Title:

Synthetic PAMPS gel activates BMP/Smad signaling pathway in ATDC5 Cells, which plays a significant role in the gel-induced chondrogenic differentiation

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Abstract: The purposes of this study were to identify signaling pathways that were specifically activated in ATDC5 cells cultured on poly (2-acrylamido-2-methylpropanesulfonic acid) (PAMPS) gel in insulin-free maintenance medium and to evaluate the significance of the determined signaling pathways in the chondrogenic differentiation induced by this gel. In this study, ATDC5 cells cultured on PAMPS gel using the maintenance medium without insulin (PAMPS Culture) were compared with cells cultured on polystyrene using the differentiation medium containing insulin (PS-I Culture). The microarray analysis, Western blot analysis, and real-time PCR analysis demonstrated that the TGF-β/BMP signaling pathway was significantly enhanced at Days 1, 2, and 3 in the PAMPS Culture when compared with the PS-I Culture. Inhibition of the BMP type-I receptor reduced the phosphorylation level of Smad1/5 and expression of type-2 collagen and aggrecan mRNA in the cells accompanied by a reduction in cell aggregation at Day 13 in the PAMPS Culture. The inhibition of the TGF-β/BMP signaling pathway significantly inhibited the chondrogenic differentiation induced by the PAMPS gel. The present study demonstrated that synthetic PAMPS gel activates the TGF-β/BMP/Smad signaling pathway in the ATDC5 cells in the absence of insulin, and that this activation plays a significant role in the chondrogenic differentiation induced by PAMPS gel.

Keywords: PAMPS hydrogel; Chondrogenic differentiation; Signaling pathway; BMP (bone morphogenetic protein); TGF (transforming growth factor).

Introduction

Articular cartilage defects are frequently made from trauma, various joint diseases, and age-related degeneration, and result in osteoarthritis of joints. It has been commonly accepted that articular cartilage tissue cannot regenerate in vivo.[1,2] Therefore, a number of investigations have studied methods to restore defects with tissue-engineered cartilage-like tissue or cell-seeded scaffold material to repair osteochondral defects.[3-6] However, functional repair of articular cartilage defects still remains a major issue in tissue regeneration medicine.

We have investigated an originally developed double-network (DN) hydrogel as a potential biomaterial to develop an innovative therapeutic method for cartilage repair;[7] this DN hydrogel is composed of poly(2-acrylamido-2-methylpropanesulfonic acid) (PAMPS) and poly(N,N'-dimethylacrylamide) (PDMAAm), and is resistant to biodegradation without any acute or sub-acute toxicity[8,9] as well as it is able to enhance chondrogenic differentiation of ATDC5 cells in in vitro conditions.[10] Further, we have found that implantation of a PAMPS/PDMAAm DN gel plug induces hyaline cartilage regeneration within 4 weeks in vivo in a large osteochondral defect created in the rabbit knee.[10]

The two independently cross-linked polymer networks in the DN gel are physically entangled with each other. The PAMPS network in the DN gel is negatively charged and has a sulphonic acid base, being similar to proteoglycans in normal cartilage. Ogawa et al.[11] established that the PAMPS gel has the ability to induce hyaline cartilage regeneration in vivo. However, the mechanism of how the synthetic PAMPS gel induces chondrogenic differentiation in vivo remains unclear. It is urgently needed to elucidate this mechanism to become able to apply the above-described discovery in novel therapeutic methods to repair osteochondral defects without cultured cells or mammalian-derived scaffolds.[12]

The present study attempts to elucidate the in vitro mechanism as to why the synthetic PAMPS gel can induce chondrogenic differentiation by using murine ATDC5 cells, representing a chondroprogenitor clone, for the following two reasons. First, the ATDC5 cell line has been utilized as a reproducible model cell line for chondrogenic differentiation studies because of its excellent characteristics including easy culture, rapid proliferation, and undifferentiation maintenance and homogeneity under usual culture conditions,[13] even though it needs stimulation with insulin to undergo chondrogenic differentiation.[14] Second, our previous in vitro study showed that the PAMPS gel can induce chondrogenic differentiation of ATDC5 cells even in an insulin-free maintenance medium.[15] Here, we conducted an in vitro study using a microarray analysis of mRNA expression together with computer-based signal-pathway analyses, Western blot analysis of candidate molecules, and real-time PCR analysis of the targets. The first purpose of this study was to identify signaling pathways that were specifically activated in the ATDC5 cells cultured on the PAMPS gel in the insulin-free maintenance medium and compare the results with culturing on polystyrene (PS) in insulin-supplemented differentiation medium. The second purpose was to evaluate the significance of the determined signaling pathways in the chondrogenic differentiation induced by the PAMPS gel by using a specific inhibitor for the pathway.

Materials and Methods

Preparation of PAMPS gel

2-acrylamido-2-methylpropanesulfonic acid (AMPS; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and 2-oxoglutaric acid (Wako Pure Chemical Industries, Ltd, Osaka, Japan) were used as purchased. N,N’-methylenebisacrylamide (MBAA; Tokyo Chemical Industry Co., Ltd.,

Tokyo, Japan) was purified by recrystallization from ethanol. PAMPS gel was synthesized by radical polymerization using MBAA as a cross-linker and 2-oxoglutaric acid as an initiator. The monomer concentration was 1 mol/l for PAMPS, 4 mol% for the cross-linker, and 0.1 mol% for the initiator. Aqueous solution containing a monomer, cross-linker, and the initiator was injected into a cell consisting of a pair of glass plates separated by a silicone rubber. After gelation, gels were immersed in 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; Sigma-Aldrich, St. Louis, MO, USA) buffer solution [$NaHCO_3$ 1.55 $x10^{-2}$ M, HEPES 5 x 10^{-3} M, NaCl 0.14 M, pH 7.4] and the HEPES solution was changed twice daily for 1 week to reach equilibrium. After sterilizing by autoclaving (120 ºC, 20 min), gel disks were punched out from a gel sheet with a hole punch having a diameter of 1.5 or 3.5 cm. The thickness of the gel disk was approximately 2 mm. The gel disks were then placed at the bottom of 6-well or 24-well PS plates.

Study design

The series of experiments described below were performed to identify the specific signaling pathways in the chondrogenic differentiation induced by PAMPS gel. First, we investigated histological and gene expression differences in chondrogenic differentiation of ATDC5 cells present during a 15-day period in cultures on PS dishes (PS Culture) and the PAMPS gel surface (PAMPS Culture) in the maintenance medium, as well as on the PS dish surface supplemented with insulin (PS-I Culture). In this experiment, histomorphological observations and Alcian blue staining were made at Days 5, 10, and 15. Real time PCR analysis was performed every two days; at Days 1, 3, 5, 7, 9, 11, and 13. Second, we performed microarray and Western blot analyses. The data were collected from the analyses at Days 1, 2, and 3, and based on the results, we additionally evaluated the gene expression of BMP4 during

the same period. Third, we evaluated the effect of inhibition of the TGF-β/BMP signaling pathway on the chondrogenic differentiation. We performed Western blot analysis for Smad1/5, and evaluated the effect of dorsomorphin on the chondrogenic differentiation induced by the PAMPS gel. The phosphorylation levels of Smad1/5 were evaluated during the 72-hour culture period; at 6, 12, 24, 36, 48, 60, and 72 hours. The effect of inhibition by dorsomorphin was evaluated at Days 7 and 13. In this study, we used dorsomorphin (Abcam Biochemicals, Cambridge, UK) because dorsomorphin selectively inhibits the BMP type I receptors such as ALK2, ALK3 and ALK6 and subsequently blocks BMP-mediated Smad1/5/8 phosphorylation.[16]

Cell culture

The ATDC5 cell line was obtained from the RIKEN cell Bank (Tsukuba, Japan). The maintenance medium consisted of 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (GIBCO Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum, 10 μg/ml human transferrin (Roche, Basel, Switzerland) and 30 nM sodium selenite (Sigma-Aldrich, St. Louis, MO, USA). The differentiation medium to induce chondrogenic differentiation were made by supplementing 10 μg/ml bovine insulin (Sigma-Aldrich, St. Louis, MO, USA) into the maintenance medium. The cells were seeded at a cell density of 5 x 10^4 cells/cm^2, at 37 ºC under 5% CO_2. The media were replaced every two days.

Alcian blue staining

ATDC5 cells were stained with Alcian blue at Days 5, 10, and 15 to compare the morphological changes among the PS, PS-I, and PAMPS Cultures. The cells were rinsed with

PBS, fixed with 4% paraformaldehyde for 20 minutes, stained with 1% Alcian Blue 8GS (Fluka, Sigma–Aldrich, St. Louis, MO, USA) in 0.1 M HCl at room temperature, rinsed with PBS, and examined with light microscopy.

Microarray analysis

Gene expression profiles of the cultured ATDC5 cells were analyzed with use of custom-made DNA microarray. Total RNA was isolated from each sample using ISOGEN with Spin Column (Nippon Gene, Tokyo, Japan). The RNA integrity number (RIN) and quantity of the RNA were measured with a 2100 Bioanalyzer (Agilent Technologies, CA, USA). Microarray experiments were conducted using SurePrint G3 Mouse GE 8 x 60K Microarray. The probes were designed to detect the directly labeled mRNA from 55681 genes from the NCBI database. The probes were loaded on a microarray in triplicate. Cy3 labeling, hybridizations, scanning with a DNA Microarray Analyzer (Agilent Technologies, CA, USA), data normalization, and Gene Ontogeny analysis were performed by DNA Chip Research Inc. (Kanagawa, Japan), an Agilent-certified Service Provider. The microarray analyses were repeated three times using the same mRNA sample to confirm the reproducibility. For hierarchical cluster analysis, the data set was filtered by removing probes having missing value and then selected showing differences in expression level at least 2-fold. The analysis to both axes was performed applying pairwise complete-linkage method by using the CLUSTER 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm#ctv). Pearson’s correlation coefficient was used to measure the similarity for gene expression data between two groups. The results were visualized and analyzed by using the TreeView (http://jtreeview.sourceforge.net). Then, we performed Gene Set Enrichment Analysis (GSEA) with the Fisher's exact test[17,18] to

clarify the signaling pathways which were significantly enhanced in the PAMPS Culture at Days 1, 2, and 3, compared with the PS and PS-I Cultures. In addition, we carried out the pathway analysis using the ConPath Software (from http://conpath.dna-chip.co.jp/).

Western blot analysis

Cells were lysed with a lysis buffer [10 mMTris-HCl (pH 7.5) with 150 mM NaCl, 1% Triton X-100, 0.1% SDS, complete protease inhibitor mixture (Roche, Basel, Switzerland), phosphatase inhibitor mixture (Sigma-Aldrich, St. Louis, MO, USA)] and centrifuged at 15,000 rpm for 5 minutes at 4 ºC. Lysate proteins (20μg) were fractionated by 10% SDS-polyacrylamide gel electrophoresis, and separated proteins were transferred to a polyvinylidenedifluoride (PVDF) filter (Immobilon, Millipore, Billerica, MA, USA). The blots were blocked to reduce nonspecific signals with 1% bovine serum albumin in TBS-Tween. PVDF filters were probed with the following antibodies (Cell Signaling Technology, USA) shown in Table 1. Immune complexes were labeled with horseradish peroxidase-conjugated mouse or rabbit antibodies (BIOSOURCE International, Camarillo, CA, USA) and visualized by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, NJ, USA) and a LAS-1000 Plus system (Fujifilm, Tokyo, Japan). To evaluate the difference of Smad1/5 phosphorylation status between the PS and PAMPS Cultures, the level of p-Smad1/5 was normalized by total Smad1 using image analysis software (NIH ImageJ 1.44p; National Institutes of Health, Bethesda, MD, USA).

Real-time PCR analysis

Total RNA was extracted from the cells, using the RNeasy mini kit (Qiagen, Valencia,

CA). RNA quality from each sample was assured by the A260/280 absorbance ratio. The RNA was reverse-transcribed into single strand cDNA using PrimeScript RT reagent kit (TakaraBio, Ohtsu, Japan). The sequences of primers used in real-time PCR analyses were shown in Table 2. The real-time PCR was performed in a Thermal Cycler Dice TP800 (TakaraBio, Ohtsu, Japan) by using SYBR Premix Ex TaqTM (TakaraBio, Ohtsu, Japan). The cDNA template (5 ng) was used for real-time PCR in a final volume of 25μl, and cDNA was amplified according to the following conditions: 95 ºC for 5 sec and 60 ºC for 30 sec at 40 amplification cycles. Fluorescence changes were monitored with SYBR Green after every cycle. A dissociation curve analysis was performed (0.5 degrees Celsius/sec increase from 60 to 95 ºC with continuous fluorescence readings) at the end of cycles to ensure that single PCR products were obtained. The results were evaluated using the Thermal Cycler Dice Real Time System software program (TakaraBio, Ohtsu, Japan). The delta-delta-Ct algorithm was used to analyze the relative changes in gene expression.[19] The gene expression level was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

All data were described as the mean and standard deviation values. The mean value of gene expression was compared among the three groups using the one-way analysis of variance (ANOVA) with the Fisher's PLSD tests for post-hoc multiple comparisons at each period. The mean value of phosphorylation or expression level was compared between the two groups using the unpaired t-test. Concerning the increase or decrease of the measured value through the culture period, Dunnett's test was performed in comparison with the value at Day 1 if the ANOVA detected a statistical significance. The significance level was set at $p=0.05$ in each

comparison.

Results

Differences in the chondrogenic differentiation process between Insulin-induction and PAMPS gel-induction

In the PS-I Culture, the cells proliferated remarkably at Days 5 and 10, and showed multi-layered proliferation at Day 15 (Figure 1), while in the PS Culture there was no cell condensation or aggregation throughout the experimental period. Proliferated cells were aggregated earlier in the PAMPS Culture than in the PS-I Culture (at Day 10) (Figure 1). In addition, cells were stained with Alcian blue to show the synthesis of sulfated glycosaminoglycans at Day 15 in the PAMPS and PS-I Cultures (Figure 1), here the area stained with Alcian blue was much larger in the PAMPS Culture than in the PS-I Culture. The real-time PCR analysis showed that type-2 collagen mRNA expression was significantly higher in the PAMPS Culture than in the PS-I Culture at Days 1-13, while the expression was significantly higher in the PS-I Culture than in the PS Culture at Days 9-11 (Figure 1). In addition, aggrecan mRNA expression was significantly higher in the PAMPS Culture than in the PS-I Culture at Days 3, 9, 11 and 13, while the expression was significantly higher in the PS-I Culture than in the PS Culture at Day 13 (Figure 1). These results showed that the chondrogenic differentiation process of the ATDC5 cells was not identical during the first 15-day period in the PAMPS and PS-I Cultures.

Microarray analysis to determine signaling pathways significantly enhanced the chondrogenic differentiation induced by PAMPS gel

The gene expression results obtained with the microarray (see supplemental material) were further examined by clustering analysis. As shown in Figure 2, a specific gene expression profile was observed in the PAMPS Culture. In the clustering analysis, the 3 PAMPS Culture samples clustered separately from both the PS and PS-I Cultures as indicated by the dendrogram. GSEA following the DNA microarray analysis showed the signaling pathways to be significantly enhanced in the PAMPS Culture at Days 1, 2, and 3, over that with the PS and PS-I Cultures (Table 3 and 4, respectively). In these pathways, we focused on pathways which are known as major signaling pathways related to chondrogenic differentiation. At Days 1, 2, and 3 the TGF-β signaling pathway was significantly enhanced in the PAMPS Culture, compared with both the PS and PS-I Cultures. The MAPK and Wnt signaling pathways were significantly enhanced in the PAMPS Culture, compared with both the PS and PS-I Cultures, both at Days 2 and 3.

Western blot analysis of the expression and phosphorylation levels of chondrogenesis-related signaling proteins

Based on the results of the GSEA, we analyzed the expression of major signaling proteins and their phosphorylation in the TGF-β/BMP, MAPK, and Wnt signaling pathways, using Western blot analysis (Figure 3). Additionally, we analyzed two major signaling pathways related to chondrogenesis, the Jak/Stat and PI3K/Akt signaling pathways. For the TGF-β/BMP signaling pathway, the phosphorylation level of Smad1/5 was clearly enhanced in the PAMPS Culture at Days 2 and 3, in comparison with the level in the PS and PS-I Cultures (Figure 3). For the MAPK signaling pathway, there was no clear change in the phosphorylation levels of SAPK/JNK, ERK, MEK1/2, p90RSK, or MSK in the PAMPS Culture, also compared with the PS and PS-I Cultures; the phosphorylation level of p38 was clearly increased in the PAMPS

Culture at Day 3 (Figure 3). There were no clear changes in the expression of the selected proteins or their phosphorylation levels in the Wnt signaling pathway or other signaling pathways.

Pathway analysis to determine highly expressed molecules in the TGF-β/BMP signaling pathway activated by PAMPS gel

To determine highly expressed molecules in the TGF-β/BMP signaling pathway activated in chondrogenic differentiation induced by PAMPS gel, a pathway analysis using the ConPath Software was performed. The result of the pathway analysis performed at Day 2 is shown in Figure 4. The pathway analysis showed that the BMP4 gene, as well as the Inhba, Ltbp1, Fst, Thbs1, Smad6, and Serpine1, were expressed by 2-fold or more in the PAMPS Culture, compared with PS-I Culture.

Expression of BMP4 mRNA in chondrogenic differentiation induced by PAMPS gel

Among the highly expressed genes in the pathway analysis, we considered BMP4 further, as BMP4 is known as the most upstream molecule in the TGF-β/BMP signaling pathway for chondrogenesis.[20] The expression level of BMP4 mRNA was significantly greater in the PAMPS Culture than in the PS and PS-I Cultures at Day 1 ($p<0.0001$ and $p<0.0001$, respectively) and Day 3 ($p=0.0001$ and $p<0.0001$, respectively) (Figure 5).

Effect of application of an inhibitor of the BMP receptor on the TGF-β/BMP signaling pathway enhanced by PAMPS gel

We investigated whether application of an inhibitor for the BMP receptor inhibited the

TGF-β/BMP signaling pathway in PAMPS Culture. First, the phosphorylation level of Smad1/5 as an indicator to monitor the degree of inhibition was investigated. As there is the possibility that the increase in the phosphorylation level of Smad1/5 described above may be a short-term alteration caused by ultradian oscillation of Smad,[21] the phosphorylation of Smad1/5 was measured together with the protein amounts at 6, 12, 24, 36, 48, 60, and 72 hours, using Western blot analysis (Figure 6). The phosphorylation levels of Smad1/5 were gradually increased in the PAMPS Culture. The results of the ANOVA demonstrated that there was a significant difference in the phosphorylation levels of Smad1/5 in the PAMPS and PS Cultures ($p=0.0001$) (Figure 6). The post-hoc test showed that there was a significant difference at each period after 36 hours ($p=0.0034$ at 36 hours, $p=0.0001$ at 48 hours, $p=0.0004$ at 60 hours, and $p=0.0003$ at 72 hours).

The effects of dorsomorphin applications of 0, 1, 5, and 10 μM on the phosphorylation levels of Smad1/5 in the PAMPS Culture were evaluated using Western blot analysis. A 10 μM dorsomorphin application reduced the phosphorylation level of Smad1/5 most (Figure 6). 10 μM dorsomorphin was applied in the PAMPS and PS Cultures, with dimethylsulfoxide (DMSO; Wako Pure Chemical Industries, Ltd, Osaka, Japan) used as the vehicle control at 24 hours. In the results, the 10 μM dorsomorphin application reduced the phosphorylation level of Smad1/5 at 24, 48, and 72 hours in the PAMPS Culture (Figure 6), and reduced the phosphorylation level of Smad1/5 at 48 and 72 hours in the PS Culture only slightly. These results demonstrated that inhibition of the BMP type-I receptor inhibited the TGF-β/BMP signaling pathway enhanced by PAMPS gel.

Effect of inhibition of the TGF-β/BMP signaling pathway on the chondrogenic differentiation induced by PAMPS gel

The possible inhibition by dorsomorphin application on chondrogenic differentiation induced by the PAMPS gel was evaluated. 10 μM dorsomorphin was applied in the PAMPS and PS Cultures at 24 and 72 hours. First, we observed the effect of the application on cell aggregation. The dorsomorphin application clearly reduced aggregation of ATDC5 cells in the PAMPS Culture, while the reduction effect was not observed in the PS Culture (Figure 7). Second, the dorsomorphin application significantly reduced type-2 collagen and aggrecan mRNA expression in the PAMPS Culture at Day 13 ($p<0.0001$ and $p=0.0165$, respectively) (Figure 7). Although the dorsomorphin application also significantly reduced these gene expressions in the PS Culture ($p=0.0111$ and $p=0.0101$) (Figure 7), the effect of the reduction by dorsomorphin on type-2 collagen and aggrecan mRNA expression, which was calculated by dividing the expression level when treated with dorsomophin by the level when treated with DMSO, were significantly higher in the PAMPS Culture than in the PS Culture ($p=0.0178$ and $p=0.0006$, respectively) (Figure 7). These results demonstrated that inhibition of the TGF-β/BMP signaling pathway significantly inhibited the chondrogenic differentiation induced by the PAMPS gel.

Discussion

The present study demonstrated that the TGF-β/BMP signaling pathway was significantly enhanced in ATDC5 cells at the early phase (Days 1, 2, and 3) in the PAMPS Culture, compared with the PS-I Culture and that inhibition of the BMP type-I receptor using dorsomorphin reduced the phosphorylation level of Smad1/5 and expression of type-2 collagen and aggrecan mRNA in ATDC5 cells, accompanied with reduction in cell aggregation, at Day 13 in the PAMPS Culture. These results showed that inhibition of the TGF-β/BMP signaling pathway significantly inhibited the chondrogenic differentiation induced by the PAMPS gel.

Previously, it has been established that the PAMPS gel can induce chondrogenic differentiation in ATDC5 cells in vitro even in insulin-free maintenance medium[14] and cartilage regeneration in an osteochondral defect in vivo.[11] However, the mechanism of how the synthetic gel functions in this manner has been unknown. The present study clarified that that the PAMPS gel can activate the TGF-β/BMP signaling pathway, which plays important roles in the chondrogenic differentiation of mesenchymal stem cells.[22-25] It is known that the BMP family plays significant roles in cartilage generation.[26-28] Further that the BMP4, which was investigated in the present study, is known as a strong stimulator of chondrogenesis both in vitro and in vivo, and it has recently attracted much attention as a potential therapeutic agent for cartilage regeneration.[29-31] The present study has provided information to explain parts of the mechanism as to how the synthetic PAMPS gel is able to induce chondrogenic differentiation and regeneration as reported in previous studies by the authors.

However, it has not been established how the PAMPS gel activated the TGF-β/BMP signaling pathway in the cells. Based on the previous studies, it is known that the highly negative charge of the PAMPS gel affects both proliferation of various cells and chondrogenic differentiation of ATDC5 cells as well as secretion of gycocalyx from vascular endothelial cells.[15,32,33] Chen et al. described that the effect of the gel charge density on cell behavior is well correlated with the total adsorbed proteins and fibronectin.[33] It is also possible that the low elastic modulus of the PAMPS gel (approximately 0.4 MPa) could activate the TGF-β/BMP signaling pathway in the ATDC5 cells which adhere on the gel surface, because it is known that the mechanical properties of a material surface significantly affect the gene expression in cultured cells and determine the direction of cell differentiation.[34,35] Engler et al.[34] reported that mesenchymal stem cells cultured on polyacrylamide gel having a low elastic modulus enhanced

gene expression of BMP and Sox9, compared with cells on PS having a high elastic modulus. Allen et al.[36] investigated whether chondrocyte differentiation is sensitive to the stiffness of the extracellular microenvironment using collagen II–coated polyacrylamide substrates with stiffness of 0.2-1.1 MPa and compared these with polystyrene plastic, and demonstrated that ATDC5 cells grown on 0.5-MPa substrates showed maximum chondrocyte gene expression relative to cells exposed to substrates of any other stiffness. Therefore, there is a strong possibility that these specific biochemical and biomechanical properties of the PAMPS gel may affect the activation of TGF-β/BMP signaling pathway and the cell behaviors.

For the above-described latter possibility, it remains largely unknown how substrate stiffness alone influences chondrogenesis. In this regard, the focal adhesion-related pathway may play an important role in the chondrogenic differentiation of the ATDC5 cells induced by the PAMPS gel. It is known that integrin forms a complex, called a “focal adhesion”, composed of specific proteins such as $p130^{cas}$, focal adhesion kinase (FAK), paxillin, vinculin, and others by stimuli from a material surface to the cellular membrane.[37-40] In the early stage of chondrogenesis, integrin-mediated FAK activation is essential for cellular condensation,[41,42] and Raghothaman et al. suggested the importance of coupling of integrin mediated cell-matrix interactions and N-cadherin/β-catenin mediated downstream signaling events in the MSC chondrogenesis.[43] In the present study, GSEA showed that the focal adhesion-related pathway and integrin-mediated pathway were enhanced in the PAMPS Culture. These results suggest that integrin played an important role in the adhesion of ATDC5 cells to the PAMPS gel surface. At the same time, it is known that FAK and/or ERK are activated in mechanosignal transduction through the focal adhesion.[40,44] However, phosphorylation of ERK was not increased in the culture on the PAMPS gel in the present study. Despite these results, there is a strong possibility

that the ATDC5 cells sense and respond to the elasticity of the PAMPS gel through the integrin-mediated pathway because integrin signaling interacts with other pathways to form a very extensive network which mediates many cellular processes.[45]

In addition to the TGF-β/BMP signaling pathway, several other pathways may contribute to the chondrogenic differentiation induced by the PAMPS gel based on the GSEA results. Among the major signaling pathways related to the chondrogenic differentiation of ATDC5 cells, the MAPK and Wnt signaling pathways were significantly enhanced in PAMPS Culture, when compared with either of the PS and PS-I Cultures. Specifically, the phosphorylation level of p38 in PAMPS Culture was higher than that with the PS and PS-I Cultures at Day 3. Recently, Greenblatt et al.[46] reported that p38, which is in the non-canonical pathway of TGF-β signaling, and also Smad1/5/8 can be simultaneously activated by TGF-β-activated kinase 1 (TAK1), resulting in induction of chondrogenic differentiation of stem cells. Related to this, Watanabe et al.[47] described that transcriptional cross-talk between the Smad, ERK and p38 pathways regulates the TGF-β-induced aggrecan gene expression in chondrogenic ATDC5 cells. However, Western blot analysis did not show an increase in the molecules related to the Wnt signaling pathway. There is some discrepancy in the results of the microarray and Western blot analysis, but this discrepancy in the expression patterns between protein and mRNA may be explained by the post-transcriptional regulation of the gene expression.[48-50] However, there is a strong possibility that these pathways play significant roles in the chondrogenic differentiation of ATDC5 cells, and it will be necessary to conduct further studies to determine this.

Among the up-regulated pathways, the inflammatory response pathway was also significantly enhanced in the PAMPS Culture. Based on the microarray data, several genes of

interest were up-regulated 2-fold or more in the PAMPS Culture, compared with the changes in the PS and PS-I Cultures (see supplemental material). Among the molecules elevated in the PAMPS Culture, MMP-2 functions as a negative regulator in chondrogenesis. Jin et al. reported that MMP-2 is involved in the regulation of cell condensation and that MMP-2-induced down-regulation of the FAK-integrin β1 interaction leads to reduced tyrosine phosphorylation of focal adhesion components, such as FAK, c- Src, and paxillin.[42] The GSEA, however, showed that the focal adhesion-related pathway was enhanced in the PAMPS Culture during the same culture period. Integrin-mediated FAK activation is essential for cellular condensation in the early stages of chondrogenesis.[41,42] Inflammatory molecules may have negative effects on the chondrogenic condensation. However, as the ATDC5 cells cultured on the PAMPS gel underwent chondrogenic differentiation through the condensation process, these negative regulators may contribute to multiple-signal-mediated control in cell differentiation because there would be feedback loops that add or remove signaling components to maintain homeostasis or regulate differentiation. Although little is known about the role of inflammatory molecules on chondrogenesis driven by the positive regulating pathways, it is possible that there is significant cross-talk between these pathways.

The present study demonstrated that inhibition of the BMP type-I receptor in the early phase (24-72 hours) reduced expression of type-2 collagen and aggrecan mRNAs in PAMPS Culture at Day 13. This result suggested that the BMP/Smad signal activated in the early phase plays a significant role in the chondrogenic differentiation of ATDC5 cells induced by the PAMPS gel. According to the literature, the process of chondrocyte proliferation and differentiation is regulated by various transcription factors, growth factors, extracellular matrices, and cell-matrix interactions.[51-53] Therefore, there is a possibility that, in the present study, the

BMP/Smad signal activated in the early phase may sequentially activate various other signals, resulting in the reduction of type-2 collagen and aggrecan mRNA expression. However, the inhibition of the BMP type-I receptor reduced expression of type-2 collagen and aggrecan mRNAs not only in the PAMPS Culture but also in the PS Culture, although the degree of inhibition was significantly greater in the PAMPS Culture than in PS Culture. This phenomenon can be explained by the previously reported fact that a small amount of BMP is constantly expressed in ATDC5 cells causing the cells to be gradually differentiated into chondrocytes at an extremely slow velocity.[54,55]

There are limitations to this study. First, we used ATDC5 cells, which are known to undergo chondrogenic differentiation under stimulation with insulin. However, the results in this study cannot be assumed to apply to different cells such as mesenchymal stem cells. Second, we did not establish which molecule was the first messenger that activated the TGF-β/BMP signaling pathway, as well as we did not evaluate the effect of insulin on the TGF-β/BMP signaling pathway. Third, we did not clarify potential changes in various signals in the very early phase within the first 24 hours of activation. Fourth, we did not examine ligands other than BMP4, which belongs to the TGF-β/BMP signaling pathway. Fifth, we cultured the ATDC5 cells only on two substrates with completely different mechanical properties. The PS dish used as a control has an elastic modulus of ~3 GPa,[56] four orders of magnitude stiffer than the PAMPS gel. However, these limitations do not negate the results, which the present study is the first to discover, that the synthetic PAMPS gel can activate the TGF-β/BMP signaling pathway in ATDC5 cells without applying any biological stimulators, such as insulin, for the chondrogenesis.

In summary, the TGF-β/BMP signaling pathway was more significantly enhanced in

ATDC5 cells at the early phase (Days 1, 2, and 3) in the PAMPS Culture than in the PS-I Culture. Further, inhibition of BMP type-I receptor using dorsomorphin reduced the phosphorylation level of Smad1/5 and the expression of type-2 collagen and aggrecan mRNA in the ATDC5 cells, accompanied with reduction in cell aggregation, at Day 13 in PAMPS Culture. The present study demonstrated that synthetic PAMPS gel activates the BMP/Smad signaling pathway in ATDC5 cells in the absence of insulin, and that it plays a significant role in the chondrogenic differentiation induced by the PAMPS gel.

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Figure legends

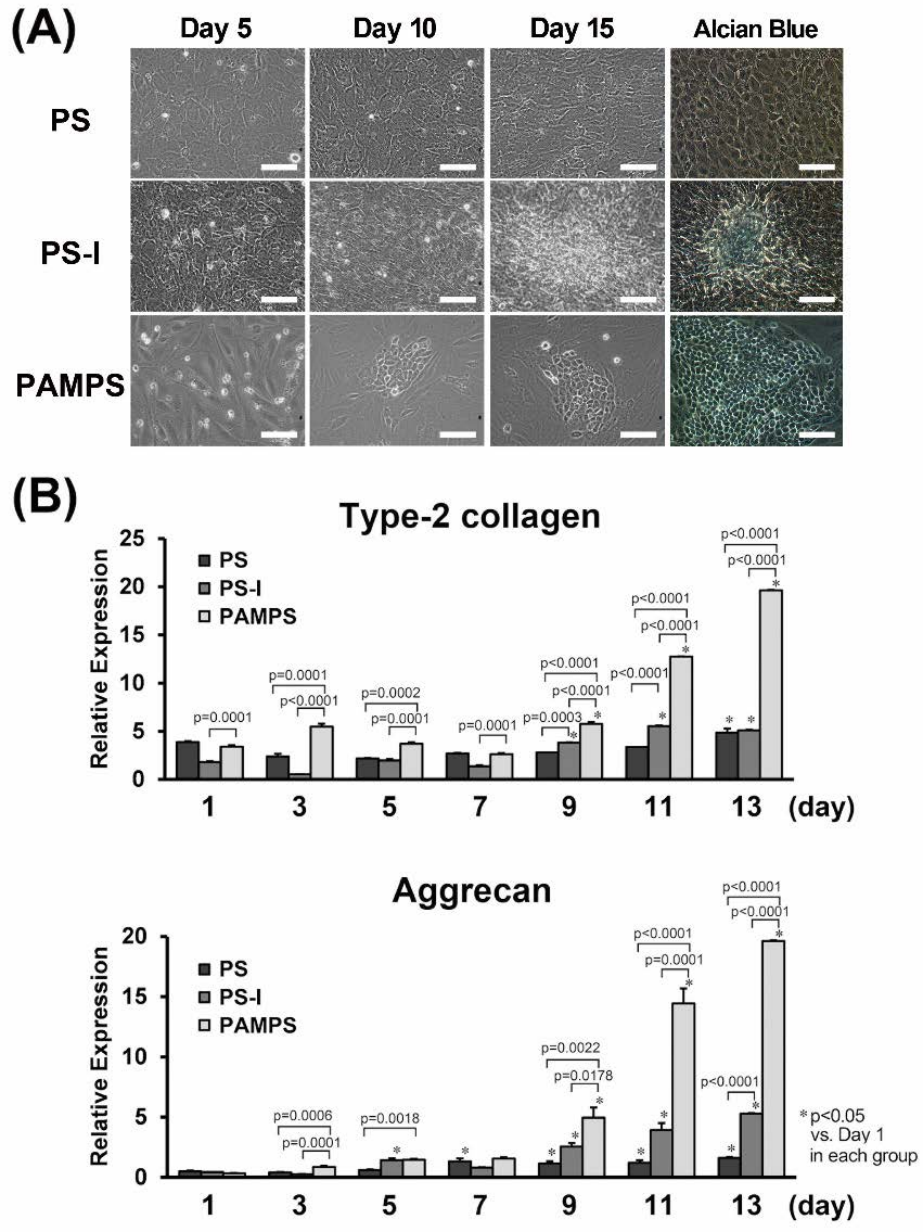

Figure 1. Differences in chondrogenic differentiation process among PS, PS-I, and PAMPS Cultures. (A) Representative phase-contrast micrographs of ATDC5 cells in the PS, PS-I, and PAMPS Cultures at Days 5, 10, and 15 after seeding, respectively. Cells stained with Alcian blue at Day 15 were shown. White scale bars show 100 μm. (B) The real-time PCR analysis showed that, in the PS-I Culture, type-2 collagen mRNA expression was significantly higher than in the

PS Culture at Days 9-11, and aggrecan mRNA expression was significantly higher than in the PS Culture only at Day 13. On the other hand, in the PAMPS Culture, type-2 collagen mRNA expression was significantly higher than in the PS-I Culture at Days 1-13, and aggrecan mRNA expression was significantly higher than in the PS-I Culture at Days 3, 9, 11 and 13. Abbreviations; PS: the culture on a polystyrene (PS) dish in the maintenance medium without insulin; PS-I: the culture on a PS dish in the differentiation medium containing insulin; PAMPS: the culture on a PAMPS gel in the maintenance medium without insulin.

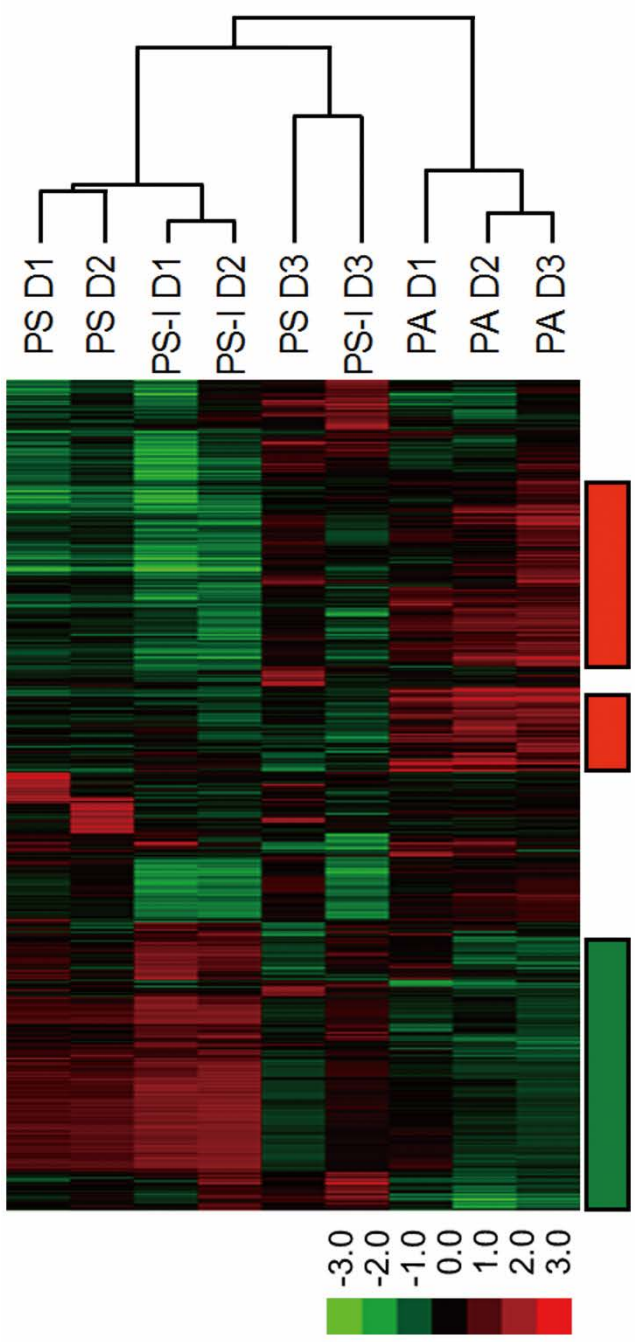

Figure 2. Hierarchical clustering of gene expression profile of PS, PS-I and PAMPS Cultures. The relative expression of 897 probes after filtering was analyzed. The level of expression of each gene is represented using a red-black-green color scale as shown in the key. The specifically up-regulated and down-regulated genes in the PAMPS Culture are indicated by red and green bar, respectively. (Lower panel): Scaled down representation of the entire cluster in the color scheme

of the heat map. (Upper panel): An experimental dendrogram of the results of the cluster analysis using TreeView program (http://jtreeview.sourceforge.net). Culture conditions were described at the top as PS; culture on a polystyrene (PS) dish in the maintenance medium without insulin, PS-I; culture on a PS dish in the differentiation medium containing insulin, and PA; culture on a PAMPS gel in the maintenance medium.

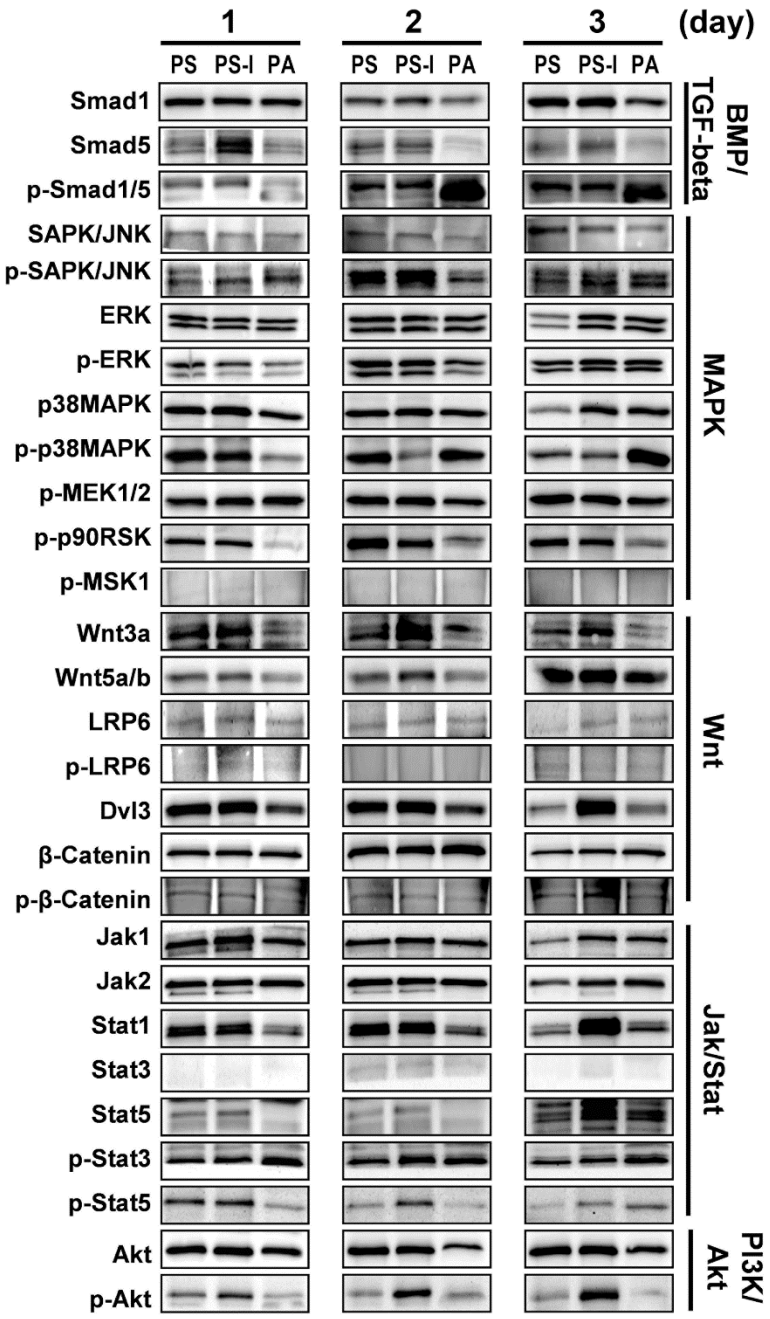

Figure 3. Western blot analysis of expression and phosphorylation levels of chondrogenesis-related major signaling proteins. Examined molecules were selected by the results of GSEA, at Days 1, 2 and 3, including signaling pathways for TGF-β/BMP, MAPK, Wnt, Jak/Stat, and PI3K/Akt indicated at the right side. Culture conditions were described at the top as PS; culture on a polystyrene (PS) dish in the maintenance medium without insulin, PS-I; culture

on a PS dish in the differentiation medium containing insulin, and PA; culture on a PAMPS gel in the maintenance medium. The names of examined molecules were indicated at the left side of the panel, and among them p- indicates phosphorylation.

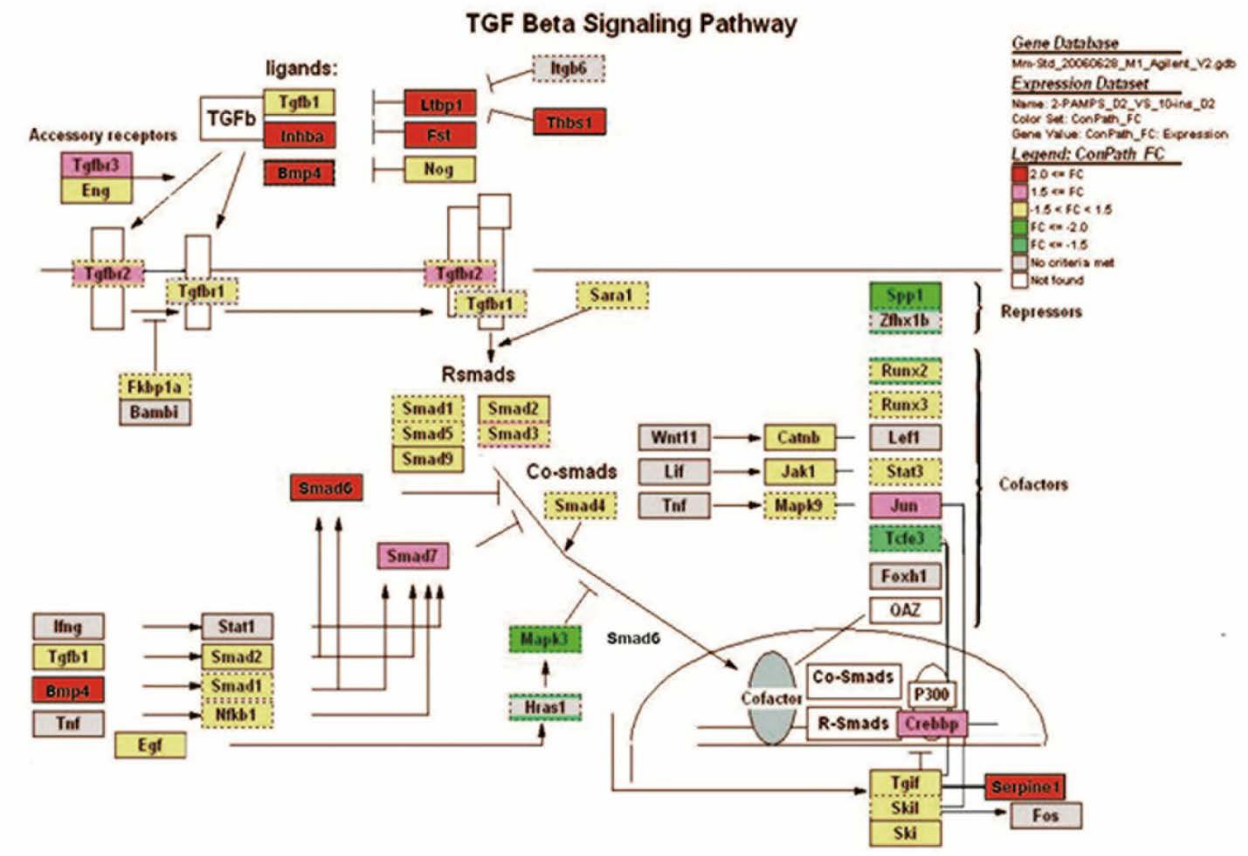

Figure 4. A representative result of the pathway analysis performed at Day 2. The pathway analysis was performed using ConPath Software to search highly expressed molecules in TGF-β/BMP signaling pathway activated in chondrogenic differentiation induced by the PAMPS gel. BMP4 gene, as well as Inhba, Ltbp1, Fst, Thbs1, Smad6, and Serpine1, was expressed by 2-fold or more (colored in red) in the PAMPS Culture, compared with the PS-I Culture.

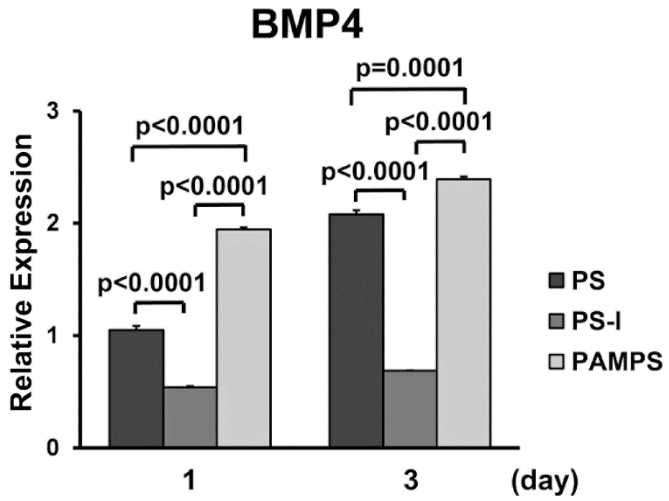

Figure 5. The expression level of BMP4 mRNA in PAMPS Culture was compared with those in PS and PS-I Cultures. An expression level of BMP4 mRNA was significantly greater in the PAMPS Culture than in the PS and PS-I Cultures at Day 1 ($p<0.0001$ and $p<0.0001$, respectively) and Day 3 ($p=0.0001$ and $p<0.0001$, respectively). Abbreviations; PS: Culture on a polystyrene (PS) dish in the maintenance medium without insulin; PS-I: Culture on a PS dish in the differentiation medium containing insulin; PAMPS: Culture on a PAMPS gel in the maintenance medium.

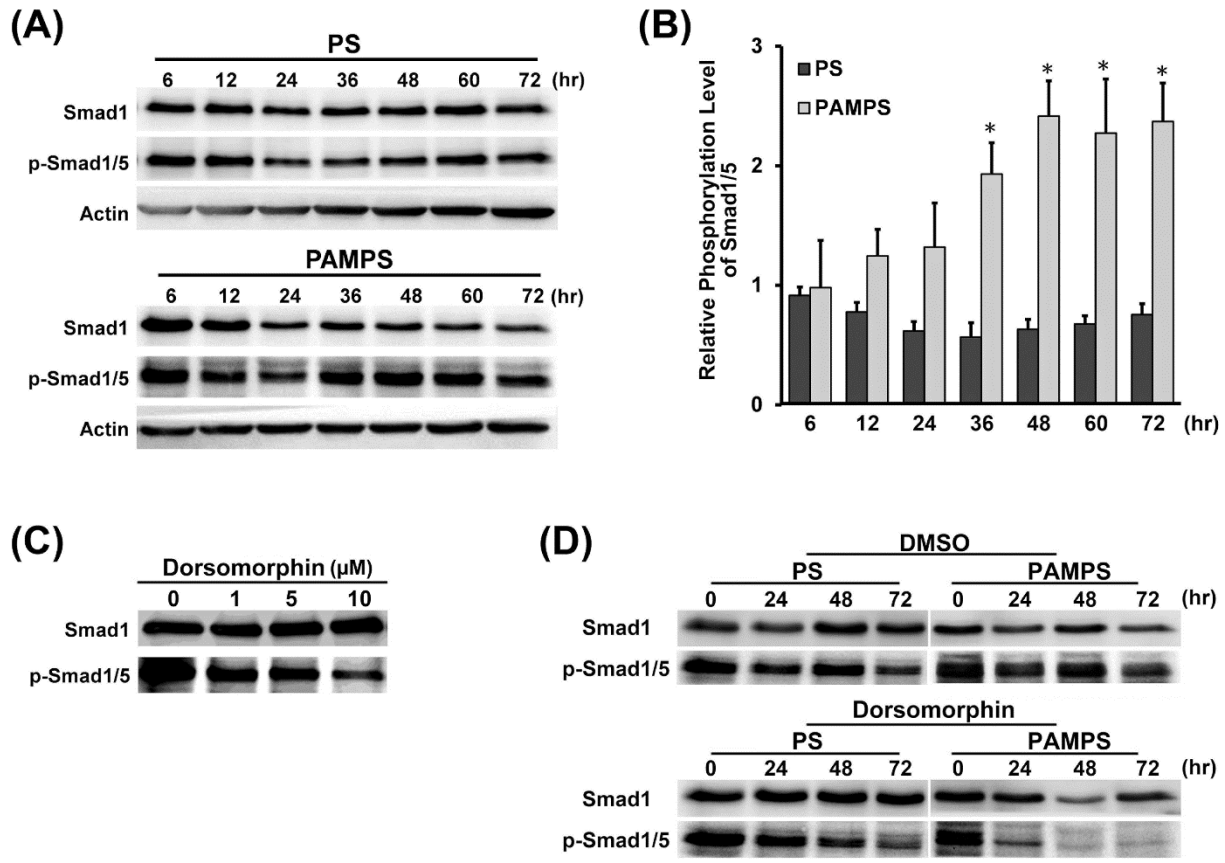

Figure 6. The effect of application of an inhibitor for BMP receptor on the TGF-β/BMP signaling pathway enhanced by PAMPS gel. (A) Western blot analysis of phosphorylated Smad1/5 (p-Smad1/5) and total Smad1 protein in ATDC5 cells in the PS and PAMPS Cultures during the 72-hour period. (B) Relative phosphorylation level of Smad1/5 was quantified using image analysis software. To evaluate the difference of Smad1/5 phosphorylation status between the PS and PAMPS Cultures, the level of p-Smad1/5 was normalized by total Smad1. The ANOVA demonstrated that there was significant differences in the phosphorylation level of Smad1/5 between the PAMPS and PS Cultures (p=0.0001). The post-hoc test showed that a significant difference (*) was calculated at each period after 36 hours (p=0.0034 at 36 hours, p=0.0001 at 48 hours, p=0.0004 at 60 hours, and p=0.0003 at 72 hours). (C) The effect of dorsomorphin applications of 0, 1, 5, and 10 μM on the phosphorylation level of Smad1/5 in the

PAMPS Culture for 48 hours. 10-μM dorsomorphin application reduced phosphorylation level of Smad1/5 to the greatest degree. (D) The 10-μM dorsomorphin application reduced the phosphorylation level of Smad1/5 at 24, 48, and 72 hours in the PAMPS Culture, although the application slightly reduced the phosphorylation level of Smad1/5 at 48 and 72 hours in the PS Culture. Dimethylsulfoxide (DMSO) was used as the vehicle control. Abbreviations; PS: Culture on a polystyrene (PS) dish in the maintenance medium without insulin; PS-I: Culture on a PS dish in the differentiation medium containing insulin; PAMPS: Culture on a PAMPS gel in the maintenance medium.

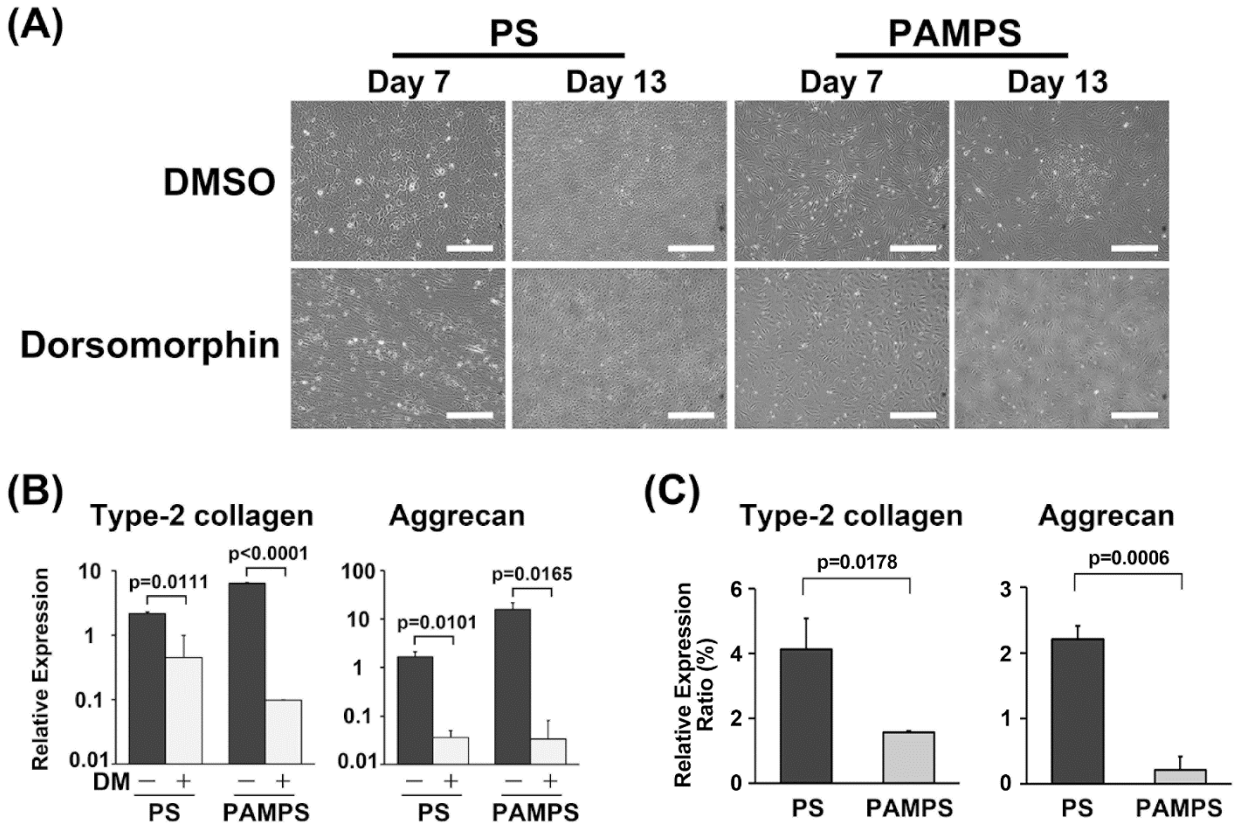

Figure 7. The effect of inhibition of TGF-β/BMP signaling pathway on the chondrogenic differentiation induced by PAMPS gel. (A) Morphological changes in ATDC5 cells treated

with 10 μM dorsomorphin (DM) and dimethylsulfoxide (DMSO) in PS and PAMPS Cultures at Days 7 and 13. White scale bars show 500 μm. (B) The dorsomorphin application significantly reduced type-2 collagen and aggrecan mRNA expression in the PAMPS Culture ($p<0.0001$ and $p=0.0165$, respectively) and in the PS Culture ($p=0.0111$ and $p=0.0101$) at Day 13. (C) The relative expression ratio (%) (the expression level treated with dorsomophin divided by that treated with DMSO) of type-2 collagen and aggrecan mRNAs were significantly greater in the PS Culture than in the PAMPS Culture ($p=0.0178$ and $p=0.0006$, respectively), which indicates that the reduction effects by dorsomorphin on type-2 collagen and aggrecan mRNA expressions were significantly greater in the PAMPS Culture than in the PS Culture. Abbreviations; PS: Culture on a polystyrene (PS) dish in the maintenance medium without insulin; PS-I: Culture on a PS dish in the differentiation medium containing insulin; PAMPS: Culture on a PAMPS gel in the maintenance medium.

Table 1. Antibodies used in Western blot analysis.

Signaling pathway	Antibody	Species	Manufacturer
	anti-Actin(C4)	Mouse	Millipore, UK
TGF-beta/BMP signaling pathway	anti-Smad1	Rabbit	Cell Signaling Technology, USA
	anti-Smad5	Rabbit	Cell Signaling Technology, USA
	anti-phospho-Smad1/5(Ser463/465)(41D10)	Rabbit	Cell Signaling Technology, USA
MAPK signaling pathway	anti-p44/42MAPK(ERK1/2)(137F5)	Rabbit	Cell Signaling Technology, USA
	anti-p38MAPK	Rabbit	Cell Signaling Technology, USA
	anti-SAPK/JNK(56G8)	Rabbit	Cell Signaling Technology, USA
	anti-phospho-p44/42MAPK(ERK1/2)(Thr202/Tyr204)(D13.14.4E)	Rabbit	Cell Signaling Technology, USA
	anti-phospho-p38MAPK(Thr180/Tyr182)(D3F9)	Rabbit	Cell Signaling Technology, USA
	anti-phospho-SAPK/JNK(Thr183/Tyr185)(81E11)	Rabbit	Cell Signaling Technology, USA
	anti-phospho-MEK1/2(Ser217/221)(41G9)	Rabbit	Cell Signaling Technology, USA
	anti-phospho-p90RSK(Ser380)(9D9)	Rabbit	Cell Signaling Technology, USA
	anti-phospho-MSK1(Thr581)	Rabbit	Cell Signaling Technology, USA
Wnt signaling pathway	anti-Wnt3a(C64F2)	Rabbit	Cell Signaling Technology, USA
	anti-Wnt5a/b(C27E8)	Rabbit	Cell Signaling Technology, USA
	anti-LRP6(C5C7)	Rabbit	Cell Signaling Technology, USA
	anti-phospho-LRP6(Ser1490)	Rabbit	Cell Signaling Technology, USA
	anti-Dvl3	Rabbit	Cell Signaling Technology, USA
	anti-β-Catenin(6B3)	Rabbit	Cell Signaling Technology, USA
	anti-phospho-β-Catenin(Ser33/37/Thr41)	Rabbit	Cell Signaling Technology, USA
Jak/Stat signaling pathway	anti-Jak1(6G4)	Rabbit	Cell Signaling Technology, USA
	anti-Jak2(D2E12)	Rabbit	Cell Signaling Technology, USA
	anti-Stat1	Rabbit	Cell Signaling Technology, USA
	anti-Stat3	Rabbit	Cell Signaling Technology, USA
	anti-Stat5	Rabbit	Cell Signaling Technology, USA
	anti-phospho-Stat3(Ser727)	Rabbit	Cell Signaling Technology, USA
	anti-phospho-Stat5(Tyr694)	Rabbit	Cell Signaling Technology, USA
PI3K/Akt signaling pathways	anti-Akt(C67E7)	Rabbit	Cell Signaling Technology, USA
	anti-phospho-Akt(Thr308)(244F9)	Rabbit	Cell Signaling Technology, USA

Table 2. List of primers used in the real-time PCR analysis for gene expression in ATDC5 cells.

Primer ID		Primers (5’-3’)
Type-2 collagen	Forward:	AGGGCAACAGCAGGTTCACATAC
	Reverse:	TGTCCACACCAAATTCCTGTTCA
Aggrecan	Forward:	AGTGGATCGGTCTGAATGACAGG
	Reverse:	AGAAGTTGTCAGGCTGGTTTGGA
BMP4	Forward:	TTCCTGGTAACCGAATGCTGA
	Reverse:	CCTGAATCTCGGCGACTTTTT
GAPDH	Forward:	TGTGTCCGTCGTGGATCTGA
	Reverse:	TTGCTGTTGAAGTCGCAGGA

Table 3. Significant pathways identified by Gene Set Enrichment Analysis for the PAMPS Culture in comparison with the PS Culture. TGF-beta signaling pathway was significantly enhanced in PAMPS Culture, as compared with PS Culture, at Day 1, 2, and 3.

Day 1		**Day 2**		**Day 3**	
Pathway name	**p-value**	**Pathway name**	**p-value**	**Pathway name**	**p-value**
Focal adhesion KEGG	4.12E-04	Focal adhesion KEGG	0.00E+00	Focal adhesion KEGG	0.00E+00
TGF Beta Signaling Pathway	1.56E-03	Smooth muscle contraction	1.00E-12	Smooth muscle contraction	2.80E-10
Inflammatory Response Pathway	8.25E-03	Insulin Signaling	1.18E-11	Insulin Signaling	4.08E-07
		MAPK signaling pathway KEGG	1.09E-09	Cell Cycle-G1 to S control Reactome	7.06E-07
		Integrin-mediated cell adhesion KEGG	1.74E-09	MAPK signaling pathway KEGG	1.82E-06
		TGF Beta Signaling Pathway	2.13E-08	Integrin-mediated cell adhesion KEGG	5.61E-06
		Alpha6-Beta4-Integrin NetPath 1	3.20E-08	Alpha6-Beta4-Integrin NetPath 1	7.79E-06
		Cell Cycle-G1 to S control Reactome	7.06E-07	TGF Beta Signaling Pathway	9.67E-06
		Regulation of Actin Cytoskeleton KEGG	2.90E-05	TNF-alpha-NF-kB NetPath 9	3.19E-04
		Wnt Signaling	1.06E-04	Wnt Signaling	1.72E-03
		TNF-alpha-NF-kB NetPath 9	1.11E-04	Inflammatory Response Pathway	1.78E-03
		Inflammatory Response Pathway	3.27E-04	Delta-Notch NetPath 3	4.02E-03
		Delta-Notch NetPath 3	1.19E-03		

Table 4. Significant pathways identified by Gene Set Enrichment Analysis for the PAMPS Culture in comparison with the PS-I Culture. TGF-beta signaling pathway was significantly enhanced in PAMPS Culture, as compared with PS-I Culture, at Day 1, 2, and 3.

Day 1		Day 2		Day 3	
Pathway name	p-value	Pathway name	p-value	Pathway name	p-value
Focal adhesion KEGG	1.96E-06	Focal adhesion KEGG	0.00E+00	Focal adhesion KEGG	0.00E+00
TGF Beta Signaling Pathway	3.25E-04	Insulin Signaling	5.75E-11	Smooth muscle contraction	6.34E-13
Inflammatory Response Pathway	1.78E-03	TGF Beta Signaling Pathway	2.13E-08	Insulin Signaling	1.18E-11
ESC Pluripotency Pathways	2.66E-03	MAPK signaling pathway KEGG	2.47E-08	Alpha6-Beta4-Integrin NetPath 1	6.25E-11
Integrin-mediated cell adhesion KEGG	3.22E-03	Integrin-mediated cell adhesion KEGG	5.31E-08	Integrin-mediated cell adhesion KEGG	2.89E-10
Smooth muscle contraction	3.43E-03	ESC Pluripotency Pathways	1.07E-07	MAPK signaling pathway KEGG	1.09E-09
Insulin Signaling	8.16E-03	Alpha6-Beta4-Integrin NetPath 1	2.18E-07	TGF Beta Signaling Pathway	2.13E-08
		Cell Cycle-G1 to S control Reactome	7.06E-07	ESC Pluripotency Pathways	2.32E-08
		Wnt Signaling	1.06E-04	Cell Cycle-G1 to S control Reactome	7.06E-07
		TNF-alpha-NF-kB NetPath 9	1.11E-04	Wnt Signaling	1.06E-04
		Regulation of Actin Cytoskeleton KEGG	7.93E-04	TNF-alpha-NF-kB NetPath 9	1.11E-04
		Delta-Notch NetPath 3	1.19E-03	Inflammatory Response Pathway	3.27E-04
				Delta-Notch NetPath 3	1.19E-03