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MiR-30a inhibits osteolysis by targeting RunX2 in giant cell tumor of bone

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ABSTRACT

RunX2 has been identified to crucially regulate the osteolysis in giant cell tumor of bone. MiR-30a is an intronic miRNA identified as tumor suppressor, but little is known about its role in giant tumor cell of bone. In our research, we reported miR-30a was down-regulated in GCT whereas RunX2 was highly expressed. Further research proved that miR-30a can regulate the expression of RunX2 by binding to its 3′-UTR, which influence the osteoclast differentiation and osteolysis formation. Thus, these results suggest that miR-30a could directly target RunX2 and participate in osteolysis in GCT.

1. Introduction

Giant cell tumor of bone (GCT) as a borderline tumor with high recurrence and malignant potential is mainly intractable for its osteolysis activity to the bone. The major three components of the GCT, that is osteoclast-like multinucleate giant cells, spindle-like stromal cells (GCTSCs) and monocytic round cells [1–4], act as different roles in the osteolysis formation such as cytokine secretion or cellular interaction [5]. Previous studies have proved that the GCTSCs as a neoplastic component of GCT originated from mesenchymal stem cells (BMSCs) secreted many cytokines to induce cell fusion, differentiation and other reaction [4,6]. It has been reported the osteoblast transcription factor 2 (RunX2) was constitutively expressed in GCTSC as well as its function in regulating matrix metalloproteinase-13 (MMP-13) [7], indicating its crucial role in the osteoclast activity in GCT. RunX2 can activate the cytokine expression of MMP-13, TWIST and PTHrP. Studies regarding RunX2 mainly focus on its regulation role on TWIST, MMP-13 and PTHrP, influencing the osteoclast differentiation and osteolysis in GCT [4,7,8]. But the mechanisms associated with posttranscriptional regulation of RunX2 are not completely defined.

MiRNAs are small non-coding RNA molecules that evolutionarily conserved and can act as post-translational regulators of gene expression [9]. MiR-30a has been reported in many tumors for its wide regulation function including suppressing proliferation and metastasis of breast cancer [10,11], inducing autophagy in chronic myelogenous leukemia [12], inhibiting the invasion and migration of Ewing tumor and colorectal carcinoma [13,14] and negatively regulating TGF-β1-induced epithelial-mesenchymal transition [15], but little is known about its role in GCT and osteoclast activities.

In our previous research, from 20 GCT specimens collected from patients, we found that RunX2 was highly expressed in GCTSC whereas miR-30a was strictly down-regulated compared with para-tumor tissues. Further research was proved that miR-30a could bind to the 3′-untranslated region (3′-UTR) of RunX2 and regulate the expression. By using dual-luciferase reporter system, we demonstrate miR-30a binding to 3′-UTR of RunX2, decreasing the expression of RunX2 as well as the down-stream target gene such as MMP-13. Collectively, we confirm that miR-30a binding to RunX2 leads to a decreased expression, indicating its crucial role in the osteolysis and may be a new target for clinical treatment.

2. Materials and methods

2.1. Cell lines and reagent

Bone marrow-derived monocyte (BMM) cells isolated from C57BL/6J mice and GCTSC were cultured as described previously

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[16]. DMEM (GIBCO, USA) was supplemented with 10% fetal bovine serum (Hyclone, USA) in the cell incubator.

The antibodies of RunX2 (sc-101145), MMP-13 (sc-30073), Actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). FuGENE HD (Promega, USA) was used for transfection according to the manufacturer's instructions. Macrophage colony-stimulating factor (M-CSF) was obtained from R&D systems (Minneapolis, USA).

2.2. Plasmids and miRNA mimics and inhibitor

RunX23′-UTR constructs were PCR amplified (forward": 5′-GAA AGATCGCCGTGTAATTCAGACAGCCTTTGACATT-3′; reverse: 5′-GGC TCTCAAGGGCATCGGGCAACATACAATACAAATAGTCCC-3′) using cDNA encoding RunX2 as templates, and sub cloned into pGL3-basic vector for Luciferase reporter assay. PCR with Quickchange site directed mutagenesis kit from Qiagen (USA) were used for construction of mutant RunX2 3′-UTR.

The miRNA mimics (ago-miR-30a), inhibitor (antago-miR-30a) and negative controls of miR-30a, purchased from RiboBio (RiboBio, Guangzhou, China) were transfected at final concentration of 50 nM. The FuGENE HD transfection agent (Promega, USA) was used according to the manufacturer's instructions.

2.3. TALENs-based stable cell lines construction

FastTALE™ TALEN Assembly kit from SiDanSai biotechnology (SiDanSai, Shanghai, China) was used to construct plasmids of TALEN targeting PPP1R12C (the AAVS1 locus). Detailed sequence information was reported previously [4].

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′-ATGCCGTCTTCAC TCGCTGGGTT-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-mir30a 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′-ATGCC GTCTTCACTCGCTGGGTT-3′; reverse, 5′-CTCCTGGGCTTGCCAAGGAC TCAA-3′) and digestion with EcoRI restriction enzyme.

2.4. In situ hybridization (ISH)

ISH was performed using the miR-30a locked nucleic acid probe (5′-digoxigenin-TCTTAAAAAATCTACACACAAATGAAGTACAAATG-3′-digoxigenin) and the microRNA ISH Optimization Kit (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. Detailed information was reported previously [4].

2.5. Real-time RT-PCR

Total RNA was extracted from cells with TRIZOL (Invitrogen). Gene transcription were quantified on 7900HT Fast Real-time PCR system (Life Technology Corporation, USA) using SYBR green dye and normalized with actin. The following primer sets were used: RunX2: forward, 5′-TACCTGAGCCAGATGACGTC-3′, reverse, 5′-CAGTGAGGGATGAAATGCTT-3′, MMP-13: forward, 5′-CTTCCCA ACCGTATTGATGC-3′; reverse, 5′-CTTCCCAACCGTATTGATGC-3′; Actin: forward, 5′-CTCCATCCTGGCCTCGCTGT-3′, reverse, 5′-GCTGT CACCTTCACCGTTCC-3′. The expression of miR-30a and U6 was examined by TaqManmiRNA Assay system (Life Technologies Corporation, USA).

2.6. Western Blot

Samples were resolved on SDS–PAGE gel. Proteins were transferred onto a PVDF membrane (Millipore, Germany) by conventional methods. Western Blot was probed with indicated antibodies and the immunoreactive protein were visualized by using BeyoECL Plus kit (Beyotime, China).

2.7. 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. Result

3.1. RunX2 is highly expressed in GCTSCs

First we analyzed RunX2 expression feature in GCT and para-tumor normal bone tissues from 20 samples collected from patients by qPCR and semi-qPCR (Fig. 1A and B). Clinical features of GCT of bone patient were presented in Table-S1. We found that, within all 20 samples, RunX2 was highly elevated compared with para-tumor normal bone tissues. The same result was also confirmed by Western Blot (Fig. 1C). Then to determine the specific component of GCT expressing RunX2, we localized RunX2 through immunohistochemistry. The result showed that GCTSCs expressed RunX2 at a high level compared with other components. Our results confirmed the GCTSCs are responsible for high expression of RunX2.

3.2. MiR-30a is down-regulated in GCT samples and directly regulate RunX2

MiR-30a has been proved to be a tumor suppressor in breast cancer, Ewing tumors, colorectal carcinoma and myelogenous leukemia [10–12]. Here, we detected the miR-30a expression in GCT and para-tumor normal bone tissues by qRT-PCR (Fig. 2A and B). Notably, miR-30a was significantly down-regulated in GCT samples. Then in situ hybridization was used to further verify the expression of miR-30a in the GCT tissues and para-tumor normal bone tissues. As the figure showed that the miR-30a was not only decreased in GCTSCs but also expressed limited in monocytes and giant cells (Fig. 2C and D). We demonstrate the reductive expression of miR-30a in GCT compared with para-tumor normal bone tissues.

To prove that miR-30a can directly target to RunX2, a dual-luciferase reporter system was practiced. We constructed a wild type (WT) RunX2 3′-UTR and two mutant sequences of RunX2 3′-UTR in possible binding sits. Luciferase activity was strictly decreased in WT-RunX2 3′-UTR with ago-miR-30a. Although the 3′-UTR analysis revealed two potential target sites for miR-30a, site 2-mutant was sufficient to account for the suppression of reporter expression by miR-30a (Fig. 3B). Except for the luciferase activity analysis, mRNA and protein of RunX2 were also determined in mimic and antago conditions. The result showed that in GCTSC

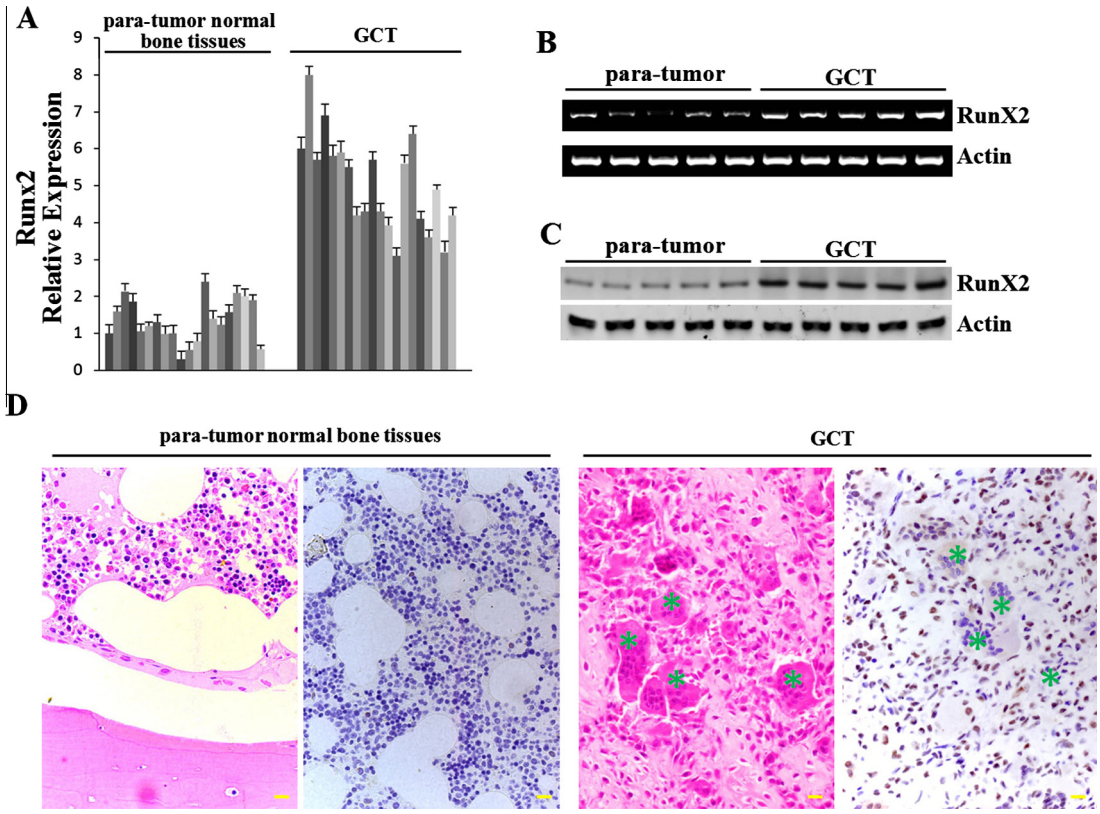

Fig. 1. (A and B) qRT-PCR and semi-qRT-PCR analysis of RunX2in GCT and para-tumor normal bone tissues. (C) Western Blot analysis of RunX2 in GCT and para-tumor normal bone tissues. (D) H&E staining and immunolocalization of RunX2in GCT and para-tumor normal bone tissues (Bar = 200 μm).

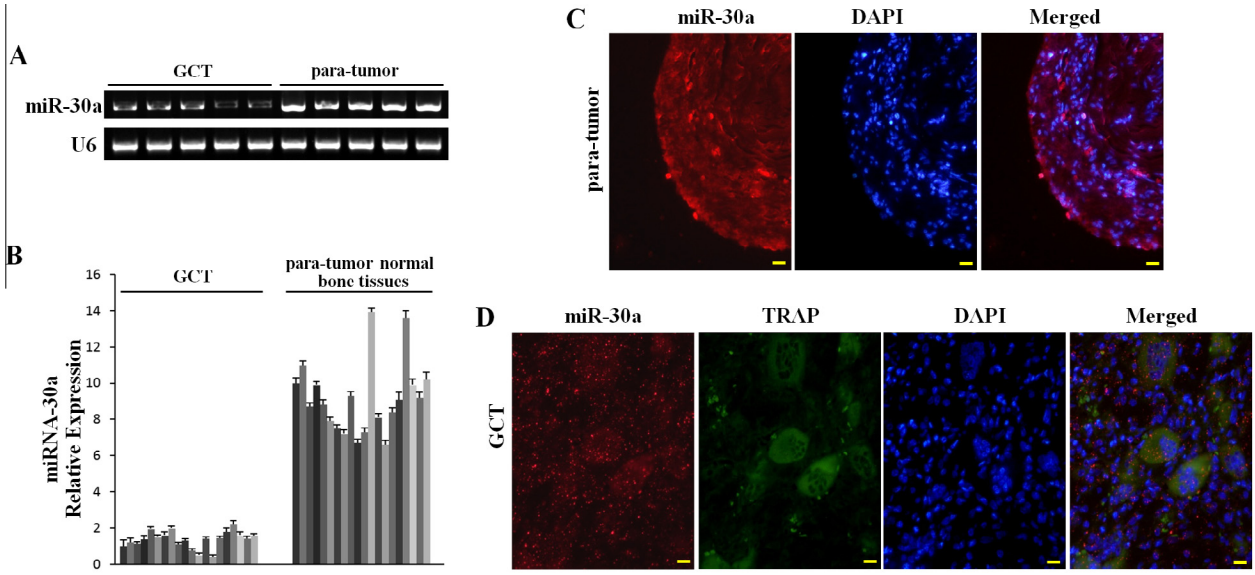

Fig. 2. (A and B) The semi-qRT-PCR and qRT-PCR of miR-30a in GCT and para-tumor normal bone tissues. (C and D) In situ hybridization analysis of miR-30a in GCTSCs and para-tumor normal tissues (Bar = 250 μm).

and MG63 cell line, mRNA level of RunX2 showed no differences while in protein level, RunX2 expression of GCTSCs with miR-30a mimic was down regulated (Fig. 3C and D). Collectively, these results reveal the negative regulatory role of miR-30a to RunX2 in GCTSCs.

3.3. TALENs-based OE-30a/controls cell lines construction

In order to investigate RunX2 function after miR-30a-over-expressing stable cell lines (OE-miR-30a and OE-controls) TALENs targeting PPP1R12C (the AAVS1 locus) and homologous sequences

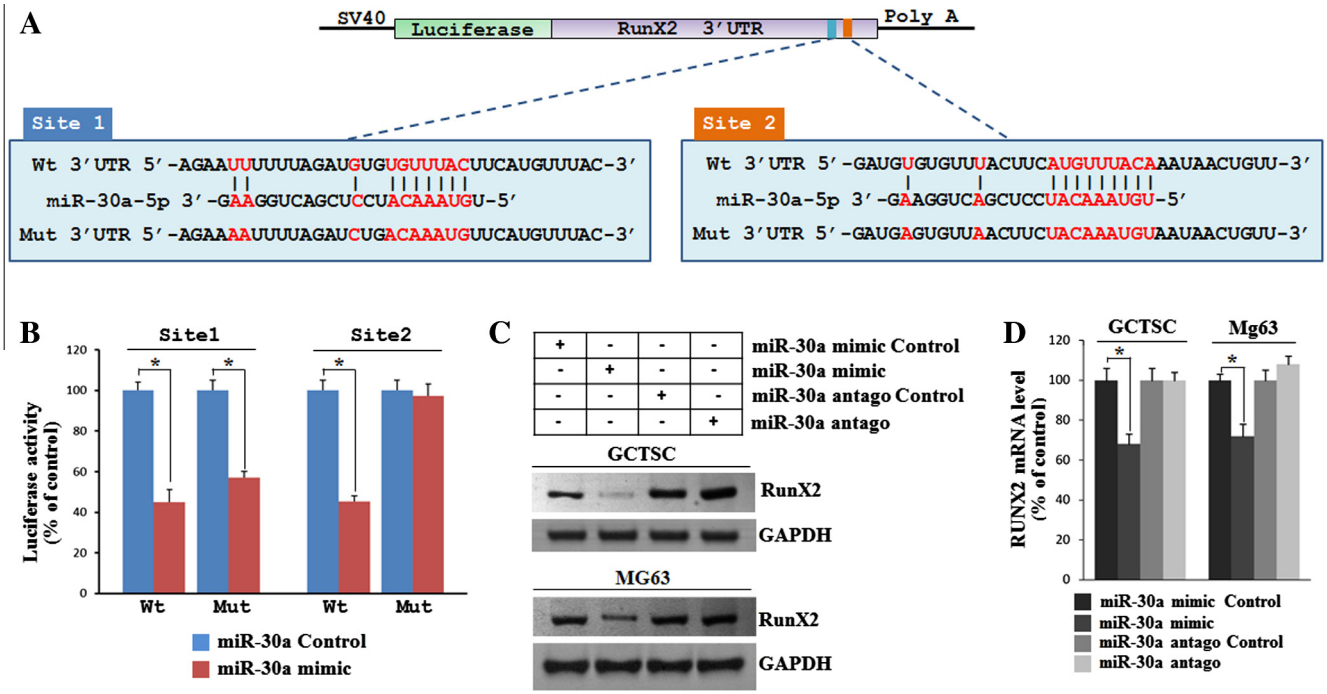

Fig. 3. (A) Schematic diagram of the design of luciferase reporter system with the WT-RunX2 3′-UTR (WT 3′-UTR) or the site-directed mutant RunX2 3′UTR (Site 1-mut 3′-UTR, Site 2-mut2 3′-UTR); (B) the effect of miR-30a control, miR-30a mimic on luciferase activity in GCTSC transfected with either the WT-RunX2 3′UTR reporter or the mutantsRunX2 3′UTR reporter; (C and D) qRT-PCR analysis and Western Blot of RunX2 in GCT and MG63 cells under treatment of miR-30a mimic and miR-30a antago and their corresponding negative controls.

in corresponding donor plasmids were used. TALEN vectors were transfected to GCTSC together with a vector containing EGFP gene and DNA fragments of pri-miR-30a. Successfully combined with pri-miR-30a and EGFP-pri-miR-30a-mutant clones were experimented by genomic PCR and restriction enzyme digestion (Fig. 4A–C). The qRT-PCR assay confirmed the OR-30a cells expressing high level of miR-30a, in which the RunX2 level was decreased, compared with OE-controls (Fig. 4D).

3.4. Over-expression of miR-30a leads to declined expression level of RunX2 and down-stream target genes

Because RunX2 is a transcription factor which regulates osteoblast and osteolysis activity [4,7], to confirm the negative regulatory role of miR-30a, we analyzed the expression level of RunX2 in OE-miR-30a and OE-Ctr cell lines as well as its down-stream target genes including MMP-13. From qRT-PCR and Western Blot results (Fig. 4E–G), we found the endogenous mRNA and protein level was down regulated in OE-miR-30a cell line. And all these findings confirm the inhibitory role of miR-30a to RunX2 and indicating the potential role of miR-30a in regulating osteoclast activities.

3.5. MiR-30a inhibits GCT-induced bone resorption and multinucleated cell formation

RunX2 is considered to regulate osteoblast and osteolysis activities in normal tissues and GCT [17,18]. As we have proved miR-30a to be a direct regulator for RunX2, we investigated the role of miR-30a during the bone resorption in GCT. BMMs were seeded on a dentin slice and cultured in conditional mediums containing M-CSF (10 ng/mL) with OR-miR-30a and OE-control GCTSCs for 7 days as in vitro osteoclast differentiation model. By calculating the number of osteoclasts and area of the pits on the dentin surface, we demonstrate that in OE-30a group, the number of osteoclasts and area of pits on the surface of the dentin slices were markedly decreased (Fig. 4H–J). Taken together, miR-30a was believed to play key roles in osteolysis in GCT by regulating RunX2.

4. Discussion

GCT is defined as an aggressive and osteolytic bone tumor with high recurrence and malignant tendency [7–9]. Though numerous cytokine was produced from GCTSCs, RunX2 as a transcription factor has been regarded as a central role for the osteolysis in GCT [6,19]. Especially its interaction with Smad and TGF-β and participating role in the RANKL signaling have been proposed for its crucial role in the regulation in osteoclast and osteoblast activities [20]. In addition, the down-stream of RunX2 which proved to be MMP-13 indicates its importance in GCT osteolysis formation [7]. Taken together, the understanding of RunX2 and its regulation may offer a new perspective for inhibits the osteoclast activity and prevent bone resorption in GCT.

Many studies have proved the suppressing effects of miR-30a in many tumors and its target genes including PIK3CD, CD99, TGF-β [10–14]. Whether miR-30a can be proved to be a regulatory role in GCT may enlighten the new therapeutic target.

In our study, by analyzing 20 specimens from patients, we found RunX2 was significantly elevated in GCTSCs whereas miR-30a was down-regulated. Furthermore, a direct but negative regulation of miR-30a on RunX2 was confirmed by luciferase reporter analysis. MiR-30a could decrease the RunX2 expression in both mRNA and protein levels, and further reduce the expression level of MMP-13. We also found the expression of miR-30a inhibits osteoclast differentiation and decrease the osteolysis in GCT.

Collectively, our findings confirmed that miR-30a can regulate the expression of RunX2 in GCT and play an important role in bone resorption of GCT. The down-regulated expression of miR-30a in

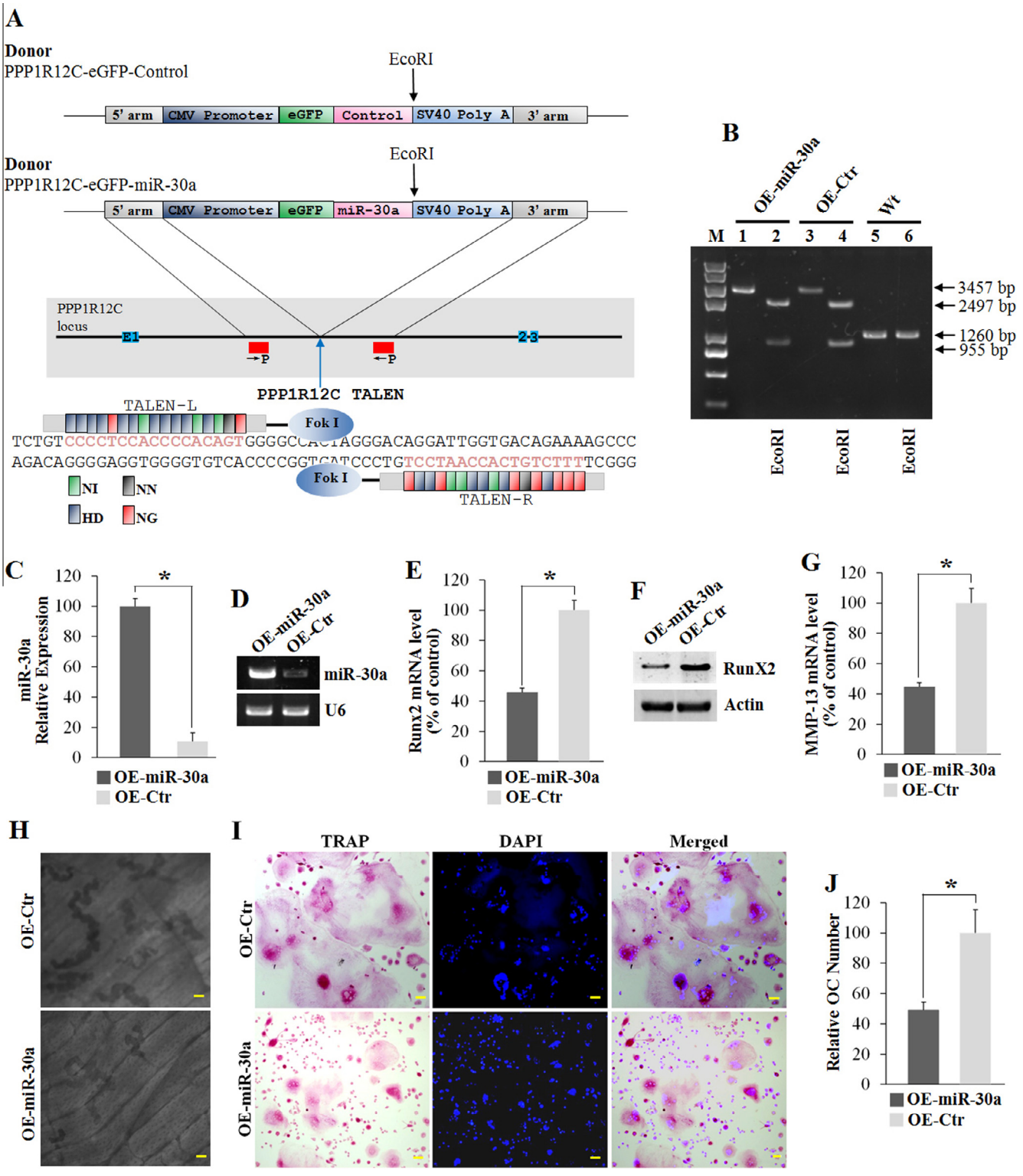

Fig. 4. (A) Schematic overview depicting the targeting strategy for PPP1R12C. Primers are shown as red boxes, exons as blue boxes; the left binding site of TALENs as TALEN-L; the right binding site of TALENs as TALEN-R; the blue arrow indicates cut site by the TALENs. Donor plasmids: CMV Promoter, human cytomegalovirus (CMV) immediate early promoter gene; eGFP, enhanced green fluorescent protein gene; pri-miR-30a, pri-miR-30a gene; control, pri-miR-30a 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); (B) genomic PCR and restriction digestion characterization of OE-miR-30a (Overexpression miR-30a), OE-Ctr (Overexpression miR-30a mutant) and Wt; (C and D) qRT-PCR and semi-qRT-PCR expression of miR-30a in OE-miR-30a and OE-Ctr; (E and F) qRT-PCR and Western Blot of RunX2 in OE-miR-30a and OE-Ctr; (G) qRT-PCR of MMP-13; (H) dentine slices were stained with Mayer's hematoxylin after removal of cells (Bar = 500 μm); (I and J) the numbers of TRAP-positive multinucleated (>3 nuclei) osteoclasts were counted (Bar = 300 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GCT indicates its potential role as tumor suppressor and new therapeutic target for bone resorption, offering more possibilities to regress GCT progression in patients.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.076.

References

[1] R. Gupta, V. Seethalakshmi, N.A. Jambhekar, et al., Clinicopathologic profile of 470 giant cell tumors of bone from a cancer hospital in Western India, Ann. Diagn. Pathol. 12 (4) (2008) 239–248.
[2] Z. Wu, X. Yang, J. Xiao, et al., Aneurysmal bone cyst secondary to giant cell tumor of the mobile spine: a report of 11 cases, Spine (Phila Pa 1976) 36 (21) (2011) E1385–90.
[3] M. Wuelling, C. Engels, N. Jesse, et al., Histogenesis of giant cell tumors, Pathology 23 (5) (2002) 332–339.
[4] W. Zhou, H. Yin, T. Wang, T. Liu, Z. Li, et al., MiR-126-5p regulates osteolysis formation and GCTSC proliferation in giant cell tumor through inhibition of PTHrP, Bone 66 (2014) 267–276 (Sep. 25).
[5] M. Devarapalli, B.J. Lawrence, S.V. Madihally, Modeling nutrient consumptions in large flow-through bioreactors for tissue engineering, Biotechnol. Bioeng. 103 (5) (2009) 1003–1015.
[6] R. Islam, H.S. Bae, W.J. Yoon, K.M. Woo, J.H. Baek, H.H. Kim, T. Uchida, H.M. Ryoo, Pin1 regulates osteoclast fusion through suppression of the master regulator of cell fusion DC-STAMP, J. Cell. Physiol. 229 (12) (2014) 2166–2174.
[7] I.W. Mak, R.W. Cowan, S. Popovic, N. Colterjohn, G. Singh, M. Ghert, Upregulation of MMP-13 via Runx2 in the GCTSC of giant cell tumor of bone, Bone 45 (2) (2009) 377–386.
[8] S. Singh, I.W. Mak, R.W. Cowan, R. Turcotte, G. Singh, M. Ghert, The role of TWIST as a regulator in giant cell tumor of bone, J. Cell. Biochem. 112 (9) (2011) 2287–2295.
[9] M.R. Fabian, N. Sonenberg, The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC, Nat. Struct. Mol. Biol. 19 (6) (2012) 586–593.
[10] J. Fu, X. Xu, L. Kang, L. Zhou, S. Wang, J. Lu, et al., MiR-30a suppresses breast cancer cell proliferation and migration by targeting Eya2, Biochem. Biophys. Res. Commun. 445 (2) (2014) 314–319.
[11] N. Zhang, X. Wang, Q. Huo, M. Sun, C. Cai, et al., MicroRNA-30a suppresses breast tumor growth and metastasis by targeting metadherin, Oncogene 33 (24) (2014) 3119–3128.
[12] Y. Yu, L. Cao, L. Yang, R. Kang, M. Lotze, D. Tang, MicroRNA 30A promotes autophagy in response to cancer therapy, Autophagy 8 (5) (2012) 853–855.
[13] M. Zhong, Z. Bian, Z. Wu, MiR-30a suppresses cell migration and invasion through downregulation of PIK3CD in colorectal carcinoma, Cell. Physiol. Biochem. 31 (2–3) (2013) 209–218.
[14] G.A. Franzetti, K. Laud-Duval, D. Bellanger, M.H. Stern, X. Sastre-Garau, O. Delattre, MiR-30a-5p connects EWS-FLI1 and CD99, two major therapeutic targets in Ewing tumor, Oncogene 32 (33) (2013) 3915–3921.
[15] Q. Zhou, M. Yang, H. Lan, X. Yu, MiR-30a negatively regulates TGF-β1-induced epithelial-mesenchymal transition and peritoneal fibrosis by targeting Snai1, Am. J. Pathol. 183 (3) (2013) 808–819.
[16] X. Wu, Z. Li, Z. Yang, et al., Caffeic acid 3,4-dihydroxy-phenethyl ester suppresses receptor activator of NF-κB ligand-induced osteoclastogenesis and prevents ovariectomy-induced bone loss through inhibition of mitogen-activated protein kinase/activator protein 1 and Ca2+-nuclear factor of activated T-cells cytoplasmic 1 signaling pathways, J. Bone Miner. Res. 27 (6) (2012) 1298–1308.
[17] M.D. Adhami, H. Rashid, H. Chen, A. Javed, Runx2 activity in committed osteoblasts is not essential for embryonic skeletogenesis, Connect. Tissue Res. 55 (2014) 102–106.
[18] D.C. Wang, H.F. Wang, Z.N. Yuan, Runx2 induces bone osteolysis by transcriptional suppression of TSSC1, Biochem. Biophys. Res. Commun. 438 (4) (2013) 635–639.
[19] J.H. Jonason, G. Xiao, M. Zhang, L. Xing, D. Chen, Post-translational regulation of Runx2 in bone and cartilage, J. Dent. Res. 88 (8) (2009) 693–703.
[20] J. Pratap, J.B. Lian, A. Javed, G.L. Barnes, A.J. van Wijnen, J.L. Stein, G.S. Stein, Regulatory roles of Runx2 in metastatic tumor and cancer cell interactions with bone, Cancer Metastasis Rev. 25 (4) (2006) 589–600.