In vivo ectopic chondrogenesis of BMSCs directed by mature chondrocytes

Xia Liu a,b,c,1, Hengyun Sun a,b,1, Dan Yan a,b, Lu Zhang a,b, Xiaojie Lv a,b, Tianyi Liu a,b, Wenjie Zhang a,b, Wei Liu a,b, Yilin Cao a,b,c,*, Guangdong Zhou a,b,*,1

a Department of Plastic and Reconstructive Surgery, Shanghai 9th People’s Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Tissue Engineering, 639 Zhi Zao Ju Road, Shanghai 200011, PR China
b National Tissue Engineering Center of China, Shanghai, PR China
b Plastic Surgery Hospital, Chinese Academy of Medical Science, Beijing, PR China

A R T I C L E   I N F O

Article history:
Received 3 August 2010
Accepted 23 August 2010
Available online 5 November 2010

Keywords:
Bone marrow stromal cells
Chondrogenesis
Chondrocytes
Co-culture
Cytokines

A B S T R A C T

In vivo niche plays an important role in determining the fate of exogenously implanted stem cells. Due to the lack of a proper chondrogenic niche, stable ectopic chondrogenesis of mesenchymal stem cells (MSCs) in subcutaneous environments remains a great challenge. The clinical application of MSC-regenerated cartilage in repairing defects in subcutaneous cartilage such as nasal or auricular cartilage is thus severely limited. The creation of a chondrogenic niche in subcutaneous environments is the key to solving this problem. The current study demonstrates that bone marrow stromal cells (BMSCs) could form cartilage-like tissue in a subcutaneous environment when co-transplanted with articular chondrocytes, indicating that chondrocytes could create a chondrogenic niche to direct chondrogenesis of BMSCs. Then, a series of in vitro co-culture models revealed that it was the secretion of soluble factors by chondrocytes but not cell–cell contact that provided the chondrogenic signals. The subsequent studies further demonstrated that multiple factors currently used for chondroinduction (including TGF-β1, IGF-1 and BMP-2) were present in the supernatant of chondrocyte-engineered constructs. Furthermore, all of these factors were required for initiating chondrogenic differentiation and fulfilled their roles in a coordinated way. These results suggest that paracrine signaling of soluble chondrogenic factors provided by chondrocytes was an important mechanism in directing the in vivo ectopic chondrogenesis of BMSCs. The multiple co-culture systems established in this study provide new methods for directing committed differentiation of stem cells as well as new in vitro models for studying differentiation mechanism of stem cells determined by a tissue-specific niche.

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1. Introduction

In vivo niche plays an important role in determining the lineage commitment and the ultimate fate of exogenously implanted stem cells [1,2]. In articular chondrogenic environments, bone marrow stromal cells (BMSCs) readily differentiate into cartilage-phenotypic cells and form steady cartilage-like tissue [3,4]. However, the lack of a proper chondrogenic niche prevents stable ectopic chondrogenesis of mesenchymal stem cells (MSCs) in subcutaneous environments [5–7], which greatly limits the application of MSC-regenerated cartilage in repairing subcutaneous cartilage defects, such as nasal or auricular cartilage defects. Recreating a chondrogenic niche in subcutaneous environment that is similar to the one found in the articular environment will contribute to solving this problem.

Chondrocytes are one of the major cell types in joints and play an essential role in the development and maintenance of the articular niche [8–10]. Many studies have demonstrated that mature articular chondrocytes can regenerate cartilage and steadily maintain the cartilage phenotype in subcutaneous environments and even in a corneal stromal environment [11–13], indicating that chondrocytes can withstand the stress of implantation into new environments and retain their own phenotypes in non-chondrogenic niches. Another study reported that the co-transplantation of BMSCs, chondrocytes, and chondrogenic factors in a hydrogel could enhance the chondrogenesis of BMSCs in subcutaneous environments [14], suggesting that chondrocytes may possess the potential of promoting chondrogenesis of BMSCs. Nevertheless, no studies have explored whether chondrocytes alone could create a chondrogenic niche similar to the articular one in non-chondrogenic sites to direct chondrogenesis of stem cells.

In consideration of the important role of chondrocytes, in the current study, articular chondrocytes were utilized to mimic the articular chondrogenic niche in subcutaneous environments to
promote chondrogenesis of BMSCs. The results demonstrated that BMSCs could form homogeneous cartilage-like tissue when co-transplanted subcutaneously with articular chondrocytes even without the addition of any chondrogenic factors, indicating that chondrocytes alone could create a chondrogenic niche in subcutaneous environments. Based on this concept, a series of co-culture models in vitro were established to further reveal the possible mechanisms of how chondrocytes recapitulate the articular chondrogenic niche and promote chondrogenesis of BMSCs.

2. Materials and methods

2.1. Isolation, culture and labeling of cells

BMSCs and articular chondrocytes from 8-week-old hybrid pigs were isolated and expanded according to previously established methods [15,16]. To trace the distribution and differentiation of cells, BMSCs were retrovirally labeled with green fluorescent protein (GFP), similar to previous reports [17,18]. All animal experiments described in this paper have been approved by an institutional review committee of Shanghai Jiao Tong University School of Medicine.

2.2. In vivo co-transplantation of chondrocytes and BMSCs

Chondrocytes and BMSCs were collected and mixed at the ratio of 3:7 (chondrocyte:BMSC). The mixed cells were resuspended in 30% Pluronic F-127 at an ultimate concentration of $5 \times 10^7$ cells/ml and injected subcutaneously into nude mice as the experimental group (Exp). Pure chondrocytes or BMSCs were likewise injected as positive control (PC) and negative control (NC), respectively. $1.5 \times 10^7$ cells/ml chondrocytes, equal to the chondrocyte concentration in Exp, were likewise injected as a low chondrocyte concentration control (LC). In addition, chondrocytes and GFP-labeled BMSCs were mixed as in Exp and injected subcutaneously (0.5 ml/dot, $n = 3$) to trace the distribution and differentiation of BMSCs in co-transplanted implants. All the implants were harvested after 8 weeks following implantation for evaluation of chondrogenesis.

2.3. In vitro mixed co-culture of chondrocytes and BMSCs on PLA/PGA scaffolds

Polylactic acid (PLA)/polyglycolic acid (PGA) scaffolds were prepared into cylinders with diameter of 9 mm and height of 3 mm according to previously established methods [19,20]. Chondrocytes and BMSCs were mixed (chondrocyte:BMSC = 3:7) and then seeded into the PLA/PGA scaffolds ($1.0 \times 10^7$ cells in 0.3 ml/scaffold, $n = 6$) to form cell-scaffold constructs as the experimental group (Exp). Pure chondrocytes and BMSCs were also seeded as a positive control (PC) and negative
control (NC), respectively. $0.3 \times 10^7$ chondrocytes in 0.3 ml (equal to the chondrocyte-scaffold constructs) were likewise seeded as a low chondrocyte concentration control (LC). Chondrocytes and GFP-labeled BMSCs were mixed similarly to Exp and seeded into the scaffolds to trace the distribution and differentiation of BMSCs in co-cultured constructs. All the constructs were cultured in regular media (DMEM plus 10% FBS) for 8 weeks followed by evaluation of chondrogenesis.

2.4. In vitro mixed co-culture of BMSCs and paraformaldehyde-treated chondrocytes

To address the potential role of cell–cell contact in initiating chondrogenic differentiation of BMSCs, chondrocytes were inactivated by a low concentration of paraformaldehyde and co-cultured with BMSCs. Similar to previously described methods [21], chondrocytes were treated with 0.5% paraformaldehyde for 2 h at 25 °C. The inactivated cells were washed three times in PBS and then mixed with BMSCs at a ratio of 1:1 (chondrocyte: BMSC). The mixture was centrifuged at 1500 rpm for 8 min to form a cell pellet. Normal chondrocytes and BMSCs were similarly mixed and centrifuged to form a cell pellet as a positive control. BMSCs alone and inactivated chondrocytes alone were also centrifuged and used as negative controls. All the pellets were cultured in regular media for 4 weeks and then evaluated for the chondrogenic phenotype.

2.5. In vitro co-culture of chondrocyte-scaffold constructs and BMSC-scaffold constructs in a transwell system

A transwell co-culture system was used to confirm the chondroinductive role of soluble factors secreted by chondrocytes [22,23]. BMSC-scaffold constructs and chondrocyte-scaffold constructs were co-cultured in a transwell system separated by a semipermeable membrane (pore size: 0.4 μm) (Fig. 4A), which allows soluble molecules but not cells to diffuse freely throughout the entire culture system. In control groups, the chondrocyte-scaffold construct was replaced by a fibroblast-scaffold construct or a BMSC-scaffold construct. After 8 weeks of in vitro culture in regular media, all of the BMSC-scaffold constructs in the chamber (n = 5 in each group) were collected for evaluation of chondrogenesis. Then, the culture media of chondrocyte-scaffold constructs were harvested as the conditional media to induce chondrogenic differentiation of BMSCs to further confirm the chondroinductive role of the soluble factors.

2.6. Quantitative detection of chondrogenic factors in conditional media and blockade of neutralizing antibodies

The amounts of transforming growth factor beta 1 (TGF-β1), insulin-like growth factor 1 (IGF-1), and bone morphogenetic protein 2 (BMP-2) in the conditional media were quantitatively detected by ELISA at different times in culture [24]. Overdosed neutralizing monoclonal antibodies specifically targeting to TGF-β1, IGF-1, and BMPs were added either altogether or separately into the conditional media as antagonists to determine the effects of the growth factors in chondrogenic differentiation of BMSCs [25]. Isotype antibodies were added into the conditional media as a control. Expression of type II collagen was examined at both protein and gene levels to validate the inductive function of these proteins.

2.7. Evaluation of chondrogenic differentiation and chondrogenesis

The wet weights and glycosaminoglycan (GAG) contents of each sample were determined to quantitatively evaluate cartilage formation in different groups [20]. Chondrogenic differentiation and chondrogenesis of BMSCs were evaluated histochemically by staining with hematoxylin and eosin (HE) and Safranin-O, and immunohistochemically with type II collagen [20]. Expression of cartilage-specific genes was detected by reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR analysis [15,26].

2.8. Detection of GFP-labeled cells in mixed cell engineered tissues

The tissues containing GFP-labeled BMSCs that were engineered both in vitro and in vivo were collected, frozen in OCT gel and sliced to 5 μm in thickness. The exact distribution of labeled cells was observed and captured by laser scanning spectral confocal microscope (TCS SP5, Leica, Germany) [15]. The chondrogenic differentiation of labeled cells was further confirmed by histological analysis.

2.9. Statistical analysis

Student’s t-test was used to analyze quantitatively the difference of wet weight and GAG content in engineered tissues among differently treated groups both in vivo and in vitro; p < 0.05 was considered statistically significant.

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Fig. 2. In vitro chondrogenesis of BMSCs induced by chondrocytes in mixed co-culture system. Both gross view and HE staining showed that the specimens in PC (A, D) and Exp (B, E) groups, but not in NC group (C, F), formed cartilage-like tissue. The specimen in Exp group also showed strong expression of type II collagen (G, immunohistochemistry). The GFP-labeled cells from BMSCs were detected in the engineered tissues of Exp group (H), which formed typical lacuna structure and were embedded in cartilage-specific matrices (I, Safranin-O staining).
3. Results

3.1. In vivo chondrogenesis of BMSCs induced by chondrocytes in subcutaneous non-chondrogenic sites

Chondrogenesis of BMSCs in subcutaneous environments is always a great challenge due to the lack of proper chondroinductive microenvironments [5–7]. In the current study, chondrocytes were used to provide a chondrogenic niche for BMSC chondrogenesis by co-transplantation in the subcutaneous space. As shown in Fig. 1, after 8 weeks of subcutaneous implantation, all the specimens in Exp (co-transplantation group), PC (high concentration chondrocyte group) and LC (low concentration chondrocyte group) formed homogeneous cartilage-like tissue with typical histological structure and specific matrix deposition similar to normal cartilage. In contrast, the specimens in NC (BMSC group) formed fibrous tissues (Fig. 1A–I). Quantitative analysis showed that the average wet weight and GAG content in Exp reached 70.3% and 95.4% of those in PC respectively, which were significantly higher than those in LC (p < 0.05) (Fig. 1L, and M), indicating that the existence of BMSCs in Exp markedly increased cartilage matrix deposition of the implants. Most importantly, the GFP-labeled BMSCs were detected in the engineered cartilage of Exp and embedded in cartilage-specific matrices (Fig. 1J, and K), providing direct evidence that the BMSCs had been transformed into chondrocyte-like cells, and that the chondrocytes mediated the formation of a chondroinductive niche in a subcutaneous environment.

3.2. In vitro chondrogenesis of BMSCs induced by chondrocytes in mixed co-culture system

Based on the above in vivo results, we further explored the feasibility of mimicking a chondroinductive niche in vitro to promote BMSC chondrogenesis by co-culture of chondrocytes and BMSCs on a 3D scaffold. Consistent with the results in vivo, the cell-scaffold constructs in both Exp (co-culture group) and PC (high concentration chondrocyte group) formed homogeneous cartilage-like tissue with typical cartilage structure and cartilage-specific matrix deposition. As expected, the specimens in NC (BMSC group) formed fibrous tissues (Fig. 2A–G). The constructs containing GFP-labeled BMSCs also formed cartilage-like tissue after 8 weeks of in vitro co-culture and abundant GFP-labeled cells were still observed in the engineered tissue (Fig. 2H). Most of the labeled cells formed mature lacuna structures and were embedded in cartilage-specific matrices (Fig. 2I), indicating that the BMSCs can be transformed into chondrocyte-like cells when exposed to chondrogenically inductive signals provided by chondrocytes.

3.3. Lack of in vitro chondrogenic induction of BMSCs by cell–cell contact between chondrocytes and BMSCs

After the chondroinductive role of chondrocytes was validated both in vivo and in vitro, the possible mechanisms were further explored. Whether cell–cell contact alone could initiate chondrogenic differentiation was first investigated by pellet co-culture of...
BMSCs and paraformaldehyde-treated chondrocytes. The treatment of a low concentration of paraformaldehyde (0.5%) can inactivate chondrocytes while allowing them to retain their original structure, thus allowing the exploration of the role of cell–cell contact without the effects of cellular actions (Fig. 3A). Similar to the results in the mixed co-culture on 3D scaffolds, groups containing chondrocytes only or BMSCs with normal chondrocytes, formed cartilage-like tissue with typical structure and strong expression of cartilage-specific matrices, while the pure BMSC group did not (Fig. 3B, D–F, H, and I), further demonstrating that chondrocytes played an important chondroductive role in the pellet co-culture system. However, the specimens of BMSCs plus inactivated chondrocytes failed to form cartilage-like tissue (Fig. 3B) with no expression of cartilage-specific matrices at both gene and protein levels (Fig. 3C, G, and I), indicating that cell–cell contact alone between chondrocytes and BMSCs is insufficient to initiate chondrogenic differentiation of BMSCs.

3.4. In vitro chondrogenic differentiation and chondrogenesis of BMSCs induced by soluble factors secreted by chondrocyte-scaffold constructs

The chondroductive role of soluble factors secreted by the chondrocyte-scaffold constructs was then explored in a transwell co-culture system. In this system, the chondrocyte-scaffold construct and the BMSC-scaffold construct were co-cultured in the same device but no direct intercellular contact, thus allowing the investigating of the role of soluble factors alone (Fig. 4A). As shown in Fig. 3, the BMSC-scaffold constructs in Exp (separated from chondrocyte-scaffold constructs by a membrane) formed cartilage-like tissue with typical cartilage structure and positive expression of cartilage-specific matrices at both gene and protein levels after 8 weeks of in vitro co-culture (Fig. 4B–F). In contrast, all the BMSC-scaffold constructs in control groups, co-cultured with either fibroblast-PGA construct (Ctrl 1) or BMSC-PGA construct (Ctrl 2), shrunk markedly during in vitro culture and showed no cartilage-like tissue formation (Fig. 4B, G–N). The quantitative analysis showed that the wet weights and GAG contents in Exp were much higher than those in control groups (data not shown). All of the results suggest that the soluble factors specifically secreted by chondrocyte-scaffold constructs induce in vitro chondrogenesis of BMSCs.

To further confirm the chondroductive role of the soluble factors, the conditional media were collected from the supernatant of chondrocyte-scaffold constructs and used for the chondrogenic induction of BMSCs. The results demonstrated that the conditional media successfully induced chondrogenic differentiation of BMSCs in both pellet and monolayer culture (Fig. 5A, and B). In the control group, the regular media failed to induce chondrogenic differentiation of BMSCs (Fig. 5C, and D).

3.5. The important role of TGF-βs, IGF-1 and BMPs in the conditional media in chondrogenic differentiation of BMSCs

To further clarify the key soluble factors in the conditional media, the chondrogenic factors currently used, such as TGF-β1, IGF-1 and BMP-2, were quantitatively detected by ELISA. As shown...
in Fig. 5, the concentrations of these factors showed a time-dependent increase within 48 h after media renewal (Fig. 5E–G). The concentrations of the growth factors also increased with time over 4 weeks of in vitro culture (Fig. 5H–J), although their maximal concentrations were still much lower than those currently used in chondrogenic induction of BMSCs [19,27].

Overdosed neutralizing antibodies specifically targeting to TGF-βs, IGF-1 and BMPs were added to the conditional media to confirm their effects on the chondrogenic differentiation of BMSCs. As expected, in CM (conditional media) and CM+Iso (isotype antibody) groups, BMSCs were successfully induced into chondrocytic phenotype with expression of collagen II (Fig. 6A, B, and H). After treatment with neutralizing antibodies both separately and in combination, the expression of collagen II was significantly suppressed compared to CM and CM+Iso groups (Fig. 6A–F, and H), indicating that all of these factors were required for chondrogenic differentiation of BMSCs. Noticeably, the expression of collagen II in group containing CM plus all three antibodies was much lower than in every single-factor blocking group and even in DMEM group (Fig. 6H), implying that these proteins exerted their chondroinductive role in a coordinated way.

4. Discussion

In vivo niche plays an important role in determining the fate of implanted mesenchymal stem cells (MSCs) by directing their committed differentiation [1,28–31]. Because of a lack of proper chondrogenic niche, regenerating cartilage with MSCs in subcutaneous environments is always a significant challenge [5–7]. Despite the facts that the articular niche plays a chondroinductive role [3,30–32], that the chondrocyte is one of the major cell types in the joint, and that the chondrocytes play an essential role in the development and maintenance of articular niche [8–10], no studies have reported whether chondrocytes alone could create a chondrogenic niche in subcutaneous environments that is similar to that in the articular environment. The current study demonstrated that BMSCs could form homogeneous cartilage-like tissue in a subcutaneous environment with no help of any chondrogenic factors when co-transplanted with articular chondrocytes, indicating that chondrocytes alone could create a chondroinductive niche. Furthermore, a series of in vitro co-culture models further confirmed that it was the soluble factors secreted by chondrocytes but not the cell–cell contact that provided sufficient levels of chondrogenic signals.

The exact mechanism of how the soluble factors promote chondrogenic differentiation of BMSCs is still unclear. According to the current results, some known chondrogenic factors, such as TGF-β1, IGF-1, and BMP-2 [33–37], were detected at certain levels in the conditional media. Furthermore, when the effects of TGF-βs, IGF-1, and BMPs were blocked, the chondrogenic differentiation of BMSCs was significantly suppressed, indicating that these proteins did play an important role in this process. Moreover, all of these factors were required and they exerted their roles in a coordinated way, because
chondrogenic differentiation of BMSCs was also significantly reduced even when TGF-βs, IGF-1 and BMPs were separately neutralized. Additionally, it is worth noting that the concentrations of these proteins in the conditional media were much lower than those used for conventional chondrogenic induction [19,27], and at levels that are too low to efficiently induce chondrogenic differentiation of BMSCs (data not shown). Therefore, there must be other soluble factors that contribute to chondrogenic differentiation of BMSCs in the conditional media. A comprehensive analysis of the soluble factors secreted by chondrocyte-PGA constructs using microarray analysis and proteomics may be helpful to elucidate the interactions of these factors and find potentially unknown chondrogenic factors. These studies are still under investigation.

Besides soluble factors, cartilage-specific matrices produced by chondrocytes may also be involved in the chondrogenic differentiation of BMSCs in the mixed co-transplantation or co-culture system. The inductive role of cartilage-specific matrices has been reported in recent years [38]. In other studies, the cartilage-specific matrix showed only a relatively low inductive effect and the best inductive effect was obtained only when in combination with chondrogenic factors [39,40]. In the current mixed co-transplantation and co-culture systems, the extracellular matrices produced by chondrocytes could be in direct contact with BMSCs and exert on their roles together with the soluble factors secreted by chondrocytes, which might provide a reasonable explanation for the fact that only 30% chondrocytes induced 70% BMSCs to form cartilage-like tissue. Recently, we have invented a method for preparing acellular matrix of cartilage, which allows the further study on the inductive role of cartilage matrices alone.

Besides the advantage that no additional chondrogenic factors are required, another important advantage of chondrocyte-mediated BMSC chondrogenesis is that the regenerated cartilage could retain the cartilage phenotype in a stable manner in subcutaneous environment. Studies have found that in vitro chondrogenically induced MSCs were apt to calcify with vascular invasion after being implanted subcutaneously [6,7]. Cui et al. also showed that low-intensity ultrasound promoted ectopic subcutaneous chondrogenesis of MSCs after 4 weeks of implantation, but the cartilage tissue eventually became ossified at 6 weeks post-implantation [5]. Therefore, the main challenge of MSC chondrogenesis in the subcutaneous environment is not the failure of chondrogenesis but the failure of maintenance of the cartilage phenotype. Chondrocytes can withstand the stress of implantation and retain their own phenotypes in non-chondrogenic niches [11–13], which is probably related to anti-angiogenic factors secreted by chondrocytes that prevent neovascularization and ossification [41–43]. In the current co-transplantation system, the co-transplanted chondrocytes may also secrete anti-angiogenic factors to prevent vascular invasion and thus retain the cartilage phenotype of the whole implants.

Fig. 6. The role of TGF-βs, IGF-1 and BMPs in the conditional media in directing chondrogenic differentiation of BMSCs. Immunofluorescent staining demonstrated that expression of collagen II was obviously enhanced in CM (A) and CM + Iso (B) groups compared to regular media group (G). The expression of collagen II was obviously suppressed in all the groups with neutralizing antibodies (C–F). Real-time PCR further quantitatively confirmed the above results at gene level (H).
5. Conclusions

In summary, this study demonstrated that paracrine signaling by chondrocytes created a chondroinductive niche similar to the articular one in a subcutaneous environment to direct chondrogenesis of BMSCs. Furthermore, TGF-β1, IGF-1 and BMPs were required for initiating chondrogenic differentiation and they fulfilled their roles in a coordinated way. Although the chondroinductive role of other potential factors and cartilage ECM is still under investigation, the co-culture system (mixed or separate) of stem cells and differentiated cells established by tissue engineering techniques provides a new way for directing committed differentiation of stem cells and a new in vitro model for studying lineage commitment directed by tissue-specific niche.

Acknowledgments

This research was supported by the National Basic Research Program of China (2005CB522702), Hi-Tech Research and Development Program of China (2006AA02A126), National Natural Science Foundation of China (30772264, 30973131, 30973130), Shanghai Aurora Program (08SC019), Shanghai Rising-star Program (9091H10600), and National Natural Science Foundation of Shanghai Jiao Tong University School of Medicine (2008XJ025). The authors appreciate the technical supports from Demin Yin, Lijuan Zong, and Juanjuan Wu in the laboratory. We also appreciate Dr. Kara Spiller (from Drexel University, Philadelphia) for language editing.

Appendix

Figures with essential colour discrimination. All figures in this article have parts that are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.08.052.

References


