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| Original Article |

Chondrogenic Differentiation and Three Dimensional Chondrogenesis of Human Adipose-Derived Stem Cells Induced by Engineered Cartilage-Derived Conditional Media

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Abstract : Due to lack of optimal inductive protocols, how to effectively improve chondrogenesis of adipose-derived stem cells (ASCs) is still a great challenge. Our previous studies demonstrated that the culture media derived from chondrocyte-scaffold constructs (conditional media) contained various soluble chondrogenic factors and were effective for directing chondrogenic differentiation of bone marrow stem cells. Nevertheless, it remains unclear whether the conditional media can induce ASCs towards chondrogenic differentiation, especially for three-dimensional (3D) cartilage formation in a preshaped scaffold. In this study, it demonstrated that the conditional media derived from chondrocyte-scaffold constructs could promote ASCs to differentiate into chondrocyte-like cells, with similar expression of type II collagen to those induced by chondrogenic growth factors. Moreover, the expression level of chondrocyte-specific genes, such as SOX9, type II collagen, and COMP, was even higher in conditional medium group (CM) than that in optimized chondrogenic growth factor group (GF), indicating that the conditional media can serve as an effective inducer for chondrogenic differentiation of ASCs. Most importantly, the conditional media could also induce ASC-scaffold constructs to form 3D cartilage-like tissue with typical lacunae structures and positive expression of cartilage specific matrices, even higher contents of GAG and type II collagen were achieved in CM group compared to GF group. The current study establishes a simple, but stable, efficient, and economical method for directing 3D cartilage formation of ASCs, a strategy that may be more closely applicable for repairing cartilage defects.

Key words: *adipose-derived stem cells, chondrogenic differentiation, chondrogenesis, chondrogenic factors, engineered cartilage-derived conditional media*

1. Introduction

Because of minimal injury to the donor site, abundant autologous cell availability, and multilineage potential,[1,2] adipose-derived stem cells (ASCs) become an attractive cell source for cartilage regeneration.[3,4] However, due to lack of an ideal inductive protocol, ASC-based cartilage engineering has achieved no significant breakthrough so far. Therefore, how to effectively direct ASCs' chondrogenic differentiation for structural cartilage construction remains a major restriction for applying ASCs in cartilage tissue engineering.

In addressing this issue, Hennig *et al*. discovered that one important cause of ASCs' weak chondrogenic potential lies in their inferior expression of key chondrogenic factors as well as their absent expression of TGF-β receptor 1 (TGF-βR1), leading to low sensitivity to conventional growth factor mediated chondrogenic inductions.[5] To compensate this effect, more types of chondrogenic growth factors including bone morphogenetic proteins (BMPs) in high doses are required,[5,6] which inevitably results in complicated manipulations, high cost, and possible side effects such as hypertrophy and *in vivo* calcification.[5,7,8]

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Thus, an alternative approach that circumvents high demand of exogenous factors and promotes potent cartilage formation of ASCs would be more applicable.

Soluble autocrine and paracrine factors secreted by chondrocytes have proved to be effective in enhancing chondrogenesis of both chondrocytes and bone marrow derived stem cells (BMSCs).[9-13] A more recent study reported that the media of monolayer cultured chondrocytes could also promote chondrogenic differentiation of ASCs during *in vitro* pellet culture,[14] which sheds a new light for chondrogenesis of ASCs without the application of high-dose growth factors. However, chondrocytes that underwent monolayer culture tend to age and de-differentiate rapidly, in which process key chondrogenic genes were significantly down-regulated and synthesis and secretion of chondrogenic factors were thus compromised, leading to an inferior chondroinductive efficacy.[15-17]

Our previous study has shown that the concentrations of chondrogenic factors in the culture media of chondrocyte-scaffold constructs (conditional media hereinafter) manifest a stable time-dependent increase in both short and long time courses. Moreover, the conditional media showed strong inductive role for chondrogenic differentiation of BMSCs.[11] Nevertheless, due to differential sensitivities to chondrogenic factors between ASCs and BMSCs,[5] it remains unclear whether the conditional media can induce ASCs towards chondrogenic differentiation. Especially, it is still uncertain whether a three-dimensional (3D) cartilage-like tissue, a practical need for repairing cartilage defects, can be regenerated using ASCs and pre-shaped scaffolds by *in vitro* induction of the conditional media. To address these issues, in the current study, we introduced the conditional medium derived from *in vitro* engineered cartilage as a novel inducer to explore its role in directing chondrogenic differentiation of ASCs. Based on this, we further explored the feasibility of regenerating 3D cartilage using ASCs and pre-shaped biodegradable scaffolds by *in vitro* induction of the conditional media.

2. Materials and Methods

2.1 Cell Isolation and Culture

Human adipose tissue was harvested from patients who underwent liposuction procedures in Shanghai 9th People's Hospital. All of the experimental protocols involving human tissue and cells were approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine. According to previous methods[1], ASCs were digested from adipose tissue with 0.075% collagenase (NB4, Serva, France) and cultured in Dulbecco's modified Eagle media (DMEM, Invitrogen Corp., Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) at 37^{o}C in a 5% CO_2 humidified incubator. Cells were subcultured when they reached 85% confluence. Cells from passage 2 were used for the following experiments.

2.2 Preparation of Conditional Media

Polyglycolic acid (PGA) / polylactic acid (PLA) scaffolds were prepared into cylinders with 5 mm diameter and 1 mm thickness according to previously established methods.[18,19] 0.3 mL articular chondrocytes from newborn pigs were seeded into the scaffold at a concentration of 5×10^7 cells/mL. The chondrocyte-scaffold constructs were cultured in regular DMEM (containing 10% FBS) for 7 days, and then the culture media were collected every other day till week 3.[11] All the media were mixed and stored at -20^{o}C. The mixed media were thawed as conditional media for the following chondroinduction.

2.3 Chondrogenic Induction in Monolayer Culture

To explore the feasibility of the conditional media to direct chondrogenic differentiation, ASCs were cultured in monolayer supplemented with (1) conditional media (CM group), (2) optimized chondrogenic media containing growth factor cocktails (regular DMEM plus 10 ng/mL transforming growth factor beta 3 (TGF-β3), 50 ng/mL insulin-like growth factor 1 (IGF-1), 10 ng/mL BMP-6, and 0.1 μM dexamethasone; R&D, Minneapolis, MN, USA),[5,20,21] (GF group), and (3) regular DMEM plus 10% FBS media (DMEM group), respectively. All the samples were cultured for 21 days (with media change every other day) and then harvested for the evaluation of chondrogenic differentiation with chondrocytes from new-born pig as a positive control.

2.4 Evaluation of Chondrogenic Differentiation in Monolayer Culture

After 21 days of culture, chondrogenic differentiation of ASCs in monolayer culture was firstly evaluated with morphological change and expression of type II collagen detected by indirect immunofluorescence.[22] Then, total RNA was extracted from the cells in different groups and expression of cartilage-specific genes was detected by reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR analysis as previously described.[23-25] The mRNA level of β-actin was quantified as an internal control. The mRNA of chondrocytes was used as a positive control in RT-PCR and real-time PCR.

2.5 Preparation of ASC-PGA/PLA Constructs

PLA coated PGA scaffold was prepared as previously

described.[24] Five milligrams of PGA unwoven fibers were compressed into a cylinder shape with 5 mm diameter and 1 mm thickness. In order to stabilize the scaffold shape, 0.1% PLA (Sigma) dichloromethane solution was used to coat the PGA fibers.

ASCs at passage 2 (5×10^6 cells in 0.1 mL) were then evenly dropped into each scaffold. After incubation for 4 hours to allow for complete adhesion of the cells to the scaffold, the conditional media were added to cover the cell-scaffold construct as experimental group (CM group), optimized chondrogenic media containing growth factor cocktails (GF group) and regular DMEM (DMEM group) were similarly added as controls. Chondrocytes at passage 2 at the same cell density were seeded into the PGA/PLA scaffold as a positive control (CHON group). The constructs were then kept in an incubator for 12 weeks at 37°C with 95% humidity and 5% CO_2 and then harvested for evaluation of chondrogenesis.

2.6 Evaluation of Structural Cartilage Formation *In Vitro*

After 12-week *in vitro* induction, the wet weight of the samples was measured by an electronic balance. ASC chondrogenesis was evaluated histologically by hematoxylin and eosin (H&E), Safranin-O, and Collagen II immunohistochemical staining.[19] The total collagen and glycosaminoglycan (GAG) contents of each sample were determined to quantitatively evaluate cartilage formation in different groups.[26,27] The quantitative amount of type II collagen was further measured by enzyme linked immunosorbent assay (ELISA) as previously described.[28]

2.7 Statistical analysis

Student's *t*-test was used to analyze quantitatively expression level of chondrogenic-related genes and the differences of contents of total collagen, type II collagen, and GAG. $p < 0.05$ was considered statistically significant.

3. Results

3.1 *In vitro* Chondrogenic Differentiation of ASCs Induced by Conditional Media

3.1.1 Morphologic Transformation of ASCs Towards Chondrocyte-like Cells Induced by Conditional Media

The effects of conditional media on chondrogenic differentiation of ASCs were first evaluated by examining changes in cell morphology. Consistent with the previous report,[1] ASCs in the DMEM (non-induced) group showed thin, spindle-like morphology (Fig 1A). However, after 21 days of induction, ASCs in both CM and GF groups (Fig 1B and C) were noticeably expanded and reshaped into a polygonal form similar to chondrocytes (Fig 1D), which provided morphological evidence that ASCs had been induced into chondrocyte-like cells by growth factors and conditional media.

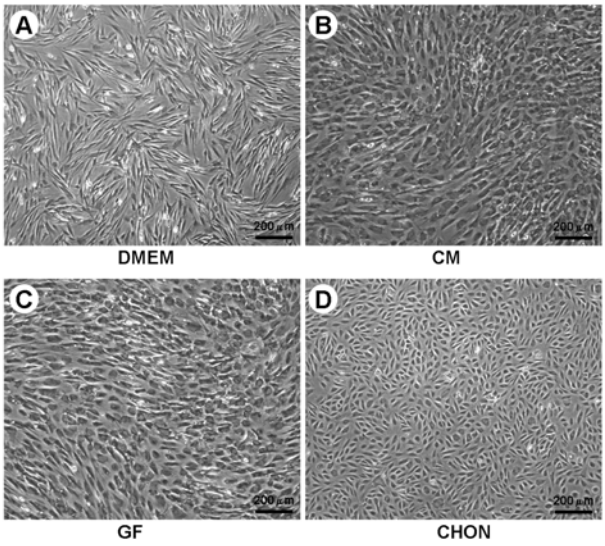

Figure 1. Morphological change of induced ASCs. ASCs in DMEM group (A) showed a spindle-like morphology. After induction with conditional media (B) and growth factors (C), ASCs were expanded and transformed into a polygonal shape similar to chondrocytes (D).

3.1.2 Expression of Type II Collagen in ASCs Induced by Conditional Media

Expression of type II collagen was detected by indirect immunofluorescence to further confirm the chondrogenic differentiation of ASCs. Moreover, green fluorescence was detected in most cells of CM and GF groups (Fig 2B, C), while no green fluorescence was observed in the cells of DMEM group (Fig 2A), indicating that type II collagen was upregulated in CM and GF groups. As expected, in the positive control, strong expression of type II collagen was detected (Fig 2D). These results indicated that conditional media had induced ASCs towards chondrogenic differentiation.

3.1.3 Expression and Quantitative Analysis of Cartilage Specific Genes in ASCs Induced by Conditional Media

The expression of cartilage specific genes was examined to further evaluate the efficiency of chondrogenic differentiation of ASCs induced by conditional media. As shown in Figure 3A, visible positive expression of type II collagen, COMP, SOX9, and aggrecan was observed in the cells of CM and GF groups, although it was still lower than that in positive control (normal

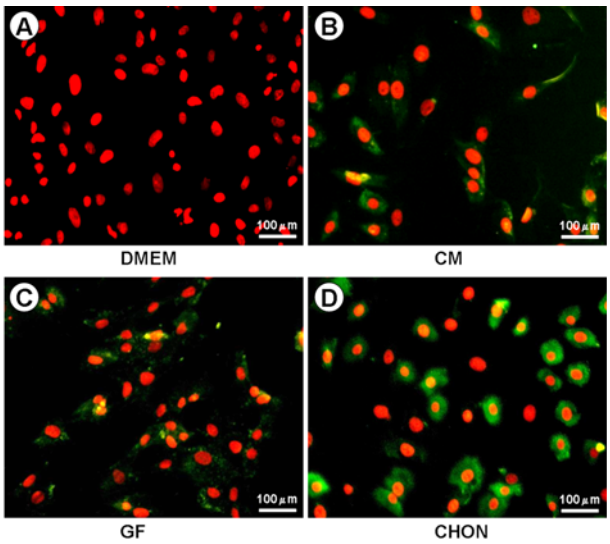

Figure 2. Expression of type II collagen in induced ASCs. In DMEM group, no visible expression of type II collagen was observed (A). Most cells in CM (B) and GF (C) groups displayed expression of type II collagen (green). As the positive control, strong expression of type II collagen was observed in most cells in the chondrocyte group (D).

articular chondrocytes) (Fig 3A). Quantitative RT-PCR results further demonstrated that cells in CM and GF groups showed significantly higher expression of SOX9 (Fig 3B), type II collagen (Fig 3C), COMP (Fig 3D), and aggrecan (Fig 3E) compared to those in DMEM group ($p < 0.05$). Consistent with the results of RT-PCR, the expression of these genes in CM and GF groups was still lower than that in the positive control ($p < 0.05$). Noticeably, a higher level of gene expression of SOX9, type II collagen, and COMP was observed in CM group than in the GF group with significant difference ($p < 0.05$) (Fig 3B-D). No significant difference was observed in the expression of aggrecan between CM and GF groups ($p > 0.05$) (Fig 3E). These results demonstrated that conditional media could remarkably upregulate the expression of cartilage specific genes of ASCs and were even more effective than conventional growth factors in chondroinduction of ASCs, according to the quantitative data.

3.2 *In Vitro* 3D Chondrogenesis of ASCs Induced by Conditional Media

3.2.1 Scaffold preparation, cell-scaffold biocompatibility, and gross view of engineered tissues

Unwoven PGA fibers were compressed into a cylinder shape with 5 mm in diameter (Fig 4A) and 1 mm in thickness (Fig 4B) and coated with PLA. After seeded onto the scaffold, ASCs secreted extracellular matrix (ECM) and had a tight adhesion with the PGA fibers (Fig 4C), which indicated that PGA/PLA scaffold had a good biocompatibility with cells. After 12 weeks of *in vitro* culture, the constructs in CM and CHON groups roughly maintained their original size and shape and formed cartilage-like tissues with an ivory-whitish appearance, but those in DMEM and GF groups demonstrated irregular edges in yellowish appearances, in spite of no obvious change of their original sizes (Fig 4D).

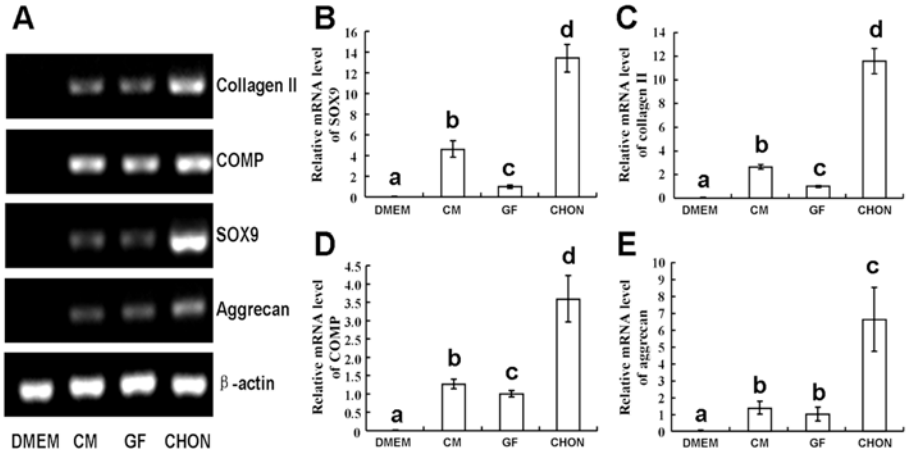

Figure 3. Expression and quantitative analysis of cartilage specific genes in induced ASCs. ASCs in CM and GF groups showed enhanced expression of type II collagen, COMP, aggrecan and SOX9 at gene level compared to DMEM group, similar to articular chondrocytes (A). Quantitative RT-PCR results showed that cells in CM and GF groups displayed significantly higher expression of SOX9 (B), type II collagen (C), COMP (D) and aggrecan (E) at gene level than those in DMEM group ($p < 0.05$), but significantly lower than articular chondrocytes ($p < 0.05$). Cells in CM group displayed significantly higher expression of SOX9 (B), type II collagen (C), and COMP (D) than those in GF group ($p < 0.05$), but no statistical difference in aggrecan (E) in between ($p > 0.05$). Different lower-case letter above each bar indicate significant difference ($p < 0.05$) between groups.

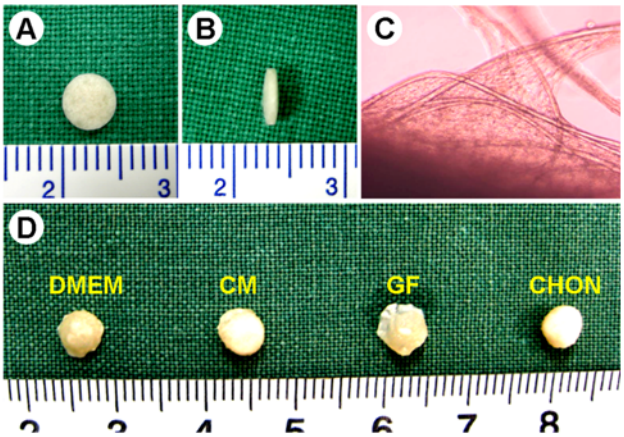

Figure 4. Gross view of PGA/PLA scaffold and cell-scaffold construct after *in vitro* culture and microscopic view of cell-scaffold biocompatibility. The PGA/PLA cylinder was 5 mm in diameter (A) and 1 mm in thickness (B). After cells were seeded into the scaffold, cells secreted ECM and had a tight attachment with the PGA fibers (C). After 12 weeks of *in vitro* culture, the constructs in CM and CHON roughly maintained their original sizes and smooth edge and formed cartilage-like tissues with an ivory-whitish appearance, but those in DMEM and GF showed an irregular edge in a yellowish appearance, in spite of roughly keeping their original size (D).

3.2.2 Histology and Immunohistochemistry of Engineered Tissues

Histological and immunohistochemical examinations were then performed for further identifying cartilage formation (Fig 5). After 12 weeks of *in vitro* induction, the specimens in both CM and GF groups formed cartilage-like tissue with typical lacuna-like structures, positive expression of type II collagen, and positive staining of safranin-O, although they still did not achieve as ideal results as CHON group. Noticeably, the specimen in CM group showed stronger safranin-O staining than that in GF group, implying a higher GAG content in CM group. As expected, no visible lacuna-like structures and positive staining of cartilage specific matrices were observed in DMEM group. These results indicated that the conditional media could induce ASCs to form 3D cartilage-like tissue *in vitro* similar to conventional growth factors.

3.2.3 Quantitative Analysis of 3D Cartilage Formation

Wet weight and contents of cartilage specific matrices were detected to quantitatively evaluate 3D cartilage formation. As shown in Fig 6, wet weights and contents of GAG, total collagen, and type II collagen in CM and GF groups were significantly higher than those in DMEM group ($p < 0.05$), although still lower than those in CHON group ($p < 0.05$).

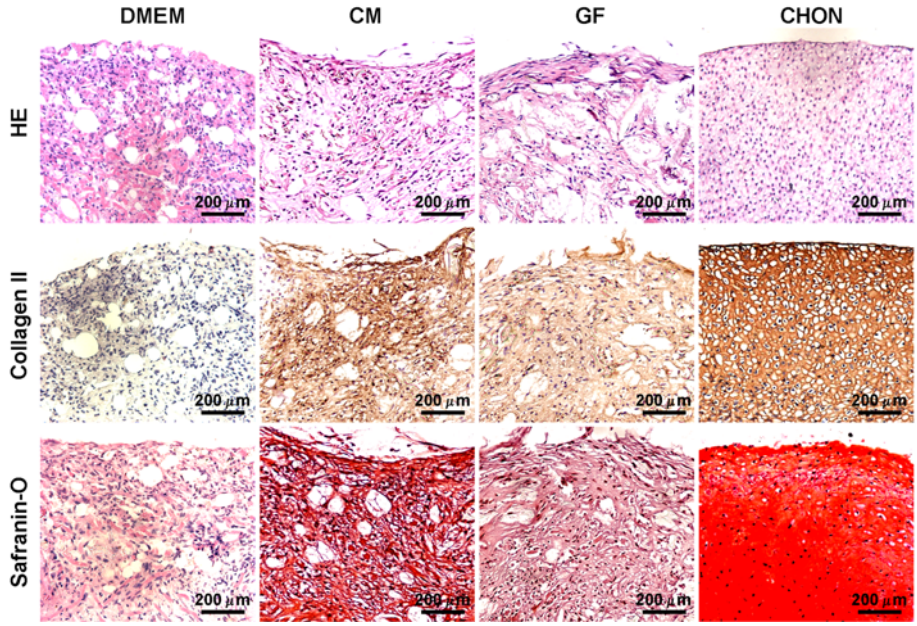

Figure 5. Histological and immunohistological examinations of engineered tissues. After 12 weeks of *in vitro* culture, the constructs in CM and GF groups formed cartilage-like tissues with typical lacunae structure and positive staining of type II collagen and Safranin-O in most areas. But those in DMEM group failed to form cartilage-like tissue with no obvious lacunae formation and positive staining of type II collagen and Safranin-O. In CHON group, a homogeneous cartilage-like tissue was observed with abundant lacunae structures and strong staining of type II collagen and safranin-O.

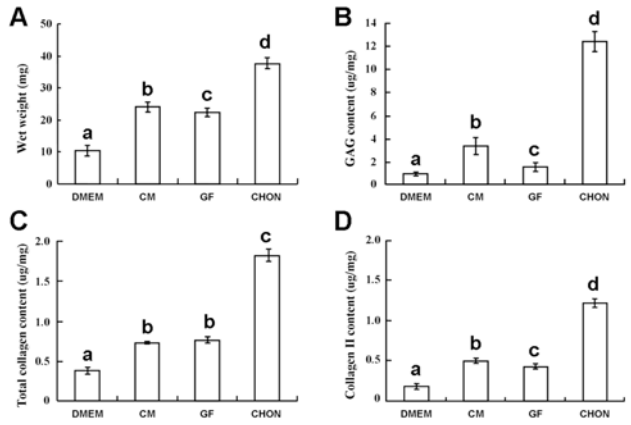

Figure 6. Quantitative analysis of 3D chondrogenesis. The wet weight (A) and contents of GAG (B), total collagen (C), and type II collagen (D) in CM and GF groups were significantly higher than those in DMEM group ($p < 0.05$) but much lower than those in CHON group ($p < 0.05$). The wet weight (A) and contents of GAG (B) and type II collagen (D) in CM group were significantly higher than those in GF group ($p < 0.05$). No statistical difference was observed in total collagen content (C) between CM and GF groups ($p > 0.05$). Different lower-case letter above each bar indicate significant difference ($p < 0.05$) between groups.

Consistent with safranin-O staining (Fig 5), GAG content in CM group was significantly higher than that in GF group (Fig 6A, B and D). Besides, wet weight and collagen II content in CM group were also significantly higher than those in GF group ($p < 0.05$) (Fig 6A and D), although there was no statistical difference in total collagen content between CM group and GF group ($p > 0.05$) (Fig 6C). These results indicated that the conditional media could efficiently direct 3D chondrogenesis of ASCs and seemed to show a stronger chondroinductive role compared to chondrogenic factor cocktails in terms of the quantitative analysis of wet weight, GAG and collagen II contents.

4. Discussion

Although ASCs have been considered as a potentially ideal cell source for cartilage engineering, how to efficiently enhance chondrogenic differentiation and 3D cartilage formation of ASCs is still a great challenge. The current results demonstrated that the culture media derived from chondrocyte-scaffold constructs (the conditional media) could efficiently direct chondrogenic differentiation of ASCs and even achieved higher expressions of cartilage specific genes compared to the current optimized growth factors. Most importantly, the conditional media were also able to promote 3D cartilage formation of ASCs on PGA/PLA scaffold *in vitro* with higher contents of some cartilage specific matrices compared to the current optimized growth factors, which provides a stable and simple inductive method for the application of ASCs in cartilage regeneration.

Lack of specific chondroinductive method is always one of the major challenges that prevent ASCs from application in cartilage regeneration. The early chondroinductive method of ASCs was mainly derived from that of BMSCs: a combination of exogenous growth factors. However, studies showed that the chondrogenesis efficiency of ASCs was much lower than that of BMSCs,[29-31] which was speculated to be related to the abnormalities of BMP profile and TGF-β receptor in ASCs, hence high doses of exogenous growth factors (such as BMPs) were required,[5,6] leading to problems such as hypertrophy and calcification *in vivo*.[5,7,8] Evidently, avoiding the application of high-dose growth factors is the key to solve these problems.

Mimicking chondroinductive niche may be a new strategy avoiding the application of high-dose growth factors. Our previous study demonstrated that chondrocytes played an essential role in chondroinduction, and that the conditional media collected from the supernatant of chondrocyte-scaffold constructs could successfully induce chondrogenic differentiation of BMSCs with no assistance from exogenous chondrogenic factors[11], which provides a clue for chondrogenesis of ASCs instead of the application of high-dose growth factors. Nevertheless, it is still uncertain whether the conditional medium is likewise efficient for chondrogenic differentiation of ASCs especially for 3D cartilage formation on a pre-shaped scaffold. After all, there are still many differences between ASCs and BMSCs, such as cell population components and their sensitivity to chondrogenic factors as mentioned above. The current results demonstrated that without exogenous growth factors, the conditional media still could efficiently direct not only chondrogenic differentiation of ASCs but also 3D cartilage formation, even better than high-dose growth factors, indicating that the conditional media may serve as a new ideal method for directing chondrogenesis of ASCs.

As a novel chondrogenic inducer, the conditional media have many advantages suitable for future application. Firstly, the conditional media may be abundantly obtained with low cost. According to the current methods, the conditional media could be collected repeatedly from *in vitro* engineered cartilage with simple manipulation and no extra growth factors were needed. Secondly, as shown in our previous results[11], the concentrations of chondrogenic factors in the conditional media showed a stable time-dependent increase in a relatively long period (over 4 weeks), which evidently facilitates to enhance chondroinductive efficiency and thus the conditional media may be sufficiently

available in a relatively long period without loss of chondroinductive efficiency. Quite different from this, if using monolayer culture system, chondrocytes tend to rapidly reach confluence and thus need to be passaged repeatedly, which inevitably results in aging and dedifferentiation and thus lose chondrocyte-specific phenotype and the function of secreting chondrogenic factors.[15-17] Thirdly, the conditional media may achieve better chondroinductive efficiency compared to the current optimized chondrogenic factors. The current results have shown that CM group achieved significantly higher expressions of some cartilage specific genes at monolayer induction and significantly higher GAG content at 3D induction compared to GF group. Finally, studies reported that soluble factors secreted by chondrocytes not only enhance chondrogenesis but also inhibit terminal differentiation and matrix calcification, which may be related to parathyroid hormone-related protein (PTHrP) secreted by chondrocytes.[9,32,33] From this point of view, using the conditional media as the inducer may help avoiding the possible side effects caused by high-dose growth factors, such as hypertrophy and calcification *in vivo*,[5,7,8] and thus contribute to stable cartilage formation.

Although the conditional media showed stable and efficient chondrogenic role, the exact chondroinductive mechanism is still unclear. In our previous studies, TGF-βs, IGF-I and BMPs were detected in the conditional media and all of them were required in the chondroinductive process according to the results of neutralizing experiments.[15] Noticeably, the concentrations of the above factors were much lower in the conditional media than that in currently used chondrogenic media,[11] indicating that there must be other chondrogenic factors or factor network involved in this process. These unknown factors obviously play a vital role in chondroinduction, since they can achieve similar efficiency of chondroinduction to high-dose growth factors. In fact, some studies have preliminarily demonstrated the important role of other factors (such as PTHrP) secreted by chondrocytes in promoting chondrogenesis and suppressing hypertrophy.[32,33] Nevertheless, the whole precise mechanisms still need to be further investigated.

5. Conclusions

In summary, this study demonstrated that the culture media of *in vitro* engineered cartilage could efficiently direct chondrogenic differentiation and 3D cartilage formation of ASCs *in vitro* and even achieved higher expressions of cartilage specific genes and higher contents of cartilage specific matrices compared to current optimized growth factors. Although the exact mechanism is still under investigation, the current study establishes a simple, but stable, efficient, and economical method for directing 3D cartilage formation of ASCs, a strategy that may be more closely applicable for repairing cartilage defects.

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