The improvement of *in vitro* chondrogenesis method and the role of focal adhesion kinase in chondrocyte differentiation

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The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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June 2007

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June 2007

ACKNOWLEDGEMENTS

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마지막으로, 하나의 만족한 결과를 얻기 위해 몇십 배 이상의 노력과 시간을 바쳐야 하는 힘들고 열악한 과학의 길에서, 자기 자신과 씨름하며 인내하고 있는 많은 후배들에게 큰 격려를 보내고 싶습니다.

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ABSTRACT

The improvement of *in vitro* chondrogenesis method and the role of focal adhesion kinase in chondrocyte differentiation

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It has been reported that chondrocyte-like cells induced from mesenchymal stem cells (MSC) by *in vitro* protocols underwent alterations related to endochondral ossification rather than adopting a stable chondrogenic phenotype *in vivo*. Differences in hypertrophy between human articular chondrocytes (HAC) and MSC pellets during *in vitro* treatment may influence the fate of cartilage-like tissue after transplantation *in vivo*.

A better method and knowledge of the molecular events in chondrogenesis is imperative for the future use of MSC in cartilage repair. Chondrocytes are surrounded by abundant type II collagen, and failure of the matrix synthesis leads to the destruction of the articulating surfaces and the development of osteoarthritis. In addition to mechanical and cytokine influences, an imbalance between the synthesis and degradation of matrix components, mainly the loss of aggrecan and type II collagen, is known to be responsible for osteoarthritis. However, there is less information on the intracellular signaling in chondrocyte

phenotypes and chondrocyte differentiation. Thus, the first objective of this study is to improve the differentiation system to minimize expression of hypertrophic genes such as type X collagen or matrix metalloproteinase-13 (MMP-13). The second, to delineate how the extracellular matrix plays a role in chondrogenesis and maintenance of chondrocyte phenotypes, the role of intracellular signaling pathways such as focal adhesion kinase (FAK), Smads, and mitogen-activated protein (MAP) kinases [extracellular-signal-regulated kinase (ERK), p38 and c-JUN N-terminal kinases（JNK)] was investigated.

Chondrogenesis was induced by three different methods (monolayer, alginate and monolayer-alginate combined method) and chondrogenic phenotypes were detected by reverse transcription-polymerase chain reaction (RT-PCR), glycosaminoglycan (GAG) secretion and Safranin-O staining. In monolayer culture system, MSCs were successfully differentiated into chondrocytes by transforming growth factor-β3 (TGF-β3) for 14 days. Type II collagen was strongly expressed whereas type I collagen was minimally expressed. Monolayer-alginate combined method also induced type II collagen expression at maximum level whereas type X collagen expression was minimized. Moreover, cell viability was increased from 30% to 65% in combined method compared to alginate bead method. Induction of type X collagen in chondrogenesis was correlated with alkaline phosphatase (ALP)/Core-binding factor alpha 1 (Cbfa1) expression level in heterogenous MSC populations. Hypertrophic marker was also detected in protein profiles by two-dimensional electrophoresis. Forty six protein spots, which consist of twenty up-regulated and twenty six down-regulated spots, were detected during *in vitro* chondrogenesis. Especially MMP-13, enzyme for degradating type I and type II collagen, was highly increased until day

14. However, treatment of MMP-13 inhibitors, MMP-9/13 inhibitor and CL82198, could not inhibit chondrogenesis, suggesting that MMP-13 might be one of hypertrophic phenotypes rather than a regulating factor during *in vitro* chondrogenesis.

Expression of Integrin α2 and FAK was significantly increased in cells grown in type II collagen-coated plates. ERK1/2 was highly activated in alginate bead culture with type II collagen. In constrast, phosphorylation of FAK and ERK1/2 was declined in dedifferentiated chondrocytes, suggesting that signaling pathway to maintain or induce chondrogenic phenotype might be mediated at least by FAK. Human chondrocytes treated with FAK-short interfering RNA (FAK-siRNA) were substantially reduced mRNA expression of type II collagen up to 50%, but there was no change in aggrecan expression. We suppressed FAK expression up to day 5 in mesenchymal stem cells. Chondrogenesis was induced in monolayer after 2 days of post transfection with FAK-siRNA. Cell morphology was changed by TGF-β3 like normal polygonal chondrocytes, but FAK-siRNA transfected cells showed more fibroblast-like cell morphology compared to the control. FAK suppression induced down-regulation of the SOX-6 and SOX-9 and also blocked differentiation into chondrocytes with loss of type II collagen expression. If the FAK signaling was blocked, type II collagen was not induced in spite of an increase of SOX transcription factors by TGF-β3. However, GAG secretion was not suppressed in FAK knock-out cells. Our results provide evidences that FAK is required to maintain chondrocyte phenotypes or induce type II collagen in chondrogenesis from human mesenchymal stem cells.

Key words : mesenchymal stem cell, chondrogenesis, proliferation, glycosaminoglycan, MAP kinase, focal adhesion kinase

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I. INTRODUCTION

1. The expression of hypertrophic markers during chondrogenesis *in vitro*

Adult mesenchymal stem cells (MSC) were proposed as an alternative cell source for cartilage repair because of their promising potential for proliferation and differentiation[1-4]. Common *in vitro* models of chondrogenesis have utilized high cell density and transforming growth factor-β (TGF-β) as differentiation-stimulating factors[5,6]. TGF-β3 can induce chondrogenic differentiation from mouse or human bone marrow-derived mesenchymal stem cells, as judged by expression of aggrecan, type II collagen and type IX collagen. As chondrocytes become postmitotic, the cells start to terminally differentiate, which is characterized by expression of type X collagen and high activity of alkaline phosphatase (ALP) in cartilage and bone development (Figure 1)[7,8].

One of the most important things for chondrogenesis *in vitro* from human mesenchymal stem cells is to find an effective and the most ideal differentiation method for clinical applications, because the chondrocytes hypertrophy, as judged by expression of type X collagen, means the possibility of bone formation by terminal differentiation shown in the growth plate or osteoarthritis (Figure 2)[9-11].

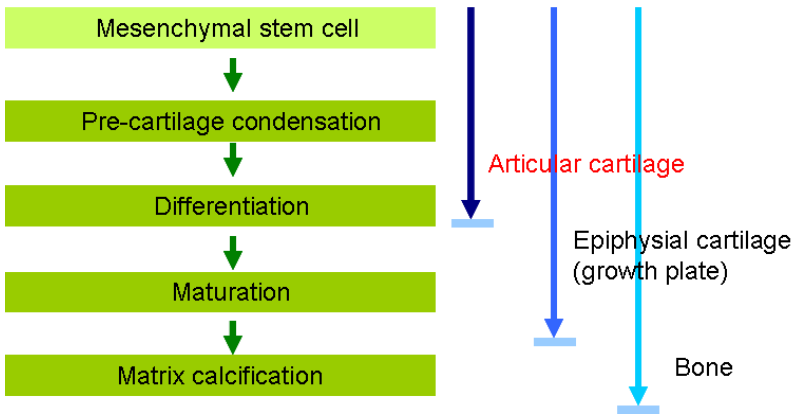

Figure 1. Cartilage and bone development

Although TGF-β is known as a growth factor to inhibit chondrocytes hypertrophy in endochondral ossification process[12], hypertrophic markers involving type X collagen were detected in chondrocytes differentiation by several *in vitro* methods such as pellet, micromass and alginate bead culture system[13-16]. Recently, chondrocyte-like cells induced from mesenchymal stem cells (MSC) by *in vitro* protocols underwent alterations related to endochondral ossification rather than adopting a stable chondrogenic phenotype *in vivo*[17]. Thus, a better knowledge of the molecular events occurring in response to stimulation with chondrogenic factors is imperative for the future use of MSC in cartilage repair. But there is less information to explain the expression of hypertrophic

markers during *in vitro* chondrogenesis. It will not guarantee a controlled induction and guidance of cell differentiation toward the desired phenotype and ensure successful tissue repair until differentiation cascades underlying chondrogenesis from MSCs are understood in detail.

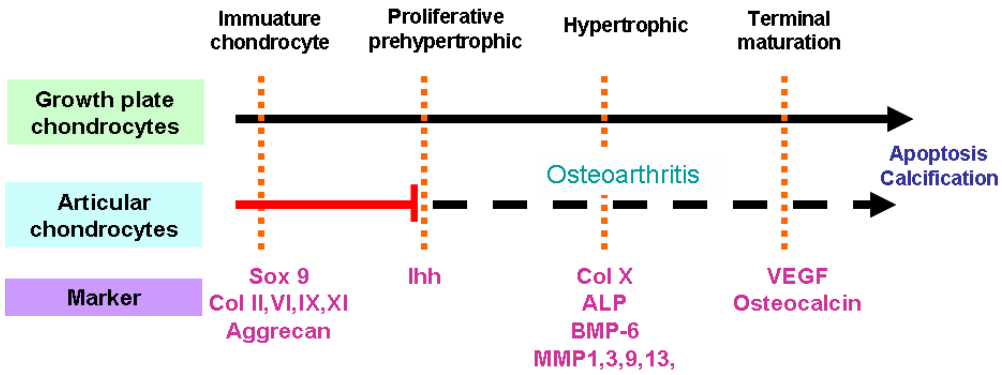

Figure 2. Molecular markers in endochondral ossification (Col:collagen, ALP: alkaline phosphatase, Ihh: Indian Hedgehog, MMP: matrix metalloproteinase).

Core-binding factor (Cbfa1) has accepted as an essential transcription factor for osteoblast differentiation and bone formation[18-21]. In Cbfa1-deficient mice, it has been shown that endochondral ossification is completely blocked and chondrocyte maturation is disturbed, suggesting that Cbfa1 is related to chondrocyte maturation[22]. Cbfa1 directly promotes transcription of osteocalcin, osteopontin, and type X collagen, which promoter regions contain binding sites of Cbfa1[23,24]. Thus, in this study we first aimed to investigate the correlation between expression level of hypertrophy-related genes in undifferentiated MSCs and chondrogenesis efficiency *in vitro.*

2. Differentiation mechanism by extracellular matrix-related signaling events

Chondrocytes are surrounded by abundant type II collagen, and failure of cartilage matrix synthesis leads to the destruction of the articulating surfaces and the development of osteoarthritis[25,26]. Human articular chondrocytes dedifferentiate to a fibroblast-like state when cultured in monolayers. This shift in cellular differentiation has been demonstrated both by morphological changes and by alterations in collagen expression patterns[27,28]. Recent studies comparing the behavior of articular chondrocytes in type I and II collagen have shown increased rates of cytokine-regulated proliferation and proteoglycan synthesis in the type II collagen[29-31]. At all time periods, significantly higher percentage of cells in a type II collagen sponge displayed a spherical morphology consistent with the chondrocytic phenotype, compared with cells in type I collagen[30,31]. Also, some studies have found positive staining of the pericellular matrix around spherical cells in a type II sponge, whereas there was no staining for type II collagen in the seeded type I sponges[32]. These results suggest a difference in the biosynthetic activity of chondrocytes in the different matrices and the importance of type II collagen as a chondrocyte microenvironment. However, the intracellular mechanisms in both chondrocyte-type II collagen interaction and *in vitro* chondrogenesis are at present only poorly understood.

After cell-ECM interactions, protein phosphorylation is one of the earliest events detected in response to integrin stimulation[33]. Studies on platelets provided the first evidence that integrin receptors can regulate tyrosine phosphorylation[34,35]. Rapid signaling processes due to integrins increase the tyrosine phosphorylation level of intracellular target proteins,

and then induce a focal adhesion complex linked to actin filaments by a number of signaling and structural molecules, including FAK, c-Src, Rho, GAP, paxillin, talin, p130CAS and caveolin-1[35]. According to recent studies, integrin-mediated activation of ERK1/2 can be divided into large two mechanisms: (i) a FAK-dependent pathway, including Src, $p130^{CAS}$ and Crk, or (ii) a FAK-independent pathway, including Shc, Fyn and caveolin-1[33,36,37]. FAK activation has been shown to be important for cell adhesion, migration, survival and proliferation[33-37]. FAK transduces signals to activate c-Jun N-terminal kinase (JNK) as well as ERK1/2, and multiple signaling connections from FAK to ERK1/2 are shown to be important for cell proliferation [33,38,39]. It has been reported that type II collagen expression was concomitant with expression of tensin, paxillin, and FAK in chondrocytes[40]. The role of Shc and ERK1/2 pathway, FAK-independent pathway, in the regulation of chondrocyte phenotype and apoptosis was demonstrated[41]. However, a role of FAK in the regulation of cartilage specific genes (type II collagen and aggrecan) during chondrocyte dedifferentiation and chondrogenesis has not been systematically investigated.

Thus, the first objective of this study was to improve the culture system minimizing the expression of hypertrophy-related genes for *in vitro* chondrogenesis. The second, the role of intracellular signaling pathways such as FAK, Smads and MAP kinases (ERK, p38, and JNK) in both chondrogenesis and dedifferentiation process was investigated.

II. MATERIALS AND METHODS

1. Culture of mesenchymal stem cell from human bone marrow

Bone marrow aspirates were obtained from posterior iliac crest of 20 healthy adult donors aged 13-60 years under the approvement of Institutional Review Board (IRB). Human bone marrow-derived mesenchymal stem cells were selected based on their ability to adhere to the tissue culture plastic. Non-adherent hematopoietic cells were removed with the culture medium after 7 days in culture. The cells were cultured in Dulbecco's Modified Eagle's medium-low glucose (DMEM-LG, Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and 1X antibiotic-antimycotic solution (Invitrogen). Cells were grown to confluence, then harvested by incubation with 0.25% trypsin/1 mM EDTA (Invitrogen), centrifuged at 1,200 rpm for 3 min and subcultured at a 1:3 split ratio in new culture flasks until 5-6 passages.

2. Isolation and culture of articular chondrocytes

Normal articular cartilage was obtained from the knee joints of pigs or human. Cartilages were shaved from the articular surface under sterile conditions, and finely minced and washed several times in phosphate-buffered saline (PBS). The tissue was then incubated at 37℃ for 4 hours in Dulbecco's Modified Eagle's medium-high glucose (DMEM-HG, Invitrogen) containing 0.1% collagenase (Worthington, Lakewood, NJ, USA) and 0.065% hyaluronidase (Worthington). Cell suspension was centrifuged at 1,200 rpm for 5 min and the pellet was washed several times with PBS. Chondrocytes were monolayer-cultured with DMEM-HG medium (Invitrogen) containing 10% FBS and 1X antibiotic-antimycotic solution (Invitrogen).

3. Alginate bead culture and collagen coating

Chondrocytes were encapsulated in 1.2% (w/v) alginate bead (Sigma-Aldrich, St. Louis, MO, USA) with 0.2 mg/ml of type II collagen (Chondrex, Seattle, WA, USA). In detail, cell suspension was passed drop-wise through a 19-gauge needle into a 6-well plate containing 102 mM $CaCl_2$ solution at a density of $2x10^6$ cells/ml. After polymerization for 5 min, the beads were washed three times with normal saline and cultured in DMEM-HG containing 10% FBS. The coating of a culture dish was performed by exposure to 2 ml of PBS containing 1 μg/ml type I collagen (Sigma-Aldrich) or type II collagen (Chondrex) in 6-well plates for 12 hours at 4℃. The residual protein absorption sites in all wells were blocked with 1% bovine serum albumin (BSA) for 1 hour at 37℃.

4. Colony-Forming Unit-Fibroblastic (CFU-F) assay

MSCs were plated into 100 cm^2 culture dishes at $1x10^3$ cells per dish in DMEM-LG medium containing 20% FBS. The medium was changed twice weekly, and the cultures were maintained for 10 days. After fixation with fixing solution (citrate buffer:acetone=2:3), the cultures were stained for ALP with alkaline dye solution (Sigma-Aldrich) for 30 min in dark. After washing with distilled water, the cells were stained with Mayer's hematoxylin soultion for 5 min and then rinsed with tap water.

5. Chondrogenic differentiation by alginate bead or monolayer culture

Alginate beads contained $2x10^6$ cells/ml were prepared in the proportion of 3 ml of cell suspension mixed with 3 ml of sterile low-viscosity alginate gel. The suspended mesenchymal stem cells were slowly expressed in a drop-wise fashion, and the gelation of the alginate beads

occurred into 102 mM $CaCl_2$ in 6-well plates. The beads were washed three times with saline and then washed once with DMEM-HG medium. The beads were cultured for 7 and 14 days in 2.5 ml of a defined medium [DMEM-HG, 1X ITS, 50 μg/ml ascorbic acid and 10 ng/ml TGF-β3 (R&D systems, Minneapolis, MN, USA)]. The culture medium was changed every 3 day. After the defined period of days, the alginate beads were washed three times with PBS and dissolved with 40 mM ethylenediaminetetraacetic acid (EDTA) for 10 min. Cell pellet was collected by centrifuging at 1,200 rpm for 5 min. In monolayer, MSCs were plated at high density ($2x10^5$ cells/plate) in 12-well plates. After attachment on culture plates, cells were differentiated into chondrocytes in defined medium for 14 days.

6. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and quantified by spectrophotometry. Total RNA was reverse-transcribed using an Omniscript kit (Qiagen). The primer sets used for gene amplification were derived from Gene Bank sequence database (Table 1). cDNA was amplified in a total volume of 50 μl containing 1X PCR buffer, 0.4 μM of each primer, 0.2 mM dNTP mix and 1 U of Taq DNA polymerase (Qiagen) at optimal temperature (Table 2). PCR products were analyzed in a 1.5% agarose gel at 100V/cm in 1X Tris-Borate-EDTA (TBE) buffer, followed by staining with ethidium bromide. The density value for the PCR products were normalized to GAPDH values to yield a semi-quantitative assessment.

Table 1. Primer sequences for RT-PCR

Genes	Sequence	Size(bp)
Type I collagen	(S)5'-AGACATCCCACCAGTCACCT-3' (AS)5'-GTGGGTGACACCTCGCCTTC-3'	295
Type II collagen	(S)5'-TTCAGCTATGGAGATGACAATC-3' (AS)5'-AGAGTCCTAGAGTGACTGAG-3'	472
Type X collagen	(S)5'-GCCCAAGAGGTGCCCCTGGA-3' (AS)5'-CCTGAGAAAGAGGAGTGGAC-3'	570
FAK	(S)5'-CAGTATTATCAGGCATGGAG-3' (AS)5'-GTGTTTTGGCCTTGACAGAA-3'	500
Aggrecan	(S)5'-GAATCTAGGAGTGAGACGTC-3' (AS)5'-CTGCAGCAGTTGATTCTGAT-3'	540
SOX-5	(S)5'-AGCCAGAGTTAGCACAATAGG-3' (AS)5'-CATGATTGCCTTGTATTC-3'	619
SOX-6	(S)5'-ACTGTGGCTGAAGCACGAGTC-3' (AS)5'-TCCGCCATCTGTCTTCATACC-3'	562
SOX-9	(S)5'-GAACGCACATCAAGACGGAG-3' (AS)5'-TCTCGTTGATTTCGCTGCTC-3'	631
Cbfa1/runx2	(S)5'-CCACCTCTGACTTCTGCCTC-3' (AS)5'-GACTGGCGGGGTGTAAGTAA-3'	172
Alkaline phosphatase	(S)5'-CTACCAGCTCATGCATAACA-3' (AS)5'-GACCCAATAGGTAGTCCACA-3'	450
DEC-1	(S)5'-CCATTTCACTAGCAGTGACC-3' (AS)5'-TGGACCAAGACAGAAGAGTC-3'	330
DEC-2	(S)5'-TTCTGCTTCCTCTCGCCTTC-3' (AS)5'-TCTTCCTGAGCAGAGCTCTC-3'	320
MMP-13	(S)5'-GTGGTGTGGGAAGTATCATC-3' (AS)5'-GCATCTGGAGTAACCGTATT-3'	400
GAPDH	(S)5'-GAAGGTGAAGGTCGGAGTC-3' (AS)5'-GAAGATGGTGATGGGATTTC-3'	220

S: sense primer, AS: antisense primer

Table 2. PCR conditions

Genes	condition	Cycle
Type I collagen	94℃ 30sec-55℃ 30sec-72℃ 30sec	31
Type II collagen	94℃ 30sec-58℃ 30sec-72℃ 30sec	30
Type X collagen	94℃ 30sec-60℃ 30sec-72℃ 30sec	29
FAK	94℃ 30sec-57℃ 30sec-72℃ 30sec	31
Aggrecan	94℃ 30sec-47℃ 30sec-72℃ 30sec	26
SOX-5	94℃ 30sec-58℃ 30sec-72℃ 30sec	30
SOX-6	94℃ 30sec-58℃ 30sec-72℃ 30sec	30
SOX-9	94℃ 30sec-58℃ 30sec-72℃ 30sec	30
Cbfa1/runx2	94℃ 30sec-59℃ 30sec-72℃ 30sec	34
Alkaline phosphatase	94℃ 30sec-52℃ 30sec-72℃ 30sec	27
DEC-1	94℃ 30sec-50℃ 30sec-72℃ 30sec	33
DEC-2	94℃ 30sec-50℃ 30sec-72℃ 30sec	33
MMP-13	94℃ 30sec-50℃ 30sec-72℃ 30sec	30
GAPDH	94℃ 30sec-57℃ 30sec-72℃ 30sec	31

7. Western blot analysis

Whole cell lysates were prepared in a lysis buffer (Promega, Madison, WI, USA) with 10 μg/ml of a protease and phosphatase inhibitor cocktail solution (Sigma-Aldrich). Lysates were clarified by centrifugation at 13,000 rpm at 4℃ for 15 min, and the protein contents of supernatants determined using a modified Bradford assay. Total 20 μg aliquots of the cell lysates were separated by 10% SDS-PAGE under reducing conditions. Separated proteins were transferred to a PVDF membrane (Amersham Pharmacia, Piscataway, NJ, USA) at 50V for 2 hours in transfer buffer containing 25 mM Tris-HCl (pH 8.3), 1.4% glycine and 20% methanol. Membranes were blocked with 5% skimmed milk dissolved in 1X TBST (50 mM Tris-HCl, 150 mM NaCl NaCl, and 0.1% Tween-20) at room temperature for 1 hour. Primary antibodies [Integrin β1, α1, α2, α5, (Chemicon, Temecula, CA, USA), FAK, p-FAK, p-Tyr (Upstate biotechnology, Lake Placid, NY, USA), ERK, p-ERK, p38, JNK (Santa Cruz, Santa Cruz, CA, USA), p-p38, p-JNK, p-Smad2, p-Smad3 (Cell signaling, Danvers, MA, USA)] were diluted in 1X TBST buffer and the membrane then incubated with the diluted antibody solutions for 3 hours at room temperature. After washing the membrane with 1X TBST, the membrane was incubated for 1 hour with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Pharmacia). The immunoblots were visualized using the ECL plus detection kit (Amersham Pharmacia). The detection of GAPDH with monoclonal antibody (Research Diagnostics, Flanders, NJ, USA) was used for internal control.

8. Immunofluorescence staining

Cultured cells were fixed in cold acetone and methanol (1:1) for 5 min and rinsed in PBS. The cells were incubated with phospho-tyrosine antibody (Upstate biotechnology) at 4℃ overnight and then washed with PBS three times for 10 min. The cells were incubated with goat-anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (FITC) (Santa Cruz, diluted 1:80) and then stained with propidium iodide (PI) (Santa Cruz, diluted 1:500) for 1 hour at room temperature.

9. Cell viability

MSCs were incubated for 14 days in chondrogenic medium with or without TGF-β3. Cell viability was determined by methylthiazoletetrazolium (MTT) assay in 24-well plates. Culture medium was removed, and 400 μl of fresh medium and MTT solution (0.5 mg/ml, Sigma-Aldrich) was then added per well and then incubated at 37℃ for 4 hours. The upper medium was then carefully removed, and the intracellular formazan was solubilized by adding 800 μl of dimethyl sulphoxide (DMSO, Sigma-Aldrich) into each well. The absorbance of produced formazan was measured at 570 nm in triplicate.

10. Cell proliferation assay

Monolayer cultured cells with or without siRNA were fixed with cold methanol and acetone (1:1) solution for 3 min. After washing it with PBS twice, the cells were stained with 20% crystal violet (Merck, Darmstadt, Germany) for 10 min at room temperature. After washing the stain with distilled water, the absorbance was detected at 595 nm after destaining with 95% ethanol. All samples was tested in triplicate.

11. Glycosaminoglycan (GAG) secretion

The amount of sulfated GAGs in the medium was determined by 1, 9-dimethy-methlene blue method using Blyscan kit (Biocolor, Newtownabbey, Northern Ireland, UK) according to the manufacturer's instruction. Total 100 μl of culture medium was mixed with 1 ml of blyscan dye reagent by shaking for 30 min to complete the GAG-dye binding. After centrifugation, the dye bound to GAG was dissolved in dissociation reagent. The recovered dye concentration was determined photometrically at 656 nm and chondroitin 4-sulfate standard solution (Biocolor) was used to generate standard curves. All samples and standards were tested in triplicate.

12. Safranin-O staining

Monolayer cultured cells were washed with PBS three times to remove the medium. Without fixing, 0.1% Safranin-O solution (Sigma-Aldrich) was added into each wells to detect proteoglycan (predominantly aggrecan), and the plates were slowly agitated for 30 mim. After washing the cells with distilled water twice, the absorbance was detected at 490 nm by destaining with 100% ethanol for 20 min.

13. RNA interference

siRNA construct was designed to target against human FAK (GenBank accession number NM_153831 and NM_005607). FAK-siRNA and scrambled (negative control)-siRNA were designed by Bioneer (Bioneer Inc., Daejeon, South Korea) and targeted the following sequences : FAK-siRNA sense: 5'-ACACCAAAUUCGAGUACUA-3', FAK-siRNA antisense : 5'-UAGUACUCGAAUUUGGUGU-3', negative -siRNA sense: 5'-CCUACGCCACCAAUUUCGU-3', Negative -siRNA antisense:

5’-ACGAAAUUGGUGGCGUAGG-3’. Each strand contained the target sequence with a 3’-terminal dTdT overhang and 5’-terminal FITC modification. Chondrocytes were plated to obtain a 70% confluency in 24-well plates and transfected with 200 nM of FAK or negative-siRNA using lipofectamine^{TM} 2000 (Invitrogen). After 5 hours of transfection, 100 ul fresh medium containing 10% FBS was added to the plates. Transfection rate was confirmed by FITC under fluorescence microscope. According to different experiments, the transfected cells were cultured in alginate bead or monolayer with or without type II collagen.

14. Two-dimensional electrophoresis (2DE)

Isoelectic focusing (IEF) was performed as described by Shevchenko et al[42]. IPG strips were used according to the manufacturer's instructions (Amersham Pharmacia). Samples containing appropriate amounts of protein were diluted to 350 μl with rehydration solution [9M urea, 4% CHAPS, 100 mM DTT and 0.5% (v/v) IPG buffer, with a trace of bromophenol blue], applied to strips (pH range of 4-7) by overnight rehydration, and 2DE was performed using 9-16% two-dimensional gels (200 x 250 x 1.0 mm) in an IsoDALT apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA) until the tracking dye reached the anode end of the gels.

15. Protein visualization and In gel digestion

Gels were silver stained according to the method of Yan et al[43]. Briefly, gels were fixed with methanol: acetic acid: water (40:10:50) for 30 min and sensitized in 30% methanol, 5% sodium thiosulfate, and 6.8% (w/v) sodium acetate for 30 min, followed by three 5 min washes in deionized water. Proteins were stained with 2.5% silver nitrate for 20 min, washed

with deionized water and stored in sealed plastic bags at 4℃. The protein patterns were recorded as digitalized images using a high resolution scanner (GS-800 Calibrated Imaging Densitometer, Bio-Rad, Hercules, CA, USA), and scanned images were analyzed using the 2DE program, Melanie III (GenBio, Geneva, Switzerland). The resulting tryptic fragments were eluted from the excised gels by diffusion into 0.5% TFA and 50% ACN, and sonicated twice. The peptide mixture was mixed with 0.1% formic acid and 50% ACN and transferred into microtiter plates.

16. MALD-TOF-MS and database searching

Mass analysis was performed with a Voyager-DE STR MALDI-TOF-MS (PerSeptive Biosystem, Framingham, CA, USA) in the reflector mode, over a mass range of 1000-3000 Da. Proteins were identified by their peptide mass fingerprint (PMF) with the search programs ProFound (http://129.85.19.192/profound bin/WebProFound. exe, Rockefeller University, version 4.10.5) and MASCOT (http:// www. matrixscience. com). The search parameters allowed for N-terminal acetylation and carboxyamidomethylation of cysteine.

17. Gelatin zymography

Total 20 μg of cell lysate or 20 μl of culture medium were mixed with non-reducing sample buffer [1M Tris-HCl (pH 6.8), 4% SDS, 10% Glycerol, 0.03% bromophenol blue]. Samples were applied to a 10% SDS-PAGE containing 1 mg/ml of type I gelatin (Sigma-Aldrich) at 4℃. The gel was washed with 2.5% Triton X-100 for 10 min and incubated in developing buffer (40 mM Tris-HCl, 50 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij35, pH 7.6) at 37℃ for 12 hours. Gels were than stained with

0.5% coomassie blue dissolved in acetic acid : methanol (1:4) and destained with acetic acid : ethanol (1:4). Concentrated serum free culture media from human tumor cells treated with interleukin-1 (AG901, Chemicon) was used as a positive controls.

18. Statistical analysis

All Data were analyzed three independent experiments performed in triplicate. Comparisons between the two groups (FAK-siRNA transfected cells and control-siRNA or non transfected cells) were analyzed by Student's t-test. Values of $p<0.05$ were considered to be statistically significant.

III. RESULTS

1. Chondrogenesis by monolayer culture system

In monolayer culture system, MSCs inoculated at high density (100% confluency) were differentiated into chondrocytes by TGF-β3 for 14 days. Chondrogenic phenotypes were confirmed by Safranin-O staining for proteoglycan synthesis or immunochemical staining and RT-PCR for type II collagen expression. Type II collagen was successfully expressed at day 14 compared to control in immunocytochemical stain (Figure 3A). Type II collagen maximally expressed at day 7 and then slowly decreased until day 14 whereas type I collagen expression was minimally expressed at day 7 in RT-PCR (Figure 3B). However, dexamethasone, as an inducer of chondrogenesis and osteogenesis, did not increase type II collagen expression. In addition, type I collagen was increased by dexamethasone at day 7 compared to non-treated control. Dexamethasone has been widely used for induction of chondrogenesis *in vitro*, but it did not show positive effect on chondrogenic phenotype. Expression of CD105, SOX-6 and SOX-9 did not show a significant increase during chondrogenesis.
Fibroblast-like cell morphology was changed to colony form by TGF-β3 during chondrogenesis. Safranin-O was positively stained in colony forming cell populations (Figure 3C).

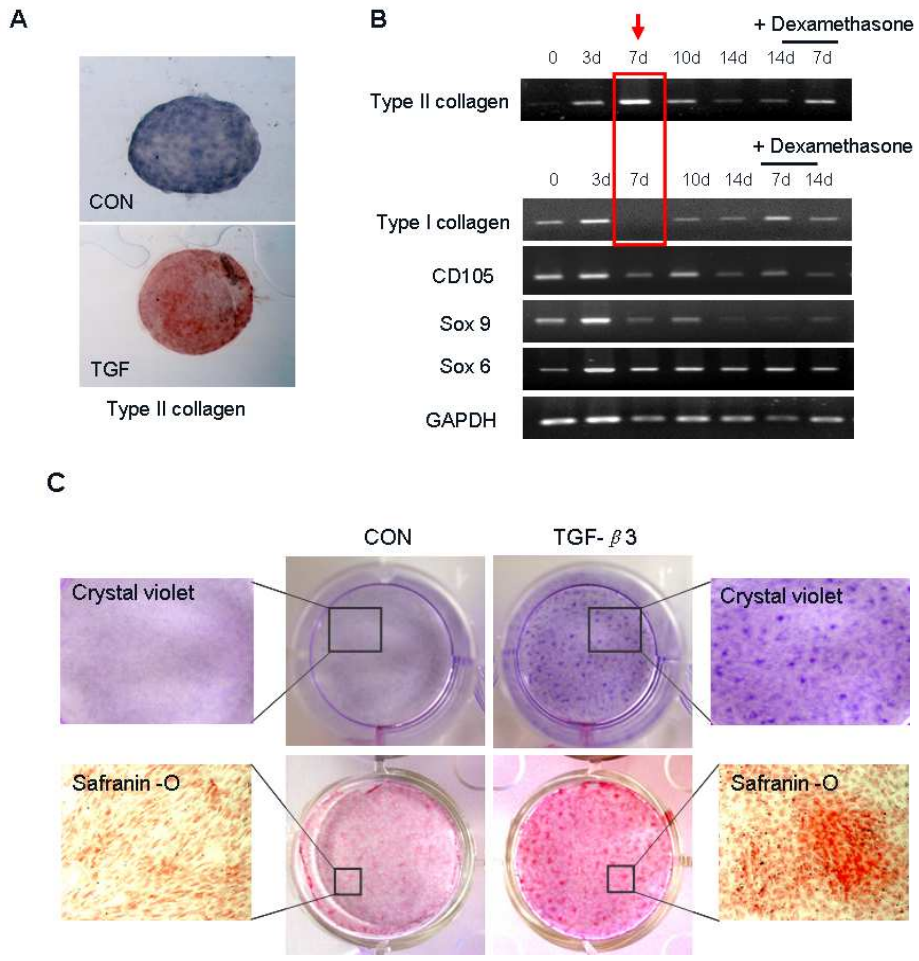

Figure 3. Chondrogenesis induced by high density-monolayer culture system. Chondrogenesis (100% confluency) was induced by 10 ng/ml of TGF-β3 with or without dexamethasone for 14 days. Chondrogenic phenotypes were detected by immunochemical staining for type II collagen (A), RT-PCR (B), and Safranin-O staining (C). Type II collagen was maximally expressed at day 7 whereas type I was minimally expressed (arrow and box).

2. Cell viability in chondrogenesis by monolayer culture system

In alginate bead culture system, viability of differentiated cells by TGF-β3 showed 85% at day 7 and 38% at day 14. In contrast, monolayer cultured cells showed 130% at day 7 and 127% at day 14 (Figure 4). Although 3D culture system has been widely used for chondrogenesis *in vitro*, there were some of problems such as low viability[4-6]. This result suggests that MSC can be differentiated into chondrocytes in high density-monolayer culture system without low viability shown in 3D culture.

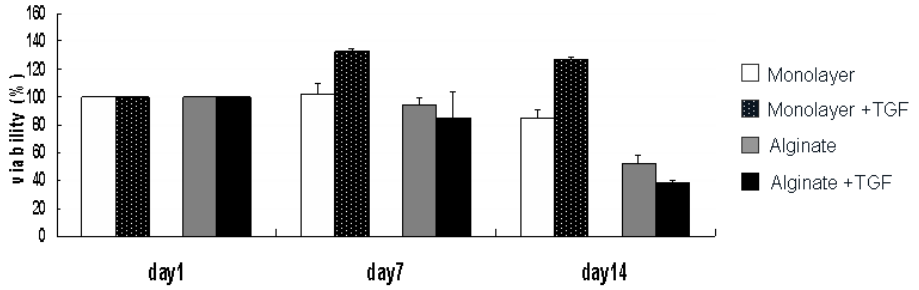

Figure 4. Cell viability during chondrogenesis by monolayer and alginate bead culture. MSCs were differentiated into chondrocytes by 10 ng/ml of TGF-β3 in serum free DMEM-HG medium containing 1X ITS-A and 50 μg/ml ascorbic acid. Each 400 μl of MTT solution (0.5 mg/ml) and fresh culture medium were added in each well and incubated for 4 hours. The absorbance of produced formazan was measured at 570 nm in triplicates.

3. Chondrogenic efficiency and cell viability by monolayer-alginate combined method

In monolayer culture system, successfully induced type II collagen and high cell viability were shown in Figure 3. However, type II collagen expession peaked at day 7 and then decreased until day 14. To optimize type II collagen expression and cell viability, MSCs were treated with TGF-β3 for 7 days in monolayer and then cultured in alginate bead for 7 days (represented as M7A7). At day 14, type II collagen was highly expressed in cells treated with TGF-β3 for 7 days (M7A7) or 11 days (M11A3), and aggrecan was expressed in all groups except monolayer culture group (M14) (Figure 5A). However, type X collagen, a hypertrophic marker, was highly expressed in M11A3 group, suggesting that pretreatment of TGF-β3 for 7 days before culturing in alginate bead could be an ideal method to enhance chondrogenic phenotypes such as type II collagen and minimize hypertrohic gene expression. Type X collagen expression was minimally induced in combined method rather than in monolayer or alginate bead system alone (Figure 5B). Cell viability was decreased time-dependently according to the period of alginate bead culture (Figure 6).

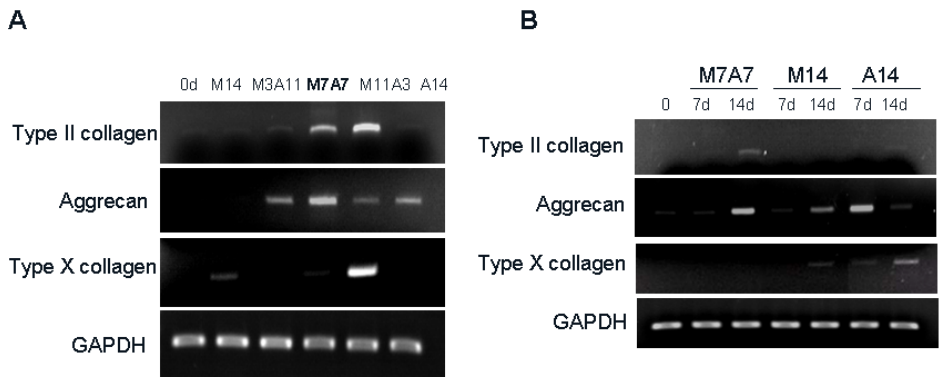

Figure 5. Expression of chondrogenic and hypertrophic markers during chondrogenesis by monolayer-alginate combined method. At day 14, chondrogenic efficiency in several combination methods according to TGF-β3 pretreatment was evaluated (A). Changes in gene expression were confirmed at day 7 and day 14 in three different methods (B). M: monolayer, A: alginate, number: culture period in each culture systems.

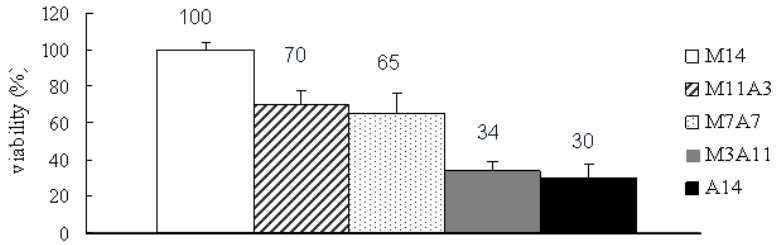

Figure 6. Cell viability during chondrogenesis by monolayer-alginate combined method. Each number represents cell viability (%). M: monolayer culture, A: alginate bead culture.

4. Expression of Type X collagen during chondrogenesis from alkaline phosphatase-positive MSCs

It has been widely accepted that ALP is an early marker of osteogenesis in bone-forming system. In addition, the enzyme express positively in mouse embryonic stem cells. In human bone marrow-derived mesenchymal stem cells, ALP activity showed at different levels among several donor populations (Figure 7A). However, ALP activity was unrelated to age and gender. ALP was stained in large-sized cells (>25-30 μm) compared to small-sized cells (5~10 μm) (Figure 8A and 8B). To evaluate whether the ALP positive or negaitve MSCs have different chondrogenesis efficiency, three donors were selected according to level of ALP activity and mRNA expression confirmed by ALP activity assay and RT-PCR, respectively (Figure 8C and 8D).

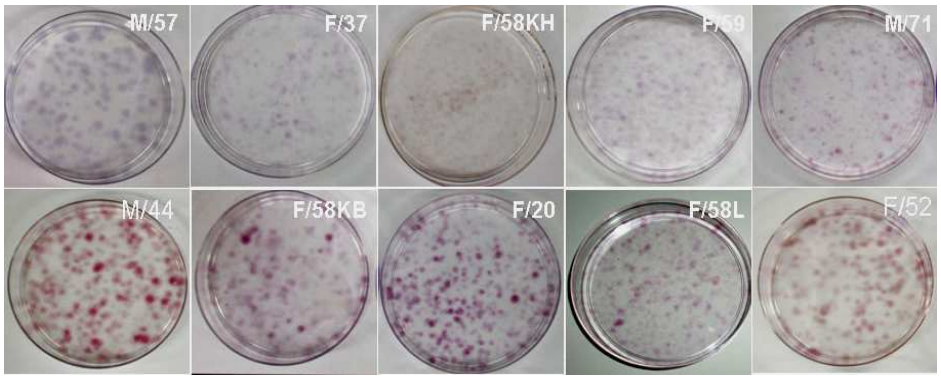

Figure 7. CFU-F assay in human bone marrow-derived MSCs. Bone marrow aspirates were cultured for 7 days and then cells were incubated at low density ($1x10^3$ cells/ culture dish) for 10 days in DMEM-LG medium containing 20% FBS. Alkaline phosphatase staining (red color) was performed, and crystal violet staining was used for standardization of each cell number.

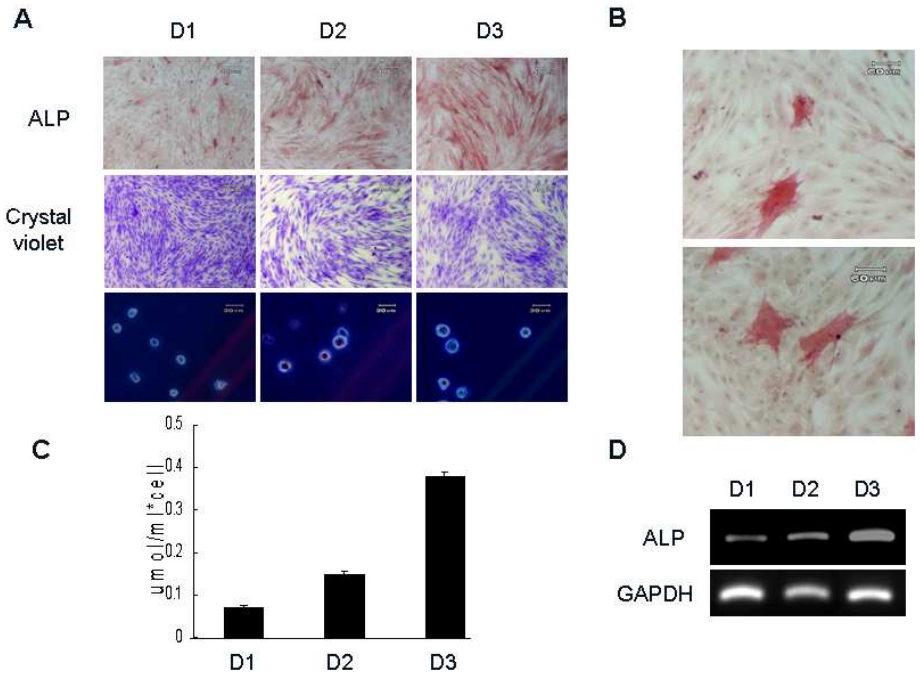

Figure 8. Level of ALP activity and mRNA expression in human bone marrow-derived MSCs (D1-D3: three donors according to the level of ALP). ALP level was checked by staining (A and B), activity (C) and mRNA expression by RT-PCR (D). ALP-positive cells showed in large sized-cells compared to small-sized cells (A: X100, B: X200).

Interestingly, ALP-positive MSCs showed increased expression of *Cbfa1/Runx2* gene, a transcription factor of type X collagen in osteogenesis, compared to ALP-negative cells (Figure 9). Each of the donors was differentiated into chondrocytes by three methods (monolayer, alginate and monolyaer-alginate combined method) evaluated in this study (Figure 3-6) for 14 days with 10 ng/ml of TGF-β3. ALP-positive cells (D3) were less differentiated into chondrocytes than ALP-negative cells (D1), because type II collagen was successfully induced in ALP-negative cells by monolayer-alginate combined (MA) or alginate bead culture

method (A) (Figure 9). Although ALP-positive cells induced type II collagen by monolayer-alginate combined method, type X collagen was also induced at high level in all methods. However, the expression of MMP-13, DEC1 and DEC2 was not correlated with ALP expression. This results suggest that ALP/Cbfa1-positive cells have a weak chondrogenic potential, and the cells are similar to osteoblast phenotypes such as gene expression, cell size and cell morphology.

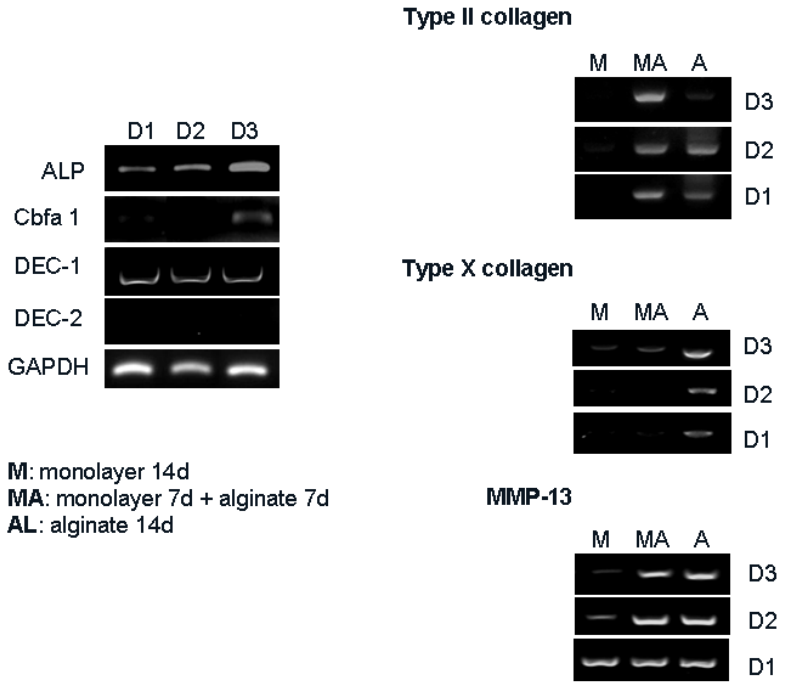

Figure 9. Expression of hypertrophic markers during chondrogenesis from ALP-positive or negative cells. Each of three MSCs (D1-D3) was differentiated into chondrocytes by monolayer, alginate and monolayer-alginate combined method for 14 days.

5. Increased MMP-13 expression as a hypertrophic marker during *in vitro* chondrogenesis

To identify differentially expressed proteins using proteome analysis during chondrogenesis of human bone marrow-derived MSCs, the cells were encapsulated into 1.2% alginate beads at a density of 2×10^6 cells/ml with 10 ng/ml of TGF-β3. Forty six protein spots, which consist of twenty up-regulated spots and twenty six down-regulated spots, were shown the changes for 14 days *in vitro*. These proteins include structural and cytoskeleton components, metabolic enzymes, protein synthesis and degradation, *etc*. Annexin 1 of the differentially regulated proteins, metabolic enzymes, was down-regulated until day 14 during chondrogenesis. Especially, MMP-13 (collagenase-3), enzyme for degradating type I and type II collagen, was highly increased at day 7 and was maintained until day 14 (Figure 10). The protein expression was confirmed in the level of cytoplasm and medium by Western blotting (Figure 11A and 11B). MMP-13 activity was higher in secreted proteins than in proteins from cytoplasm (Figure 11C). Treatment of MMP-13 inhibitors (MMP-9/13 inhibitor and CL82198) could not inhibit chondrogenesis in monolayer culture, suggesting that MMP-13 might be expressed as one of hypertrophic phenotypes rather than a regulating factor in chondrogenesis (Figure 12).

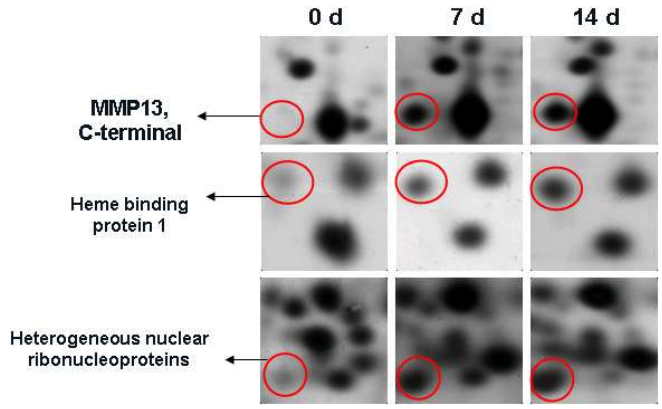

Figure 10. *De novo* synthesized proteins in chondrogenesis. Cell pellets were harvested at each day with 40 mM EDTA solution to dissolve alginate beads. Three proteins not expressed in undifferentiated mesenchymal stem cells were gradually increased until day 14.

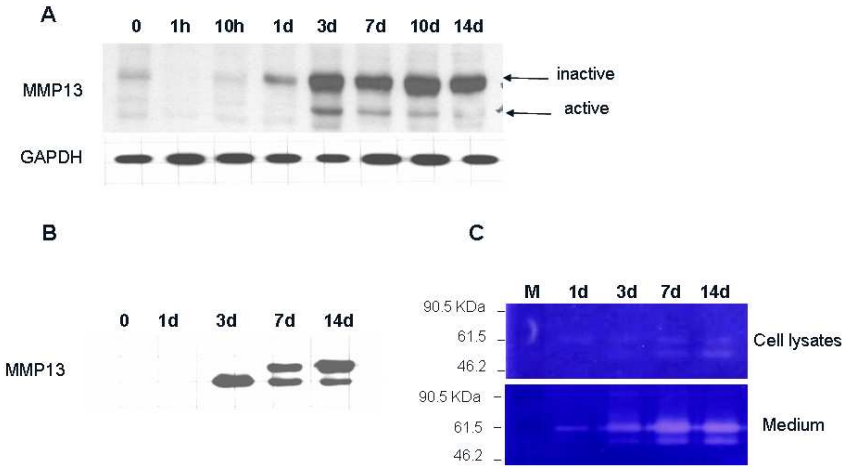

Figure 11. Confirmation of MMP-13 expression (A and B) and activity (C) during chondrogenesis. Whole cell lysates were obtained by 1X passive lysis buffer and then total 30 μg of proteins or 10 μl of culture medium was used in Western blot and gelatin zymography. The MMP-13 expression and activity were increased until day 14. M: molecular size marker.

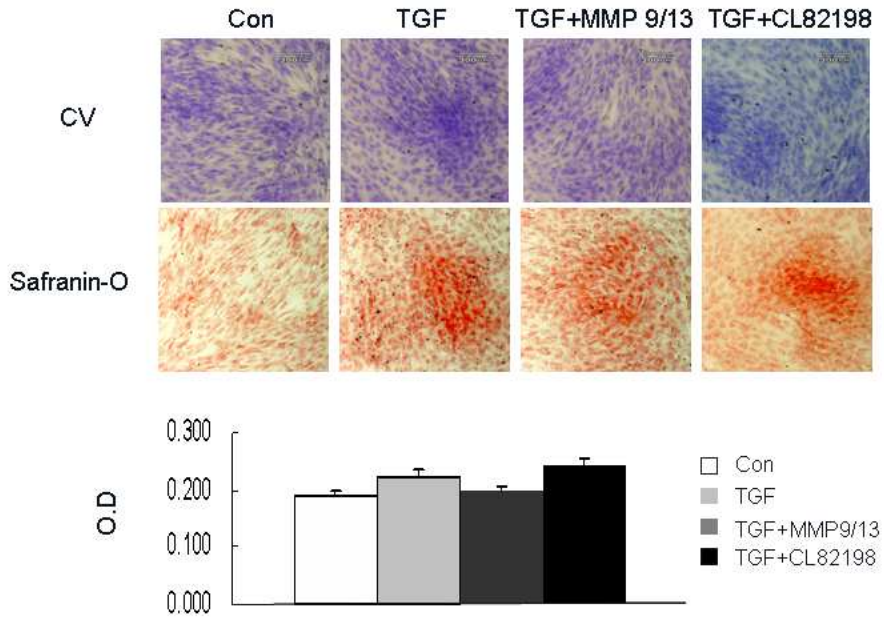

Figure 12. Safranin-O staining after treatment of MMP13 inhibitors in chondrogenesis. Chondrogenesis was induced by 10 ng/ml of TGF-β3 with or without MMP-13 inhibitors (MMP-9/13 inhibitor and CL82198). The absorbance was detected at 490 nm by destaining with 100% ethanol for 20 min.

6. Expression of integrin subunits in articular chondrocytes-type II collagen interaction

Most cells are anchorage-dependent for their growth, meaning that proliferation does not occur unless the cells are attached to ECMs by integrins[44]. Integrins are critical for the intracellular integrin-mediated response in many cellular function by activating various protein tyrosine kinase, including FAK and Src family kinase, a serine-threonine kinase, and integrin linked kinase[45,46].

At first, we examined which integrin subunits are changed in chondrocyte-type II collagen interactions. Integrin β1 and α2 expression were significantly increased 1.5-fold ($p<0.05$) and 2.2-fold ($p<0.05$) at day 7 in cells grown in type II collagen coated plates (Figure 13A). It should be noted that the FAK was significantly expressed 20-fold ($p=0.005$) in the cells attached to type II collagen compared to the TCP at day 7, but the integrin β1 expression showed no difference. And also, the integrin α 5 expression was increased 1.3-fold ($p<0.005$) in TCP attached cells at day 4 and day 7. There were differences in the morphology after attachment to type II collagen and TCP. The chondrocytes in the type II collagen coated plates showed a round shape, whereas TCP attached cells showed a fibroblast-like morphology (Figure 13B).

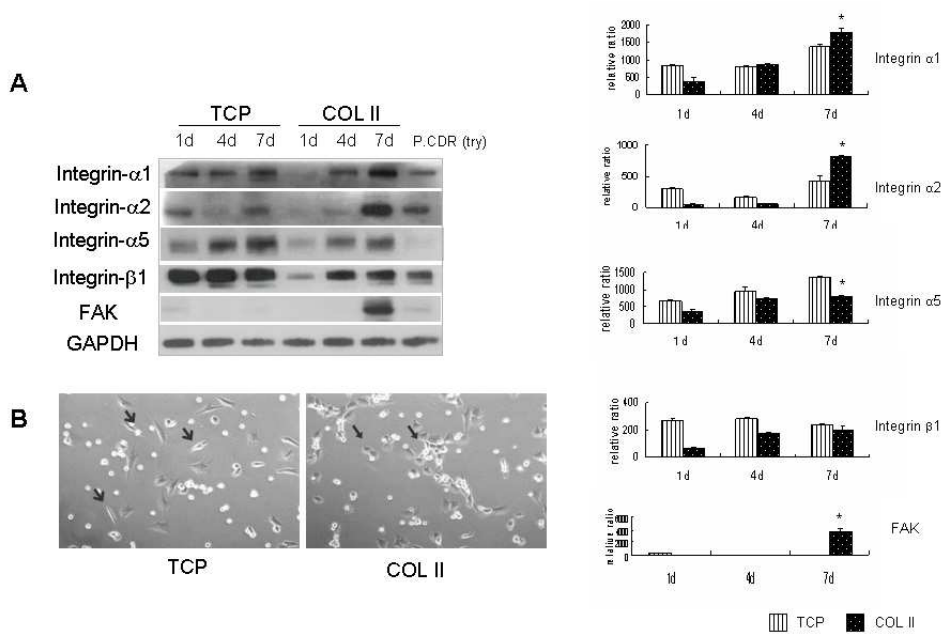

Figure 13. Integrin expression in chondrocyte-type II collagen interaction. After trypsinization of porcine articular chondrocytes [P.CDR (try)], the cells were replated in 1 μg/ml of type II collagen-coated plates or tissue culture plate (TCP) for 7 days. Total 20 μg aliquots of the cell lysates were used to detect integrin expression in Western blot analysis (A). Morphology of cells have a difference between fibroblast-like cell phenotype in TCP and polygonal cell shape when grown in type II collagen-coated plates (COL II) (B).

7. Tyrosine phosphorylation by chondrocytes-type II collagen interaction

In many cell types, adherence to the matrix stimulates large two cellular events. The first event is protein tyrosine phosphorylation[47]. To determine whether protein tyrosine phosphorylation occurs in chondrocytes-type II collagen interactions, the cells were allowed to adhere to the type II collagen for 5 hours. After attachment, the tyrosine phosphorylation was maximally increased at 40 min in the proteins around 120, 80 and 60 kDa and then decreased both in Western blotting and immunofluorescence staining (Figure 14A and 14B). Integrin receptor activation through cell-ECM interactions can lead to focal adhesion complex as a second event[47]. FAK can be recruited to focal adhesions because it interacts, either directly or through the cytoskeletal proteins, with the cytoplasmic tail of integrin β subunits[48]. Therefore, we investigated changes of integrin β1 and FAK following adhesion to type II collagen. Integrin β1 was constantly expressed until 5 hours, whereas FAK auto-phosphorylation at Tyr-397 (p-FAK_{Y397}) showed a peak at 40 min (Figure 14C).

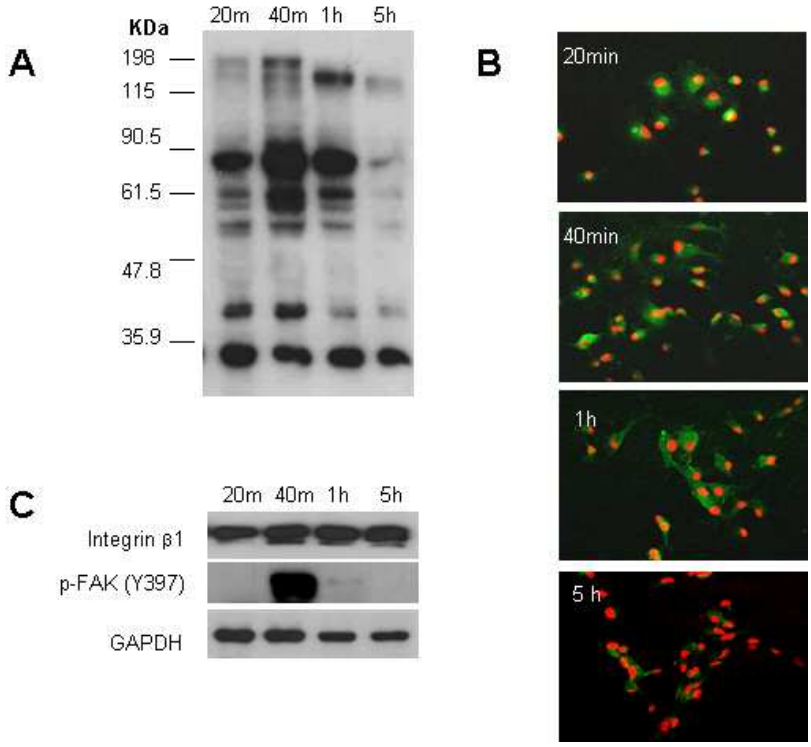

Figure 14. Tyrosine phosphorylation in chondrocyte-type II collagen interaction. Porcine articular chondrocytes were cultured in type II collagen-coated plates for 5 hours and then tyrosine phosphorylation was detected in Western blot analysis (A) or confocal microscopy (B). Expression of integrin β1 and p-FAK_{Y397} was checked until 5 hours after attachment to each plate (C).

8. Activation of FAK and MAP kinase by chondrocyte-type II collagen interaction

Rapid signaling processes by integrins includes tyrosine phosphorylation of FAK, activation of MAP kinase (ERK, p38 and JNK) pathways and changes in membrane fluxes of divalent cations and hydrogen ions[33,47]. In present study, FAK expression was increased until day 3 in the type II collagen-coated plates compared to the TCP and the p-FAK397 was increased from day 3 to day 5 (Figure 15A). In similar to FAK phosphorylation, ERK1/2 activity was increased at day 3 and day 5 in type II collagen-coated plates compared to TCP (Figure 15B). We therefore compared the ERK1/2 phosphorylation both in alginate bead system and monolayer culture system with type II collagen (Figure 15C). ERK1/2 phosphorylation was gradually increased until 10 hours in two culture systems. Interestingly, we could detect that ERK1/2 was highly activated in alginate bead culture with type II collagen all the time compared to alginate alone or monolayer culture with or without type II collagen. ERK1/2 activation was started at 30 min in only alginate bead with type II collagen. It suggest that ERK signaling by type II collagen may be more effectively transmitted in 3D culture system such as alginate beads rather than 2-dimensional culture system. This result suggests that the specific signaling by type II collagen might be mediated, at least in part, via a signaling pathway that proceeds through FAK and ERK1/2. We could not detect JNK and p38 activation after attachment to both TCP and type II collagen (data not shown).

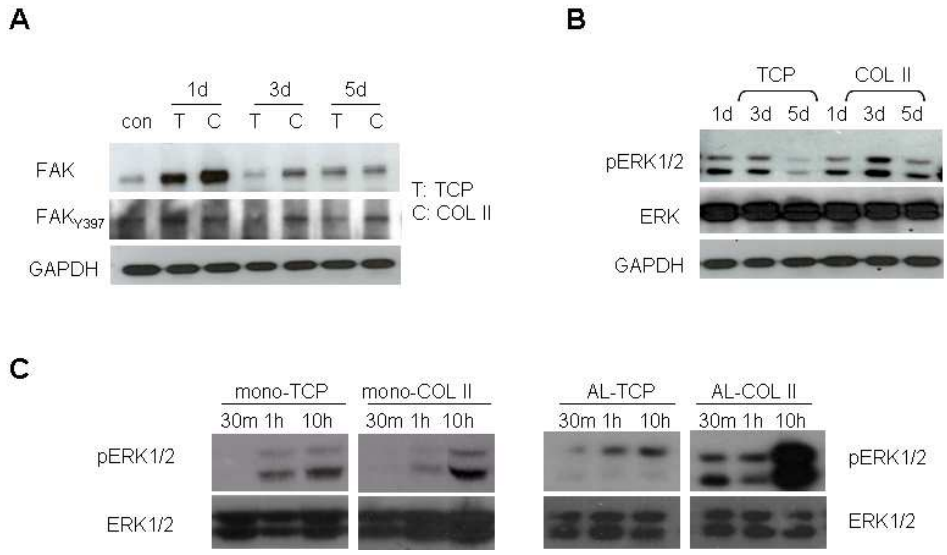

Figure 15. FAK (A) and ERK1/2 activation (B) by addition of type II collagen in monolayer or alginate bead system (C). AL: alginate, TCP: tissue culture plastic, COL II: type II collagen-coated plates.

9. Activation of FAK, MAP kinase and Smads in dedifferentiated chondrocytes

We demonstrated that FAK and ERK1/2 activation was induced by chondrocyte-type II collagen interactions in Figure 8. Dedifferentiated chondrocytes induced by low density-monolayer culture showed morphological change from a polygonal morphology to a fibroblast-like morphology after day 50 (Figure 16A). Type II collagen mRNAs was expressed strongly in freshly isolated cells, but had decreased up to 40% in monolayer cultured cells after 3 weeks. In contrast, type I collagen expression was increased from day 21 and kept increasing for the 86 days of study. In Western blot analysis, p-FAK_{Y397} and p-ERK1/2 were decreased in dedifferentiated chondrocytes time-dependently, and their expression were equally maintained for 40 days. The p-FAK_{Y397} and p-ERK1/2 showed correlation with loss of the type II collagen mRNA expression during chondrocyte dedifferentiation (Figure 16B).

A few years ago, other group reported Smad2/3 activation by type II collagen in chondrocytic cells[49]. We therefore questioned whether the Smad activation as well as MAP kinase was changed in dedifferentiating chondrocytes without type II collagen. In these conditions, p-Smad2 and p-Smad3 were constantly expressed for 60 days. Therefore, stability of the chondrocyte phenotypes might be correlated with FAK-ERK signaling rather than other pathway such as p38, JNK and Smad2/3 (Figure 16C).

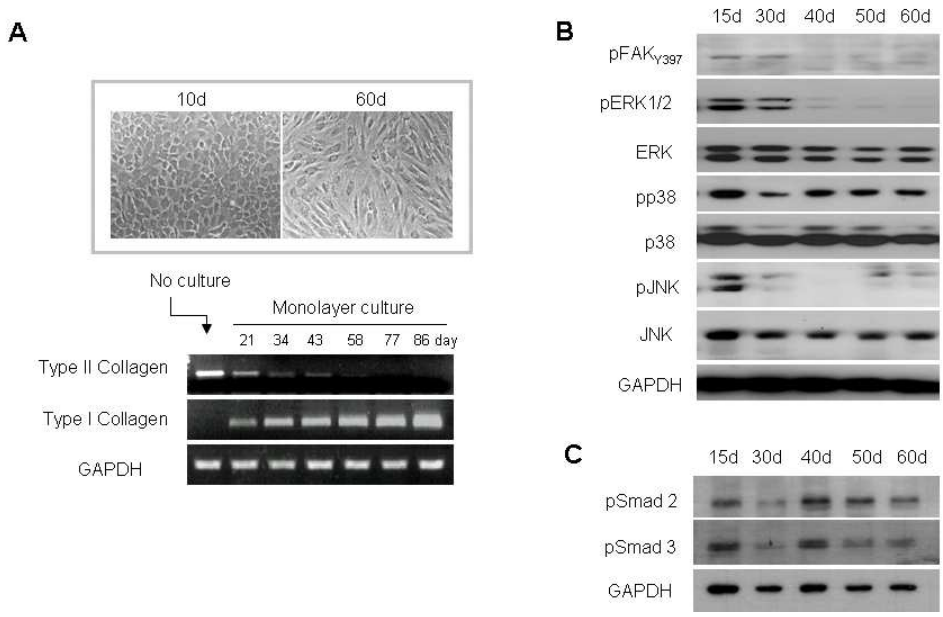

Figure 16. Activation of FAK, MAP kinase and Smad2/3 during dedifferentiation. Dedifferentiation phenotypes were detected in changes of cell morphology and collagen mRNA expression by RT-PCR analysis (A). Changes of the phosphorylation level of FAK, MAP kinase and Smad2/3 were detected by Western blotting (B and C).

10. Change in cell morphology and ERK activation by FAK inhibition

Human articular chondrocytes were transfected with FAK-siRNA or negative control-siRNA (ctrl-RNA), and allowed the cells to proliferate in DMEM-HG medium containing 10% FBS for 5 days. FAK specific siRNA completely blocked the expression of FAK (Figure 17A). FAK-knock out cells showed fibrous morphology compared to the ctrl-siRNA transfecd cells and non-transfected cells (Figure 17B). The morphology changed by FAK-siRNA was similar to fibroblast-like cells in dedifferentiating process as shown in Figure 16, suggesting that FAK might involve in signaling mechanism to regulate chondrocyte morphology. However, FAK and ERK1/2 activation did not precisely correlate (Figure 17C). ERK1/2 activity was completely reduced by FAK-siRNA at day 1 and then reactivated at day 4 even though FAK was completely blocked. This is consistent with previous reports that ERK activation was not dependent on FAK phosphorylation, because MAPKs could bind to many types of proteins including protein kinase, protein phosphatase, cytoskeletal proteins and transcriptional proteins. It has been shown that the differences observed in kinetics of FAK and ERK activation might reflect differences between cell types, detection method, and lysis buffer[50]. Therefore, our results suggest that ERK activation might be not exclusively regulated by FAK. In spite of disappeared FAK activity, the reactivation of ERK1/2 suggests that some other factors such as growth factors in serum may intervene to increase ERK activity during proliferation.

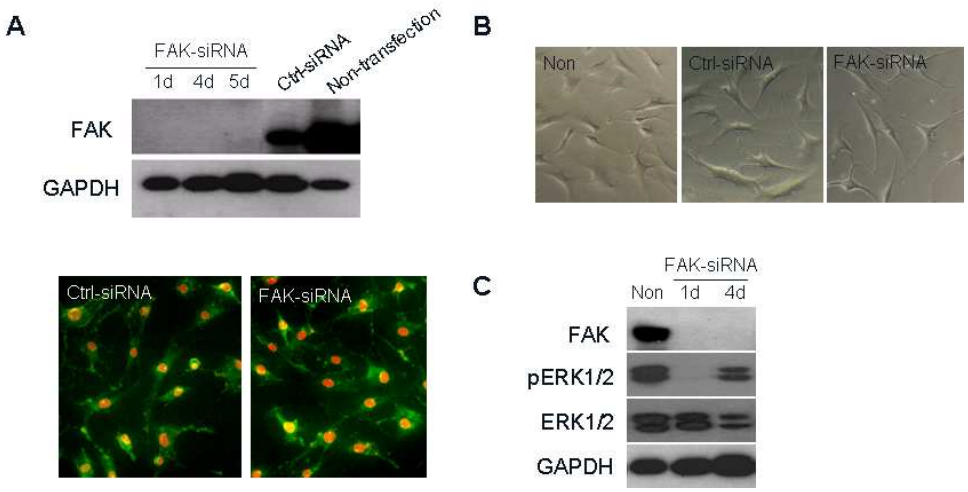

Figure 17. FAK inhibition by siRNA in human articular chondrocytes. FAK expression (A) and cell morphology (B) were changed by transfection at day 1. ERK activation was inhibited and then reactivated at day 4 after FAK-siRNA transfection (C).

11. Effects of silencing FAK on the chondrocyte proliferation and GAG secretion

FAK plays a key role in proliferation of normal and tumor cells by recruiting other signaling molecules to the submembranous region[35-37,39,47,48]. To verify that FAK blocking induces growth-suppressing effect on normal articular chondrocytes, the cells after pre-treatment with or without siRNA for 2 days were cultured on type II collagen for 3 days in DMEM-HG containing 10% FBS. FAK expression was specifically blocked in FAK-knock out cells until day 3 compared to the negative control cells or non-transfected cells (Figure 18A). The chondrocytes cultured on type II collagen showed 3.5-fold increases in proliferation for 3 days. However, the proliferation of FAK-siRNA transfected cells only increased to 1.7-fold and it was significant compared to both control-siRNA transfected cells ($p=0.001$) and non-transfected cells ($p=0.041$) (Figure 18B). ERK1/2 was only inhibited by FAK inhibition, suggesting that chondrocytes proliferation could be regulated by FAK via ERK not p38 or JNK pathway (Figure 18D). There was no inhibitory effect on GAG secretion in FAK-knock out cells (Figure 18C).

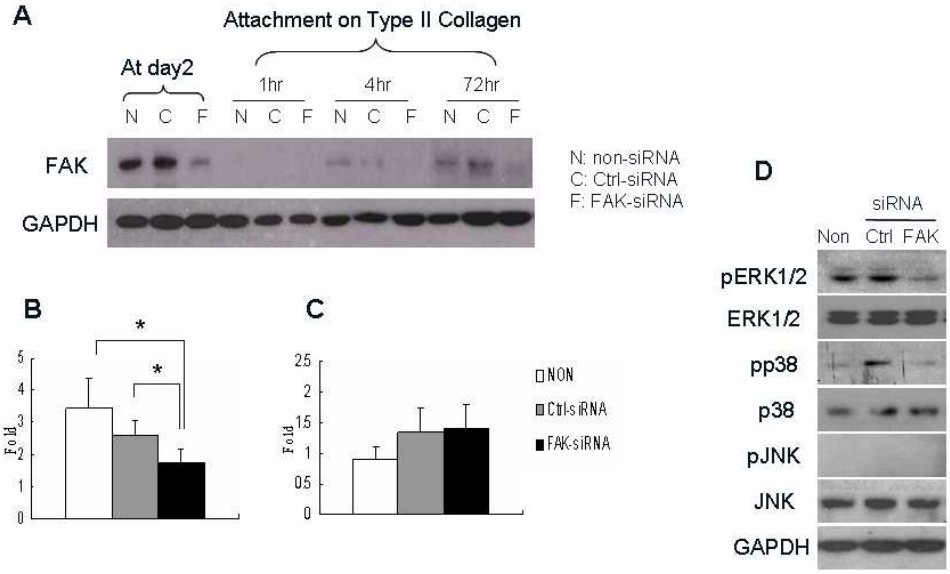

Figure 18. Proliferation and GAG secretion by FAK-siRNA transfection. FAK expression (A) was specifically blocked at day 3 and the proliferation rate (B) was decreased in FAK-siRNA transfeced cells compared to the control-siRNA transfected cells (Ctrl-siRNA). But, there was no inhibitory effect on proteoglycan secretion in FAK-knock out cells (C). At that time, only ERK1/2 was inactivated by FAK inhibition, suggesting that chondrocytes proliferation could be regulated by FAK through ERK pathway (D).

12. The role of FAK in redifferentiation of human articular chondrocytes

The dedifferentiating chondrocytes are able to redifferentiate and upregulate the expression of cartilage specific genes when cultured in 3D culture[28,51]. We further determined whether FAK was essential for chondrocyte specific gene expression during redifferentiation. At day 2 of post transfection with siRNA, the cells were recapsulated in alginate bead with type II collagen or TGF-β1 as extracellular stimulators (Figure 19A). When untransfected chondrocytes were transferred to alginate bead system, type II collagen expression gradually increased in all groups. Especially, type II collagen expression showed 6.7-fold increase in cells cultured in alginate bead with exogenous type II collagen compared to monolayer cultured cells (Figure 19B). The expression was more increased by exogenous type II collagen (6.7±0.2 fold) than by TGF-β1 (3.5±0.3 fold). FAK expression was also highly increased by exogenous type II collagen and TGF-β1 up to 5.1-fold, and there is no significant difference in both cells. We also found that type II collagen could be induced up to 3.0-fold in alginate bead system without any stimulators such as TGF-β1 or ECM. However, FAK-siRNA transfected cells did not increase type II collagen and FAK expression by TGF-β1 or exogenous type II collagen, suggesting that type II collagen could not be recovered without FAK signaling even though TGF-β1 or ECM was treated. In contrast to type II collagen, aggrecan was not inhibited by FAK-siRNA transfection for 2 days in monolayer cultured cells (Figure 19C). When the siRNA-transfected cells were transferred to alginate bead system, there was no changes in aggrecan expression, suggesting that FAK signaling might be required in regulation of type II collagen but not aggrecan.

SOX family, SOX-5, SOX-6 and SOX-9, are specific transcription factors that regulate the expression of chondrocyte specific genes and play a major role in chondrocyte differentiation[52]. Therefore, we tested the effect of FAK on the expression of SOX genes. Interestingly, SOX-6 was only down-regulated by FAK inhibition, but SOX-5 or SOX-9 was not affected, suggesting that down-regulation of type II collagen in FAK-knock out cells might be predominantly mediated by SOX-6 not SOX-5 or SOX-9 (Figure 19D).

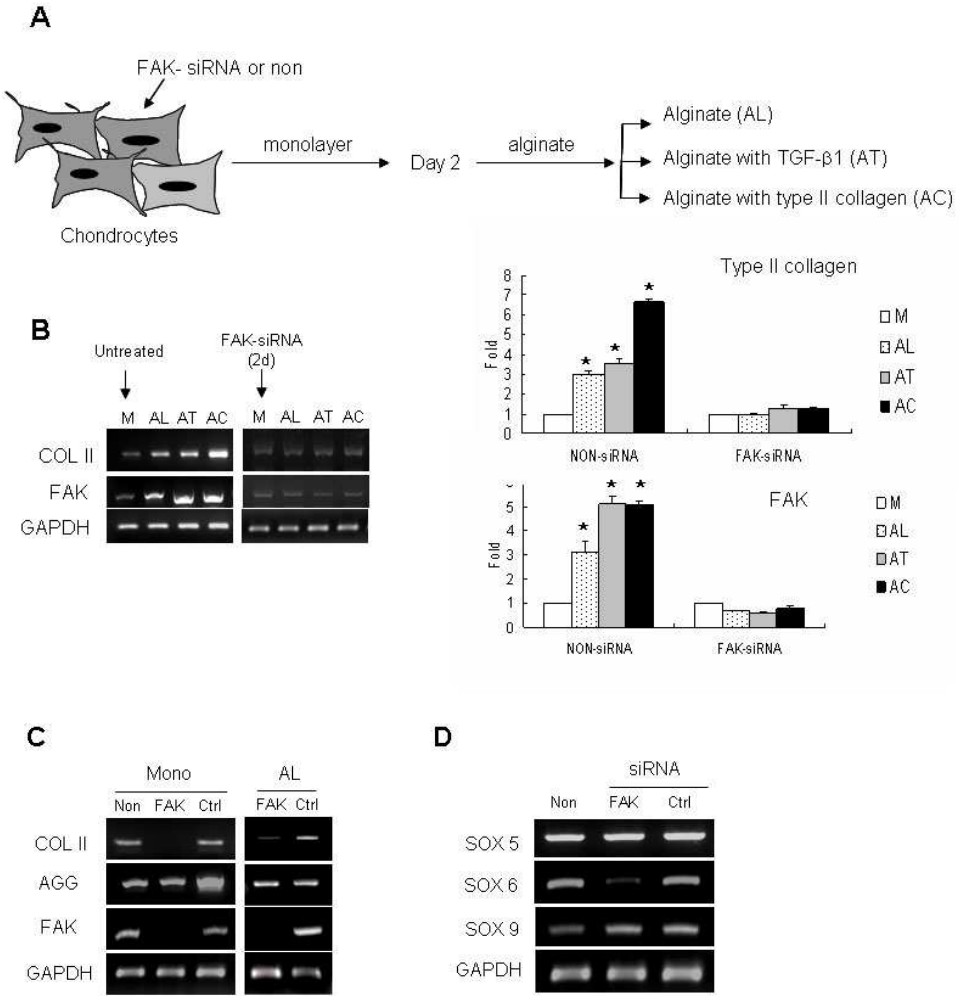

Figure 19. Gene expression during redifferentiation of human articular chondrocytes. At day 2 of post transfection with FAK-siRNA, the cells were recapsulated in alginate bead with type II collagen or TGF-β1 (A and B). siRNA-transfect cells blocked the up-regulation of type II collagen and FAK expression by TGF-β1 or exogenous type II collagen (B) whereas aggrecan expression was not affected by siRNA transfection in both alginate bead and monolayer system (C). SOX-6 among three SOX genes was only down-regulated by FAK inhibition (D).

13. Inhibition of chondrogenesis by FAK-siRNA transfection

Finally, we questioned whether FAK inhibition could down-regulate the type II collagen expression during chondrogenesis of the human bone marrow-derived MSCs. At day 2 of post transfection with siRNA, the undifferentiated MSCs were differentiated into chondrocytes by high density-monolayer culture method for 5 days. Cell morphology was changed by TGF-β3 treatment like normal polygonal chondrocytes (Figure 20A). However, FAK-siRNA transfected cells showed more fibroblastic cell morphology compared to the control-siRNA transfected cells. FAK suppression resulted in down-regulation of the SOX-6 and SOX-9 in undifferentiated MSCs. Similar to chondrocytes, FAK-knock out cells blocked differentiation into chondrocytes by suppression of the type II collagen expression (Figure 20B). When chondrogenesis was induced by TGF-β3, type II collagen and FAK expression were increased in control-siRNA transfected cells. It suggest that if the FAK signaling was blocked, type II collagen was not induced in spite of increased SOX transcription factors by TGF-β3. However, There was no inhibitory effect on proteoglycan secretion in FAK-knock out cells (Figure 21A and 21B).

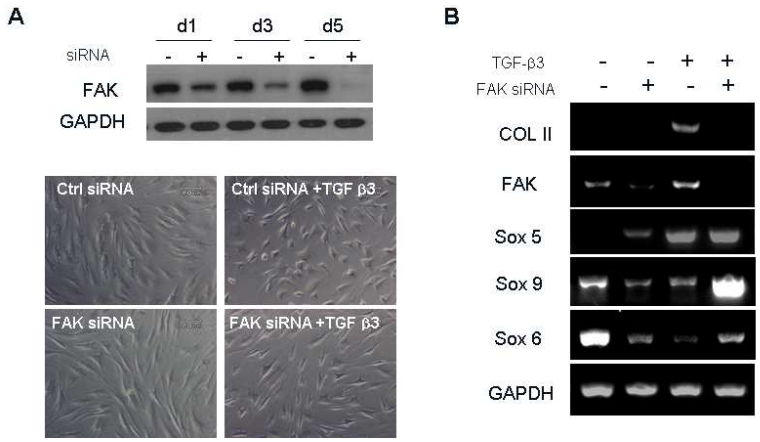

Figure 20. Chondrogenic phenotyes and cell morphology by FAK inhibition during chondrogenesis. Transfected cells with FAK or control (Ctrl)-siRNA were treated by 10 ng/ml TGF-β3 in high density-monolayer culture for 5 days. FAK expression and other chondrogenic markers were detected by Western blotting (A) and semi-quantitative RT-PCR, respectively (B).

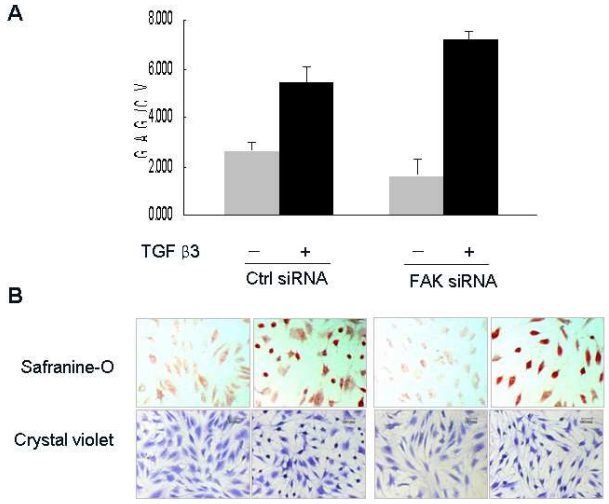

Figure 21. GAG secretion (A) and Safranin-O staining (B) during chondrogenesis by FAK-siRNA transfection. At day 7, GAG amount was checked at 656 nm.

IV. DISCUSSION

MSC have been shown to undergo chondrogenesis *in vitro* using a high cell density pellet culture or alginate system, which mimics the cellular condensation requirements for embryonic mesenchymal chondrogenesis and provides the physical and biochemical environmental factors conductive to cartilage formation[3-5,53,54]. Previous studies have reported that tyape II collagen was expressed prior to type X collagen expression during chondrogenesis[15,16,55]. Type X collagen and MMP-13, markers of chondrocyte hypertrophy, are expressed in human chondrocytes during fetal ossification and degenerative joint diseases[56]. Thus, caution must be exercised when using type X collagen in association with adult human mesenchymal stem cell differentiation. Most remarkably, no aspects of hypertrophy and terminal differentiation were induced when human articular chondrocytes were expanded in monolayer. Human articular chondrocytes may down-regulate cartilage differentiation markers during monolayer culture[27,28], it appears that they do not 'forgot' their history as stable articular chondrocytes and are not prone to hypertrophic differentiation during expansion[57] or in the presence of TGF-β as an inhibitor of hypertrophy of chondrocytes[58]. Therefore, the first aim of this study was to improve the culture system minimizing the expression of hypertrophy-related genes during *in vitro* chondrogenesis.

In this results, MSCs could be successfully differentiated into chondrocytes by TGF-β3 in monolayer culture system. Type II collagen was strongly expressed whereas type I collagen was minimally expressed. Moreover, monolayer-alginate combined method induced type II collagen expression at maximum level whereas type X collagen expression was minimized. And also, cell viability was more increased from 30% to 65% in monolayer-alginate combined method compared to alginate bead system. It suggested that pretreatment of TGF-β3 for 7 days before

culturing in alginate bead could be an ideal method to enhance chondrogenic phenotypes such as type II collagen and minimize hypertrohic gene expression. However, MMP-13 mRNA expression was induced at day 14 by all methods. We could also detect the MMP-13 expression in proteome analysis and Western blotting. MMP-13 is involved in triple helical collagen degradation during matrix remodeling, and its activity was reported to be enhanced in osteoarthritis cartilage[59]. Recent data suggest that proteolysis involving MMP-13 is required for chondrocyte differentiation into a hypertrophic state and occurs together with up-regulation of type X collagen but before osteocalcin expression[60]. In this result, the treatment of MMP-13 inhibitors (MMP-9/13 inhibitor and CL82198) could not inhibit chondrogenesis, suggesting that MMP-13 might be induced as one of hypertrophic phenotypes rather than a regulating factor in chondrogenesis.

Cbfa1 is one of members of the runt-related transcription factor family, which proteins share a unique 125-amino acid motif called the 'runt' DNA binding domain[61]. Forced expression of Cbfa1 in chondrocytes stimulated the expression of hypertrophic phenotypes such as ALP and matrix calcification[23,24,62]. We could show here that Cbfa1 was highly expressed in ALP-positive undifferentiated MSCs (large-sized cells) compared to ALP-negative undifferentiated MSCs (small-sized cells). When the ALP-positive or negative MSCs were differentiated into chondrocytes for 14 days, type X collagen was induced at high level by all methods (monolayer, alginate and monolayer-alginate combined method) in ALP-positive MSCs compared to ALP-negative MSCs. Therefore, it could suggest that increased type X collagen expression during *in vitro* chondrogenesis might be dependent on level of ALP/Cbfa1 expression in undifferentiated MSCs. ALP or Cbfa1 in

undifferentiated MSCs could be used as a marker predicting chondrocyte hypertrophy during *in vitro* chondrogenesis. In the future, the development of selective techniques of ALP-negative MSCs is required for cartilage regeneration without chondroycte hypertrophy in clinical application.

Type II collagen is a major protein in articular cartilage to maintain biological and mechanical characteristics. Several studies have suggested that type II collagen could maintain the chondrocyte phenotype in 3D culture not in monolayer[63,64]. We have focused the effect of type II collagen on the expression of cartilage specific genes, DNA synthesis and proteoglycan synthesis for several years[65,66]. However, less is known about mechanisms of intracellular signaling of chondrocytes initiated by matrix protein such as type II collagen. In the present study, the role of FAK-ERK signaling in the intracellular events by exogenous type II collagen and maintenance of the chondrocyte phenotypes was investigated. The addition of type II collagen to porcine chondrocytes induced the change of integrin expression. Integrin α2 expression was significantly increased in the cells grown on type II collagen-coated plates, but integrin α1, α5 and β1 subunits was not affected by type II collagen. Articular chondrocytes express receptors, including the integrins α1β1, α3β1, α5β1, α10β1, αvβ1, αvβ3, and αvβ5, which may facilitate binding to these ligands[67,68]. Some studies reported that adult human articular chondrocytes expressed integrin α1β1 but not integrin α2β1, while human fetal articular chondrocytes, human chondrosarcoma cell lines, and bovine calf chondrocytes expressed integrin α2β1 and α210β1, but little or no integrin α1β1[69,70]. Therefore, this discrepancy may depend on differences in species, antibody specificities or the detection techniques used. Interestingly, in our data, the FAK expression and phosphorylation were

significantly increased in the cells attached to type II collagen compared to TCP. Thus, it is proposed that integrin α2β1 may dominantly transmit extracellular signaling to FAK on binding to type II collagen.

Cell adhesion via integrin engagement has been shown to result in two large changes: (i) enhanced intracellular protein tyrosine phosphorylation and (ii) the formation of focal adhesion complexes[47]. Indeed, we found that tyrosine phosphorylation was peaked at 40 min and FAK phosphorylation was only shown at 40 min following adhesion to type II collagen. However, there was no changes in integrin β1 expression in all periods and its expression seemed to be maintained during chondrocyte-type II collagen interaction. FAK is autophosphorylated predominantly on Tyr397 and phosphorylated Tyr397 conforms to the consensus binding for SH2 domain for c-Src[71]. Interaction between Src and FAK leads to phosphorylation of FAK at several sites including Tyr^{576}, Tyr^{577}, Tyr^{861} and Tyr^{925}, which then results in activation of MAPK[72]. In this study, ERK1/2 was sharply risen in alginate with type II collagen compared to TCP or alginate alone, suggesting that type II collagen-stimulated ERK1/2 might be activated effectively in 3D culture system than in monolayer. ERK1/2 activation increased in cartilage explants was decreased by IL-1, and inhibition of ERK induced apoptosis of chondrocytes[73,74]. Therefore, we speculate that type II collagen-stimulated ERK1/2 activation in cartilage can be changed by inflammatory cytokines in pathogenesis of osteoarthritis and also ERK activation by type II collagen may be important to maintain normal chondrocyte phenotypes. This hypothesis could be proven in dedifferentiated chondrocytes induced by low-density monolayer culture. It is well known that chondrocytes grown in monolayer culture

dedifferentiate into fibroblast-like cells, which fail to produce their specific markers including type II collagen and aggrecan[27,28]. In this study, interestingly, the FAK and ERK1/2 activation were observed to be correlated with the loss of the expression of type II collagen during dedifferentiation of porcine chondrocytes. The FAK and ERK1/2 phosphorylation were decreased, and became absent after day 40. Our finding is consistent with previous observation by *Vinall RL* et al[36]. They demonstrated that type II collagen expression in monolayer of bovine chondrocytes is concomitant with the expression of cytoskeletal components such as tensin, talin, paxillin and FAK. ERK inhibition compeletly inhibited the TGF-β1-induced gene expression of aggrecan, as well as collagen type II and SOX-9 in chondrogenesis[75]. Recently, type II collagen-stimulated Smad2/3 activation, one of TGF signaling cascades, was also found in chondrocytic cells and the activation led to an increase in type II collagen transcription[49]. However, our results here showed that Smad2/3 activation was not decreased during dedifferentiation, suggesting that chondrocyte dedifferentiation might be predominantly controlled by FAK-ERK signals but not Smad2/3 signals.

Many studies have reported that dedifferentiated chondrocytes could be recovered their phenotypes by culturing in 3D culture system with or without TGF-β. However, the roles of integrin signaling in both dedifferentiation and redifferentiation are not clearly known yet. We could knock out FAK expression completely by siRNA transfection. In proliferation assay, ERK1/2 activity was influenced by FAK inhibition and FAK-siRNA transfected cells showed only 1.5-fold increase for 3 days compared to control-siRNA transfected cells (2.6-fold increase) and untransfected cells (3.5-fold increase). Previous studies have shown that

expression of the mutant of FAK results in a decrease in cyclin D1 expression, whereas overexpression of wild type FAK increased transcription of cyclin D1 gene[76]. Furthermore, ERK activity was necessary for FAK promotion of cell proliferation and active ERK induces transcription of cyclin D1[77,78]. We could show that supression of FAK expression result in ERK1/2 inactivation and decreased chondrocyte proliferation whereas GAG secretion was not affected by FAK inhibition. FAK-siRNA transfected cells reduced type II collagen expression whereas aggrecan was not changed, suggesting that aggrecan expression was not predominantly mediated by FAK-ERK signaling. These results was consistent with previous results that p38 MAPK play a crucial role in mediating IL-1 induced down-regulation of aggrecan in SW1353 cells, chondrocyte cell lines[79]. They showed that IL-1 induced a massive down-regulation of the aggrecan expression was prevented by p38 inhibitor whereas ERK1/2 inhibitor had no effect[79]. Therefore, it is supposed that the aggrecan expression was not decreased in FAK knock out cells, because p38 activation was still remained after siRNA transfection in our experiments. We could significantly induce type II collagen expression in alginate system with exogenous type II collagen (6.7-fold) or TGF-β1 (3.5-fold). FAK expression was also induced by exogenous type II collagen or TGF-β1, indicating that FAK might be essential for type II collagen expression during chondrocyte redifferentiation in 3D culture system. Our hypothesis was proven here that the treatment of FAK-siRNA suppressed exogeneous type II collagen or TGF-β1-stimulated type II collagen expression in alginate bead system.

Futhermore, we could find that the block of type II collagen expression by FAK inhibition was mediated by SOX-6 transcription factor but not SOX-5 or SOX-9. We could also suppress FAK expression up to day 5

in mesenchymal stem cells. Cell morphology was changed to chondrocyte-like shape by TGF-β3. FAK suppression induced down-regulation of the SOX-6 and SOX-9, and it blocked differentiation into chondrocytes by suppressing type II collagen expression. If the FAK signaling was blocked, type II collagen was not induced in spite of increased SOX transcription factors by TGF-β3. However, GAG secretion was not suppressed in FAK knock-out cells, suggesting that FAK might be required in chondrogenesis from human mesenchymal stem cells. Numerous studies have showed that SOX-9 is an essential transcription factor for chondrocyte specific genes such as type II collagen and aggreacan. However, SOX-9 does not positively correlate with type II collagen expression in adult human articular chondrocytes[80]. Co-expression of three SOX transcription factors, SOX-5, SOX-6 and SOX-9 was more effective than any of the transcription factors alone in activation type II collagen gene[52]. Therefore, we checked mRNA expression of three SOX transcription factors in human articular chondrocytes after transfecting with FAK-siRNA. We found that SOX-6, among three SOX transcription factors, is only affected by FAK, suggesting that decrease of type II collagen by FAK inhibition might be mediated by SOX-6 transcription factor.

In conclusion, we provide molecular evidences that type II collagen-stimulated FAK-ERK signaling as a positive regulator of chondrocyte phenotype in both dedifferentiation and redifferentiation as well as proliferation. Finally, we showed for the first time that the lack of SOX-6 by FAK inhibition might result in decrease of type II collagen expression but not aggrecan.

V. CONCLUSION

1. Improvement of the differentiation system to minimize the expression of hypertrophy-related genes

In monolayer culture system, MSCs were successfully differentiated into chondrcytes by TGF-β3 for 14 days. Type II collagen was strongly expressed whereas type I collagen was minimally expressed. Monolayer-alginate combined method induced type II collagen expression at maximum level whereas minimized type X collagen expression. And also, cell viability was more increased from 30% to 65% in combined method compared to alginate bead system. It suggested that pretreatment of TGF-β3 for 7 days before culturing in alginate bead could be an ideal method to enhance chondrogenic phenotypes such as type II collagen and minimize hypertrohic gene expression. Induction of type X collagen in chondrogenesis was correlated with ALP/Cbfa1 expression level in heterogenous MSC populations (Figure 22).

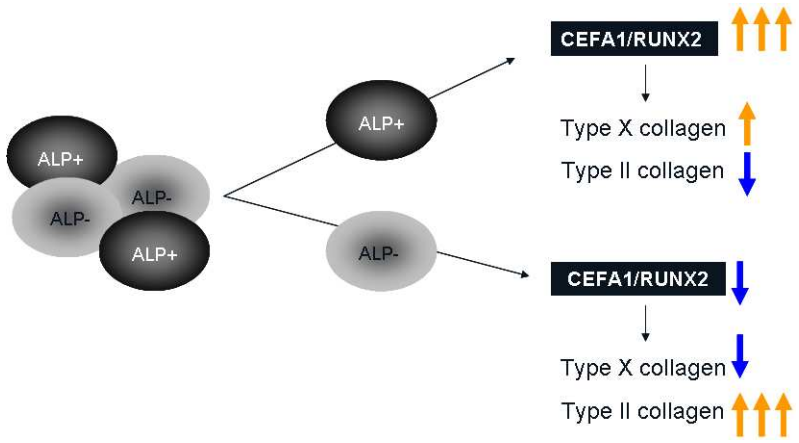

Figure 22. Role of type X collagen induction during chondrogenesis from bone marrow-derived mesenchymal stem cells.

2. Role of intracellular signaling pathways such as FAK, Smad, and MAP kinase in chondrogenesis and maintenance of chondrocyte phenotypes

Integrin α2 and FAK expression were significantly increased in cells grown in type II collagen-coated plates. ERK1/2 was highly activated in alginate bead culture with type II collagen. In constrast, FAK and ERK1/2 activity were declined in dedifferentiated chondrocytes, suggesting that signaling pathway to maintain or induce chondrogenic phenotype might be mediated at least by FAK. FAK suppression by siRNA transfection blocked type II collagen mRNA expression stimulated by exogenous type II collagen or TGF-β1 in alginate bead system. But there was no change in aggrecan expression. We also suppressed FAK expression by siRNA transfection up to day 5 in human bone marrow-derived MSCs. Cell morphology was changed by TGF-β3 treatment like normal polygonal chondrocytes. However, FAK-siRNA transfected cells showed more fibroblastic cell morphology. FAK suppression induced down-regulation of the SOX-6 and SOX-9 and blocked differentiation into chondrocytes by suppressing type II collagen expression. If the FAK signaling was blocked, type II collagen expression was not induced in spite of increased SOX transcription factors by TGF-β3. However, GAG secretion was not suppressed in FAK knock-out cells. Our results provide evidences that FAK is required in chondrogenesis of human mesenchymal stem cells as well as the maintenance of chondrocyte phenotypes such as type II collagen expression and proliferation.

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ABSTRACT IN KOREAN

시험관적 연골 분화법의 향상과 연골 세포 분화 과정에서 focal adhesion kinase의 기전

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발생과정에서 연골은 골 형성 과정과 동반되며, 연골분화(differentiation)-성숙 (hypertrophy)-석회화 (calcification)-골화의 과정에서 생체 조절 인자에 의해 분화가 조절됨으로써 연골 및 골의 특이적 조직 형성이 이루어진다. 그러나, 중간엽 줄기세포로부터 분화된 연골세포를 이식하였을 경우, 안정적인 연골 특이적 표현형을 나타내기 보다는 석회화 및 신혈관 생성 등의 골화와 관련된 표현형으로 교체되는 등의 문제점이 보고되고 있다. 이러한 원인은 줄기세포로부터 분화시킨 연골세포는 고유의 관절 연골 세포에서는 발현하지 않는 제 10형 교원질 및 기질분해효소 (MMP-13) 등의 연골성숙인자(hypertrophy-related genes)가 발현하는 것으로 밝혀졌다. 따라서, 중간엽 줄기세포를 이용하여 골 관절염 등의 치료법에 응용하기 위해서는 중간엽 세포로부터 연골세포 분화 방법 개선 및 기전에 관한 이해가 매우 중요하다. 관절 연골세포는 제 2형 교원질 등의 세포외기질로 구성되어 있으며, 세포외기질의 파괴는 골 관절염을 유발하는 원인이 된다. 세포외기질과 세포와의 결합은 인테그린이라는 리셉터를 통해 세포내로 신호가 전달되어진다. Focal adhesion kinase (FAK)는 인테그린 신호전달기전의 중심적인 역할을 하는 단백질로서, FAK를

통한 세포신호는 c-Src, Rho GAP, paxillin, talin, p130CAS 및 caveolin-1 등을 통해 이루어진다. 그러나, 제 2형 교원질과 연골세포와의 결합을 통한 세포신호전달기전에 관하여 보고된 바가 적다.

따라서 본 연구의 첫 번째 목적은 연골세포 분화 방법의 개선 즉, 연골 성숙인자의 발현 및 세포사멸의 최소화와 성숙인자의 발현 원인을 분석하고자 하였다. 또한 연골 분화 과정 및 연골 특이적 표현형 유지에 있어서 인테그린 하위 신호전달기전인 FAK, Smad 및 MAP kinase의 역할을 살펴보고자 하였다.

성인 중간엽 줄기세포는 골수로부터 채취하여 계대 배양한 후, 3 종류의 배양법 (단층배양법, alginate bead 배양법 및 단층-alginate bead 혼합배양법)으로 분화를 유도하였다. 연골화 표현형 확인은 RT-PCR, GAG 분비 및 Safranin-O 염색을 통해 분석하였다. 분화과정에서의 단백질 변화는 프로테오믹스를 통해 관찰하였고, FAK는 siRNA기법을 통해 억제시킨 후, 세포증식 및 유전자 발현과 GAG 분비를 측정하였다.

TGF-β3을 이용하여 단층배양법을 통해 제 2형 교원질 유전자 발현을 유도하고, 제 1형 교원질 유전자 발현을 억제함으로써, 연골 분화를 유도할 수 있었다. 또한 혼합 분화방법을 통해 제 2형 교원질 발현을 최적화하고, 제 10형 교원질 발현을 최소화 하였다. 세포 생존률은 혼합배양법을 통해 30% (alginate bead 배양법)에서 65%로 증가시켰다. 연골 분화 과정에서 제 10형 교원질 발현 유도는 중간엽 줄기세포의 알칼리 탈인산화 효소 및 Cbfa1발현 정도와 상관관계를 보였다. 이러한 성숙 관련 인자는 프로테옴 분석을 통해 발현이 증가하는 것을 관찰하였다. 분화 과정에서 총46개의 단백질 (20개의 증가 단백질 및 26개의 감소 단백질) 변화를 관찰하였으며, 특히, MMP-13의 발현이 현저히 증가하였다. MMP-13의 발현은 Western blot을 통해 분화 14일까지 꾸준히 증가하는 것을 관찰하였으며, 분비되는 MMP-13의 활성화는 gelatin zymography를 통해 14일까지 증가함을

관찰하였다. 그러나, MMP-13 특이적 억제제를 처리한 경우, 연골화를 억제하지 않았으며, 이러한 결과는 MMP-13이 연골 분화과정에서 조절인자로서 증가된 것이 아니라, 연골 성숙인자로서 발현이 유도된 것으로 사료된다. FAK 발현은 연골분화 과정 및 탈분화 과정에서 각각 증가 및 감소하였고, FAK가 연골세포의 유전자 발현과 상관관계가 있음을 보여주었다. 따라서, FAK를 siRNA법을 통해 억제하였으며, 억제된 세포의 재분화를 유도하였다. FAK가 억제된 연골세포의 제 2형 교원질 발현이 40%로 억제되었으나, aggrecan유전자 발현에는 영향을 미치지 않았다. 또한 전사인자 SOX-6 및 SOX-9를 억제시킴으로써, 제 2형 교원질의 억제가 이들 전사인자에 의해 영향을 받은 것으로 사료된다. FAK의 억제는 연골세포 및 미분화 중간엽 줄기세포의 세포 모양을 섬유아세포와 유사한 형태로 변화시켰으며, 연골세포의 증식을 억제시켰으나, 단백다당의 분비는 억제시키지 않았다. 연골 분화과정에서 FAK의 억제는 제 2형 교원질 유전자를 억제시켰으며, 전사인자 SOX의 발현이 TGF-β3에 의해 증가하였음에도, FAK의 발현이 억제되었을 경우, 제 2형 교원질 유전자를 유도하지 못하였다. 따라서, 중간엽 줄기세포로부터 연골세포의 분화 및 연골세포의 표현형 유지에서 FAK는 제 2형 교원질 유전자 발현을 조절하는 중요한 세포신호전달 역할을 하는 것으로 사료된다.

핵심되는 말 : 중간엽 줄기세포, 연골세포, 세포증식, 단백다당, MAP kinase, focal adhesion kinase