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## The Effect of Hydrostatic Pressure on 3-D Chondroinduction of Human Adipose-Derived Stem Cells

Rei Ogawa <sup>1,2</sup>, Shuichi Mizuno <sup>3</sup>, George F. Murphy <sup>4</sup>, Dennis P. Orgill<sup>1</sup>

1: Division of Plastic Surgery, Department of Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, USA

2: Department of Plastic, Reconstructive and Aesthetic Surgery, Nippon Medical School, Tokyo, Japan

3: Department of Orthopedic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, USA

4: Program in Dermatopathology, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, USA

### Authors contribution;

**Rei Ogawa:** Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing

**Shuichi Mizuno:** Provision of study material, Collection and/or assembly of data

**George F. Murphy:** Data analysis and interpretation, Manuscript writing

**Dennis P. Orgill:** Review of data, Manuscript writing

### Address correspondence to:

Rei Ogawa, MD, PhD

Division of Plastic Surgery, Department of Surgery, Brigham and Women's Hospital, Harvard Medical School,

75 Francis Street, Boston, MA02115, USA

TEL: +1-617-732-7874

E-Mail: r.ogawa@nms.ac.jp

## Abstracts

**Background:** The optimal production of three-dimensional (3-D) cartilage *in vitro* requires both inductive factors and specified culture conditions (e.g., hydrostatic pressure [HP], gas concentration, and nutrient supply) to promote cell viability and maintain phenotype. In this study, we optimized the conditions for human cartilage induction using human adipose derived stem cells (ASCs), collagen scaffolds and cyclic HP treatment. **Methods:** Human ASCs underwent primary culture and three passages prior to being seeded into collagen scaffolds. These constructs were incubated for one week in an automated bioreactor using cyclic hydrostatic pressure (HP) at 0-0.5 MPa, 0.5 Hz, and compared to constructs exposed to atmospheric pressure (AP). In both groups, chondrogenic differentiation medium including transforming growth factor-beta 1 (TGF $\beta$ 1) was employed. One, two, three and four weeks after incubation, the cell constructs were harvested for histological, immunohistochemical, and gene expression evaluation. **Results:** In histological and immunohistochemical analyzes, pericellular and extracellular metachromatic matrix was observed in both groups and increased over 4 weeks, but accumulated at a higher rate in the HP group. Cell number was maintained in the HP group over 4 weeks but decreased after 2 weeks in the AP group. Chondrogenic specific gene expression of type II and X collagen, aggrecan and Sox 9 was increased in the HP group especially after 2 weeks. **Conclusion:** Our results demonstrate chondrogenic differentiation of ASCs in a 3-D collagen scaffolds with treatment of a cyclic HP. Cyclic HP was effective in enhancing accumulation of extracellular matrix and expression of genes indicative of chondrogenic differentiation.

## Introduction

Cartilage is an avascular tissue found in articular surfaces, the tracheobrochial tree, ribs, eyelids, ears and the nasal skeleton. Tissue engineered three-dimensional (3-D) chondrocyte constructs can be potentially used to repair or replace damaged cartilage. It is widely recognized that chondrocytes, particularly those in articular cartilage, develop and are maintained physiologically by mechanical forces, including hydrostatic pressure<sup>1,2</sup> and shear stress<sup>3,4</sup>. These mechanical forces may regulate chondrocyte differentiation, maturation, and tissue formation<sup>5,6</sup>. For purposes of tissue engineering, mechanical forces, are necessary to develop and maintain cartilage-like

constructs *in vitro*. We reported a high pressure hydrostatic perfusion culture system that is useful for constructing 3-D engineered cartilage using bovine chondrocytes<sup>7-9</sup> and human dermal fibroblasts<sup>10</sup> with collagen gel/sponge scaffolds.

In this study, we formed 3-D cartilage using human adipose-derived stem cells (ASCs) with a collagen scaffold under cyclic HP treatment. To date, ASCs are the most rapidly accessible for harvesting the large quantities of adult stem cells. We have previously reported the extraordinary multipotency inherent in murine ASCs with respect to their ability to undergo chondrogenic<sup>11</sup>, osteogenic<sup>11</sup>, adipogenic<sup>12</sup> and neurogenic<sup>13</sup> differentiation of mouse ASCs *in vitro*. We have also demonstrated *in vivo* adipose tissue regeneration<sup>14</sup> using mouse ASCs and *in vivo* periodontal tissue regeneration<sup>15</sup> using rat ASCs.

Some reports suggest that ASCs, unlike with bone marrow derived mesenchymal stem cells (MSCs), are not suitable for cartilage regeneration<sup>16,17</sup>. These papers addressed the differential expression profile of chondrogenic specific genes and extracellular matrix in MSCs or ASCs in chondrogenic differentiation. Melhorn et al.<sup>16</sup> reported that collagen type II and X were secreted more strongly by MSCs than by ASCs in chondrogenic induction using TGF $\beta$ 1, and Afizah et al.<sup>17</sup> reported that collagen type II and proteoglycans were synthesized only in MSCs in chondrogenic induction using TGF $\beta$ 3.

In order to test the hypothesis that this perceived limitation of ASCs may be related to the potential necessity of external microenvironmental factors such as hydrostatic pressure, this present study was undertaken.

## Materials and Methods

### *1. Isolation and Culture of Human Adipose-Derived Stem Cells*

Under an approval of an IRB protocol, discarded human adipose tissues (~5 g) harvested from three different donors were used in this study. Adipose tissue was washed with phosphate-buffered saline (PBS) extensively. The fat was finely minced and digested with 0.075% type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ) dissolved in Dulbecco modified Eagle medium (DMEM) / Hams' F-12 (Invitrogen, Carlsbad, CA) with gentle shaking for 30 minutes at 37°C using a 50 ml

conical tube. Then, the digested cell suspension was diluted with an equal volume of growth medium: DMEM/Hams' F-12 (Invitrogen) with 10% fetal bovine serum (FBS) and 1% antibiotics-antimicrobials (Invitrogen). After centrifugation for 5 min at 390 x g, the cell pellet was resuspended with the growth medium and viable cells were counted with Trypan blue. Five hundred thousand cells were seeded to a 100 mm tissue culture dish and incubated in the growth medium at 37°C and 5% CO<sub>2</sub>. This primary culture was defined as passage 0 (P0). The cells were expanded in the growth media until 80–90% confluence (approximately 5-7 days in culture), then underwent three passages (P3).

### **2. Characterization of The Adipose-Derived Stem Cells**

Expression of cell surface protein was evaluated in P3 cells using a FACS Caliber flow cytometer with CELLQuest acquisition software (Becton-Dickinson, Franklin Lakes, NJ). Data analysis was performed using Flow Jo software (Tree Star, Ashland, OR).  $5 \times 10^5$  cells were incubated with saturating concentrations of a fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanine (APC)-conjugated antibodies: CD13, CD14, CD31, CD34, CD44, CD45, and CD90 (BD Biosciences, San Diego, CA) for 60 min at 4°C. After incubation, the cells were washed three times with PBS supplemented with 1% FBS. Then, the cells were resuspended in 0.3 ml of cold PBS with 1% FBS for the evaluation.

### **3. Construction of Collagen Scaffolds**

Porous collagen sponges were made as previously reported<sup>10</sup>. In brief, a solution of 0.5% pepsin-digested collagen from bovine skin: Cellagen (ICN Biomedical, Costa Mesa, CA) was neutralized with HEPES and NaHCO<sub>3</sub>. Two hundred fifty ml of the collagen solution was poured into a mold and frozen at -20°C. Moist tissue paper was placed over the surface of the frozen collagen to avoid formation of a membranous collagen skin during lyophilization. After lyophilization, the tissue paper was removed and each side of the porous collagen sponge was irradiated by UV light for 3 h at a distance of 30 cm. The final dimensions of the sponge were 7 mm diameter and 1.5 mm thickness. The sponges were placed in a seeding device that was sealed with O-rings.

#### 4. 3D Cell Culture using A Bioprocessor

The P3 cells were harvested and  $4.5 \times 10^7$  cells were re-suspended with 2.5 ml of the cold neutralized type I collagen solution. A 50- $\mu$ l of the cell suspension ( $1 \times 10^6$  cells) was placed on a sterile Teflon dish (Fisher), absorbed with the collagen sponge (total 45 sponges), and incubated at 37°C for gelation (**Fig.1**). After a day of incubation in the growth medium at 37°C and 5% CO<sub>2</sub> in air, 5 sponges were harvested for samples of Day 0. 40 sponges were divided into two groups; Group HP, incubation during the first one week with treatment of cyclic HP at 0-0.5 MPa (3750 mm Hg, 4.93 atm), 0.5 Hz, with a medium replenishment rate of 0.1 ml/min, at 37°C, 3% O<sub>2</sub>, and 5% CO<sub>2</sub> in air using a bioprocessor TEP-P02 (Takagi Industrial, Shizuoka, Japan) followed by no HP for 3 weeks and Group AP, incubation without HP at 37°C, 3% O<sub>2</sub>, and 5% CO<sub>2</sub> for 4 weeks. In both groups, the cells were incubated in a differentiation medium defined as DMEM/Ham's F-12 with 2% FBS and 1% antibiotics-antimycotics, transforming growth factor- $\beta$  (TGF) (10 ng/ml; R&D Systems, Minneapolis, MN), dexamethasone ( $1 \times 10^{-7}$  mol), ascorbic acid 2-phosphate (50  $\mu$ g/ml), sodium pyruvate 1 mM, and l-proline (40  $\mu$ g/ml).

#### 5. Histological and Immunohistochemical Analysis

Constructs were embedded in glycomethacrylate: JB-4 (Polysciences, Warrington, PA) and paraffin and cut into 10- $\mu$ m sections and stained with 0.2% toluidine blue-O (Fisher) at pH 4.0. Immunohistological staining was performed using a Vectastain ABC kit and a 3,38-diaminobenzidine (DAB) kit (Vector Laboratory, Burlingame, CA). 5  $\mu$ m paraffin sections were dewaxed with xylene and then rehydrated in a graded series of alcohol to PBS. After rinsing with PBS, the sections were blocked with 3% normal horse serum at room temperature for 20 min in a humidified chamber. For collagen type II staining, the sections were incubated with a rabbit anti-human collagen type II antibody diluted 1: 50 (Chemicon, Temecula, CA) with 1% normal horse serum for 60 min at room temperature. After three rinses with PBS, the sections were incubated with biotinylated goat anti-rabbit IgG antibody (Vector Laboratory) and followed by manufacturer's instruction of the ABC kit. Color was developed with DAB. For keratin sulfate staining, the rehydrated sections were incubated for 60 min with biotinylated monoclonal anti-keratin sulfate (Seikagaku corporation, Tokyo, Japan) in a 1: 250 diluted with PBS at room temperature. After three rinses with PBS, color was developed

with DAB (Vector Laboratory). Nuclei were counter-stained with hematoxylin.

### **6. Semi-quantitative Histological Evaluation**

Toluidine blue stained sections were used for evaluating cellularity in the sponge (**Fig.2**). Total number of nuclei on a surface and a deep zone of the sponge were counted. Three fields in each zone of 40x images were randomly chosen.

### **7. Real Time Reverse Transcriptase - Polymerase Chain Reaction (Real Time RT-PCR)**

Freshly collected samples (three sponges in each group at each time point) were washed with PBS and total RNA was extracted using the RNeasy mini kit (Qiagen, Chatsworth, CA) following the manufacturer's instructions. Briefly, samples were finely homogenized with handheld homogenizer and QIA shredder with buffer RLT including beta-mercaptoethanol. After adding 70% ethanol and mixed well, samples were centrifuged using RNeasy spin column. Then, Buffer RW1 and RPE were sequentially added into the column and centrifuged. Total RNA was extracted with RNase free water. Total RNA was quantified using the NanoDrop (NanoDrop Technologies, Wilmington, DE) method.

Complementary deoxyribonucleic acid (cDNA) was synthesized using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA). Total RNA (< 1 ug) was mixed with random hexamers (50 ng/ul) and dNTP (10 mM), then incubated at 65 °C for 5 min. Tubes were cooled on ice, then RT buffer (10 x), MgCl<sub>2</sub> (25 mM), DTT (0.1 M), RNaseOUT (40 U/ul) and SuperScript III RT (200 U/ul) were added, giving a final volume of 21 ul. Samples were then incubated at 25 °C for 10 min, 50 °C for 50 min, and 85 °C for 5 min, and cooled on ice. Then, E.coli RNase H (2 U/ul) was add and incubated at 37 °C for 20 min.

Real time RT-PCR was performed in an ABI Prism7300 system (Applied Biosystems, Foster City, CA) using The RT<sup>2</sup> SYBR Green / ROX qPCR master mix (SA Biosciences, Frederick, MD) with primers designed for this study (**Table 1**).

Amplifications of the cDNA samples were performed in triplicate in 96-well plates in a final volume of 20  $\mu$ l at 40 PCR cycles consisting of a denaturation step at 95 °C for 15 sec and an anneal/extension step at 60 °C for 1 min. Fluorescence measurements were used to generate a dissociation curve utilizing the system software program v1.4 (Applied Biosystems, Foster City, CA). Relative quantity of each gene was determined from the standard curve. Signal levels were normalized to the expression of a constitutively expressed gene, GAPDH and shown as a relative ratio. We verified the constant GAPDH expression with another internal control gene for 28S ribosomal RNA.

### 8. Statistical Analysis

All quantitative data reported here was analyzed using one-way analysis of variance (ANOVA), with  $p < 0.05$  being considered significantly different. Replicates were done for each experimental group.

## Results

### *Characterization of Stem Cells*

By flow cytometric analysis of cultured cells, the percentage of positive cells a) for stromal associated markers CD13, 44 and 90 were  $98.8 \pm 0.6$ ,  $95.9 \pm 1.4$ ,  $96.4 \pm 2.3$ , respectively; b) for the macrophage and neutrophilic granulocyte marker CD14 was  $0.5 \pm 0.2$ ; c) for the endothelial-associated marker CD31 was  $2.6 \pm 0.8$ ; d) for the stem cell-associated marker CD34 was  $4.4 \pm 0.6$ ; and e) for the pan-hematopoietic marker CD45 was  $0.2 \pm 0.1$ . These results were consistent with a previous report of human ASCs cultured with identical growth media<sup>18</sup>. Although primary ASCs are a heterogeneous cell population<sup>18</sup>, this heterogeneity decreased after three passages in culture.

### *Histological and Immunohistochemical Evaluation*

Cells were distributed throughout the sponges. An accumulation of pericellular and extracellular metachromatic matrix stained with Toluidine blue was seen in both groups and increased over 4 weeks (**Fig.3**). Accumulation of the matrix in



the HP group was much denser than AP groups, particularly after 2 weeks. Immunohistochemical evaluation revealed that the production of type II collagen and keratin sulfate in both groups increased with time, with HP groups showing greater production than AP groups at each time point. Cell number in AP groups achieved a peak at two weeks of incubation and decreased thereafter on both the middle and surface zone of sponges (**Fig.4**). In contrast, cell number in the HP groups increased gradually in the middle zone, and peaked at one week after incubation, maintaining their number for the entire course on surface zone.

### ***Gene expression evaluation***

Gene expression of chondrogenic specific genes collagen type II (*COL 2A1*), collagen type X (*COL 10A1*), aggrecan (*ACAN*) and Sox 9 (*Sox 9*) were identified by real time RT-PCR (**Fig.5**) in both groups. However, there existed significant differences between HP and AP groups after 2 weeks of culture for aggrecan and Sox 9 expression, after 3 weeks of culture for type X collagen, and at the 2 and 4 weeks time points point for type II collagen.

## **Discussion**

### ***Adipose-Derived Stem Cells***

Adipose tissue represents an alternative source of adult stem cells that also may be harvested from bone-marrow<sup>19</sup>, skin<sup>20</sup> and skeletal muscles<sup>21</sup>. Subcutaneous adipose depots are accessible, abundant, and replenishable<sup>18</sup>. Moreover, adipose-tissue is routinely discarded after cosmetic surgical procedures such as liposuction, providing a fertile opportunity for adipose-tissue banking<sup>22</sup>. ASCs previously have been well characterized by many groups<sup>18,23,24</sup>. In these studies it has been suggested that over 90% of cultured ASCs express CD13, 44, 73 and 90, but do not express hematopoietic markers CD 14 and 45, and our data are consistent with these results. Differences exist, however, among previous studies with regard to CD34 expression by ASCs<sup>18,23,24</sup>, and it has been suggested that CD34 detection may be influenced by culture methods and antibodies used for detection. In the present study, CD34 expression was less than 5% at the time point of passage 3 of culture, a finding compatible with the previous results of Mitchell et al<sup>18</sup> where an identical culture medium was utilized.

The chondrogenic potential of ASCs have been previously demonstrated<sup>25,26</sup>,

although some have indicated that ASCs are less suitable for cartilage regeneration when compared with bone marrow-derived mesenchymal stem cells (MSCs)<sup>16,17, 27, 28</sup>. Hennig et al.<sup>28</sup> reported characteristics of chondrogenesis of ASCs. Spheroid cultured ASCs were supplemented with various human recombinant growth factors, and increased concentrations of TGF $\beta$  did not improve chondrogenesis of ASCs, and BMP6 treatment induced TGF $\beta$ -receptor-I expression. Henning et al.<sup>28</sup> speculated that a distinct BMP and TGF $\beta$ -receptor repertoire may explain the reduced chondrogenic capacity of ASCs *in vitro*, which could be compensated by exogenous application of lacking factors.

### ***Effect of Hydrostatic Pressure on Chondro-induced Stem Cells***

For tissue engineering applications, modified cell culture systems (e.g., a follow-fiber<sup>29</sup>, a spinner flask<sup>30,31</sup>, a rotating vessel<sup>32,33</sup>, a direct displacement<sup>34,35</sup>, and a perfusion culture<sup>36-40</sup>) have been developed for optimization of culture conditions. Such devices are effective for mass transfer between culture medium and the cell constructs<sup>10</sup>. We recently developed a perfusion culture system that promoted osteogenesis<sup>41</sup>, hematopoiesis<sup>42</sup>, and epithelial formation<sup>43</sup>. However, perfusion at a defined flow rate was not consistently beneficial for chondrogenesis by articular chondrocytes in 3-D collagen sponge constructs<sup>7</sup>. Thus, we developed a hydrostatic pressure/perfusion culture system that applied HP directly to the medium fluid phase<sup>44</sup>. We confirmed that this bioprocessor is useful for constructing 3-D engineered cartilage using bovine chondrocytes<sup>7-9</sup> and human dermal fibroblasts<sup>10</sup> with collagen gel/sponge scaffolds.

Comparing chondrogenic gene expression profile by chondro-induced ASCs affected by either AP or HP, type II and X collagen, aggrecan and Sox 9 were expressed in both groups. This is considered to relate to the chondrogenic media, including TGF $\beta$ 1, that was employed for both groups (HP alone did not have chondrogenic differentiation effects on ASCs: data not shown). However, this expression profile was significantly enhanced by HP loading compared with the AP control group. Sox9 gene expression is crucial for the induction of the cartilage phenotype during skeletal development, and

most of the major cartilage matrix proteins, including type II collagen and aggrecan, contain binding sites for this transcription factor in their promoters<sup>45</sup>. We concluded that HP accelerates chondrogenic differentiation of ASCs and/or maturation of differentiated chondrocytes. Type X collagen is regarded as a hypertrophic chondrocyte marker that is upregulated by mechanical force<sup>45</sup>, and our results are compatible with this established function. It has been suggested that type X collagen expression is undesirable for articular cartilage regeneration, because hypertrophic chondrocytes reminiscent of endochondral bone formation<sup>46</sup>. Steck et al.<sup>46</sup> suggested that type X collagen expression is a common feature induced by protocols for *in vitro* chondrogenesis, and speculated that it can be prevented by avoiding pre-differentiation of stem cells prior to transplantation *in vivo*. Further *in vivo* experiments will be required to confirm this.

The mechanism(s) responsible for the direct effects of HP on cell viability, proliferation, and differentiation of ASCs remains unclear. ASCs may be able to convert a mechanical stimulus into an electrical signal through mechanoreceptors (mechanosensors) such as mechanosensitive ion channels<sup>47</sup>. Previously, we attempted to characterize the change in intracellular calcium concentration ( $[Ca^{2+}]_i$ ) in response to the application of HP to cultured bovine articular chondrocytes using a fluorescent indicator, a custom-made pressure-proof optical chamber, and laser confocal microscopy<sup>9</sup>. The peak  $[Ca^{2+}]_i$  in cells showed a significant increase after the application of HP at constant 0.5 MPa for 5 min, and it was considered that HP stimulates calcium mobilization through stretch-activated ion channels<sup>9</sup>. We hope to do future studies to examine the potential influence of mechanosensitive ion channels on chondrodifferentiation of ASCs.

### ***Future Prospects of Cartilage Regeneration using Adipose-Derived Stem Cells***

In this study, we elucidated that HP followed by static culture promoted production of cartilage-specific ECM by chondroinduced ASCs. For reconstructing cartilage, the desired quality of chondroinduced ASCs should be optimized with mechanophysiological factors including HP, oxygen concentration, scaffold materials, and medium components including cytokine and morphogen e.g., BMP-6<sup>28</sup>, serum<sup>48</sup>. Our study addresses possible options to augment shorted fibrous cartilage. Lineage and histological integration of the chondroinduced ASCs with adjacent cartilage should be studied *in vivo*.

With respect to the waveform of cyclic HP, we chose a sinusoidal profile between 0 and 0.5 MPa, 0.5 Hz used in the experiments of cultivating bovine chondrocytes<sup>7-9</sup> and human dermal fibroblasts<sup>10</sup>. We should explore other profiles and algorithms of the HP *in vitro* to optimize quality of the cell construct including integration of post implantation.

The cell location within the scaffold influences diffusion of nutrients and waste products from the perfusion media associated with more cells proliferating near the periphery than deeper in the scaffold. The newly proliferated cells and matrix impact the material/structure of the scaffold, nutrient availability and waste dissipation in the partitioned cell construct. HP/medium perfusion may also be useful for improved mass transfer of necessary medium supply and waste exclusion.

In summary, our results indicate that HP loading using a HP/medium perfusion culture system clearly enhances accumulation of extracellular matrix and expression of chondrogenic specific genes by ASCs *in vitro*, potentially obviating previously perceived disadvantages<sup>16,17</sup> in cartilage regeneration using ASCs. Combining the latest technologies provides hope that ASCs might be useful for cartilage engineering.

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## Figure legends

### **Fig.1 Stem cell seeding into the collagen scaffold**

Cultured passage 3 adipose-derived stem cells were harvested and re-suspended with neutralized type I collagen solution, and each 50  $\mu$ l of cell suspension ( $1 \times 10^6$  cells) was absorbed with each collagen sponge scaffold and incubated at 37°C for gelation.

### **Fig.2 Semi-quantitative histological analysis**

**SZ: Surface zone**

**MZ: Middle zone**

Toluidine blue – hematoxylin stained sections were analyzed for cell proliferation quantification on both surface and middle zone. High-power digital images of sections were used to measure the total number of hematoxyline positive nuclei. The degree of proliferation was quantified over the entire sponge using 3 fields in each zone at 40 magnification.

### **Fig.3 Histological analysis**

**HP: hydrostatic pressure treated groups**

**AP: atmospheric pressure control groups**

Accumulation of pericellular and extracellular metachromatic matrix that stained with Toluidine blue within collagen scaffolds was observed in both groups and increased over 4 weeks. Accumulation of the matrix in the HP group was much greater than AP groups especially after 2 weeks. Immunohistochemical evaluation revealed that expression of type II and keratan sulfate of both groups increased with time, and HP groups showed greater expression than AP groups at each time point.

### **Fig.4 Semi-quantitative analysis of histology**

**MZ: Middle zone**

**SZ: Surface zone**

**HPF: High-power field (40x images)**

**AP: Atmospheric pressure group**

**HP: Hydrostatic pressure group**

Cell number in AP groups achieved a peak at two weeks of incubation and decreased after peaking on both middle and surface zone of sponges. On the other hand, that of HP groups increased gradually on middle zone, and peaked at a week after incubation and kept their number in the entire course on surface zone.

### **Fig.5 Realtime RT-PCR**

**ACAN: Aggrecan, Sox9: SRY-box9, COL2A1: Type II collagen alfa-1, COL10A1: Type X collagen alfa-1**

**AP: Atmospheric pressure group**

**HP: Hydrostatic pressure group**

Three sponges in each group at each time points were analyzed by real time RT-PCR. Chondrogenic specific gene expression of type II and X collagen, aggrecan and sox9 were observed on both groups. However, there was significant differences between HP and AP groups after 2 weeks of culture on aggrecan and sox 9, after 3 weeks of culture on type X collagen, and at 2 and 4 weeks of time point on type II collagen.

## Figure legends

### **Fig.1 Stem cell seeding into the collagen scaffold**

Cultured passage 3 adipose-derived stem cells were harvested and re-suspended with neutralized type I collagen solution, and each 50  $\mu$ l of cell suspension ( $1 \times 10^6$  cells) was absorbed with each collagen sponge scaffold and incubated at 37°C for gelation.

### **Fig.2 Wave-form of cyclic hydrostatic pressure produced by bioprocessor**

Cell constructs were incubated on the first week with treatment of cyclic hydrostatic pressure (HP) at 0-0.5 MPa (3750 mmHg, 4.93 atm), 0.5 Hz, with a medium flow rate of 0.1 ml/min, at 37°C, 3% O<sub>2</sub>, and 5% CO<sub>2</sub> using an automated pressure/perfusion bioprocessor TEP-P02 (Takagi Industrial, Shizuoka, Japan).

### **Fig.3 Quantitative histological analysis**

#### **SZ: Surface zone**

#### **MZ: Middle zone**

Truidine blue – hematoxylin stained sections were analyzed for cell proliferation quantification on both surface and middle zone. High-power digital images of sections were used to measure the total number of hematoxiline positive nuclei. The degree of proliferation was quantified over the entire sponge using 3 fields in each zone at 40 magnification.

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Accumulation of pericellular and extracellular metachromatic matrix that stained with Toluidine blue within collagen scaffolds was observed in both groups and increased over 4 weeks. Accumulation of the matrix in the HP group was much greater than AP groups especially after 2 weeks. Immunohistochemical evaluation revealed that expression of type II and keratan sulfate of both groups increased with time, and HP groups showed greater expression than AP groups at each time point.

### **Fig.5 Quantitative analysis of histology**

**MZ: Middle zone**

**SZ: Surface zone**

Cell number in AP groups achieved a peak at two weeks of incubation and decreased after peaking on both middle and surface zone of sponges. On the other hand, that of HP groups increased gradually on middle zone, and peaked at a week after incubation and kept their number in the entire course on surface zone.

### **Fig.6 Realtime RT-PCR**

**1A: AP group at 1 week after incubation, 1H: HP group at 1 week after incubation,**

**2A: AP group at 2 week after incubation, 2H: HP group at 2 week after incubation,**

**3A: AP group at 3 week after incubation, 3H: HP group at 3 week after incubation,**

**4A: AP group at 4 week after incubation, 4H: HP group at 4 week after incubation**

**ACAN: Aggrecan, Sox9: SRY-box9, COL2A1: Type II collagen alfa-1, COL10A1:**

**Type X collagen alfa-1**

Three sponges in each group at each time points were analyzed by real time RT-PCR.

Chondrogenic specific gene expression of type II and X collagen, aggrecan and sox9 were observed on both groups. However, there was significant differences between HP and AP groups after 2 weeks of culture on aggrecan and sox 9, after 3 weeks of culture on type X collagen, and at 2 and 4 weeks of time point on type II collagen.

Gene	Gene Bank Code	Forward	Reverse
Type II collagen alfa-1 (COL2A1)	NM_001844	5-CAACACTGCCAACG TCCAGAT-3'	5-CTGCTTCGTCCA GATAGGCAAT-3'
Type X collagen (COL10A1)	NM_000493	5-TGCTAGTATCCTTG AACTTGGTTCAT-3'	5-CTGTGTCTTGGT GTTGGGTAGTG-3'
Aggrecan (ACAN)	NM_001135	5-AAGTATCATCAGTC CCAGAATCTAGCA-3'	5-CGTGGAATGCA GAGGTGGTT-3'
SRY-box9 (Sox9)	NM_000346	5-ACACACAGCTCACT CGACCTTG-3'	5-GGAATTCTGGT TGGTCCTCTCTT-3 ,
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_002046	5-GAAGGTGAAGGTC GGAGTC-3'	5-GAAGATGGTGA TGGGATTTTC-3'

**Tab. 1 Primers for realtime RT-PCR**

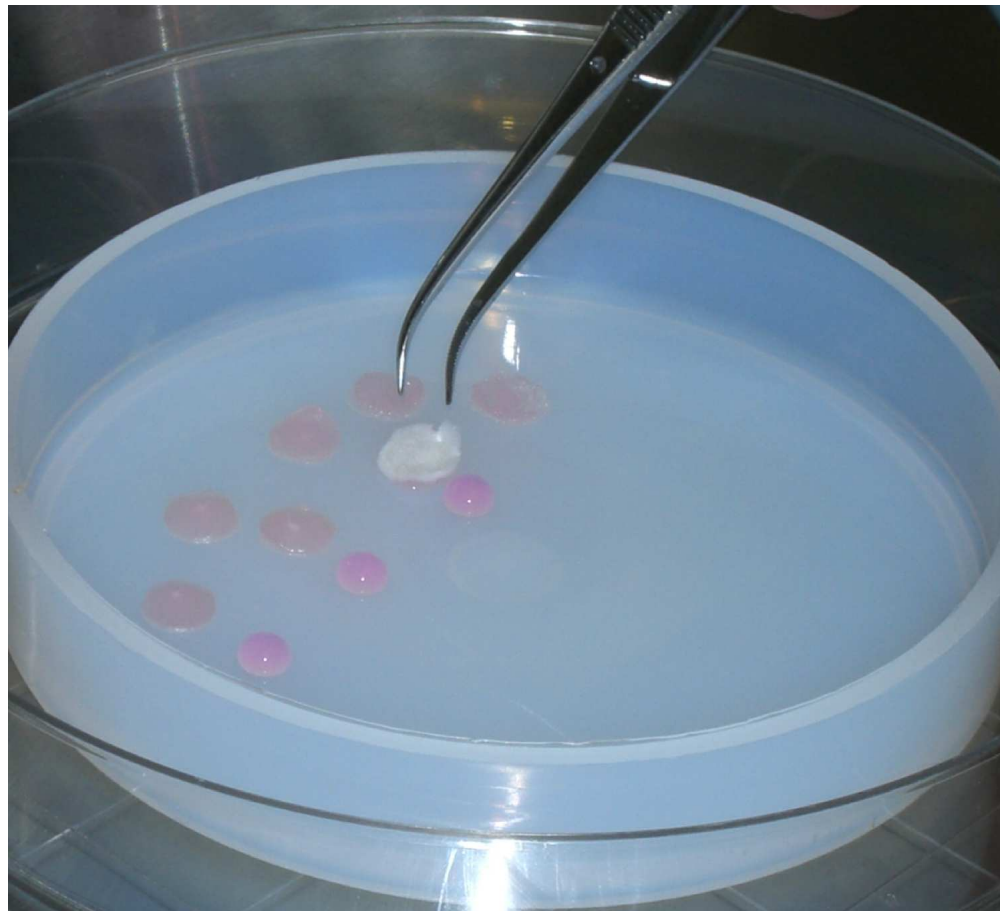


Fig.1 --- Stem cell seeding into the collagen scaffold --- Cultured passage 3 adipose-derived stem cells were harvested and re-suspended with neutralized type I collagen solution, and each 50  $\mu$ l of cell suspension ( $1 \times 10^6$  cells) was absorbed with each collagen sponge scaffold and incubated at  $37^{\circ}\text{C}$  for gelation.

127x115mm (300 x 300 DPI)

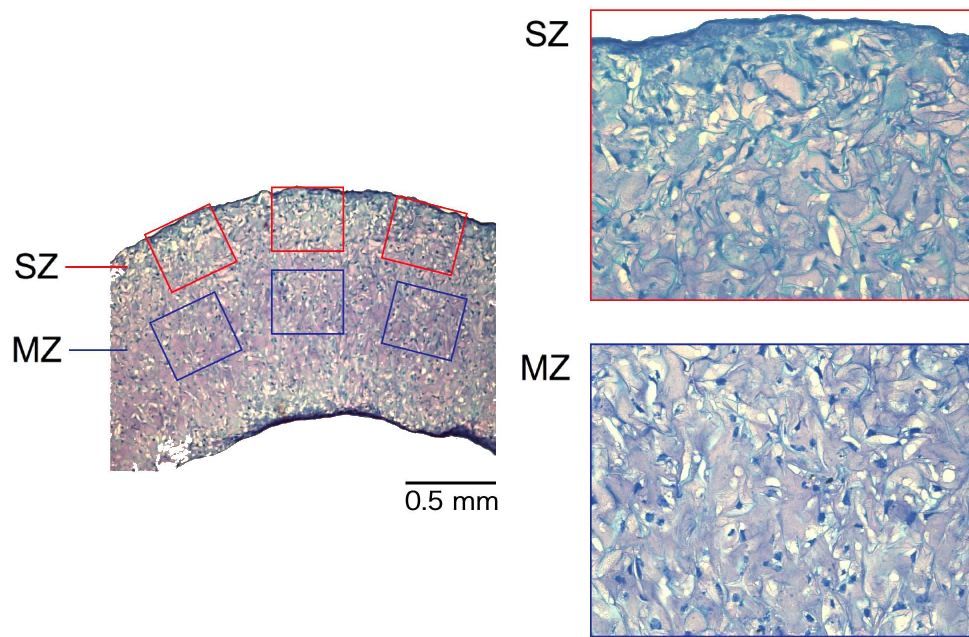


Fig.2 --- Semi-quantitative histological analysis --- SZ: Surface zone --- MZ: Middle zone --- Truidine blue - hematoxylin stained sections were analyzed for cell proliferation quantification on both surface and middle zone. High-power digital images of sections were used to measure the total number of hematoxiline positive nuclei. The degree of proliferation was quantified over the entire sponge using 3 fields in each zone at 40 magnification.

169x114mm (300 x 300 DPI)



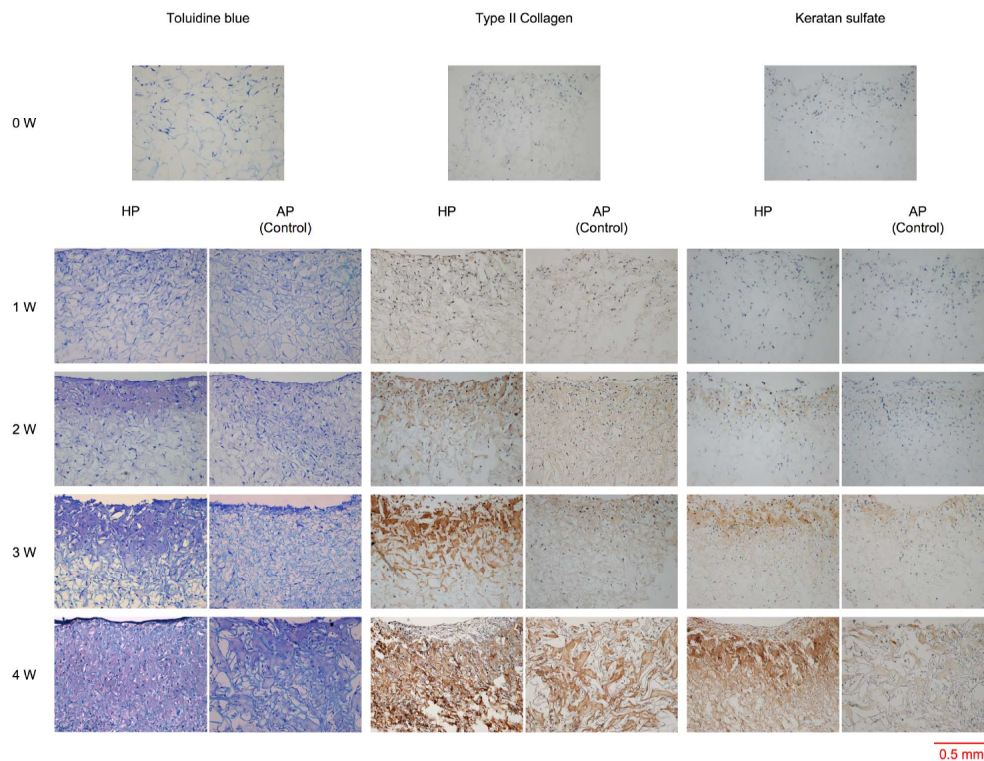


Fig.3 --- Histological analysis --- HP: hydrostatic pressure treated groups --- AP: atmospheric pressure control groups --- Accumulation of pericellular and extracellular metachromatic matrix that stained with Toluidine blue within collagen scaffolds was observed in both groups and increased over 4 weeks. Accumulation of the matrix in the HP group was much greater than AP groups especially after 2 weeks. Immunohistochemical evaluation revealed that expression of type II and keratan sulfate of both groups increased with time, and HP groups showed greater expression than AP groups at each time point.

169x133mm (300 x 300 DPI)

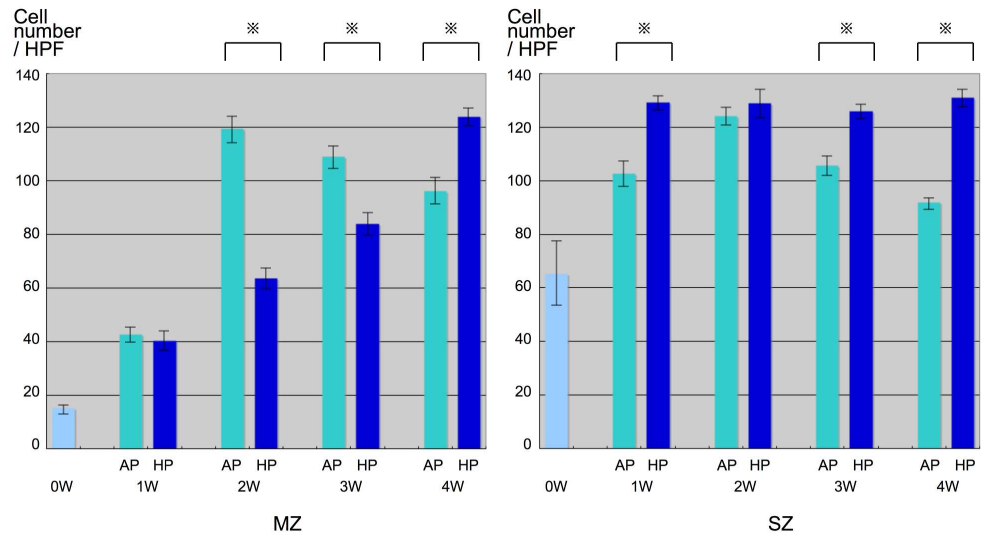


Fig.4 --- Semi-quantitative analysis of histology --- MZ: Middle zone --- SZ: Surface zone --- HPF: High-power field (40x images) --- AP: Atmospheric pressure group --- HP: Hydrostatic pressure group --- Cell number in AP groups achieved a peak at two weeks of incubation and decreased after peaking on both middle and surface zone of sponges. On the other hand, that of HP groups increased gradually on middle zone, and peaked at a week after incubation and kept their number in the entire course on surface zone.

169x97mm (300 x 300 DPI)

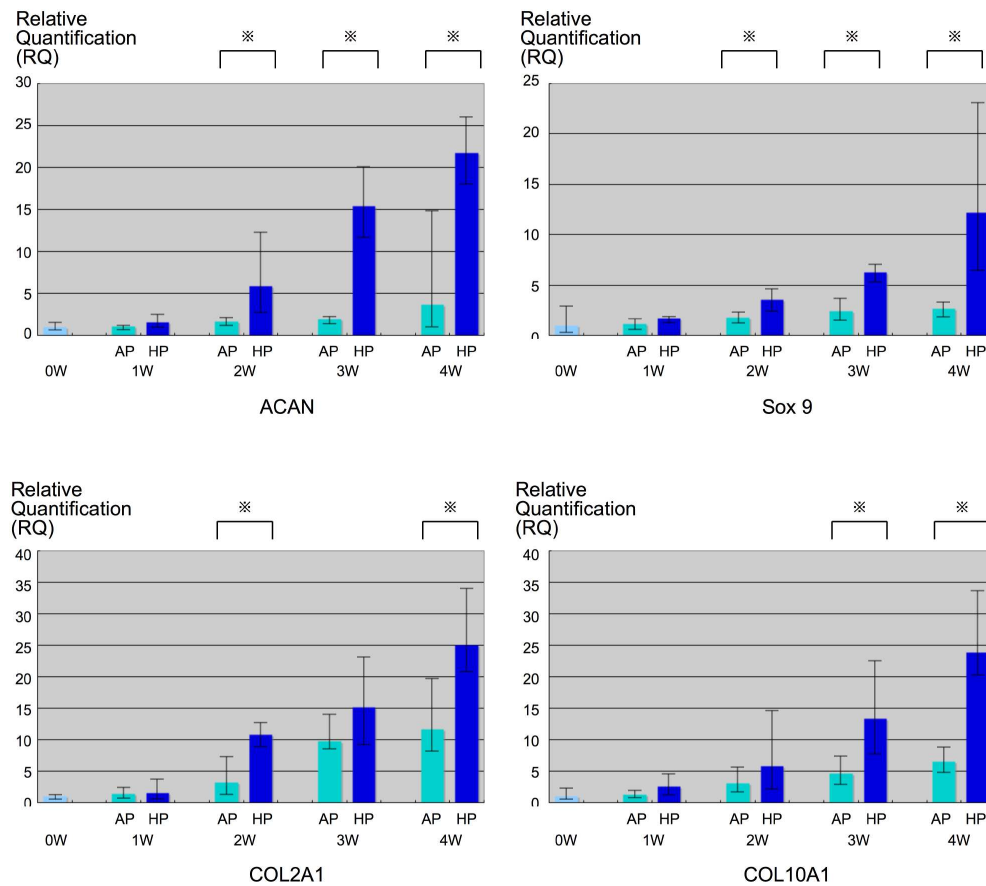


Fig.5 --- Realtime RT-PCR --- ACAN: Aggrecan, Sox9: SRY-box9, COL2A1: Type II collagen alfa-1, COL10A1: Type X collagen alfa-1 --- AP: Atmospheric pressure group --- HP: Hydrostatic pressure group --- Three sponges in each group at each time points were analyzed by real time RT-PCR. Chondrogenic specific gene expression of type II and X collagen, aggrecan and sox9 were observed on both groups. However, there was significant differences between HP and AP groups after 2 weeks of culture on aggrecan and sox 9, after 3 weeks of culture on type X collagen, and at 2 and 4 weeks of time point on type II collagen.

169x156mm (300 x 300 DPI)