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Collagen Type II Enhances Chondrogenesis in Adipose Tissue–Derived Stem Cells by Affecting Cell Shape

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Ideally, biomaterials have inductive properties, favoring specific lineage differentiation. For chondrogenic induction, these properties have been attributed to collagen type II. However, the underlying mechanisms are largely unknown. This study aimed to investigate whether collagen type II favors chondrogenic induction by affecting cell shape through $\beta 1$ integrins and Rho A/Rock signaling. For this purpose, adipose tissue–derived stem cells (ASCs) were encapsulated in collagen type I or II gels and cultured in plain and chondrogenic medium. It was demonstrated that (i) ASCs showed more efficient chondrogenic induction (higher *collagen X*, *aggrecan*, *sox6*, *sox9*, and *collagen II* gene expression) in both plain and chondrogenic media in collagen type II versus collagen type I gels; (ii) ASCs showed lower *Rock 2* gene expression and a more rounded cell shape in collagen type II versus type I gels when grown in plain medium; (iii) Rock inhibitor (Y27632) more effectively enhanced chondrogenic gene expression of ASCs in collagen type I than in collagen type II gels, and diminished differences in chondrogenic gene expression and cell shape of ASCs between the two gel types; and (iv) $\beta 1$ integrins blocking not only reduced the differences of chondrogenic gene expression but also eliminated the differences of *Rock 1* and *Rock 2* gene expressions and cell shape when comparing ASCs embedded in collagen type I and II gels. We conclude that collagen type II provides the inductive signaling for chondrogenic differentiation in ASCs by evoking a round cell shape through $\beta 1$ integrin–mediated Rho A/Rock signaling pathway.

Introduction

DAMAGED CARTILAGE TISSUES (e.g., articular cartilage and nucleus pulposus of the intervertebral disc) related to degenerative diseases and traumatic injury are a major health problem around the world,^{1,2} because of their limited self-repair capability, low cell content, and low supply of progenitor cells.^{3,4} Despite the recent advances in surgical and nonsurgical interventions, no treatments that can effectively restore or repair the damaged cartilage are yet available.⁵ Tissue engineering offers a unique therapeutic opportunity for the repair of damaged tissues.^{4,5} There are three key elements in the concept of tissue engineering: cells, scaffolds (biomaterials), and bioactive factors. Their proper combination might create a surgically transplantable product that promotes tissue regeneration and restores the intrinsic biological function of the tissue.

Among the three key elements of tissue engineering concept, the choice of scaffolds (biomaterial) is critical for a successful tissue engineering approach. The ideal biomaterial

should be able to provide suitable biomechanical and biocompatibility properties to the implanted tissue, and also a favorable environment for cell attachment. Moreover, in particular in the case of tissue engineering using stem cells, biomaterials should preferably have inductive properties that favor a specific lineage differentiation.^{6,7} For instance, bone matrix extracts favor osteoblastic differentiation of human bone marrow–derived mesenchymal stem cells (MSCs)⁸; cartilaginous extracellular matrix (ECM) components, however, favor the chondrogenic differentiation of MSCs.^{9,10} Therefore, it is imperative to know the underlying mechanisms by which the different biomaterial-based scaffolds exert their distinct lineage differentiation induction.

Cell shape has recently been identified as an important determinant for stem-cell differentiation,^{11,12} and chondrogenesis benefits from round cell shape.^{13–15} When cells are seeded in/on a three-dimensional scaffold, cell shape can be largely affected by cell membrane receptor–scaffold (ECM) interactions.^{16,17} $\beta 1$ integrins are one of the main cell surface receptors to interact with ECM molecules. The

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extracellular domain of $\beta 1$ integrins recognizes and binds to ECM proteins, whereas the intracellular domain associates with cytoskeleton elements and subsequently regulates cell shape and the differentiation of MSCs.¹⁴ As an upstream regulator of cytoskeleton dynamics, Rho A/Rock signaling, which stimulates stressed fibril formation and promotes elongated cell shape, has been shown to inhibit the multiple stages of chondrogenic differentiation and instead promotes chondrocyte proliferation.^{13,18} In our previous study, we proposed that $\beta 1$ integrin-mediated Rho A/Rock signaling pathway modulates the shape of the cells and subsequently influences the chondrogenic differentiation.

Collagen type II, one dominant component in a pool of ECM molecules of cartilage tissue, has the preference for both maintaining and inducing chondrogenic phenotype.^{9,19–21} In this study, we hypothesized that collagen type II may enhance chondrogenic differentiation by supporting round cell shape through $\beta 1$ integrin-mediated Rho A/Rock signaling pathway. To test this hypothesis, first, chondrogenic induction properties of collagen type I and II were compared by embedding adipose tissue-derived stem cells (ASCs) in collagen type I and II gels, and *Rock 1* and *2* gene expressions and cell shape (characterized by actin cytoskeleton visualization) of ASCs were also compared; second, the effects of Rock inhibitor (Y27632) on chondrogenic differentiation and cell shape between ASCs in collagen I or II gels were further analyzed and compared; finally, the roles of $\beta 1$ integrins in ASCs in determining chondrogenesis, Rock signaling, and cell shape were investigated by blocking ASCs with neutralizing $\beta 1$ integrin antibodies before collagen type I/II embedment.

Materials and Methods

ASC isolation and culture

Retrieval of all human specimens was approved by the human ethical committee of the VU University Medical Center, and informed consent was obtained.

ASCs were isolated from subcutaneous adipose tissue obtained from patients undergoing elective surgical procedures as described previously.²² Briefly, harvested tissue was washed several times with phosphate-buffered saline (PBS) and then enzymatically dissociated with 0.5 U/mL Liberase Blendzyme 3 solution (Roche Diagnostics, Almere, The Netherlands). The dissociated tissue was then passed through a 100- μ m mesh filter (Stokvis & Smith B.V., IJmuiden, The Netherlands) to remove debris, and the resulting single-cell suspension was centrifuged. Pelleted stromal cells were then washed several times with PBS. The stromal cells were plated in 25-cm² tissue culture flasks. Plating and expansion medium consisted of Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, United Kingdom) supplemented with 500 μ g/mL streptomycin sulfate (Sigma, St. Louis, MO), 600 μ g/mL penicillin (Sigma), 50 μ g/mL gentamycin (Gibco), 2.5 μ g/mL fungizone (Gibco), and 10% fetal bovine serum (Hyclone, Logan, UT). Cultures were grown in a humidified incubator at 37°C in an atmosphere of 5% CO₂. ASCs used for the experiments were at passages 2–4.

Embedment of ASCs in collagen type I/II hydrogels and in vitro chondrogenesis

Collagen type I/II hydrogels were prepared by mixing five parts of collagen type I (rat tail; R&D Systems, Minneapolis,

MN) or collagen type II (chicken sternal cartilage; Sigma), one part of 10 $\times$ DMEM, one part of reconstitution buffer (2.2 g NaHCO₃ in 100 mL of 0.05 N NaOH and 200 mM HEPES), one part of 20 mM acetic acid, and two parts of cell suspension in culture medium. The final concentration of collagen type I/II was 2.5 mg/mL, and the cell density was 1.5 $\times$ 10⁶ cells/mL gel. All the components above were mixed on ice, and 100 μ L mixtures were seeded onto six-well plates for the study. The collagen gel lattices were formed by placing the plates in a humidified incubator at 37°C in an atmosphere of 5% CO₂ for 1 h. Thereafter, the culture medium, either plain medium (DMEM supplemented with ITS+™ Premix [final concentration in medium when diluted 1:100 insulin, 6.25 μ g/mL; transferrin, 6.25 μ g/mL; selenous acid, 6.25 ng/mL; bovine serum albumin, 1.25 mg/mL; linoleic acid, 5.35 μ g/mL; R&D Systems], 500 μ g/mL streptomycin sulfate [Sigma], 600 μ g/mL penicillin [Sigma], and 2.5 μ g/mL fungizone [Gibco]) or chondrogenic medium (plain medium supplemented with 7.5 ng/mL transforming growth factor beta 1 [TGF- β 1; R&D Systems]) was carefully overlaid on each gel. The medium was refreshed every 3–4 days till day 14.

$\beta 1$ integrins blocking and supplement of Rock inhibitor

In $\beta 1$ integrin blocking studies, ASCs were preincubated for 45 min with plain medium supplemented with either mouse nonimmune IgG (50 μ g/mL; Vector Labs, Burlingame, CA) as control or neutralizing $\beta 1$ integrin-blocking antibody (25 μ g/mL, CD29; R&D Systems) as experimental group before embedding in collagen type I/II gels. Thereafter, the pretreated cells were used for the preparation of collagen gels.

For Rock inhibiting experiments, the medium was supplemented with either dimethyl sulfoxide (DMSO) as vehicle control (1 μ L in 1 mL) or 10 μ M Y27632 (dissolved in DMSO) as experimental group. The inhibitors were added every 24 h, and the medium was changed every 3 days until harvesting.

Confocal microscopy

For observing F-actin distribution of ASCs, ASC-loaded gels were fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2) for 20 min and subsequently treated with 2% glycine/1% bovine serum albumin in PBS for 30 min. Thereafter, cells were permeabilized with 0.5% Triton X-100 in PBS for 20 min. Actin fibers within the ASCs were stained with 2.5 U rhodamine phalloidin (Invitrogen, Breda, The Netherlands) in PBS in the dark for 1 h and subsequently observed by confocal fluorescence microscopy with a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Real-time polymerase chain reaction analysis

Total RNA was extracted from cultured cells using Trizol (Invitrogen) according to the manufacturer's instructions. Seven hundred and fifty nanograms of total RNA was reverse transcribed in 20 μ L of reverse transcriptase (RT)-polymerase chain reaction (PCR) mix (5 U transcriptor reverse transcriptase [Roche Diagnostics], 0.08 A260 units random primers [Roche Diagnostics], 1 mM of each of dNTP [Invitrogen], 10 U protector RNase inhibitor [Roche Diagnostics], and 1 $\times$ transcriptor RT buffer) at 55°C for 30 min and

TABLE 1. THE PRIMERS USED FOR REAL-TIME POLYMERASE CHAIN REACTION

Gene		Oligonucleotide sequence	Product size (bp)
18S	Forward	5'-GTA ACC CGT TGA ACC CCA TT-3'	151
	Reverse	5'-CCA TCC AAT CGG TAG TAG CG-3'	
Collagen type II	Forward	5'-GGA TGG GCA GAG GTA AAT G-3'	255
	Reverse	5'-GGT CCT TTG GGT CCT ACA A-3'	
Collagen type X	Forward	5' CAC TAC CCA ACA CCA AGA CA-3'	225
	Reverse	5'-CTG GTT TCC CTA CAG CTG AT-3'	
Aggrecan	Forward	5'-CAA CTA CCC GGC CAT CC-3'	160
	Reverse	5'-GAT GGC TCT GTA ATG GAA CAC-3'	
sox5	Forward	5'-CTC ACT TGA CAG GTT CAG-3'	208
	Reverse	5'-CGT CAC TCT CCT CTT CTT C-3'	
sox6	Forward	5'-CTC CCA CCC ACA AAT TAA CC-3'	207
	Reverse	5'-GAG ACC GTC CCT GCT GTG TT-3'	
sox9	Forward	5'-CCC AAC GCC ATC TTC AAG G-3'	242
	Reverse	5'-CTG CTC AGC TCG CCG ATG T-3'	

then at 85°C for 5 min to inactivate the transcriptase. Real-time PCR was performed to determine the gene expression of *aggrecan*, *collagen type X*, *collagen II*, *sox5*, *sox6*, *sox9*, *Rock 1*, and *Rock 2* by lightcycler (Roche LC 480). All the target genes were normalized to housekeeping gene *18S* to obtain the relative gene expression. Primers used in real-time PCR are listed in Table 1.

Statistical analysis

Data were obtained from five independent donors. For statistical analysis, Levene's test was firstly performed to determine the homogeneity of variance for all the data, and then the data were analyzed by repeated measures in general linear model followed by Tukey *post hoc* analysis. When the data are not equally distributed, log transformation was used. SPSS 13.0 program (Chicago, Illinois) was employed for all statistic analysis, and differences were considered significant if $p < 0.05$.

Results

Collagen type II favors chondrogenic induction in ASCs compared with collagen type I

ASCs embedded in collagen type I or II gels were grown in either plain or chondrogenic medium for 4 and 14 days, and chondrogenic gene expression of ASCs (*collagen X*, *aggrecan*, *sox5*, *sox6*, *sox9*, and *collagen type II*) was analyzed. Our results showed that the responses of ASCs to chondrogenic induction largely depended on the collagen type in which they were encapsulated: for ASCs in collagen type II gels, TGF- β 1 (chondrogenic condition) significantly upregulated the gene expression of *collagen type X* (Fig. 1A) but not of other chondrogenic markers (*aggrecan*, *sox5*, *sox6*, and *sox9*; Fig. 1B–E). TGF- β 1 also advanced the induction time of *collagen type II* gene expression from day 14 in plain medium to day 4 in chondrogenic medium (Table 2). However, for ASCs in collagen type I gels, TGF- β 1 (chondrogenic condition) significantly upregulated the gene expressions of *collagen type X*, *sox6*, and *sox9* (Fig. 1A, D, E), and it also increased the induction frequency of *collagen type II* gene expression among the five donors (Table 2).

Chondrogenic gene expression was also compared between ASCs in collagen type I and II gels. In both plain and chondrogenic medium, ASCs in collagen type II gels displayed

more efficient chondrogenic induction than those in collagen type I gels: significant higher *aggrecan*, *sox6*, and *sox9* gene expressions at day 14 in plain medium, and higher *collagen type X* and *sox9* gene expressions at day 14 in chondrogenic medium (Fig. 1F). Under both plain and chondrogenic conditions, *collagen type II* gene expression was also induced more frequently and at an earlier time point when comparing type II and type I collagen gels (Table 2).

Collagen type II gel embedment of ASCs results in lower *Rock* gene expression and more round cells presented than collagen type I gel embedment

To address the question why collagen type II gel favors chondrogenic differentiation of ASCs, gene expression of *Rock 1* and *Rock 2* as well as the ASC cell shape in collagen type I and II gels were determined. When ASCs embedded in either collagen type I or II gels were cultured in plain or chondrogenic medium for 4 and 14 days, the following comparisons were made.

- The effects of chondrogenic condition on *Rock* gene expression of ASCs in either collagen type I or II gels: it was found that only at the 14-day time point, *Rock 1* and *Rock 2* gene expressions of ASCs in collagen type I gels were significantly downregulated by chondrogenic condition but not in collagen type II gels (Fig. 2A, B).
- The effects of collagen gel type embedded on *Rock* gene expression of ASCs under plain or chondrogenic conditions: under plain medium, a significant down-regulation was observed for *Rock 2* at day 4 and a similar trend for *Rock 1* at the same time point in collagen type II gels versus collagen type I gels, and expression differences were lost after 14 days. However, chondrogenic conditions leveled out the differences observed under plain conditions (Fig. 2C).

Previously, we reported that ASCs seeded in collagen type II gels, but not in collagen type I gels, are prone to aggregate after several days of culturing in plain medium⁹ and that only small amounts of ASCs in collagen type I gels displayed rounded cell shapes, whereas most of them remained elongated with stressed actin cytoskeleton across the whole cell.²³ The latter observations were confirmed in this study (Fig. 3A). In contrast, most of the ASCs embedded in collagen

FIG. 1. Comparisons of chondrogenic gene expression between adipose tissue-derived stem cells (ASCs) embedded in collagen type I and type II three-dimensional (3D) gels. After ASCs were embedded in either collagen type I or II gels and cultured for 4 and 14 days, ASCs showed different chondrogenic gene expression profiles in response to chondrogenic induction and the type of collagen used. (A) ASCs in both collagen type I and II gels displayed significant higher induction of *collagen X* gene expression under chondrogenic conditions. (B) There was no significant induction of *aggrecan* gene expression in ASCs in either collagen type I or II gels under chondrogenic conditions. (C) There was no significant induction of *sox5* gene expression of ASCs in either collagen type I or II gels under chondrogenic conditions. (D) *sox6* gene expression of ASCs was only significantly induced in collagen type I gels at day 14 under chondrogenic conditions. (E) *sox9* gene expression of ASCs was only significantly induced in collagen type I gels at day 14 under chondrogenic conditions. (F) ASCs in collagen type II gels showed higher chondrogenic gene expressions than those in collagen type I gels in both plain (*aggrecan*, *sox6*, and *sox9* at day 14) and chondrogenic (*collagen X* and *sox9* at day 14) medium. Data were represented as mean $\pm$ standard error of mean (SEM), $n = 5$ [(A–E) relative gene expression; (F) ratios of relative gene expression of ASCs in collagen type II gels vs. ASCs in collagen type I gels; dashed line, no difference]. Col, collagen. * $p < 0.05$.

type II were characterized with round cell shapes, and relaxed actin cytoskeleton smoothly distributed underneath of the cell membranes (Fig. 3D). Under chondrogenic conditions, cell shapes between ASCs in collagen type I and II gels did not show apparent differences; in other words, chondrogenic conditions induced round cell shapes of ASCs in collagen type I gels but did not further change the round cell shapes of ASCs in collagen type II gels except that they prevented ASC cell aggregation (Fig. 3G, J).

Rock inhibitor differently affects chondrogenic gene expression and cell shape of ASCs in collagen type I or II gels

Next, it was examined whether collagen type II exerted its chondrogenic induction properties through the modulation of Rock signaling. ASCs embedded in either collagen type I or II gels were cultured in medium supplemented with Rock inhibitor (Y27632) or vehicle (DMSO) for 4 and

TABLE 2. INDUCTION FREQUENCY OF COLLAGEN TYPE II GENE EXPRESSION OF ADIPOSE TISSUE-DERIVED STEM CELLS EMBEDDED IN DIFFERENT COLLAGEN GELS AND WITH DIFFERENT TREATMENTS

Gel type	Treatments	Plain medium			Chondrogenic medium		
		Day 4	Day 14	Total	Day 4	Day 14	Total
Collagen type I	Control	0	2	2	2	3	4
	Y27632	4	4	4	5	3	5
	β 1 integrin blocking	1	4	4	5	2	5
Collagen type II	Control	1	4	4	4	2	5
	Y27632	4	5	5	5	3	5
	β 1 integrin blocking	4	5	5	5	3	5

14 days. Differential responses to Y27632 were found for ASCs in collagen type I and II gels with respect to chondrogenic gene expression.

In collagen type I gels, Y27632 significantly upregulated *sox6* gene expression in ASCs cultured in plain medium (Fig. 4A) and also exhibited increased induction frequency and advanced induction time (from day 14 to day 4) of *collagen type II* gene expression in ASCs embedded in both plain and chondrogenic medium (Table 2). For ASCs in collagen type II gels, however, Y27632 did not significantly affect chondrogenic marker expression in ASCs except that it advanced the

induction time (from day 14 to day 4) of *collagen type II* gene expression of ASCs cultured in both plain and chondrogenic medium (Fig. 4B and Table 2). The significant differences of chondrogenic gene expression (*collagen type X*, *aggrecan*, *sox6*, and *sox9*) observed in ASCs embedded in collagen type II versus type I gels (Fig. 1F) were largely diminished after Y27632 was added in the culturing media (Figs. 1F and 4C; Table 2).

In concert with the effects of Y27632 on chondrogenic gene expression of ASCs, Y27632 also had differential effects on the cell shapes of ASCs in collagen type I and II gels. In plain medium in collagen type I gels, Y27632 induced some

FIG. 2. Comparisons of *Rock 1* and *2* gene expression between ASCs embedded in collagen type I and collagen type II 3D gels. After ASCs were embedded in either collagen type I gels or II gels and cultured for 4 and 14 days, ASCs showed different *Rock 1* and *2* gene expression profiles in response to chondrogenic induction and the type of collagen used. (A) *Rock 1* gene expression of ASCs was only significantly downregulated in collagen type I gels at day 14 under chondrogenic conditions. (B) *Rock 2* gene expression of ASCs was only significantly downregulated in collagen type I gels at day 14 under chondrogenic conditions. (C) In plain medium, *Rock 2* gene expression of ASCs in collagen type II gels was significantly lower than in collagen type I gels. Data were represented as mean $\pm$ SEM, $n = 5$ [(A, B) relative gene expression; (C) ratios of relative gene expression of ASCs in collagen type II gels vs. ASCs in collagen type I gels; dashed line, no difference]. Col, collagen. * $p < 0.05$.

FIG. 3. Comparisons of cell shape or actin cytoskeleton distribution between ASCs in collagen type I or II 3D gels before and after treatment with Rock inhibitor (Y27632) or $\beta 1$ integrin blocking. (A–F) In plain medium, most of the ASCs in collagen type I gels remained elongated, containing stressed actin cytoskeleton across the whole cell (A); in contrast, most of the ASCs in collagen type II were characterized with round cell shapes and relaxed actin cytoskeleton smoothly distributed underneath the cell membranes (D); Y27632 induced some round ASCs in collagen type I gels with less stressed and/or granule-like actin cytoskeleton (B); however, Y27632 did not further change the round cell shape of ASCs but induced some granule-like actin cytoskeleton as well (E); $\beta 1$ integrin blocking induced a round cell shape in ASCs in collagen type I gels (C), but it did not further change the distribution of the actin cytoskeleton and round cell shape of ASCs in collagen type II gels (F). (G–L) Under chondrogenic conditions, all the ASCs in collagen type I and II gels looked similar and displayed round cell shapes irrespective of whether they were embedded in collagen type I or II gels or treated with Y27632 or $\beta 1$ integrin blocking. Col, collagen. Scale bars: 50 μ m. Color images available online at www.liebertonline.com/ten.

irregular ASCs, and these cells were characterized with less stressed and/or granule-like actin cytoskeleton compared with control incubations (Fig. 3A, B). For ASCs in collagen type II gels, however, Y27632 did not further change the round cell shapes of ASCs except to induce some granule-

like actin cytoskeleton (Fig. 3D, E). In chondrogenic medium, ASCs in collagen type I or II gels still kept round cell shapes, but some actin cytoskeleton protruded from the cellular membranes of ASCs in both collagen type I and II gels (Fig. 3H, K).

FIG. 4. Effects of Rock inhibitor on chondrogenic differentiation in ASCs in collagen type I or II 3D gels. After ASCs were embedded in either collagen type I or II gels and cultured in medium supplemented with Rock inhibitor (Y27632) for 4 and 14 days, ASCs in collagen type I and II gels showed different responses to Y27632 with respect to chondrogenic gene expression. (A) Y27632 significantly upregulated *sox6* gene expression of ASCs when they were embedded in collagen type I gels. (B) Y27632 did not significantly affect any chondrogenic marker in ASCs embedded in collagen type II gels. (C) Only *collagen type X* gene expression was significantly higher in ASCs in collagen type II gels compared with those in collagen type I gels at day 14. Data were represented as the ratios of mean $\pm$ SEM, $n = 5$ [(A, B) relative gene expression of experimental group treated with Y27632 vs. control group treated with dimethyl sulfoxide; (C) relative gene expression of ASCs in collagen type II gels vs. ASCs in collagen type I gels; dashed line, no difference]. Col, collagen. * $p < 0.05$.

$\beta 1$ integrins blocking in ASCs differently affects chondrogenic and Rock gene expression and ASC shape in collagen type I and II gels

Finally, we investigated whether $\beta 1$ integrins participated in collagen type II-mediated chondrogenic induction by regulating Rock signaling and cell shape. Comparisons of chondrogenic and *Rock 1/2* gene expression and cell shape between ASCs in collagen type I and II gels were performed after ASCs were preincubated with neutralizing $\beta 1$ integrin antibodies before collagen gel embedment. The significant differences of chondrogenic (*collagen type X*, *aggrecan*, *sox6*, and *sox9*) and *Rock* gene expressions of ASCs resulted from collagen type I and II gel embedment were eliminated after $\beta 1$ integrins in ASCs were blocked by neutralizing $\beta 1$ integrin antibodies (data not shown), although *collagen type II* gene expression of ASCs in collagen type II gels was still more frequently induced than those in collagen type I gels (Table 2).

In plain medium, as previously reported, $\beta 1$ integrin blocking in ASCs dramatically changed the shapes of ASCs and induced round cell shapes when ASCs were embedded in collagen type I gels (Fig. 3C). However, $\beta 1$ integrin blocking did not further change the round cell shape or actin cytoskeleton distribution of ASCs in collagen type II gels (Fig. 3F). In chondrogenic medium, ASCs in collagen type I or II gels remained round cell shapes with actin cytoskeleton distributed underneath of cellular membranes after $\beta 1$ in-

tegrins were blocked by $\beta 1$ integrin neutralizing antibodies (Fig. 3I, L).

Discussion

The function of scaffolds has been extended to bio-functionality: not only the fundamental function of supporting cell attachment and cell proliferation but also the inductive function of driving stem cells into specific lineage differentiation. Many efforts have been made to reveal the mechanisms of scaffold-mediated inductive potential, for example, that the stiffness of substrate or the pore size of scaffold can dramatically affect the differentiation of stem cells attached.^{11,24,25} In this study, we demonstrated that collagen type II possessed a higher chondrogenic inductive property compared with collagen type I, and that collagen type II gel embedment of ASCs downregulated *Rock 2* gene expression and supported round cell shape. Further, we showed that Rock inhibitor (Y27632) largely diminished the differences of chondrogenic gene expression and cell shape of ASCs induced by collagen type I and type II gel embedment. Finally, our data showed that $\beta 1$ integrin blocking in ASCs also largely eliminated the differences of chondrogenic and *Rock* gene expression and cell shape of ASCs resulted from collagen type I and II gel embedment.

The stronger inductive property of collagen type II over collagen type I for maintaining and inducing a chondrogenic

phenotype was shown by our group in various different circumstances (chondrogenic induction by soluble factors secreted by nucleus pulposus cells or by TGF- β 1) and also by other groups.^{9,19,20} This study was performed in an attempt to reveal the mechanisms underlying this phenomenon. We previously reported that ASCs seeded in collagen type II gels are prone to aggregate at around day 7 after they were cultured in plain medium.⁹ In the current study, a similar phenomenon was observed. Also it was found that most of the ASCs in collagen type II were characterized with round cell shapes, and actin cytoskeleton smoothly distributed underneath the cell membranes. It is very interesting to compare these results with data recently published by Wu *et al.*,¹⁰ which showed that a chondroitin sulfate-based microenvironment also promotes cell aggregation and subsequent chondrogenic differentiation. Since both collagen type II and chondroitin sulfate are main components of the cartilaginous ECM, combining these datasets suggests that the ECM microenvironment in cartilage can provide a good "soil" for guiding the chondrogenic differentiation of stem cells and that the ECM does so by influencing the shape of the encapsulated cells.

The stronger chondrogenesis-inductive property of collagen type II over collagen type I prompted us to evaluate its underlying mechanisms. Cell shape-dependent control of lineage commitment is believed to be mediated by Rho A signaling pathway, specifically via its effects on Rock-mediated cytoskeletal tension.^{12,26,27} The evidence of the role of Rho A/Rock-mediated cytoskeleton (especially for actin cytoskeleton) organization in chondrogenic differentiation has accumulated over the last 20 years.¹⁴ It is well known that chondrocytes plated in monolayer culture tend to change their morphology to flattened cells and cease the production of collagen II and glycosaminoglycans (GAGs).^{28,29} The mere addition of inhibitors of actin polymerization (dihydrochalcasin B or cytochalasin D) can stimulate rounding of dedifferentiated chondrocytes and reexpression of these same chondrogenic markers.^{30,31} First, we showed that encapsulating ASCs in collagen type II gels, on one hand, decreased gene expression of *Rock 1/2* and caused less stressed actin cytoskeleton (round cell shape), and on the other hand promoted chondrogenic commitment. Second, although we did not observe that Rock inhibitor (Y27632) induced round cell shape and cortical distribution of cytoskeleton in ASCs as reported in other studies^{13–15} (might be because of less sensitivity of ASCs to the stimulation of Y37632), Y37632 did exert its chondrogenic induction function by upregulating *sox6* gene expression of ASCs in collagen type I gels and also increased the induction frequency of *collagen type II* gene expression of ASCs in both collagen type I and II gels. In addition, Y27632 largely diminished the differences of chondrogenic gene expression found after collagen type I and II embedment. Based on these data, we postulate that collagen type II enhances chondrogenic differentiation and supports round cell shape largely through Rho A/Rock-mediated actin cytoskeleton signaling pathway. However, it is notable that Rho A/Rock signaling might not be the only mechanism underlying the preferential chondrogenic induction property of collagen type II, because ASCs in collagen type II gels still displayed slightly more efficient chondrogenic induction than those in collagen type I gels as shown by more frequent induction of *collagen type II* gene expression.

We previously proposed a model for chondrogenic differentiation of ASCs induced by soluble inductive factors, cell shape, and cell-matrix interactions²³: cell-matrix interaction is able to promote the chondrogenic commitment of stem cells by affecting cell shape but is not sufficient for mature chondrogenic differentiation, and this effect can be largely replaced by the normal chondrogenic induction of growth factors (TGF- β 1) through the Rho/Rock signaling pathway or direct Rho/Rock signaling inhibition (β 1 integrins blocking or Rock inhibitor). The results of the current study were in concert with this proposal: there were significant differences between ASCs in collagen type I and II gels when they were cultured in plain medium, but these differences were largely eliminated by the introduction of chondrogenic conditions, β 1 integrins blocking, or the addition of Y27632.

The roles of β 1 integrins in chondrogenesis have been extensively studied but so far remain elusive.^{32–36} Our previous study showed that β 1 integrins play a more important role in the later stages than in the earlier stages of chondrogenesis, and Rho A/Rock signaling appeared to participate in this process.²³ This study further provided the evidence that ECM can modulate chondrogenesis through β 1 integrins-Rho/Rock signaling pathway and showed that β 1 integrins blocking eliminated or largely eliminated the differences of chondrogenic and *Rock* gene expression resulting from collagen type I and II embedment. However, because of using a general β 1 integrin neutralizing antibody in our previous and current study, we could not deduce which particular subset of the $\alpha\beta$ 1 integrin family was involved in this induction process. Our current, comparative studies on the differential effects of both types of collagen (I and II), combined with the fact that α 1 β 1, α 2 β 1, and α 3 β 1 integrins are the main receptors for collagen type I^{37,38} and α 1 β 1, α 2 β 1, and α 10 β 1 integrins are the main receptors for collagen type II^{34,39,40} suggest a pivotal role for the α 10 β 1 integrin. Unfortunately, since no neutralizing antibodies for the α 10 subunit are available, direct evidence is currently lacking, although some interesting *in vitro* and *in vivo* studies are in support of this hypothesis: (i) the α 10 β 1 integrin is known to be highly expressed in chondrocytes⁴¹; (ii) α 10 β 1 integrin is increasingly expressed in the progress of chondrogenic differentiation^{42,43}; and (iii) α 10 β 1 integrin knockdown results in the failed maturation of chondrocytes *in vivo*.^{42,43} Additional studies using different approaches, such as blocking α 10 expression blocking studies using siRNA technology, are needed to provide more insight into the potential roles of the α 10 β 1 integrin receptor. Interestingly, the fact that the differences of chondrogenic gene expression between ASCs in collagen type I and II gels were not completely eliminated by β 1 integrin blocking may also suggest that so far unidentified additional collagen receptors may be involved in chondrogenic induction processes.

Conclusions

In summary, this study shows that collagen type II scaffolds not only provide support for ASCs but also mediate inductive signaling for chondrogenic differentiation by provoking a round cell shape through β 1 integrin-mediated Rho A/Rock signaling. Moreover, collagen type II enhances the action of chondroinductive factors such as TGF- β 1. These

data highlight the importance of the coordination among cells, scaffolds, and bioactive factors, the three main components in the concept of tissue engineering, and they also have an important implication for designing appropriate scaffolds for cartilage tissue engineering, which not only support the cells but also shape the round cells.

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Disclosure Statement

No competing financial interests exist.

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