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MiR-126-5p regulates osteoclast differentiation and bone resorption in giant cell tumor through inhibition of MMP-13

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ABSTRACT

Giant cell tumor (GCT) of bone is an aggressive skeletal tumor characterized by localized bone resorption. Matrix metalloproteinase-13 (MMP-13) is the principal proteinase expressed by the stromal cells of GCT (GCTSCs) and also considered to play a crucial role in formation of the osteolytic lesion in GCT. However, the exact mechanism of the regulation of MMP-13 expression in GCTSCs was unknown. In this study, we identified miR-126-5p was significantly downregulated in GCTSCs and affect osteoclast (OC) differentiation and bone resorption by repressing MMP-13 expression at the post-transcriptional level. Thus, our studies show that miR-126-5p plays an important physiological role in multinucleated giant cell formation and osteolytic lesion in GCT.

1. Introduction

Giant cell tumor (GCT) of bone, a common aggressive skeletal tumor, typically arises in the metaphyseal regions of long bones and less commonly occurs in sacrum, pelvis, and spine [1]. Metastasis and malignant transformation are not common in the benign lesion, but GCT does produce substantial localized osteolysis which results in regional pain and the predisposition to pathologic fractures [2–4]. Surgical resection is the preferred treatment of GCT, but local recurrence rates remain high [5,6]. Thus, further understanding of the mechanisms leading to significant localized osteolysis is helpful for developing new treatment options for this aggressive tumor.

Histologically, GCT is a heterogeneous tumor and consists of three major cell types: osteoclast-like multinucleated giant cells, spindle-like stromal cells, and monocytic round cells [7,8]. Spindle-like stromal cells of GCT (GCTSC) which originate from mesenchymal stem cells in the bone marrow are neoplastic component of GCT and play a crucial role in the occurrence and progression of GCT by secreting various chemokines [9]. Although the characteristic bone destruction in GCT is often attributed to the multinucleated giant cells [10–12], many studies show that GCTSCs play a crucial role in the formation of localized osteolysis by secreting matrix metalloproteinases (MMPs), particularly the high elevated expression of MMP-13 [3,13–16].

MMP-13 is consistently and highly expressed by the GCTSCs in GCT and also considered to play a vital role in osteolytic lesion formation through a complex process [3,15–19]. MMP-13 is a kind of interstitial collagenase and can effectively degrade the type I collagen which is the primary organic component of bone ECM [17]. The characteristic of MMP-13 to stimulate osteoclast differentiation and enhance RANKL/RANK/OPG signaling as well as TGF-β signaling to promote the formation of osteolytic lesion indicates its crucial role in GCT [3,18,19]. MMP-13 expression can be regulated at the level of both transcription and posttranscription [3,14,15,18,20]. Elegant cellular studies have shown that a set of cytokines and transcription factors, including IL-1α, PTH, PTHrP, TGF-β and Runx2 can regulate MMP-13 expression [3,15,18]. However, the mechanism of the posttranslational processes which are vital for the final protease activity [12], has not been completely defined.

MicroRNAs (miRNA) are small, evolutionarily conserved noncoding RNA molecules that act as posttranslational regulators of gene expression [21]. MiR-126-5p is an intronic miRNA located in the epidermal growth factor-like domain 7 (EGFL7) gene and has been identified as a tumor suppressor in many tumors such as prostate cancer, melanoma, breast cancer and non-small cell lung cancer [22–26]. In this study, we examined the expression level of miR-126-5p in the samples of GCT and normal cancellous bone tissue, and analyze the role of miR-126-5p in multinucleated giant cell formation and bone resorption in GCT by regulating MMP-13 expression.

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2. Materials and methods

2.1. Cell lines and reagents

For primary cell culture, BMM cells isolated from C57/BL6 mice and GCTSCs isolated from GCT samples were cultured as described previously [31]. HEK293 and MG63 cells were maintained in DMEM (GIBCO) supplemented with 10% fetal bovine serum (HyClone) in the cell incubator (37 °C, 5% CO_2). BMM and GCTS cells were maintained in α-MEM (GIBCO) supplemented with 10% fetal bovine serum.

The antibodies of MMP-13 (sc-30073), GAPDH (sc-32233) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Transfections were performed using FuGENE HD (Promega, USA) according to the manufacturer's instructions. M-CSF was from R&D Systems (Minneapolis, USA).

2.2. Plasmids

MMP-13. 3′-UTR constructs were PCR amplified using cDNA encoding MMP-13 (NM_002427) as templates, and subcloned into pGL3-Basic vector for Luciferase reporter gene assay. The construction of the MMP-13 3′-UTR mutant were done by PCR using the Quickchange site directed mutagenesis kit from Qiagen (USA).

2.3. Transfection of miRNA mimics and inhibitors

The miRNA mimics (miR-126 mimic), inhibitors (antago-miR-126) and negative controls of miR-126-5p were purchased from RiboBio. The cells were transfected with a mimic, an inhibitor and a negative control at final concentrations of 50 nM. The FuGENE HD transfection agent (Promega, USA) was used according to the manufacturer's instructions.

2.4. Stable cell lines constructed using TALENs

The plasmids of TALENs targeting the PPP1R12C (the AAVS1 locus) was constructed using FastTALE™ TALEN Assembly kit from SiDanSai biotechnology (SiDanSai, Chain). The detailed sequence information was reported previously [30]. The PPP1R12C locus homologous sequences were PCR amplified using genomic DNA extracted from HEK293 cells as template, and cloned in pEASY-T1 vector (TransGen, China) as Donor-1. The following primer sets were used: forward, 5′-ATGCCGTCTTCACTCGCTGGGTT-3′; reverse, 5′-CTCCTGGGCTTGCCAAGGACTCAA-3′. Then, Donor-BamHI mutant vector were constructed using Donor-1 vector as template by PCR, and using Quickchange site directed mutagenesis kit. After that, human cytomegalovirus (CMV) immediate early promoter gene, enhanced green fluorescent protein (EGFP) gene, pri-mir126 gene, SV40 early mRNA polyadenylation signal gene were inserted into BamHI site from Donor-BamHI vector in turn. All clones were constructed using In-Fusion™ advantage PCR cloning Kit (Clontech, USA).

The two TALENs and corresponding Donor plasmids were transfected into GCTSCs, after selection with puromycin, resistant colonies with green fluorescent were picked up, and examined by genomic PCR (the following primers were used: forward, 5′-ATGC CGTCTTCACTCGCTGGGTT-3′; reverse, 5′-CTCCTGGGCTTGCCAAGGACTCAA-3′) and digestion with EcoRI restriction enzyme.

2.5. In vitro osteoclastogenesis assay

BMMs were seeded on a dentin slice and cultured with conditional mediums containing MCFS (10 ng/mL) for 7 days with media changed every 2 days. For TRAP staining, the cells were fixed and stained for TRAP activity kit (Sigma, USA).

2.6. Real-time RT-PCR

For the real-time RT-PCR analysis, we extracted total RNA from cells with TRIZOL (Invitrogen). MMP-13 transcripts were quantified on 7900HT Fast Real-Time PCR System (Life Technologies Corporation, USA) using SYBR green dye and normalized with β-actin. The following primer sets were used: MMP-13: forward, 5′-CTTCCCAACCGTATTGATGC-3′; reverse, 5′-CTTCCCAACCGTATTGATGC-3′; GAPDH: forward, 5′-TTTGGAAGACCCAGTTCAGA-3′; reverse, 5′-AGTCCTTCCACGATACCAAAGT-3′. The expression of miRNA126-5p and U6 was examined by TaqMan miRNA Assay system (Life Technologies Corporation, USA).

2.7. Immunoblot analysis

The samples were resolved on SDS–PAGE gel, and proteins were transferred onto a PVDF membrane (Millipore) by conventional methods. Immunoblotting was probed with indicated antibodies. The immunoreactive proteins were visualized by using BeyoECL Plus kit (Beyotime).

2.8. Statistical analyses

All of the measurements were collected in triplicate for each independent preparation. The results were statistically analyzed using Student's *t*-test and ANOVA. The SPSS software, version 16.0, was used for all of the statistical analyses, and differences with a p value less than 0.05 were considered statistically significant ($^{*} = p < 0.05$).

3. Results

3.1. MMP-13 is secreted by GCTSCs at high level in GCT

MMP-13 is considered to be expressed at a high level and play a crucial role in bone resorption in GCT [15–19]. Western blot and qRT-PCR assay showed that MMP-13 was expressed at low level in the normal cancellous bone tissues and U2OS cells, whereas MMP-13 was significantly over expressed in GCT (Fig. 1A–C).

In order to figure out which cell is responsible for high MMP-13 expression in GCT, we localized MMP-13 in GCT tissue samples using immunohistochemistry. Five GCT samples were stained and graded to show expression profiles of MMP-13 and we found that both GCTSCs and its surrounding showed cellular staining of MMP-13 (Fig. 1D). These results suggested that MMP-13 was expressed by GCTSCs at high level.

3.2. MiR-126-5p is dramatically down-regulated in GCT specimen and directly targets MMP-13

MiR-126-5p is an intronic miRNA located in the epidermal growth factor-like domain 7 (EGFL7) gene and has been identified as a tumor suppressor [24,25]. We examined the expression level of miR-126-5p in GCTSCs and normal cancellous bone tissues from 10 patients. The qRT-PCR result showed that miR-126-5p was significantly down-regulated in GCTSCs (Fig. 1E). More importantly, miR-126-5p was predicted to have a binding site at the 3′-UTR of MMP-13 with an 8 mer perfect match. So, we chose miR-126-5p as object to investigate its regulatory role on MMP-13 expression as well as its function in GCT.

To test whether miR-126-5p directly targets MMP-13, we constructed luciferase reporters that had either a wild-type (WT)

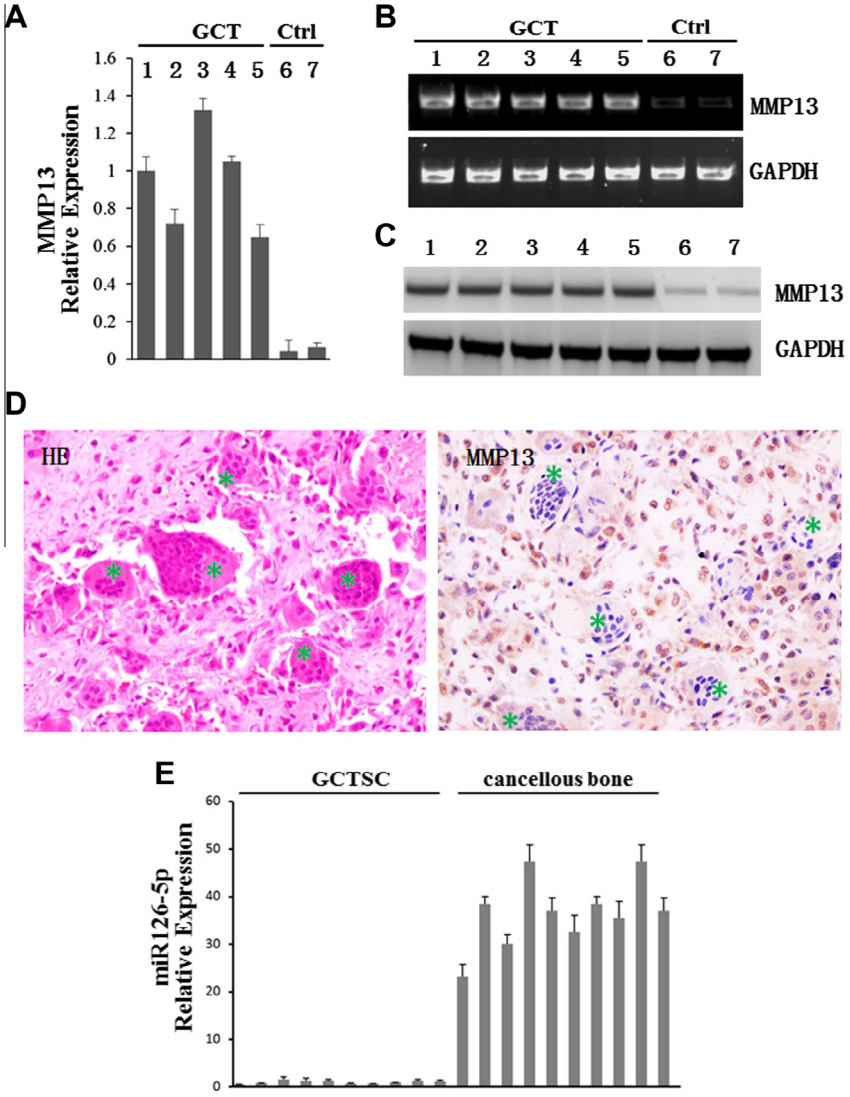

Fig. 1. Expression of MMP-13 and miR-126-5p in GCT. (A) qRT-PCR analysis of MMP-13 expression in GCT samples and normal cancellous bone tissues. (B) Expression of MMP-13 mRNA in samples from five cases of GCT (lanes 1–5), normal cancellous bone (lane 6), U2OS cells (lane 7). The sizes of MMP-13 and GAPDH PCR products were 143 and 113 bp, respectively. The GAPDH housekeeping gene determines the variation of loading in the gel. (C) Western blot analysis of MMP-13 expression in GCT samples and normal cancellous bone. (D) HE staining and immunolocalization of MMP-13 in human specimens of GCT formalin-fixed-paraffin-embedded tissue. Giant cells are marked with asterisks. (E) qRT-PCR analysis of miR-126-5p expression in GCT samples and cancellous bone tissues.

MMP-13 3′ UTR or an MMP-13 3′ UTR containing mutant sequences in the miR-126-5p binding site (Fig. 2A). We found that agomir-126-5p inhibited the luciferase reporter activity of the WT-MMP-13 3′ UTR but not mutated 3′ UTR (Fig. 2B). Furthermore, to identify the action of miR-126-5p on MMP-13, we transfected GCTSCs and MG-63 cells with miR-126-5p mimic (agomir-126-5p) or inhibitor (antago-126-5p) and measured the mRNA and protein levels of MMP-13 by qRT-PCR, ELISA and Western blot. Relative to the control, agomir-126-5p down-regulated endogenous MMP-13 expression at both mRNA and protein level (Fig. 2C–E).

3.3. MiR-126-5p regulates GCTSC induced bone resorption and multinucleated cell formation

In order to reach a more comprehensive understanding of the regulatory role of miR-126-5p on MMP-13, we established the stable cell lines (OE-miR126 and OE-control) using TALENs targeting the PPP1R12C (the AAVS1 locus) and corresponding donor plasmids bearing homologous sequences (Fig. 3A). Briefly, GCTSCs were transfected with two TANEN vectors [30] in conjunction with a targeting vector containing the EGFP gene and DNA fragments of pri-miR126. Clones with a successful recombination in pri-miR126 and EGFP-pri-miR126-mutant were identified by genomic PCR and restriction digestion (arrows for primers in Fig. 3A). As expected, OE-miR126 cells expressed high level of miR126-5p compared to OE-control by qRT-PCR assay. Relative to the control group, endogenous MMP-13 expression was dramatically down-regulated in OE-miR126 GCTSCs at not only protein level but also mRNA level, which was consisted with the western blot data (Fig. 4C).

To investigate the role of miR-126-5p during bone resorption in GCT, we used BMMs with M-CSF stimulation as in vitro osteoclast (OC) differentiation model. OE-miR126 and OE-control GCTSCs were used to examine the effects of miR126-5p on bone resorption and osteoclast precursor differentiation (Fig. 4A). Two groups of BMMs were seeded on a dentin slice and cultured with conditional mediums containing M-CFS (10 ng/mL) from OE-miR126 and OE-Control GCTSCs respectively for 7 days. The number and area of

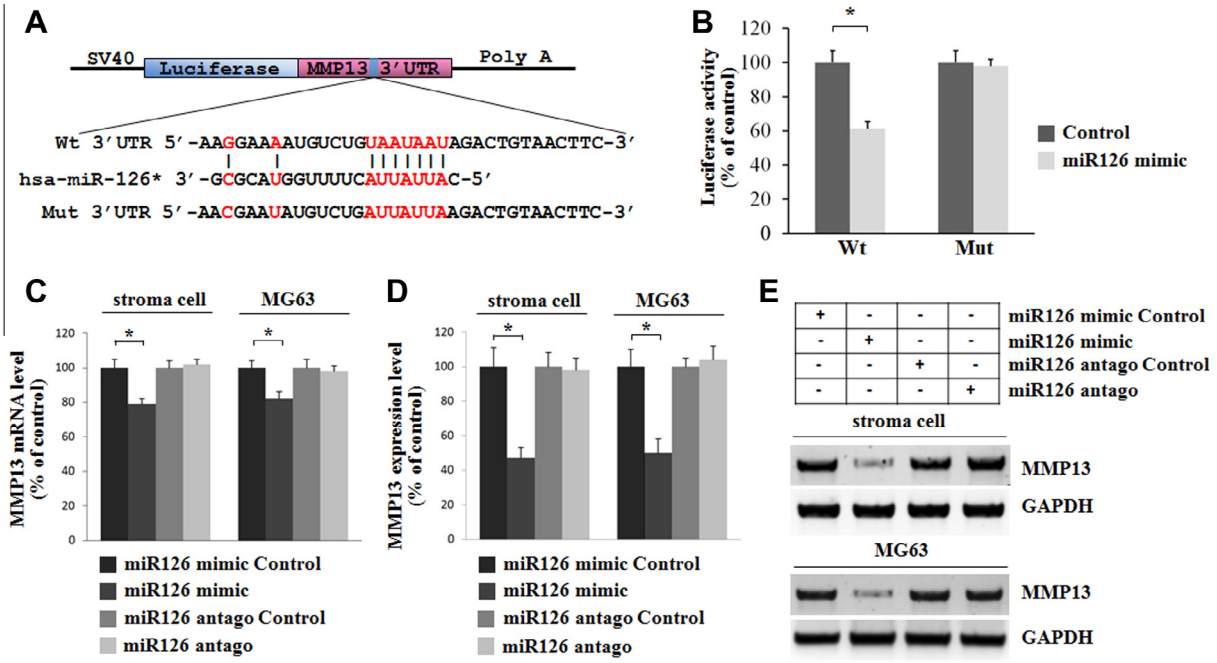

Fig. 2. miR-126-5p directly binds to the 3′ UTR of MMP-13. (A) Schematic diagram illustrating the design of luciferase reporters with the WT-MMP-13 3′ UTR (WT 3′-UTR) or the site-directed mutant MMP-13 3′ UTR (Mut 3′-UTR). (B) The effect of agomir-Control, agomir-126-5p on luciferase activity in HEK293 cells transfected with either the WT MMP-13 3′ UTR reporter (left) or the mutant MMP-13 3′ UTR reporter (right). (C) Real-time PCR analysis of MMP-13 mRNA levels in GCTSCs and MG63 cells under the treatment of agomir-126-5p or antagomir-126-5p or their corresponding negative controls. (D) ELISA analysis of MMP-13 secretion level. (E) Western blot analysis of MMP-13 expression level.

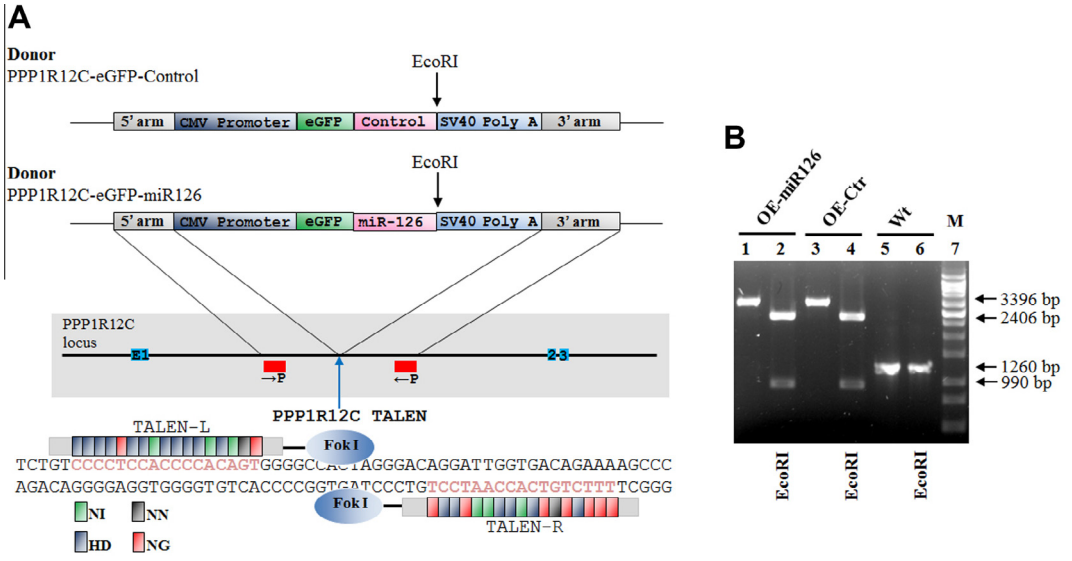

Fig. 3. Genetic engineering of GCTSCs using TALENs. (A) Schematic overview depicting the targeting strategy for PPP1R12C. Primers are shown as red boxes, exons as blue boxes; the arrow indicates cut site by the TALENs. Donor plasmids: CMV Promoter, human cytomegalovirus (CMV) immediate early promoter gene; eGFP, enhanced green fluorescent protein gene; miR-126-5p, pri-miR126 gene; Control, pri-miR126 binding site mutant gene; SV40 Poly A, SV40 early mRNA polyadenylation signal gene. Below, scheme of PPP1R12C TALENs and their recognition sequence. TALE repeat domains are colored to indicate the identity of the repeat variable diresidue (RVD); each RVD is related to the cognate targeted DNA base by the following code (NI = A, HD = C, NN = G, NG = T) [30]. (B) Genomic PCR and restriction digestion characterization of OE-miR126 (Overexpression miR-126-5p) and OE-control (Overexpression miR-126-5p mutant) cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pits on the surface of the dentin slices were markedly decreased in OE-miR126 conditional mediums group. We also found that culture with OE-miR126 conditional medium strongly inhibited BMM differentiation by TRAP staining, TRAP activity was also inhibited (Fig. 4E). These results indicated that miR126-5p regulate MMP-13 secretion from GCTSCs to modulate osteoclast resorption and differentiation.

4. Discussion

Abundant bone resorption is the most prominent feature of GCT. Significant osteolysis causes severe pain and pathologic fractures which bring great distress to the patients and increase the difficulty in treatment [2–4]. Moreover, in our previous study we have found that inhibition of bone resorption could significantly

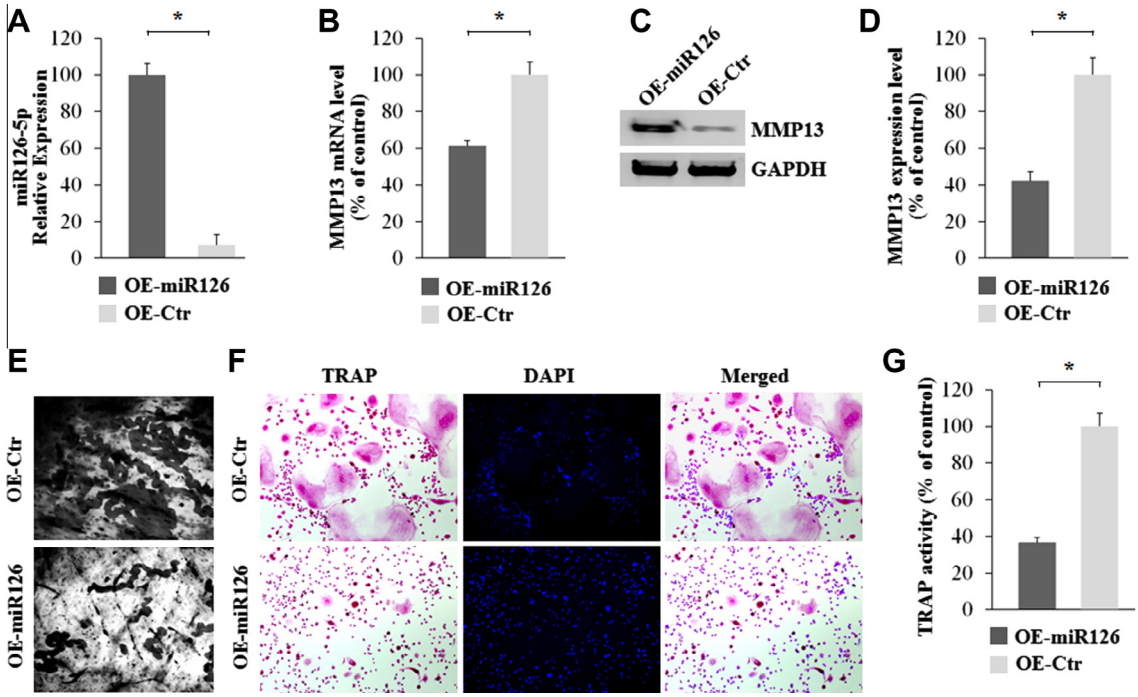

Fig. 4. miR-126-5p regulates GCTSC induced bone resorption and multinucleated cell formation. (A, B) qRT-PCR analysis of miR-126-5p and MMP-13 expression in OE-miR126 and OE-Control cells. (C) Western blot analysis of MMP-13 expression level. (D) ELISA analysis of MMP-13 secretion level. (E, F) Mouse BMMs were seeded on dentin slices or empty walls and cultured with conditional mediums containing MCFS (10 ng/mL) from OE-miR126 or OE-miR126-Control GCTSCs respectively for 7 days. (E) Dentine slices were stained with Mayer's hematoxylin after removal of cells. The resorption pits were visualized with light microscopy. (F) TRAP staining of BMM cells. (G) TRAP activity assay of BMM cells.

reduce the recurrence rate of GCT [6]. Although bone resorption poses a very important role in the occurrence and treatment of GCT, the mechanisms leading to the significant osteolysis are poorly defined.

The role of MMP-13 in bone resorption in GCT is a complex process which involves multiple inflammatory factors and several pathways. Bone resorption requires specific proteases to remove the organic matrix (predominantly fibrillar type I collagen) [12,27]. MMP-13 is one of the two proteases (MMP-13, cathepsin K) which could effectively cleave the native triple helical region of fibrillar type I collagen and meanwhile are actively expressed at high level in GCT [3,12,17].

MMP-13 can stimulate osteoclast (OC) differentiation and promote OC bone-destructive activity [18]. MMP-13 can indirectly induce OC differentiation by activating pro-MMP-9 and stimulate the OC activity to directly degrade bone matrix by cleaving the osteoclastogenesis inhibitor galectin-3 [18]. Also, it can further contribute to the osteolytic process by regulating RANKL/RANK/OPG and TGF-β signaling pathways which are important in osteoclast activation and subsequent bone resorption [19]. In addition, MMP-13 may have an ability to stimulate the formation of multinucleated cells from mononuclear cells [28,29]. This may help to explain the phenomenon that the GCTSCs not the giant cells are present at the margins of the tumor where bone resorption occurs [16].

We found that miR-126-5p can directly and negatively regulate the MMP-13 expression. MiR-126-5p is an intronic miRNA located in the epidermal growth factor-like domain 7 (EGFL7) gene and was found to be significantly down-regulated in GCT in our study (Fig. 1E). MiR-126-5p could decrease the MMP-13 expression in both mRNA and protein levels (Fig. 2C–E).

We further found that miR-126-5p overexpression could inhibit OC differentiation and decrease the osteolysis formation in GCTSCs. Relative to the control group, OC differentiation was significantly inhibited in OE-miR126 group (Fig. 4E). Similar results were also obtained in bone resorption assay (Fig. 4F). As miR-126-5p could significantly and directly downregulate MMP-13 expression, we considered that the inhibitory effect was achieved through the negative regulation of MMP-13 expression. Meanwhile the OC differentiation and bone resorption in GCT involve a great deal factors, miR-126-5p might influence some other factors expression in the process through a direct or indirect way, which requires further study and is also the next goal of our work.

In summary, in this study we found that the expression of miR-126-5p was at low level in GCT, while MMP-13 was high expressed by GCTSCs. MiR-126-5p could negatively regulate MMP-13 expression, and plays an important role in the regulation of OC differentiation and osteolysis formation in GCT, which provided a potential therapeutic target for GCT.

Acknowledgments

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