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以中胚層間葉幹細胞及膠原蛋白間質進行軟骨組織工程及軟骨形成機轉之研究

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中文摘要

隨著人類平均壽命的延長，關節病變成為年長者最常罹患的退化性疾病，而且好發的年齡更有向下發展的趨勢，尤其是運動傷害所造成的關節病變。這使得關節炎的研究及其治療成為本世紀最被重視的課題之一。傳統治療骨關節炎的方式有兩種，一為被動式的藥物治療主要用於抑制疼痛及發炎反應，另一種方式是使用外科手術的方式清除壞死組織以週邊健康的軟骨組織移植到傷口處或向下穿刺製造傷口以引導骨髓到傷口處進而促進組織修復。

由於近年間再生醫學的快速進展，以組織工程的方式建構體外培養的新生組織以為移植之用進而促使組織再生，成為另一種更好的關節病變治療方式，本研究的目的即是以第一型膠原蛋白為骨架將軟骨細胞培養在其中，以建構一種可作為移植之用的新生軟骨組織。這一種新型態的三度空間培養模式除了可提供軟骨組織臨床移植之用也提供研究軟骨發生的體外模型。此軟骨組織的細胞型態、分布及其所含軟骨細胞外間質的基因及蛋白表現將為本研究的重點。藉由組織切片及組織染色後來探討軟骨細胞的型態和分布情況，再以免疫組織染色來觀察新生軟骨組織的分化情況。此外本研究亦於探討膠原蛋白與細胞激素的協同作用，其對軟骨細胞表現軟骨特性及中胚層幹細胞進行軟骨分化之影響，以細胞層面探討此軟骨修復材料的分子特性。並以紐西蘭大白兔為模型進行顳顎關節之軟骨修復，已為將來的關節病治療帶來新的思維及方式。

關鍵詞：軟骨細胞，膠原蛋白，軟骨組織，組織染色，繼代培養，組織工程，組織培養。

Abstract

Tissue engineering of cartilage brought the newest medical technologies in the therapy of with osteoarthritis (OA). Osteoarthritis is a joint disease that is characterized by focal degradation of articular cartilage. It is the most common joint disease, which cause of extremities deformities. Approximately one-third of people aged over 25 years have radiographic evidence of osteoarthritis involving at least one joint in the USA.(Arthritis Rheum 1998; 41:778±799) In fact, large cartilage defects (>4mm diameter 2-3mm thick) due to congenital abnormality, trauma as well as OA do not normally heal spontaneously. In the severe cases, surgical removal of the damaged on dysfunctional joint and the installation of an artificial joint prosthesis indicated.

Many studies have focused on OA and established many therapeutic strategies, including anti-inflammatory drug therapy[1-3] and surgical treatment. Because there is only one cell type, the chondrocyte, in cartilage, in vitro cartilage tissue engineering becomes feasible and one of the strategies in OA treatment. Despite cartilage tissue engineering has been reported by using primary chondrocytes[15;16]; however, the difficulty of phenotype maintaining and deficiency of chondrocyte source, problems of *in vitro* tissue engineering of cartilage were still remained to be solved. Collagen matrix anchored by chondrocytes was reported to produce a cartilage-like artificial tissue in several laboratory [19; 20]. When the advanced medical technology successfully develops a biodegradable implant to induce joint regeneration in patients with severe joint disease, the complications of surgery and medical expenses would be reduced to the minimum.

This research was focused on the manufacture and characterization of a neo-cartilaginous tissue, which made *in vitro*. By using chondrocytes as a cell source and type I collagen as a scaffold, a tissue-engineered neo-cartilage was made. Efforts were also made to characterize the neo-cartilage with histological examination with H&E and alcian blue staining. Furthermore, we examined the chondrogenic differentiation effects of type II collagen on the mesenchymal progenitor cell. Temporomandibular Joint (TMJ) repair by using neo-cartilage and the histological evaluation were made in a rabbit model. The data generated might provide important information for further study on cartilage repair and further artificial cartilage tissue implantation.

Keywords: Chondrocyte, Collagen, tissue staining, consecutive passage, Cartilage, Tissue engineering, Tissue culture

I. Introduction

Articular cartilage in adults is a comparatively acellular tissue, with cell volume averaging only approximately 2% of the total cartilage volume in human adults. The remainder is occupied by an extensive extracellular matrix that is synthesized by these cells that are called chondrocytes. In adult, mature articular cartilage several zones are apparent in histological sections of areas free from destructive lesions. The zones form a stratified series of layers: a superficial zone immediately beneath the synovial cavity, in which some of the cells are elongated and lie with their long axes parallel to the articular surface; an intermediate zone; and a deep zone. In certain joints the cells of the deep zone group into columns aligned vertical to the articular surface.

Articular cartilage is among the most incredible materials that nature has engineered. It provides a nearly friction-free load-bearing joint surface so humans can move about smoothly and without pain. Although the composition of most of the matrix molecules are known, the material scientist has not been able to duplicate the properties of native articular cartilage. Unfortunately, articular cartilage is not immune to factors that can lead to its eventual breakdown and diseases. Joint trauma and local mechanical factors can accelerate the degeneration process of the articular cartilage. Articular cartilage destruction is suggested to be an age-correlated process. However, it cannot be regarded as a uniform or generalized pattern of aging change. The exact causes of osteoarthritis are unknown; however, genetic and environmental factors have been implicated including obesity, female, age, previous trauma and surgery to the involved joint, and estrogen deficiency.

Collagen and proteoglycan are the major components of articular cartilage, providing it with its inherent mechanical properties of a composite structure. Collagen is the main fibrous component and, in addition to providing tensile strength, it contributes to the compressive properties of cartilage by resisting the swelling pressure created by the glycosaminoglycan chains of the proteoglycan molecules. In normal adult cartilage, these matrix macromolecules are continuously being broken down and new ones synthesized, albeit at a slow rate, particularly in the case of collagen.

The symptomatic degeneration of articular cartilage and associated arthritis is among the most prevalent chronic conditions all over the world and the population most at risk is increasing. Clinically, several strategies are used to treat the patients with osteoarthritis including non-operative and non-pharmacological therapy, pharmacological therapy, alternative medicine, and surgical therapy. Although physical modalities may be beneficial in selective situations, Nonsteroidal anti-inflammatory drugs (NSAIDs) treatment are among the most widely treatment in the world. Main consideration in the treatment of patients with osteoarthritis is that it is not a fatal disease and to date no treatment has been shown to modify its long-term natural history. Therefore, NSAIDs are the first choice for medication for osteoarthritis as determined by patient

over-the-counter use and by physician prescription. The goals of alleviating pain and improving function without having a joint replacement push patients to try any new treatment. One of these modalities being used more frequently is viscosupplementation. In osteoarthritis, the hyaluronan molecular weight and concentration is diminished. This has led to the concept of viscosupplementation in which hyaluronan based products are injected into the articular space. Food supplement of glucosamine sulfate is also recommended as another therapy of alternative medicine. In fact, studies of glucosamine sulfate for the treatment of osteoarthritis has shown it to be as good as NSAIDs for osteoarthritis of the knee. However, under severe cartilage degeneration and full-thickness loss in specific areas, surgical therapy is one of the last choices to alleviate the symptom.

II. Article Review

A. The effect of growth factor

1) TGF-beta1

The transforming growth factor beta (TGF-beta) family of hormonally active polypeptides have attracted much attention because of their ability to control cellular functions that underwrite animal embryo development and tissue homeostasis. TGF-beta family members act by modifying the expression of specific sets of target genes, and biologists pursuing the elucidation of TGF-beta signaling mechanisms have turned up a fairly simple system, linking membrane TGF-beta receptors to such genes.

TGF-beta is the prototypic member of the TGF-beta superfamily. This factor is synthesized in an inactive pro-form due to its binding to the latency-associated peptide (LAP). In response to proteolytic activity or extremes in pH, among other stimuli, TGF-beta is released from LAP and becomes activated. LAP is synthesized by chondrocytes, and articular cartilage contains large amounts of latent TGF-beta. Significant levels of active TGF-beta are found in the synovial fluids of patients who have osteoarthritis [J Exp Med 1989; 169:291, Clin Exp Rheumatol 1996; 14:155.].

For all of the diversity and physiological importance of the responses that this family can elicit, a simple system is the core of its signaling pathways. The basic signaling engine consists of two receptor serine/threonine protein kinases (receptor types I and II) and a family of receptor substrates (the Smad proteins) that move into the nucleus. The ligand assembles a receptor complex that activates Smads, and the Smads assemble multi-subunit complexes that regulate transcription. Two general steps carry the TGF-beta stimulus signaling to regulate target genes.

TGF-beta appears to inhibit cartilage degradation and to promote cartilage repair. TGF-beta preferentially stimulates proteoglycan synthesis in osteoarthritic chondrocytes rather than in chondrocytes from normal cartilage [Br J Rheumatol 1993; 32:281, J Rheumatol 1997; 24:536.]. TGF-beta 1 was proposed to stimulate the expression of type II collagen in three-dimensional tissue culture of chondrocyte.

2) IGF-I

Although the early stages of osteoarthritis are characterized by enhanced synthesis of matrix molecules, a deficiency in IGF-I signaling has been proposed to be a mechanism involved in degeneration of the articular cartilage. IGF-I was functioned through binding with IGF binding protein (IGFBP) and IGF-I and IGFBP are expressed in chondrocytes. The action of IGF-I is controlled by a number of IGF binding proteins, with both carrier and IGF blocking activity, which are synthesized by articular chondrocytes [Inflam Res 1998; 47:90]. In fetal bovine, IGF-I and IGF-II expression occurs mainly in proliferating chondrocytes, although others have identified IGF-I mRNA in hypertrophic and proliferating chondrocytes or in all zones of the

postnatal rat (J Clin Invest 93:1078, 1994; Bone 15:563, 1994). IGF-I was suggested to have important direct effects on proteoglycan synthesis and cell proliferation (*Pediatr Res* 52: 137, 2002). It has also been suggested [Arthritis Rheum 1994; 37:253] that osteoarthritis chondrocytes produce increased quantities of IGF-binding proteins, thereby blocking the anabolic effects of this growth factor in osteoarthritic cartilage.

IGF-I is an essential growth factor for the maintenance of articular cartilage homeostasis. This factor stimulates matrix synthesis and inhibits matrix breakdown (Biochem J 1989; 260:543). In human serum and synovial fluid, IGF-I is the main stimulator of chondrocyte proteoglycan synthesis (Arthritis Rheum 1989; 32:66). In osteoarthritic cartilage, increased expression of IGF-I might be involved in the increased synthesis of matrix molecules that is observed early during the course of osteoarthritis (Ann Rheum Dis 1992; 51:440, J Rheumatol 1999; 26:870).

3) FGF-2

FGF-2 was previously shown: (1) to stimulate chondrocyte growth in monolayers and glycosaminoglycan (GAG) synthesis [(1985) *J. Cell Biol.* **100,** 477]; (2) to induce disassembly of the actin microfilament architecture [(1995) *J. Bone Miner.Res.* **10,** 735], which in turn may induce the expression of chondrogenic phenotype [(1984) *J. Cell Biol.* **99,** 115–123.]; and (3) to preserve the ability of bone marrow derived mesenchymal progenitor cells expanded in monolayers to subsequently differentiate into bonelike and cartilaginous tissues, *in vivo* and *in vitro* [*Endocrinology* (1997) **138,** 4456; *J. Orthop. Res.* **16,** (1998) 181]. Martin et al reported that FGF-2 maintains the chondrogenic potential during chondrocyte expansion in monolayers, possibly due to changes in the architecture of F-actin elements and allows more efficient utilization of harvested tissue for cartilage tissue engineering (Cell. Biochem. 83:121, 2001).

Although FGF-2 had been reported to use wildly in the culture of primary chondrocytes. Little is known about the theoretical risk of malignant transformation associated with FGF-2 stimulation of chondrocytes. Kamil et al (TE 8(1)85-92, 2002) reported that FGF-2 as well as TGF-beta1 might not introduce malignancy to cultured chondrocytes.

B. Tissue Engineering of Cartilage

Cartilage differs from most other tissues in the body in its response to injury. Due to its avascularity, the inflammatory and reparative phases, of the classic healing response, which are mediated by the vascular system, are not available to damaged cartilage (Am J Sports Med 1998; 26:309–24.).

Several studies have focused on OA and established many therapeutic strategies, including anti-inflammatory drug therapy {Fioravanti, Storri, et al.} {Ausiello & Stafford, et al.} {Makarowski, Zhao et al.} and surgical treatment. Several surgical approaches have been proposed or reported, including cell transplantation {Hayes & Averett, et al.} {Robinson, Ash, et

al.}, mosaicplasty {Hangody, Kish, et al.} {Garrett 1986}, periosteal implantation {O'Driscoll}, biomaterial implantation {An, Woolf, et al. }, growth factors treatment {Hunziker, et al.} {O'Connor, Botti, et al.} {Perka, Schultz, et al.}, hole-drilling through subchondral bone {Garces, Mugica-Garay, et al.} {Shapiro, Koide, et al. }, and tissue engineering {Freed, Vunjak-Novakovic, et al } {Freed, Grande, et al. } {Martin, Obradovic, et al. } {Jackson, Scheer, et al.}.

Autologous chondrocyte implantation (ACI) is a form of tissue engineering that is being used increasingly to treat damaged articular cartilage. Roberts et al (ARTHRITIS & RHEUMATISM Vol. 44, No. 11, November 2001, pp 2586–2598) reported that ACI is capable of not only cartilage repair but, in some cases, regeneration. This might be achieved by the turnover and remodeling of an initial fibro-cartilaginous matrix via enzymatic degradation and synthesis of newly formed type II collagen. In humans, the clinical symptoms appear to resolve satisfactorily up to 9 years post-treatment (Clin Orthop 2000; 374:212–34.), but there has been limited study in humans of the composition and cellular processes of the repair tissue in the region of the ACI graft (J Bone Joint Surg Am 1999; 81-B: 1064–8.). Peterson et al (Clin Orthop 2000; 374:212–34.) reported that hyaline-like repair tissue correlated with most favorable clinical results, whereas in patients with graft failure, only fibrous tissue was seen.

Tissue engineering of articular cartilage has been motivated by the limitations of current articular cartilage repair techniques and could potentially benefit an estimated 1 million patients per year. (Lancet **354,** Suppl. 1, SI32, 1999.) Previous studies reported that cartilaginous constructs have been engineered by seeding bovine calf articular chondrocytes on poly-glycolic acid (PGA) meshes and culturing the cell–polymer constructs *in vitro* in bioreactors for up to 7 months (Bio/Technology **20,** 689, 1994; Biotechnol. Prog. **14,** 193, 1998).

C. Chondrogenic Induction of MPCs (Mesenchymal Progenitor Cells)

The clinical need for improved treatment options for patients with cartilage injuries has encouraged scientists to investigate in vivo implantation of isolated MPCs. MPCs can undergo differentiation to regenerate either cartilage[103] or bone[97,134] in defects. Implantation of MPCs has been demonstrated to affect tissue regeneration. Then different environmental factors can be introduced to trigger differentiation into specific phenotypes.

Chondrogenic differentiation has been induced in vitro by cytokines TGF-β1 and dexamethasone in MPCs derived from bone marrow of human[136] or animal models[109, 117, 135]. ECM has been demonstrated to regulate the chondrogenic or osteogenic differentiation induced by MPCs[108, 119]. How the mechanisms of ECM affect MPC differentiation are still not well understood. This study was undertaken to further identify how collagen regulated *rh*TGF-β1's and dexamethasone's effects on chondrogenic differentiation. Differential expression of MPC-induced glycosaminoglycan (GAG) and alkaline phosphatase (ALP) exposed to different concentrations of dexamethasone, *rh*TGF-β1, and collagens was measured by spectrophotometry.

The levels of mRNA expression of Sox9, aggrecan, and type II collagen were found to be identifiers of certain mechanisms of chondrogenic differentiation by MPCs.

III. Materials and Methods

A. The Isolation and Cultivation of Chondrocytes:

Human cartilage obtained from hips or knees of patients during surgeries were dissected and minced into 1-mm^3 slices. Tissues were transferred into a 50 ml tube in 30 ml Enzyme solution (1mg/ml Type II and 1mg/ml IV collagenases in DMEM-F12 w/ 10% FBS). After enzyme-digestion over night, the dissociated chondrocytes in the suspension were then collected by centrifugation at 1,000 × g for 5 min. The cell pellet was resuspended in Dulbecco modified Eagle/F12 medium (HYCLONE) with 10% FBS, 50 μg/ml gentamicin sulfate, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml fungizone. An aliquot of 8×10^5 cells was plated per 10-cm petri dish and cultured in 5 % CO_2 incubator at 37 degrees Celsius. The cells reached confluent and were harvested with 0.25% trypsin/EDTA (GIBCO) after 10 to 14 days. For storage chondrocytes were frozen with 10% DMSO and 90% FBS at -80℃.

B. Extraction of type I collagen:

Type I collagen was prepared according to our previous study {Lai, Choong, et al. 2000 7 /id}. Rat-tail tendon was sliced and washed with 0.5 M NaCl, and 20mM EDTA in 0.05M Tris buffer (pH 7.4), and then dissolved in 0.5 M acetic acid. The extracted type I collagen was precipitated by adding NaCl to reach a final concentration of 0.9 M. The pellet was washed with 70% alcohol several times to totally remove the acid and salt. After washing out alcohol, the type I collagen pellet was redissolved in 10 mM acetic acid to make the final concentration of 2 mg/ml.

C. Three dimensional culture of chondrocyte - collagen constructs:

Aliquots of 5×10^6 cells per ml of human chondrocytes in 2× concentration of DMEM/F12 media (HYCLONE) and FBS was mixed with equal volumes of collagen solution. The chondrocyte-collagen-media mixtures were placed in 15 ml sterilized culture tubes to enhance cell-matrix contraction. Spherical constructs of 2-3 mm diameter formed and media (5 ml) were changed every 2-3 days. At the end of 7, 14, 21, 28-day intervals, 3 constructs were withdrawn and fixed in 4% neutrally buffered formaldehyde for the subsequent analysis of MSC chondrogenic differentiation by histological examination.

D. Histological and/or immunohistochemical analysis of chondrogenesis:

The fixed constructs and cell pellets were embedded in paraffin and serially sectioned at 5-8 μm thickness (performed by Department of Pathology, TMU). After deparaffined and rehydrated, sections were stained with hematoxylin and eosin to reveal cell morphology and

distribution. Alcian blue (SIGMA) staining was performed to examine proteoglycan changes. The distributions of cells within tissue constructs or cell pellet were assessed by microscopic evaluation of hematoxylin/eosin (H/E)-stained cross sections.

E. MPC Subjects

Consenting bone marrow donors were selected from patients admitted to the Orthopedic Section of Taipei Municipal Chung-Hsin Hospital, Taipei, Taiwan. None had endocrine disease or was receiving hormone replacement therapy. Bone marrow was obtained from femur fracture site by proximal femur aspiration during surgical treatment procedures.

F. Isolation and cultivation of MPCs

MPCs were isolated from rabbit and human bone marrow. They were mixed with sodium-heparin, and diluted with equal five volumes of PBS. The cell suspension was fractionated on a Percoll gradient (40% initial density, Phamacia). The MPC-enriched interface fraction was collected and cultured in Dulbecco's modified Eagle medium with 1 g/ml glucose (DMEM/LG, Sigma D5523), 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone under normal conditions. The medium was changed every four days. During the primary culture, adherent cells formed colonies that were passaged when cells proliferated subconfluently. Second- and third- passage cells were selected to identify the mechanisms of differentiation.

G. mRNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA harvested from subconfluent monolayer cultures (approximately10^6 MPCs) was extracted using TRIzol® Reagent (Invitrogen life technologies, Carlsbad, CA). Extracted RNA was dissolved in sterilized ddH_2O and stored at −80 °C. Reverse transcription was performed with SuperScript™ III (Invitrogen life technologies) and Oligo $d(T)_{12–18}$ primer. Four micrograms of RNA was added into a final solution of 21 μl 10 mM dNTP mix, 10 X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNase Inhibitor and RNase H. Six μg of RT products were used in PCR amplification in a final 50 μl solution containing 2.5 mM dNTP, 25 mM $MgCl_2$, upstream/downstream primers and *Taq* DNA polymerase (Invitrogen life technologies). Following an initial denaturation at 95 °C for 5 min, the DNA was amplified in the Touchgene Gradient (TECHINE, UK). Thirty-five cycles of 1 min at 94 °C were applied for denaturation and 1 min at 72 °C for extension. A final extension was applied at 72 °C for 5 min. PCR products were visualized on 1% agarose gel (Agarose I, AMRESCO, Ohio) and stained with EtBr. PCR products were confirmed by size verification and analyzed using FloGel-I (Fluorescent Gel Image System, TOPBIO). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primer sets were following:

(1)Sox9-5′-GGCAGCTGTGAACTGGCCA-3′ (sense primer) and 5′-GCACACGGGGAACTTGTCC-3′ (antisense primer) which gives a 408 bp product;

(2)COL2A1-5′-CACGCAGAAGTTCACCAAGAA-3′ (sense primer) and 5′-CTGCAGGATCAGCCATGGTAGA-3′ (antisense primer) which gives a 501 bp products;

(3)Aggracan-5′-TGAGGAGGGCTGGAACAAGTACC-3′ (sense primer) and 5′-GGAGGTGGTAATTGCAGGGAACA-3′ (antisense primer) which gives 466 bp product;

(4)GADPH-5′-GCTCTCCAGAACATCATCCCTGCC-3′ (sense primer) and 5′-CGTTGTCATACCAGGAAATGAGCTT-3′ (antisense primer) which gives 346 bp product.

H. Analysis of chondrogenic differentiation in monolayer culture

Rabbit and human MPCs were seeded into six-well plates with 10% FBS and 50 μg/ml ascorbic acid in DMEM/LG. After four days of cultivation, the medium of rabbit MPCs was changed with dexamethasone (10^{-7} M, Sigma D2915, St. Louis), *rh*TGF-β1 (0.1, 1, and 10 ng/ml, R & D Systems 240-B, Minneapolis, MN), and collagen (10, and 100 μg/ml). Six wells were employed for each condition. Differentiation was analyzed by GAG synthesis and ALP activity after 14 days. GAG synthesis and ALP activity were measured using spectrophotometry of the absorption of OD_{600} and OD_{405} respectively after alcian-blue staining[91]. The medium of human MPCs, after four days of cultivation, was changed with dexamethasone (10^{-7} M), *rh*TGF-β1 (1 ng/ml), and collagen (100 μg/ml). Three wells were employed for each condition. The mRNA for aggrecan, type II collagen, and Sox9 was analyzed using RT-PCR as described above.

I. Statistical analysis

Expressions of each GAG and ALP from chondrocyte cultures exposed to different concentrations of dexamethasone, *rh*TGF-β1, and collagens were analyzed using Mann–Whitney test. Dexamethasone-, *rh*TGF-β1-1, and collagen-treated and untreated control cultures at different concentrations were also compared. Data are reported as the mean ± SD. $P < 0.05$ was considered statistically significant.

IV. Results

A. The Cytokine Effects on Human Articular Chondrocyte

1.) Isolation and purification of human chondrocytes

Human cartilages were obtained from hips or knees of patients during surgeries. Unlike rabbit chondrocytes, human chondrocytes are more difficult to extract from cartilage tissue. Therefore, the isolation protocols were modified. Cartilages were transferred into 50 ml tube containing 30 ml Enzyme solution (1 mg/ml Type II and 1 mg/ml IV collagenases in DMEM-F12 and 10% FBS). Cartilages were digested over night, collected, resuspended and then plated on patri dishes at the density of 8×10^3 cells/ml. Every 10 ml of cells were seeded in a 100 mm dish and cultured in 5 % CO_2 incubator at 37 degrees Celsius. The cells were harvested with 0.25% trypsin/EDTA (GIBCO) after getting confluence on 10 to 14 days.

2.) Monolayer expansion of human chondrocytes

Human chondrocytes were cultured and became more and more uniform morphologically under standard condition. The cells were becoming spindle-shaped after serial passages of culture. Cells also grew larger and larger after serial passages. Human chondrocytes at P0 appeared round at the beginning and gradually became polygonal. After serial passages, the cell morphology also became more uniform and appeared spindle-shaped (Figure 1). The flow cytometric analysis also showed that the granularity of cells after serial passage became more complex with increasing cell size (Figure 2).

3.) The effects of IGF-I, FGF-2, and TGF-beta 1 on human chondrocyte proliferation and differentiation

The effects of different growth factors were determined on monolayer-cultured chondrocytes under standard culture condition. P2 cells were re-plated in a 6-well plate in a density of $1.5x10^4$ cells/well. IGF-I (insulin-like growth factor I), FGF-2 (fibroblast growth factor 2) and TGF-beta 1 (transforming growth factor 1) were included in the culture medium, separately, and the cells were followed on day one and day five after adding growth factors. As early as day one, the cells treated with 4 ng/ml of TGF-beta 1 were slightly aggregated as compared to those of the other groups. On day five, the cells treated with 10 ng/ml of FGF-2 appeared fibroblast-like in morphology with obviously extended shape and their proliferation rate appeared the highest. Cells in 4 ng/ml of TGF-beta 1 exhibited a more aggregated phenomenon with the lowest proliferation rate (Figure 3). On the other hand, the morphology and number of the cells treated with 100 ng/ml of IGF-I did not show significant difference from those of the control group (Figure 4A).

The GAG expression and mRNA expression of cells treated with different growth factors were also evaluated on day five (Figure 4B).In brief, TGF-beta 1 greatly reduced chondrocyte proliferation rate while FGF-2 stimulated it. As to accumulate GAG levels, the three growth factors used in the study did not show significantly effect. TGF-beta 1-treated cells showed the highest level of type II, type X, aggrecan mRNA expression (Figure 5), while those of the FGF-2-treated group were the lowest (Figure5). Especially the levels of type X collagen and aggrecan m-RNA expression in the TGF-beta 1 treated cells were significantly greater than those in other groups. As to type I collagen mRNA expression, FGF-2-treated group showed the highest expression level while that of the TGF-beta 1- and IGF-I-treated groups were lower. All these data confirmed the greater positive effects of TGF-beta 1 and IGF-I on chondrocytes phenotype expression than those of FGF-2.

4.) Three dimensional culture of chondrocyte-matrix constructs

Three-dimensional constructs of chondrocytes were fabricated and analyzed. Three-dimensional constructs were cultivated for 7, 14 and 28 days(Figure 6). After harvested and fixed in 4% formaldehyde, constructs were then subjected to H/E staining. The cells with chondrogenic phenotype was slightly observed on H/E stained of Day 14 constructs, and became enhanced by day 28 (Figure 7). Based on H/E stain data, the ECM was more mature on day 28 than days 7 and 14. A pale-stained and hyaline-like section could be observed on the 28 day-cultured cell constructs. In alcian blue stain sections, some lacuna-like structures were well recognized. These features indicated that the chondrogenic maturation occurred in the 3D-cultured cell constructs.

B. Type I and II collagen regulation of chondrogenic differentiation by mesenchymal progenitor cells

1.) Type I and II collagen regulated rhTGF-β1 and dexamethasone effects on GAG expression of rabbit MPCs

Significantly increased GAG expression was found in the culture supplemented with 10 ng/ml rhTGF-β1. GAG expression was 2.6 ± 0.2 times greater than those of untreated cells. However, GAG synthesis did not increase with 0.1 and 1 ng/ml concentrations of rhTGF-β1. Treatment with 10^{-7} M dexamethasone alone increased GAG expression 2.25 ± 0.18 times in rabbit MPCs compared to the untreated group. The increased rate of GAG expression remained the same as in the untreated group after adding 0.1 and 1 ng/ml concentrations of rhTGF-β1. A synergistic effect was found when incorporated with both 10 ng/ml rhTGF-β1 and 10^{-7} M dexamethasone. The maximal GAG expression was 3.75 ± 0.32 times higher than that of control cells (Fig. 9(A)).

Type II collagen alone at the concentration of 10 μg/ml did not significantly change GAG expression, whereas at the concentration of 100 μg/ml, GAG expression increased 2.5 times. Synergistic increases in GAG expression by MPCs to be up to 5.8 times when adding 100 μg/ml type II collagen (Fig. 9(B)). When adding either 10 μg/ml or 100 μg/ml concentration of type II collagen (10 or 100 μg/ml), the osteogenic marker ALP activity of MPCs did not significantly increase. Increases in ALP activity were only 1.2 and 1.4 times, respectively, higher than the control. With treatment of 10 ng/ml TGF-β1 and 10−7 M dexamethasone, ALP expression increased 2.4 times. Further addition of type II collagen ALP caused the same increased expression of 2.4 times (at 10 μg/ml concentration) and 2.7 times (at 100 μg/ml concentration). Thus, type II collagen did not significantly regulate the ALP activity of MPCs with or without TGF-β1 and dexamethasone (Fig. 9(C)).

Incorporated with 100 μg/ml of type II collagen, the population of MPCs was reduced by 40%. However, GAG expression increased 2.5 times. In contrast, with the addition of 100 μg/ml of type I collagen, the population and GAG expression of MPCs appeared to not significantly change (Fig. 9(D)).

2.) Type I and II collagen regulated rhTGF-β1 and dexamethasone effects on aggrecan, Sox9 and collagen mRNA expression

RT-PCR using RNA obtained from human MPCs showed no signal for aggrecan, type II collagen, and Sox9. An mRNA expression of type II collagen and Sox9 was induced when treated with dexamethasone. With dexamethasone and rhTGF-β1 MPCs increased mRNA expression of type II collagen and Sox9. In addition to dexamethasone and rhTGF-β1, type II collagen induced mRNA expression of aggrecan and enhanced levels of Sox9 mRNA. In contrast, incorporation with type I collagen, dexamethasone and rhTGF-β1 MPCs reduced levels of aggrecan, and Sox9 mRNA, showed no type II collagen mRNA (Fig. 11).

3.) Structural effects of collagen on chondrogenic differentiation induced by MPCs

To determine whether the structure of collagen affects chondrogenic differentiation, native and denatured collagens were introduced to MPCs. With pretreatment using rhTGF-β1 and dexamethasone, MPCs increased GAG synthesis 4.3 times after adding native type II collagen. The enhancement of GAG synthesis was downgraded to 2.9-fold increase, when denatured type II collagen was added instead. In contrast, no significant difference was noted between the increase in GAG synthesis in the presence of native (2.5 times) or denatured type I collagen (2.1 times) (Fig. 10(A)). Intact α1(I), α2(I), and α1(II) bands were clearly identified with native type 1 and II collagens. Contrarily, a smear phenomenon was noted with denatured type I and II collagens (Fig. 10(B)). These results indicate that type II collagen-triggered chondrogenic differentiation is type-specific, and correlates with collagen's native triple-helical structure.

4.) Type II and I collagen effects on morphological changes in MPCs treated with dexamethasone and rhTGF-β1 in monolayer cultures

MPC cultures of all six wells showed a phenomenon of contact inhibition with fibroblast-like morphology after 14 days (Fig. 12(A)). A mild pile-up was noted in MPCs of all six wells when supplemented with rhTGF-1 and dexamethasone (Fig. 12(D)). In all cultures incorporating type I collagen, cells showed a shift to elongated spindle-shaped fibroblastic appearance independent of the absence or presence of TGF-β1 and dexamethasone (Fig. 12(B) and (E)).

Incorporated with type II collagen, MPCs of all six wells showed more cuboidal-, and less spindle-shaped morphology (Fig. 12(C)). Semi-transparent fibrous like substance was noted on cells. Cells predominantly appeared rectangular after pretreatment with type II collagen, dexamethansone and rhTGF-β1. Cell-collagen matrix aggregates were found in four of six wells (Fig. 12(F)). Abundant GAG, which found in the cartilage-like cell-collagen matrix aggregate was then identified by alcian-blue staining (Fig. 12(G)).

C. Artificial Articular Disc Implantation in TMJ Repair :

1) Control Group

Figure 13 shows the TMJ section of control group animal (non-surgery performed group). There is a full-thinckness fibrocartilage presented on the condyle surface. Type I collagen is presented in the fibril layer of the fibrocartilage surface and the interface between the fibril surface layer and the hyaline cartilage layer (Figure 14). As to type II collagen, the strongest staining of the protein is located at the hyaline cartilage layer (Figure 14).

Figure 15 shows the chondroblast in the TMJ disc section. There is an obvious type II collagen expression around these cells, but not type I collagen (Figure 16).

Figure 17 shows a one month disc-removal TMJ without artificial disc implantation (surgery group without artificial disc implantation). The section shows several fibrillation and degeneration features of TMJ. A rough cartilage surface is observed and part of the cartilage is detached at the tempolar fossa.

At the degenerative cartilage surface, the chondrocytes proliferate and form cell clusters (Figure 18). Type I and type II collagen protein expression are detected around these hyperplasia cells (Figure 19).

Figure 20 shows a three month disc-removal TMJ without artificial disc implantation (surgery group without artificial disc implantation). A degeneration condition could be observed

in the section. The covering cartilage surface is eroded / destructed and leading to the expose of the subchondral bone. Clots are also observed indicates the bleeding in the synovial cavity.

2) Experimental Group

Figure 21 shows a three month disc-removal TMJ with artificial disc implantation (surgery group with artificial disc implantation). The section shows a rough cartilage surface but no detachment of cartilage is observed.

At the degenerative cartilage surface, type I collagen protein expression are detected. However type II collagen protein expression in the hyaline cartilage layer exhibits a largely decreasing as compared to the control group (Figure 22).

Figure 23 shows a three month disc-removal TMJ with artificial disc implantation (surgery group with artificial disc implantation). The implanted artificial disc covers the full articular cartilage surface. No obvious degeneration condition is observed in the section. Type I and type II collagen protein expression can be detected in the sections (Figure 24).

V. Discussion

A. The Cytokine Effects on Human Articular Chondrocyte

Human cartilages were obtained from hips or knees of patients during surgeries. Human chondrocytes were more difficult to extract from cartilage tissue by using rabbit chondrocytes isolation methods. The modified isolation protocols were much simpler. The P0 human chondrocytes morphology appeared as heterogeneous cell population. After serial passages (Figure 1), cultured cells became more and more homogeneous and fibroblast-like. Previous studies proposed that the chondrocyte would lose its phenotype during monolayer culture. The data here were similar to previous reports. The flow cytometry analysis supported that granularity of cells after serial passages became more complex with increased and uniform cell size (Figure 2).

After treated with TGF-beta 1 in monolayer culture, chondrocytes exhibited obviously different phenotype from untreated group. According to previous report,[90] chondrocytes may express cell membrane molecules such as N-Cadherin. The expression of such ECM binding/cell-cell contact molecules might result in changing cell phenotype in culture, such as clustering of cells, or polygonal shape of the TGF-beta 1 treated cells.

Former studies reported that IGF-I and TGF-beta 1 could promote ECM expression of chondrocytes.[39,42,43] The data of Figure 3B supported the results of previous reports. In Figure 3B, cells treated with IGF-I and TGF-beta1 expressed more ECM molecules than control group. The higher ECM molecule expression might explain why cells treated with IGF-I and TGF-beta 1 became poorly proliferated. When chondrocytes became more and more “differentiated”, they

produced more ECM molecules; the cells would rather not proliferate themselves than produce more extracellular proteins. The proliferation rate of cells treated with FGF-2 was the fastest one. It is comparable with the results of the other studies that FGF-2 promoted chondrocyte proliferation.[56,57] Although previous reports indicated that FGF-2 might not affect the phenotype of chondrocytes. However, the cell morphology of FGF-2 treated group on day 5 did not support previous observation (Figure3). It is questionable that how chondrocytes with dramatic changes in morphology could ever keep identical phenotype as untreated cells. The data of semi-quantitative RT-PCR also support the speculations of the function of FGF-2, IGF-I and TGF-beta 1.

In all sections of neocartilage (Figure 7), no obvious cell apoptosis was observed. This indicated that RTT provided good surviving environment for those embedded human chondrocytes. On H&E stained section of day 7, chondrocytes exhibited a non-uniform morphology, with a high nuclear/cytoplasmic ratio. A fibrous ECM could be observed on the section of day 7. The fibrous ECM might be the remaining RTT type I collagen fibers. In fact, the fibrous ECM disappeared on the section of day 28. On day 14, the H&E staining revealed a myxoid ECM. It indicated that chondroid ECM was produced and at the same time, RTT type I collagen was also partially digested by the embedded chondrocytes. This might be inferred from less fibril tissue on the section. The RTT type I collagen could be replaced by endogenous cartilage ECM molecules, such as type II collagen or aggrecan. This phenomenon was considered as the maturation of the chondroid cells presented on the section of day 14 constructs.

A pale stained and hyaline-like section was seen on day 28. The translucent ECM indicated the totally replacement of RTT type I collagen by the newly formed type II collagen. Some lacunae-like structures were also observed. The chondrocytes cultivated in three-dimension for 28 days exhibited an obvious smaller nuclear/cytoplasmic ratio and spindle-like phenotype. This indicated the maturation of both ECM and chondrocytes themselves. More cartilaginous features could be observed in the sections of this time points.

During culture period, the proliferation rate of primary human chondrocytes was relatively slower than rabbit cells. This could be a limitation in clinical use because there might not have enough cell number to engineer a neocartilage. According to the present data, FGF-2 significant promoted the proliferation rate of cultured chondrocytes and could be used as a proliferation promoter in coming clinical use. However, FGF-2 also significantly reduced the gene expression of type II collagen and significantly promoted the gene expression of type I collagen. In another words, the possibility of losing the re-differentiation capacity of human chondrocytes during monolayer expansion did exist. Former studies[27] proposed that FGF-2 could be used to proliferating chondrocytes without losing their capacity of re-differentiation. But, the cells used in the reference were bovine chondrocytes. It still needed further experiments, whether the phenomena remained in cultured human cells or not. Conservatively speaking, TGF-beta 1 and IGF-I might not good candidates for clinical usages.

Compared with the former study of the rabbit neocartilage in this laboratory (Figure 8), the re-differentiation status of human chondrocytes in the neocartilage was poor because of the unobvious chondrogenic phenotype. On the sections of rabbit neocartilage, significant lacunae structure could be observed as early as day 14 during culture periods. But, translucent chondroid-like ECM could be observed only on the sections of day 28 of human chondroctes cultures (Figure 8D). The most likely explanation was that the human cells were obtained from elder patients during knee or hip surgeries from the ages of 40 to 65. The human cells moderately lost their capacity of re-differentiation after monolayer expansion. The cells for rabbit neocartilage were obtained from newborn animals. The rabbit cells were too young to lose their capacity of re-differentiation. Other explanation might lie in the original native of human cells and individual differences. However, more human samples must be done to clarify the speculations in further studies.

By the observation of the histological stain, "proper" clinical implantation timing might be the culture period between day 14 to 28. The embedded human cells gradually recovered their chondrogenic phenotype (start to express chondroid matrix) according to the observation of myxoid ECM in the H&E stain. Further experiments of immunohistological and biomechanical tests should be done to estimate a "proper" timing for the implantation of artificial tissue.

Previous studies reported that cartilage did not heal itself under mild and severe injuries. Tissue engineering of cartilage gives a chance to those who are suffering from cartilage diseases, especially patients with osteoarthritis. Whether or not the engineered neocartilage could ever repair defect or restore the original functions of native tissue when implanted still remained unclear. However, in this thesis, the engineered neocartilage seemed to work *in vitro*. The optimal time point for the implantation required further study. It would take a great deal of further efforts to evaluate weather the implanted engineered cartilage would restore joint function.

B. Type I and II collagen regulation of chondrogenic differentiation by mesenchymal progenitor cells

Bone marrow-derived MPCs are highly proliferative, multipotential cells that have been considered ideal cells for use in repair of injured cartilage and fractures of bone. It is known that repair tissue arises from differentiation of local MPCs. Both periosteum and bone marrow contain these cells, which preserve the ability to differentiate into both chondrocytes and osteoblasts[101, 114, 118]. Chondrogenic differentiation can be triggered, if the environmental factors such as ECM and local cytokines are facilitative. Local MPCs enable accumulation, proliferation, and terminal differentiation into hypertrophic chondrocytes. If environmental factors are not facilitative; however, local MPCs differentiate into fibrochondrocytes and form fibrocartilage[99, 128].

In addition to the potential for multidifferentiation, MPCs are relatively easy to extract from bone marrow and expand in culture. Development of an in vitro chondrogenic differentiation model of marrow-derived MPCs presents an opportunity to explore the extracellular matrix's guiding effects on chondrogenesis that is of potential therapeutic utility.

Previous study showed that TGF-β1 induces proliferation of osteoblasts, chondrocytes, and mesenchymal stem cells[130]. The present study also showed an increase in the proliferation of mesenchymal progenitors. Additionally, other study have shown that synthesis of ECM was found to be enhanced by TGF-β1[129]. Similarily, this study also showed a consistency in this regard, in that TGF-β1 increased synthesis of GAG in MPCs. TGF-β1 controls the morphology and differentiation of epithelial cells[137, 93, 120]. The morphological changes in MPCs induced by TGF-β1 in this research were elongation and extension of cell shapes.

Chondrogenesis of chondroprogenitors can be stimulated in the presence of the dexamethasone[102]. Chondrogenesis was induced by dexamethasone. That was revealed by increased GAG expression. There was a synergistic effect with TGF-β1. This study imply that dexamethasone may be the fundamental factor that triggers chondrogenic differentiation, whereas TGF-β1 further enhances the process of the synthesis of extracellular matrix. Levels of mRNA for type II collagen and Sox9 were also increased when treated with dexamethasone in this study. This was correlated with previous study that an enhancement of Sox mRNA by dexamethasone was showed in chondrocytes[126].

The regulatory effect of collagen seems to be correlated with its triple-helical structure. Our data showed that enhancement of GAG synthesis by native type II collagen (4.3 times) was significantly downgraded when using denatured type II collagen (2.9 times). These findings agree with previous studies that denatured type II collagen dimilished native type II collagen effect on chondrocyte regulation[123, 131, 132.]

Exogenous type II collagen could maintain the phenotype of chondrocytes. This increased the syntheses of type II collagen and GAG [127, 122, 123]. Cell-matrix interactions via cell surface receptors transduce extracellular signals inwards to regulate the cell phenotype[124]. In this research by adding type II collagen the semi-transparent fibrous-like substance became noted on MPCs. This indicated the type II collagen receptor might be induced on cell surfaces. Integrin α2β1, a major receptor for type II collagen, plays important roles during chondrogenic differentiation by MPCs[107, 113]. Binding affinity and signaling of type II collagen are mediated by integrin receptor[125]; however, the signaling of specific domains of α or β integrins for type II collagen is not well understood. Further study will identify the specific receptor for type II collagen, their downstream signals, and how it regulates chondrogenic differentiation of MPCs.

Type I collagen, in contrast to cartilage-specific type II collagen, exists ubiquitously in bone, tendon, cornea, and skin and acts as a structural protein in mammals[112]. During cartilage repair of large osteochondral defects, bone marrow-derived MPCs are induced to differentiate into fibroblasts or osteoblasts within fibrin clots containing fibronectin and type I collagen[92, 128].

Consistent with previous research, type I collagen treated MPCs did not increase GAG expression, but induced an elongated spindle-shaped fibroblastic appearance. Therefore, type I collagen matrix may provide the suitable microenvironment, which is better for fibrogenic differentiation. This matrix-guided mesenchymal progenitor cell differentiation in situ predominantly induces regeneration towards fibro-cartilage formation.

The current study substantiates the concept that chondrogenic differentiation by MPCs can be synergistically triggered by cytokines and further regulated by the ECM. A comprehensive understanding of the regulation of chondrogenic differentiation by MPCs may enable the intentionally engineering of cartilage development in vitro. Subsequent results may yield further information for the purpose of a direct repair of large cartilage defects.

C. Artificial Articular Disc Implantation in TMJ Repair :

The H.E. staining data indicates the repair / regeneration effects of the artificial disc implant. Disc-removal TMJ without implantation undergoes degenerative progression of the joint. However, disc-removal TMJ with artificial disc implantation undergoes a slighter degenerative progression of the joint. Different cartilage regeneration features can be seen especially at the three months sections between implantation group and non-implantation group. In the implantation group, regenerated tissue protected by the artificial disc encounters less physical stress thus has a better repairing outcome.

After one month of disc-removal, fibrillation occurs at the articular cartilage erosion surface. Large amount of type I collagen production can be detected at the eroding region, with type II collagen expression around the proliferated chondrocytes.

In the implantation group, articular cartilage undergoes slight breakdown of the matrix and chondrocytic hyperplasia. In the three months section, degenerative progress of the articular cartilage slows down because of the protection effects of the artificial implantation disc. Repairing of the cartilage thus can be proceeded, and the lower degree of matrix fragmentation reduces the inflammatory reaction of the wounded site.

The artificial disc template from our laboratory consists of type I collagen, one of the dominant ECM contain existed in the articular disc. Type I collagen has great affinity with fibro-cartilage cells, through binding of the integrin receptor, to accelerate the regeneration of the articular cartilage.

The proliferating cells attach the implant as a scaffold and replace the scaffold with their new-synthesized ECM. With the replacement of the matrix, the ECM composition of the implant is more like an original disc tissue. Furthermore, this artificial implant also can be applied in other articular defects like osteoarthritis or large defect of long bones. Our future works should focus on this issue.

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VII. Figures and Tables:

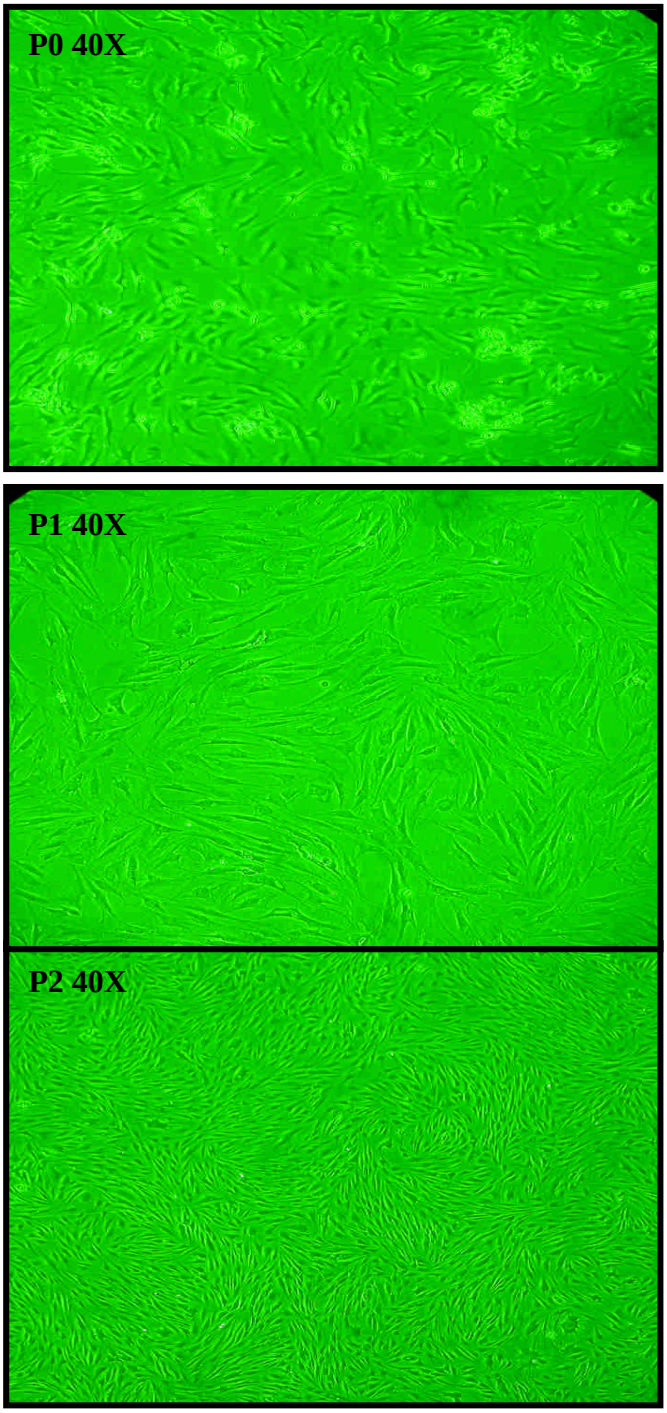


Figure 1: A) Morphology of primary chondrocytes after serial passages in monolayer culture. Human chondrocytes at P0 appeared round at the beginning and gradually became polygonal. After serial passages, the cell also became more uniform and appeared spindle-shaped.

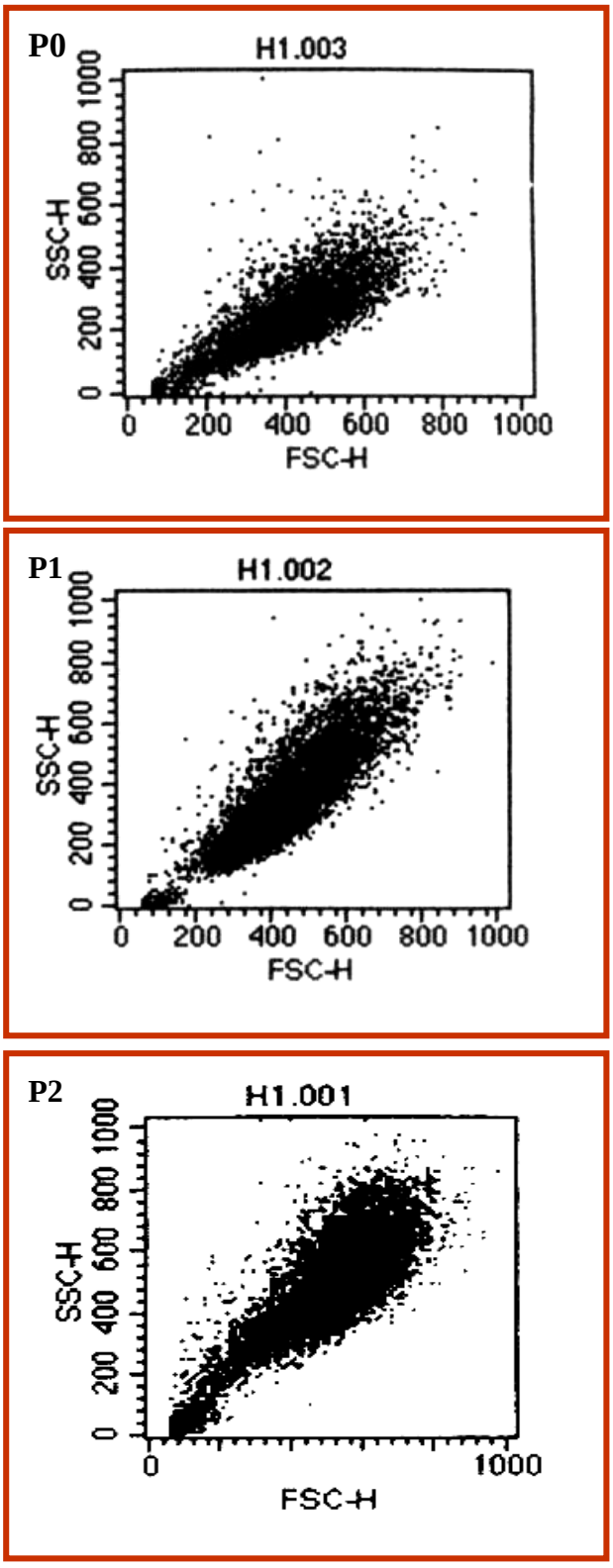


Figure 2: Flow cytometric analysis of primary chondrocytes after serial passages.The flow cytometric analysis showed that the granularity of cells after serial passage became more complex with increasing cell size.

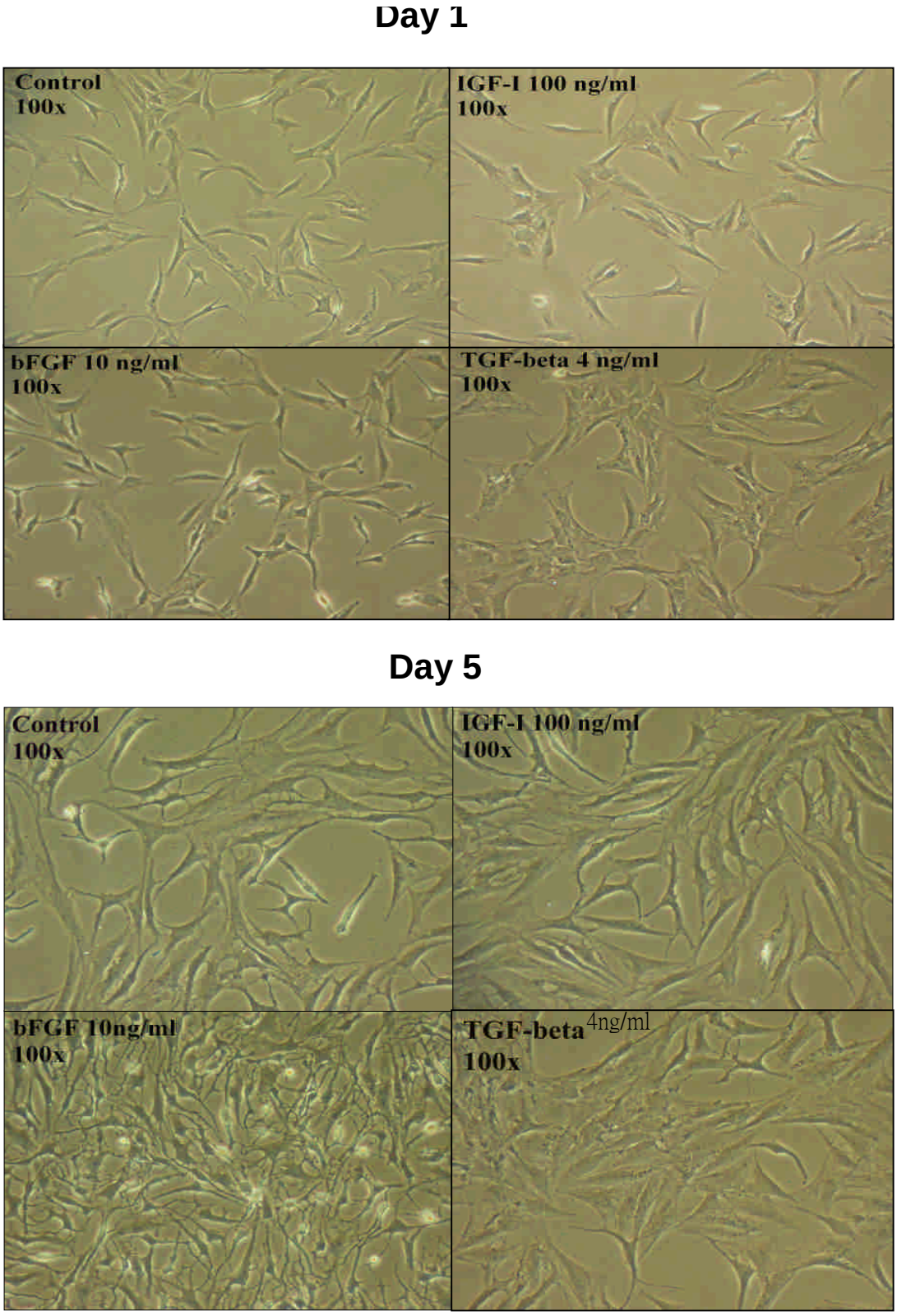
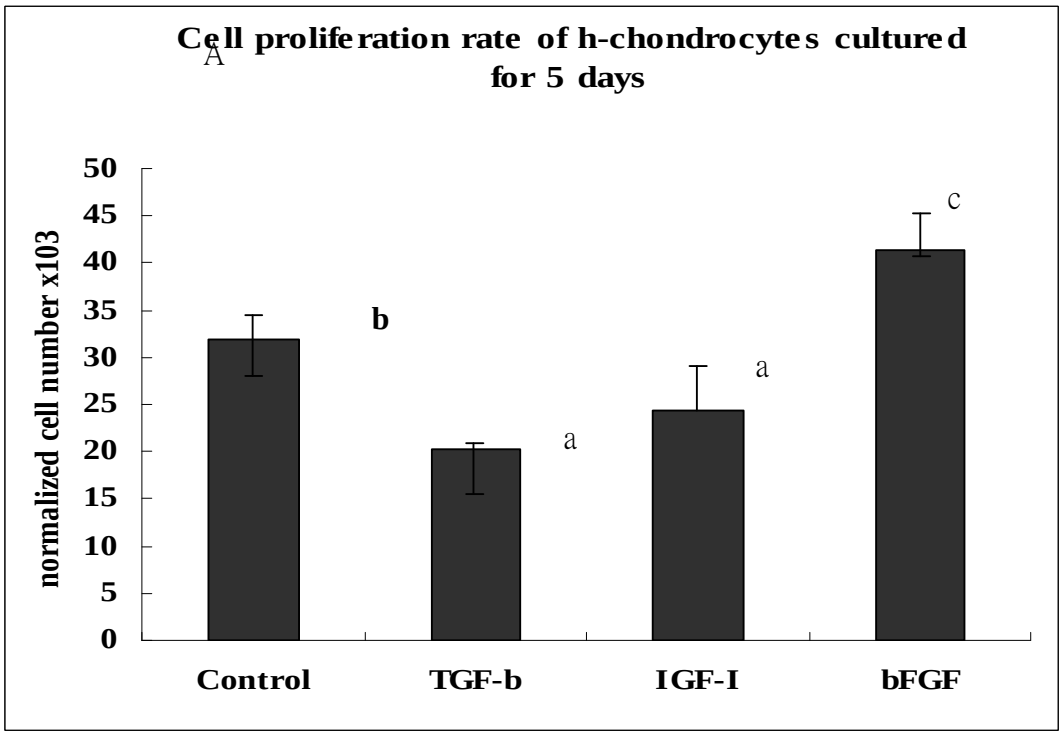


Figure 3: Morphology of chondrocytes treated with IGF-I, FGF-2, and TGF-beta 1. As early as day one, the cells treated with TGF-beta 1 were slightly aggregated as compared to those of the other groups. On day five, the cells treated with FGF-2 appeared fibroblast-like morphology with obviously extended shape.



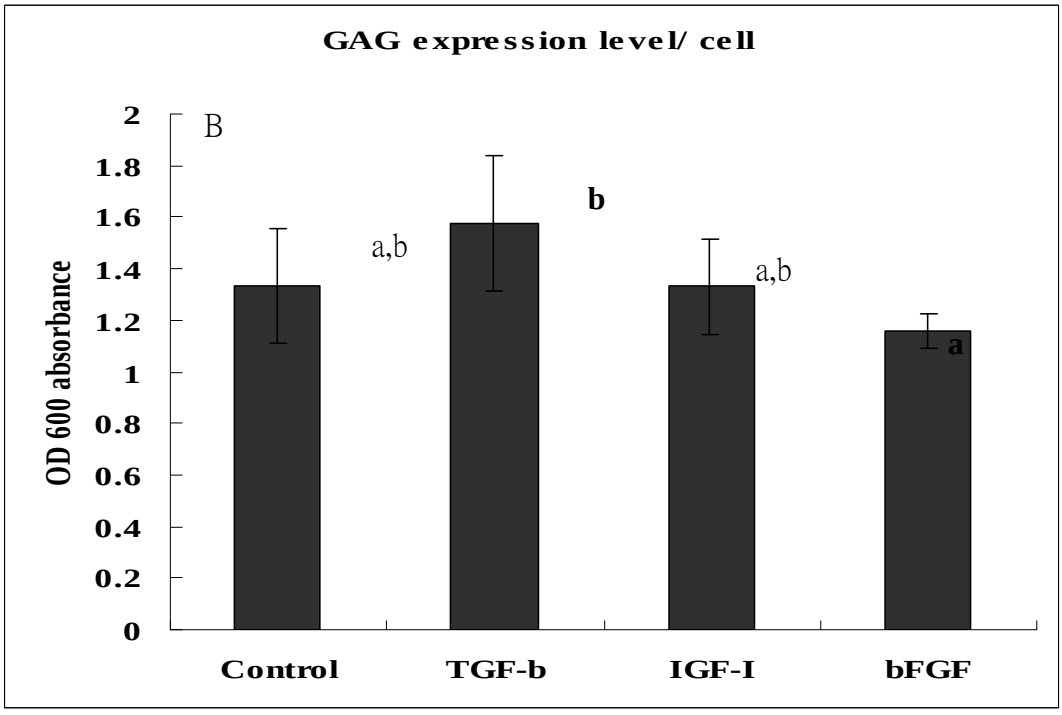


Figure 4: A) Cell proliferation rate and B) GAG expression level of chondrocytes treated with IGF-I, FGF-2, and TGF-beta 1.

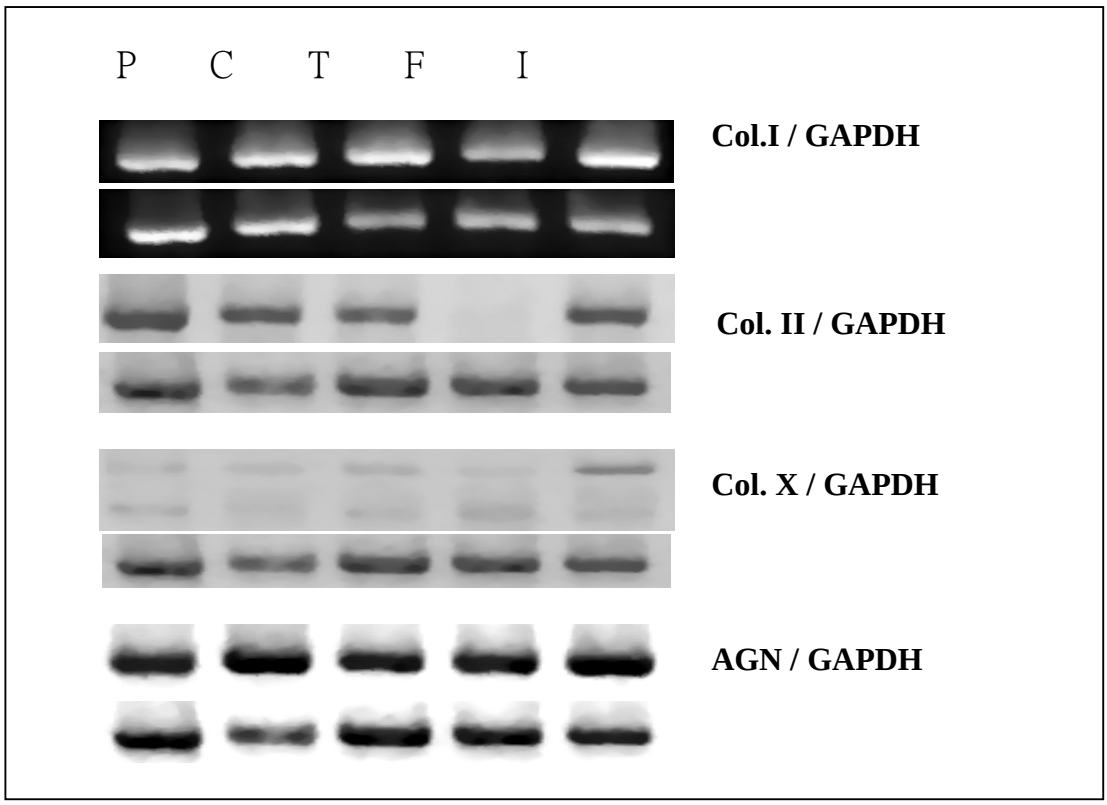


Figure 5 A): RT-PCR analysis of chondrocyte m-RNAs after treated with IGF-I, FGF-2, and TGF-beta 1; P: Positive control (P1 condrocytes); C: Control (P3 chondrocytes), without growth factor treated; T: TGF beta 1-treated; I: IGF-I-treated; F: bFGF-treated.

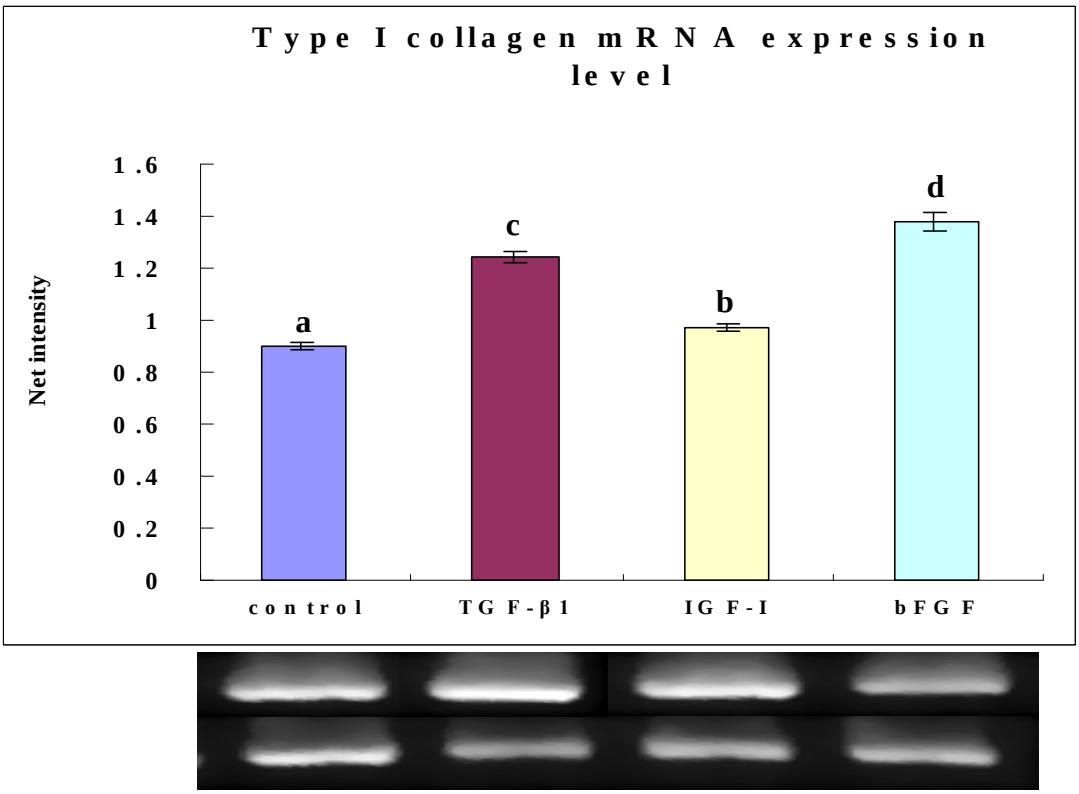


Figure 5B): type I collagen m-RNA expression after treated with IGF-I, FGF-2, and TGF-beta 1.

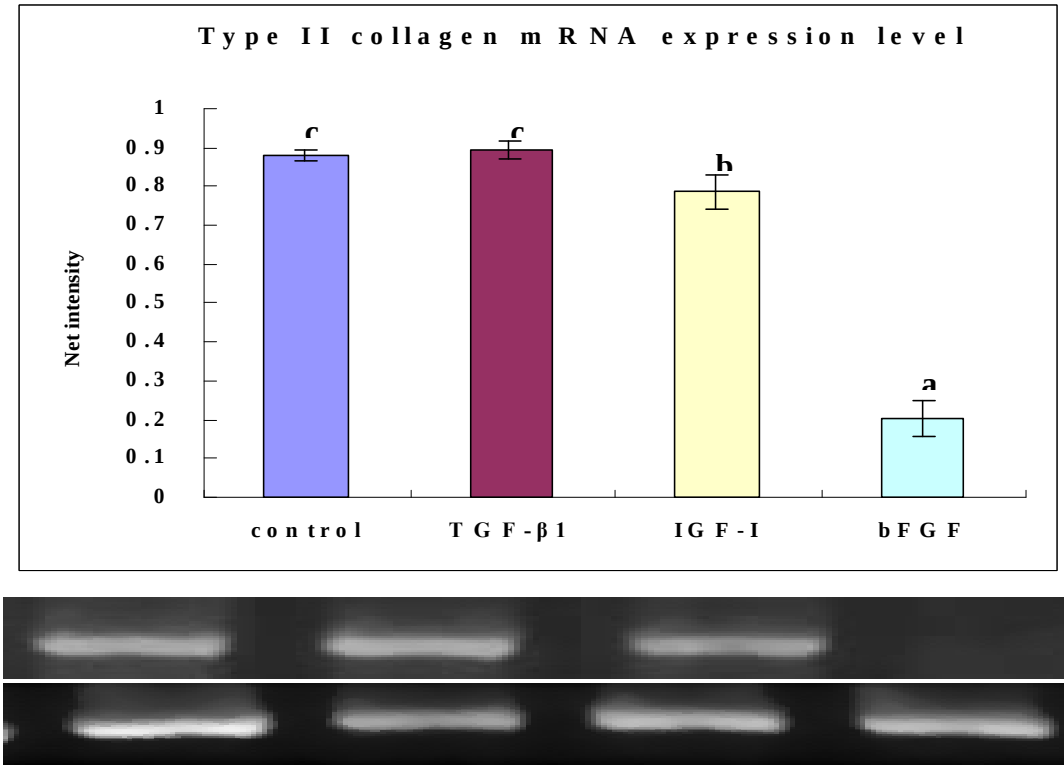


Figure 5C): type II collagen m-RNA expression after treated with IGF-I, FGF-2, and TGF-beta 1.

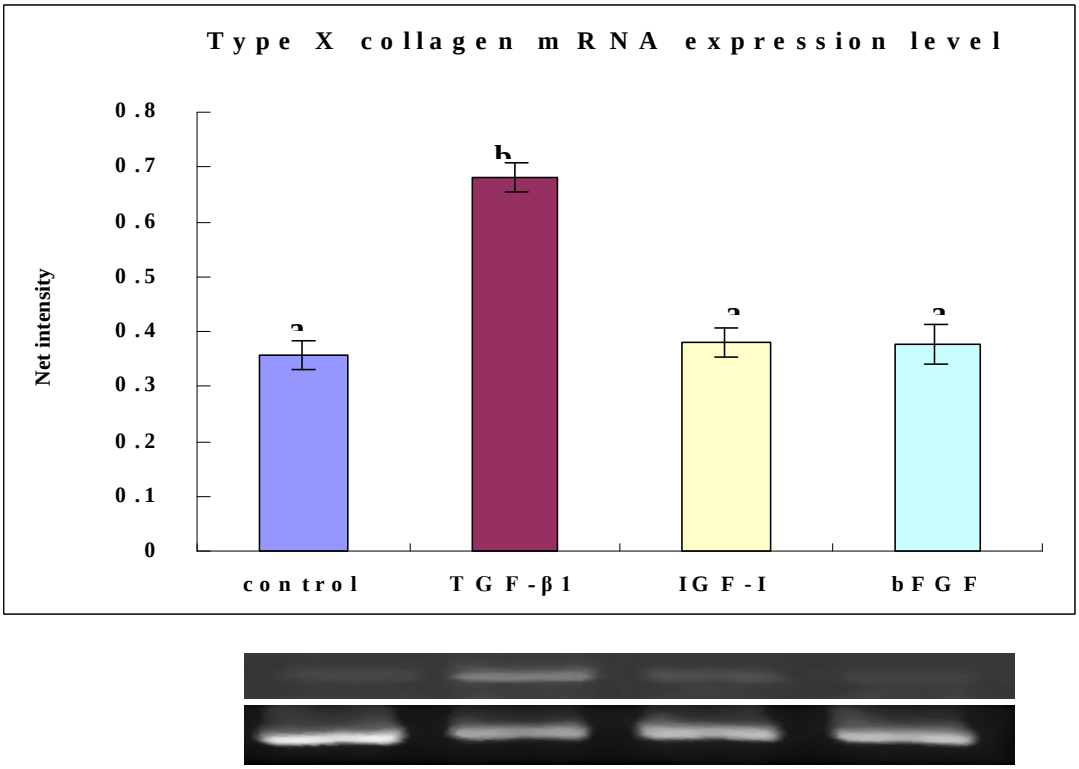


Figure 5D): typeX collagen m-RNA expression after treated with IGF-I, FGF-2, and TGF-beta 1.

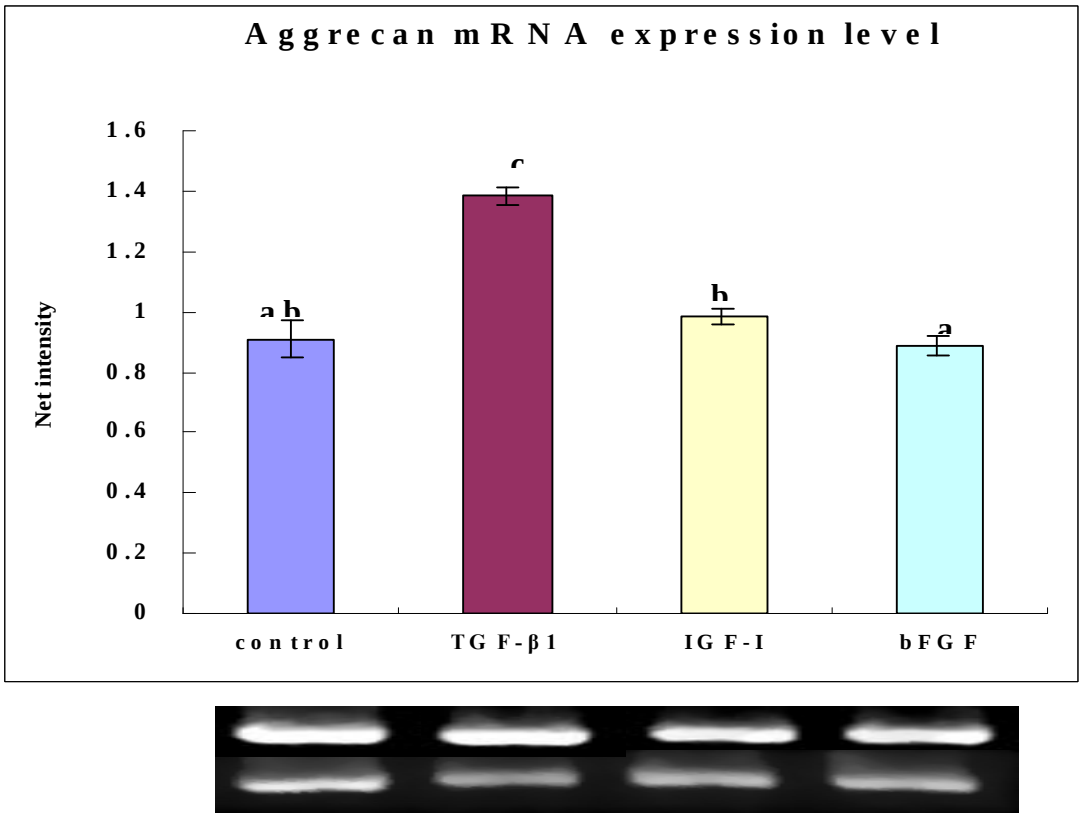


Figure 5E): Aggregan m-RNA expression after treated with IGF-I, FGF-2, and TGF-beta 1.

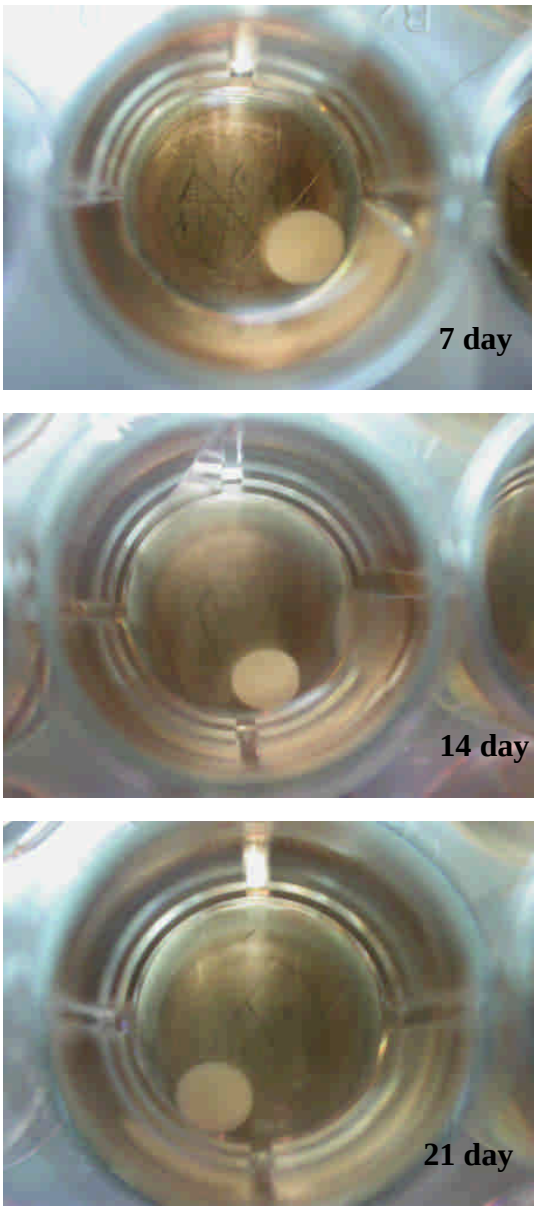


Figure 6: Fabrication of neocartilage constructs were cultured for 7, 14, 28-days as indicated in the graph.

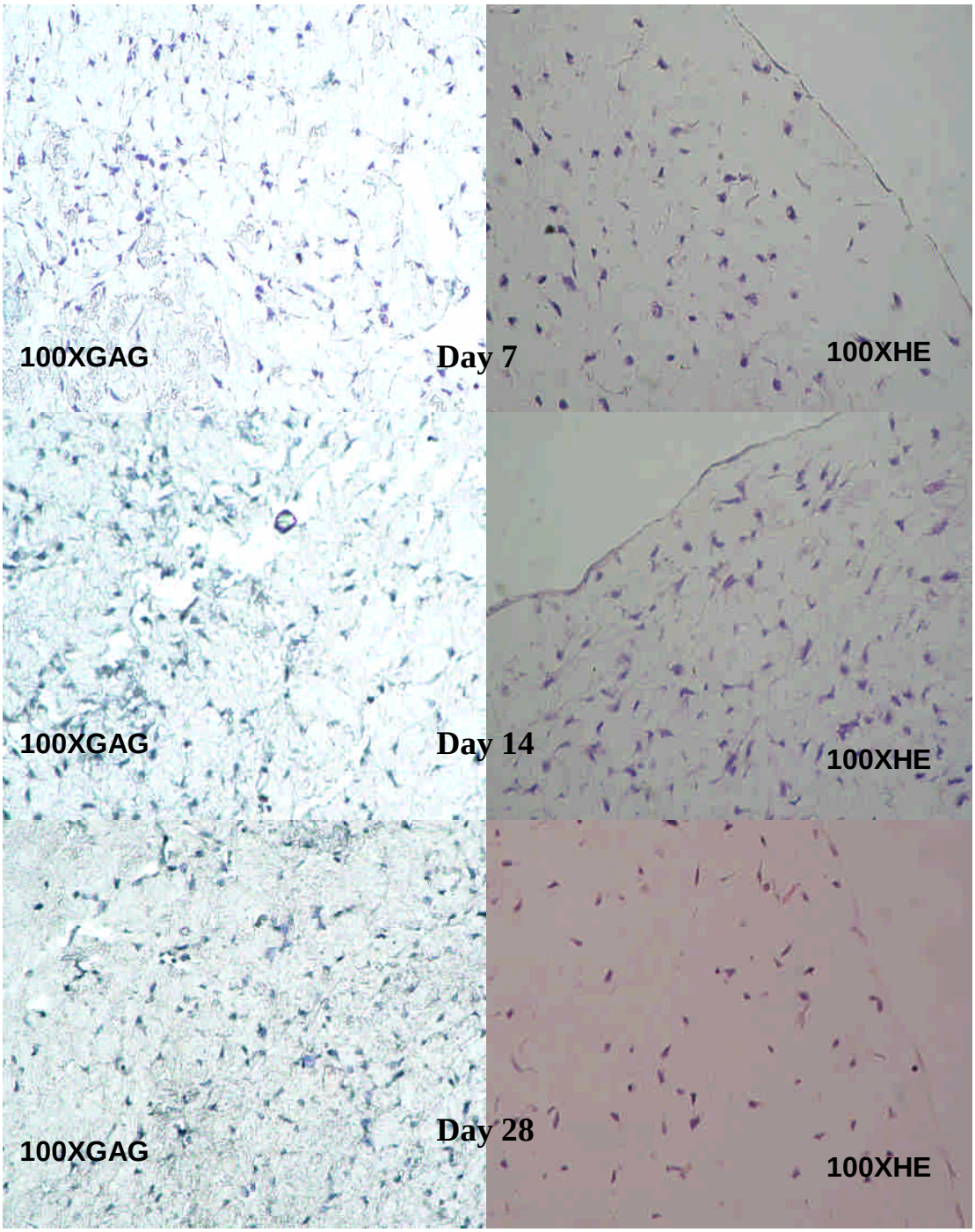


Figure 7: Fabrication and analysis of neocartilage

Neocartilage constructs were cultured for 7, 14, 28-days ,HE stain (right panel) and GAG stain (left panel) of the cross sections of neocartilage constructs are presented.

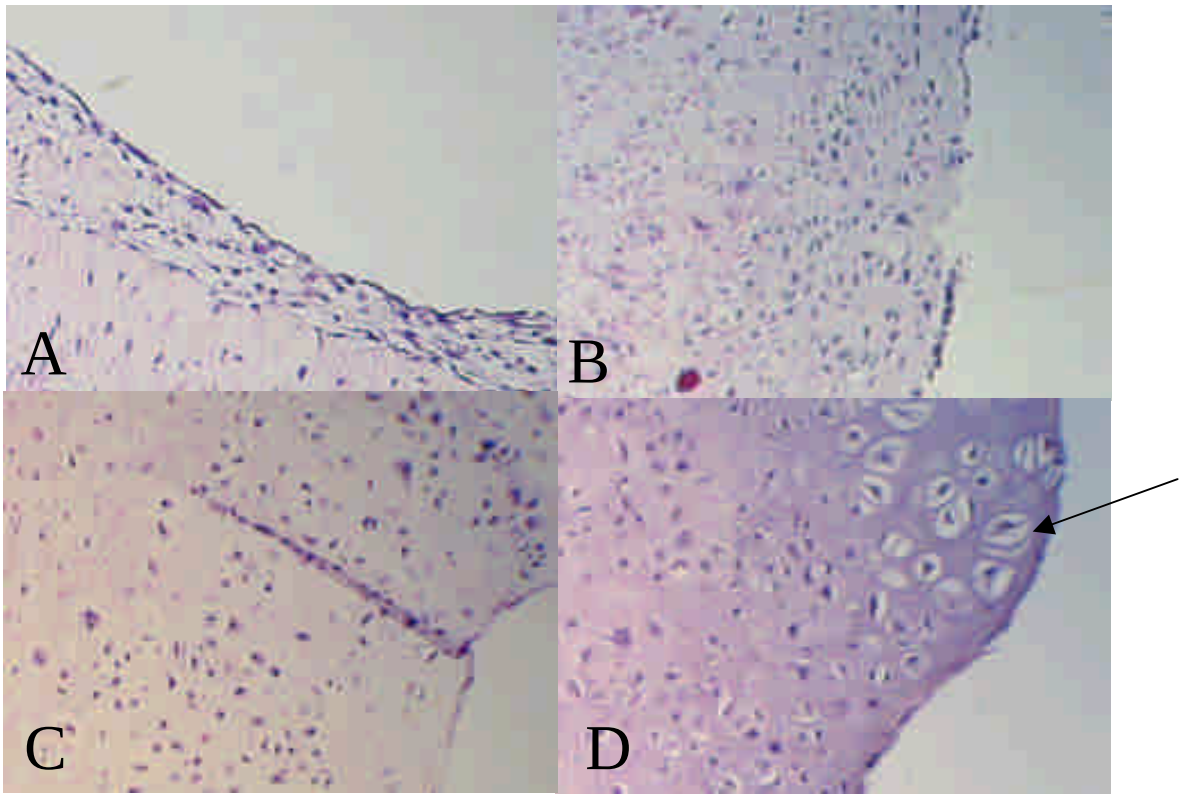


Figure 8: H&E staining of 3-D tissue culture of newborn rabbit chondrocyte-collagen constructs. Three dimensional tissue culture of chondrocyte-matrix neocartilage were harvested on day 7 (A), 14 (B), 21 (C) and 28 (D) and stained with Hematoxyline and eosin. Frozen chondrocytes of passage one were used in these constructs. Arrow in D indicates hypertrophic chondrocyte. (100X)

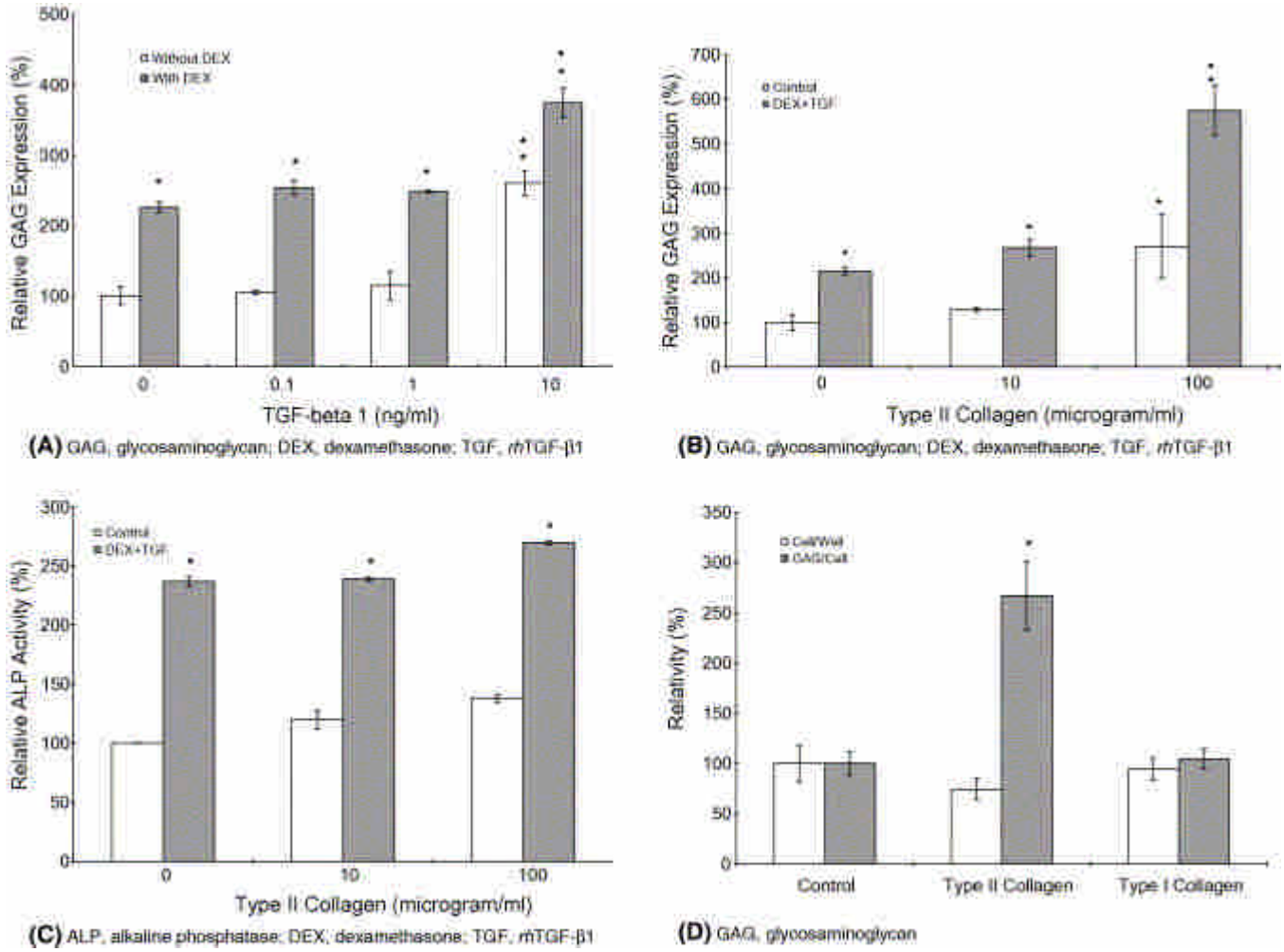


Fig. 9: GAG and ALP expression of MPCs exposed to varying concentrations of *rh*TGF-β1, DEX, and collagen, measured by spectrophotometry. Means ± S.D; (*n* = 6). (A) TGF-β1 and dexamethasone induced an increase in GAG by MPCs. An asterisk indicates a significant ($p < 0.05$) difference in DEX compared to the untreated control. A diamond indicates a significant ($p < 0.05$) difference among different TGF-β1 concentrations. (B) Type II collagen up regulated GAG synthesis with and without dexamethasone. An asterisk indicates a significant ($p < 0.05$) difference in DEX compared to the untreated control. A diamond indicates a significant ($p < 0.05$) difference among different type II collagen concentrations. (C) Type II collagen, in contrast to dexamethasone, did not significantly increase ALP activity by mesenchymal progenitor cells. An asterisk indicates a significant ($p < 0.05$) difference in TGF-β1 and DEX compared to the untreated control. (D) Only type II collagen significantly increased GAG synthesis, but not cell proliferation, whereas type I collagen did not change GAG synthesis or cell proliferation. An asterisk indicates a significant ($p < 0.05$) difference in type II collagen compared to type I collagen and the untreated control.

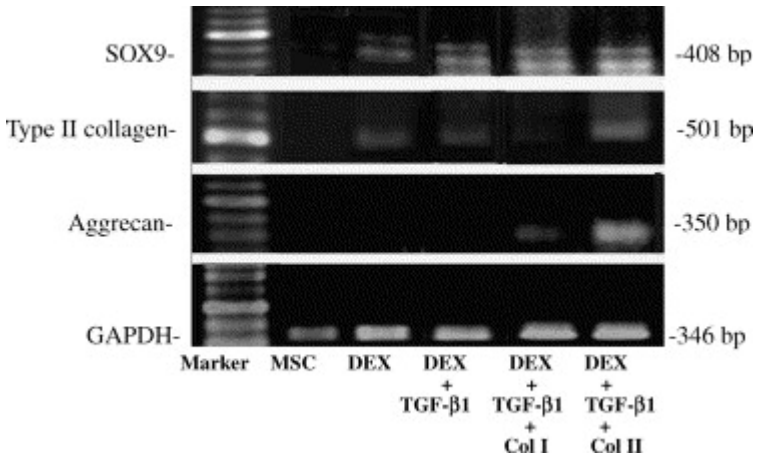
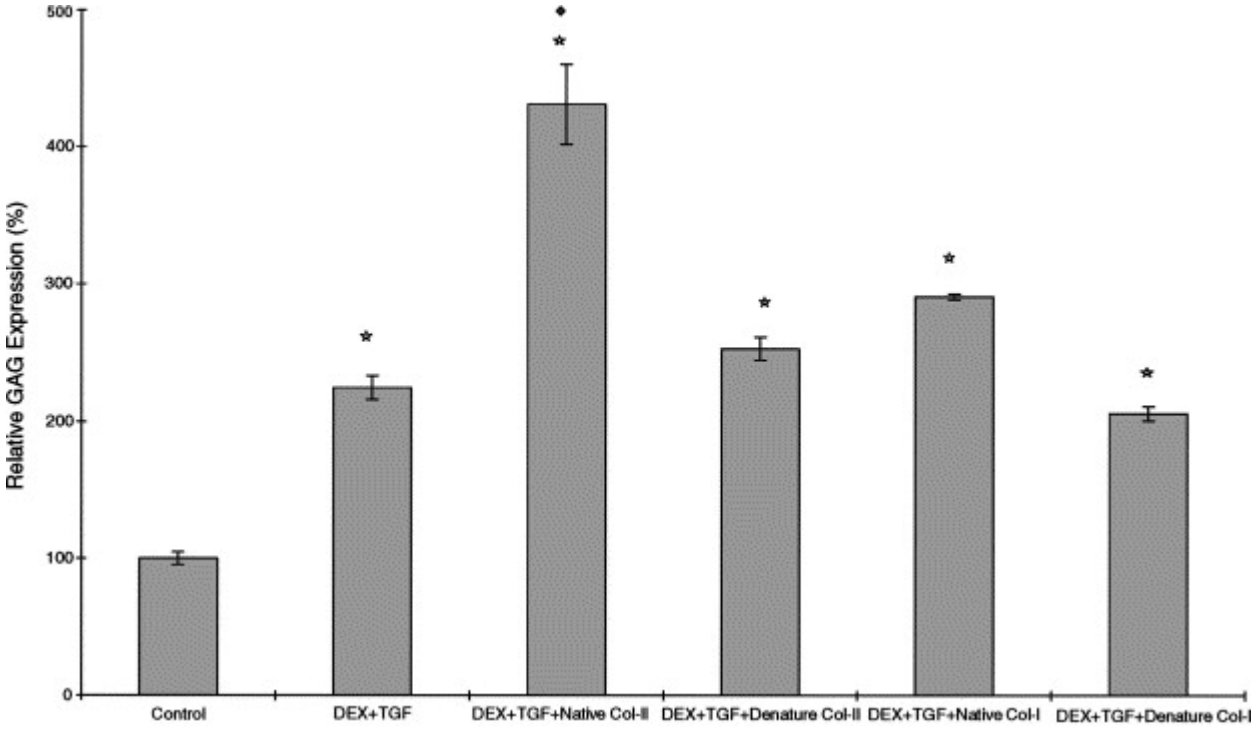
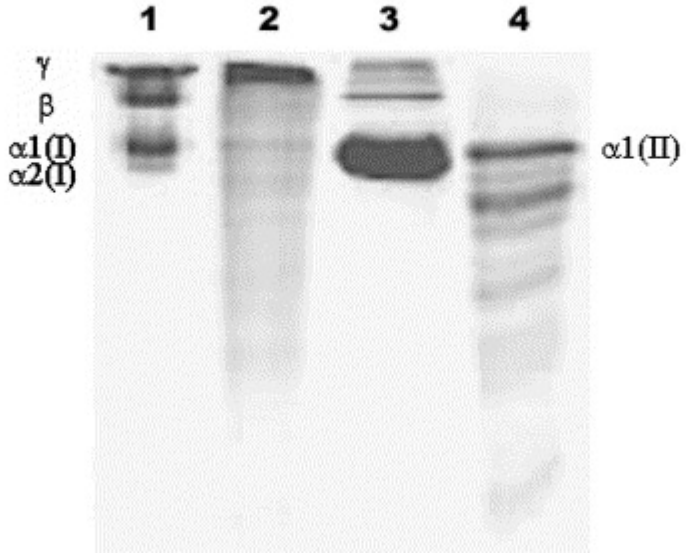


Fig. 11: Type I and II collagens regulated *rh*TGF-β1 and dexamethasone effects on aggrecan, Sox9 and collagen mRNA expression. Total RNA was isolated from MPCs and used for PT-PCR to detect expression of type II collagen and aggregan. Expression of GADPH was used as control. Lane 1: DNA standards; lane 2: MPCs showed no signal for aggrecan, type II collagen, and Sox9; lane 3: With dexamethasone MPCs induced mRNA expression of type II collagen and Sox9; lane 4: With dexamethasone and *rh*TGF-β1 MPCs increased mRNA expression of type II collagen and Sox9; lane 5: Cultured with type I collagen, dexamethasone and *rh*TGF-β1 MPCs showed no type II collagen mRNA; lane 6: Cultured with type II collagen, dexamethasone and *rh*TGF-β1 MPCs induced mRNA expression of aggrecan and enhanced levels of Sox9 and type II mRNA.



(A) GAG, glycosaminoglycan; DEX, dexamethasone; TGF, *rh*TGF-β1



(B)

Fig. 10: Structural effects of collagen on chondrogenic differentiation by MPCs. (A) Native type II collagen remarkably increased 4.3 times GAG synthesis, the increase of GAG was reduced to 2.9 times with the denatured type II collagen. Noted no significant difference of GAG synthesis among groups of native type I collagen, denatured type I and II collagens, and dexamethasone and *rh*TGF-β1 treated. (B) SDS-polyacrylamide gel electrophoresis of native and denatured collagens. Note the clearly identifiable bands of type I native collagen in lane 1 and of type II collagen in lane 3. The denatured type I collagen showed a smear phenomenon in lane 2, and small fragments of denatured type II collagen was showed in lane 4.

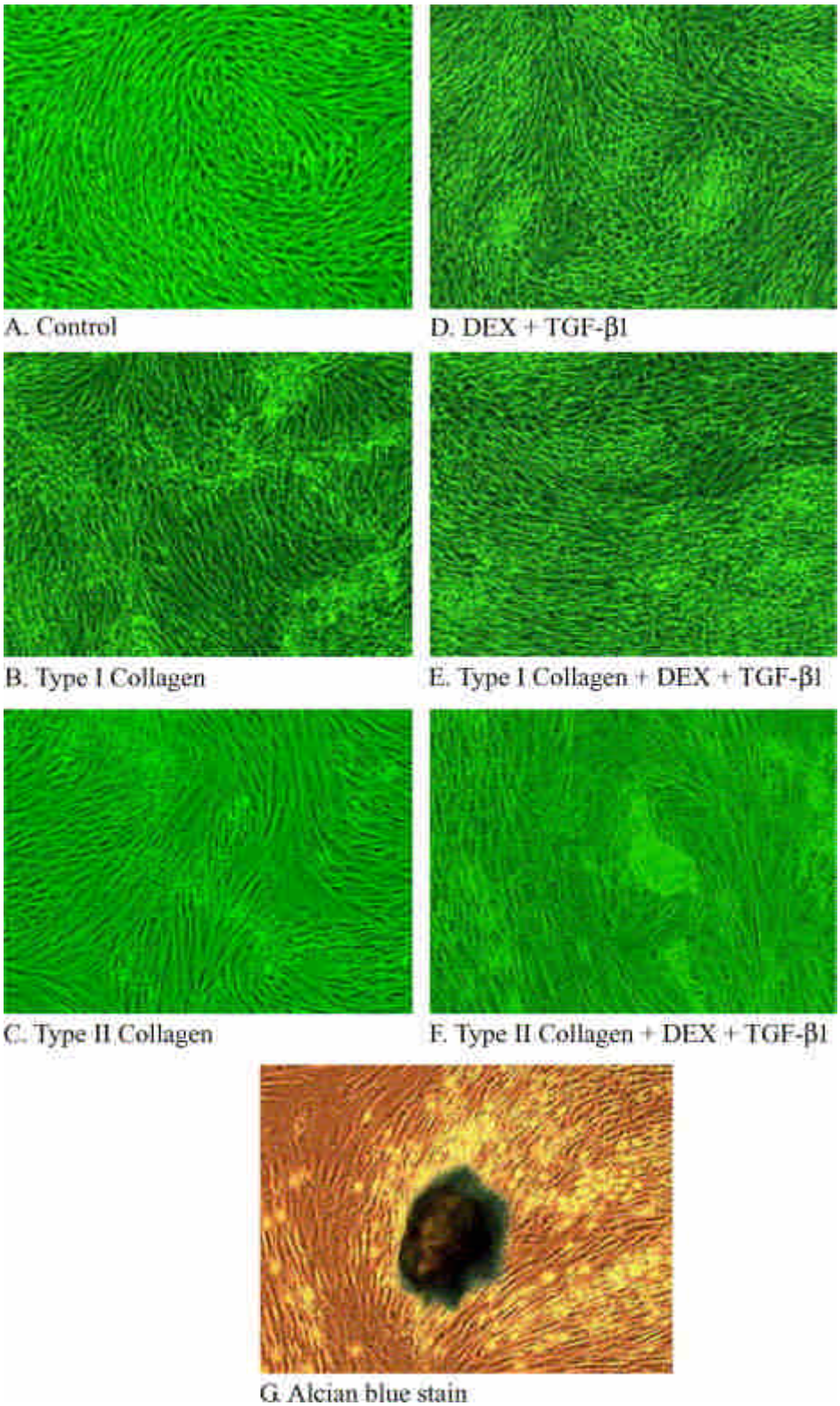


Fig. 12: Type I and II collagen effects on morphological changes in MPCs treated with dexamethasone and *rh*TGF-β1 in monolayer culture (100×). (A and D) MPCs appeared fibroblast-like morphology and mild pile-up 14 days after cultured with dexamethasone and TGF-β1. Incorporation with extracellular type I collagen cells showed an elongated spindle-shaped fibroblastic appearance in the absence (B) or presence (E) of *rh*TGF-β1 and dexamethasone. (C) Cells showed more cuboidal-, and less spindle-shaped morphology after

pretreatment with type II collagen. (F) Cells appeared predominantly rectangular after pretreated with type II collagen in addition to dexamethansone, and *rh*TGF-β1. (G) Note abundant GAG in the cartilaginous-like cell-collagen matrix aggregate (alcian-blue staining).

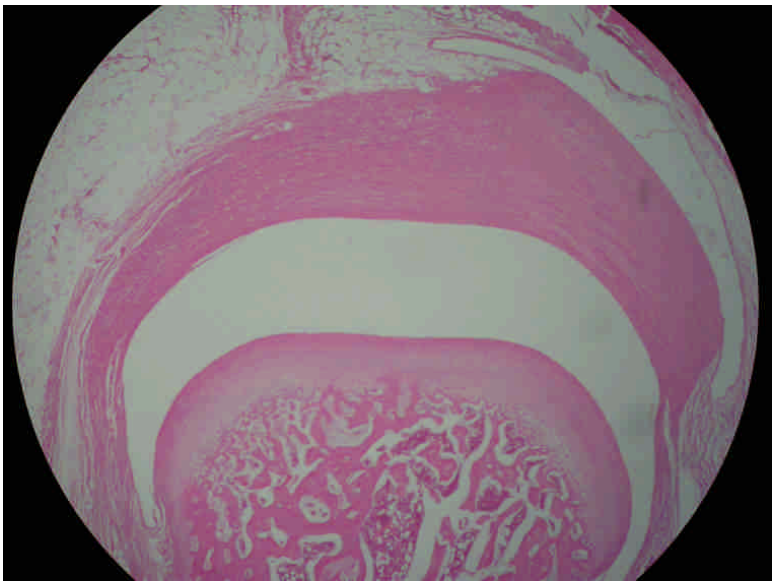


Figure 13: H.E. staining of control group TMJ (non-surgery performed group)(4X)

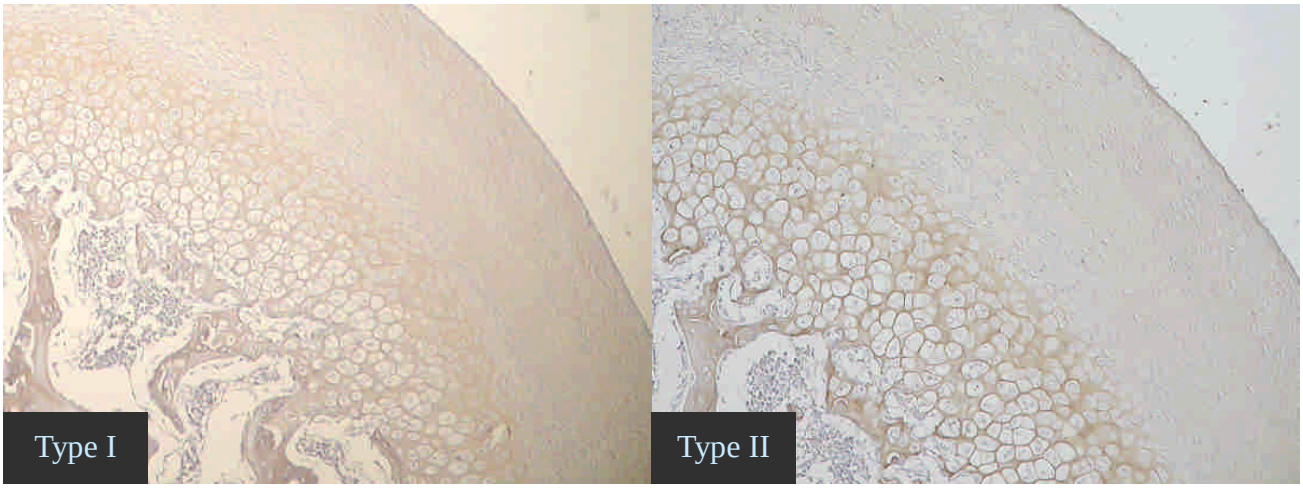


Figure 14: Collagen expression of cartilage surface from control group TMJ. (20X)

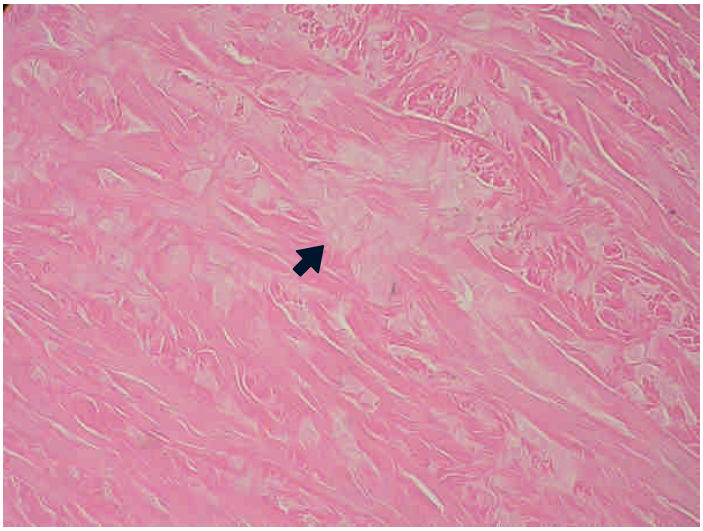


Figure 15: Chondroblast presented in the articular disc of control group TMJ. (arrow) (40x)

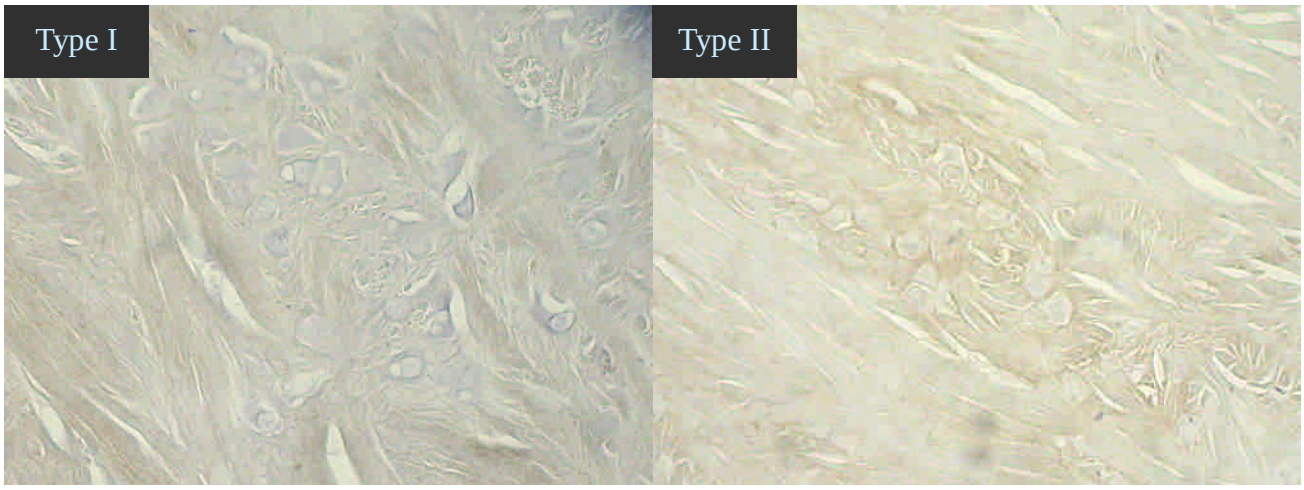


Figure 16: Collagen expression around the cells presented in the articular disc of control group TMJ. (40X)

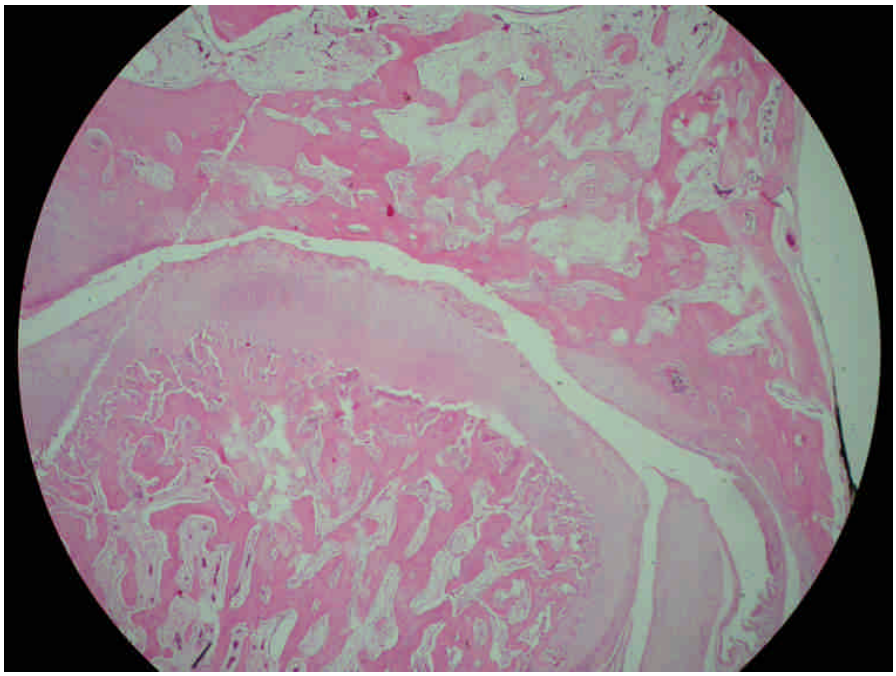


Figure 17: H.E. staining of one month disc-removal TMJ without artificial disc implantation (surgery group without artificial disc implantation) (4X)

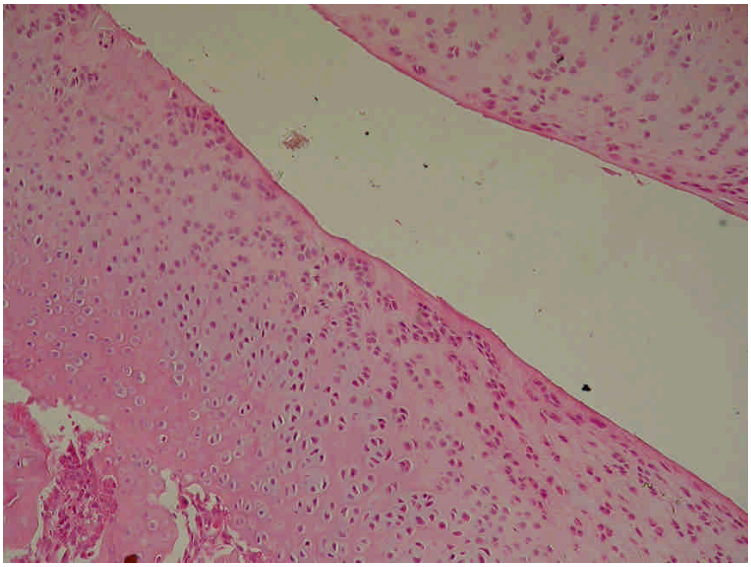


Figure 18: The proliferating cell clusters at the degenerative cartilage surface (20x)

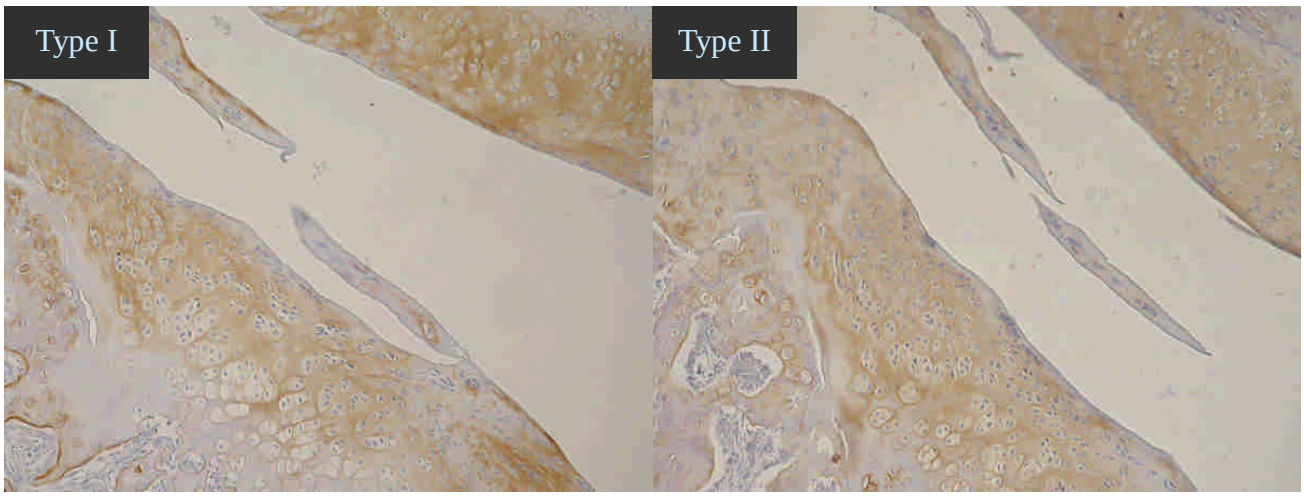


Figure 19: Collagen expression of cartilage surface from three month disc-removal TMJ without artificial disc implantation. (surgery group without artificial disc implantation) (20X)

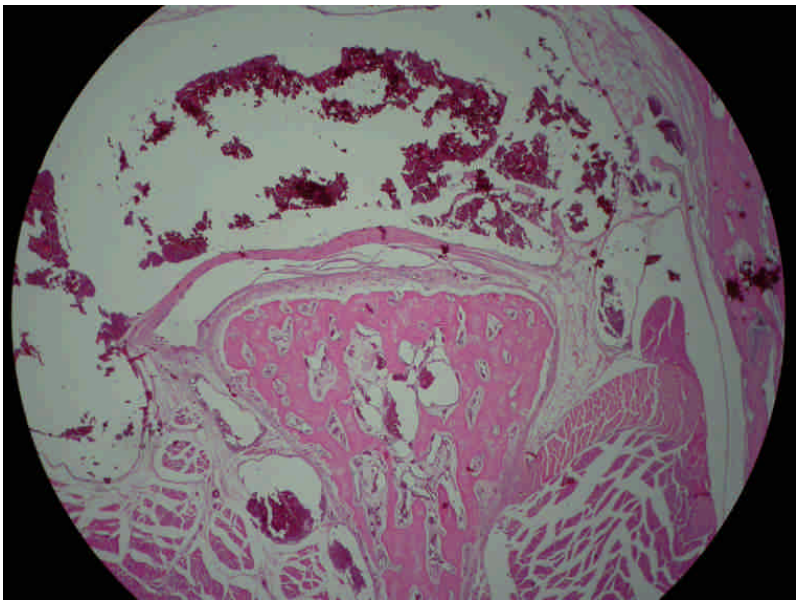


Figure 20: H.E. staining of a three month disc-removal TMJ without artificial disc implantation (surgery group without artificial disc implantation). (4x)

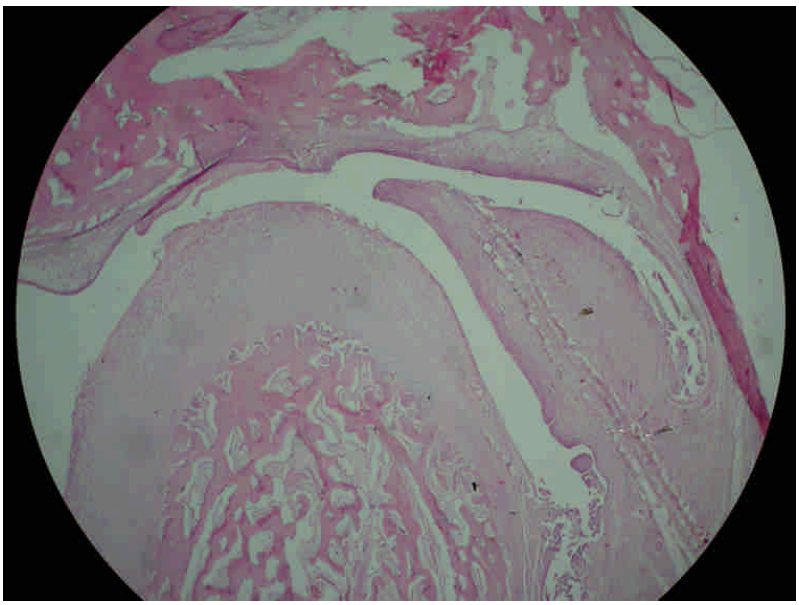


Figure 21: H.E. staining of one month disc-removal TMJ with artificial disc implantation (surgery group with artificial disc implantation) (4X)

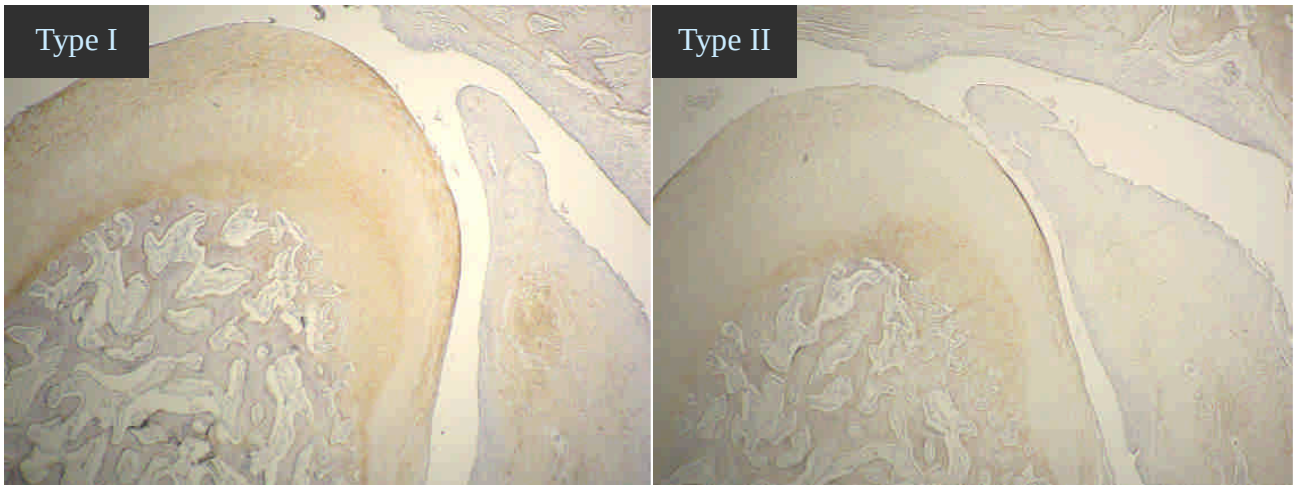


Figure 22: Collagen expression of cartilage surface from one month disc-removal TMJ with artificial disc implantation. (surgery group with artificial disc implantation) (20X)

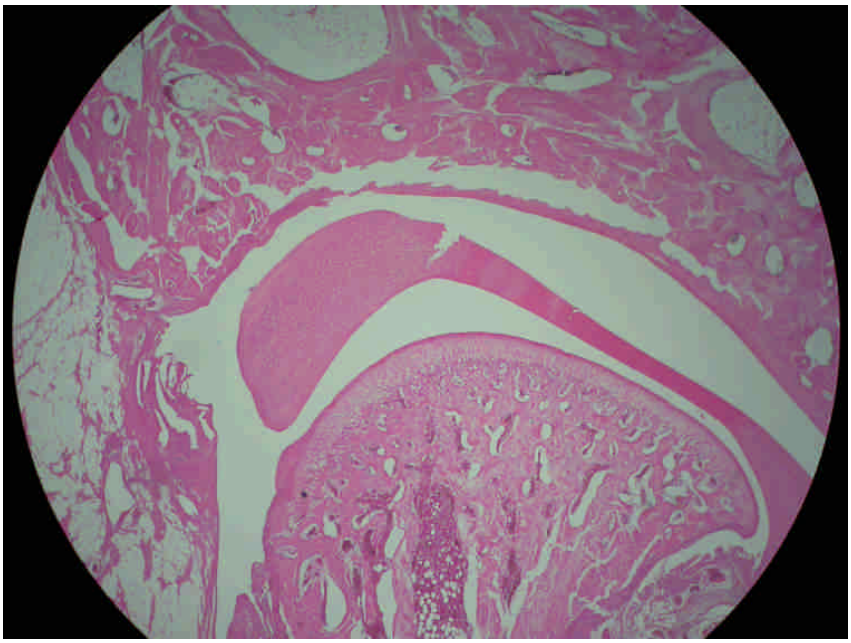


Figure 23: H.E. staining of a three month disc-removal TMJ with artificial disc implantation (surgery group with artificial disc implantation). (4x)

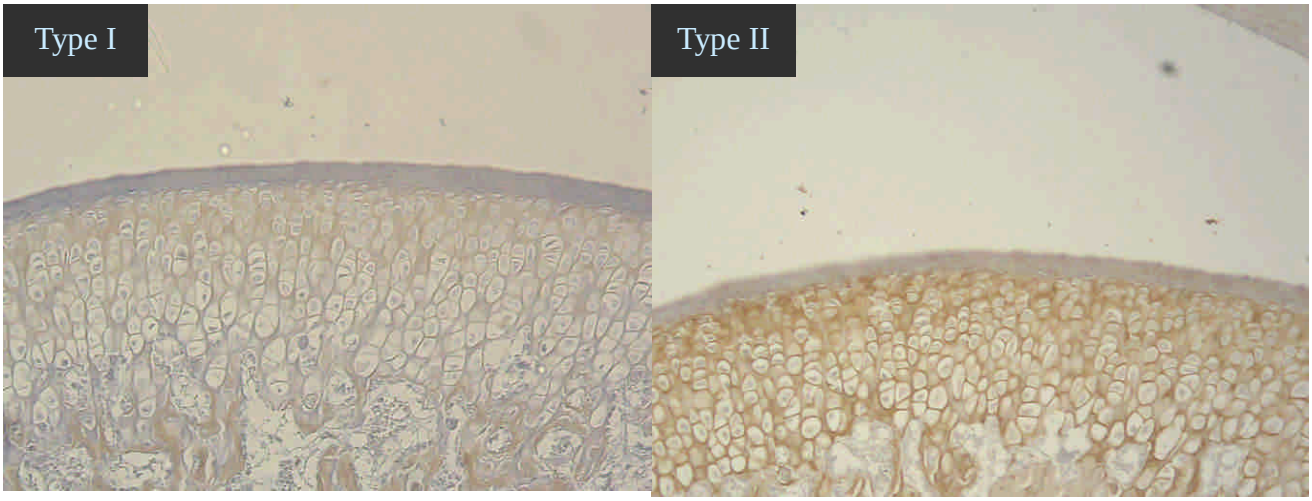


Figure 24: Collagen expression of cartilage surface from three month disc-removal TMJ with artificial disc implantation. (surgery group with artificial disc implantation) (20X)

VIII. 計畫成果自評:

Publications:

A) Conference Abstract:

1. Chen Chun-Wei, Tsai Yu-Hui, and **Lai WF**: The Differential Effects of Type II and Type I Collagens on the Regulation of Alkaline Phosphatase Activity in Osteoblasts. 31th Annual Meeting of Society for Biomaterials April 30-May3, Nevada, 2003.

The inclusion of native**,** but not heat-denatured, type II collagen in culture medium enhanced ALP activity of U2-OS cells about 1.7 folds in DMEM/F12 medium, and about three folds in McCoys'5A medium. On the other hand, treatment of cells with native or fragmented type I collagen did not significantly influence ALP activity. Furthermore, the addition of native type II collagen to primary chondrocytes slightly increased ALP activity to about 130 percent, while native type I collagen was invalid. It is known that during the hypertrophy and early ossification stage of embryonic development, the extension of blood vessels into cartilage structure provides osteo-progenitors as well as osteoblasts sources from adjacent tissues. Our results implicated that type II collagen could up-regulate ALP activity of chondrocytes as well as those of osteoblasts, and eventually promote endochondral ossification. Data from other laboratories also demonstrated that defects in type II collagen caused abnormal skeletal development (Talts et al, 1998; metsaranta et al,1992). It is, thus, suggested that the matrices of cartilaginous tissue under certain conditions not only serve as simple scaffolds occupying the space for bone formation, but also provides signals to modulate the calcification progression. It is speculated that type II collagen maybe utilized as tissue regeneration materials to promote differentiation, mineralization, and healing of the bone.

2. Chen Chun-Wei, Tsai Yu-Hui, Deng WP, Shih HN, Fang CL, and **Lai WF**: The regulation of chondrogenic differentiation in mesenchymal stem cells by type II and type I collagen. Nov.2002, The 4th Asian-Pacific Organization for Cell Biology Congress.

Previous studies reported an in vitro chondrogenic differentiation of bone-marrow derived mesenchymal stem cell (MSCs) in the culture system. Cytokines such as TGF-beta 1 and dexamethasone (Dex) were shown to trigger chondrogenic differentiation of mesenchymal stem cells. However, mechanisms of differentiation are still not well understood. The purpose of this study was to further investigate the effect of extracellular matrix (ECM) on the regulation of chondrogenic differentiation of rabbit MSCs. Current results demonstrated that 10 ng/ml TGF-beta 1 increased cell proliferation and expression of GAG (glycosaminoglycan), a marker of osteo-chondrogenic differentiation, after cultivation of MSCs for 14 days. With the treatment of Dex (10^{-7}M) and TGF-beta 1, the increase of cell proliferation and GAG expression was enhanced. GAG was found to accumulate around MSCs 14 days after cultivation in the presence

of rabbit type II collagen. On the contrary, the alkaline phosphatase (ALP), an osteogenic marker, did not increase at the same time interval. In the three-dimensional tissue cultivation, collagen matrices were mixed with primary chondrocytes. In contrast to type I collagen matrices, type II collagen matrices significantly promoted and prolonged the cartilaginous phenotype preservation of chondrocytes. In conclusion, in additional to cytokine effects, extracellular matrix, type II and type I collagen, may play an important role in the regulation of Chondrogenic differentiation of MSCs.

3. **Cartilage engineering by using marrow-derived stromal cells in Type I collagen matrix** (The XVII Joint Annual Conference of Biomedical Sciences (2002) #071) by *CM Chang, **WFT Lai**, CL Fang, CW Chen and YH Tsai*

Marrow-derived stromal cells (MSCs) have the ability to differentiate into several lineages and are used as the cell source for *in vitro* tissue engineering. In this experiment, it was intended to seed MSCs into rat-tail tendon type I collagen matrix, and cultivate the cells with differentiation factors to generate a neocartilage. The MSCs were obtained from the iliac crest of New Zealand White rabbits and type I collagen was extracted from rat-tail tendon. An aliquot of MSCs was mixed with type I collagen in 15ml culture tube. The contracted, near spherical cell-matrix constructs were further incubated with dexamethasone and TGF-β1. The chondrogenesis of MSCs was observed based on histological examination. The contracted MSCs-embedded collagen matrix (MECM) might play a role in promoting cell-cell interaction. Meanwhile, the presences of proper concentrations of dexamethasone and TGF-β1 induced chondrogenesis in MECM. The periphery area of the specimen in differentiation group showed apparent chondroblast formation as early as on day 7, according to histological and immunohistochemical studies. The present system provides a convenient way to study various aspects of chondrogenesis. The use of type I collagen as a scaffold in tissue engineering may facilitate the clinical efficacy of neocartilage implantation for its biocompatibility and intoxicity. This culture system may provide a new model to study chondrogenesis as well as a new strategy to engineer a cartilage replacement device.

4. Chen Chun-Wei, Tsai Yu-Hui, and **Lai WF**: Type II Collagen Matrix and bFGF Supports the Migration, Differentiation, and GAG Expression of Chondrocytes– *In Vitro* 3D Cartilage Wound Healing Model. 14th Annual Meeting of Wound Healing Society May 23-26, Atlanta, 2004.

Cartilage preserves limited regenerative capacity and results in insufficient healing after joint injury. Previous researches showed that biocompatible materials and growth factors enable to facilitate cartilage repair; however, the mechanisms of cartilage regeneration are still not completely understood. The purpose of this study was to determine the effects of extracellular

matrices and growth factor on chondrocyte migration and phenotype during cartilage regeneration. Neocartilage, 35mm in diameter and 1mm in thickness, was fabricated by using rabbit primary chondrocytes embedded in an *in vitro* three-dimensional collagen matrix. Five mm diameter defects were made. The effects of collagen matrices and basic-FGF on three-dimensional cartilage wound healing were determined. Results showed both type I and type II collagen matrices (1mg/ml) facilitated migration of chondrocytes from surrounding cartilage into the defect area in the presence of 10ng/ml basic-FGF. The average of migration speed was 91.5micron/day for type I, and 88.1micron/day for type II collagen matrix in the period of 26 days. Incorporation with basic-FGF, chondrocytes increased approximately 1.5 times motility in the both collagen matrices. However, the cartilaginous phenotype was noted only when applied with type II collagen matrix. The results indicate that type II collagen matrix may trigger the redifferentiation after the migration of chondrocytes. Extracellular matrix such as type I and type II collagen, in addition to cytokines such as basic-FGF, play an important role in the process of cartilage wound healing in a three-dimensional defect model.

5. Charn-Bing Yang, Yu-Huei Tsai, Win-Ping Deng, Wen-Fu Lai. Frontiers of Cartilage Repair, Regeneration, and Early Diagnosis. The 9th Biomedical Material & Technology Symposium, R.O.C. Taipei, Taiwan. R.O.C. May 18-19, 2005.

Cartilage is featured by one cell type, low oxygen requirement and the capacity of being stored for relatively long periods of time. These characteristics make challenges in cartilage repair and regeneration. Current therapies for cartilage regeneration include placements of carbon plugs periosteum, periochondrium, autologous chondrocyte transplantation, and subchondral drilling. Success rates varied. Most methods of therapy had serious limitations in clinical use. In vitro chondrogenic differentiation from mesenchymal stem cell and in vivo implantation of isolated mesenchymal stem cells were also attempted. Committed mesenchymal stem cells are employed either to differentiate into regeneration of cartilage or bone in defect. Cytokines, in combination with scaffold matrix, play the important role to regulate the differentiation and proliferation of mesenchymal stem cells. More recently, attempts were made in the early detection of arthritis using molecular imaging such as near infrared fluorescent probe in order to repair joint defect more completely. Future efforts in the engineering should emphasize the development of methods to allow adequate human chondrocyte proliferation in short periods of time while preserving cellular phenotype. Biomechanical and physical properties of the constructs at different time points give a clue in the clinical feasibility. Studies related to the ability of tissue-engineered cartilage generated in specific applications, as well as its potential to provide long- term functional improvement need to be carried out. It also believes that advances in the engineering of cartilage will provide useful information for other tissues.

B) Original Research Article:

1. Chen CW, YH Tsai YH, Deng WP, Shih SN, Fang CL, Burch JG, Chen WH **Lai WF**. Type I and type II collagen Regulation of Chondrogenic Differentiation in Mesenchymal Progenitor Cells. J Orthopaedic Res. 23(2):446-53, 2005 (SCI: 3/46, PMID: 15734261)

2.

C) Personnel Training:

The personnel involved in this grant have been well trained and become skillful in chondrocyte and MPC isolation, cultivation, and growth factor- & ECM-guided differentiation of MPC-tissue engineering.

Dr. Charng-Bin Yang (楊長彬) promoted to the chief surgeon of the surgical department of Taipei City Hospital.

Chung-Wei Chen (陳俊偉) applied and worked in McLean Hospital, Harvard.

Tsung-Tan Tsai (蔡宗潭) was graduated from Graduate Institute of Biomedical Materials of Taipei Medical University.

Li-Shuan Chiu (邱立軒) applied and now are working in the PhD program in Graduate Institute of Cell and Molecular Biology of Taipei Medical University.

D) Research Contribution:

A. Cellular responses of primary human chondrocytes to various commercial available bone repair related cytokines were determined. The results are valuable as a database for cell therapy of osteoarthritic defects in the clinical trial.
B. Effects of type I, type II collagen, and combined with cytokines on the chondrogenic differentiation was further evaluated. The data could contribute to the new design of matrices for cartilage engineering.
C. To define the regeneration mechanisms of cartilage and meniscus, extracellular matrix changes of artificially induced osteoarthritic rabbit TMJs with collagen template implantation were further evaluated using immunohistochemistry. The data reveals the reconstituted collagen template facilitate cartilage and meniscal regeneration at the wound site of TMJ. These findings will yield the useful information for the osteoarthritis therapy in the future.