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Original Article

Effects of GPNMB on proliferation and odontoblastic differentiation of human dental pulp cells

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Abstract: Glycoprotein (transmembrane) nonmetastatic melanoma protein b (GPNMB) plays crucial roles in odontogenesis. However, the role of GPNMB in human dental pulp cells (hDPCs) is still unclear. Therefore, in this study, we investigated the expression and function of the GPNMB in odontoblastic differentiation of hDPCs. Cells were cultured in odontoblast differentiation-inducing medium; the expression of the GPNMB was assessed by reverse transcriptase polymerase chain reaction and Western blot analysis. We performed gene knockdown of GPNMB in hDPCs using lentivirus-mediated small interfering RNA (siRNA)-GPNMB. The proliferation of cells was measured by the MTT assay, and the differentiation of cells was detected with alkaline phosphatase (ALP) activity assay, qRT-PCR and Western blot were used to determine the expression levels of dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1). The expression level of GPNMB was significantly increased during odontoblastic differentiation of hDPCs. Suppression of GPNMB expression by siRNA-GPNMB obviously promoted the proliferation of hDPCs. Furthermore, siRNA-GPNMB significantly inhibited the activity of ALP and expression levels of DSPP and DMP-1 during odontoblastic differentiation of hDPCs. Our results show that GPNMB plays an important role in regulating the expression of key pluripotency genes in hDPCs and modifying odontogenic differentiation.

Keywords: Glycoprotein (transmembrane) nonmetastatic melanoma protein b (GPNMB), human dental pulp cells (hDPCs), proliferation, odontoblastic differentiation

Introduction

Dental pulp plays a critical role in the reparative regeneration of tooth tissue. In response to dental injury and irreversible caries damage, dental pulp can produce new odontoblasts secreting a reparative dentine matrix [1]. Dental pulp cells (DPCs) are composed of ectodermic and mesenchymal components containing neural crest-derived mesenchymal progenitors endowed with plasticity and multipotency [2]. DPCs are easily obtained from extracted teeth, possess high proliferative ability, and can be reprogrammed into induced pluripotent stem cells at relatively high rates [3, 4]. Cultured DPCs have the ability to differentiate into odontoblast-like cells under certain culture conditions *in vitro* [5]. Although the differentiation process from DPCs to odontoblasts has been reported to be regulated by complex signaling pathways [6, 7], the molecular mechanisms controlling odontoblastic differentiation of DPCs are yet not understood.

Glycoprotein (transmembrane) nonmetastatic melanoma protein b (GPNMB), also known as HGFIN, osteoactivin, and DC-HIL, is a type I membrane glycoprotein involved in various biological processes, including inflammation, invasion and metastasis of malignant tumors, cell differentiation, and tissue regeneration [8-10]. Previous studies showed that GPNMB acts as an osteogenic factor that stimulates osteoblast differentiation *in vivo* and *in vitro*. Abdelmagid SM et al. showed that the ability of osteoblasts to differentiate was decreased in DBA/2J mouse with a loss-of-function mutation in GPNMB, compared to that in wild-type mice [11]. In addition, Hu X et al. demonstrated that recombinant GPNMB dose-dependently increased the differentiation of human bone marrow stromal cells (hBMSCs) into osteoblasts, as well

as the mRNA levels of osteoblasts marker alkaline phosphatase (ALP) and osteocalcin [12]. The odontogenic differentiation of DPCs is a biological process similar to hBMMSC osteogenic differentiation, which suggests that GPNMB may be involved in the odontogenic differentiation of hDPCs. However, the role of GPNMB in hDPCs is still unclear. Therefore, in this study, the expression of GPNMB was examined during the odontoblastic differentiation of hDPCs *in vitro*, and then GPNMB was knocked-down in hDPCs to determine its function on odontoblastic differentiation of hDPCs. Our results show that GPNMB inhibits the proliferation and promotes the differentiation of hDPCs.

Materials and methods

Isolation and culture of hDPCs

This study was performed according to an informed protocol approved by the Ethics Committee of the School of Stomatology, Shandong University. The primary cultured hDPCs came from pulp tissues of extracted healthy human third molars and cultured in maintenance medium containing Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin (Sigma, St Louis, MO, USA). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

For odontoblastic induction, hDPCs were plated in six-well plates (Takara, Dalian, China) at an initial density of 2×10^5 cells/well and cultured in DMEM supplemented with 10% fetal bovine serum, antibiotics, 50 mg/mL ascorbic acid, 10 mmol/L sodium β-glycerophosphate, and 10 nmol/L dexamethasone (Sigma, St Louis, MO, USA). The culture medium was changed every 3 days [13].

Small interfering RNA (siRNA) transfection

hDPCs expressing the GPNMB protein were transfected with siRNA-GPNMB or the control siRNA (Takara, Dalian, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The siRNA-GPNMB target sequences were 5'-GCACGGGUUUCUAUAAACAdTdT-3' (sense) and 5'-UGUUUAUAGAAACCCGUG-CdTdT-3' (antisense).

Cell proliferation assay

The effect of GPNMB on the proliferation of hDPCs was examined with the MTT (3-[4, 5-dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide) assay. After 1, 3, 5 or 7 days of incubation, MTT reagent (diluted from a 4 mg/ml solution in PBS) (Sigma, St. Louis, MO, USA) was added to all the wells at a final concentration of 0.8 mg/ml and the cells were further incubated for 4 h at 37°C. The formazan was dissolved in dimethyl sulfoxide (DMSO) (200 μl/well; Sigma, St. Louis, MO, USA) and absorbance was read at 570 nm in an ELISA plate reader.

Alkaline phosphatase (ALP) activity assay

Cells were seeded at a density of 2×10^5 cells/well in 24-well plates and cultured in mineralisation-inducing media containing 100 μM/ml ascorbic acid, 2 mM of β-glycerophosphate and 10 nM of dexamethasone. The cells were cultured for 7 and 14 days, and an ALP activity kit (Sigma, St. Louis, MO, USA) was used to analyze the activity of ALP according to the manufacturer's instructions. The absorbance was detected in the microplate reader at 520 nm wavelength. The protein content was quantified using a BCA protein assay (Beyotime, Haimen, China). The amount of ALP in the cells was normalized against the total protein content.

Real-time quantitative PCR (qRT-PCR) analysis

Total RNA was extracted from hDPCs using the RNA plus kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. 2.0 μg of the total RNA was primed with Oligo dT primer and reverse transcribed using the AMV Reverse Transcription System (Takara Bio Inc., Otsu, Japan). The levels of gene mRNA transcripts were analyzed by using the specific primers and SYBR Green I reagent and the RT-PCR kit, according to the manufacturer's instructions, on Bio-Rad iQ5 Quantitative PCR System (Takara Bio Inc., Otsu, Japan). The specific primers for GPNMB were sense, 5'-TCTGAACCGAGCCCTGACATC-3' and 5'-AGCAGTAGCGGCCATGTGAAG-3'; for dentin sialophosphoprotein (DSPP) were sense, 5'-GCAGTGATGAATCTAATGGC-3' and antisense, 5'-CTGATTTGCTGCTGTCTGAC-3'; for dentin matrix protein-1 (DMP-1) were sense, 5'-CAGGAGCACAGGAAAAGGAG-3' and antisense, 5'-CTGGTGGTATCTTCCCCCAG-

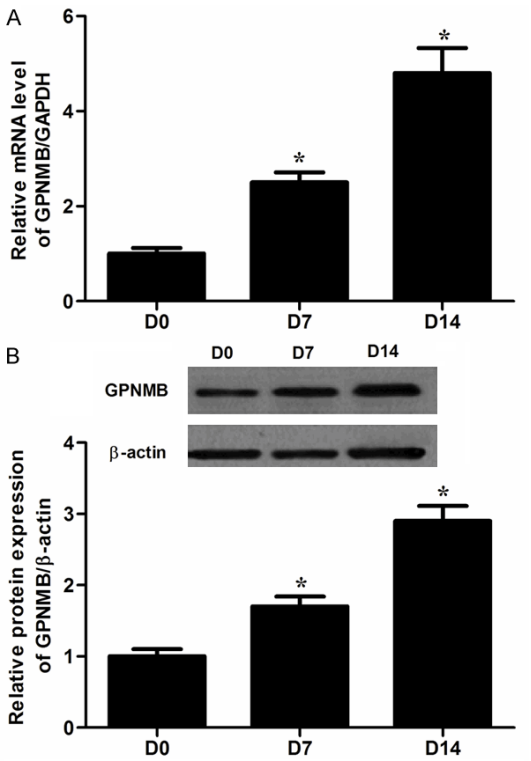

Figure 1. Expression of GPNMB during odontoblastic differentiation of hDPCs. A. GPNMB mRNA expression is detected by qRT-PCR; B. Protein level of GPNMB on days 0, 7, and 14. Data are normalized with β-actin values and are expressed as fold changes of β-actin. All experiments were repeated at least three times. *$P < 0.05$ compared with the control group.

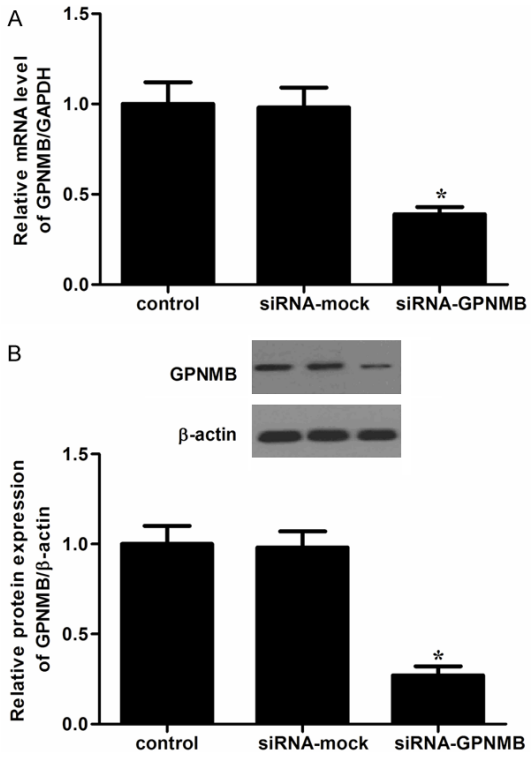

Figure 2. The determination of transfection efficiency. A. qRT-PCR showed that the expression level of GPNMB RNA was lower in the siRNA-GPNMB group compared with the siRNA-mock; B. Western blot analysis showing that the expression level of GPNMB protein in the siRNA-GPNMB group was lower than that of the siRNA-mock. Each experiment was repeated in triplicate. *$P < 0.05$ compared with the siRNA-mock group.

GAG-3'; and for GAPDH were sense, 5'-TGCACCACCAACTGCTTAGC-3' and antisense, 5'-GGCATGGACTGTGGTCATGAG-3'. Results were normalized to GAPDH expression. Then, the differential expression of these genes was analyzed by the ΔCt method and expressed as the fold changes.

Western blot

Cells were lysed in RIPA buffer (Bioteke, Beijing, China). The soluble protein was obtained from the lysate and protein concentration was determined by BCA. The mixture of total protein was separated by 10% SDS-PAGE electrophoresis, and then the proteins were transferred to PVDF membrane (Amersham, Little Chalfont, UK). The membranes were treated using the following procedure with shaking and blocking at room temperature with 2% non-fat dry milk in Tris-buffered saline (TBS) for 1 h followed by incubation in the primary antibodies (anti-GPNMB, anti-DMPP, anti-DMP-1 and anti-β-actin; from Invitrogen) overnight at 4°C. After washing thoroughly with TBST, the membrane was incubated with the second antibody for 2 h at 37°C. The bound antibodies were visualized using ECL reagent (Boehringer Mannheim, Mannheim, Germany). All experiments were repeated three times.

Statistical analysis

All results are reported as means ± SD. Statistical significance was evaluated by one-way analysis of variance using SPSS software (Version 10.0; SPSS, Chicago, IL). Values of $P < 0.05$ were considered statistically significant.

Results

Expression of GPNMB in cultured hDPCs

We first analyzed the expression levels of GPNMB in hDPCs during cell differentiation.

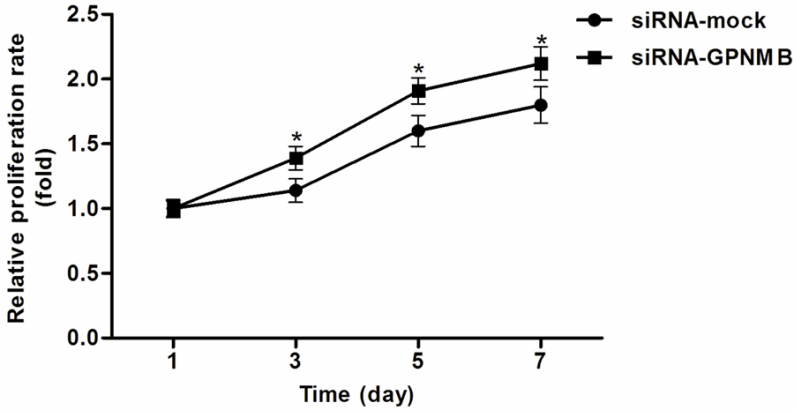

Figure 3. SiRNA-GPNMB promotes the proliferation of hDPCs. Growth curves of hDPCs transfected with siRNA-GPNMB or siRNA-mock analyzed by the MTT assay. Each experiment was repeated in triplicate. *$P < 0.05$ compared with the siRNA-mock group.

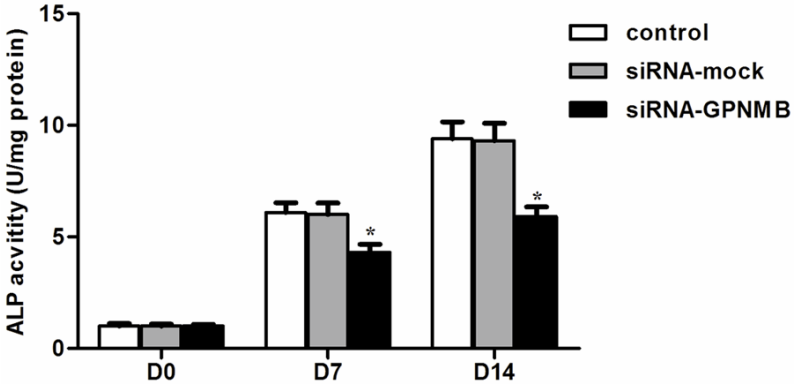

Figure 4. Effect of GPNMB on ALP activity during odontoblastic differentiation of hDPCs. ALP activity on days 0, 7 and 14 after odontoblastic induction. The activity of ALP activity was significantly lower in the siRNA-GPNMB group than in the control group. All experiments were repeated at least three times. *$P < 0.05$ compared with the siRNA-mock and control groups.

When hDPCs were cultured with odontoblastic induction medium, the mRNA level of GPNMB showed an obvious upregulation on day 7 and continued to rise until day 14 (**Figure 1A**). Consistent with the results of qRT-PCR, the corresponding increase in GPNMB protein level was also confirmed by Western blot (**Figure 1B**). These results showed that the odontoblastic differentiation of hDPCs was associated with up-regulation of GPNMB.

Establishment of GPNMB knockdown cells

To investigate the functions of GPNMB *in vitro*, a siRNA experiment was performed in hDPCs. As shown in **Figure 2**, the mRNA level and protein expression of GPNMB in the siRNA-GPNMB transfected group were significantly lower than those in the siRNA-mock group and in the control group. The results demonstrated that GPNMB knockdown was successful.

SiRNA-GPNMB promotes the proliferation of hDPCs

To determine the importance of GPNMB in the proliferation of hDPCs, MTT assays were performed on cells transfected with siRNA-GPNMB at different time points (days 1, 3, 5, and 7) after transfection. As shown in **Figure 3**, siRNA-GPNMB significantly promoted the proliferation of hDPCs, as compared with the control cell transfected with siRNA-mock group. These results demonstrated that GPNMB inhibited the proliferation of hDPCs.

SiRNA-GPNMB inhibits odontoblastic differentiation of hDPCs

To explore the role of GPNMB in hDPCs odontogenic differentiation, hDPCs were treated with siRNA-GPNMB together with mineralization media for 7 or 14 days. ALP activity assays was performed and the activity of ALP activity was significantly lower in the siRNA-GPNMB group than in the control group (**Figure 4**). Furthermore, we used qRT-PCR and Western blot analysis to examine the expression levels of DSPP and DMP1. The results showed lower DSPP and DMP1 expression levels in the siRNA-GPNMB group than those in the siRNA-mock group and in the control group (**Figure 5**). All these results indicated that GPNMB promoted the odontoblastic differentiation of hDPCs.

Discussion

During dentinogenesis, DPCs proliferate and differentiate into dentin-forming odontoblasts [14-16], but molecular mechanisms for controlling odontoblastic growth and differentiation is currently poorly understood. In this study, we

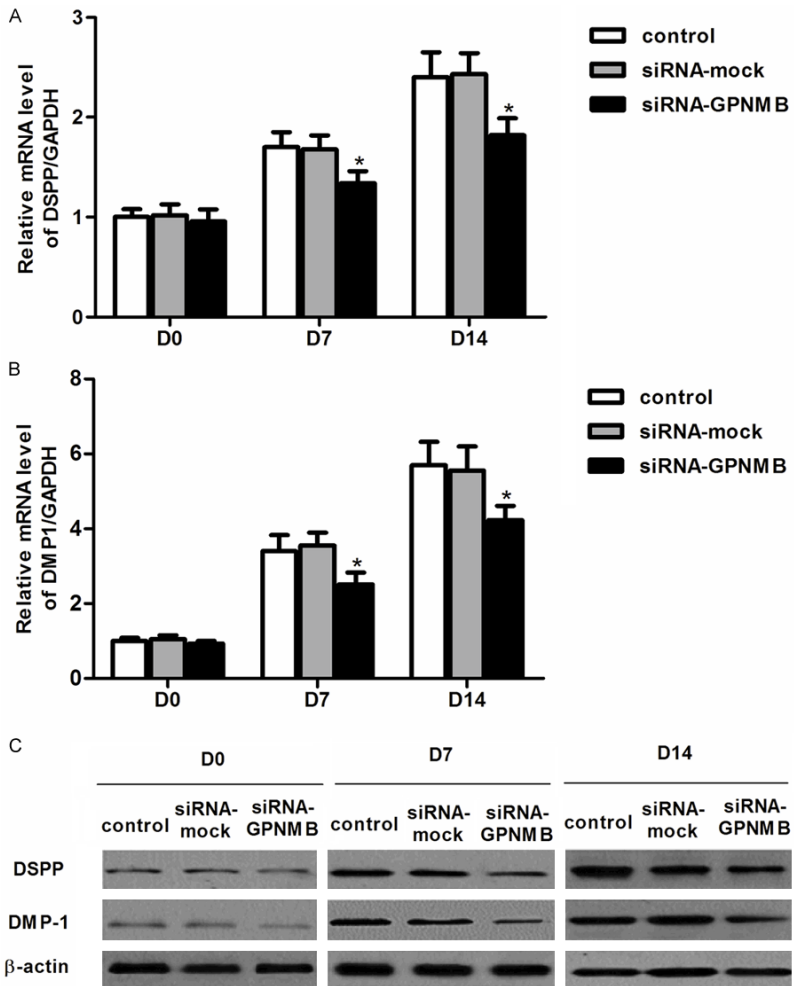

Figure 5. Effects of GPNMB on the expression of mineralization-related markers in hDPCs after 0, 7, and 14 days of culture. A, B. DSPP and DMP1 mRNA expression by qRT-PCR analysis on days 0, 7, and 14; C. DSPP and DMP1 protein expression by Western blot analysis on days 0, 7, and 14. Data are normalized with β-actin values and are expressed as fold changes of β-actin. All experiments were repeated at least three times. *$P < 0.05$ compared with the siRNA-mock and control groups.

investigated the effects of GPNMB on the proliferation and differentiation of hDPCs *in vitro*, and the key findings of this study were as follows: (1) GPNMB expression progressively increased during odontoblast differentiation; (2) GPNMB inhibited the proliferation of hDPCs; (3) GPNMB promoted odontoblast differentiation of hDPCs.

Previous studies showed that expression of GPNMB is associated with differentiation and maturation of primary rat osteoblasts *in vitro* [14], and that bone morphogenetic protein-2 (BMP-2) significantly stimulates GPNMB expression in rat osteoblasts [11]. In addition, treatment of osteoclasts precursors with receptor-activator of NF-κB ligand (RANKL) could result in hundreds- or even thousands-fold increase in GPNMB expression [15]. Odontoblast-like differentiation of DPCs has been assumed to be similar to that observed with osteoblasts [17]. In this study, we analyzed the expression levels of GPNMB in hDPCs during cell differentiation, and found that GPNMB expression progressively increased during odontoblast differentiation. These results led to our conclusion that the odontoblastic differentiation of hDPCs was associated with up-regulation of GPNMB.

Cell proliferation was indispensable for tissue development. In the present study, we found that siRNA-GPNMB significantly promoted the proliferation of hDPCs. This was in accordance with one study that showed that ectopic overexpression of GPNMB obviously attenuated cell proliferation in prostate carcinoma cells [18]. These results suggest that GPNMB inhibited the proliferation of hDPCs during odontoblast differentiation.

Differentiation from the undifferentiated state to functional active odontoblasts is a series of steps involving a number of proteins expressed at each stage. It is known that ALP is a functional marker for osteoblast and odontoblast differentiation [14]. DMP-1 is an acidic extracellular matrix protein that is primarily found in dentin and bone and has been implicated in dentin mineralization and signal transduction in the process of odontogenesis [19, 20]. *DMP-1* is initially expressed in differentiating odontoblasts and osteoblasts and facilitates the expression of the osteoblast and odontoblast differentiation marker genes [21]. DSPP is a major non-collagenous protein found in dentin that is essential for odontoblast differentia-

tion and dentin mineralization [22]. It has been reported that DSPP expression of odontoblasts was higher during primary dentinogenesis than during secondary dentinogenesis, suggesting more aggressive roles of DSPP for odontoblast differentiation [23]. In the present study, we found that the knockdown of GPNMB in hDPCs significantly decreased the activity of ALP and the expression levels of odontoblastic differentiation markers, including DSPP and DMP1. These results are consistent with a recent report, showing that suppressing the GPNMB functional activity with a blocking antibody reduced differentiation and bone formation activity of rat osteoblasts [24]. These data suggest that GPNMB has a positive effect on hDPCs differentiation into odontogenic/osteogenic cells.

In conclusion, our findings demonstrated the involvement of GPNMB in regulating the proliferation and differentiation of hDPCs and suggested that GPNMB might function as a potential regulator involved in the specific differentiation of hDPCs.

Disclosure of conflict of interest

None.

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