Effect of GARP on osteogenic differentiation of bone marrow mesenchymal stem cells via the regulation of TGFβ1 in vitro

Ruixue Li*, Jian Sun*, Fei Yang, Yang Sun, Xingwen Wu, Qianrong Zhou, Youcheng Yu and Wei Bi

Department of Stomatology, Fudan University Zhongshan Hospital, Shanghai, Shanghai, China

*These authors contributed equally to this work.

ABSTRACT

Mesenchymal stem cells (MSCs), which have multipotential differentiation and self-renewal potential, are possible cells for tissue engineering. Transforming growth factor β1 (TGFβ1) can be produced by MSCs in an inactive form, and the activation of TGFβ1 functions as an important regulator of osteogenic differentiation in MSCs. Recently, studies showed that Glycoprotein A repetitions predominant (GARP) participated in the activation of latent TGFβ1, but the interaction between GARP and TGFβ1 is still undefined. In our study, we successfully isolated the MSCs from bone marrow of rats, and showed that GARP was detected in bone mesenchymal stem cells (BMSCs). During the osteogenic differentiation of BMSCs, GARP expression was increased over time. To elucidate the interaction between GARP and TGFβ1, we downregulated GARP expression in BMSCs to examine the level of active TGFβ1. We then verified that the downregulation of GARP decreased the secretion of active TGFβ1. Furthermore, osteogenic differentiation experiments, alkaline phosphatase (ALP) activity analyses and Alizarin Red S staining experiments were performed to evaluate the osteogenic capacity. After the downregulation of GARP, ALP activity and Alizarin Red S staining significantly declined and the osteogenic indicators, ALP, Runx2, and OPN, also decreased, both at the mRNA and protein levels. These results demonstrated that downregulated GARP expression resulted in the reduction of TGFβ1 and the attenuation of osteoblast differentiation of BMSCs in vitro.

INTRODUCTION

Implant supported dentures have become a mainstream treatment in repairing dentition defects and deletions (Alkan et al., 2018; Greenberg, 2017). However, anodontia caused by serious decay, periodontal disease, congenital anodontia, and trauma may result in severe alveolar bone resorption, which can be a disadvantage of implantation. Although autogenous bone grafts are generally thought to be the gold standard for bone regeneration (Moses et al., 2007; Sbordone et al., 2014; Zhang et al., 2014), this procedure is limited due to local complications and an insufficiency of bone volume in donor sites (Jensen, Jensen & Worsaae, 2016; Nkenke & Neukam, 2014). A novel and easily available treatment is therefore needed.

How to cite this article Li R, Sun J, Yang F, Sun Y, Wu X, Zhou Q, Yu Y, Bi W. 2019. Effect of GARP on osteogenic differentiation of bone marrow mesenchymal stem cells via the regulation of TGFβ1 in vitro. PeerJ 7:e6993 http://doi.org/10.7717/peerj.6993
necessary for bone regeneration. Mesenchymal stem cells (MSCs), which can be isolated from tissues such as blood, adipose tissue, bones, and teeth, have the multipotential to differentiate into osteoblasts, chondrocytes, and adipocytes, and have become a promising vector for bone tissue engineering (Ma et al., 2017). Because they are easy to cultivate and expand, and maintain their pluripotency after serial subcultivation in vitro, bone marrow MSCs (BMSCs) are an ideal vehicle for tissue regeneration in severe bone defects and bone remodeling (Yang, FMV & Putnins, 2010; Han et al., 2013).

However, bone formation is a complex process controlled by many factors (Loeffler et al., 2017; Schroeder & Mosheiff, 2011), especially transforming growth factor β1 (TGFβ1). TGFβ1, which is secreted by bone marrow stromal cells and hematopoietic progenitors, is abundant in bone matrix, and functions as a modulator of cell growth, inflammation, and matrix synthesis (Kirk & Kahn, 1995; Kim & Niyibizi, 2001; Rahman et al., 2015; Majidinia, Sadeghpour & Yousefi, 2018), so we assumed that the use of TGFβ1 may be an alternative approach in stem cell-based tissue engineering. However, TGFβ1 is secreted as an inactive complex, which includes the mature TGFβ1 dimer and the latency-associated proteins (LAPs). Furthermore, LAPs remain inactive. It is the removal of TGFβ1 from LAPs that activates the TGFβ1 function. It is generally accepted that the latent TGFβ1-binding proteins (LTBPs) participate in the transportation and activation process. LTBPs, associated with latent TGFβ1, assist the complex transfer and then are anchored to the extracellular matrix (ECM). The involvement of protease, thrombospondin-1, and integrin, and changes in the condition of reactive oxygen species and pH then trigger the activation process of latent TGFβ1.

Another hypothesis is that membrane Glycoprotein A repetitions predominant (GARP) also participates in the activation of TGFβ1. GARP, which encodes a leucine-rich repeat containing 32 (LRRC32) protein, was first isolated from a breast carcinoma (Ollendorff et al., 1992). Most proteins of GARP with the LRR motif are membrane bound, and the remaining proteins can be secreted to extracellular sites or are localized to the cytoplasm or nucleus (Rothberg et al., 1990; Ohkura & Yanagida, 1991). Many relevant studies have focused on the biological function of GARP, and have proposed that GARP is involved in the activation of latent TGFβ1. It was reported that GARP and latent TGFβ1 are co-localized on the membrane of BMSCs (Carrillo-Galvez et al., 2015; Niu et al., 2017), but the interaction between them and whether GARP regulates the bioactivities of TGFβ1 in BMSCs still remain to be elucidated. Furthermore, whether GARP can affect the osteogenic differentiation ability of BMSCs is unknown. We therefore downregulated the expression of GARP in BMSCs, and found that the secretion of TGFβ1 was decreased, and the osteogenic results showed attenuated osteogenesis while the expression of GARP was knocked-down.

**MATERIALS AND METHODS**

**Animals**
All the BMSCs were isolated from 4-week-old male Sprague-Dawley (SD) rats under SPF raising conditions. The experiments were approved by the Animal Research Committee of Zhongshan Hospital, Fudan University, Shanghai, China (2016-128).
Isolation and culture of BMSCs

Rats were sacrificed by cervical dislocation. The femur and tibia were isolated, and both ends of the bones were removed to expose the marrow, which was flushed with a 10 mL syringe filled with alpha-Minimum Essential Medium (α-MEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific), and 1% penicillin/streptomycin. The fresh marrow suspension was filtered through a 70 µm cell strainer and centrifuged at 500×g for 5 min. The supernatant was discarded, and the sediment was resuspended and seeded in 25 cm² culture flasks. The culture medium was replaced after 48 h. BMSCs were trypsinized using 0.25% trypsin/1mM EDTA (Gibco; Thermo Fisher Scientific) to subculture the cells when they grew to 80% confluency. Cells at passages 3–6 were used for all experiments.

Identification and GARP expression of rat BMSCs by using flow cytometry

To identify the target cell, we used fluorescein isothiocyanate (FITC)-conjugated CD90 (561973; 1:100; BD biosciences; Franklin, Lakes, NJ, USA), CD45(561867; 1:100; BD biosciences), CD105(NB500-453; 1:100; novus biologicals; USA) and phycoerythrin (PE)-conjugated CD 44 (MA5-16908; 1:100; Thermo Fisher Scientific Inc.) to label the BMSC membranes. For analysis of GARP expression, we used a LRRC32/GARP antibody (NBP2-24664; 1:1,000; Novus Biologicals) followed by Alexa Fluor 647 AffiniPure secondary antibody (111-605-003; 1:100; Jackson Immuno Research Laboratories, West Grove, PA, USA). When cells grew to 80% confluency, they were harvested by gently washing once with phosphate-buffered saline (PBS), trypsinizing for 2 min at 37 °C , and centrifuging for 5 min at 500×g. The cells were resuspended to a concentration of 1×10⁶ cells/mL in PBS, and 100 µL suspensions were used for each sample. One µg of antibody was added to each sample, then incubated on ice in the dark for 1 h. One mL of PBS was added to stop the reaction, then the suspension was centrifuged for 5 min at 500×g, and the supernatant was discarded. The pellet was washed twice by resuspending the cells in PBS and centrifuging for 5 min at 500×g, followed by discarding the supernatant. The pellet was resuspended in 500 µL of PBS per sample, and used for the flow cytometry (BD Biosciences) analysis.

Immunofluorescent staining

The third passage BMSCs were seeded in a 6-well culture plate with prepared cell sheets at a density of 1.5×10⁴ cells/mL, and the cells were incubated overnight. When the cells were 50% confluent, the cell sheets were washed three times by PBS. The cells were then fixed with 4% formalin for 30 min, followed by washing three times with PBS, and blocked with 5% bovine serum albumin for 30 min. The experimental and control group cells were then incubated with anti-GARP antibody (NBP2-68740; 1:100; Novus Biologicals) and homologous anti-IgG antibody (A7016; 1:100; Beyotime Institute of Biotechnology, Haimen, China) at 4 °C in the dark overnight respectively, and the cell sheets washed three times with PBS. The cells were then incubated with FITC secondary antibody (A0562; 1:100; Beyotime Institute of Biotechnology) at room temperature for an hour. Cell sheets were washed three times with PBS. Then cells were incubated with 4',
This table showed the primers for RT-qPCR in the present study and GARP shRNA sequences.

| Gene     | Forward (3′–5′)          | Reverse (5′–3′)          |
|----------|--------------------------|--------------------------|
| GARP     | GGCAGAGAACAGCCTCACTC     | AAGGCAACATCCTCAATGTC     |
| ALP      | GATGGACAAGTTCCCCCTTGT    | CTTCAAGCACAACAAAGTAG     |
| Runx2    | CCTCTGACTTCTGGCTCTGG     | CTTCAAGCACAACAAAGTAG     |
| OPN      | CCAAGGCTGGAAAACACAGGCC   | GGTCTTGGAATCCTGGACTG     |
| β-actin  | GCAGGATACGATGGAGTGCCG    | ACAGCTGACATCACTG         |
| GARP-sh1 | GGCTCAACCTACAGGGAAA      | GGCTCAACCTACAGGGAAA      |
| GARP-sh2 | GGGTTAAAGGCTCAGAGAGAC    | GGGTTAAAGGCTCAGAGAGAC    |
| GARP-sh3 | GGCTGTACTTGCAGAGAGAC    | GGGTTAAAGGCTCAGAGAGAC    |
| GARP-sh4 | GCACTTCGCCACCTGGATTTA    | GCACTTCGCCACCTGGATTTA    |
| GARP-sh5 | GCAACAGATTGGACCTTCCG     | GCAACAGATTGGACCTTCCG     |

**Notes.**
GARP, Glycoprotein A repetitions predominant; ALP, Alkaline phosphatase; OPN, Osteopontin; Runx2, Runt-related transcription factor 2.

6-diamidine-2′-phenylindole dihydrochloride (DAPI; Invitrogen, Carlsbad, CA, USA) for 10 min in the dark, and then washed as previously described. The cell sheets were imaged using an immunofluorescence microscope (Olympus, Tokyo, Japan).

**Lentivirus gene vector production and transfection of BMSCs**

The GARP short hairpin RNA (shRNA)-encoded lentivirus vector was packaged by Shanghai Hanyin (Shanghai, China). Lentiviral vectors for rat GARP-shRNA carried a green fluorescent protein (GFP) sequence. The target sequences for rat GARP knockdown are listed in Table 1. The recombinant GARP-shRNA lentivirus and the negative control lentivirus were prepared and titered to 10⁹ TU/mL (transfection unit). Before transfection, BMSCs were seeded at a density of 5 × 10⁴ cells per well in a 6-well culture plate and incubated overnight. The cells were approximately 50% confluent on the day of infection. A mixture of medium with polybrene (Shanghai Hanyin) was prepared at a final concentration of 5 µg/mL. The medium was removed from the plate wells and replaced with 1 mL of this polybrene/media mixture. The cells were infected by adding 10 μL shRNA lentivirus to the culture. The plate was gently swirled to mix and incubated in 5% CO₂ at 37 °C. The medium was changed after 8 h. Stable clones expressing a GARP shRNA (GARP-sh) were selected using 3 µg/mL puromycin dihydrochloride (Shanghai Hanyin). At the same time, negative control cells (NC) were infected by the same dose of empty vector virus.

**Cell Counting Kit-8 (CCK-8) analysis of transfected BMSCs**

To further investigate the differences of proliferation and viability between transfected and controlled BMSCs, CCK-8 experiments were performed. The transfected and controlled BMSCs were seeded in a 96-well culture plate at a density of 1 × 10³ cells/mL. At the same time, there were six wells with only culture medium, which were used as the blank controls. The plates were then incubated at 37 °C. According to the manufacturer’s instructions, 10 μL CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added to
each well. The optical density (OD) value was measured at a wavelength of 450 nm, with the results presented as the mean.

**Quantitative real time-polymerase chain reaction (RT-qPCR)**

Extractions of total RNA were obtained from each group using TRIzol (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer’s instructions. Reverse transcription of RNA was conducted using the PrimeScript RT Master Mix (Takara Bio, Ostu, Japan). RT-qPCR analyses were performed by using the SYBR-Green Real-Time PCR Master Mix (Takara Bio). Rat β-actin, a single housekeeping gene, was selected as an internal control for normalization. All primers used in the RT-qPCR are listed in Table 1. The RT-qPCR procedure was conducted using the following program: one cycle at 95 °C for 30 s; 40 cycles at 95 °C for 5 s; and one cycle at 60 °C for 30 s. The total volume of the reaction was 20 µL, and the relative expression of the target gene was analyzed by the comparative cycle threshold \( (2^{-\Delta\Delta CT}) \) method.

**Western blot analysis**

For western blot analysis, cells from each group were washed with PBS on ice. Cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology). The lysate was collected and then centrifuged at 12,000× g for 5 min. The supernatant was collected and the protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). Twenty µg of protein lysate from each sample was resolved using a 10% SDS-PAGE gel and then transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% nonfat milk for 2 h at room temperature (RT), and the membrane was incubated with primary antibodies including anti-GARP (NBP2-24664; 1:1,000; Novus Biologicals), anti-ALP (08337; 1:10,000; Abcam, Cambridge, UK), anti-OPN (91655; 1:1,000; Abcam), anti-Runx2 (23981; 1:1,000; Abcam), and anti-β-actin (8227; 1:1,000; Abcam) at 4 °C overnight. The next day, the membranes were washed in TBST (20 mM Tris-HCL, 137 mM NaCl and 0.1% Tween-20; pH 7.6) three times, for 10 min each time. The membrane was then blocked with goat-anti-rabbit IgG-HRP antibody (SC-2370; 1:5,000; Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h, followed by washing in TBST three times, with 10 min for each wash. Proteins were detected by Chemiscope5600 (Shanghai Clinx Science Instruments, Shanghai, China) using BeyoECL Plus Reagent (Beyotime Institute of Biotechnology).

**TGFβ1 analysis by ELISA of transfected BMSCs**

When cells grew to 80% confluency, the medium was changed to SD rat mesenchymal stem cell osteogenic differentiation medium (Cyagen Biosciences, Santa Clara, CA, USA). Supernatants were collected at 6 h, 12 h, 18 h, 24 h, and 48 h after the change of medium. All samples were centrifuged at 2,500 rpm/min for 20 min, and cell debris was discarded to obtain the supernatant. TGFβ1 levels were analyzed using a rat TGFβ1 ELISA kit (Dakewe Bioengineering, Shenzhen, China), according to the manufacturer’s instructions.

**Osteogenic differentiation**

Cells were seeded at a density of 5 × 10^4 cells per well in a 6-well culture plate and incubated. When cells grew to 80% confluency, the medium was replaced with SD rat mesenchymal
stem osteogenic differentiation medium (Cyagen Biosciences). The differentiation medium was changed every 3 days.

**Alkaline phosphatase (ALP) activity analysis**
After osteogenic differentiation, cells were washed gently with PBS, fixed with 4% formalin for 30 min, and stained with a BCIP/NBT alkaline phosphatase color development kit (Beyotime Institute of Biotechnology) for 30 min at RT. Finally, they were washed with PBS and then visualized with a microscope (Olympus, Tokyo, Japan).

**Alizarin Red S staining experiment**
On the days 14 after the osteogenic differentiation, cells were gently washed with PBS, fixed with 4% formalin for 30 min and then washed with ddH$_2$O. Each well was added about 1mL Alizarin Red S staining reagent (Cyagen Biosciences) for 3–5 min at RT. Then cells were gently washed with ddH$_2$O and visualized with a microscope (Olympus, Tokyo, Japan).

**Statistical analysis**
All data are expressed as the mean ± standard deviation. $P$ values < 0.05 were considered as statistically significant differences. The comparisons of two groups were performed using the two-tailed $t$-test, and the results of more than two groups were analyzed by one-way analysis of variance. Statistical analysis was performed using Prism 6.0 software (GraphPad, La Jolla, CA, USA).

**RESULTS**

**Identification of rat BMSCs and GARP expression of BMSCs**
The mature BMSCs adhered to the bottom of the culture flask and showed a spindle shape and typical fibroblast-like appearance (Fig. 1). To identify the purity of BMSCs, we assessed the expression of bone marrow mesenchymal and hematopoietic stem cell membrane epitopes, CD44, CD90, CD105 and CD45. Based on the cytometry results (Fig. 2), positive percentage of BMSCs for the CD44 was 95.82%, the CD90 99.85%, the CD105 99.09%, while the CD45 positive ratio was 5.53%. This indicated that we obtained a high purity of BMSCs. For the detection of GARP expression of BMSCs, we used anti-GARP antibody to react with GARP protein, homologous anti-IgG antibody as isotype control, and FITC-conjugated secondary antibody for immunofluorescence staining, followed by imaging with a fluorescence microscope, which showed that GARP was localized to both the membrane and cytoplasm of BMSCs (Fig. 3).

To verify the expression level of GARP during osteogenic differentiation, RT-qPCR and western blot experiments were performed at different stages of osteogenic differentiation on days 0, 7 and 14. GARP expression levels increased significantly with the increasing osteogenic differentiation time, indicating that GARP may play an important role in osteogenic differentiation (Fig. 4).

**GARP expression was knocked-down by lentivirus transfection**
To further investigate the bioactivities of GARP, we constructed the GARP-shRNA encoded lentivirus vector to silence the expression of GARP. After selection of successfully transfected
cells by puromycin dihydrochloride, samples were extracted to analyze the GARP mRNA and protein levels via RT-qPCR and western blotting, respectively. We tested five targets for RNA interference to choose the most the effective one. Based on the RT-qPCR results, mRNA levels of GARP were significantly decreased with transfection of GARP-sh4 and GARP-sh5 (Fig. 5). The subsequent experiments were therefore conducted using GARP-sh5. GARP total protein expression levels were examined by western blotting. The results showed a dramatic decline of total protein expression (Fig. 6). We hypothesized that the membrane localized GARP, which bound latent TGFβ1 to the cell surface, was a key component in regulating the bioactivities of BMSCs, so we performed flow cytometry to determine GARP membrane protein levels of the treatment and control groups, which showed that the GARP membrane protein expression level in the NC group was 26.1%, and 10.7% in the GARP-sh group. There was a 15.4% expression difference between the transfected and NC groups (Fig. 7).

Based on the CCK-8 analysis result, cells of GARP-sh group and NC group were in the slow growth period on the first two days after seeding in the plate. On the days 3, the growth pattern of cells began to display an exponential growth phase. On the days 6, the cells reached to the plateau phase (Fig. 8). Compared to the NC group, No significant difference was showed and the silence of GARP didn’t affect the viability and proliferation of BMSCs.
Figure 2  **Cytometry results of BMSCs.** Cytometry results demonstrated that BMSCs were positive for CD 44 (95.82%), CD 90 (99.85%), CD 105 (99.09%) and negative for CD 45 (5.53%). BMSCs, bone marrow mesenchymal stem cells; FITC, fluorescein isothiocyanate; PE, phycoerythrin. (A) count; (B) Blank Control; (C) CD 44; (D) CD 90; (E) CD 105; (F) CD 45; (t test).

Full-size DOI: 10.7717/peerj.6993/fig-2

Figure 3  **Immunofluorescent staining experiment.** For immunofluorescent staining experiment, we used GARP antibody to show the expression of GARP protein, homologous anti-IgG antibody as isotype control and DAPI to show the location of nucleus. GARP, Glycoprotein A repetitions predominant; DAPI, diamidino-phenyl-indole. (A) and (D) showed the merge image of two groups; (B) and (E) showed the FITC immunofluorescent staining; (C) and (F) showed the DAPI staining; 200X; Bar = 50 µm.

Full-size DOI: 10.7717/peerj.6993/fig-3
According to the qRT-PCR and western blot experiments results, on days 7 and 14 after osteogenic differentiation, GARP mRNA and protein levels increased significantly, compared to the days 0. (A) qRT-PCR experiment of GARP mRNA expression; (B) western blot experiment of GARP protein expression; (t test, * P < 0.05).

Decreased levels of active TGFβ1 were detected after transfection.

It was reported that GARP bound latent TGFβ1 on the cell surface (Tran et al., 2009), so we proposed that a decreased GARP protein level may affect TGF β1 secretion. To confirm this possibility, we developed an ELISA to determine the total TGFβ1 expression of supernatants from the control and GARP-sh groups. We collected supernatants at 6 h, 12 h, 24 h, and 48 h after the change of fresh medium. The results showed that the TGFβ1 secretion level was gradually increased before 24 h and then reached a plateau (Fig. 9). Based on comparison of the two groups, silencing of GARP expression decreased the extracellular secretion of active TGFβ1.
Western blot experiments of GARP expression. Western blot experiments showed that GARP total protein expression in GARP-sh group was decreased, compared to control group.

Inhibition of osteogenic differentiation of BMSCs after transfection with the GARP-sh lentivirus

Numerous factors were involved during bone ossification, and it was confirmed that ALP, Runx2, and OPN were essential to this process. A high concentration of ALP denoted the start of ossification. Runx2 is the main osteogenic differentiation component of BMSCs. OPN participates in the proliferation and calcification of BMSCs. Thus, to evaluate the osteogenic differentiation ability of BMSCs, we performed ALP activity staining, RT-qPCR, and western blotting to analyze ALP, Runx2, and the OPN protein expression levels, respectively, at different stages of osteogenic differentiation on days 0, 7, and 14.

RT-qPCR results showed that ALP, Runx2, and OPN expressions showed no difference on days 0 of differentiation. But on days 7, the expressions of these factors increased to varying degrees, when compared with those on days 0. In addition, the expressions of these factors in the GARP-sh group were lower than the NC group. On days 14, ALP, Runx2, and OPN expressions were higher than on days 0 and days 7, and there was a significant decline of these factors in the GARP-sh group (Fig. 10).

Western blot analysis indicated that ALP, Runx2, and OPN protein levels showed no significant difference between the two groups during the pre-induction of osteogenesis. On days 7 and 14, the results displayed remarkable differences in the ALP, Runx2, and OPN expression levels between the GARP-sh and NC groups (Fig. 11).

On the days 7, the ALP activity staining assay showed that the color of ALP staining was much more obvious than that of the GARP-sh group. On days 14, the Alizarin Red S staining level of NC group was much darker than GARP-sh group. And there are more mineralized nodule formation in the NC group, compared to the GARP-sh group (Fig. 12). Together, the results showed that ALP levels and mineralizing ability were decreased in the GARP-sh group during osteogenic differentiation.

DISCUSSION

BMSCs show a multilineage potential, allowing differentiation into osteoblasts, chondrocytes, adipocytes, and other tissue cells, which are easily isolated and cultured.
Thus, BMSCs could be suitable cells for bone tissue engineering as osteogenic stem cells (Cancedda et al., 2000). The osteogenic differentiation process is activated by a series of signal transduction processes, of which the most important is the TGFβ pathway (Jian et al., 2006). Regulation of TGFβ may therefore provide a novel approach for the differentiation of BMSCs.

GARP, which has been mostly reported in the study of activated Treg cells, is expressed on the cell surface of activated functional FOXP3+ Tregs, and functions as a receptor for latent TGFβ1. Binding of TGFβ1 to the membrane is essential for the functioning of Tregs (Tran et al., 2009). With the assistance of GARP, latent TGFβ1 is localized on the cell surface.
Figure 8  Cell Counting Kit-8 (CCK-8) analysis of transfected BMSCs. CCK-8 analysis result demonstrated that cells of GARP-sh group and NC group were in the slow growth period on the first two days after seeding in the plate. On the days 3, the growth pattern of cells began to display an exponential growth phase. On the days 6, the cells reached to the plateau phase. There was no significant differences between two groups (t test.).

Figure 9  ELISA experiments of mature TGFβ1 level in GARP-sh and NC groups. ELISA experiments showed that mature TGFβ1 level was down-regulated in GARP-sh group. (t test, * P < 0.05).

cell membrane, and is then ready to release its active form (Stockis et al., 2009). However, the exact mechanism of activation of membrane latent TGFβ1 in Tregs is not known. GARP mRNA expression has been recently detected in MSCs (Barbet et al., 2011). The interaction between GARP and latent TGFβ1 has also been described in a follow-up report (Carrillo-Galvez et al., 2015). In the present study, we conducted further studies to determine the effect of GARP on the osteogenic development of BMSCs via the regulation of latent TGFβ1.

Our study determined the existence of GARP in BMSCs. To further investigate the bioactivity of GARP, we performed transfection experiments to silence the expression of GARP. The results showed that both total and membrane GARP protein expressions were knocked down. Furthermore, we showed that the secretion of TGFβ1 was decreased
Figure 10  Osteogenic differentiation related factors’ mRNA expression results. Osteogenic differentiation results demonstrated ALP, Runx 2 and OPN mRNA expression was decreased in GARP-sh group at osteogenic differentiation 7 days and 14 days. (t test, * P < 0.05); (A) mRNA expression of ALP; (B) mRNA expression of Runx2; (C) mRNA expression of OPN.

Figure 11 Western blot analysis of osteogenic related factors’ protein expression. Western blot analysis showed that ALP, Runx2 and OPN protein expressions were decreased in GARP-sh group.

Li et al. (2019), PeerJ, DOI 10.7717/peerj.6993/fig-10
Figure 12  ALP and Alizarin Red S staining analysis of osteogenic differentiation. After 7 days osteogenic differentiation, ALP staining experiments showed that the staining of NC was much higher than the GARP-sh group. on 14 days after osteogenic differentiation, Alizarin Red S staining results showed that there was more mineralized nodule formation in the NC group, compared to the GARP-sh group. (A) ALP and Alizarin Red S staining with the gross appearance. (B) ALP and Alizarin Red S staining under the microscopic view (40X). Bar = 500 µm.

with the reduction of GARP. Previous studies have reported that TGFβ1 is secreted as an inactive form (latent TGFβ1) (Annes, Munger & Rifkin, 2003), and both LTBP5 and GARP bind latent TGFβ1. LTBP5 bind latent TGFβ1 by disulfide bonds. In this manner, LTBP5s assist the transmembrane transportation and anchorage in the ECM, of latent TGFβ1. Furthermore, the storage of latent TGFβ1 in the ECM is functionally activated under specific conditions (Taipale et al., 1994). GARP can anchor latent TGFβ1 noncovalently to the cell membrane. The membrane GARP/latent TGFβ1 complex serves as a source of active TGFβ1. The membrane latent TGFβ1 can be activated by mediation of integrins αvβ6 (Wang et al., 2012). In our study, the decrease of membrane GARP provided an insufficient amount of latent TGFβ1, so eventually the secretion of TGFβ1 was lower than untreated BMSCs. In contrast to the results of Carrillo-Galvez et al., our data indicated that silencing of GARP resulted in the reduction of TGFβ1. It was reported that GARP competed better with LTBP5 when binding to latent TGFβ1 (Wang et al., 2012). We speculated that membrane-associated GARP plays a more important role than the LTBP5s in the process of TGFβ1 activation.

TGFβ1 can be produced by BMSCs and participates in osteoblast differentiation of stem cells as a pleiotropic molecule. The activation of the SMAD signaling pathway by TGFβ1 can increase the osteoblast differentiation (Jian et al., 2006; Kulzerer et al., 2007; Ng et al., 2008; Miron et al., 2013). A previous study reported that TGFβ1 actions can be attributed to the foundation of bone mass and quality through the regulation of perilacunar/canalicular remodeling (Dole et al., 2017). In the present study, we showed that osteogenic-related genes of ALP, Runx2, and OPN were significantly downregulated with decreased secretion of TGFβ1, and that the suppression of osteogenic differentiation was also confirmed at the translational level. According to our western blot results, all ALP, Runx2, and OPN
protein expression levels were significantly attenuated compared to the untreated group. Moreover, the ALP activity of GARP-sh cells was lower than that of untreated BMSCs. These results indicated that the decreased amount of TGFβ1 inhibited the osteogenesis of BMSCs. However, the biological mechanism remains to be definitively elucidated. Further animal experiments are necessary to demonstrate that the osteogenic differentiation is affected by the GARP/latent TGFβ1 complex in vivo.

It has been shown that MSCs reside in the periodontal ligament, which may be a promising reservoir for regeneration (Beertsen, McCulloch & Sodek, 1997). In addition, TGFβ1 can cooperate with other growth factors, such as bone morphogenetic proteins (BMPs), to induce osteoblast differentiation (De Gorter et al., 2011; Hyun et al., 2017). In the present study, GARP was responsible for the regulation of the activation and secretion processes of latent TGFβ1. Furthermore, GARP indirectly regulated osteogenic differentiation of BMSCs. These results may provide promising applications in the field of tissue engineering.

CONCLUSIONS
We verified that the downregulation of GARP decreased the level of mature TGFβ1 and the osteogenic ability in rat BMSCs. However, bone regeneration is a rather complicated process that is regulated by various factors. It still remains to be elucidated whether a decrease in the levels of mature TGFβ1 reduces the osteogenic ability in vivo. Based on the results above, we hypothesize that the osteogenic ability can be improved by increased TGFβ1 levels via the upregulation of GARP. The regulation of GARP on levels of TGFβ1 may therefore provide a novel solution to challenges in tissue engineering and bone regeneration.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding
This work was supported by Science Researching Project of the National Science Foundation of China “Study on the effect of GARP-TGF-beta1 complex in inhibiting the inflammatory alveolar absorption by bone marrow mesenchymal stem cells”, 2017/01-2020/12. (No. 8167040094), Science Researching Project of the National Science Foundation of China “The effect of AdipoRon in improving the osseointegration of dental implant in diabetic patients and its relative mechanisms”, 2019/01-2022/12.(No.81870793), and the Shanghai Science Commission project “Biological effects on osteoblast of the structure of femtosecond laser produced micro-grooves on the surface of zirconia dental implants”, 2016/07-2019/06.(No.16520710400). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures
The following grant information was disclosed by the authors: Science Researching Project of the National Science Foundation of China: 2017/01-2020/12. (No. 8167040094), 2019/01-2022/12. (No.81870793).
Shanghai Science Commission project, 2016/07-2019/06: 16520710400.

**Competing Interests**
The authors declare there are no competing interests.

**Author Contributions**
- Ruixue Li conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Jian Sun conceived and designed the experiments, performed the experiments, prepared figures and/or tables, approved the final draft.
- Fei Yang conceived and designed the experiments, prepared figures and/or tables, approved the final draft.
- Yang Sun analyzed the data, contributed reagents/materials/analysis tools, approved the final draft.
- Xingwen Wu contributed reagents/materials/analysis tools, approved the final draft.
- Qianrong Zhou performed the experiments, contributed reagents/materials/analysis tools, approved the final draft.
- Youcheng Yu conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Wei Bi conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.

**Animal Ethics**
The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):
The experiments were permitted by the Animal Research Committee of Zhongshan hospital, Fudan University, Shanghai, China (2016-128).

**Data Availability**
The following information was supplied regarding data availability:
The raw data is available in the Supplemental Files.

**Supplemental Information**
Supplemental information for this article can be found online at [http://dx.doi.org/10.7717/peerj.6993#supplemental-information](http://dx.doi.org/10.7717/peerj.6993#supplemental-information).

**REFERENCES**

Alkan EA, Lian PM, Schoolfield J, Guest GF, Cochran DL. 2018. Prevalence of dental implants and evaluation of peri-implant bone levels in patients presenting to a dental school: a radiographic cross-sectional 2-year study. *International Journal of Oral and Maxillofacial Implants* 33(1):145–151 DOI 10.11607/jomi.5756.

Annes JP, Munger JS, Rifkin DB. 2003. Making sense of latent TGFbeta activation. *Journal of Cell Science* 116(Pt 2):217–224 DOI 10.1242/jcs.00229.
Barbet R, Peiffer I, Hatzfeld A, Charbord P, Hatzfeld JA. 2011. Comparison of gene expression in human embryonic stem cells, hESC-derived mesenchymal stem cells and human mesenchymal stem cells. Stem Cells International 2011:Article 368192 DOI 10.4061/2011/368192.

Beertsen W, McCulloch CA, Sodek J. 1997. The periodontal ligament: a unique, multifunctional connective tissue. Periodontol 2000 13:20–40 DOI 10.1111/j.1600-0757.1997.tb00094.x.

Cancedda R, Castagnola P, Cancedda FD, Dozin B, Quarto R. 2000. Developmental control of chondrogenesis and osteogenesis. International Journal of Developmental Biology 44(6):707–714.

Carrillo-Galvez AB, Marien Cobo C-G, Cuevas-Ocana S, Gutierrez-Guerrero A, Sanchez-Gilabert A, Bongarzone P, Garcia-Perez A, Munoz P, Benabdellah K, Toscano MG, Martin F, Andersonet P. 2015. Mesenchymal stromal cells express GARP/LRRC32 on their surface: effects on their biology and immunomodulatory capacity. Stem Cells 33(1):183–195 DOI 10.1002/stem.1821.

De Gorter DJJ, Van Dinther M, Korchynskyi O, Ten Dijke P. 2011. Biphasic effects of transforming growth factor beta on bone morphogenetic protein-induced osteoblast differentiation. Journal of Bone and Mineral Research 26(6):1178–1187 DOI 10.1002/jbmr.313.

Dole NS, Mazur CM, Acevedo C, Ritchie RO, Mohammad KS, Alliston T. 2017. Osteocyte-Intrinsic TGF-beta Signaling Regulates Bone Quality through Perilacunar/Canalicular Remodeling. Cell Reports 21(9):2585–2596 DOI 10.1016/j.celrep.2017.10.115.

Greenberg AM. 2017. Advanced dental implant placement techniques. Journal of Istanbul University Faculty of Dentistry 51(3 Suppl 1):S76–S89.

Han X, Liu H, Wang D, Su F, Zhang Y, Zhou W, Li S, Yang R. 2013. Alveolar Bone Regeneration around Immediate Implants Using an Injectable nHAC/CSH Loaded with Autogenic Blood-Acquired Mesenchymal Progenitor Cells: an Experimental Study in the Dog Mandible. Clinical Implant Dentistry and Related Research 15(3):390–401 DOI 10.1111/j.1708-8208.2011.00373.x.

Hyun S-Y, Lee J-H, Kang K-J, Jang Y-J. 2017. Effect of FGF-2, TGF-beta-1, and BMPs on Teno/Ligamentogenesis and Osteo/Cementogenesis of Human Periodontal Ligament Stem Cells. Molecular Cell 40(8):550–557 DOI 10.14348/molcells.2017.0019.

Jensen SS, Jensen AT, Worsaae N. 2016. Complications related to bone augmentation procedures of localized defects in the alveolar ridge. A retrospective clinical study. Oral and Maxillofacial Surgery 20(2):115–122 DOI 10.1007/s10006-016-0551-8.

Jian H, Shen X, Liu I, Semenov M, He X, Wang X. 2006. Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells. Genes and Development 20(6):666–674 DOI 10.1101/gad.138806.

Kim MK, Niyibizi C. 2001. Interaction of TGF-beta1 and rhBMP-2 on human bone marrow stromal cells cultured in collagen gel matrix. Yonsei Medical Journal 42(3):338–344 DOI 10.3349/ymj.2001.42.3.338.
Kirk MD, Kahn AJ. 1995. Extracellular matrix synthesized by clonal osteogenic cells is osteoinductive in vivo and in vitro: role of transforming growth factor-beta 1 in osteoblast cell–matrix interaction. *Journal of Bone and Mineral Research* 10(8):1203–1208.

Kulterer B, Friedl G, Jandrositz A, Sanchez-Cabo F, Prokesch A, Paar C, Scheideler M, Windhager R, Preisegger K-H, Trajanoski Z. 2007. Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *BMC Genomics* 8:70 DOI 10.1186/1471-2164-8-70.

Loeffler J, Duda GN, Sass FA, Dienelt A. 2017. The metabolic microenvironment steers bone tissue regeneration. *Trends in Endocrinology and Metabolism* 29(2):99–110 DOI 10.1016/j.tem.2017.11.008.

Ma G, Zhao JL, Mao M, Chen J, Dong ZW, Liu YP. 2017. Scaffold-Based Delivery of Bone Marrow Mesenchymal Stem Cell Sheet Fragments Enhances New Bone Formation In Vivo. *Journal of Oral and Maxillofacial Surgery* 75(1):92–104 DOI 10.1016/j.joms.2016.08.014.

Majidinia M, Sadeghpour A, Yousefi B. 2018. The roles of signaling pathways in bone repair and regeneration. *Journal of Cellular Physiology* 233(4):2937–2948 DOI 10.1002/jcp.26042.

Miron RJ, Saulacic N, Biser D, Iizuka T. 2013. Osteoblast proliferation and differentiation on a barrier membrane in combination with BMP2 and TGFbeta1. *Clinical Oral Investigations* 17(3):981–988 DOI 10.1007/s00784-012-0764-7.

Moses O, Nemcovsky CE, Langer Y, Tal H. 2007. Severely resorbed mandible treated with iliac crest autogenous bone graft and dental implants: 17-year follow-up. *International Journal of Oral and Maxillofacial Implants* 22(6):1017–1021.

Ng F, Boucher S, Koh S, Sastry KSR, Chase L, Lakshmipathy U, Choong C, Yang Z, Vemuri MC, Rao MS, Tanavde V. 2008. PDGF, TGF-β, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. *Blood* 112(2):295–307 DOI 10.1182/blood-2007-07-103697.

Niu J, Yue W, Lele Z, Bin L, Hu X. 2017. Mesenchymal stem cells inhibit T cell activation by releasing TGF-beta1 from TGF-beta1/GARP complex. *Oncotarget* 8(59):99784–99800.

Nkenke E, Neukam FW. 2014. Autogenous bone harvesting and grafting in advanced jaw resorption: morbidity, resorption and implant survival. *European Journal of Oral Implantology* 2(Supl 2):S203–S217.

Ohkura H, Yanagida M. 1991. S. pombe sds22+ essential for a midmitotic transition encodes a leucine-rich repeat protein that positively modulates protein phosphatase-1. *Cell* 64(1):149–157 DOI 10.1016/0092-8674(91)90216-L.

Ollendorff F, Szepetowski P, Mattei M-G, Gaudray P, Birnbaum D. 1992. New gene in the homologous human 11q13-q14 and mouse 7F chromosomal regions. *Mammalian Genome* 2(3):195–200 DOI 10.1007/BF00302877.
Rahman MS, Akhtar N, Jamil HM, Banik RS, Asaduzzaman SM. 2015. TGF-beta/BMP signaling and other molecular events: regulation of osteoblastogenesis and bone formation. Bone Research 3:Article 15005 DOI 10.1038/boneres.2015.5.

Rothberg JM, Jacobs JR, Goodman CS, Artavans-Tsakonas S. 1990. slit: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains. Genes and Development 42(12A):2169–2187.

Sbordone C, Toti P, Guidetti F, Califano L, Pannone G, Sbordone L. 2014. Volumetric changes after sinus augmentation using blocks of autogenous iliac bone or freeze-dried allogeneic bone. A non-randomized study. Journal of Cranio-Maxillo-Facial Surgery 42(2):113–118 DOI 10.1016/j.jcms.2013.03.004.

Schroeder JE, Mosheiff R. 2011. Tissue engineering approaches for bone repair: concepts and evidence. Injury 42(6):609–613 DOI 10.1016/j.injury.2011.03.029.

Stockis J, Colau D, Coulie PG, Lucas G. 2009. Membrane protein GARP is a receptor for latent TGF-beta on the surface of activated human Treg. European Journal of Immunology 39(12):3315–3322 DOI 10.1002/eji.200939684.

Taipale J, Miyazono K, Heldin C-K, Keski-Oja J. 1994. Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. Journal of Cell Biology 124(1–2):171–181 DOI 10.1083/jcb.124.1.171.

Tran DQ, Andersson J, Wang R, Ramsey R, Unutmaz D, Shevach EM. 2009. GARP (LRRC32) is essential for the surface expression of latent TGF-beta on platelets and activated FOXP3+ regulatory T cells. Proceedings of the National Academy of Sciences of the United States of America 106(32):13445–13450 DOI 10.1073/pnas.0901944106.

Wang R, Zhu J, Dong X, Shi M, Lu C, Timothy A. 2012. GARP regulates the bioavailability and activation of TGFbeta. Molecular Biology of the Cell 23(6):1129–1139 DOI 10.1091/mbc.e11-12-1018.

Yang Y, Rossi FMV, Putnins EE. 2010. Periodontal regeneration using engineered bone marrow mesenchymal stromal cells. Biomaterials 31(33):8574–8582 DOI 10.1016/j.biomaterials.2010.06.026.

Zhang W, Zhu C, Ye D, Xu L, Zhang X, Wu Q, Zhang X, Kaplan DL, Jiang X. 2014. Porous silk scaffolds for delivery of growth factors and stem cells to enhance bone regeneration. PLOS ONE 9(7):e102371 DOI 10.1371/journal.pone.0102371.