하였다. 배양된 세포를 알리자린레드에스(Alizarin red S; ARS)를 이용하여 염색하였고, 세포의 칼슘농도를 직접 측정하였다.

4일 동안 배양한 미분화셀시트와 골분화셀시트는 형질전환성장인자-베타(transforming growth factor- β)의 메신저리보핵산(mRNA)의 발현정도가 미분화세포보다 유의적으로 높게 나타났다 ($p < 0.05$). 골형성단백질-7 (bone morphogenetic protein 7)의 메신저리보핵산의 발현량도 4일동안 배양한 골분화셀시트가 유의적으로 상승했으며, 7일 동안

배양한 골분화셀시트는 미분화세포와 7일동안 배양한 미분화셀시트보다 유의적인 상승을 보였다. 7일 동안 배양한 두 시트에서 Runt-related transcription factor-2의 메신저리보핵산의 발현정도가 유의적으로 상승했다. 알칼라인포스파타제의 메신저리보핵산의 발현 정도는 7일동안 배양한 두 시트에서 모두 유의적인 상승을 보였으며, 알칼라인포스파타제의 활성은 미분화 셀시트는 14일, 골분화셀시트에서는 7일차가 가장 높았다 ($p < 0.05$). 두 시트의 osteocalcin 메신저리보핵산은 21일 차에 유의적인 상승을 보였다. 알리자린레드에스는 모든 세포에서 염색되지 않았으나, 21일 동안 배양한 두 시트의 칼슘농도는 미약한 상승을 보였다. 골분화셀시트의 세포들은 배양 10 - 12 일 이후 부터 세포끼리 부분적으로 뭉치기 시작하였으며, 배양 21일에는 시트형태가 부분적으로만 남아있었다. 골분화와 관련된 유전자발현의 상승, 알칼라인포스파타제의 활성과 시트의 형태학적인 변화를 고려했을 때, 골분화셀시트는 7일에서 10일 사이, 미분화 셀시트는 배양 14일 이후에 적용하는 것이 이상적이다.

주요어: 개, 골분화능, 중간엽줄기세포, 셀시트, 골분화셀시트, 개 지방세포, 골 재생

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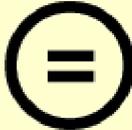
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Introduction

Mesenchymal stromal cells (MSCs) show great potential for therapeutic use in tissue engineering due to their immunoregulatory and paracrine effects as well as their ability to transdifferentiate (Parekkadan et al., 2010; Uccelli et al., 2008). However, injection of a dissociated single-cell suspension of MSCs to deliver cells to target tissues has the disadvantage of the cells disappearing from the system soon after transplantation (Matsuura et al., 2014). To overcome this obstacle, Okano and coworkers developed a cell sheet technique to improve the therapeutic potential of MSCs. These cell sheets could be detached intact from the culture dish at lower temperatures (Egami et al., 2014; Kim et al., 2005), enabling the retention of abundant endogenous extracellular matrix (ECM) and proteins on the cell surface. In addition, an intact cell sheet structure ensures homeostasis of the cellular microenvironment (Akahane et al., 2008; Egami et al., 2014; Guo et al., 2015; Inagaki et al., 2013; Ma et al., 2010; Nakamura et al., 2010;

Pirracco et al., 2011; Yang et al., 2007) while delivering growth factors and cytokines over a prolonged period of time to promote tissue repair (Matsuura et al., 2014).

The cell sheet technique has been applied to repair several diseased organs such as large lesions of the esophagus, impaired periodontal ligaments, and various thin-layered tissues, including the cornea (Nishida et al., 2004; Ohki et al., 2012). Cell sheet transplantation has been used to repair damage in three-dimensional thickened tissues such as the heart muscle (Sekine et al., 2011). Recently, functional recovery of the liver and pancreas was evaluated following cell sheet transplantation therapy (Ohashi et al., 2007; Saito et al., 2011). Osteogenically differentiated MSC sheets exhibit osteogenic potential *in vitro* (Akahane et al., 2008; Guo et al., 2015; Ma et al., 2010; Nakamura et al., 2010; Wei et al., 2012). In addition, direct application of osteogenic cell sheets to damaged bone promoted bone repair (Inagaki et al., 2013; Nakamura et al., 2010; Pirracco et al., 2011; Uchiyama et al., 2011), and osteogenic cell sheets implanted under subcutaneous tissues

demonstrated *in vivo* bone formation (Akahane et al., 2008; Ma et al., 2010). However, the optimal time needed to culture cell sheets for therapeutic applications as well as the requirement of osteogenic differentiation medium to culture MSCs for therapeutic use have not been elucidated. Therefore, in the present study I determined the culture time needed to ensure the highest osteoblastic activity of cells as well high levels of matrix formation. I also compared the osteogenic potential of osteogenic differentiated and undifferentiated cell sheets.

Materials and Methods

Isolation and culture of adipose tissue – derived (Ad)-MSCs

Canine Ad-MSCs were harvested according to the methods described in our previous paper (Ryu et al., 2009). In brief, adipose tissues were collected aseptically from the gluteal subcutaneous fat of 2-year-old beagle dogs (4 females). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-141210-1), Korea. Adipose tissue (approximately 1 g) was washed extensively with Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY, USA) and then minced with scissors. The minced tissue was digested with 1 mg/ml collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37 °C. The samples were then washed with DPBS and centrifuged at $980 \times g$ for 10 min. The resulting pellet of the stromal vascular fraction was resuspended by DPBS and filtered through a 100- μm nylon mesh,

followed by overnight incubation in low-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone) at 37 °C in a 5% CO₂ humidified atmosphere. After 24 h, the unattached cells and residual non-adherent red blood cells were removed by washing with DPBS. The medium was changed at 48-h intervals until the cells became confluent. The cells were subcultured after they reached 90% confluence and the cells at passage 3 were used for the experiments (Kang et al., 2013).

Cell seeding and harvesting

Cells were seeded in tissue culture polystyrene dishes and cultured in low-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (HyClone) at 37 °C in a 5% CO₂ humidified atmosphere (basal medium). After the cells reached 60–70% confluence, the basal medium was changed to a different medium based on the desired experimental conditions. The undifferentiated cells (UC) were cultured in basal medium until reaching 100% confluence. The MSCs for

undifferentiated cell sheets (U-CS) were cultured in basal medium supplemented with 50 µg/ml L-ascorbic acid 2-phosphate (Asc; Sigma-Aldrich). The MSCs for osteogenic differentiated cell sheets (O-CS) were cultured in high-glucose DMEM supplemented with 10% FBS, antibiotics, 10^{-7} M dexamethasone (Dex; Sigma-Aldrich), and 50 µg/ml Asc. Both the cells and cell sheets were harvested at 4, 7, 14, and 21 days of culture.

Morphological examination

ECM formation and morphological changes occurring in cells during culture were monitored under an inverted light microscope (model CKX41SF, Olympus, Tokyo, Japan) and images were captured with a digital camera (model E-330, Olympus, Tokyo, Japan).

Reverse transcription – polymerase chain reaction

Total RNA was extracted using a Hybrid-R RNA Extraction Kit (GeneAll, Seoul, Republic of Korea) and the RNA concentrations were

determined by measuring light absorbance at 260 nm using ImplenNanoPhotometer (model 1443, Implen GmbH, Munich, Germany). PrimeScript II First-strand cDNA Synthesis Kit (Takara, Otsu, Japan) was used to synthesize cDNA using 1000 ng of total RNA as template, and 1 μ l of cDNA was amplified by PCR using the T3000 Thermocycler (Whatman, Biometra, Biomedizinische Analytik GmbH, Goettingen, Germany). The PCR mixture (25 μ l) contained 12.5 μ l of PCR Premix (EmeraldAmpTM PCR Master Mix, Takara), 0.2 μ M forward primer and 0.2 μ M reverse primer (Standard Oligo, Bioneer, Daejeon, Korea), 1 μ l of cDNA, and 9.5 μ l of distilled water. The primer sequences used for transforming growth factor- β (*TGF- β*), bone morphogenetic protein-7 (*BMP7*), runt-related transcription factor-2 (*RUNX2*), alkaline phosphatase (*ALP*), collagen1 α -1 (*COL1 α -1*), and osteocalcin (*OCN*) are shown in Table 1. The mRNA levels for each gene were normalized to that of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The PCR conditions used were: denaturation at 94 °C (5 min), 27 to 37 cycles at 94 °C (1 min), annealing at 50–60 °C

(1 min), extension at 72 °C (1 min), and a final elongation at 72 °C. The PCR products were analyzed by electrophoresis on a 2% agarose gel (LE agarose, Dongin, Seoul, Republic of Korea). Redsafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Korea) was used to stain the 2% agarose gel. The longitudinal sections of the visualized gel were analyzed using ImageJ (version 1.37, National Institutes of Health, Bethesda, MD, USA). All transcripts obtained from cultured cells were compared to those obtained from 4-day cultures of UC.

Table 1. Specific primers for reverse-transcription polymerase chain reaction with amplicon sizes and optimal annealing temperatures.

Gene		Primer sequence (5'-3')	Amplicon size (base pair)	Ann. Temp. (°C)
<i>GAPDH</i>	Forward	CATTGCCCTCAATGACCACT	104	58
	Reverse	TCCTTGGAGGCCATGTAGAC		
<i>TGF-β</i>	Forward	CTCAGTGCCCACTGTTCCCTG	215	58
	Reverse	TCCGTGGAGCTGAAGCAGTA		
<i>BMP7</i>	Forward	TCGTGGAGCATGACAAAGAG	370	58
	Reverse	AACTTGGGGTTGATGCTCTG		
<i>RUNX2</i>	Forward	CGCATTCCTCATCCCAGTAT	768	58
	Reverse	GGCCACTGCTGAGGAATTT		
<i>ALP</i>	Forward	CCGAGACACAAGCACTCTCA	843	58
	Reverse	GCTGGCCATCTGTCATAGGT		
<i>COL1a-1</i>	Forward	GTAGACACCACCCTCAAGAG	119	58
	Reverse	TTCCAGTCGGAGTGGCACAT		
<i>OCN</i>	Forward	CTGCTCACAGACCCAGACAG	449	60
	Reverse	GATGACAAGGACCCCACT		

GAPDH: glyceraldehyde-3-phosphate dehydrogenase, *TGF-β*: transforming growth factor-β, *BMP7*: bone morphogenetic protein-7, *RUNX2*: runt-related transcription factor-2, *ALP*: alkaline phosphatase, *COL1a-1*: collagen1α-1, *OCN*: osteocalcin

Alkaline phosphatase activity measurement

ALP activity in cells and cell sheets cultured in 100 × 20-mm cell culture dishes was measured using an ALP activity colorimetric assay kit (Biovision, San Francisco, CA, USA). Cells were washed with DPBS, detached using a cell scraper, and sonicated in 300 μl of assay buffer. After sonication, the solution was centrifuged at 13,000 ×g for 3 min at 4 °C, and 80 μl of the supernatant was added to 50 μl of *p*-nitrophenyl phosphate substrate in a 96-well plate followed by incubation for 1 h at room temperature (22–25 °C) in the dark. Stop solution (20 μl) was added to the colored samples and the absorbance of each well was measured at 415 nm with a microplate reader (model 680, Bio-Rad, Hercules, CA, USA).

Detection of mineralization

In Alizarin Red S (ARS; Sigma-Aldrich) staining, cells and cell sheets cultured in 6-well plates were washed with DPBS twice and fixed in 4% paraformaldehyde (Wako, Tokyo, Japan) at room temperature for 10 min. The cells were then washed twice with distilled water, and 1 ml of 40 mM 2% ARS (pH 4.1–4.3) was added to each well. The plates were then incubated at room temperature for 20 min with gentle shaking. Any unincorporated dye was aspirated from the wells and the wells were washed four times with distilled water (Gregory et al., 2004).

The calcium content per well was measured using a calcium colorimetric assay kit (Biovision) based on the ortho-cresolphthalein complexone method. Cells and cell sheets cultured in 6-well plates were washed in DPBS twice and incubated in 0.5 ml of 10% formic acid (Sigma-Aldrich) and 0.5 ml of 0.2% IGEPAL CA-630 (Sigma-Aldrich) containing 1 mM MgCl₂ for 24 h at 4 °C with gentle shaking (Akahane et al., 2008). The chromogenic reagent (90 µl) and calcium assay buffer (60 µl) were added to 50 µl of each extract and mixed well. Samples

were incubated for 10 min at room temperature and the optical density was measured at 540 nm using a microplate reader (Kang et al., 2013).

Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). The Kruskal–Wallis test was used to analyze differences among the groups. A post-hoc test and the Mann–Whitney U test were used to confirm the differences among groups. A *P*-value < 0.05 was considered to be statistically significant.

Results

Morphological changes in cell sheets

UC reached confluence at 4 days of culture (Fig. 1A), whereas U-CS and O-CS cells formed a sheet between 2 and 4 days of culture (Fig. 1B, D). However, the O-CS cells started to aggregate partially at days 10–12 (Fig. 1E) and some intact O-CS remained at 21 days of culture (Fig. 1F). On the other hand, the U-CS maintained an intact matrix form until day 21 (Fig. 1C).

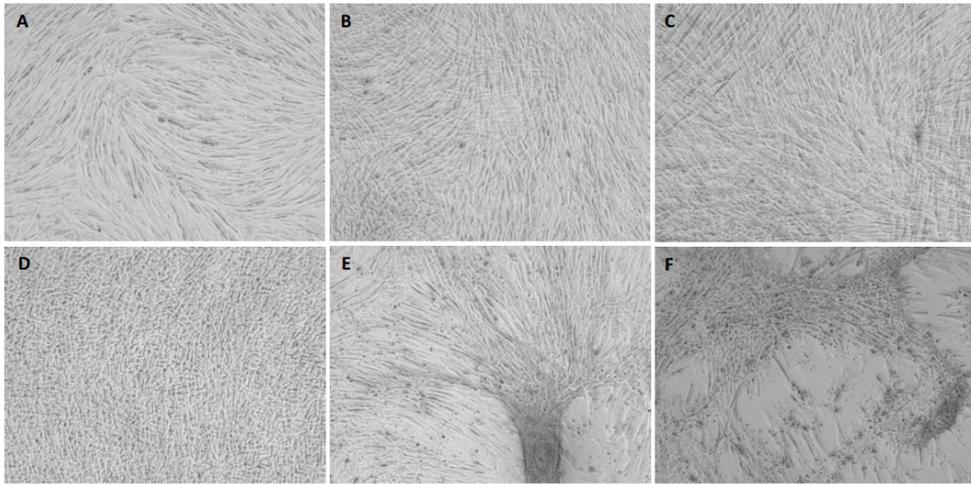


Figure 1. Morphology of canine adipose-derived mesenchymal stromal cells in different media. An inverted microscope was used; magnification, 400X. (A) Undifferentiated cells (UC) cultured in basal medium at 4 days. (B) Undifferentiated cell sheets (U-CS) cultured in basal medium containing 50 µg/ml L-ascorbic acid 2-phosphate (Asc) at 4 and (C) 21 days. (D) Osteogenic differentiated cell sheets (O-CS) cultured in basal medium containing 50 µg/ml Asc and 10^{-7} M dexamethasone (Dex) formed a matrix after 4 days. The U-CS after 21 days showed an intact cell matrix. (E) After 10 days, cells in the O-CS showed partial aggregation. (F) After 21 days, only partial matrix formation was observed in the O-CS.

Expression of genes related to osteogenic differentiation

TGF-β mRNA expression in U-CS and O-CS at 4 days of culture was significantly higher than that of UC ($p < 0.05$, Fig. 2A). The up-regulation in *TGF-β* expression in U-CS and O-CS was maintained until days 14 and 7, respectively. *BMP7* mRNA expression was significantly higher in O-CS after 4 days of culture than in UC, and the highest expression was observed at day 7 ($p < 0.05$, Fig. 2B). *BMP7* expression was also up-regulated in U-CS after 7 days of culture. After 14 days of culture, there was no significant difference in the *BMP7* expression in O-CS and U-CS. There was a significant increase in *RUNX2* mRNA expression at day 7 in both U-CS and O-CS compared to that in UC. *RUNX2* expression was significantly higher in O-CS at days 14 and 21 than in U-CS ($p < 0.05$, Fig. 2C). *ALP* mRNA expression in U-CS and O-CS was significantly up-regulated after 7 days of culture compared to that in UC ($p < 0.05$, Fig. 2D). *ALP* expression in O-CS peaked at day 7, whereas that in U-CS increased gradually until day 21. Up-regulation of *COL1a-1* mRNA expression was observed in U-CS only at 14 days of

culture ($p < 0.05$, Fig. 2E). *COL1a-1* expression of O-CS was up-regulated from day 7. Significant up-regulation of *OCN* expression was observed in U-CS at 4 days of culture compared to that in UC ($p < 0.05$, Fig. 2F). Significant up-regulation of *OCN* expression was observed in both U-CS and O-CS at day 21.

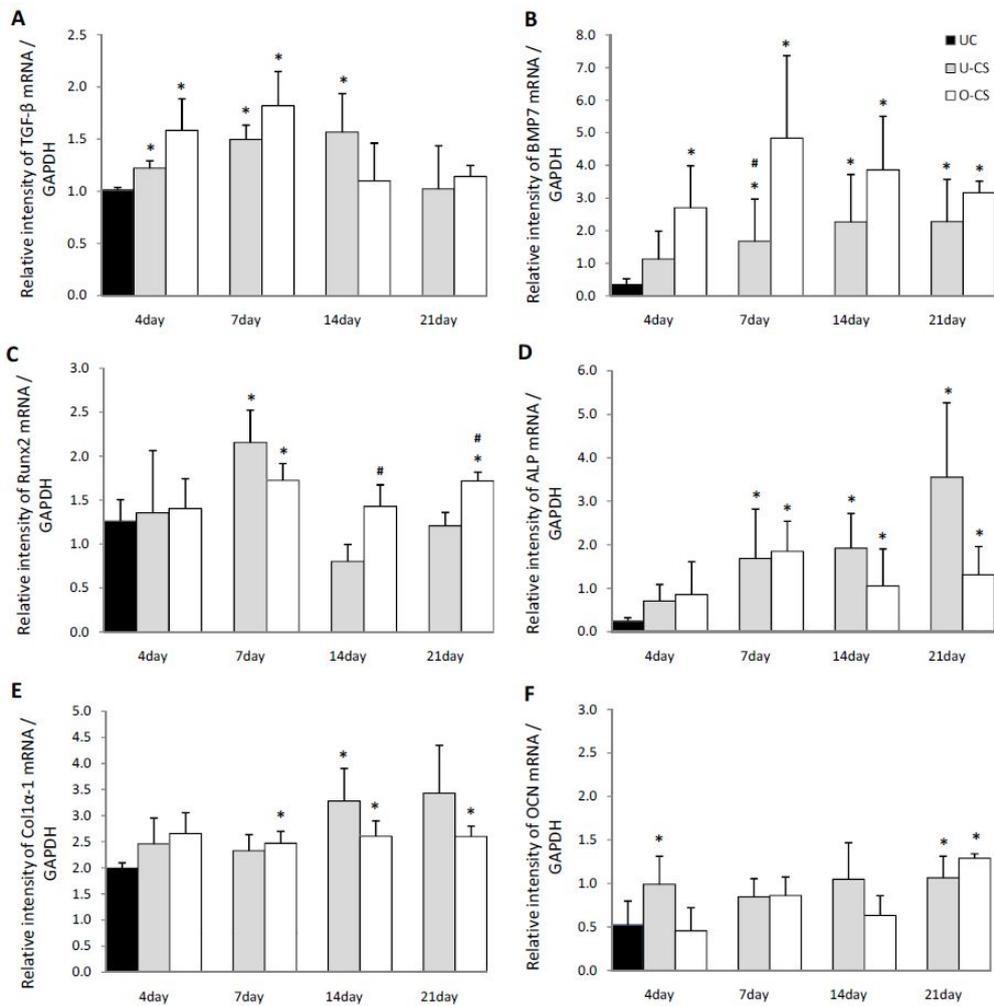


Figure 2. Osteogenic differentiation-associated gene expression profiles of undifferentiated cells (UC), undifferentiated cell sheets (U-CS), and osteogenic differentiated cell sheets (O-CS) at 4, 7, 14, and 21 days. The effect of different media compositions on mRNA expression relative to *GAPDH* was assessed using semi-quantitative reverse-transcription

polymerase chain reaction (RT-PCR) for (A) transforming growth factor- β (*TGF- β*), (B) bone morphogenetic protein 7 (*BMP7*), (C) runt-related transcription factor 2 (*RUNX2*), (D) alkaline phosphatase (*ALP*), (E) collagen 1 α -1 (*COL1 α -1*), and (F) osteocalcin (*OCN*) mRNA expression at 4, 7, 14, and 21 days. Each bar represents the mean \pm SD. *, # represent a statistically significant difference compared to UC at 4 days and between U-CS and O-CS at each day, respectively ($p < 0.05$).

Expression of genes related to osteogenic differentiation

ALP activity was significantly higher in U-CS and O-CS at 7 and 14 days of culture compared to that in UC at 4 days of culture ($p < 0.05$, Fig. 3). ALP activity in U-CS at 14 days of culture was significantly higher than that in O-CS ($p < 0.05$).

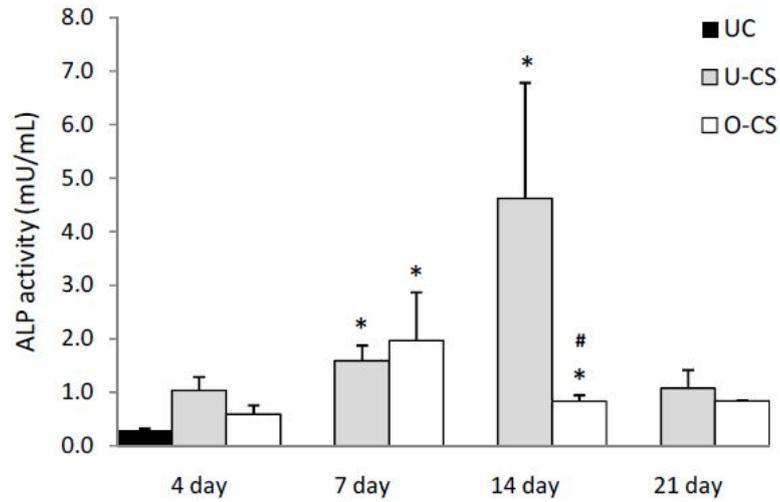


Figure 3. Alkaline phosphatase (ALP) activity of undifferentiated cells (UC), undifferentiated cell sheets (U-CS), and osteogenic differentiated cell sheets (O-CS) at 4, 7, 14, and 21 days. ALP activity of U-CS increased gradually from 4 days and peaked at 14 days. ALP activity of O-CS peaked at 7 days and decreased after 7 days. Each bar represents the mean \pm SD. * represents a statistically significant difference between two groups, and # indicates a statistically significant difference between U-CS and O-CS at day 14 ($p < 0.05$).

Alizarin red S staining and calcium contents

No ARS reactivity was observed at 21 days of culture in either U-CS or O-CS (Fig. 4B, C). Mineral deposition in both sheets was not detected until 14 days of culture (Fig. 4A). Small amounts of calcium were detected in O-CS and U-CS (0.23 ± 0.02 mg/dl and 0.74 ± 0.71 mg/dl, respectively), but not in UC at 21 days of culture.

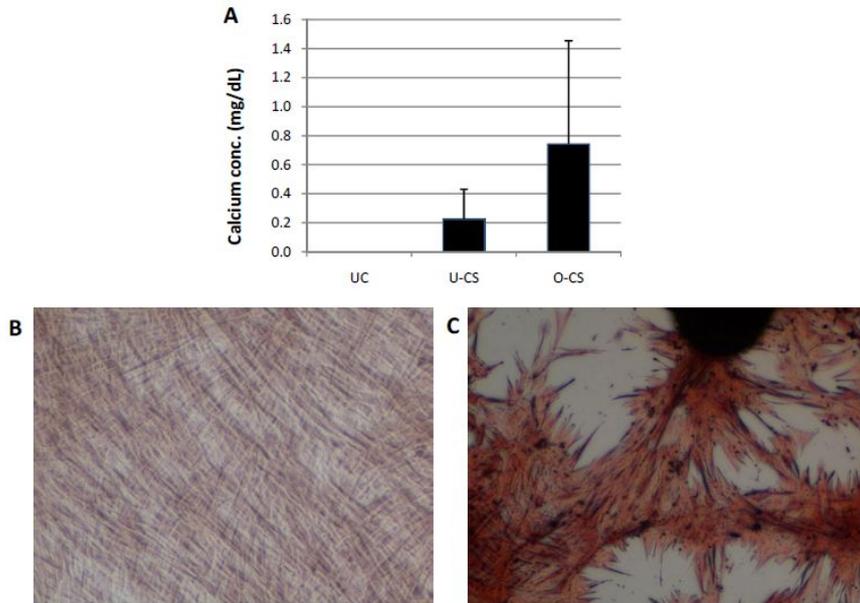


Figure 4. The calcium contents and alizarin red S (ARS) staining at 21 days. (A) Calcium contents of undifferentiated cell sheets (U-CS) and osteogenic cell sheets (O-CS) increased marginally. (B) ARS stained negatively at day 21 in U-CS and (C) O-CS.

Discussion

The addition of 50 µg/ml of Asc to the culture medium stimulated the formation of cell sheets between 2 and 4 days of culture. Previous studies have shown that Asc supplementation to culture medium stimulated cell sheets to form multiple layers and secrete abundant ECM (Guo et al., 2015; Wei et al., 2012; Yu et al., 2014). Culturing cells in tissue culture polystyrene dishes preserves the structure of the ECM even after detaching cells using a cell scraper (Akahane et al., 2008; Guo et al., 2015; Inagaki et al., 2013; Ma et al., 2010; Nakamura et al., 2010). Similar to some previous reports, we observed that the edges of the cell sheets started to fold away from the cell culture dish after 7–10 days of culture (Guo et al., 2015). Further, the cells in O-CS started to aggregate at 10–12 days and only a partial matrix was observed by 21 days of culture. The cellular aggregation that occurs during osteogenic differentiation *in vitro* (Hakki et al., 2014; Vieira et al., 2010) could have contributed to the partial loss of the matrix observed in the present

study. Therefore, the O-CS sheets cannot be used for therapeutic application after 10–12 days of culture.

MSCs express many growth factors, including TGF- β (Uccelli et al., 2008). TGF- β 1 promotes the proliferation of MSCs and regulates the formation and degradation of the ECM (Chin et al., 2004; Phillips et al., 1992). TGF- β isoforms are stored in the ECM and interact with ECM proteins (Dallas et al., 2002). The significant increase in *TGF- β* expression observed in both U-CS and O-CS at 4 days of culture compared to UC was probably due to higher TGF- β secretion into the ECM from the MSCs.

BMP7 expression in O-CS was up-regulated after 4 days of culture, demonstrating that osteogenic medium facilitates osteogenic differentiation. In addition, the up-regulation of *BMP7* in U-CS at 7 days of culture could be attributed to TGF- β -driven osteogenic differentiation. The coordination of *RUNX2* and BMP/TGF- β signaling is critical for the early stages of bone formation. TGF- β and *BMP7* signaling induce osteoblast differentiation through several pathways

such as SMAD, MAPK, and Wnt, all of which converge at the *RUNX2* gene to facilitate mesenchymal precursor cell differentiation (Chen et al., 2012; Dallas et al., 2002; Komori et al., 2005; Tou et al., 2003). In the present study, *RUNX2* expression in both U-CS and O-CS was up-regulated at day 7, followed by up-regulation of *TGF- β* and *BMP7*. *RUNX2* regulates the commitment of mesenchymal cells to an osteoblast lineage and induces the expression of genes encoding bone matrix proteins such as *COL1 α -1* and ALP (Komori et al., 2005; Komori et al., 2006; Lee et al., 2000). The ALP mRNA expression and activity at the protein level as well as *COL1 α -1* expression were up-regulated from 7 days of culture in O-CS.

The level of ALP activity increases during ECM maturation; the initiation of mineralization correlates with the peak in ALP activity, and mineralization correlates with a decrease in ALP activity (Chang et al., 2006; Chang et al., 2000; Lian et al., 1992; Mathews et al., 2012; Sabokbar et al., 1994). The peaks of ALP activity in U-CS and O-CS were observed at 14 and 7 days of culture, respectively. This suggests

that the osteogenic ECM maturation of O-CS was earlier than that of U-CS. MSCs cultured in osteogenic medium without Dex have been reported to exhibit higher ALP activity and to mineralize, although after a delay of 1 week relative to Dex-treated cultures (Hoemann et al., 2009). Dex in osteogenic medium could accelerate RUNX2-related transcriptional changes by facilitating DNA binding of RUNX2 (Komori et al., 2005). In the present study, the use of O-CS medium resulted in faster differentiation compared to the use of U-CS medium.

After ECM maturation, mineralization occurs by deposition of calcium and phosphate (Park et al., 2007). However, in the present study, the small amount of mineral deposition in both sheets was not sufficient to be detected by ARS staining. RUNX2 inhibits the expression of OCN during the maturation phase of osteoblasts (Komori et al., 2006). O-CS and U-CS, which progressed later than O-CS, might differentiate until the maturation phase, so that mineralization of neither sheet had yet occurred.

In the present study, ALP activity and the mRNA expression of *RUNX2*,

ALP, and *COL1a-1* indicated that U-CS and O-CS show osteogenic potential in the stage of ECM maturation. Considering the observed peak in ALP activity and morphological changes, the optimal times for application of O-CS and U-CS are between 7 and 10 days and after 14 days of culture, respectively.

References

- Akahane, M.; A. Nakamura, H.; Ohgushi, H.; Shigematsu, Y.; Dohi, Y.; Takakura, Y. Osteogenic matrix sheet-cell transplantation using osteoblastic cell sheet resulted in bone formation without scaffold at an ectopic site. *J. Tissue Eng. Regen. Med.* 2(4):196-201; 2008.
- Chang, P. L.; Blair, H. C.; Zhao, X. C.; Chien, Y.-W.; Chen, D.; Tilden, A. B.; Chang, Z. J.; Cao, X.; Faye-Petersen, O. M.; Hicks, P. Comparison of fetal and adult marrow stromal cells in osteogenesis with and without glucocorticoids. *Connect Tissue Res.* 47(2):67-76; 2006.
- Chang, Y. L.; Stanford, C. M.; Keller, J. C. Calcium and phosphate supplementation promotes bone cell mineralization: Implications for hydroxyapatite (HA)-enhanced bone formation. *J. Biomed. Mater. Res.* 52(2):270-278; 2000.
- Chen, G. Q.; Deng, C. X.; Li, Y. P. TGF-beta and BMP signaling in osteoblast differentiation and bone formation. *Int. J. Biol. Sci.* 8(2):272-288; 2012.
- Chin, D.; Boyle, G. M.; Parsons, P. G.; Coman, W. B. What is transforming growth factor-beta (TGF-beta)? *Br. J. Plast. Surg.* 57(3):215-221; 2004.

- Dallas, S. L.; Rosser, J. L.; Mundy, G. R.; Bonewald, L. F. Proteolysis of latent transforming growth factor-beta (TGF-beta)-binding protein-1 by osteoclasts - A cellular mechanism for release of TGF-beta from bone matrix. *J. Biol. Chem.* 277(24):21352-21360; 2002.
- Egami, M.; Haraguchi, Y.; Shimizu, T.; Yamato, Y.; Okano, T. Latest status of the clinical and industrial applications of cell sheet engineering and regenerative medicine. *Arch. Pharm. Res.* 37(1):96-106; 2014.
- Gregory, C. A.; Gunn, W. G.; Peister, A.; Prockop, D. J. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal. Biochem.* 329(1):77-84; 2004.
- Guo, P.; Zeng, J. J.; Zhou, N. A novel experimental study on the fabrication and biological characteristics of canine bone marrow mesenchymal stem cells sheet using vitamin C. *Scanning* 37(1):42-48; 2015.
- Hakki, S. S.; Bozkurt, B.; Hakki, E. E.; Kayis, S. A.; Turac, G.; Yilmaz, I.; Karaoz, E. Bone morphogenetic protein-2, -6, and -7 differently regulate osteogenic differentiation of human periodontal ligament stem cells. *J. Biomed. Mater. Res. B Appl. Biomater.* 102(1):119-130; 2014.

- Hoemann, C. D.; El-Gabalawy, H.; McKee, M. D. In vitro osteogenesis assays: Influence of the primary cell source on alkaline phosphatase activity and mineralization. *Pathol. Biol(Paris)*. 57(4):318-323; 2009.
- Inagaki, Y.; Uematsu, K.; Akahane, M.; Morita, Y.; Ogawa, M.; Ueha, T.; Shimizu, T.; Kura, T.; Kawate, K.; Tanaka, T. Osteogenic matrix cell sheet transplantation enhances early tendon graft to bone tunnel healing in rabbits. *Biomed. Res. Int.* 2013:842192; 2013.
- Kang, B.-J.; Kim, Y.; Lee, S. H.; Kim, W. H.; Woo, H.-M.; Kweon, O.-K. Collagen I gel promotes homogenous osteogenic differentiation of adipose tissue-derived mesenchymal stem cells in serum-derived albumin scaffold. *J. Biomater. Sci. Polym. Ed.* 24(10):1233-1243; 2013.
- Kim, Y. S.; Lim, J. Y.; Donahue, H. J.; Lowe, T. L. Thermoresponsive terpolymeric films applicable for osteoblastic cell growth and noninvasive cell sheet harvesting. *Tissue Eng.* 11(1-2):30-40; 2005.
- Komori, T. Regulation of skeletal development by the runx family of transcription factors. *J. Cell. Biochem.* 95(3):445-453; 2005.
- Komori, T. Regulation of osteoblast differentiation by transcription factors. *J. Cell. Biochem.* 99(5):1233-1239; 2006.

- Lee, K. S.; Kim, H. J.; Li, Q. L.; Chi, X. Z.; Ueta, C.; Komori, T.; Wozney, J. M.; Kim, E. G.; Choi, J. Y.; Ryoo, H. M.; Bae, S. C. Runx2 is a common target of transforming growth factor beta 1 and bone morphogenetic protein 2, and cooperation between runx2 and smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol. Cell. Biol.* 20(23):8783-8792; 2000.
- Lian, J. B.; G. S. Stein. Concepts of osteoblast growth and differentiation: Basis for modulation of bone cell development and tissue formation. *Crit. Rev. Oral Biol. Med.* 3(3):269-305; 1992.
- Ma, D.; Ren, L.; Liu, Y.; Chen, F.; Zhang, J.; Xue, Z.; Mao, T. Engineering scaffold-free bone tissue using bone marrow stromal cell sheets. *J. Orthop. Res.* 28(5):697-702; 2010.
- Mathews, S.; Bhonde, R.; Gupta, P. K.; Totey, S. Extracellular matrix protein mediated regulation of the osteoblast differentiation of bone marrow derived human mesenchymal stem cells. *Differentiation* 84(2):185-192; 2012.
- Matsuura, K.; Utoh, R.; Nagase, K.; Okano, T. Cell sheet approach for tissue engineering and regenerative medicine. *J. Control. Release.* 190:228-239; 2014.

- Nakamura, A.; Akahane, M.; Shigematsu, H.; Tadokoro, M.; Morita, Y.; Ohgushi, H.; Dohi, Y.; Imamura, T.; Tanaka, Y. Cell sheet transplantation of cultured mesenchymal stem cells enhances bone formation in a rat nonunion model. *Bone* 46(2):418-424; 2010.
- Nishida, K.; M. Yamato, M.; Hayashida, Y.; Watanabe, K.; Yamamoto, K.; Adachi, E.; Nagai, S.; Kikuchi, A.; Maeda, N.; Watanabe, H.; Okano, T.; Tano, Y. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N. Engl. J. Med.* 351(12):1187-1196; 2004.
- Ohashi, K.; Yokoyama, T.; Yamato, M.; Kuge, H.; Kanehiro, H.; Tsutsumi, M.; Amanuma, T.; Iwata, H.; Yang, J.; Okano, T.; Nakajima, Y. Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. *Nat. Med.* 13(7):880-885; 2007.
- Ohki, T.; Yamato, M.; Ota, M.; Takagi, R.; Murakami, D.; Kondo, M.; Sasaki, R.; Namiki, H.; Okano, T.; Yamamoto, M. Prevention of esophageal stricture after endoscopic submucosal dissection using tissue-engineered cell sheets. *Gastroenterology* 143(3):582-U556; 2012.
- Parekkadan, B.; Milwid, J. M. Mesenchymal stem cells as therapeutics. *Annu. Rev. Biomed. Eng.* 12:87-117; 2010.

- Park, B.-W.; Hah, Y.-S.; Kim, D. R.; Kim, J.-R.; Byun, J.-H. Osteogenic phenotypes and mineralization of cultured human periosteal-derived cells. *Arch. Oral. Biol.* 52(10):983-989; 2007.
- Phillips, C. L.; Tajima, S.; Pinnell, S. R. Ascorbic-acid and transforming growth factor-beta-1 increase collagen biosynthesis via different mechanisms: Coordinate regulation of pro-alpha-1(I) and pro-alpha-1(III) collagens. *Arch. Biochem. Biophys.* 295(2):397-403; 1992.
- Pirracò, R. P.; Obokata, H.; Iwata, T.; Marques, A. P.; Tsuneda, S.; Yamato, M.; Reis, R. L.; Okano, T. Development of osteogenic cell sheets for bone tissue engineering applications. *Tissue Eng. Part A.* 17(11-12):1507-1515; 2011.
- Ryu, H.-H.; Lim, J.-H.; Byeon, Y.-E.; Park, J.-R.; Seo, M.-S.; Lee, Y.-W.; Kim, W. H.; Kang, K.-S.; Kweon, O.-K. Functional recovery and neural differentiation after transplantation of allogenic adipose-derived stem cells in a canine model of acute spinal cord injury. *J. Vet. Sci.* 10(4):273-284; 2009.
- Sabokbar, A.; Millett, P. J.; Myer, B.; Rushton, N. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. *Bone Miner.* 27(1):57-67; 1994.
- Saito, T.; Ohashi, K.; Utoh, R.; Shimizu, H.; Ise, K.; Suzuki, H.; Yamato, M.; Okano, T.; Gotoh, M. Reversal of diabetes by the creation of

- neo-islet tissues into a subcutaneous site using islet cell sheets. *Transplantation* 92(11):1231-1236; 2011.
- Sekine, H.; Shimizu, T.; Dobashi, I.; Matsuura, K.; Hagiwara, N.; Takahashi, M.; Kobayashi, E.; Yamato, M.; Okano, T. Cardiac cell sheet transplantation improves damaged heart function via superior cell survival in comparison with dissociated cell injection. *Tissue Eng. Part A.* 17(23-24):2973-2980; 2011.
- Tou, L.; Quibria, N.; Alexander, J. M. Transcriptional regulation of the human *runx2/Cbfa1* gene promoter by bone morphogenetic protein-7. *Mol. Cell. Endocrinol.* 205(1-2):121-129; 2003.
- Uccelli, A.; Moretta, L.; Pistoia, V. Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.* 8(9):726-736; 2008.
- Uchiyama, H.; Yamato, M.; Sasaki, R.; Sekine, H.; Yang, J.; Ogiuchi, H.; Ando, T.; Okano, T. In vivo 3D analysis with micro-computed tomography of rat calvaria bone regeneration using periosteal cell sheets fabricated on temperature-responsive culture dishes. *J. Tissue Eng. Regen. Med.* 5(6):483-490; 2011.
- Vieira, N. M.; Brandalise, V.; Zucconi, E.; Secco, M.; Strauss, B. E.; Zatz, M. Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. *Cell Transplant.* 19(3):279-289; 2010.

- Wei, F. L.; Qu, C. Y.; Song, T. L.; Ding, G.; Fan, Z. P.; Liu, D. Y.; Liu, Y.; Zhang, C. M.; Shi, S. T.; Wang, S. L. Vitamin C treatment promotes mesenchymal stem cell sheet formation and tissue regeneration by elevating telomerase activity. *J. Cell. Physiol.* 227(9):3216–3224; 2012.
- Yang, J.; Yamato, M.; Shimizu, T.; Sekine, H.; Ohashi, K.; Kanzaki, M.; Ohki, T.; Nishida, K.; Okano, T. Reconstruction of functional tissues with cell sheet engineering. *Biomaterials* 28(34):5033–5043; 2007.
- Yu, J. S.; Tu, Y. K.; Tang, Y. B.; Cheng, N. C. Sternness and transdifferentiation of adipose-derived stem cells using L-ascorbic acid 2-phosphate-induced cell sheet formation. *Biomaterials* 35(11):3516–3526; 2014.

국문초록

세포시트는 자가 유래 중간엽 줄기세포를 이용해서 손쉽게 만들 수 있으며, 지지체를 이용하지 않고 골재생을 유도할 수 있다. 본 연구는 중간엽 줄기세포를 이용해 만든 세포시트들의 골분화 능력을 평가하였다. 개의 지방세포 유래의 중간엽 줄기세포로 미분화셀시트(Undifferentiated cell sheets)와 골분화셀시트(Osteogenic differentiated cell sheets)를 만들었다. 미분화세포(Undifferentiated cells)는 대조군으로 사용되었다. 골분화의 평가는 배양 후 4, 7, 14, 21일에 알칼리인포스파타제(alkaline phosphatase activity; ALP activity)의 활성 정도와 역전사 중합효소 연쇄반응을 이용한 골분화와 관련된 유전자가 발현되는 정도로 평가하였다. 배양된 세포를 알리자린레드에스(Alizarin red S; ARS)를 이용하여 염색하였고, 세포의 칼슘농도를 직접 측정하였다.

4일 동안 배양한 미분화셀시트와 골분화셀시트는 형질전환성장인자-베타(transforming growth factor- β)의 메신저리보핵산(mRNA)의 발현정도가 미분화세포보다 유의적으로 높게 나타났다 ($p < 0.05$). 골형성단백질-7 (bone morphogenetic protein 7)의 메신저리보핵산의 발현량도 4일동안 배양한 골분화셀시트가 유의적으로 상승했으며, 7일 동안

배양한 골분화셀시트는 미분화세포와 7일동안 배양한 미분화셀시트보다 유의적인 상승을 보였다. 7일 동안 배양한 두 시트에서 Runt-related transcription factor-2의 메신저리보핵산의 발현정도가 유의적으로 상승했다. 알칼라인포스파타제의 메신저리보핵산의 발현 정도는 7일동안 배양한 두 시트에서 모두 유의적인 상승을 보였으며, 알칼라인포스파타제의 활성은 미분화 셀시트는 14일, 골분화셀시트에서는 7일차가 가장 높았다 ($p < 0.05$). 두 시트의 osteocalcin 메신저리보핵산은 21일 차에 유의적인 상승을 보였다. 알리자린레드에스는 모든 세포에서 염색되지 않았으나, 21일 동안 배양한 두 시트의 칼슘농도는 미약한 상승을 보였다. 골분화셀시트의 세포들은 배양 10 - 12 일 이후 부터 세포끼리 부분적으로 뭉치기 시작하였으며, 배양 21일에는 시트형태가 부분적으로만 남아있었다. 골분화와 관련된 유전자발현의 상승, 알칼라인포스파타제의 활성과 시트의 형태학적인 변화를 고려했을 때, 골분화셀시트는 7일에서 10일 사이, 미분화 셀시트는 배양 14일 이후에 적용하는 것이 이상적이다.

주요어: 개, 골분화능, 중간엽줄기세포, 셀시트, 골분화셀시트, 개 지방세포, 골 재생

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