In Vitro Cell.Dev.Biol.—Animal (2016) 52:356–364
DOI 10.1007/s11626-015-9969-9

IGF-I induces adipose derived mesenchymal cell chondrogenic differentiation in vitro and enhances chondrogenesis in vivo

Quan Zhou[1] · **Baojun Li**[2] · **Jiali Zhao**[1] · **Wei Pan**[1] · **Jin Xu**[1] · **Sumei Chen**[1]

Received: 1 June 2015 / Accepted: 16 October 2015 / Published online: 28 January 2016 / Editor: Tetsuji Okamoto

Abstract Recent studies have demonstrated that insulin-like growth factor-1 (IGF-I) modulates bone mesenchymal stem cell chondrogenic differentiation independent of transforming growth factor beta (TGF-β) signaling in vitro. However, it is unclear whether IGF-I can solely modulate human adipose-derived mesenchymal cell (hAMC) chondrogenic differentiation, or whether it has additive effects with TGF-β1 to induce chondrogenic differentiation in vitro and development of mature cartilage in vivo. We investigated the effect of IGF-I on the induction of hAMC chondrogenic differentiation in the presence or absence of transforming growth factor beta 1 (TGF-β1) in vitro, and chondrogenesis of the induced hAMC in vivo. The results showed that IGF-I alone induced collagen type II, aggrecan, and Sox9 mRNA expression and collagen type II and aggrecan proteins expressions in hAMCs. Notably, there was greater mRNA expression of collagen type II, aggrecan and Sox9, and greater protein expression of collagen type II and aggrecan following TGF-β1 + IGF-I treatment, compared to either TGF-β1 or IGF-I-treated hAMCs. These results were confirmed in cartilage tissues derived from induced hAMCs. These findings indicate that IGF-I alone has the ability to induce chondrogenic differentiation and has additive effects with TGF-β1 to induce chondrogenic differentiation in vitro and in vivo.

Keywords Chondrogenic differentiation · Insulin-like growth factor · Multipotentmesenchymal cells · Tissue engineering

Introduction

Tissue engineering is an attractive approach to cartilage repair (Hendriks *et al.* 2007; Correia *et al.* 2014). In spite of many advantages, tissue engineering for cartilage repair still faces several challenges, such as finding appropriate cell source and suitable method to induce chondrogenic differentiation. Human adipose-derived mesenchymal cells (hAMCs) are undifferentiated pluripotent cells capable of differentiating into osteoblasts, chondrocytes, epithelial cells, adipocytes, neurons, or myocytes (Zuk *et al.* 2002; Brzoska *et al.* 2005; Kingham *et al.* 2007; Sowa *et al.* 2013; Gao *et al.* 2014). Moreover, hAMCs are an attractive cell source of cartilage tissue engineering for their abundance, accessibility, and strong chondrogenic ability (De Ugarte *et al.* 2003). Compared with bone marrow-derived mesenchymal stem cells (BMSCs), hAMCs have less chondrogenic potential, which can be overcome by bone morphogenetic protein 6 or great doses of growth factor (Hennig *et al.* 2007; Kim and Im. 2009).

Quan Zhou and Baojun Li contributed equally to this work.

✉ Sumei Chen
wuque1@hotmail.com

Quan Zhou
wuque1@126.com

Baojun Li
lbjesd@163.com

Jiali Zhao
Nick0908@163.com

Wei Pan
wuque197928@163.com

Jin Xu
wuque182@163.com

[1] Department of Orthopaedics, Huai'an Hospital Affiliated of Xuzhou Medical College and Huai'an Second Hospital, No. 62 Huaihai Road South, Huai'an 223002, China

[2] Department of Joint Surgery, Second People's Hospital of Hunan Province, Changsha 410007, China

The TGF-β superfamily plays a central role in the chondroinductive differentiation of mesenchymal stem cells (MSCs) derived from bone marrow, adipose, or other mesenchymal tissue (Worster *et al.* 2001; Zuk *et al.* 2002; Fukumoto *et al.* 2003; Kim and Im. 2009). TGF-β1-3 mRNA is expressed throughout the process of chondrogenesis (Pelton *et al.* 1991; Derynck and Zhang. 2003). TGF-β1 or TGF-β3 is known to primarily induce chondrogenesis (Goude *et al.* 2014). IGF-I is regarded as one of the most critical growth factors in cartilage development and homeostasis (Nixon *et al.* 1998; Bonnevie *et al.* 2014; Madry *et al.* 2013). IGF-I has been reported to increase in arthritic cartilage to recruit local chondrocytes for cartilage repair (Middleton and Tyler. 1992; Olney *et al.* 1996; Madry *et al.* 2013); therefore, decreases in IGF-I may lead to severe osteoarthritis (Denko *et al.* 1990; Schouten *et al.* 1993). Several recent studies demonstrated that IGF-I enhances chondrocyte metabolism while maintaining the differentiated phenotype and the chondrogenic ability of the differentiated bone mesenchymal cells (Worster *et al.* 2001) and periosteal cells (Fukumoto *et al.* 2003) in a three-dimensional matrix or micromass culture condition in vitro (Nixon *et al.* 1998). Recent studies have shown that IGF-I can modulate bone mesenchymal stem cell chondrogenic differentiation, independent from transforming growth factor beta (TGF-β) signaling in vitro (Longobardi *et al.* 2006; Li *et al.* 2012). However, whether IGF-I can solely modulate hAMC chondrogenic differentiation, and the possibility of additive effects with TGF-β1 to induce hAMC chondrogenic differentiation in vitro and chondrogenesis in vivo, remains unknown. Insulin is often used as an important component in the chondrogenic inducing media at 100 times above physiological levels. Insulin has an affinity that is approximately two orders of magnitude lower for the IGFIR (insulin-like growth factor-I receptor) compared with IGF-I, so high levels of insulin may depress the biological activity of IGF-I (Longobardi *et al.* 2006). In this study, we evaluated the effects of IGF-I, alone or in combination with TGF-β1, on hAMC chondrogenic differentiation in vitro and chondrogenesis in vivo in the absence of insulin.

Materials and Methods

hAMCs isolation and culture. Subcutaneous fat was obtained from six 18–25-yr-old male subjects by hip surgery. This study was approved by the ethics committee of Huai'an Hospital of Xuzhou Medical College, Huai'an, China, and informed consents were obtained from all the subjects. hAMCs were isolated as described previously (Bogdanova-Jantniece *et al.* 2014). Briefly, the adipose was extensively washed with phosphate-buffered saline (PBS). Macroscopic blood vessels were removed and the adipose was sheared with a pair of scissors. The sheared adipose was digested at 37°C with an equal volume of 0.075% collagenase I (Sigma, St. Louis, MO) for 2 h, filtered through a 100-μm mesh to remove undigested tissues and centrifuged at 1,200×*g* for 10 min. The pellet was resuspended in 160 mM NH_4Cl at room temperature for 10 min to lyse red blood cells and centrifuged again at room temperature for 10 min. The pelleted cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) with low glucose (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution (Invitrogen), and incubated in a tissue culture flask at 37°C in a humidified atmosphere containing 5% CO_2. The media was replaced every 2 d. The cells were passaged at 80% confluency.

Induction of hAMCs with growth factors. Stem cells generate high-density condensations during chondrocyte differentiation, suggesting that a 3D culture model is ideal to induce stem cells chondrogenesis. However, condensation is difficult to observe in 3D culture, and previous studies have shown that induction of hAMCs in 2D culture is sufficient to obtain cartilaginous tissue formation in vivo (Merceron *et al.* 2011). Therefore, in our study, we performed chondrogenic differentiation in monolayer to observe stem cells condensation in vitro. hAMCs (5×10^6) at passage 6 were passaged onto T-25 flasks and cultured under the same condition as above. To observe the condensation during chondrogenic induction, the hAMCs were cultured in monolayer. The old media were removed and treated with chondrogenic media or mock treated with fresh media for 30 min. Then the media was replaced with chondrogenic media containing DMEM high glucose and supplemented with 1% FBS, 1% antibiotic–antimycotic solution (Invitrogen), 0.1 mM ascorbic acid-2-phosphate, 10^{-7} dexamethasone, 6.25 μg/ml transferrin, 6.25 ng/ml selenous acid (Sigma), 10 ng/ml recombinant human TGF-β1 (Pepretech, London, UK), 100 ng/ml recombinant human IGF-I (Pepretech), and TGF-β1 + IGF-I or without any growth factor. The chondrogenic media was changed at a 2-d interval for 14 d.

Histology and immunohistology of the induced hAMCs and cartilage tissues. The hAMCs were harvested 14 d after the induction, and 50 μL of cells suspended in DMEM with low glucose supplemented with 10% FBS and 1% antibiotic–antimycotic solution (1×10^5 cells/ml) were transferred to slides and incubated for 30 min under the same culturing condition to allow the cells adhere to slides. Then the hAMCs on the slides were either used for toluidine blue metachromatic or immunohistochemical staining of collagen type II. For toluidine blue metachromatic staining, the hAMCs were fixed with 10% paraformaldehyde solution at room temperature for 10 min, stained with toluidine blue (Amresco, Solon, OH) for 10 min and washed with double distilled water three times to detect proteoglycans of the induced hAMCs. For immunohistochemical staining of

collagen type II, the hAMCs were blocked with normal goat serum at 37°C for 30 min and incubated with polyclonal goat anti-human collagen type II (1:100 in 1% Tween-20/PBS) (Amresco) for 60 min followed by washing with PBS (Amresco) three times. Then the hAMCs were incubated with mouse anti-goat antibody (1:100 in 0.01 M PBS) (Amresco) at 37°C for 60 min in a humidified chamber followed by the same wash as above, and incubated with streptavidin–perosidase at 37°C for 30 min. Diaminobenzidine (DAB) method was used for color development.

Reverse transcription polymerase chain reaction (RT-PCR) of collagen type II, aggrecan, and Sox 9. The hAMCs were harvested 14 d after the induction, and total RNAs from the hAMCs were obtained by TRIzol extraction according to the manufacture's protocol (Invitrogen). β-actin, collagen type II, aggrecan, and Sox9 were amplified using a PCR kit according to the manufacturer's instructions (TaKaRa, Otsu, Shiga, Japan). The primer sequences (Lin *et al.* 2005) are listed in Table 1. The abundance of mRNA was determined by densitometry and the data are expressed as relative values to the amounts of β-actin mRNA.

Western blot analysis of collagen II and aggrecan. The hAMCs were washed with PBS twice and lysed with 500 μL of cell lysis buffer (50 mMTris-cl, 150 mMNaCl, 0.02% sodium azide, 0.1% SDS, 100 mg/l PMSF, 1 mg/l Aprotinin, 1% Triton X-100, and 0.5% Na-deoxycholate). After centrifugation at 13,000×*g* at 4°C for 30 min, each supernatant was collected and the protein concentrations were determined by a BCA Protein Assay Reagent Kit (Pierce Corporation, Rockford, IL). Equal amounts of protein extracts were run on 7% sodium dodecyl sulfate (SDS)–polyacrylamide gels and electrotransferred to a nitrocellulose membrane. The membranes were blocked with 3% skimmed milk and incubated with mouse monoclonal antibodies (1:100 in PBS) (Amresco) against collagen type II, aggrecan, and β-action, followed by washing three times with TTBS (0.5 ml Tween 20 in 1 L tris buffered saline). Then the membranes were incubated with peroxidase -labeled secondary goat anti-mouse antibody, followed by the same wash as above, and visualized by an ECL detection kit (Pierce) according to the manufacturer's instructions.

In vivo study of chondrogenesis of the induced hAMCs. Poly lactic-co-glycolic acid (PLGA) (latide: glycolide = 75:25; molwt: 66,000–107,000; porosity ≥ 93%; pore diameter varies from 100 to 200 μm.) was used as scaffolds. PLGA scaffolds were exposed to UV light, soaked in 75% alcohol for 24 h, thoroughly washed with PBS, and stored at 40°C. 5×10^5 hAMCs were induced with IGF-I, TGF-β1, or both for 14 d, trypsinized with 0.25% trypsin-EDTA, and the induced hAMCs were adjusted to 1×10^7cells/ml, then 0.05 ml hAMCs suspension were seeded on PLGA scaffolds. Then the PLGA scaffolds were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2 h to allow hAMCs to adhere to the PLGA scaffolds. The cells were cultured with chondrogenic media (containing IGF-I, TGF-β1, or both) for 2 d.

Male athymic nude mice were anesthetized with amylobarbitone sodium (30 mg/kg). A 2-cm incision was made on the dorsum of the mice and the hAMCs-attached PLGA scaffolds were subcutaneously transplanted onto the dorsum of the mice ($n = 10$ per group). Twelve weeks later, the cartilage tissues were harvested and the maxium diameter of the cartilage tissues were measured with an electronic balance. The tissues were fixed in 10% formalin in PBS at room temperature overnight, dehydrated by treatment with a series of alcohol, embedded in paraffin, and cut into sections 5 μm thick. The sections were stained with hematoxylin–eosin or the collagen type II antibody, following the same procedure as above. The animal experiments were performed in compliance with the guidelines of Huai'an Hospital of Xuzhou Medical College, Huai'an, China.

Statistical analysis. All results were expressed as means ± standard deviation. Statistical significance was evaluated by *t* test using the SPSS 11.0 statistics software and a statistical significance was set at $P < 0.05$.

Table 1. Primer sequences and PCR conditions

Gene	Primers	Annealing temperature (°C)	Fragment (bp)	Cycle no.	GenBank no.
β-actin	5′- ACTCTTCCAGCCTTCCTTCC-3′ 5′- ACTCGTCATACTCCTGCTTGC-3′	55	313	30	BC013835
SOX9	5′-GAACGCACATCAAGACGGAG-3′ 5′-TCTCGTTGATTTCGCTGCTC-3′	60	631	28	NM_000346
aggrecan	5′-GCAGAGACGCATCTAGAAATTG-3′ 5′-GGTAATTGCAGGGAACATCATT-3′	55	504	31	NM_013227
Col-II	5′-TTCAGCTATGGAGATGACAATC-3′ 5′-AGAGTCCTAGAGTGACTGAG-3′	55	472	31	BC007252

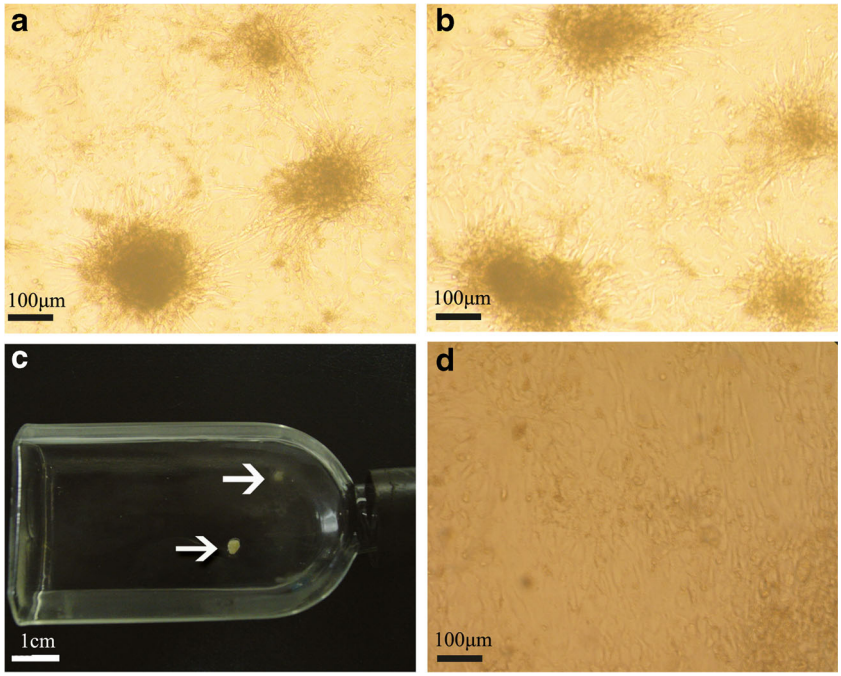

Figure 1. hAMCs formed cell clusters and detached at 7 d post induction. All the hAMCs treated with either or both of the growth factors formed cell clusters and detached 7 d after the treatments (*a*, TGF-β1; *b*, IGF-I; *c*, IGF-I + TGF-β1), but no cell clusters were found in the mock-treated hAMCs (*d*).

Results

Morphology of hAMCs induced with TGF-β1, IGF-I, or TGF-β1 + IGF-I. To investigate the effect of IGF-I on the chondrogenic differentiation of hAMCs, hAMCs were induced with TGF-β1, IGF-I, TGF-β1 + IGF-I, or growth factor mock treated. All of the hAMCs treated with either or both of the growth factors formed cell clusters and detached 3 d after the treatments, but no cell clusters were found in the mock-treated hAMCs (Fig. 1). The clusters in the tissue culture flask induced with TGF-β1 + IGF-I formed many macroscopic cells masses and could be seen with naked eye (Fig. 1*c*), while those induced with either of the two growth factors could only be seen under a light microscope (Fig. 1*a* and *b*). These results indicated that IGF-1 can induce hAMCs condensation alone and enhance hAMCs condense in vitro.

Histology and immunochemistry of collagen type II of induced hAMCs. Toluidine blue metachromatic staining and collagen type II immunohistochemical staining were positive in the IGF-I, TGF β1, and IGF-I + TGF β1 groups, but negative in the growth factor mock-treated group (Fig. 2). hAMCs induced with IGF-I, TGF-β1, or TGF-β1 + IGF-I were round or polygonal morphous (Fig. 2*a*, *b*, *c*, *e*, *f*, *g*), while the growth factor hAMC mocked-treated group maintained their flat appearance (Fig. 2*d*, *h*). These results indicated that hAMCs induced with IGF-1 or TGF-β1 express collagen type IIand proteoglycans.

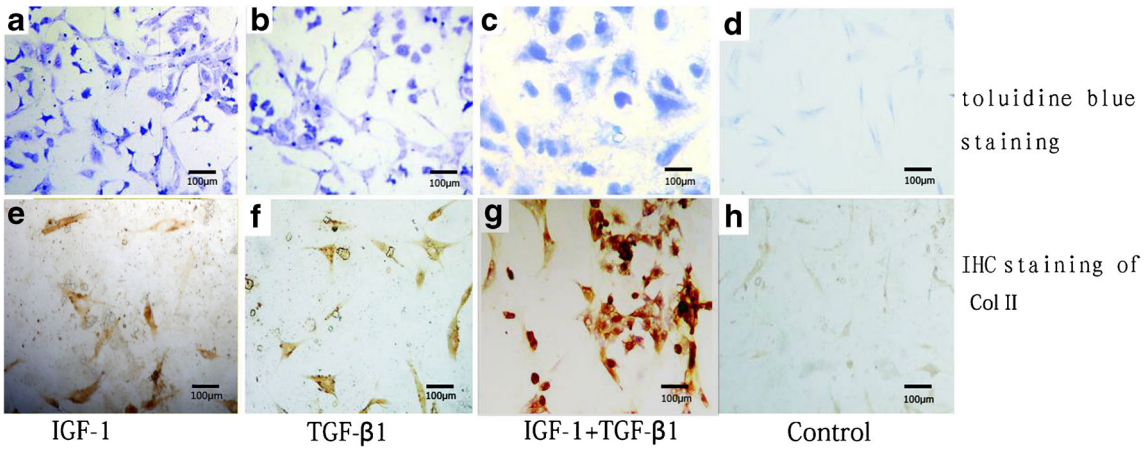

Figure 2. Toluidine blue metachromatic staining of hAMCs and immunohistochemical staining of collagen type II in hAMCs. hAMCs induced with IGF-I (*a*, *c*), TGF-β1 (*b*, *f*), IGF-I + TGF-β1 (*c*, *g*), or mock treated without any growth factor (*d*, *h*). IHC, immunohistochemical.

Collagen II, aggrecan and Sox9 mRNA, and collagen II, aggrecan protein expressions of induced hAMCs. The collagen type II, aggrecan, and Sox9 mRNA expressions were detected in all the growth factor-treated groups, but not in the growth factor mock-treated group (Fig. 3*a*). The optical density of the mRNA of collagen type II, aggrecan, or Sox9 was the greatest in the TGF-β1 + IGF-I group ($P < 0.05$), while no statistically significant difference was found between TGF-β1 and IGF-I groups ($P > 0.05$, Fig. 3*b*).

The results of Western blot revealed that the optical density of collagen type II or aggrecan was the greatest in the TGF-β1 + IGF-I group ($P < 0.05$), but no significant difference was found between TGF-β1 and IGF-I-treated groups with respect to collagen type II or aggrecan levels ($P > 0.05$, Fig. 4). These results indicated that collagen type II, aggrecan, and Sox9 mRNA, and collagen type II and aggrecan proteins were expressed in hAMCs induced with IGF-I alone, and greater levels of collagen type II, aggrecan, and Sox9 mRNA, and collagen type II and aggrecan proteins were expressed in hAMCs induced with IGF-I and TGF-β1.

In vivo study of cartilage tissues formed from induced hAMCs. Twelve weeks following implantation, new tissue was formed in the IGF-I, TGF-β1, or IGF-I + TGF-β1-treated hAMCs, while no new tissue was formed in the growth factor mock-treated group (Fig. 5). The maxium diameter of tissue derived from IGF-I + TGF-β1-treated hAMCs was significantly greater compared to either IGF-I or TGF-β1-treated hAMCs ($P < 0.05$). Hematoxylin and eosin staining revealed that cartilage lacunas were formed in the tissue from IGF-I + TGF-β1, IGF-I, or TGF-β1-treated hAMCs, while no cartilage lacuna was

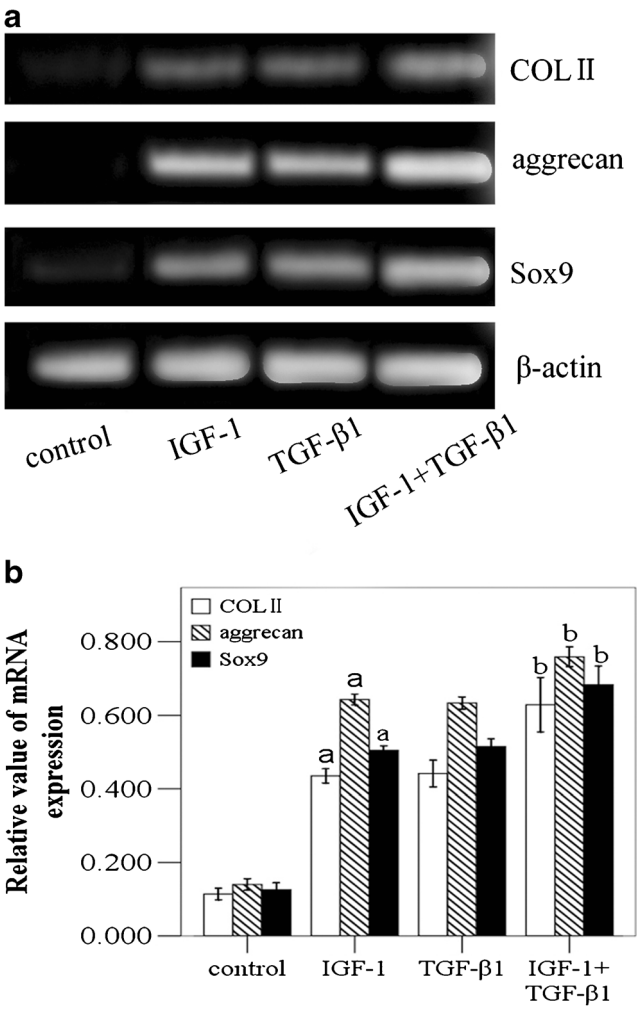

Figure 3. IGF-I induced and enhanced hAMCs chondrogenic-specific genes collagen type II (COL II), aggrecan, and Sox9 mRNA expression after induction with IGF-I, TGF-β1, IGF-I + TGF-β1 or mock treated without any growth factor for 14 d. (*a*) RT-PCR and, (*b*) analysis of mRNA expressions. *a*, $P < 0.01$ vs growth factor mock-treated group; *b*, $P < 0.01$vs TGF-β1-treated group.

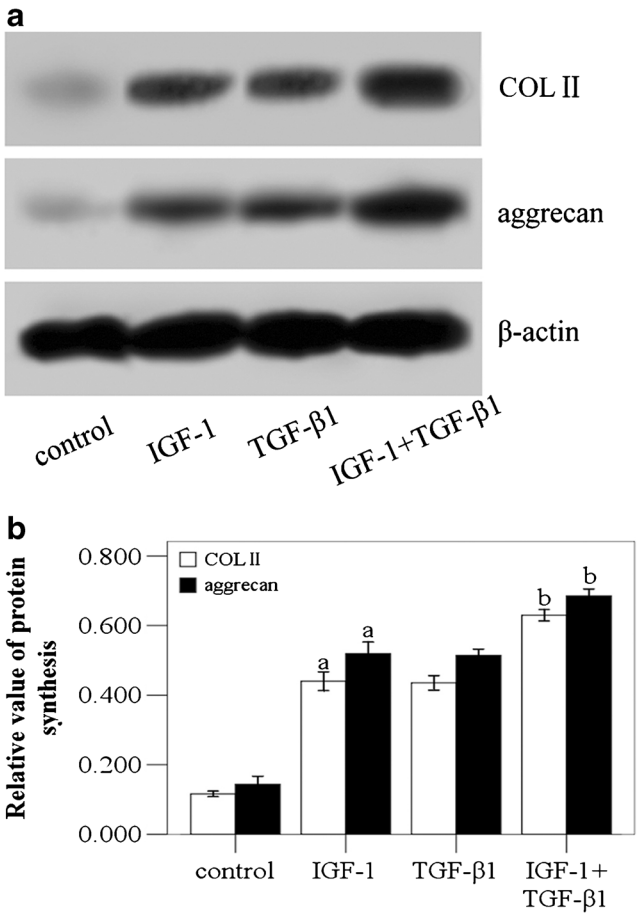

Figure 4. IGF-I induced and enhanced hAMCs to express chondrogenic-specific proteins COL II and aggrecan after induction with IGF-I, TGF-β1, IGF-I + TGF-β1 or mock treated without any growth factor for 14 d. (*a*) Western blot and (*b*) analysis of protein expressions. *a*, $P < 0.01$ vs growth factor mock-treated group; *b*, $P < 0.01$vs TGF-β1-treated group.

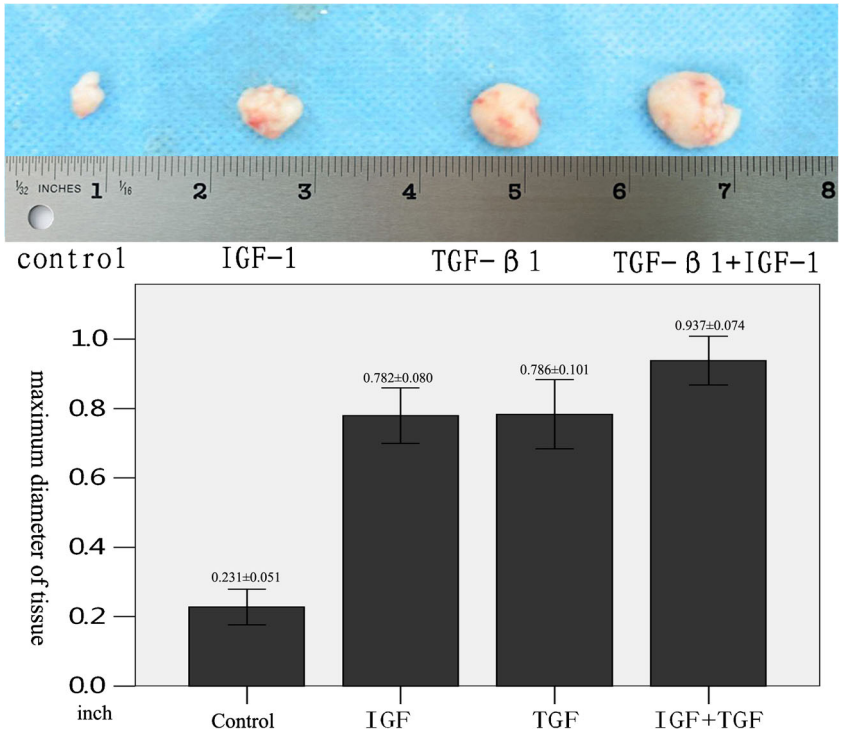

Figure 5 Tissues formed after hAMCs induced with IGF-I, TGF-β1, IGF-I + TGF-β1 or mock treated without any growth factor and transplanted into mice for 12 wk in a PLGA scaffold.

associated with growth factor mock-treated hAMCs (Fig. 6). Collagen type II immunohistochemical staining and toluidine blue metachromatic staining confirmed that collagen type IIand proteoglycans were expressed in all the growth factor treated but not growth factor mock-treated hAMCs. These results indicated hAMCs induced with IGF-1 can form new cartilage and IGF-1 can enhance cartilage formation in vivo.

Discussion

To investigate whether IGF-I promotes hAMCs chondrogenic differentiation alone, or may have additive effects on chondrogenic differentiation by TGF-β1, we treated hAMCs with TGF-β1, IGF-I, or TGF-β1 + IGF-I. We found that IGF-I solely induced hAMC chondrogenic differentiation and had an additive effect with TGF-β1 on chondrogenic differentiation*in vitro*, and mature cartilage formation from hAMCs treated with TGF-β1 in vivo.

IGF-I induced hAMC chondrogenic differentiation in vitro and chondrogenesis in vivo. Histological analysis of hAMCs and the immunohistochemistry of collagen type II in hAMCs demonstrated that IGF-I induced hAMCs to express proteoglycan and collagen type II. Correspondingly, the results of RT-PCR and Western blot showed that collagen type II, aggrecan, and Sox9 mRNA, and collagen type II and aggrecan proteins were expressed in hAMCs induced with IGF-I, respectively. These findings indicate that IGF-I alone induces hAMC chondrocytic differentiation in vitro. Furthermore, the in vivo study demonstrated that cartilage lacuna or collagen type II expression was found in the presence of PLGA compounds in the induced hAMCs with IGF-I. These findings indicate that the hAMCs induced with IGF-I have the ability to induce chondrogenic differentiation in vitro and form mature hyaline cartilage from inoculated hAMC tissue. Taken together, we found that IGF induces chondrogenic differentiation in vitro and in tissue independent of TGF-β1. In fact, prior reports have suggested a robust effect of IGF-I on cartilage repair and regeneration (Schouten *et al.* 1993; Milne *et al.* 1998; Nixon *et al.* 1998; Madry *et al.* 2013), as well as the ability to enhance extracellular matrix synthesis by cartilage chondrocytes (Nixon *et al.* 1998; Fortier *et al.* 2002) and inhibit chondrocyte apoptosis (Schouten *et al.* 1993; Worster *et al.* 2001; Fortier *et al.* 2002; Madry *et al.* 2002; Yi *et al.* 2013). IGF-I also regulates chondrogenesis of mesenchymal cells and anabolism of cartilage matrix molecules (Martel-Pelletier *et al.* 1998; Deng *et al.* 2013). However, several studies showed that IGF-I has no effect on chondroinduction of mesenchymal stem cells alone (Baddoo *et al.* 2003; Indrawattana *et al.* 2004), which is different from the findings in the current study. In accordance with the present study, other studies have demonstrated that IGF-I induces chondrogenic differentiation of mesenchymal cells isolated from limb buds of Hamburger-Hamilton stage 23/24 chicken embryos (Oh and Chun. 2003) and modulates bone mesenchymal stem cells chondrogenesis independent of TGF-β signals (Longobardi *et al.* 2006). While inducing media is very

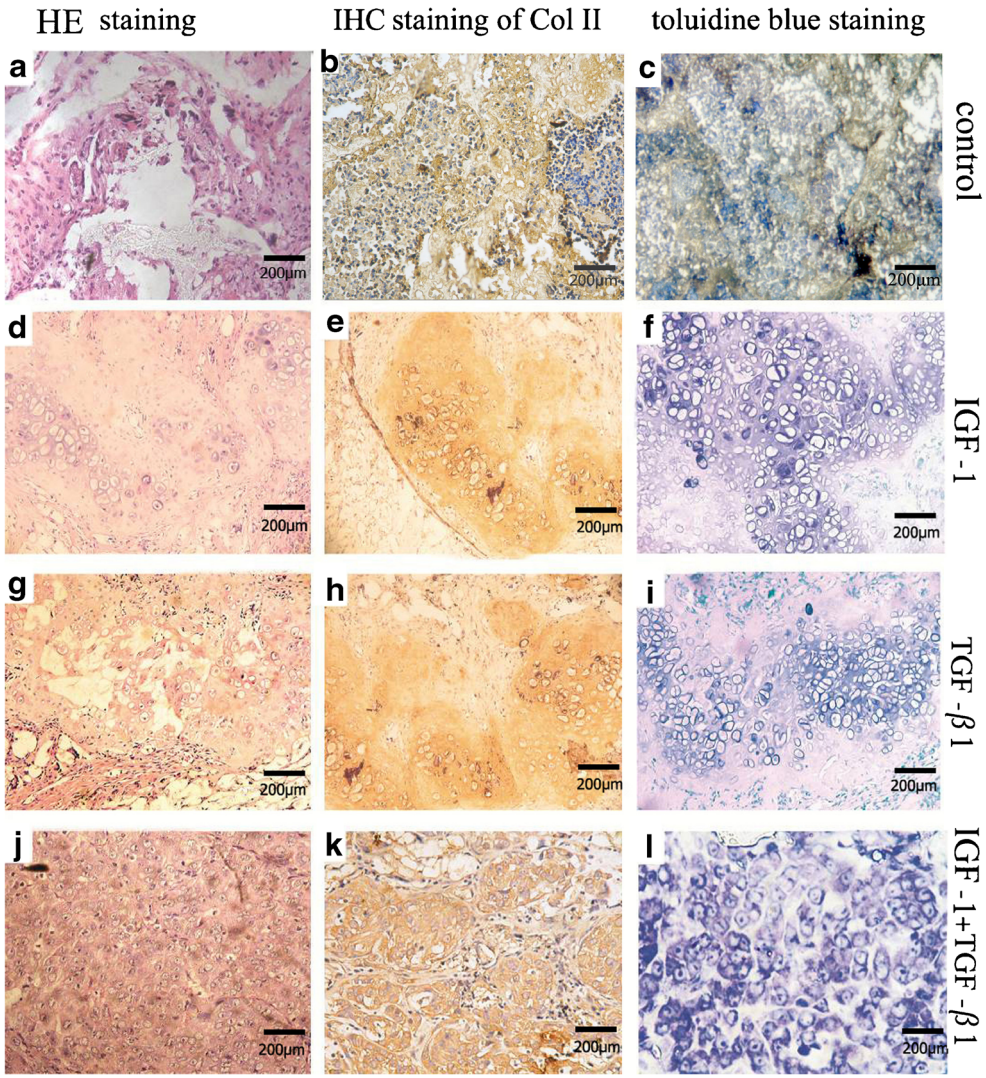

Figure 6 Hematoxylin and eosin (HE) and toluidine blue metachromatic staining and immunohistochemical (IHC) staining of collagen type IIof the cartilage tissues. Cartilage lacunas were formed in the TGF-β1 + IGFgroup (*e*, *f*), TGF-β1 (*c*, *d*), or IGF-I-treated group, while no cartilage lacuna was found in growth factor mock-treated group (*a*, *b*). Extracellular matrix (ECM) of TGF-β1 + IGF-I (*f*), TGF-1 (*e*), or IGF-I treated group was positive in collagen type II immunohistochemical staining and toluidine blue metachromatic staining.

important in chondrogenic induction, insulin is a widely used component of chondrgenic inducing media. High levels of insulin may depress biological activity of IGF-I (Longobardi *et al.* 2006). In the present study, insulin-absent chondrogenic media were used to avoid the influence of insulin. In absence of this potential cofounder, our results indicated IGF-1 could induce hAMCs chondrogenic differentiation.

IGF-I had additive effect on TGF-β1 induced hAMC chondrogenic differentiation in vitro and chondrogenesis in vivo. We found that hAMCs treated with TGF-β1 + IGF-I formed significantly more remarkable macroscopic cells masses than hAMCs treated with either TGF-β1 or IGF-I, indicating that IGF-I promoted hAMC condensation, which is an essential step of chondrogenic differentiation. This is consistent with the fact that more collagen type II, aggrecan, and Sox9 mRNA, and greater collagen type II and aggrecan protein expression was found in TGF-β1 + IGF-I compared to either TGF-β1 or IGF-I-treated hAMCs. These findings indicate that IGF-I has an additive effect on TGF-β1-induced chondrogenic differentiation. The maximum diameter of cartilage formed from TGF-β1 + IGF-I-treated hAMCs was significantly greater compared to either IGF-I of TGF-β1-treated hAMCs. The IGF-I additive effects on TGF-β1-induced hAMC chondrogenesis was only in tissue level without direct effect once in vivo because the growth factor treatment was not present once the hAMCs tissue was implanted in vivo.

Indeed, several studies have demonstrated that IGF-I can enhance chondrocytes and MSCs chondrogenesis of TGF-β1 via enhancing metabolically active and depressing cytotoxic activity of TGF-β1 (Worster *et al.* 2001; Blunk *et al.* 2002; Fukumoto *et al.* 2003; Chiou *et al.* 2006; Sakimura *et al.* 2006). Cartilage formation is initiated by mesenchymal cells differentiation into chondrocytes, and chondrogenesis is triggered by aggregation of mesenchymal cells that develop into cartilage nodules that can be enhanced by higher inoculum cell density (Takagi *et al.* 2007). IGF-I regulates the chondrogenesis of mesenchymal cells and maintains differentiated articular chondrocytes phenotype (Oh and Chun. 2003).

In conclusion, the present study demonstrated that IGF-I induced chondrogenic differentiation of hAMCs in the absence of TGF-β1 and has an additive effect on TGF-β1-induced hAMC chondrogenic differentiation in vitro and chondrogenesis in vivo. Our findings may provide an attractive approach to effective tissue engineering for cartilage repair.

Acknowledgments This research was supported by grants from the scientific research fund of the Bureau of Public Health of Jiangsu province (no. H201254) and the Huai'an Technology Support Program (social development) funded projects (no. HAS2013046).

Conflict of Interest The authors declare that they have no conflict of interest.

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