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ORIGINAL RESEARCH PAPER

Mechano-growth factor enhances differentiation of bone marrow-derived mesenchymal stem cells

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Abstract
Objectives To investigate the effect of mechano-growth factor (MGF) on the differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs) in vitro.
Results Flow cytometry assay identified the isolated cells were human bone marrow mesenchymal stem cells, which had differentiation ability when cultured with specific induction culture media. Alizarin Red S, Oil Red O and Alcian Blue staining showed osteogenic, adipogenic and chondrogenic differentiation were significantly increased after hBMSCs were treated with MGF E peptide. Collagen II expression was considerably increased after hBMSCs were induced with chondrogenic induction culture medium supplemented with TGF-β3 and MGF E peptide. Overexpression of MGF by an expression plasmid further confirmed the MGF could enhance tri-lineage differentiation of hBMSCs. Moreover, we found that hBMSCs proliferation rate was decreased and G1 phase of the cell cycle was lengthened after MGF treatment when compared to the control group.
Conclusions MGF can enhance differentiation of hBMSCs during specific induction culture media induction by lengthening G1 phase of cell cycle.

Keywords Bone marrow mesenchymal stem cells · Mechano-growth factor · Differentiation · Cell cycle

Introduction

In cell therapy and tissue engineering, stem cells need to differentiate into various cells. Bone marrow mesenchymal stem cells (BMSCs), capable of self-renewal and differentiation into multiple cell lineages, have boosted interest in the field of regenerative medicine. Application of BMSCs promotes tissue repair, including in bone, cartilage, liver, airway, skeletal muscle imperfecta or defects, etc. (Dominici et al. 2006). At present, the efficiency of tissue repair using BMSCs is, however, limited by using the current differentiation methods.

Insulin-like growth factor 1(IGF-1) is a crucial factor that regulates growth in many tissues (Liu et al. 1993). In humans, *IGF-1* pre-mRNA generates three

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isoforms including *IGF-1Ea*, *IGF-1Eb* and *IGF-1Ec* (*MGF*) by alternative splicing. These three isoforms contain the same mature IGF-1 peptide but a different E domain. A 49 base pair insert during the splicing of exons 5 of *IGF-1* can give rise to a unique E domain of MGF (MGF E peptide) (Yang et al. 1996).

MGF E peptide has unique physiological properties distinct from IGF-1. It is a positive regulator of skeletal myoblast proliferation and differentiation leading to muscle hypertrophy or regeneration (Yang and Goldspink 2002). In addition, MGF E peptide promotes the migration ability but attenuates proliferation of hBMSCs (Collins et al. 2010). Injecting the peptide into the bone defect gap results in accelerated bone healing (Deng et al. 2011). However, whether and how MGF can influence the differentiation of hBMSCs remains largely elusive.

In this study, we obtained primary hBMSCs and characterized their differentiation ability. By application of MGF E peptide or overexpression of MGF, we tested the role of MGF in osteogenic, adipogenic and chondrogenic differentiation of hBMSCs. Accompanying hBMSCs differentiation, we also detected cycle phase of hBMSCs which was treated by MGF. Our results indicate that MGF significantly enhanced trilineage differentiation of hBMSCs by reducing the cell proliferation and lengthening the cell cycle G1 phase, thus providing a potential application of MGF for cell therapy and tissue engineering.

Materials and methods

Cell culture

hBMSCs were generated from healthy human bone marrow samples (ranging from 22 to 42 years old in age). Tissue usage was approved by donors and Ethics Committee of Chongqing University, China. Bone marrow aspirates were cultured in low-glucose Dulbecco's Modified Eagle Medium supplemented with 10 % (v/v) FBS, penicillin (100 U ml^{-1}), streptomycin (100 U ml^{-1}), and basic fibroblast growth factor (bFGF, 1 ng ml^{-1}; Invitrogen, Carlsbad).

Cell differentiation

hBMSCs were seeded at 5×10^3 cells cm^{-2} for osteogenic differentiation or 10^4 cells cm^{-2} for adipogenic differentiation. Osteogenic induction medium (OIM) contained 10 mM β-glycerophosphate, 100 nM dexamethasone and 0.2 mM ascorbic acid in low-glucose-DMEM supplemented with 10 % (v/v) FBS. Adipogenic induction medium (AIM) contained 40 μM dexamethasone, 20 ng insulin ml^{-1}, 20 μM 3-isobutyl-1-methyl-xanthine (IBMX; Sigma), and 100 μM indomethacin (Sigma). For chondrogenesis, 2×10^5 cells were centrifuged at $300 \times g$ in 15 ml polypropylene conical tubes. Aggregates were cultured with chondrogenic induction medium (CIM): high-glucose-DMEM supplemented with 10 % (w/v) recombinant human insulin/human transferrin/sodium selenite (ITS) (Sigma), 10 μM dexamethasone, 100 μM sodium pyruvate and 100 μM L-proline. 20 ng MGF E peptide (Phoenix Pharmaceuticals, Inc. Burlingame) ml^{-1} was added to induction medium every day.

Alkaline phosphatase (ALP) activity assay

ALP activity quantitative analysis was assessed by using a biochemical assay kit (JianCheng, Nanjing, China). Cultured cells were lysed with 0.2 % v/v Triton X-100, and incubated with *p*NPP substrate. Colored end product was detected at 520 nm on a microplate reader.

Alizarin Red S staining

hBMSCs undergoing osteoblastic differentiation were fixed with 10 % (v/v) formalin for 30 min at room temperature and were then stained with Alizarin Red S solution for 5 min after wash. Images were taken with an inverted microscope (Olympus, Japan).

Oil Red O staining

hBMSCs undergoing adipogenic differentiation were fixed in 4 % (v/v) formalin for overnight, washed with 60 % (v/v) 2-propanol and air dried. Then the cells were stained with Oil Red O working solution for 20 min, washed with 70 % (v/v) ethanol and distilled water. After being incubated in 2-propanol for 1 h, the samples were measured at 490 nm on the microplate reader.

Histological and immunohistochemical analysis

The chondrogenic cell pellets were frozen at the optimum cutting temperature and cut into 5 μm

sections. For histological examination, sections were fixed with methanol and stained with 0.5 % Alcian Blue for 30 min. To quantify the intensity of the staining, the dye was dissolved with guanidine hydrochloride and the absorption was measured at 630 nm. For immunostaining, the sections were incubated with the primary antibody (anti-human Collagen II, 1:100; Abcam, Hongkong) and Alexafluor 488-conjugated secondary antibody (Life Technology), followed by DAPI (1 mg ml^{-1}; Life Technology) counterstaining. All samples were imaged on a fluorescence microscope (Nikon, Tokyo).

ELISA and spectrophotometry

Total protein was extracted from hBMSCs which were exposed to chondrogenic medium for 15 days. Collagen II expression was detected by using a commercially available ELISA kit. The absorbance was measured on a microplate reader. A DNA extraction kit (Bioteke, China) was used to extract the total cellular DNA, which was then measured by spectrophotometry.

Overexpression of MGF in hBMSCs

The whole length *MGF* gene fragment was cloned into a *pc*DNA3.1 (+) vector. The sequences of forward and reverse primers for *MGF* were: 5′-CGAAGTCTC AGAGAAGGAAAGG-3′ and 5′-ACAGGTAACTCG TGCAGAGC-3′. The MGF-plasmid (pcMGF) or control plasmid was transfected into hBMSCs using a lipofectamine kit (Invitrogen). Expression of *MGF* in the hBMSCs was assessed by GFP and RT-PCR (see Fig. 3 below).

Semi- quantitative RT-PCR and quantitative real-time PCR

Total RNA was isolated using the RNeasy Plus mini kit (Qiagen, China) according to the manufacturer's instructions. Semi-quantitative PCR and real-time PCR were performed by using a PCR kit (MBI) or QuantiTect SYBR Green PCR kit (Qiagen, China), respectively. *GAPDH* was used as an internal control to normalize the data. The PCR primers are shown in Supplementary Table 1.

Proliferation assay

hBMSCs were seeded into 96 well plates at 2×10^3 cells/well. The following day, 20 ng MGF E peptide ml^{-1} or 20 ng IGF-1 ml^{-1} or MGF E peptide + IGF-1 were added into different wells. Cell viability was determined by using a Cell Counting Kit-8 (Beyotime, Wuhan). The absorbance of each well was measured with a microplate reader set at 450 nm.

Flow cytometry analysis

To analyze the phenotypic characterization of hBMSCs (P3), collected cells were incubated with FITC-conjugated antibodies against CD29, CD90, CD34, CD45 (all from BioLegend, San Diego) for 30 min and detected by fluorescence-activated cell sorting (FACS). For cell cycle analysis, hBMSCs were cultured in low glucose-DMEM (containing antibiotics and 2 % v/v FBS) with or without 20 ng ml^{-1} MGF E peptide. After 24 h, the cells were collected and stained with 0.5 ml propidium iodide. Analysis was performed on a BD FACSAria I flowcytometer (BD Biosciences, San Jose). G1, S, and G2 ratios were calculated using CellQuest Pro Software (version 5.1, BD Biosciences).

Statistical analysis

All the experiments were performed at least three times. Data are expressed as mean ± SD. Samples were compared by one-way ANOVA using Origin Pro 7.5 software. In each analysis, significance level was set to be $P < 0.05$.

Results

Characterization of hBMSCs

Flow cytometry was used to characterize the phenotypic identification of isolated mesenchymal cells. Over 98 % of cells expressed mesenchymal stem cells markers CD29, CD90 but did not express the hematopoietic markers CD34 and CD45 (Fig. 1a). To verify the differentiation ability of isolated mesenchymal cells, cells were cultured with OIM, AIM or CIM. Compared to the none-induced cells represented by phase- contrast microscopy image, the isolated

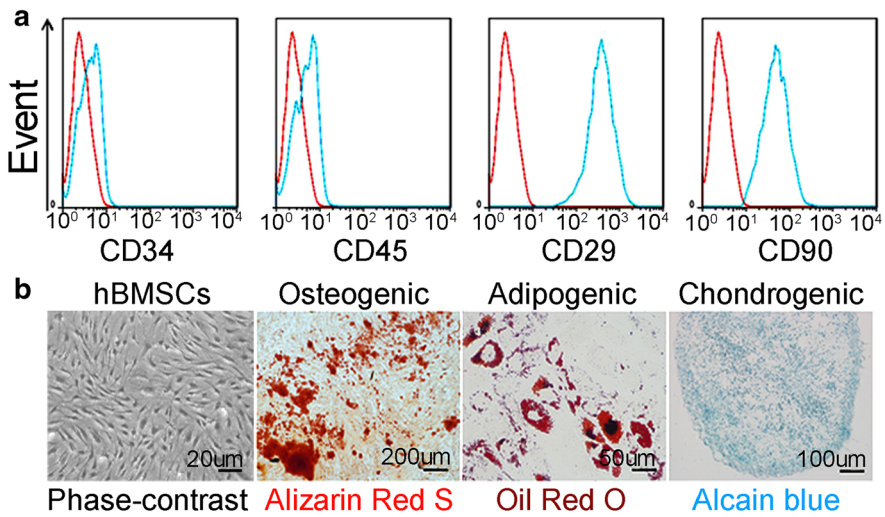

Fig. 1 Characterization of human bone marrow-derived mesenchymal stem cells (hBMSCs). **a** The expression profiles of hBMSCs surface marker. **b** The osteogenic, adipogenic and chondrogenic differentiation potential of hBMSCs

mesenchymal cells were differentiated into osteoblastic cells, adipogenic cells, and chondrogenic cells after culture for 2–3 weeks shown by Alizarin Red S, Oil Red O and Alcian blue stainings, respectively (Fig. 1b). These studies indicate that the isolated mesenchymal cells were hBMSCs.

Effects of MGF E peptide on differentiation of hBMSCs to osteogenic, adipogenic and chondrogenic cells

To determine the effect of MGF E peptide on the osteogenic differentiation potential of hBMSCs, cells were cultured in OIM supplemented with MGF E peptide for 9 days. Alizarin Red S staining revealed that the mineral deposition was significantly higher in OIM + MGF E peptide-treated group than that in OIM control group (Fig. 2a). The average ALP activity level was also dramatically increased (1.25-fold) after MGF E peptide treatment compared to OIM control group (Fig. 2b).

The effect of MGF E peptide on adipogenic differentiation ability of hBMSCs was next evaluated. After the cells were treated for 8 days, Oil Red O staining showed that larger amount of lipid droplets were accumulated in AIM + MGF E peptide-treated group than in that of AIM control group (Fig. 2c). Statistical analysis revealed that significantly increased absorbance value of Oil Red O in AIM + MGF E peptide-treated group (1.36 times higher) than that of the AIM control group (Fig. 2d). RT-PCR and statistical analysis revealed the mRNA levels of two adipogenic markers, peroxisome proliferator-activated receptor γ (*PPAR γ*) and fatty acid binding protein 2 (*aP2*), were 1.2- and 2.4-fold higher in the AIM + MGF E peptide-treated group than in the AIM control group (Fig. 2e–g).

TGF-β3 can enhance and accelerate chondrogenesis and neocartilage regeneration (Lee et al. 2010; Mackay et al. 1998; Moioli et al. 2006). Here we also used TGF-β3 to investigate whether MGF E peptide acts synergistically with TGF-β3 in chondrogenesis of hBMSCs. Cells were incubated in CIM, TGF-β3 + CIM or TGF-β3 + CIM + MGF E peptide for 15 days, respectively. Immunostaining showed that, compared to CIM control group, collagen II (marker of mature chondrocytes) expression was significantly increased after TGF-β3 + CIM or TGF-β3 + CIM + MGF E peptide treatment (Fig. 2h). This was especially true for TGF-β3 + CIM + MGF E peptide-treated group. ELISA assay was further used to detect collagen II induction after different treatments (Fig. 2i). The relative induction of Collagen II was normalized to DNA content among different experimental groups (Fig. 2j–k). The results showed that collagen II expression was significantly increased by 5.4- (13.7 ng/ml) and 7.6-fold (18.8 ng/ml) after TGF-β3 + CIM or TGF-β3 + CIM + MGF E peptide treatment, respectively, when compared to the CIM control group (2.5 ng/ml). Notably, collagen II expression level was 1.4-fold higher in TGF-

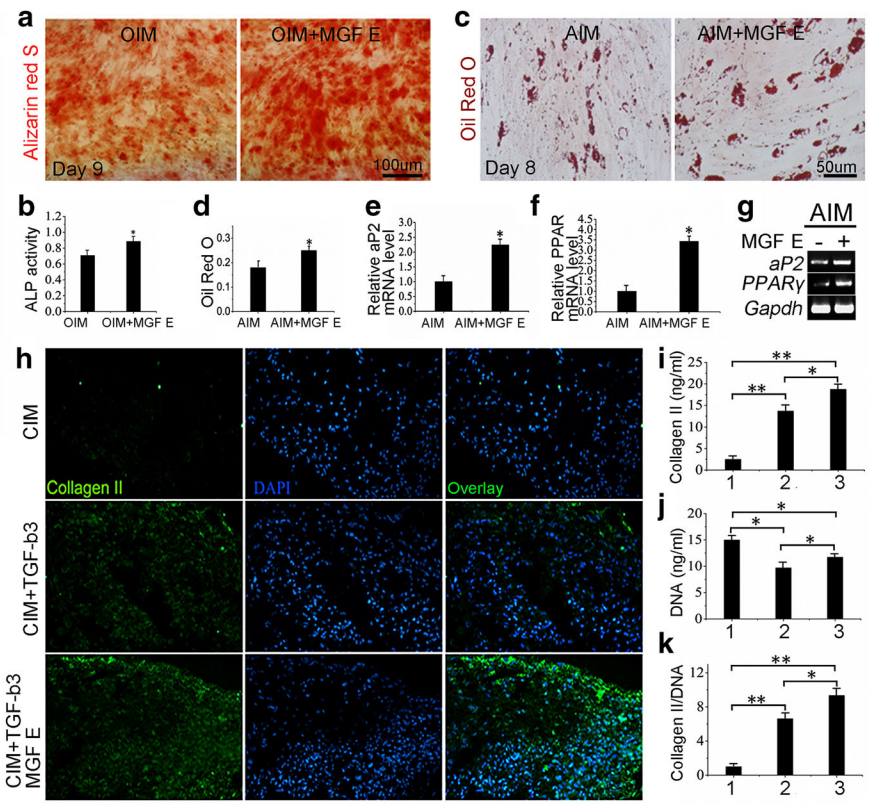

Fig. 2 Effects of MGF E peptide on osteogenic, adipogenic and chondrogenic differentiation of hBMSCs. **a** Alizarin *Red S staining* shows osteogenic differentiation in OIM and OIM + MGF E peptide-treated groups. *Scale bar* = 100 μm. **b** ALP activity analysis of OIM and OIM + MGF E peptide-treated groups. **c** Oil *Red O staining* shows adipogenic differentiation in AIM and AIM + MGF E peptide-treated groups. *Scale bar* = 50 μm. **d** Quantification of lipid drops in Oil *Red O staining* assay. **e** and **f** The expression levels of *aP2* and *PPAR γ* genes. Data was normalized against the control. **g** RT-PCR analysis of *aP2* and *PPAR γ* expression. *GAPDH* was used as reference gene. **h** Immunostaining of collagen II (*green*) deposition in different groups. Nuclei were stained by DAPI (*blue*). *Scale bar* = 50 μm. (i) ELISA assay for quantification of collagen II. **j** DNA contents levels in different groups. **k** Total content of collagen II was normalized to total DNA content. (i–k) *1* CIM group; *2* CIM + TGF-β3 group; *3* CIM + TGF-β3 + MGF E group. *P < 0.05, **P<0.01

β3 + CIM + MGF E peptide-treated group than that of TGF-β3 + CIM group (Fig. 1k).

Overepression of MGF improves osteogenesis, adipogenesis and chondrogenesis of hBMSCs

To identify the roles of MGF in hBMSCs differentiation, the *MGF* expression plasmid was cloned and then transfected into hBMSCs. Fluorescent microscopy images for GFP showed about 50 % of the cells were successfully transfected with control or pcMGF plasmids (Fig. 3a). RT-PCR analysis further confirmed significantly higher expression of *MGF* in hBMSCs after being transfected with pcMGF plasmid, when compared to the control plasmid group (Fig. 3b). The transfected hBMSCs were then cultured in induction medium to induce mesenchymal tri-lineage differentiation. Consistent with the MGF E peptide induction studies, Alizarin Red S, Oil Red O and Alcian blue stainings showed that overexpression of *MGF* significantly enhanced calcium nodule deposits (Fig. 3c), lipid droplets accumulation (Fig. 3e) and cartilage matrix formation (Fig. 3g), when compared with empty vector- treated group under induction media, respectively. Statistical analysis revealed that calcium deposits, lipid droplets accumulation and cartilage sulfated proteoglycan matrix in *MGF* overexpression group were increased by 29, 61 and 77 %, respectively, when compared to the empty vector-treated group (Fig. 3d, f, h). These

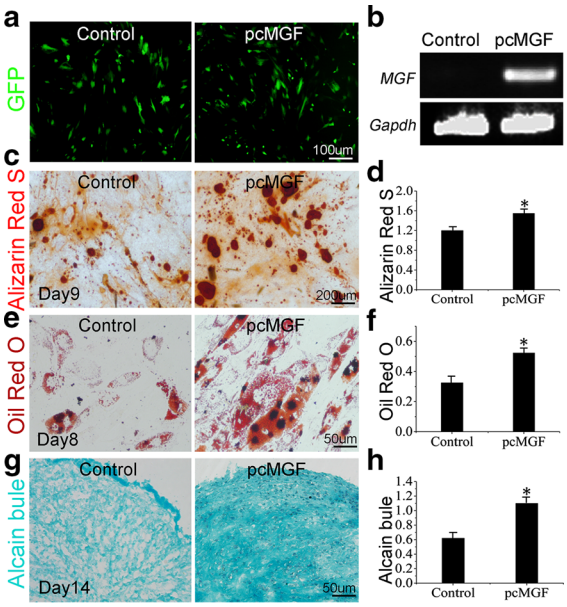

Fig. 3 Effects of *MGF* overexpression on differentiation of hBMSCs. **a** Fluorescent images show successful transfection of control or *MGF* plasmid into hBMSCs (*Scale bar* = 100 μm). **b** RT-PCR shows mRNA level of *MGF* in hBMSCs (product size, 531 bp). **c** Alizarin *Red S staining* shows osteogenic differentiation of hBMSCs after MGF overexpression (*Scale bar* = 200 μm). **d** Quantification of Alizarin *Red S staining*. **e** Oil *Red O staining* shows adipogenic differentiation of hBMSCs after MGF overexpression (*scale bar* = 50 μm). **f** Quantification of Oil *Red O staining*. **g** Alcian *blue staining* shows chondrogenic differentiation of hBMSCs after MGF overexpression (*Scale bar* = 50 μm). **h** Quantification of Alcian *Blue staining*. *P < 0.05 with control

data further confirm MGF can promote hBMSCs differentiation.

Effects of MGF E peptide on cell cycle

To study how MGF affects hBMSCs differentiation, we then test the cell viability of hBMSCs after 20 ng ml^{-1} MGF E peptide or 20 ng ml^{-1} IGF-1 treatments. MGF E peptide-treated cells showed significantly lower cell viability while IGF-1-treated cells showed significantly higher cell viability from 12 up to 48 h, when compared to that of the control (Fig. 4a). These results suggest that MGF E peptide may retard cell cycle in hBMSCs. Accordingly, flow cytometry assay regarding the cell cycle showed that, MGF E peptide treatment induced a significant inhibition of transformation from G1 phase to S phase in hBMSCs cultured in growth medium as compared to that of the control group at 24 h (Fig. 4b, c). The percentage of cells in G1 phase was increased by 12 % (88.4 ± 1.4 vs. 78.6 ± 2.1) after MGF E peptide treatment when compared to the control group (Fig. 4d). To further confirm the inhibition of MGF E peptide on cell cycle of hBMSCs, the expression of the cyclin (*Cyclin E*) and the cyclin-dependent kinase 2 (*CDK2*) were analyzed by quantitative real-time PCR experiment. The results showed that the mRNA level of *cyclin E* and *CDK2* in hBMSCs was significantly decreased by 25 % and 18 %, respectively, after MGF E peptide treatment when compared to the control groups (Fig. 4e, f).

Discussion

MGF plays different roles in cell differentiation (Hill and Goldspink 2003; Xin et al. 2014; Olesen et al. 2006). So far, little is known about the effect of MGF on hBMSCs differentiation. In this study, we showed that MGF significantly enhanced osteogenic, adipogenic and chondrogenic differentiation of hBMSCs. Moreover, we demonstrated that MGF enhance stem cell differentiation by lengthening G1 phase of cell cycle.

hBMSCs have to pass through several stages to differentiate into osteoblasts. ALP levels are up-regulated during early stages of bone formation (Jikko et al. 1999). In our study, ALP expression was up-regulated under osteogenic conditions with MGF E peptide. We found increased ALP expression by adding MGF E peptide compared with OIM group. The mineralization phase is the last stage of bone formation. MGF E peptide can significantly suppress osteoblast differentiation of rat BMSCs (Cui et al. 2014) although our study showed that the hBMSCs produce much more calcium nodules after MGF E peptide treatment or MGF overexpression, indicating that there was enhanced conversion of hBMSCs into osteoblasts. The different results could be due to different cell lines or concentrations of MGF E peptide being used. Therefore, we conclude that MGF E peptide could enhance hBMSCs osteogenic differentiation under OIM condition.

TGF-β3 plays broad roles in cell adhesion, differentiation and homing (Lee et al. 2010; Moioli et al. 2006). The IGF-1 signal can enhance the effect of TGF-β3 on chondrogenesis induction of hBMSCs

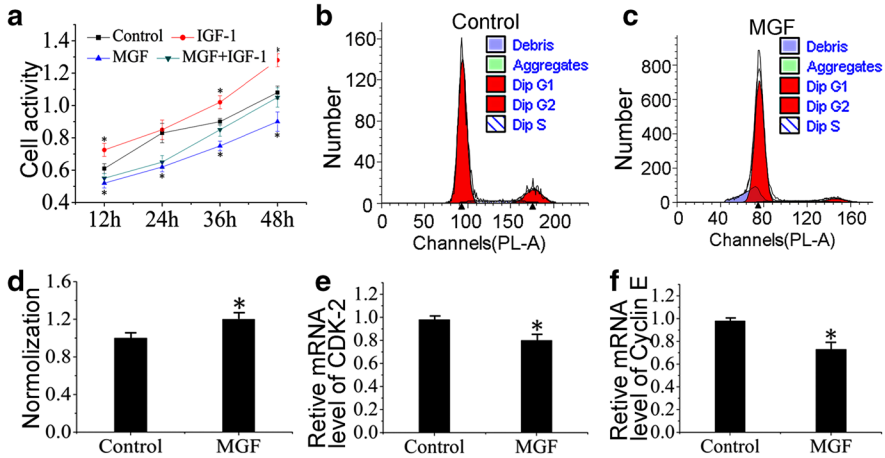

Fig. 4 Effects of MGF on cell cycle. **a** Effects of MGF E peptide of IGF-1 on the proliferation of hBMSCs. **b** and **c** Flow cytometry analysis of hBMSCs after MGF E peptide treatment (Control: G1:78.53 %, G2:14.74 %, S: 6.74 %; MGF E peptide group: G1:89.81 %, G2: 5.24 %, S: 4.95 %). **d** Quantification of flow cytometry analysis. **e** and **f** Relative mRNA expression levels of *cyclin E* and *CDK2*. *GAPDH* was used as reference gene. *P < 0.05 with control

(Indrawattana et al. 2004). Our results also showed that TGF-β3 + MGF E peptide group has more collagen II synthesis compared to other groups. Alcian blue staining indicated that MGF increased the expression of cartilage sulfated proteoglycan matrix. These findings together suggest that MGF E peptide might act synergistically with TGF-β3 to enhance chondrogenesis of hBMSCs. Interestingly, we found that the total DNA content was decreased in both TGF-β3 + MGF E peptide and MGF E peptide-treated groups. Apoptosis may occurr in the pellet cultures of chondrogenesis (Wang et al. 2010). Therefore, combining increased differentiation with apoptotic event of MGF-treated hBMSCs, the DNA content will be decreased in chondrogenesis of hBMSCs.

How does MGF enhance hBMSCs differentiation? Usually cell differentiation will be promoted if the cell cycle is blocked at the G1 phase. The cell cycle arrested in G0/G1 phase promotes MSCs differentiation (Liu et al. 2014; Sharma et al. 2014) and our results showed that the cell proliferation rate was decreased and the expression of *cyclin E* and *CDK2* was down-regulated after MGF E peptide treatment. MGF E peptide may also cause activation of the MAPK/Erk1/2 signaling pathway (Mills et al. 2007; Armakolas et al. 2010). MAPK signal pathways are largely involved in the regulation of cell proliferation in mammalian cells (Zhang and Liu 2002). Whether and how MGF regulates MAPK pathway to block the G1 phase of cell cycle in hBMSCs needs further study.

In conclusion, our study provides direct evidence that MGF can promote hBMSCs differentiation during osteogenic, adipogenic and chondrogenic differentiation. It also provides a potential strategy of employing MGF for cell therapy and tissue engineering.

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Supporting information Supplementary Table 1—Primer sequences and PCR product sizes for each PCR.

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