Supplementary Figures

GATA4-driven miR-206-3p signatures control orofacial bone development by regulating osteogenic and osteoclastic activity

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Figure S1. Exosomes derived from OMSCs promote osteoclastic differentiation and function.

(A) Images of OMSCs stained with DAPI to identify nuclei, and immunostained for the mesenchymal stem cell-associated markers (CD73 and SCA1). Scale bar: 200 µm.

(B) The size and the nuclei numbers of TRAP-positive multinucleated cells (TRAP⁺MNCs) in Figure 2A were measured (n = 5).

(C) M-CSF and RANKL treated bone marrow-derived macrophages (BMMs) from Gata4fl/fl (WT) mice and Wnt1-cre;Gata4fl/fl (cKO) mice, and then the cells were stained for TRAP. The size and the nuclei numbers of TRAP⁺MNCs were measured (n = 5). Scale bar: 200 µm.

(D) OMSCs infected with lentivirus was assessed by fluorescence microscopy. Scale bar: 200 µm.

(E) Efficiency of GATA4 knockdown in the protein and mRNA level after infection with lentivirus and quantitative analysis of western blotting and
qRT-PCR analysis (n = 5). (F) The size and the nuclei numbers of TRAP⁺MNCs in Figure 2G were measured (n = 5). (G) RAW264.7 cells were cultured in a medium with exosomes isolated from shCtr or shGATA4 OMSCs (OMExo-Ctr, OMExo-G4). After 24 h, cell viability was measured by CCK-8 assay (n = 5). (H) RAW264.7 cells were cultured in osteoclastogenic medium in the presence of OMExo-Ctr or OMExo-G4. Cell mobility was measured by wound healing assay. Scale bar: 200µm. Two-tailed Student’s t test. Each experiment was repeated at least three times with the same conditions. Data are shown as mean ± SD. **P < 0.01; ns, not significant.
Figure S2. GATA4-miR-206-3p-Bmp3 signaling in regulating osteogenic differentiation of OMSCs.

(A) The ALP activity and the number of mineral nodules of OMSCs from Gata4<sup>lo/lo</sup> (WT) mice or Wnt1-cre;Gata4<sup>lo/lo</sup> (cKO) mice were accessed by ALP and ARS assay (n = 5). Two-tailed Student’s t test. (B) Indicated osteogenic gene expression analysis of shGATA4-transfected OMSCs as compared with shCtr OMSCs after 5 days following mineralization induction (n = 5). Two-tailed Student’s t test. (C) Protein expression levels of several osteogenic markers in shGATA4-transfected OMSCs were detected by western blotting as compared with shCtr OMSCs after 5 days following mineralization induction (n = 5). Two-tailed Student’s t test. (D) qRT-PCR analysis showed the knockdown and over expression efficiency of miR-206-3p in OMSC-derived osteoblasts (n = 5). Ordinary one-way ANOVA. (E) The ALP activity
and semi-quantitative estimation of calcium from Figure 4A were measured (n = 5). Ordinary one-way ANOVA. (F) OMSCs were co-transfected with shGATA4 and miR-206-3p mimic, the mRNA levels of *Opn*, *Ocn*, *Runx2*, *Alp* and *Osx* in the indicated cells were detected using qRT-PCR analysis (n = 5), and (G) ALP and ARS staining to detect osteogenic induction (n = 5). Ordinary one-way ANOVA. (H) miR-206-3p control inhibitor /inhibitor/ control mimic/ mimic transfected OMSCs were subjected to qRT-PCR analysis to detect the mRNA levels of Bmp3 (n = 5). Ordinary one-way ANOVA. (I) Protein and mRNA levels of Bmp3 from OMSCs of WT and cKO mice were examined by using western blotting and qRT-PCR analysis (n = 5). Two-tailed Student’s t test. (J) OMSCs were co-transfected with miR-206-3p inhibitor and siBmp3, the mRNA levels of *Bmp3*, *Opn*, *Ocn*, *Runx2*, *Alp* and *Osx* in the indicated cells were detected using qRT-PCR analysis (n = 5). Ordinary one-way ANOVA. control inhibitor, Ctr-inhi; inhibitor, inhi; control mimic, Ctr-mimic. Each experiment was repeated at least three times with the same conditions. Data are shown as mean ± SD. *P < 0.05, **P < 0.01.
Figure S3. Exosomal miR-206-3p from OMSCs regulates osteoclast activity by targeting NFATc1.

(A) The exosomes from OMSCs were treated with miR-206-3p inhibitor or mimic, respectively, and then co-cultured with RANKL-treated RAW264.7 cells. The knockdown and over expression efficiency of miR-206-3p in osteoclasts was examined by qRT-PCR (n = 5). Ordinary one-way ANOVA. (B) The size and the nuclei numbers of TRAP-positive multinucleated cells (TRAP+MNCs) in Figure 5A were measured (n = 5). Ordinary one-way ANOVA. (C) Protein and mRNA levels of NFATc1 of bone marrow-derived macrophages (BMMs) from Gata4fl/fl (WT) mice or
*Wnt1-cre;Gata4*0/β* (cKO) mice were examined by using western blotting and qRT-PCR analysis (n = 5). Two-tailed Student’s t test. (D) RANKL-treated RAW264.7 cells were co-transfected with miR-206-3p inhibitor and siNFATc1, mRNA levels of osteoclast related genes including NFATc1, Car2, Mmp9, Ctsk and TRAP were determined by using qRT-PCR assay. Ordinary one-way ANOVA. Each experiment was repeated at least three times with the same conditions. control inhibitor, Ctr-inhi; inhibitor, inhi; control mimic, Ctr-mimic. Data are shown as mean ± SD. *P < 0.05, **P < 0.01.
| Gene   | Primer   | Sequence (5’-3’)                  |
|--------|----------|----------------------------------|
| NFATc1 | Forward  | CGAGTTCACATCCACACAG              |
|        | Reverse  | GACAGCCACATCTTTCTTC              |
| Car2   | Forward  | ATCCCTTGCTCCCTTCTTC              |
|        | Reverse  | ATCCAGGTCACACATTCC               |
| Mmp9   | Forward  | TCACTTTCCTTCACCTTC               |
|        | Reverse  | ATTTGCGCTCTTTATCGT               |
| Ctsk   | Forward  | CCCATCTCTGTGTCCCATC              |
|        | Reverse  | AGTGCTTGCTTTCCCTTCT              |
| TRAP   | Forward  | CAGCAGCCAAGGAGGACTAC             |
|        | Reverse  | ACATAGCCCAACACGTTCTC             |
| Opn    | Forward  | ACCATGCAGAGAGGCCAGATT            |
|        | Reverse  | GGGACATCGACTGTTAGGGACG           |
| Ocn    | Forward  | ACTCTTGCTCTCGTCCACT              |
|        | Reverse  | GGTCTCTTCACTACCTCGCT             |
| Runx2  | Forward  | AGTTCCCAAGGCTTTCATC              |
|        | Reverse  | GGCAGGTAGGTGGTAGT                |
| Alp    | Forward  | CAGTGCGGTTCCAGACATAG             |
|        | Reverse  | GAACAGAAACTGTGGTGAATAG           |
| Osx    | Forward  | CTACCCATCTGACTTTGCTC             |
|        | Reverse  | CACTATTCCCACCTGCTT              |
| Rankl  | Forward  | AACAGGCCCTTTCAA GGAGCTGTGC      |
|        | Reverse  | AAGAGGACAGACTCACTTTATGGGG       |
| Bmp3   | Forward  | AACGATGCTGCCATTTTCT              |
|        | Reverse  | CTTCCTCCTCTCAACCGA              |
| GAPDH  | Forward  | GAAGGTGAAGGTCGGAGTC              |
|        | Reverse  | GAGATGGTGATGGGATTTC             |
Table S2. Differential genes

| Upregulated miRNAs | Downregulated miRNAs |
|--------------------|-----------------------|
| miR-6908-5p        | miR-409-3p            |
| miR-711            | miR-6970-5p           |
| miR-6366           | mir-5126              |
| miR-2137           | miR-6987-5p           |
| miR-6349           | miR-7081-5p           |
| miR-7003-5p        | miR-221-3p            |
| miR-99b-5p         | miR-652-3p            |
| miR-8101           | miR-1981-5p           |
| miR-145a-5p        | miR-8119              |
| miR-674-5p         | miR-714               |
| miR-3102-5p,2-5p   | miR-7653-5p           |
| miR-7654-3p        | miR-5100              |
| miR-7046-5p        | miR-7684-5p           |
| miR-671-5p         | miR-466f-5p           |
| miR-425-5p         | miR-16-1-3p           |
| miR-5112           | miR-1195              |
| miR-30c-5p         | miR-297a-5p           |
| miR-3072-5p        | miR-466h-5p           |
| miR-7671-3p        | miR-5129-3p           |
| miR-3067-3p        | miR-669a-5p           |
| miR-34b-3p         | miR-669p-5p           |
| miR-574-3p         | miR-669c-5p           |
| miR-1249-5p        | miR-574-5p            |
| miR-151-3p         | miR-3082-5p           |
| miR-34c-3p         | miR-669l-5p           |
| miR-100-5p         | mir-1194              |
| miR-28a-3p         |                        |
| miR-7687-5p        |                        |
| miR-700-3p         |                        |
| miR-6239           |                        |
| miR-193b-3p        |                        |
| miR-3473f          |                        |
| miR-3104-5p        |                        |
| miR-211-3p         |                        |
| miR-423-5p         |                        |
| miR-7221-3p        |                        |
| miR-329-3p         |                        |
| miR-6368           |                        |
| miR-504-3p         |                        |
| miR-222-3p         |                        |
| miR-6910-5p        |                        |