Ghrelin promotes the osteogenic differentiation of rMSCs via miR-206 and the ERK1/2 pathway

Nan Ye · Yifeng Yang · Zhongping Ma · Jian Huang

Received: 21 February 2020 / Accepted: 19 July 2020 / Published online: 22 July 2020
© Springer Nature B.V. 2020

Abstract

Objective Mesenchymal stem cells (MSCs) can differentiate into chondroblasts, adipocytes, or osteoblasts under appropriate stimulation. Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (GHSR), stimulates growth hormone (GH) secretion and exerts both orexigenic and adipogenic effects. The ERK1/2 signaling pathway is known to trigger osteogenic differentiation of rabbit bone marrow-derived mesenchymal stromal cells. In the present study, the function of miR-206 in the ghrelin-mediated osteogenic differentiation of rabbit bone marrow-derived mesenchymal stromal cells (rMSCs) was explored.

Methods The expression of miR-206 was detected by qPCR, and phosphorylated ERK1/2 and the protein expression levels of ALP, RUNX2, and Osterix were assessed by western blotting. Results: Ghrelin inhibited the expression of miR-206 to promote the osteogenic differentiation of rMSCs. Moreover, ghrelin increased the phosphorylation of ERK1/2, while overexpression of miR-206 suppressed ERK1/2 phosphorylation, indicating that miR-206 can regulate the ERK1/2 pathway. Further, inhibition of ERK1/2 had no influence on miR-206 expression; however, the

phosphorylation of ERK1/2 was decreased, and the protein expression levels of ALP, RUNX2, and Osterix were downregulated. Conclusions: Ghrelin promotes the osteogenic differentiation of rMSCs via miR-206 and the ERK1/2 pathway.

Keywords Ghrelin · miR-206 · rMSCs · ERK1/2

Introduction

Ghrelin is a peptide composed of 28 amino acids; and as a gastric hormone, it can regulate many functions such as food intake and energy metabolism (Howick et al. 2020; Karim et al. 2020). Ghrelin was identified as the first endogenous ligand for the growth hormone secretagogue receptor (GHSR). Recently, it has been reported that ghrelin can inhibit apoptosis (Hao et al. 2016; Yu et al. 2015) and promote cell Survival (Li et al. 2014) of cardiomyocytes (Yang et al. 2011). In addition, many studies have reported that ghrelin can regulate the differentiation of mesoderm-derived precursor cells such as osteoblasts and preadipocytes (Xu et al. 2008; Liu et al. 2009; Ye and Jiang 2015).

Micro(mi)RNAs, non-coding small RNAs, play a crucial role in the regulation of many genes (Zhang and Cohen 2013; Tay et al. 2014). MiRNAs target the 3'UTR of mRNAs with imperfect base-pairing, resulting in decreased translation or altered mRNA degradation (Beckman 2007). A recent study revealed that

N. Ye · Y. Yang · Z. Ma · J. Huang (✉)
Department of Cervical Surgery, The Second Affiliated
Hospital of Inner Mongolia Medical University, Muslims
Camp Square Road No 1, Hohhot, China
e-mail: huangjian1122@126.com

miR-206 is downregulated in osteoblast cells during osteogenesis, suggesting that miR-206 has a regulatory function during the differentiation of mesenchymal stem cells (MSCs) (Chen et al. 2019).

In previous studies (Ye and Jiang 2015; Ye et al. 2018), we have shown that ghrelin triggers the osteogenic differentiation of rabbit bone marrow-derived mesenchymal stromal cells (rMSCs) through the ERK1/2 signaling pathway; however, the function of miR-206 in this process has not been explored. Thus, in the present study, we investigated the function of miR-206 in the ghrelin-mediated osteogenic differentiation of rMSCs.

Materials and methods

Unless otherwise specified, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against IgG, GAPDH, ALP, Runx2, U0126 (an inhibitor of phospho-ERK1/2), ERK1/2, and phospho-ERK1/2 were obtained from Abcam (Cambridge, MA, USA). The ECL reagent was procured from Pierce (Rockford, IL, USA).

Isolation and culture of rMSCs

The femur was isolated from a neonatal New Zealand white rabbit, the ends of the femur were opened, and

the bone marrow was flushed out with low-glucose Dulbecco's modified Eagle's medium (DMEM) using a 1-mL syringe. Cells were harvested into a culture dish, resuspended using a Pasteur pipette, and seeded into a flask containing DMEM supplemented with 15% fetal bovine serum. Subsequently, cells were cultured in an incubator with 5% CO₂ at 37 °C, and the medium was replaced every two days. At a confluence of approximately 85%, cells were passaged using 0.25% trypsin and 0.1% EDTA (1:2).

The animal protocol was approved by the Inner Mongolia Medical University Experimental Animal Management Committee.

Determination of miRNA levels by RT-qPCR

Total miRNA, isolated with Tiangen reagent, was employed as a template for complementary DNA (cDNA) synthesis using the Universal Reverse Transcription Kit (M-MLV) reverse transcriptase. The Rotor-Gene 6000 system with SYBR Premix Ex Taq (Mansoori et al. 2016) was used for RT-qPCR. The primers were as follows: hsa-miR-206 forward, 5'-GCGCGTGAATGTAAGGAAGT-3'; hsa-miR-206 reverse, 5'-AGTGCAGGGTCCGAGGTATT-3'; U6 (internal reference miRNA) forward, 5'-CTCGCTTCGGCAGCAC-3'; and U6 reverse 5'-AACGCTTCACGAATTTGCGT-3'. Data were analyzed using the comparative cycle threshold (CT)

Fig. 1 The downregulation of miR-206 following ghrelin treatment in BMSCs. **a** Relative miR-206 expression in BMSCs as measured by qPCR following ghrelin treatment (0, 20, 40, and 60 min with 600 ng/mL ghrelin); * $p < 0.05$ vs. 0 min; # $p < 0.05$ vs. 20 min. **b** Relative miR-206 expression as measured by qPCR following ghrelin treatment (40 min with

600 ng/mL) and miR-206 mimic transfection. * $p < 0.05$ vs. blank without ghrelin; # $p < 0.05$ vs. blank + ghrelin; & $p < 0.05$ vs. NC mimic without ghrelin; \$ $p < 0.05$ vs. NC mimic + ghrelin; @ $p < 0.05$ vs. miR-206 mimic without ghrelin. Five replicates of each experiment were performed

Fig. 2 Effects of ghrelin treatment on ERK1/2 phosphorylation. **a** Representative western blots showing p-ERK1/2, ERK1/2, and GAPDH protein expression following ghrelin (600 ng/mL) treatment for 0, 20, 40, and 60 min. **b** Histograms summarizing the results shown (**a**) for ERK1/2. * $p < 0.05$ vs. 0 min; # $p < 0.05$ vs. 20 min. **c** Representative western blots showing p-ERK1/2, ERK1/2, and GAPDH protein expression

following ghrelin (600 ng/mL) treatment after miR-206 mimic transfection. **D**: Histograms summarizing the results shown in (**c**) for ERK1/2. * $p < 0.05$ vs. blank without ghrelin; # $p < 0.05$ vs. blank + ghrelin; & $p < 0.05$ vs. NC mimic without ghrelin; \$ $p < 0.05$ vs. NC mimic + ghrelin; @ $p < 0.05$ vs. miR-206 mimic without ghrelin. Five replicates of each experiment were performed

($2^{-\Delta\Delta CT}$) method. Five replicates of each experiment were performed.

Western blotting

rMSC lysates were subjected to 12% SDS-PAGE, following which the separated proteins were transferred to nitrocellulose membrane. After blocking in 5% milk, the membranes were incubated with primary antibodies overnight at 4 °C. The next day, the membranes were washed three times in PBS and incubated with species-compatible peroxidase-

conjugated secondary antibodies. ECL (Pierce, Rockford, IL, USA) was used to detect protein bands. Five replicates of each experiment were performed.

Osteogenic differentiation

rMSCs were seeded at a density of 5000 cells/cm² and cultured in standard differentiation-induction medium (SigmaAldrich; Merck KGaA) for 21 days. The medium (with ghrelin added) was changed twice per week. Osteogenic differentiation was achieved following standard in vitro protocols (Zhang et al. 2014).

Fig. 3 Effects of ghrelin treatment on the protein expression levels of ALP, Runx2, Osterix, and GAPDH. **a** Representative western blots showing ALP, Runx2, Osterix, and GAPDH protein expression following ghrelin treatment (600 ng/mL) for 0, 20, 40, and 60 min. **b** Histograms summarizing results from (A) for ALP, Runx2, and Osterix. * $p < 0.05$ vs. 0 min; # $p < 0.05$ vs. 20 min. **c** Representative western blots showing ALP, Runx2, Osterix, and GAPDH protein expression following

ghrelin treatment (600 ng/mL) after miR-206 mimic transfection. **d** Histograms summarizing the results shown in (C) for ALP, Runx2, and Osterix. * $p < 0.05$ vs. blank without ghrelin; # $p < 0.05$ vs. blank + ghrelin; & $p < 0.05$ vs. NC mimic without ghrelin; \$ $p < 0.05$ vs. NC mimic + ghrelin; @ $p < 0.05$ vs. miR-206 mimic without ghrelin. Five replicates of each experiment were performed

Cell transfection

The miR-206 mimic was obtained from GenePharma (Shanghai, China). LipofectamineTM 2000 was used for transfection of cells with plasmids or oligonucleotides according to the manufacturer's protocol.

Statistical analysis

Comparisons between groups were made using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) with Tukey-HSD analysis was employed to determine differences in gene expression levels. Five replicates were included in the statistical model. Data are shown as the mean $\pm$ SD.

Results

The influence of ghrelin on miR-206 expression

The expression of miR-206 following stimulation with ghrelin was measured by qPCR. The fold change in miR-206 expression was decreased as the stimulation time increased, as shown in Fig. 1a. After a 40-min ghrelin stimulation, the fold change in miR-206 expression was the lowest. When miR-206 was overexpressed, the fold change in miR-206 expression was higher than that in the blank and negative control (NC) mimic groups, in the presence or absence of ghrelin (Fig. 1b).

The influence of ghrelin-mediated miR-206 overexpression on ERK1/2

To investigate the effects of ghrelin-mediated miR-206 expression on ERK1/2 signaling, the phosphorylation level of ERK1/2 was determined. As shown in Fig. 2a, b, the fold change in ERK1/2 phosphorylation was higher following a 40-min or 60-min ghrelin treatment as compared with 20 min or no treatment. When miR-206 was overexpressed, the fold change in ERK1/2 phosphorylation in response to ghrelin treatment was lower ($p < 0.05$, Fig. 2c, d).

The influence of ghrelin-mediated miR-206 overexpression on ALP, RUNX2, and Osterix protein expression

To explore the function of miR-206 in rMSC-derived osteoblasts, rMSCs were allowed to differentiate into osteoblasts and treated with ghrelin for 0, 20, 40, and 60 min. The fold change in ALP, RUNX2, and Osterix protein expression was subsequently examined. The fold change in these proteins was higher at 40 min than that at the other time points (Fig. 3a, b). When miR-206 was overexpressed, the fold change in these proteins was lower in the presence of ghrelin (Fig. 3c, d).

The effect of ERK inhibition in the presence of ghrelin on expression during the osteogenic differentiation of rMSCs

ERK1/2 was inhibited by the ERK1/2 inhibitor, U0126, to explore its function during osteogenic differentiation. The fold change in miR-206 expression was lowest in the U0126 + ghrelin group, with no difference from the blank in the presence of ghrelin (Fig. 4). The fold change in ERK1/2 phosphorylation (Fig. 5a, b) and the fold change in ALP, RUNX2, and Osterix protein expression (Fig. 5c, d) were lowest in the U0126 + ghrelin group.

Discussion

BMSCs are the progenitor cells for osteoblasts, adipocytes, and chondrocytes. The osteogenic differentiation of BMSCs involves a complex regulatory network (Chen et al. 2019). Ghrelin is produced mainly in the stomach, although smaller amounts are also produced in the intestine, pancreas, pituitary, kidney, and placenta. The ghrelin receptor, GHSR, is a typical G protein-coupled receptor (Fujitsuka et al. 2012). miRNAs play important roles in a variety of cellular processes including proliferation, migration,

Fig. 4 Relative miR-206 expression as measured by qPCR following ghrelin (40 min with 600 ng/mL) treatment after U0126 (ERK1/2 inhibitor) addition. * $p < 0.05$ vs. blank without ghrelin; # $p < 0.05$ vs. blank + ghrelin; & $p < 0.05$ vs. U0126 without ghrelin. Five replicates of each experiment were performed

Fig. 5 Effects of ghrelin treatment on BMSCs following U0126 (ERK1/2 inhibitor) addition. **a** Representative western blots showing p-ERK1/2, ERK1/2, and GAPDH protein expression following ghrelin treatment (600 ng/mL) after U0126 (ERK1/2 inhibitor) addition. **b** Histograms summarizing results shown in (a) for ERK1/2. * $p < 0.05$ vs. blank without ghrelin; # $p < 0.05$ vs. blank + ghrelin; & $p < 0.05$ vs. U0126 without ghrelin.

c Representative western blots showing ALP, Runx2, Osterix, and GAPDH protein expression following ghrelin treatment (600 ng/mL) after U0126 addition. **d** Histograms summarizing results shown in (c) for ALP, Runx2, and Osterix. * $p < 0.05$ vs. blank without ghrelin; # $p < 0.05$ vs. blank + ghrelin; & $p < 0.05$ vs. U0126 without ghrelin. Five replicates of each experiment were performed

differentiation, and apoptosis (Zhou et al. 2019). Moreover, miRNAs are also involved in the diverse pathology of human bone diseases. miR206 has been shown to be involved in the development of bronchoalveolar dysplasia by downregulating fibronectin in premature infants (Zhou et al. 2019), in addition to playing a critical role in BMSC differentiation (Zhai et al. 2018; Yang et al. 2019); miR-206 is significantly downregulated in BMSCs (Chen et al. 2019). In the

present study, we found that ghrelin inhibited the expression of miR-206 to promote the osteogenic differentiation of rMSCs. It has also been demonstrated that miR-206 levels may inhibit hematopoiesis commitment of human induced pluripotent stem cells by promoting muscle cell differentiation programs (Flamant et al. 2019).

The ERK1/2 signaling pathway plays a regulatory role in numerous cellular processes, such as cell

growth, apoptosis, and differentiation (Flamant et al. 2019; Pedini et al. 2019; Hao and Fan 2017). In the present study, ghrelin increased ERK1/2 phosphorylation, while overexpression of miR-206 suppressed the phosphorylation of ERK1/2, indicating that miR-206 can regulate the ERK1/2 pathway. It has previously been shown that miR-206 mimics can significantly inhibit the phosphorylation of ERK1/2 in papillary thyroid carcinoma (Liu et al. 2019). Further, in the present study, ERK1/2 inhibition had no influence on miR-206 expression. The phosphorylation level of ERK1/2 was decreased and the protein expression of ALP, RUNX2, and Osterix was down-regulated. We found that miR-206 regulates ghrelin to promote the osteogenic differentiation of rMSCs via the ERK1/2 pathway. Previous studies have only shown that ghrelin promotes the osteogenic differentiation of rMSCs via the ERK1/2 pathway (Xu et al. 2008).

In conclusion, although our *in vitro* study in rMSCs has limitations, these results also suggest that ghrelin promotes the osteogenic differentiation of rMSCs via miR-206 and the ERK1/2 pathway.

Acknowledgements This work was supported by the Inner Mongolia Natural Science Foundation (No. 2017BS0813).

References

- Beckman M (2007) MicroRNAs found cavorting with p53. *J Natl Cancer Inst* 99:1664–1665
- Chen Y et al (2019) miR-206 inhibits osteogenic differentiation of bone marrow mesenchymal stem cells by targeting glutaminase. *Biosci Rep* 39:3
- Flamant S et al (2019) Global MicroRNA profiling uncovers miR-206 as a negative regulator of hematopoietic commitment in human pluripotent stem cells. *Int J Mol Sci* 20:7
- Fujitsuka N et al (2012) Efficacy of ghrelin in cancer cachexia: clinical trials and a novel treatment by rikkunshito. *Crit Rev Oncog* 17:277–284
- Hao Y et al (2016) Ghrelin protects against depleted uranium-induced apoptosis of MC3T3-E1 cells through oxidative stress-mediated p38-mitogen-activated protein kinase pathway. *Toxicol Appl Pharmacol* 290:116–125
- Hao XZ, Fan HM (2017) Identification of miRNAs as atherosclerosis biomarkers and functional role of miR-126 in atherosclerosis progression through MAPK signalling pathway. *Eur Rev Med Pharmacol Sci* 21:2725–2733
- Howick K et al (2020) Behavioural characterization of ghrelin ligands, anamorelin and HM01: appetite and reward-motivated effects in rodents. *Neuropharmacology* 108:11
- Karim R et al (2020) Association of hot flushes with ghrelin and adipokines in early versus late postmenopausal women. *Menopause* 27:512–518
- Li E et al (2014) Ghrelin stimulates proliferation, migration and differentiation of neural progenitors from the subventricular zone in the adult mice. *Exp Neurol* 252:75–84
- Liu J et al (2009) [Effects of ghrelin on the proliferation and differentiation of 3T3-L1 preadipocytes and its possible mechanisms]. *Zhongguo Dang Dai Er Ke Za Zhi* 11:69–73
- Liu F et al (2019) Over-expression of miR-206 decreases the Euthyrox-resistance by targeting MAP4K3 in papillary thyroid carcinoma. *Biomed Pharmacother* 114:108605
- Mansoori B et al (2016) HMG1-C suppressing induces P53/caspase9 axis to regulate apoptosis in breast adenocarcinoma cells. *Cell Cycle* 15:2585–2592
- Pedini F et al (2019) Joint action of miR-126 and MAPK/PI3K inhibitors against metastatic melanoma. *Mol Oncol* 13:1836–54
- Tay Y et al (2014) Characterization of dual PTEN and p53-targeting microRNAs identifies microRNA-638/Dnm2 as a two-hit oncogenic locus. *Cell Rep* 8:714–722
- Xu G et al (2008) Ghrelin and cell differentiation. *Acta Biochim Biophys Sin (Shanghai)* 40:841–847
- Yang J et al (2011) Ghrelin promotes differentiation of human embryonic stem cells into cardiomyocytes. *Acta Pharmacol Sin* 32:1239–1245
- Yang TR et al (2019) Resina draconis inhibits the endoplasmic-reticulum-induced apoptosis of myocardial cells via regulating miR-423-3p/ERK signaling pathway in a tree shrew myocardial ischemia-reperfusion model. *J Biosci* 44:2
- Ye N, Jiang D (2015) Ghrelin accelerates the growth and osteogenic differentiation of rabbit mesenchymal stem cells through the ERK1/2 pathway. *BMC Biotechnol* 15:51
- Ye N et al (2018) Ghrelin accelerates the cartilaginous differentiation of rabbit mesenchymal stem cells through the ERK1/2 pathway. *Cytotechnology* 70:415–421
- Yu J (2015) Ghrelin protects MES235 cells against rotenone via inhibiting mitochondrial dysfunction and apoptosis. *Neuropeptides* 56:69–74
- Zhai W et al (2018) MiR-532-5p suppresses renal cancer cell proliferation by disrupting the ETS1-mediated positive feedback loop with the KRAS-NAP1L1/P-ERK axis. *Br J Cancer* 119:591–604
- Zhang W, Cohen SM (2013) The Hippo pathway acts via p53 and microRNAs to control proliferation and proapoptotic gene expression during tissue growth. *Biol Open* 2:822–828
- Zhang Z et al (2014) Bone marrow stromal cell-derived extracellular matrix promotes osteogenesis of adipose-derived stem cells. *Cell Biol Int* 39:291–9
- Zhou J et al (2019) miR-206 regulates alveolar type II epithelial cell Cx43 expression in sepsis-induced acute lung injury. *Exp Ther Med* 18:296–304

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.