Enhancement of the Chondrogenic Differentiation of Mesenchymal Stem Cells and Cartilage Repair by Ghrelin

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ABSTRACT: Transforming growth factor beta (TGF-β) is commonly utilized in chondrogenic differentiation protocols, but this often results in incomplete maturation of the derived chondrocytes. Gene expression analysis, quantitation of sulfated glycosaminoglycan and collagen, and histological staining were performed to assess the effects of ghrelin. The signaling pathways involved were investigated with inhibitors or targeted by shRNAs. Joint cavity delivery of TGF-β with or without ghrelin, within a rat cartilage defect model was performed to evaluate the in vivo effects of ghrelin. Ghrelin dramatically enhanced gene expression levels of *SOX9*, *ACAN*, and *COL II* and resulted in increased synthesis of sulfated glycosaminoglycan (sGAG) and collagen in vitro. Combined treatment with TGF-β and ghrelin synergistically enhanced the phosphorylation of ERK1/2 and DMNT3A, which accounted for increased expression of chondrogenic genes. Delivery of ghrelin in combination with TGF-β after MSC implantation within a rat osteochondral defect model significantly enhanced de novo cartilage regeneration, as compared to delivery with TGF-β alone. In conclusion, ghrelin could significantly enhance MSC chondrogenic differentiation in vitro and can also enhance cartilage regeneration in vivo when used in combination with TGF-β. © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: chondrogenic differentiation; MSCs; ghrelin; ERK1/2

Mesenchymal stem cells (MSCs) are a potential source of chondrogenic cells for the treatment of cartilage injuries. For MSCs to generate a stable hyaline rich cartilage in vivo, they require the efficient delivery of bioactive factors at a suitable dose over appropriate time durations to induce chondrogenesis of MSCs, and to maintain the cartilage phenotype. Transforming growth factor beta (TGF-β) is a potent stimulator of proteoglycans and of type II collagen synthesis in chondrocytes.[1] In vivo, TGF-β delivered by slow-release from microspheres can induce the chondrogenic differentiation of MSCs to form ectopic cartilage and repair full-thickness cartilage defects.[2,3] However, direct injection of TGF-β produced side effects even in healthy joints, resulting in osteophyte formation, swelling and synovial hyperplasia, and this phenomenon is exacerbated during osteoarthritic development.[4,5]

Most commonly utilized chondrogenic differentiation protocols attempt to mimic embryonic chondrogenesis through cell condensation and supplementation of transforming growth factor-β (TGF-β).[6] TGF-β initiates chondrogenic differentiation through intracellular signaling cascades including the cross-talk of SMAD2/3,[7] as well as pathways involving the mitogen-activated protein (MAP) kinases particularly p38, and extracellular signal-regulated kinase (ERK).[8] ERK signaling is required for precartilage condensation during the early stages of chondrogenesis.[9] On the other hand, increased phosphorylation of ERK has also been implicated in chondrocyte hypertrophy.[10] Although chondrogenic differentiation of stem cells have been successfully induced with TGF-β supplementation, the cartilage equivalents derived are found to possess inferior biochemical content and mechanical strength when compared to tissues generated with mature chondrocytes,[11,12] with the tendency for fibrogenesis[13] and development of hypertrophy.[14] Various biological factors, particularly proteins and peptides such as FGF, IGF, BMP, and Wnt, have been investigated to further enhance the chondrogenic effects of TGF-β.[15,16]

Ghrelin, also known as "hunger hormone," is expressed and secreted by ghrelinergic cells located within the stomach, cerebra, and growth plate. It physiologically promotes secretion of growth hormone and plays a key role in the distribution and usage of energy through binding to the growth hormone secretagogue receptor (GHSR).[17] Ghrelin is also involved in multiple cell differentiation pathways and organ functions. It promotes proliferation of epithelial cells via the mitogen-activated protein kinases (MAPK) signaling pathway.[18] Ghrelin serum levels was found to be correlated with osteoarthritis, and was shown to increase the formation of cartilage matrix by inhibiting IL-1β-induced NFκB and AKT signaling in osteoarthritis.[19] In growth plate cartilage, ghrelin is localized predominantly in the proliferative and maturative zone of the epiphysism.[20] These studies suggest that ghrelin might play a role in the chondrogenic differentiation of MSCs during either differentiation, maturation, or matrix formation.

In this study, the in vitro chondrogenic effects of ghrelin was evaluated, in the absence and presence of TGF-β using standard chondrogenic pellet culture. The temporal effects of ghrelin delivery in enhancing TGF-β induced MSC chondrogenesis was investigated

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and the underlying mechanisms involved was explored. Lastly, we evaluated the in vivo effects of delivering ghrelin on MSC-based cartilage regeneration in a rat cartilage defect model.

MATERIALS AND METHODS

Isolation and In Vitro Expansion of Rat MSCs and Chondrocytes

MSCs and chondrocytes were harvested from 6 weeks old Sprague Dawley (SD) rats. After the animals were sacrificed, the tibia and femur of the back leg were carefully dissociated and bone marrow cells were flushed off the bone cavities. The marrow cells were cultured in expansion medium composed of Dulbecco's modified Eagle's medium (DMEM, Gibco, Suzhou, China) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), and penicillin/streptomycin (PS, Amresco, Solon, OH), within a humidified incubator at 37°C with 5% CO_2. Cells were trypsinized with 0.25% (w/v) trypsin (Invitrogen, Carlsbad, CA) upon reaching 85% confluence. MSCs at passage 4 were used for further experiments. Forty rats were used for MSCs isolation, and the cells from individual donors were mixed together to constitute one cell line.

The articular cartilage was cut into small pieces by a lancet and digested in 0.2% (w/v) Collagenase II (17101-015, Gibco) at 37°C overnight. The harvested chondrocytes were cultured under the same condition as MSCs. Expanded chondrocytes were used at passage 4. Twenty rats were used for chondrocyte isolation, and the cells from each donor were mixed together to constitute one cell line.

Optimization of Ghrelin Concentration for In Vitro Chondrogenic Differentiation of MSCs

Chondrogenic differentiation was carried out in a 3D pellet culture system as previously described.[21] Briefly, MSCs were resuspended in serum-free chondrogenic differentiation medium (CM) composed of high glucose DMEM (Gibco), 10^{-7} M of dexamethasone (dexamethasone; D4902, Sigma-Aldrich (St. Louis, MO), 50 μg/ml of ascorbic acid (A5960, Sigma), 1 mM of sodium pyruvate (P2256, Sigma), 4 mM of proline (P5607, Sigma), 1% (v/v) of ITS (41400045, Gibco), with/without varying concentrations (1, 10, 100 nM) of ghrelin (031-30, PHOENIX BIOTECH, Beijing, China) and/or 10 ng/ml of TGF-β3 (R&D Systems, Minneapolis, MN).[22] The MEK inhibitor PD98059 (10 μM; ab146592, Abcam, London, UK) was added into CM to inhibit phosphorylation of ERK1/2. Aliquots containing 2.5×10^5 MSCs were centrifuged in 15 ml polypropylene tubes at 200*g* for 10 min, prior to culture at 37°C within a humidified 5% CO_2 atmosphere. The culture medium was changed every 3 days.

qRT-PCR Analysis of Gene Expression

Total RNA were extracted with Trizol Reagent (206101, New Industry) following the manufacturer's protocol. Reverse transcription reactions were performed with 2000 ng of total RNA with the iScript® cDNA synthesis kit (Bio-Rad, Foster City, CA). Quantitative real-time PCR was performed with the Pikoreal 96 system (Thermo, Waltham, MA) utilizing the Real-time Master Mix SYBR Green (FP202, Tiangen, Beijing, China) following the manufacturer's instructions. The gene-specific primer sequences are listed in Tables S-1 and S-2. The relative expression levels of genes were expressed as fold-differences with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels. There were four replicate samples for each group. The qRT-PCR analyses were conducted on 7 or 14 days post-chondrogenic differentiation. Gene expression levels of ghrelin and GHSR-1α, were analyzed on the days 3, 6, 9, and 12 time points.

Quantification of Sulfated Glycosaminoglycan (sGAG) and Collagen

After culture in chondrogenic differentiation medium for 14 days, cell pellets were digested in 0.5 mg/ml proteinase K (Sigma) at 56°C for 12 h, and then conjugated with dimethyl-methylene blue (DMMB, 341088, Sigma). The digestive solution was agitated for 30 min, and centrifuged at 10,000*g* for 10 min. The centrifugal sediment was dissolved in a decomplexation solution and the absorbance was measured at 630 nm. Additionally, the samples were hydrolyzed at 100°C with 6 N HCL. Hydroxyproline content was then determined using the Hydroxyproline Assay Kit (BC0255-100, Solarbio, Beijing, China). The DNA content of the pellets was measured by Hochest 33258 at 350 nm. Triplicate samples were performed for each experimental group.

Histology Staining and Immunohistochemistry

After 14 days of culture, the pellets were fixed in 4% (w/v) paraformaldehyde solution (Life Technologies Corporation, Carlsbad, CA) and dehydrated with an ascending ethanol gradient before being embedded in paraffin and sectioned with a thickness of 5 μm. For alcian blue staining, the tissue sections were incubated with 0.5% (w/v) alcian blue (DG0041, leagene, Beijing, China). For HE staining, the sections were incubated with hematoxylin and eosin (DH0006, leagene). Histological staining of the pellets was carried out after 14 days of chondrogenic differentiation in vitro. For in vivo evaluation, histological staining was carried out at 4 and 12 weeks post-surgery.

For immunohistochemistry staining, hydrogen peroxide was used to block endogenous peroxidase in the pellet sections before pepsin treatment. Monoclonal antibodies specific for collagen type I (ab34710, Abcam), collagen type II (ab34712, Abcam) and collagen type X (orb10444, Biorbyt, Shanghai, China) were applied, followed by incubation with biotinylated goat anti-mouse or rabbit anti-goat secondary antibodies. For in vivo evaluation, the immunohistochemistry staining of the samples was carried out at 4 and 12 weeks post-surgery.

Immunofluorescence

Cells were fixed with 4% (w/v) paraformaldehyde for 15 min and then washed twice with PBS. The cells were then blocked with 5% (w/v) bovine serum albumin (BSA) prior to being incubated with primary antibodies against ghrelin (ab104307, Abcam) or GHSR-1α (sc-10359, Santa Cruz Biotechnology, Dallas, TX). After washing off the primary antibodies, the cells were incubated with FITC-conjugated goat anti-rabbit IgG (0114, Cwbio, Beijing, China) or FITC-conjugated rabbit anti-goat IgG (BA1110, Boster, Wuhan, CN) secondary antibodies. The nuclei were stained with DAPI. Cells were then imaged by confocal laser scanning microscopy (CLSM, LSM510, Zeiss, Oberkochen, Germany).

Western Blot

Western blot analyses of pellets were conducted 1 day after chondrogenic differentiation unless otherwise indicated. Pellets were lysed with RIPA lysis buffer (R0020, Solarbio) supplemented with fresh protease inhibitor of 0.1% (w/v) phenylmethanesulfonyl fluoride (PMSF, Solarbio) and phosphatase inhibitor (Beyotime, Shanghai, China). The pellets were ultrasonicated and then kept for 30 min at 4°C, before being centrifuged at 8,000*g* for 20 min at 4°C. Western blotting was performed as previously described.[23] Rabbit polyclonal antibodies against P38 MAPK (4511P, Cell Signaling, Danvers, MA), P38 MAPK (8690P, Cell Signaling), ERK1/2 MAPK (4695P, Cell Signaling), p-ERK1/2 MAPK

(4370P, Cell Signaling), Smad-3 (ab40854, Abcam), p-Smad-3 (ab195837, Abcam), DNMT3A (ab188470, Abcam), and p-DNMT3A (ab87763, Abcam) were incubated with 30 ng of the lysed proteins, and then incubated with secondary HRP-conjugated anti-rabbit IgG (BA1081, Boster). Chemiluminescence was evaluated with the ChemiDoc XRS 1 Molecular Imager (Bio-Rad), prior to analysis with the Tanon Gis image software (Tanon, Shanghai, China).

Implantation in Articular Cartilage Defects

All animal experiments were approved by the Institutional Animal Care and Use Committee of Peking University. Articular cartilage defects were created in both knees of thirty 10-week-old male Sprague–Dawley (SD) rats. Rats were anesthetized intraperitoneally with 10% (w/v) chloral hydrate (23100, Sigma) constituted in PBS. Defects of 1.0 mm depth and 1.5 mm diameter were created in the middle of the articular cartilage of the femoral trochlea. Five hundred thousand MSCs constituted in 20 μl of alginate (1% v/v) were implanted into the defects, followed by 10 μl of calcium chloride (10% w/v) to gel the alginate. After the operated joints were closed with suture, 200 μl of chondrogenic differentiation medium containing either TGF-β (100 or 1,000 ng/ml) alone, or TGF-β with ghrelin (10 or 100 nM, respectively) were injected into the joint cavity of the knees. Rats were sacrificed at 4 ($n = 5$), and 12 ($n = 5$) weeks after surgery. The whole knee joints were dissected and fixed in 10% formalin for 24 h. Subsequently, the tissues were decalcified with 10% (w/v) EDTA for 2 weeks and embedded in paraffin. Sections were cut at a thickness of 5 μm and utilized for immunohistochemistry.

The Macroscopic Cartilage Evaluation Score and Histological Score of Cartilage Defect Healing

The macroscopic images of cartilage repair were collected at 4 and 12 weeks post-surgery. The International Cartilage Repair Society (ICRS) macroscopic score of the macroscopic images were evaluated by three independent observers. The histological sections of each defect were scored according to the ICRS visual histological score by three independent observers.

Statistical Analysis

All experiments were repeated multiple times (n). One-way ANOVA analysis was performed with the least significant difference (LSD) test using the SPSS 13.0 software (SPSS Inc., Chicago, IL). p values ≤ 0.05 were considered significant, and p values ≤ 0.01 were considered highly significant. The net optical density of the western blot was quantified by the Tanon Gis software. Optical densities of immunohistochemical staining images were quantified by the Image J software.

RESULTS

Gene and Protein Expression Levels of Ghrelin and Its Receptor GHSR-1α

To investigate the expression of ghrelin and GHSR-1α in MSCs and chondrocytes, RT-PCR and immunocytochemistry were carried out. The gene expression levels of ghrelin in rat chondrocytes was higher than rat MSCs. On the other hand, the ghrelin receptor, GHSR-1α, was only expressed in MSCs, and was not detectable in chondrocytes (Fig. 1A and B). Immunocytochemistry also showed the different ghrelin protein expression levels of MSCs and chondrocytes, localized within the cytoplasm (Fig. 1C). GHSR-1α was strongly expressed by MSC, while it was not detectable in chondrocytes (Fig. 1D).

Ghrelin Enhanced Chondrogenic Differentiation Induced by TGF-β and BMP

The effects of ghrelin (1, 10, and 100 nM) on MSC chondrogenic differentiation was assayed in the presence or absence of 10 ng/ml TGF-β. Treatment with ghrelin alone, induced similar expression of *Col II* and *ACAN* as treatment with TGF-β, without any dose-dependent effect (Fig. S-1). In the presence of TGF-β, ghrelin synergistically increased the chondrogenic effect of TGF-β, in an inversely dose-dependent manner. Ghrelin at the lowest tested dose of 1 nM significantly increased gene expression levels of *SOX9*, *COL II*, and *ACAN* by at least threefolds, while it also significantly doubled the gene expression levels of *COL X*. Other concentrations of ghrelin exerted weaker synergistic effects on chondrogenic differentiation, as seen by mild to negligible elevation of the gene expression levels of *SOX9* and *COL II* (Fig. S-1). Based on the above results, 1 nM ghrelin was chosen for subsequent experiments.

To investigate whether the synergistic effect of ghrelin was time-dependent, the delivery of ghrelin was fine-tuned to optimize its effects on chondrogenic differentiation. Ghrelin (G) was delivered alone, or in combination with TGF-β (T) at either the first week, second week, or at all time points, and the effect was screened by analyzing the expression of chondrogenic differentiation genes (Figs. 2A and S-2). Ghrelin was observed to promote chondrogenic differentiation, with a better effect being observed when used only during the first week. Upregulation of the gene expression levels of *SOX9*, *COL II* and *ACAN*, and the ratio of *COL II*/*I* was highest in the TG-T group (TGF-β + ghrelin for the first week and TGF-β alone for the second week), as compared to the T-T group (continuous TGF-β usage) and other groups. Although expression of *SOX9*, *COL II*, and the ratio of *COL II*/*I* were also enhanced significantly in the TG-TG group (TGF-β + ghrelin for the whole 2 weeks), as well as the G-G group (continuous ghrelin usage), they were at significantly lower levels than the TG-T group (Fig. 2B). The levels of cartilaginous matrix formation were analyzed to further ascertain the effect of treatment. Significant increases in sGAG and collagen content were displayed by the TG-TG and TG-T groups (Fig. 2C–E), with ~20% higher sGAG expression in the TG-TG and TG-T group, as compared with the T-T group (Fig. 2C). However, there was no difference in sGAG and collagen content between the TG-TG and TG-T group. Further investigation showed that ghrelin can also promote chondrogenic gene expression levels of BMP-2 induced MSCs (Fig. S-3). In addition, chondrogenic differentiation of ESCs (embryonic stem cells) is sensitive to ghrelin treatment, as attested by the upregulation of chondrogenic genes (Fig. S-4).

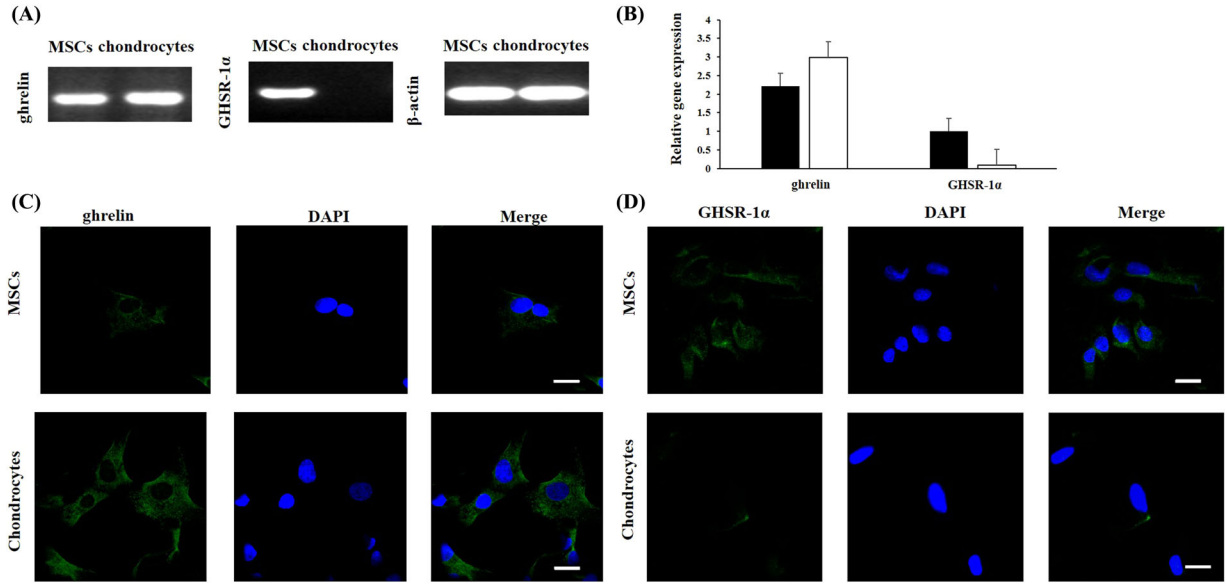

Figure 1. Gene and protein expression levels of ghrelin and GHSR-1α in MSCs and chondrocytes. (A) Agarose gel electrophoresis of ghrelin and GHSR-1α expression in rat MSCs and chondrocytes. (B) Gene expression levels of ghrelin and GHSR-1a in rat MSCs and chondrocytes were analyzed by qRT-PCR, $n = 4$ for each group. (C) Protein expression levels of ghrelin in MSCs and chondrocytes. (D) Protein expression levels of GHSR-1α in MSCs and chondrocytes. Scale bar = 10 μm.

Ghrelin Promotes Phosphorylation of ERK1/2 and DNMT3A

To study the mechanism of ghrelin induced chondrogenic differentiation, activation of signaling pathways that have been associated with MSC chondrogenic differentiation such as Smad,[24] and mitogen-activated protein kinases, p38, and ERK1/2[25] were investigated (Fig. 3A). TGF-β and ghrelin alone both enhanced phosphorylation of ERK1/2, with the latter being more effective. When applied together, TGF-β and ghrelin synergistically enhanced phosphorylation of ERK1/2. Both TGF-β and ghrelin, alone or together had little effects on the phosphorylation of p38. Activation of Smad3 and Smad1/5/8 (Fig. S-5) were detected with TGF-β, but not with ghrelin, and addition of ghrelin to TGF-β treatment did not further enhance the effect of

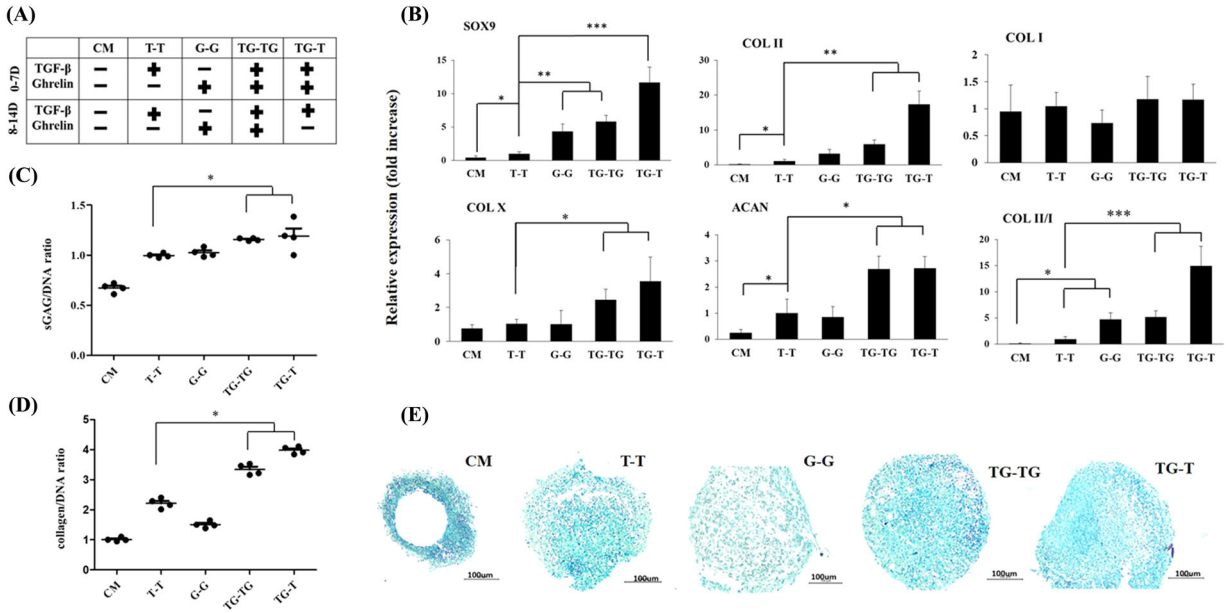

Figure 2. Chondrogenic differentiation of MSCs treated with various combinations of TGF-β and ghrelin. (A) The timeline for supplementation of ghrelin and TGF-β. (B) qRT-PCR analyses of *SOX9*, *COL II*, *COL I*, *COL X*, *ACAN*, and *COL II*/*I* expression were conducted after 14 days of chondrogenic differentiation. $n = 5$ per group. Quantification of (C) sGAG and (D) collagen deposition induced by pulse delivery of ghrelin was conducted after 14 days of chondrogenic differentiation. $n = 4$ per group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (E) Alcian blue staining of sGAG formation in pellets was conducted after 14 days of chondrogenic differentiation. $n = 4$ per group. Scale bar = 100 μm.

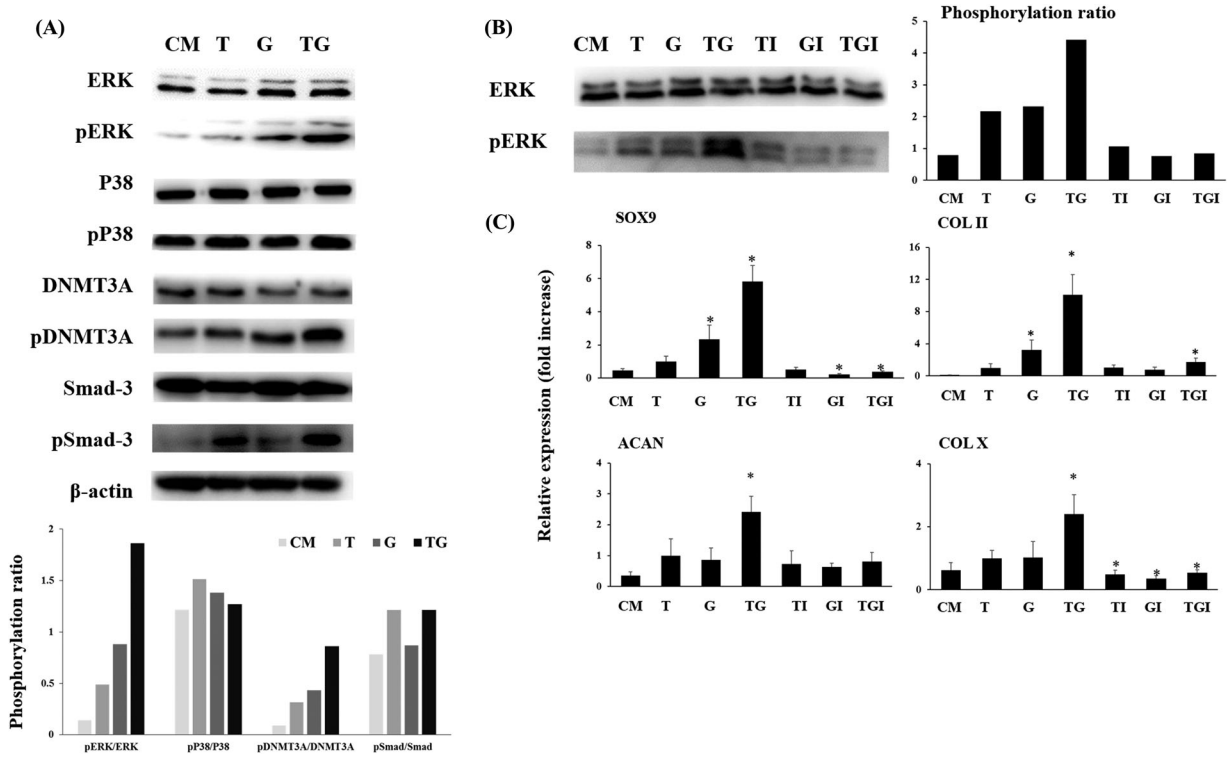

Figure 3. Analysis of signaling pathway activation in MSCs treated with TGF-β and ghrelin. MSCs were cultured in chondrogenic differentiation medium for 1 day in the presence of TGF-β and/or ghrelin. (A) Western blot analysis of ERK, P38, DNMT3A, and Smad phosphorylation. The phosphorylation ratio was analyzed by Tanon Gis. (B) ERK phosphorylation in the absence or presence of PD98059. (C) The gene expression levels of *SOX9*, *ACAN, COL II*, and *COL X* in the absence or presence of PD98059 were analyzed by qRT-PCR at 7 days. $^{*}p < 0.05$ compared with the control (CM) condition, $n = 3$ per group. CM = control, chondrogenic basal media without growth factor; T = TGF-β; G = ghrelin; TG = TGF-β + ghrelin. I denotes culture in the presence of PD98059 (Phosphorylation inhibitor of ERK).

TGF-β. DNA methytransferase, DNMT3A, was recruited to the *SOX9* promoter and inhibited gene expression level of *SOX9*. Activated ERK1/2 has been shown to phosphorylate DNMT3A, thereby blocking the recruitment of DNMT3A to the *SOX9* promoter, reversing DNMT3A inhibition of *SOX9* expression.[26] In this study, TGF-β and ghrelin alone both induced DNMT3A phosphorylation, which together exert a synergistic effect on DNMT3A phosphorylation.

Inhibition of ERK1/2 Phosphorylation and Knockdown of ERK1/2 and DNMT3A

To confirm that ERK1/2 activation was involved in chondrogenic differentiation induced by TGF-β and ghrelin, we investigated the effects of inhibiting ERK1/2 during chondrogenic differentiation using the phosphorylation inhibitor, PD98059. Inclusion of PD98059 inhibited phosphorylation of ERK1/2 induced by TGF-β, ghrelin or TGF-β + ghrelin (Fig. 3B), which in turn resulted in inhibition of *SOX9*, *COL II*, *COL X*, and *ACAN* expression induced by TGF-β and/or ghrelin (Fig. 3C).

To further dissect the ERK1/2-DNMT-3A signaling axis in mediating chondrogenesis of MSCs, we knocked down ERK1/2 or DNMA-3A in MSCs with the respective shRNA, shRNA-E or shRNA-D. ShRNA-E and shRNA-D efficiently knocked down the expression of ERK1/2 and DNMT3A, and subsequently that of pERK1/2 or pDNMT3A, respectively. Furthermore, shRNA-D had no effect on the phosphorylation of ERK1/2. However, the phosphorylation of DNMT3A was dramatically downregulated by shRNA-E (Fig. 4A and B). This result confirmed that DNMT3A was phosphorylated by ERK1/2, and with the knockdown of ERK1/2, the ratio of pDNMT3A/DNMT3A declined. Analysis of the expression of the chondrogenic genes (Fig. 4C) showed that shRNA-C had no effects on gene expression, while shRNA-E downregulated the gene expression levels of *SOX9*, *ACAN*, and *COL II*. ShRNA-D, on the other hand, resulted in upregulation of *SOX9* through downregulating expression of *DNMT3A* (Fig. 4D). However, no significant effect was observed on *COL II*, and *ACAN* expression.

Gene Expression Levels of Ghrelin, GHSR-1α, and ERK Phosphorylation During Chondrogenic Differentiation

We investigated the gene expression levels of ghrelin and GHSR-1α in relation to the different chondrogenic differentiation stages (Fig. 5A). In the TGF-β (T) group, ghrelin increased moderately during the first 6 days of culture, before subsequently declining to original levels in the undifferentiated MSCs (onefold gene expression). While in the TGF-β + ghrelin (TG) group, there was a moderate and consistent increase

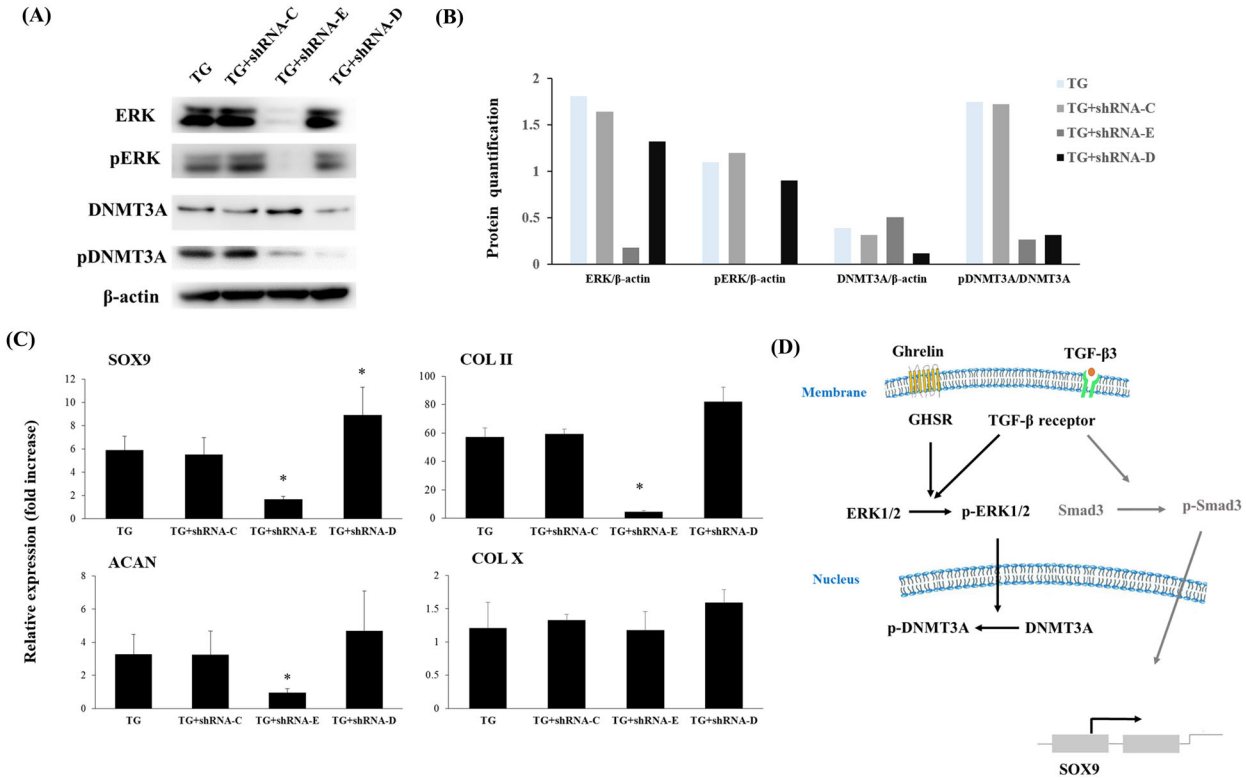

Figure 4. shRNA silencing of ERK and DNMT3A. (A) Western blot analysis of ERK and DNMT3A phosphorylation. (B) Protein quantification using Tanon Gis. (C) The expression levels of *SOX9*, *COL II*, *COL X*, and *ACAN* expression in the presence of shRNA were analyzed by qRT-PCR at 7 days. $^{*}p < 0.05$, compared to TG condition, $n = 4$ per group. (D) Schematic illustration of the possible mechanism of TGF-β + ghrelin in modulating chondrogenic differentiation of MSCs.

in ghrelin expression over 9 days of culture, which declined to the same levels as in the undifferentiated MSCs by the 12th day of culture. Expression of GHSR-1α dropped to the lowest level on day 6 for all groups and then gradually increased, but never returned to the original levels even by day 12. In the TGF-β group, GHSR-1α expression was upregulated on the 9th day but declined to same level as the undifferentiated MSCs by the 12th day.

To uncover much more details about why ghrelin can enhance the TGF-β effect, we investigated the activation of ERK1/2 during the process of chondrogenic differentiation (Fig. 5B). Ghrelin, in combination with TGF-β, induced increased phosphorylation of

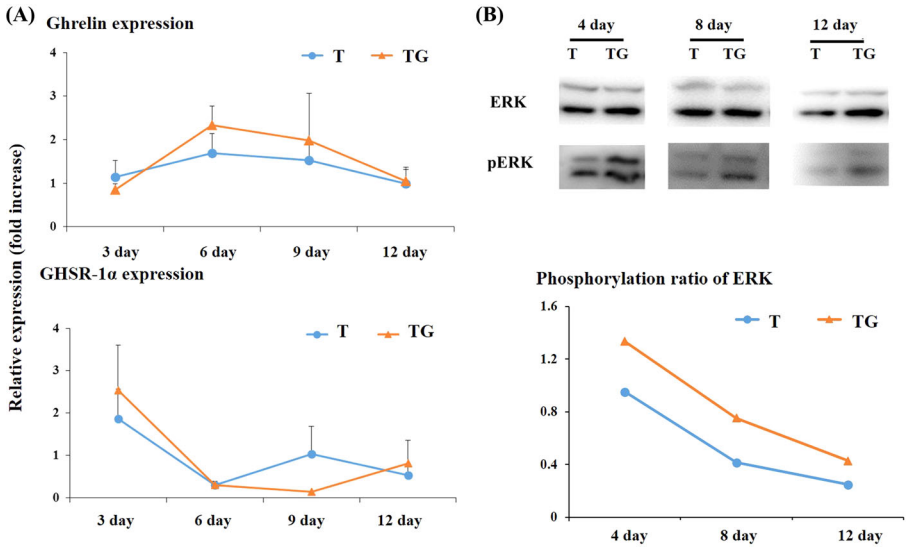

Figure 5. Gene expression levels of ghrelin, GHSR-1α, and ERK activation during chondrogenic differentiation. (A) qRT-PCR analyses of ghrelin and GHSR-1α gene expression at various time points during chondrogenic differentiation. $n = 3$ per group. (B) Western blot analyses of ERK phosphorylation during chondrogenic differentiation on days 4, 8, and 12.

ERK1/2, as compared to TGF-β alone on day 4. While ERK1/2 phosphorylation induced by TGF-β alone declined after day 4, continuous addition of ghrelin with TGF-β maintained a higher level of phosphorylation of ERK1/2 on day 8 that was sustained up to day 12.

Regeneration of Articular Cartilage Defects

The synergistic enhancing effect of ghrelin on TGF-β-induced MSC chondrogenesis was evaluated using a rat osteochondral defect model. Cartilage defects were implanted with MSCs, followed by delivery of TGF-β with or without ghrelin to the joint cavity. Two concentrations of TGF-β were delivered, 100 and 1,000 ng/ml, and the amount of ghrelin delivered in conjunction with TGF-β is at the ratio of 1:10 (nM: ng/ml), in accordance to the optimum combination condition derived from the in vitro study (Fig. S-1). At 4 weeks post-surgery, the ICRS macroscopic score (Fig. 6A and B) and ICRS visual histological score (Fig. 6C and D) showed that co-administration of ghrelin with TGF-β was able to yield significantly higher scores compared to TGF-β alone. Overall, the 10 nM ghrelin-addition group exhibited partial cartilage tissue repair compared to the lack of repair with TGF-β alone. Although 1,000 ng/ml of TGF-β with 100 nM of ghrelin showed no difference with 1,000 ng/ml TGF-β alone in ICRS score performance, the Alcian blue and collagen II staining were stronger in the combination group, with no difference in collagen X and collagen I staining between the groups. At 12 weeks post-surgery, all groups showed better ICRS macroscopic score with more continuous tissue and much smoother cartilage surface (Fig. 7A and C). There were no significant differences between the high concentration groups, but again, the 10 nM ghrelin-addition group displayed a higher score in the ICRS macroscopic score, as compared with TGF-β alone (Fig. 7B and D). Importantly, at 12 weeks post-surgery, there was more intense staining of collagen I in high concentration TGF-β groups. There were also no differences in the Young's moduli of the cartilage defects between the four groups at 12 weeks post-surgery (Fig. S-6).

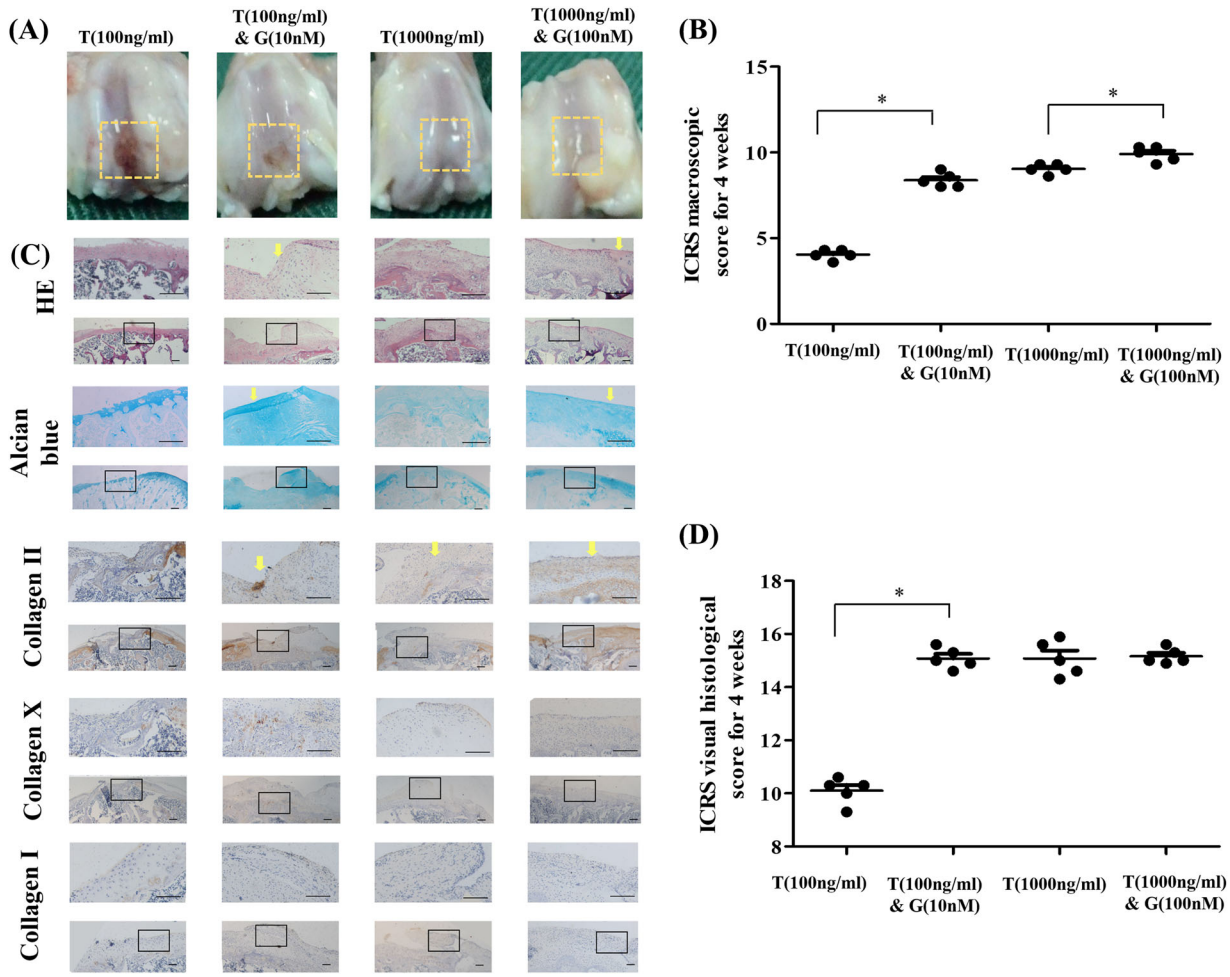

Figure 6. In vivo effects of ghrelin and TGF-β on cartilage regeneration in a rat osteochondral defect model (at 4 weeks post-implantation of MSC). (A) Macroscopic view of cartilage repair. (B) ICRS Macroscopic score of joints. $n = 5$ per group. (C) HE staining, Alcian blue staining, immunohistochemical staining of sections for collagen type II, collagen type X, and collagen type I. Scale bar = 200 μm. (D) ICRS visual histological score of tissue sections. $n = 5$ per group. T denotes TGF-β, and G denotes ghrelin. $^{*}p < 0.05$.

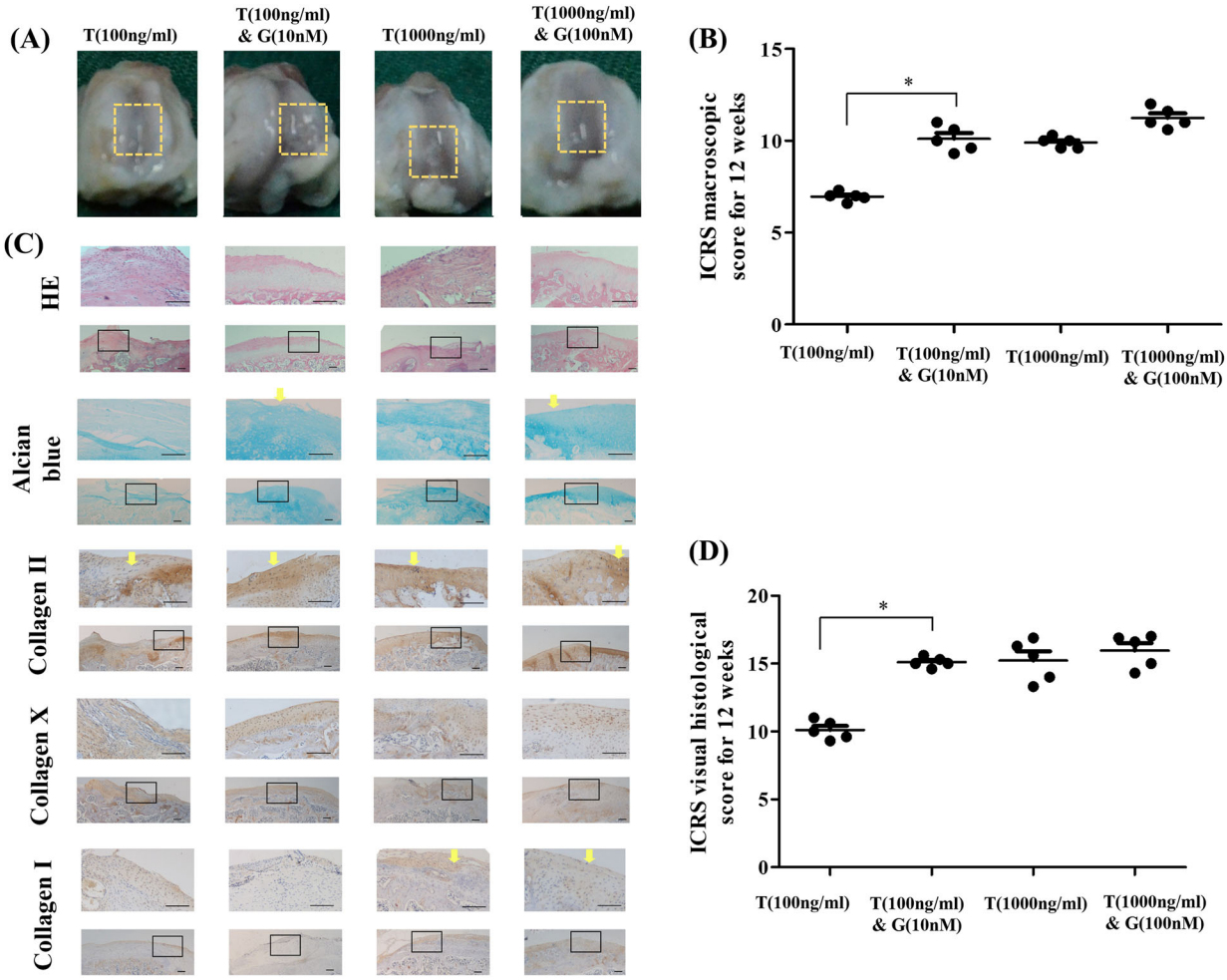

Figure 7. In vivo effects of ghrelin and TGF-β on cartilage regeneration in a rat osteochondral defect model (at 12 weeks post-implantation of MSCs). (A) Macroscopic view of cartilage repair. (B) ICRS Macroscopic score of joints. $n = 5$ per group. (C) HE staining, Alcian blue staining, immunohistochemical staining of tissue sections for collagen type II, collagen type X, and collagen type I. Scale bar = 200 μm. (D) ICRS visual histological score of tissue sections. $n = 5$ per group. T denotes TGF-β, and G denotes ghrelin. $^{*}p < 0.05$.

DISCUSSION

The rationale for temporal delivery of ghrelin instead of continuous delivery to enhance chondrogenic differentiation lies in the differential expression of ghrelin and its receptor GHSR-1α over different stages of chondrogenic differentiation. The gap between relatively low expression of ghrelin[27] and relatively high expression of GHSR-1α in MSCs is the rationale for adding ghrelin during the early stage of chondrogenic differentiation of MSCs, while gradual down-regulation of the GHSR-1α as well as up-regulated expression of ghrelin by the differentiated MSCs is the rationale for limited delivery of ghrelin during later stages of chondrogenic differentiation, as previously reported.[28]

Besides increasing expression of chondrogenic genes, delivery of ghrelin and TGF-β also enhanced the gene expression level of *COL X*, which is the marker of hypertrophy and matured chondrocytes. TGF-β promoted hypertrophy and terminal differentiation of chondrocytes derived from MSCs.[29] However, ghrelin failed to offset the hypertrophy induced by TGF-β. Given that our in vitro intracellular signaling pathway analysis indicated that both TGF-β alone, or in combination with ghrelin, induced similar levels of P38 (Fig. 3A) and Smad-1/5v (Fig. S-5) actiation, both pathways being associated with chondrogenic hypertrophy,[30] it is thus not unexpected that co-treatment with ghrelin did not mitigate TGF-β-induced hypertrophy development. Further studies to investigate other therapeutic combinations with ghrelin for hypertrophy suppression will be required to optimize ghrelin application in cartilage regeneration. Various combinations of growth factors can enhance chondrogenic differentiation of MSCs, but often result in upregulated gene expression level of collagen X,[31,32] except for forskolin, which can significantly enhance chondrogenic differentiation of MSCs without any significant up-regulation of *Col X*.[33]

The aforementioned synergistic effects of TGF-β and ghrelin on the chondrogenic differentiation of mesenchymal stem cells could potentially be expanded

to a larger scope: Core component (X) + adjunct component (Y) on multiple stem cells (Z). Besides TGF-β, BMP2/4, GDF-5 (BMP-14), and pTHrP can also induce chondrogenic differentiation. BMP-2/4 can induce hypertrophy,[34,35] but not GDF-5 and pTHrP.[36,37] We have confirmed the synergistic effects of BMP-2 with ghrelin on the chondrogenic differentiation of MSCs, which was comparable to TGF-β with ghrelin (Fig. S-3). Ghrelin and TGF-β were demonstrated to synergistically activate the ERK1/2 signaling pathway in this study. Besides phosphorylation of ERK, multiple mechanisms are potentially linked to the enhancing effects of TGF-β and other chondrogenic factors, such as factors that fine-tune ligand-receptor interaction, DNMT3A phosphorylation, etc. Multiple growth factors and small molecules have been reported, including forskolin, epidermal growth factor, and leptin.[33,38,39] Furthermore, the synergistic effects of TGF-β and ghrelin on the chondrogenic differentiation of Embryonic Stem Cells can also be extrapolated to induced pluripotent stem cells and other adult stem cell types (Fig. S-4). With further optimization of these X × Y × Z components, multiple parameters could be fine-tuned, such as efficiency, hypertrophy, etc.

The rat osteochondral defect model utilized in this study are often utilized for preliminary in vivo investigations. However, the rat osteochondral defect model has its limitation and do not completely reflect cartilage lesions in the human clinical model. The joints of rats have stronger capacity to heal spontaneously, when compared to the human model.[40,41] The defect created in rats is osteochondral which result in marrow infiltration, while human cartilage lesions pre-dominantly have their osteochondral bone intact. Additionally, the rat knee differs anatomically to that of human knee,[41] with different mechanical loading to the joint. Both these inherent factors are known to affect cartilage repair, and might affect the in vivo effects of TGF-β and ghrelin on cartilage regeneration.

It is of great significance that ghrelin, when used in combination with a relatively low dose of TGF-β, can dramatically enhance de novo cartilage regeneration, as this could circumvent the use of high doses of TGF-β, which could result in osteophyte formation and synovial hyperplasia.[4,5] Although a large number of different agents have been reported to promote chondrogenic differentiation, very few of these have the potential to be effective within in vivo studies.[42] Furthermore, ghrelin at the dose used have been shown to reduce inflammatory cytokine induced expression of matrix proteinases and ameliorated degradation of type II collagen and aggrecan.[43] Moreover, we observed an increase in collagen I deposition (which is a marker of fibrocartilage formation) in animals treated with high concentrations of TGF-β (Fig. 7). This was not observed in the neo-cartilage of combined ghrelin + TGF-β treatment groups. Despite significant improvement in cartilage regeneration, no differences in the compressional property of the repaired cartilage were detected, and hypertrophy was not completely avoided, indicating that our resulting repaired cartilage tissue with one time delivery of TGF-β and ghrelin was probably inadequate. Further studies with controlled longer term delivery of growth factors could further improve the regeneration outcome.

In conclusion, this study not only fine-tuned current chondrogenic differentiation protocols, but also laid the foundation for further development with the "X × Y × Z" model, with a more comprehensive understanding of the multiple factors involved in chondrogenic differentiation. As a naturally occurring human peptide, ghrelin has high potential for utilization in clinical applications of cartilage regeneration and early intervention, in combination with TGF-β, or other growth factors, together with appropriate scaffolds and delivery protocols.

AUTHORS' CONTRIBUTIONS

Litong Fan: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; Jiaqing Chen: Data analysis and interpretation; Yanmeng Tao: Construction of shRNA; Boon Chin Heng: Editing of manuscript; Jiakuo Yu: Editing of manuscript; Zheng Yang: Data interpretation, manuscript writing; Zigang Ge: Conception and design, data interpretation, manuscript writing. All the authors have read and approved the final submitted manuscript.

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ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

All animal experiments were approved by the Institutional Animal Care and Use Committee of Peking University.

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SUPPORTING INFORMATION

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