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| Original Article |

# A Thermosensitive Chitosan/Corn Starch/β-Glycerol Phosphate Hydrogel Containing TGF-β1 Promotes Differentiation of MSCs into Chondrocyte-Like Cells

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**Abstract :** Our previous study showed that thermosensitive chitosan/corn starch/β-glycerol phosphate (C/S/β-GP) hydrogel was an effective carrier for chondrocytes and their transforming factor, TGF-β1. In the present study, MSCs were grown in C/S/β-GP hydrogels as an effective tool for chondrocyte-like cell differentiation. The MSCs –encapsulated hydrogel was prepared by blending chitosan solution (1.70% w/v in 0.1 M HCl) with pregelatinized corn starch solution (1.70% w/v). The total final concentration of the blended polymers was 1.53% w/v, and the weight ratio of chitosan to corn starch was 4 to 1. The TGF-β1 (final concentration of 25 ng/mL) and $5 \times 10^5$ MSCs were added to 500 μL chitosan/starch solution. Finally, β-GP (60% w/v) was added to obtain 6.0% w/v final concentration. The C/S/β-GP hydrogel changed from a liquid at room temperature to a gel at $37 \pm 2^{o}$C. It converted the fibroblast-like MSCs into spheroid cells. In hydrogels containing TGF-β1, these cells further differentiated into chondrocyte-like cells. This was shown by their expressions of type II collagen and aggrecan mRNA. Type I collagen mRNA was initially expressed but this disappeared by 6 weeks in culture suggesting a complete chondrocyte differentiation by that time. Type II collagen protein production was detected by immunohistochemistry and immunofluorescence, and successively increased after 4-6 weeks in culture. Neither the mRNA nor the collagen expression could be detected in the absence of TGF-β1. The data indicate that MSCs would be an appropriate chondrocyte precursor in conjunction with our hydrogel loading TGF-β1 which is able to sustain chondrocyte function.

**Key words:** *thermosensitive hydrogel, chitosan/corn starch/β-glycerol phosphate hydrogel, mesenchymal stem cells, transforming growth factor-β1, chondrocyte-like cells*

## 1. Introduction

Over past decade, many studies on chondrocyte incorporation into tissue engineered scaffolds have been undertaken in order to generate new functional cartilage. The major problems presenting in this area include insufficient cell numbers, donor site morbidity, and tendency for cells transform in the two dimensional conditions.[1,2] To circumvent these problems, mesenchymal stem cells (MSCs) have been considered as an alternative cell source.[3-5] They are unspecialized progenitor cells that reside in various connective tissues,[6] and by optimizing the appropriate growth factors or mediators, MSCs can differentiate into a variety of connective tissue lineages including chondrocytes. Thus, it would be possible to accumulate large stocks of MSCs as a source of chondrocytes.[7-9]

Transforming growth factor-β (TGF-β) family members are expressed in most cell types including chondrocytes and MSCs.[10,11] TGF-β, particularly TGF-β1 plays the major role in chondrogenic differentiation by promoting MSC condensation and increasing the production of aggrecan and type II collagen.[1,12] For this reason, TGF-β1 has attracted interest as a chondrogenic mediator.[13-16]

Previously, we developed a thermosensitive hydrogel based on chitosan/corn starch/β-glycerol phosphate (C/S/β-GP).[17,18] The C/S/β-GP solution could be injected and transformed into a gel at body temperature. *In vitro*, it was shown that the C/S/β-

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GP hydrogel could maintain a constant release of TGF-$\beta$1 over a 14 day period.[18] Furthermore, rat chondrocytes cultured in C/S/$\beta$-GP containing TGF-$\beta$1 could retain their phenotype and function.[18] However, these experiments were limited by the restricted availability of chondrocytes. To overcome this, the present study used MSCs as primary source of cells while again relying on the sustained release of TGF-$\beta$1 from the C/S/$\beta$-GP hydrogel for their differentiation into chondrocytes. This was preceded by an *in vitro* evaluation of the C/S/$\beta$-GP hydrogel as an effective tool for chondrocyte-like cell differentiation as shown by the expression of type II collagen and aggrecan.

## 2. Materials and Methods

### 2.1 Preparation of C/S/$\beta$-GP Hydrogel With or Without TGF-$\beta$1

The thermosensitive hydrogel was prepared as described in our previous study.[17,18] Briefly, chitosan solution was prepared by dissolving crab chitosan (molecular weight of $10^5$ - $10^6$ Da, degree of deacetylation > 90%, Bannawach Bio-Line Co., Ltd., Chonburi, Thailand) as 1.70% w/v in 0.1 M HCl. Corn starch (amylose : amylopectin, 30.23 : 69.77, Tawan Chemical, Bangkok, Thailand) was dissolved in deionized water as 1.70% w/v and then heated up to 90°C until gelatinization. The crosslinking agent, $\beta$-glycerol phosphate ($\beta$-GP, Fluka Chemie GmbH, Buchs, Switzerland), was prepared as a 60% w/v solution in distilled water. The chitosan and starch solutions were autoclaved, and $\beta$-GP solution was sterilized through a 0.22 μm filter. After solution preparation, corn starch solution was added to chitosan solution (weight ratio of chitosan to corn starch, 4 to 1), and the total final concentration of the blended polymers was 1.53% w/v. The blended solution was constantly stirred at 600 rpm (IKAMAG®, IKA®Werke GmbH & Co.KG, Staufen, Germany) until it was homogenous. To obtain a hydrogel containing recombinant human transforming growth factor-$\beta$1 (TGF-$\beta$1, BioSource International, Inc., California, USA), TGF-$\beta$1 solution was added to the chitosan/starch (C/S) solution giving a final concentration of 25 ng/mL, as determined in our previous work.[18] The sterilized $\beta$-GP solution was then added drop-wise to obtain 6.0% w/v final concentration and constantly stirred for 10 min to give a clear and homogenous solution. The hydrogel solution was freshly prepared for all studies.

### 2.2 Expansion of MSCs

MSCs used in this study were the commercial stem cells from Lonza Walkersville, Inc., Maryland, USA. Cells in passage 3-7 were used. MSCs were expanded in Dulbecco's modied Eagle's medium (DMEM, high glucose, Sigma-Aldrich Co., Missouri, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, California, USA), maintained at 37°C in a humidified atmosphere containing 5% $CO_2$, and the medium refreshed every three days. When the cultures reached 80-90% confluence, they were trypsinized and sub-cultured. The cells maintained their elongated fibroblast-like morphology and colony forming behavior indicating that they were still pluripotent.

### 2.3 Viability of MSCs Cultured With C/S/$\beta$-GP Hydrogel

Fifty microliters of C/S/$\beta$-GP solution with MSCs ($5 \times 10^4$ cells) was added on each well of 96-well plate and incubated at 37°C. The cultured medium, DMEM supplemented with 10% FBS were added after gel formation, and the cells with hydrogel were then incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. The cultured medium was changed every 3 days. After culturing for 1 day or 7 days, the old medium was replaced with 200 mL of fresh DMEM without FBS. Cell viability was determined by adding 50 mL of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT, Roche Diagnostics, Mannheim, Germany) as labelling reagent.[19] The absorbance was measured at 490 nm after incubation at 37°C in a humidied atmosphere containing 5% $CO_2$ for 4 h. The absorbance value obtained is directly proportional to the number of living cells. The percentage of cell viability was normalized to the absorbance of cells ($5 \times 10^4$ cells/well) directly seeded on the 96-well plate at day 0 (control).

### 2.4 Determination of *In Vitro* Chondrogenesis and Activity of TGF-$\beta$1

To determine the role of TGF-$\beta$1 in MSC differentiation into chondrocytes, the experiment was divided into 2 groups (n=3/week): (1) C/S/$\beta$-GP hydrogel containing MSCs and TGF-$\beta$1 (25 ng/mL) and (2) as above but without TGF-$\beta$1. Five hundred microliters of hydrogel solution (with or without TGF-$\beta$1) suspending MSCs ($5 \times 10^5$ cells) was seeds in 24-well plates and incubated at 37°C for 10 min to allow hydrogel formation. After gelation, the chondrogenic medium (high glucose DMEM supplemented with 100 nM dexamethasone (Sigma-Aldrich Co.), 50 μg/mL ascorbate-2-phosphate (Sigma-Aldrich Co.), 100 μg/mL sodium pyruvate(Sigma-Aldrich Co.), 40 μg/mLproline (Sigma-Aldrich Co.) and insulin-transferrin-selenium-plus premix diluted 1:100 (Sigma-Aldrich Co))[13-15] was added on the top of hydrogel. The culture was performed at 37°C, 5% $CO_2$ for 6 weeks with a medium change every 3 days. Three batches of cultures were run, and after 2, 4, or 6 weeks, the hydrogel was removed, and its upper and lower surfaces were

stroked across a glass microscope slide to deposit hydrogel and MSCs. The samples were collected for immunohistochemistry, immunofluorescence, and the remaining hydrogels were pooled and used for RT-PCR.

### 2.5 Determination of Chondrogenic Gene Expression by RT-PCR

To analyze chondrogenic mRNA, Trizol® reagent (Invitrogen) was added to the hydrogel containing cells, and total mRNA were amplified by RT-PCR using 40 cycles under the following conditions: 15 s at 94°C for denaturation, 30 s at 56°C for annealing and 1 min at 68°C for extension. The PCR products were separated by gel electrophoresis with 1.4% w/v agarose gel, and $\beta$-actin was used as the house keeping gene. The oligonucleotide primers (Invitrogen) were designed, according to previous studies[20,21] and were: type II collagen; 5′-AGT GGA AGA GCG GAG ACT A-3′ and 5′-GAC AGG CCC TAT GTC CAC AC-3′ (579 base pairs), aggrecan; 5'-TCA GGA ACT GAA CTC AGT GG-3' and 5'-GCC ACT GAG TTC CAC AGA-3' (487 base pairs), type I collagen; 5′-GCC AGC AGA TTG AGA ACA TCC-3′ and 5′-AAC CTT CGC TTC CAT ACT CG-3′ (312 base pairs), and $\beta$-actin; 5′-GAC CTT CAA CAC CCC AGC CAT G-3′and 5′-GGG CCGGACTCA TCG TAC TCC T-3′ (726 base pairs).

### 2.6 Immunohistochemistry Analysis of Type II Collagen

Immunohistochemistry staining was performed to demonstrate type II collagen protein being produced by chondrocytes-like cells within the C/S/$\beta$-GP hydrogel with or without TGF-$\beta$1. The sample slides were fixed in ice-cold methanol for 5 min and then rinsed 3 times with tris-buffered saline (TBS). The samples were blocked with 10% bovine serum albumin (BSA, Sigma-Aldrich Co.) for 2 h and incubated overnight with the mouse primary antibody against type II collagen (1:250 v/v, Abcam plc, Cambridge, UK) at 4°C. They were then rinsed twice with TBS and incubated in 0.3% $H_2O_2$ in TBS for 15 min. After 15 min, HRP-conjugated rabbit polyclonal against mouse IgG (Abcam plc) was applied, and the samples were incubated for 1 h at room temperature and rinsed 3 times with TBS. The color was developed with 3,3'-diaminobenzidine (DAB, Sigma-Aldrich Co.) for 10 min at room temperature. After rinsing under running tap water for 5 min, the samples were counterstained with hematoxylin and mounted in Premount™ mounting medium (BioSource International, Inc.). The samples were viewed by light microscopy.

### 2.7 Immunofluorescence Analysis of Type II Collagen

The samples were dried and then lightly coated with ice-cold methanol to fix the cells/hydrogel for 15-20 min. Cells were permeabilized with 0.1% Triton X-100 in PBS pH 7.4 for 5 min, blocked with 3% BSA in PBS for 30 min and incubated with mouse monoclonal against type II collagen (1:250 v/v) at room temperature in the dark for 2 h. The samples were washed with PBS for 4 times and incubated with secondary antibody (FITC conjugated goat polyclonal to mouse IgG, 1:250 v/v, Abcam plc) at room temperature in the dark for 1 h. After washing with PBS for 4 times, the samples were counterstained with propidium iodide (Sigma-Aldrich Co.) diluted in PBS (1:200 v/v) and again incubated for 10 min. Finally, the samples were washed with PBS for 4 times, mounted in Premount™ mounting medium and then photographed under a fluorescence microscope (Observer. A1, Carl Zeiss, Aalen, Germany).

## 3. Results

### 3.1 An Appearance of C/S/$\beta$-GP

Fig 1 shows the phase changes of C/S/$\beta$-GP thermosensitive hydrogel which changed from a liquid at room temperature to a gel at 37 ± 2°C within 10 min. The transition temperature and time, and gel characteristic were unaffected by the presence of MSCs and/or TGF-$\beta$1 (data not shown).

### 3.2 Morphology and Viability of MSCs Cultured With C/S/$\beta$-GP Hydrogel

When MSCs were cultured on bare plastic plates, they exhibited a flat, extended, and fibroblast-like morphology (Fig 2). In contrast, MSCs in the hydrogel exhibited distinctly spherical, and by focusing through the optically clear hydrogel, the cells appeared to evenly distribute at all depths.

Fig 3 shows the viability of MSCs when tissue culture plate or hydrogel was acting as substrate. The value obtained from control, cells seeded on the plastic plate at day 0 was adjusted to 100%. After culturing for 7 days, percent MSC viability on plastic plate slightly increased (106.1 ± 1.2%) from the beginning day (day 0). Percent MSC viability in hydrogel after

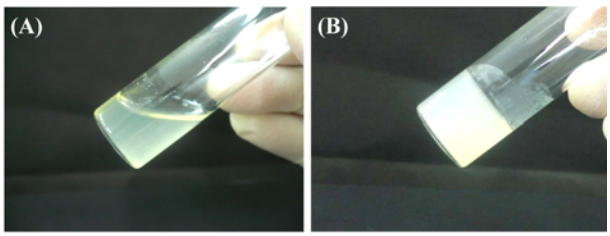


Figure 1. Photographs of C/S/$\beta$-GP thermosensitive hydrogels demonstrating liquid phase at room temperature (A) and gelling at 37 ± 2°C (B).

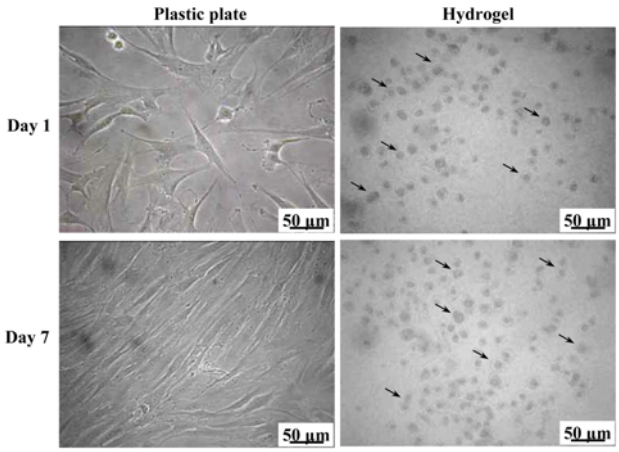


Figure 2. MSCs morphology cultured on plastic plates and in hydrogel for 1 and 7 days and in the cultured medium but without TGF-$\beta$1. The arrows point to MSCs.

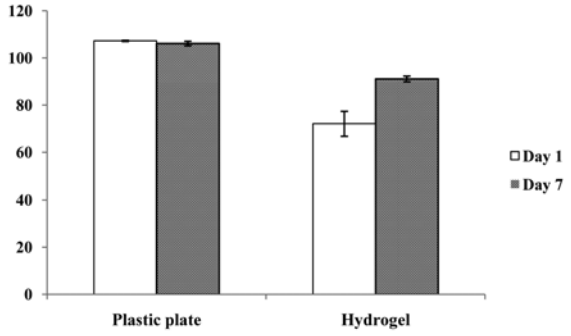


Figure 3. Percent viability of MSCs cultured on plastic plate and in hydrogel for 1 and 7 days and in the cultured medium but without TGF-$\beta$1(n = 3).

cultivation for 1 day remained to 72.1 ± 0.2% of the control and followed by increasing of viability up to 91.1 ± 1% after 7 days of cultivation.

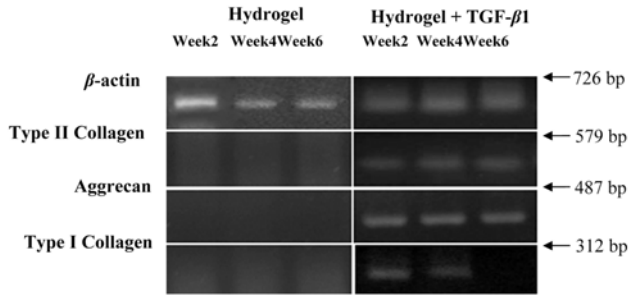


Figure 4. Expression in MSCs of chondrogenic mRNAs cultured in hydrogels alone and with TGF-$\beta$1 for 2, 4 and 6 weeks.

### 3.3 Chondrogenic Expression *In Vitro*

3.3.1 RT-PCR

Cells grown without TGF-$\beta$1 showed no expression of the chondrogenic mRNAs; only $\beta$-actin constructs were apparent (Fig 4). In contrast, the cell specific type II collagen and aggrecan mRNAs were consistently expressed throughout the 2-6 weeks in the hydrogel with TGF-$\beta$1. Type I collagen mRNA was expressed at 2 and 4 weeks but disappeared at 6 weeks.

3.3.2 Immunohistochemistry and Immunofluorescence Analysis of Type II Collagen

There was no discernible trace of any type II collagen protein expression when MSCs were incubated in the absence of TGF-$\beta$1 (Fig 5). In contrast, type II collagen staining was evident for all time points as indicated by the brown deposits (Fig 5). Furthermore, this was clearly greater at 4 and 6 weeks. While much of this appears to be intracellular, the photograph at 6 weeks shows a substantial area of type II collagen matrix outside the cells. Immunofluorescence of MSCs in hydrogel in the absences of TGF-$\beta$1 showed red staining of cell nuclei but no evidence of green staining collagen in and around these

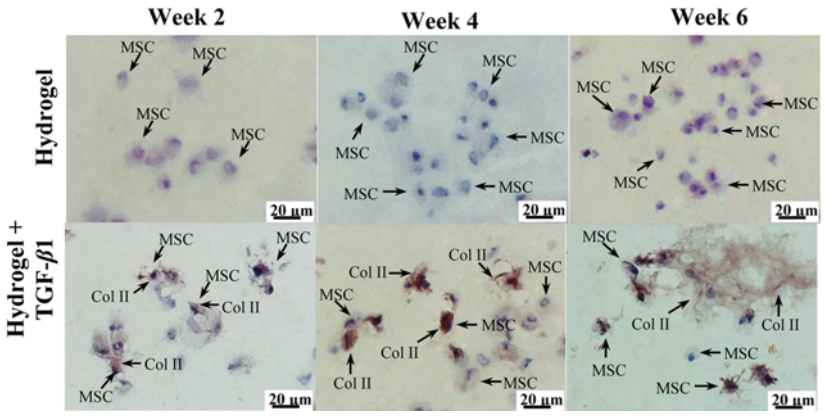


Figure 5. Immunohistochemistry staining of type II collagen (Col II) in MSCs cultured in hydrogel alone and with TGF-$\beta$1 for 2, 4 and 6 weeks.

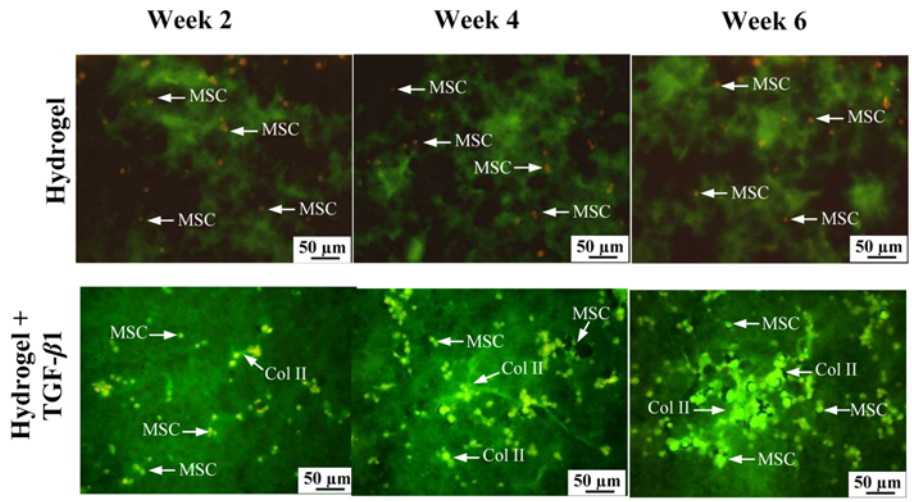


Figure 6. Immunofluorescence staining of type II collagen (Col II) in MSCs cultured in hydrogel alone and with TGF-$\beta$1 for 2, 4 and 6 weeks.

chondrocytes at any time point (Fig 6). These observations accord with the immunohistochemistry and the PCR data. This is in clear contrast with MSCs incubated with TGF-$\beta$1 which showed intense green staining around the cells, and this was increased in weeks 4 and 6 where it was found also within the hydrogel.

## 4. Discussion

The MSCs appears grow well and thus provided an abundant source of highly proliferative and multi-potent cells. Here, we needed a chondrogenic lineage which proliferated, survived, rounded up, and showed the appropriate phenotype within the C/S/$\beta$-GP hydrogel by responding to TGF-$\beta$1. It is common to use the cell pellet method to promote *in vitro* MSC chondrogenesis.[22,23] However, this is not suited to articular cartilage as cells grown under this condition may have high expression of type I collagen which may cause its differential into bone. Another approach for MSC chondrogenesis was seeding them in a 3D scaffolds, such as agarose,[5] calicium alginate,[24] and hybrid hyaluronic acid and collagen hydrogel.[25]

Our results showed that during expansion MSCs on monolayer, the morphology of MSCs exhibited as fibroblast-like cells and adhered to a plate. However, percent number of living cells slightly increased after cultivation for 7 days. This coincides with previous study indicating that MSCs culture on plastic plate exhibited the initial lag phase of 2-4 days and followed by a log phase of 6-10 days of cultivation.[26]

Focusing on cells cultured in C/S/$\beta$-GP hydrogel, MSC morphology exhibited as spherical shape and gradually proliferated within C/S/$\beta$-GP hydrogel. The hydrogel forced them to round up which is critical step in the MSC chondrogenesis and the maintenance of chondrocytic phenotype and functions as noted in other studies.[3-5,16,24] However, the spherical shape of cell would affect the initial adhesion. We found that at day 1, the number of living cells in the hydrogel decreased to 72.1 ± 0.2% of number of cells initially seeded on plastic plate. However, after incubation for 7 days, cell number increased to 91.1 ± 1%. The obtained results correspond to previous studies. They suggested that the spherical shape of cell affected to rate of cell mitosis, and number of cell adhesion was less.[27,28] Other studies reported that cells could adapt themselves and proliferate within the 3D environment after 7-14 days of cultivation.[29,30] Based on the results obtained, we possibly concluded that the C/S/$\beta$-GP hydrogel is not cytotoxic to MSCs.

Our previous study[18] showed that TGF-$\beta$1 released by *in vitro* from C/S/$\beta$-GP hydrogel at the beginning time and constantly released for 14 days. Moreover, the results showed biologically active of TGF-$\beta$1 released. From these obtained results, we expected that the sustained release of TGF-$\beta$1 from the C/S/$\beta$-GP hydrogel would be applicable for MSCs differentiation into chondrocytes. In the present study, the results from RT-PCR indicate the expression of chondrogenicm RNAs (type II collagen and aggrecan) throughout the 2-6 weeks in the hydrogel with TGF-$\beta$1. For type I collagen mRNA, its expression disappeared at 6 weeks. This might arise because initially, some MSCs were undifferentiated but differentiated by 6 weeks also suggesting adequate active TGF-$\beta$1.

Immunohistochemistry and immunofluorescence were conducted to confirm the expression of type II collagen protein in

the hydrogel. Corresponding to the RT-PCR results, type II collagen protein was greater expression at 4 and 6 weeks, particularly at 6 weeks, a substantial area of type II collagen matrix was found in the hydrogel with TGF-$\beta$1. This implies that TGF-$\beta$1 is maintaining differentiation of MSCs into chondrocytes and that the supply of TGF-$\beta$1 is enough to sustain this.

All above results show that MSCs were efficiently transformed into chondrocyte-like cells by the presence of TGF-$\beta$1. This is in agreement with report showing human-boned embryonic transformation within the first 10 days of exposure.[31] MSCs initially produced type I collagen but as they subsequently by differentiate into chondrocytes, the cells became rounded and expressed aggrecan and type II rather than type I collagen. At 14 days, such chondrocytes stop proliferating and begin to elongate to form the prehypertrophic chondrocytes that still produce type II collagen-rich ECM. Therefore, serving TGF-$\beta$1 at the initial stage with optimal concentration should be a promising way for MSC differentiation.

Our collective results show that the C/S/$\beta$-GP hydrogel can be used as an effective delivery system for MSCs and TGF-$\beta$1. It not only preserved the chondrocytes-like cell phenotype but also promoted cell functions such as production of cartilage ECM. Nevertheless, the results from our study were only the qualitative results. Before these findings can be effectively translated, the effect of many variables such as source of MSCs, age, passages number, cell density, etc., need further assessment. Nevertheless, this approach shows promise in the application of cartilage tissue engineering.

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