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RESEARCH PAPER

Chondrogenic Priming Adipose-Mesenchymal Stem Cells for Cartilage Tissue Regeneration

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ABSTRACT

Purpose Chondrocytes lose their ability to produce cartilaginous matrix during multiplication in culture through repeated passages, resulting in inferior tissue phenotype. To overcome the limited amount of primary chondrocytes, we aimed to determine the optimal culture condition for *in vitro/in vivo* cartilage regeneration using human adipose-derived mesenchymal stem cells (AMSCs).

Methods To evaluate the effects exerted by the chondrocytic culture condition on AMSC, we utilized chondrocyte conditioned medium (CM) and/or co-culture methods to prime and differentiate AMSCs. We evaluated ultimate *in vivo* engineered cartilage with primed AMSCs with that of chondrocytes. To examine the link between conditioned factors and proliferation/differentiation, cell cycle progression of AMSCs were examined using 5-ethynyl-2′-deoxyuridine (EdU), and gene expression was monitored.

Results We report that AMSCs can be stimulated to become chondrogenic cells when expanded with chondrocyte CM. Polymeric scaffolds co-seeded with CM-expanded AMSCs and primary chondrocytes resulted in *in vivo* cartilaginous tissues with similar biochemical content to constructs seeded with chondrocytes alone.

Conclusion These results indicate that chondrocyte CM consists of suitable morphogenetic factors that induce the chondrogenic priming of AMSCs for cartilage tissue engineering.

KEY WORDS auricular chondrocytes · cartilage · co-culture · human adipose derived mesenchymal stem cells · tissue engineering

INTRODUCTION

Cartilage is an avascular tissue that has limited regenerative capacity. Recently, many tissue engineering strategies have been developed for cartilage regeneration that may greatly benefit the treatment of various musculoskeletal disorders that cannot be treated by conventional methods (1–3). Even though autologous chondrocytes are widely utilized in clinical settings for cartilage regeneration, many treatments utilizing autologous chondrocytes are limited to filling small defects or an injectable plaque of the affected cartilages (4). In order to broaden tissue engineering application to major disorders such as total external ear reconstruction for traumatic amputation or congenital microtia, cartilaginous matrix should be abundantly produced by sufficient numbers of autologous chondrocytes seeded onto three-dimensional (3D) scaffolds that can provide mechanical support. Since the number of the chondrocytes that can be isolated primarily from the native cartilage is limited, recent studies have demonstrated promising use of mesenchymal precursor cells for cartilage tissue engineering (5–8).

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Although biochemical factors such as growth factors and hormones are known to induce chondrogenic differentiation of these mesenchymal precursor cells *in vitro*, their use for *in vivo* cell commitment and survival remains questionable (9). Furthermore, these cells require an active and prolonged supply of differentiation and growth factors for spontaneous chondrogenic differentiation, which may not be cost effective.

Chondrogenesis is a highly regulated process. Cartilage development during embryogenesis begins with mesenchymal cell condensation, which ultimately differentiates into chondrocytes (10). Differentiated chondrocytes proliferate rapidly and secrete a cartilage-specific extracellular matrix to form cartilage. Many of these sequential events in cartilage development are precisely regulated by various growth factors and soluble factors released from cartilage elements and perichondrium. Previous studies have investigated that chondrocyte-secreted factors may influence the mesenchymal cells via paracrine, juxtacrine or gap-junction signaling pathways (11–13). In addition, results of those studies have suggested that the chondrocyte-secreted factors can be utilized for tissue engineering applications by stimulating extracellular matrix by the precursor cells (14). Furthermore, recent literature suggests the co-transplantation of mesenchymal precursor cells and chondrocytes is considered a reasonable approach for the regeneration of cartilaginous tissues (13,15,16). However, there remain inevitable limitations, including induction of hypertrophic differentiation and limited biochemical content of cartilages engineered with stem cells, which lead to inferior cartilaginous tissues.

The objectives of this study were to develop a clinically practical method for engineering cartilage that can overcome the limitations imposed by stem cells. Worster and colleagues demonstrated that pretreatment of mesenchymal stem cells (MSCs) with TGF-β1 in monolayer followed by exposure of cells to IGF-1 in 3D culture significantly increased the formation of cartilaginous tissues (17). However, proteoglycan and procollagen type II production by MSCs remained lower than parallel chondrocytes culture. We initially determined optimal expansion and commitment scheme for AMSCs *in vitro* and further developed a co-seeding methodology to create phenotypically stable cartilage *in vivo*. Finally, we evaluated the properties of tissue-engineered cartilage by the chondrocyte-AMSC co-seeded scaffolds to examine whether this strategy can be applied to cartilage regeneration by circumventing the cell source problems.

MATERIALS AND METHODS

Cell Isolation and Expansion

Ovine auricular chondrocytes were isolated from 10-month-old Finn sheep. Briefly, cartilage was minced into 1 mm^3 pieces using razor blades and digested with type II collagenase (0.1%, Worthington, Lakewood, NJ) solution overnight at 37°C. The digested contents were then filtered through a 100 μm filter and washed with phosphate-buffered saline (PBS, Invitrogen). The isolated chondrocytes were then plated at 5,000 cells per cm^2 and grown to confluence with DMEM:F12 (GIBCO) containing 2 mM L-glutamine and 10% FBS (Hyclone), 50 U/mL penicillin, 50 μg/mL of streptomycin, 50 μg/ml ascorbic acid and 0.1 mM nonessential amino acids. Chondrocyte-conditioned medium (CM) was collected for 48 h by incubating chondrocytes with high glucose DMEM (GIBCO) and further supplemented with 10% FBS and 1% pen/strep. Human adipose-derived mesenchymal stem cells (AMSCs) were purchased from Invitrogen and expanded with mesenchymal stem cell growth medium (GM) composed of high glucose DMEM supplemented with 10% FBS and 1% pen/strep. AMSCs were plated at 5,000 cells per cm^2 and were expanded with either GM or CM. Serum-free chondrogenic medium was prepared with DMEM (GIBCO) containing 2 mM L-glutamine, 100 nM dexamethasone (Sigma), 50 μg/ml ascorbic acid phosphate (Sigma), 1 mM sodium pyruvate (Invitrogen), 40 μg/ml praline (Sigma), 1% ITS+ (BD Biosciences) with or with out TGF-β1.

Scaffold Preparation

Poly (l-lactic acid)/poly (lactide-co-ε-caprolactone) (PLLA/PLCL, Polysciences, Worrington, PA) scaffolds were fabricated via salt leaching technique as previously described (18). Briefly, PLLA and PLCL (1:1 ratio) were dissolved in chloroform to yield a solution of 5% (wt/vol) polymer; 0.25 ml of polymer solution was loaded into molds packed with 0.4 g of sodium chloride particles. The solvent was allowed to evaporate overnight, and the sponges were subsequently immersed for 12 h in distilled water (changed every 2 h) to leach the salt and create pore structures. The polymer sponges were soaked in 75% (vol/vol) ethyl alcohol overnight, washed three times with PBS, and coated with fibronectin (10 ng/ml, Sigma) for 3 h before cell seeding. For SEM photomicrographs, scaffolds were prepared and cut in half with a razor blade. The cross-sections were coated with platinum using a sputter coater. The samples were observed with a scanning electron microscope (JEOL 6700F).

Co-Culture Experiments

Primary auricular chondrocytes were seeded onto Transwell™ inserts at 1×10^4 cells/cm^2 (six-well plates, BD Biosciences) with 0.4 μm porous membrane and lowered into the wells with AMSCs for co-culture. Prior to co-culture, AMSCs were initially plated at 1×10^4 cells/cm^2. AMSCs co-cultured for 7 days in GM or GM supplemented with

TGF-β1 (10 ng/ml, R&D Systems). Control AMSCs were maintained with GM or GM supplemented with TGF-β1 (10 ng/ml). Medium was aspirated and exchanged twice a week.

EdU Proliferation Assay

Proliferating cells were quantified by utilizing the click-iT EdU Cytometry Assay Kit (Invitrogen). In the EdU incorporation experiments, AMSCs were cultured with chondrocyte-conditioned medium or the control medium for 48–73 h in 12-well plates. EdU was added at a 10 μM final concentration 45 min before harvesting the cells. Cells were then fixed, permeabilized, and stained for EdU according to the kit's protocol. The proportion of nucleated cells incorporating EdU was determined by flow cytometry (BD FACScan).

Real-Time PCR

Real-time PCR was performed to determine if the AMSCs were producing cartilage-specific proteins. Total RNA was isolated from AMSCs using the TRIzol method (Invitrogen). Total mRNA (0.5 μg) was reverse-transcribed using SuperScript reverse transcriptase with random hexamer. The real-time PCR was performed suing SYBR Green PCR Mastermix and the StepOnePlus™ Real-Time PCR System (Applied Biosystems). cDNA samples were analyzed for gene of interest and for the reference β-actin. Sequences of primers were 5′ TGGCACCACACCTTCTACAATGAGC 3′ and 5′ GCACAGCTTCTCCTTAATGTCACGC 3′ for β-actin; 5′ GCCTTGAGCAGTTCACCTTC 3′ and 5′ CTCTTCTACGGGGACAGCAG 3′ for aggrecan (ACN); 5′ GAAACCATCAATGGTGGCTTCC 3′ and 5′ CGA TAACAGTCTTGCCCCACTT 3′ for type II collagen (COL2A1); 5′ CAGGACGACTTTGATGCAGA 3′ and 5′ AAGCTGGAGCTGTCCTGGTA 3′ for cartilage oligomeric matrix protein (COMP). The level of expression of each target gene was then calculated as $-2^{\Delta\Delta Ct}$ as previously described (19).

Subcutaneous Transplantation into Athymic Nude Mouse

Scaffolds were seeded with auricular chondrocytes (three million cells/scaffold) alone, chondrocyte-conditioned medium-expanded AMSCs, AMSCs alone, or a mixture of AMSCs and auricular chondrocytes. Cells were mixed with 0.1% (w/v) atellocollagen and seeded onto the scaffolds for efficient seeding. Prior to implantation, all cell-seeded constructs were cultured *in vitro* for 10 days in serum-free chondrogenic medium with TGF-β1. Cell-seeded constructs were then implanted into 6-week-old athymic nude mice. Induction of anesthesia was achieved using intraperitoneal tribromoethanol (400 mg/kg). A 1 cm incision was made in the dorsum, and subcutaneous pockets were created by blunt dissection. After construct implantation, the incision was closed using surgical staples that were removed after 10 days. After 6 weeks, the constructs were harvested and histologically and biochemically analyzed for cartilaginous tissue formation.

Immunostaining and Histology

For immunostaining of the monolayer expanded AMSCs, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were then blocked with 5% normal goat serum in PBS for 30 min and incubated with rabbit polyclonal anti type II collagen or anti-type I collagen (RDI, Flanders, NJ) with 1:100 dilutions. Samples were then incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:100 dilutions, Jackson ImmunoResearch laboratory, West Grove, PA) and TRITC-conjugated phalloidin (1:1000 dilution, Chemicon) for 1 h. Nuclei were counterstained with DAPI (Chemicon) for 10 min, and images were collected with Zeiss LSM Metal Confocal microscope. For histological analysis, portions of *in vivo* generated constructs were fixed in 4% paraformaldehyde overnight, dehydrated in serial ethanol dilutions, and embedded in paraffin. Constructs were then cut into 5 μm sections. Sections were de-paraffinized and stained with hematoxylin and eosin to evaluate construct morphology, Safranin-O/fast green to assess the production of glycosaminoglycan, and Elastica Verhoeff-van Gieson (EVG) staining for elastin production. For immunostaining of *in vivo* constructs, the de-paraffinized sections were treated with chondroitinase ABC (Sigma) in a humidified environment to enhance permeability of the extracellular matrix by removal of chondroitin sulfate. Slides were rinsed with PBS, quenched of peroxidase, and blocked with goat serum for 1 h. Sections were then incubated with a rabbit monoclonal anti-type X collagen antibody (Abcam, Cambridge, MA), anti-type II collagen antibody (RDI), or anti-type I collagen antibody (Chemicon) and then incubated with FITC-conjugated goat anti-rabbit secondary antibody. The localizations were observed with Zeiss LSM Metal Confocal microscope.

Biochemical Analyses

In vivo generated constructs were biochemically analyzed after harvest. For analysis, *in vivo* samples were lyophilized and digested with papain, as previously described (14). After digestion of the constructs for 18 h at 60°C, cell debris and insoluble materials were removed by centrifugation at 6,000 g for 5 min. Portions of the digest were analyzed for DNA using PicoGreen (Invitrogen), sulfated glycosaminoglycan (sGAG)

by 1,9 dimethylmethylene blue (DMMB, Sigma), and collagen by hydroxyproline assays (20–22). Hydroxyproline content was converted to collagen content by assuming a mass ratio of collagen to hydroxyproline of 7.14 (22).

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA single factor) with * $P<0.05$ or ** $P<0.01$.

RESULTS

Effects of Conditioned Medium

The initial goal of this study was to determine the chondrogenic commitment of AMSCs during the expansion. In order to evaluate the effects exerted by the chondrocytic culture condition on AMSC, we utilized conditioned medium (CM). Furthermore, to examine the link between conditioned factors and proliferation, cell cycle progression of AMSCs was examined using 5-ethynyl-2′-deoxyuridine (EdU), a thymine analogue, which is incorporated by proliferating cells. EdU incorporation was measured after 72 h of CM treatment. The results indicate that the proportion of EdU incorporation was higher with GM compared to the CM-expanded AMSCs, indicating CM inhibited the proliferation (Fig. 1a, b). Indeed, only 5.51% cells in CM were positive for EdU, while 13.2% of GM-expanded cells were positive for EdU. The calculated population doubling time for the GM-expanded AMSCs was approximately 24 h, while CM-expanded AMSCs was around 45 h. After 7 days of CM expansion, the chondrocytic commitment of AMSCs was investigated by immunostaining for type I and type II collagens. The majority of AMSCs expanded with CM expressed type I collagen (Fig. 1c) and had markedly less cell area compared to the GM-expanded AMSCs (data not shown). Furthermore, type II collagen was detected after 7 days of CM expansion of AMSCs, indicating that the chondrocyte-secreted factors induced chondrogenic commitment during expansion (Fig. 1d).

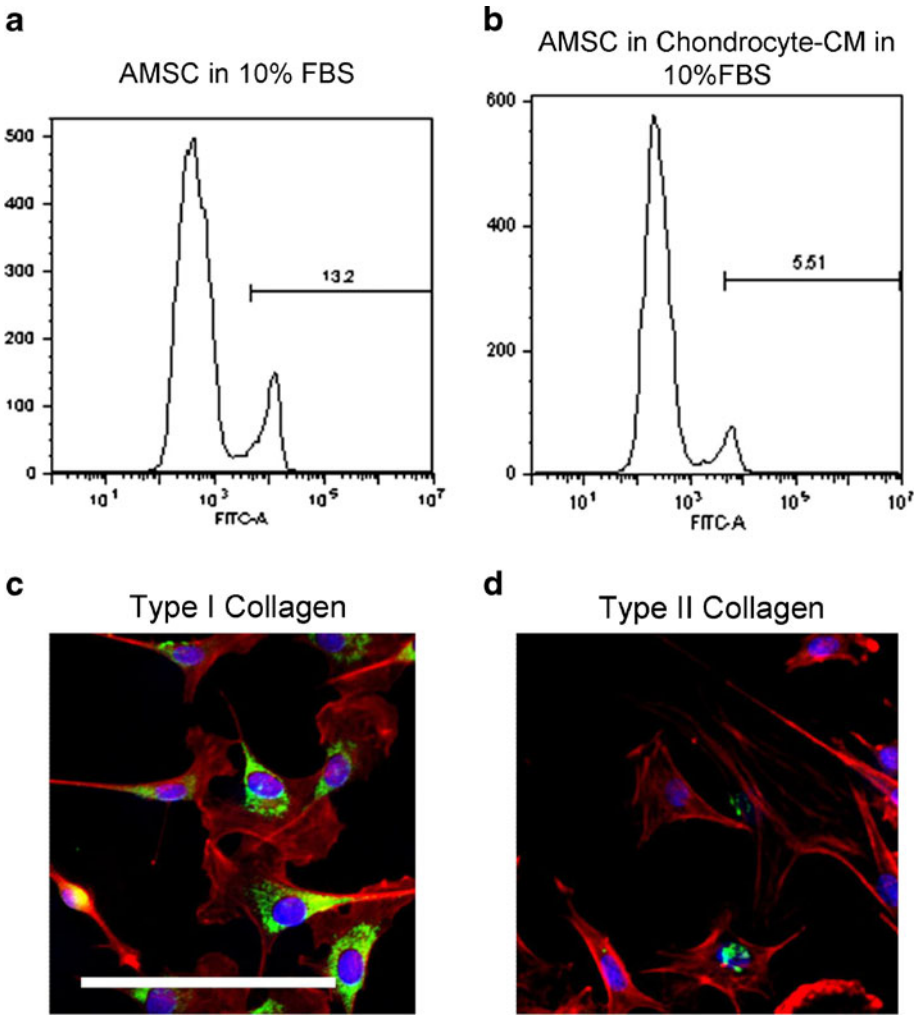

Fig. 1 Effects of chondrocyte-conditioned medium (CM) on AMSCs. (**a**) Flow cytometry analysis of EdU incorporated AMSCs. (**b**) Flow cytometry analysis of EdU incorporated CM-expanded AMSCs for proliferating cells. (**c**) Type I collagen staining of CM-expanded AMSCs (*Green*: collagen type I, *Red*: actin, *Blue*: nucleus). (**d**) Type II collagen staining of CM-expanded AMSCs (*Green*: collagen type II, *Red*: actin, *Blue*: nucleus).

Synergistic Effects of CM and TGF-β1

We further investigated the paracrine effects of chondrocyte-secreted factors on the AMSCs by establishing the co-culture system using Transwell™ inserts with 0.4 μm porous membranes (Fig. 2a). AMSCs were plated at high cell density (1×10^4 cells/cm^2) and co-cultured with chondrocytes (1×10^4 cells/cm^2) for seven days with GM. The relative expression of ACN mRNA, COL2A1 mRNA, and COMP mRNA was significantly higher in the co-cultured AMSCs compared to the control AMSCs at day 7 (Fig. 2b). TGF-β1 is a multifunctional polypeptide that regulates a variety of biological functions including chondrogenic differentiation of stem cells. Previous reports showed that partial chondrogenic commitment was observed in TGF-β1-treated MSCs monolayer culture (23). Therefore, we supplemented GM with TGF-β1 and observed the enhancement of cartilage-specific gene expressions. Our result demonstrated that the supplementation of TGF-β1 in the co-culture system significantly upregulated cartilage-associated genes (Fig. 2c). In the presence of TGF-β1, ACN and COMP expressions were upregulated 75-fold and 20-fold, respectively, in co-cocultured AMSCs. In addition, COL2A1 expression was also significantly enhanced by the TGF-β1 supplementation.

Growth Factor Effects of Primed AMSCs in 3D Culture

Previously, spontaneous chondrogenic differentiation of MSCs was observed in high-cell-density pellet culture system, indicating the importance of culture conditions prior to chondrogenesis (24). To observe the growth factor response of GM- or CM-expanded AMSCs toward the chondrogenic differentiation, cells were seeded in high density onto the 3D PLLA/PLCL scaffolds with atellocollagen as carriers. Prior to seeding on the scaffolds, AMSCs were expanded with GM or CM for three passages (14 days). The cell-seeded constructs were then cultured for 14 days in serum-free chondrogenic medium with or

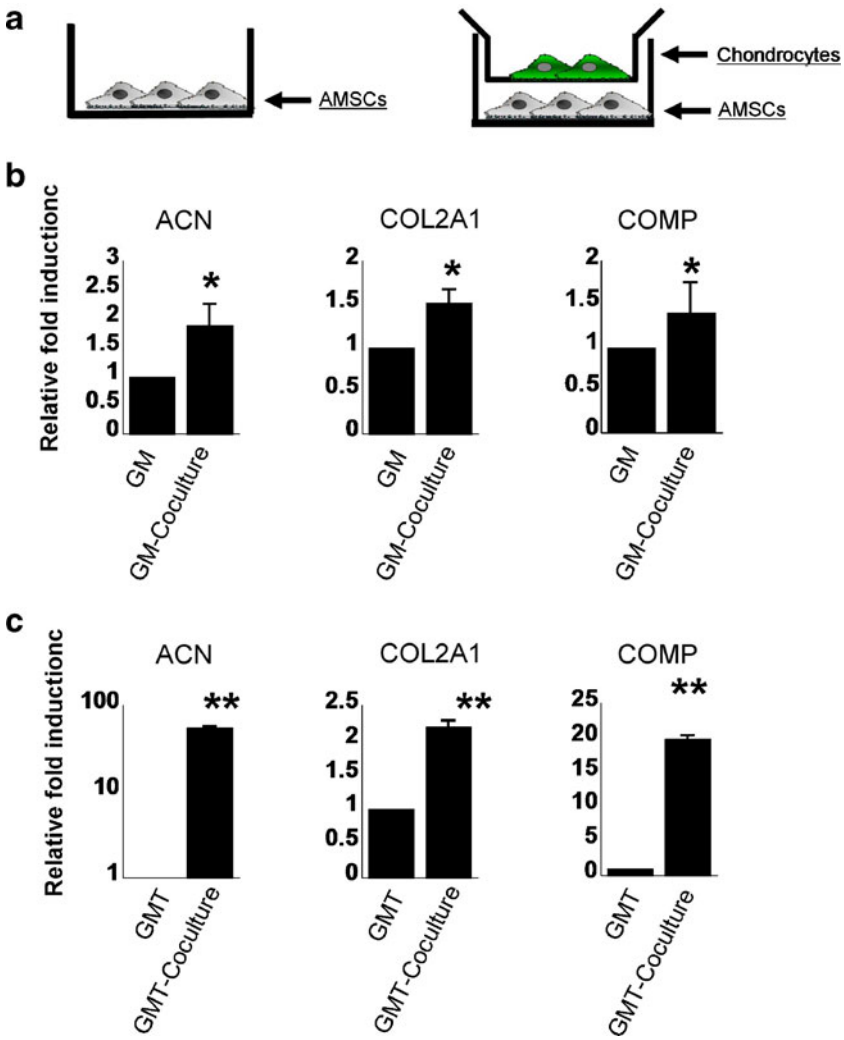

Fig. 2 Paracrine effects of chondrocyte-secreted factors on AMSCs. (**a**) Cells were transwell co-cultured for 7 days and analyzed for chondrogenic markers and messenger RNA expression of ACN, COL2A1, and COMP was measured and normalized against β-actin. (**b**) Real-time PCR analysis of AMSCs co-cultured with chondrocytes. (**c**) Real-time PCR analysis of AMSCs co-cultured with or without medium containing TGF-β1 (10 ng/ml). Results are reported as relative expression to β-actin using the Ct method. *Error bars* represent the SD. GM: growth medium, CM-Coculture: AMSCs co-cultured with chondrocytes in GM, GMT: GM + 10 ng/ml TGF-β1, GMT-Coculture: AMSCs co-cultured with chondrocytes in GM + 10 ng/mg TGF-β1. ACN: aggrecan, COL2A1: collagen type II, COMP: cartilage oligomeric matrix protein.

without the supplementation of TGF-β1. Relative expression of COL2A1 indicated that CM-expanded AMSCs responded significantly higher compared to the GM-expanded cells after 14 days on PLLA/PLCL scaffolds. For GM-expanded cells, serum-free chondrogenic medium with TGF-β1 supplementation resulted in only a 9-fold increase of COL2A1 expression compared to the GM-expanded cells without the TGF-β1. In contrast, CM-expanded cells resulted in 232-fold increase in COL2A1 expression even without the TGF-β1 supplementation. Furthermore, TGF-β1 supplementation resulted in 1488-fold increase of COL2A1 expression of CM-expanded cells compared to the GM-expanded cells without the TGF-β1 (Fig. 3).

In Vivo Engineering Cartilaginous Tissues

The *in vitro* experiments suggest that CM expansion is an effective method to chondrogenically prime the AMSCs. Furthermore, responsiveness of the AMSCs to TGF-β1 was augmented by CM treatment. We further examined whether priming of AMSCs by CM is sufficient to induce chondrogenic commitment *in vivo* (Fig. 4). GM- or CM-expanded AMSCs were seeded onto PLLA/PLCL scaffolds or co-seeded with primary chondrocytes in 1:1 ratio (Fig. 5a). For positive controls, primary auricular chondrocytes were seeded onto the scaffold. Since TGF-β1 can augment chondrogenic response of CM-expanded AMSCs, all the constructs were *in vitro* cultured in serum-free chondrogenic medium containing TGF-β1 for 10 days prior to *in vivo* transplantation. Cell-seeded scaffolds were transplanted subcutaneous region of athymic nude mouse for 6 weeks. After 6 weeks, the engineered tissues appeared glossy and opaque similar to native cartilage, except for the GM-expanded AMSCs (Fig. 5b).

Histologically, the transplanted AMSCs expanded with GM showed no cartilaginous tissues even with 10 days of *in vitro* stimulation (Fig. 5c). For CM-expanded AMSCs, cells formed small clusters of cartilage throughout the constructs, and the pericellular region of individual cells stained positive for Safranin-O were distributed throughout the construct (Fig. 5c). Co-seeded constructs with AMSCs and auricular chondrocytes resulted in larger clusters of cartilaginous tissue in the constructs, and the AMSCs adopted a chondrocyte-like phenotype and produced a cartilage-like matrix. Furthermore, co-seeded construct with chondrocytes and CM-expanded AMSCs resulted in even more homogeneous Safranin-O staining throughout the construct, with a more even distribution of cartilage ECM. It was possible to observe a clear chondrocyte phenotype with cell lacunae formation, particularly in intense Safranin-O positive regions in co-seeded constructs. Chondrocyte-only construct showed intense matrix staining, although it was more localized to central section of the construct. Detectable level of elastin was observed near the tissue-scaffold interface in all engineered constructs except the ones with GM-expanded AMSCs (Fig. 5c).

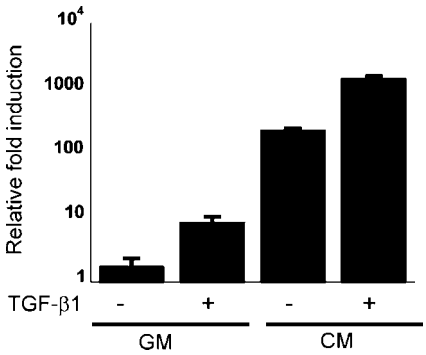

Fig. 3 Growth factor effects on primed AMSCs. AMSCs expanded with CM or GM were seeded on PLLA/PLCL scaffolds and cultured for 14 days in serum-free chondrogenic medium with or with out TGF-β1 (10 ng/ml), and messenger RNA expression of COL2A1 was measured by real-time PCR.

Immunostaining for types I, II, and X collagen showed that the transplanted chondrocytes retained their chondrocyte phenotype and formed cartilage-like matrix that was positive for type II collagen but negative for types I and X collagens (Fig. 6). To some degree, the CM-expanded AMSCs adopted chondrocytic phenotype with type II collagen expression (Fig. 6), indicating that *in vitro* expansion of AMSCs with CM resulted in chondrogenic priming of the cells *in vivo*. However, types I, and X collagen-positive matrices were visible in the CM-expanded AMSCs, indicating hypertrophy of cells. Co-seeded constructs with GM- or CM-expanded AMSCs both resulted in intense type II collagen stained regions. However, extent of type X collagen staining was less compared to AMSC-CM construct, indicating that co-seeding with chondrocytes may inhibit hypertrophic differentiation (Fig. 6).

Properties of the *In Vivo* Engineered Cartilage from Primed AMSCs

We next examined the properties of the tissue-engineered cartilage with or without primed AMSCs (Fig. 7). DNA was measured to assess cell number and sulfated glycosaminoglycan (sGAG), and collagens were assayed to determine the production of cartilage-specific extracellular matrix. The biochemical analysis indicates that GM-expanded AMSCs resulted in the least sGAG and collagen produced (Fig. 7b, c). However, GM-expanded AMSCs resulted in the highest DNA content among all samples (Fig. 7a). This is probably due to the migration of the host cells into the constructs, as GM-expanded AMSCs demonstrated least ECM accumulation. sGAG and collagen level normalized by the dry weight of the construct significantly increased by 72% and 479%, respectively, in AMSCs expanded with CM. In addition, the

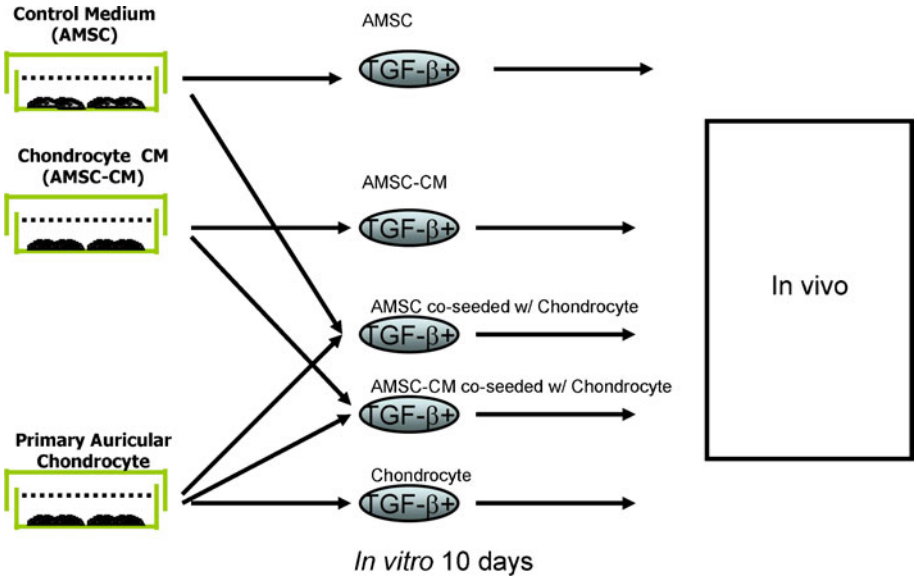

Fig. 4 Schematic methods used to engineer cartilage constructs with AMSCs and chondrocytes. AMSCs were expanded with growth medium (GM) or conditioned medium (CM) followed by 10 days three-dimensional culture with the atellocollagen gel and the PLLA/PLCL scaffolds with or without chondrocytes. For 10 days *in vitro* stimulation serum-free chondrogenic medium with TGF-β1 was utilized.

biochemical contents of co-seeded constructs were similar to those of scaffolds seeded with chondrocytes alone, indicating that primed AMSCs made a similar biochemical contribution as the chondrocytes. This demonstrates that CM-primed AMSCs can undergo *in vivo* chondrogenic commitment. Interestingly, when normalized to the amount of DNA, constructs co-seeded with primed AMSCs and chondrocytes resulted in the highest sGAG and collagen levels (Fig. 7d, e).

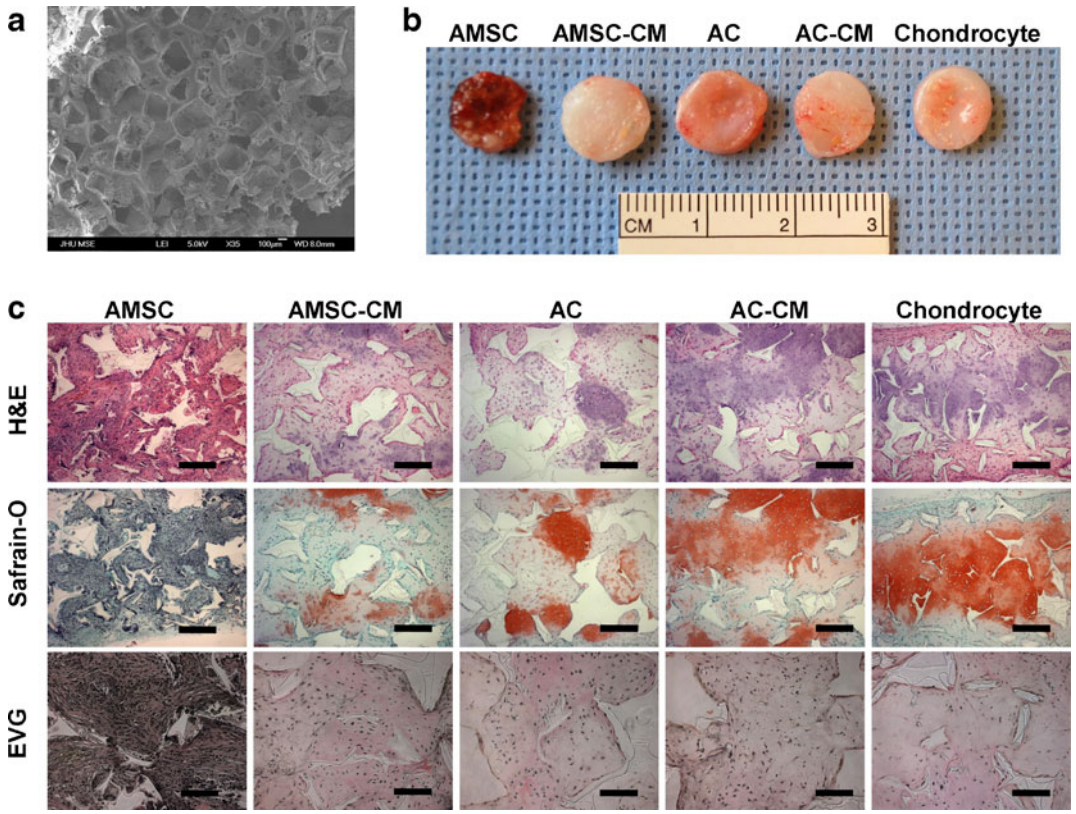

Fig. 5 *In vivo* engineered cartilage with primed AMSCs. (**a**) SEM of the scaffold. (**b**) The gross appearance of engineered tissues after 6 weeks *in vivo*. (**c**) Histological finding stained with hematoxylin and eosin (H&E), Safranin-O/fast green (Safranin-O), and Elastica Verhoeff-van Gieson (EVG) after 6 weeks *in vivo*. AMSC: GM-expanded AMSCS, AMSC-CM: CM-expanded AMSCS, AC: Co-seeded constructs with GM-expanded AMSCs and chondrocytes, AC-CM: Co-seeded constructs with CM-expanded AMSCs and chondrocytes. *Bar* = 100 μm.

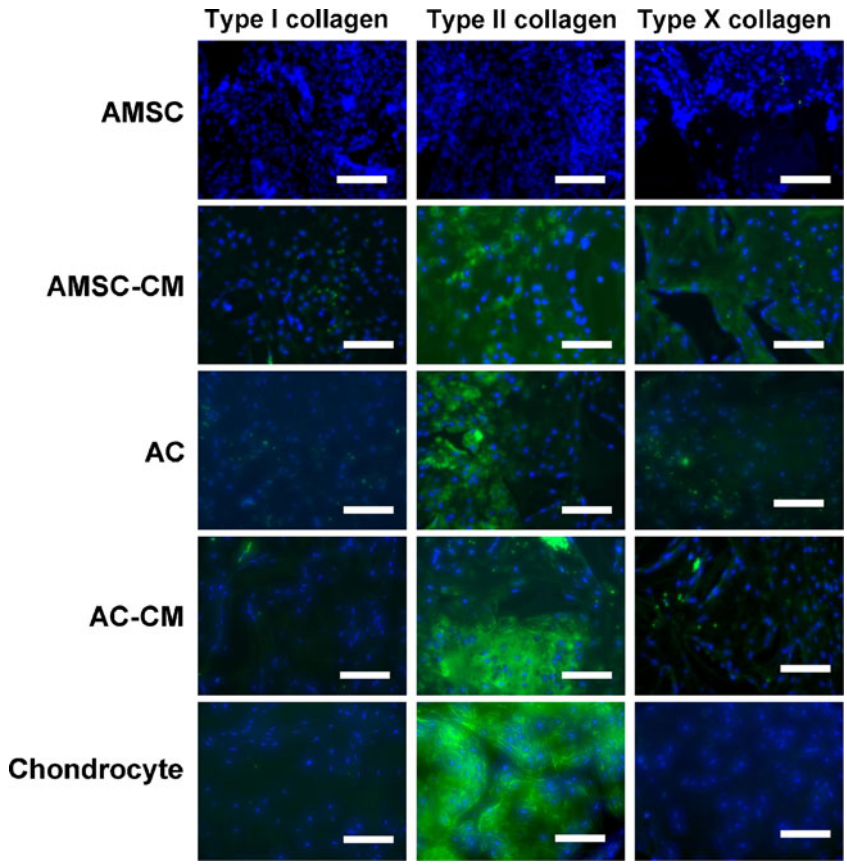

Fig. 6 Immunostaining of engineered cartilage. I mmunostaining of anti-type I collagen, anti-type II collagen, and anti-type X collagen antibodies were performed in the middle area of the implants. AMSC: GM-expanded AMSCS, AMSC-CM: CM-expanded AMSCS, AC: Co-seeded constructs with GM-expanded AMSCs and chondrocytes, AC-CM: Co-seeded constructs with CM-expanded AMSCs and chondrocytes. *Bar* = 100 μm.

DISCUSSION

Mesenchymal stem cells isolated from adipose tissue (AMSCs) are multipotent cells capable of differentiating into connective tissues, and several attempts have been made to achieve engineered cartilage utilizing AMSCs (25–27). The objectives of this study were to evaluate the chondrogenic differentiation of AMSCs and their *in vivo* chondrogenic potential through chondrocyte-mediated paracrine signaling. The chondrogenic differentiation process is tightly coupled with the action of multitude growth and differentiation factors, and without such stimuli, mesenchymal cells would lack the capacity for spontaneous chondrogenic differentiation. Recently, several studies revealed that chondrocytes are capable of producing and releasing numerous growth factors and cytokines, including cytokine-like protein 1 (Cytl1), bone morphogenetic protein-2 (BMP-2), parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), and TGF-β1 (28–31). Consequently, they are likely present in the chondrocyte CM utilized in the current study. Factors that cause chondrogenic commitment of MSCs have been shown to inhibit MSC proliferation. For example, Worster et al. studied the effects of TGFs on equine MSCs, and they demonstrated that the pretreatment with 5 ng/ml of TGF-β1 promoted subsequent chondrogenesis of MSCs while suppressing proliferation (23). Similar to TGF-β1, our data suggest that CM treatment allowed chondrogenic commitment of AMSCs and resulted in the slower growth rate of AMSCs.

TGF-β1 is a multifunctional polypeptide that regulates a variety of biological functions. Previous reports showed that partial chondrogenic differentiation was observed in TGF-β1-treated MSCs monolayer culture (17). Furthermore, TGF-β1 mediated upregulation of sGAG, and collagen biosynthesis has been demonstrated (23,32). However, when considering chondrogenic differentiation of AMSCs, the use of TGF-β1 alone had a limited value. Indeed, our results indicate that CM-expanded AMSCs on the 3D scaffold showed increased expression of CO2A1 compared to the GM-expanded AMSCs treated with TGF-β1. This study also demonstrated that these chondrocyte-secreted factors are synergistic with TGF-β1. Priming of AMSCs with CM was achieved as the CM-expanded cells showed increased sensitivity to the TGF-β1 treatment.

Recently, Gelse and colleagues reported a dual role for transplanted chondrocytes (12). First, the transplanted chondrocytes contributed to neo cartilage formation (12). In addition, the transplanted chondrocytes have the capability to act as a source of potent chondrogenic factors by significantly stimulating cell invasion and chondrogenic

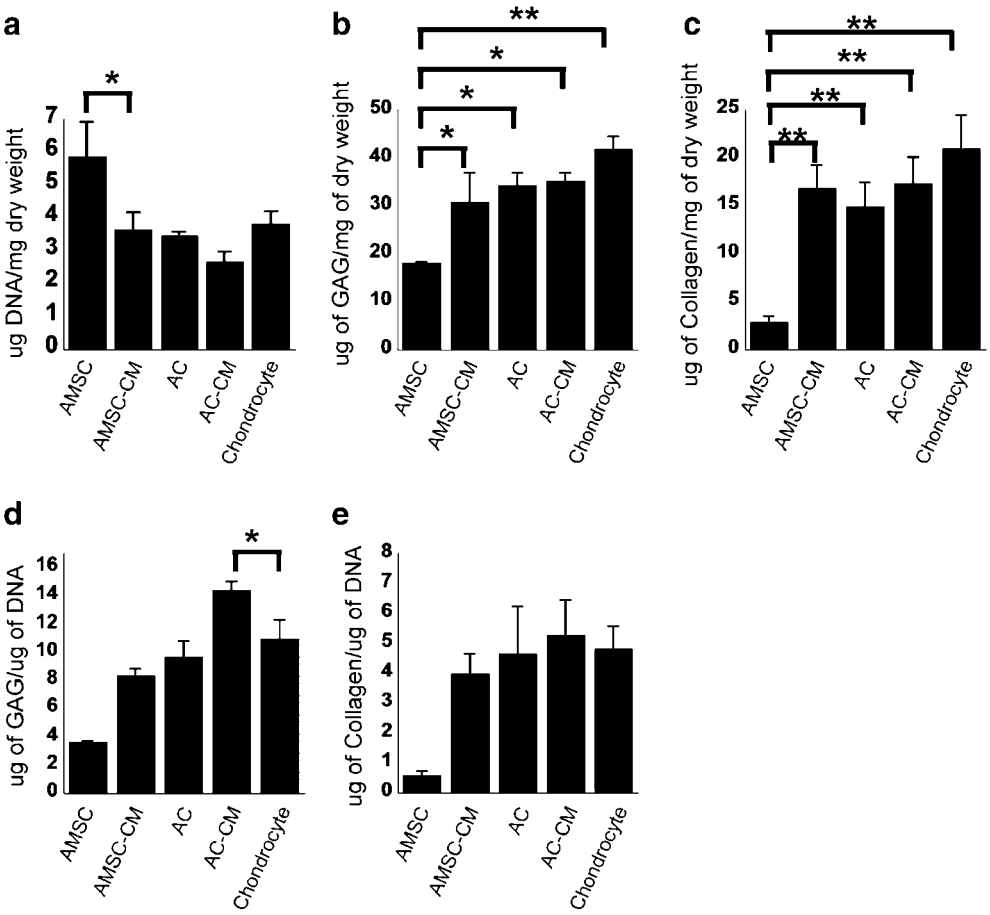

Fig. 7 Analysis of the tissue-engineered cartilage implants harvested after 6 weeks *in vivo*. DNA amounts (**a**), glycosaminoglycan (**b**), and collagen (**c**) are normalized against the dry weight of engineered tissues. In addition, glycosaminoglycan (**d**) and collagen (**e**) contents are normalized against total DNA. Values represent the mean ± standard error of four different samples. AMSC: GM-expanded AMSCS, AMSC-CM: CM-expanded AMSCS, AC: Co-seeded constructs with GM-expanded AMSCs and chondrocytes, AC-CM: Co-seeded constructs with CM-expanded AMSCs and chondrocytes. $*p < 0.05$, $**p < 0.01$.

differentiation of MSCs from the bone marrow, thereby inducing secondary cartilaginous tissues (12). These secondary cartilaginous tissues were characterized by high proteoglycan content, and they stained positive for type II collagen but negative for type I collagen. In addition, a study by Xie and colleagues also reported that co-transplantation of chondrocytes and progenitor cells mediated differentiation of stem cells through paracrine signaling (33). They sorted out Flk-1-positive cells from mouse embryonic stem cells (ES), and these cells were seeded onto polyglycolic acid (PGA) discs with chondrocytes and implanted subcutaneously into nude mice. Flk-1-positive cells formed pure cartilage with mature chondrocytes, whereas Flk-1-positive cells alone could not form cartilage in the subcutaneous environment, indicating that the chondrocyte-secreted factors mediated chondrogenic commitment of Flk-1-positive cells. Similarly, our current study demonstrates that the co-culture of primed AMSCs with chondrocytes had a profound effect on their differentiation and tissue forming abilities *in vivo*. Co-transplantation with chondrocytes resulted in biochemical contents that were similar to the cartilage engineered with primary chondrocytes. In addition to co-transplantation, our biochemical analysis of sGAG and collagen from engineered cartilage support the claim that the chondrocyte CM can be utilized for the efficient cartilaginous tissue formation by AMSCs *in vivo*. The cellular morphology of the engineered cartilage with primed AMSCs or co-seeded constructs were similar to that of the chondrocyte-seeded constructs. Even though there are localized areas with intense Safranin-O staining, probably due to the localization of chondrocytes, the co-seeded AMSCs showed an improved ability to form cartilage tissues by the presence of cartilage-specific ECM production throughout the engineered tissue.

For the researchers to direct chondrogenic differentiation with exogenous cytokines, growth factors, and ECM substratum would require a prolonged duration of *in vitro* culture (34,35). Our preliminary studies have demonstrated that chondrocyte-secreted morphogenetic factors can be used for directing and generating efficient *in vivo* cartilaginous tissue from AMSCs. However, previous reports have indicated that the microenvironment produced during chondrogenesis regulates the subsequent osteogenic differentiation of MSCs, suggesting that soluble factors from chondrocytes may play a significant role in multiple steps of endochondral ossification (36,37). Likewise, *in vitro* differentiation of mesenchymal stem cells into cartilaginous tissues has been shown to undergo hypertrophy and eventual calcification, leading to failure after transplantation (34,38).

Therefore, use of AMSCs may result in inevitable problems, such as induction of hypertrophic differentiation of transplanted cells, which can lead to endochondral ossification resulting in stiff and brittle tissues and ultimate failure. However, no hypertrophy of engineered cartilage was observed, indicating that co-culture with fully differentiated chondrocytes may provide inhibitory effects on the hypertrophy and endochondral ossification process. Indeed, a number of researchers have performed co-culture experiments with articular chondrocytes and MSCs and reported that chondrocytes may secrete factors, such as PTH/PTHrP, to prevent hypertrophy of mesenchymal cells (13,39). Similarly, we observed that CM-expanded AMSCs resulted in hypertrophic matrices, while chondrocyte co-seeded constructs resulted in no detectable level of type X collagen.

Despite this significantly increased chondrogenic potential via CM expansion and co-culture, there is a need to further investigate the morphogenetic factors responsible for chondrogenic priming of AMSCs. Indeed, a recent paper by Kim *et al.* showed Cytl1 as a novel chondrocyte-secreted factor that may induce chondrogenic commitment of mesenchymal cells (28). A pilot study in our laboratory with recombinant Cytl1 demonstrated that these factors synergistically enhanced chondrogenic differentiation of AMSCs in pellet culture (data not shown). Therefore, chondrocyte-secreted factors may be one strategy to *in vivo* engineer cartilage tissues. We are currently harnessing these chondrocyte-secreted factors for controlled delivery into cartilage lesions.

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