Megakaryocytes promote bone formation through coupling osteogenesis with angiogenesis by secreting TGF-β1

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Supplemental information

Materials and Methods

Mice

CAG-LoxP-ZsGreen-Stop-LoxP-tdTomato (Rosa26-mT/mG) mice were purchased from Nanjing biomedical research institute of Nanjing University (Nanjing, China). Pf4-cre⁺; Rosa26-mT/mG mice were generated by crossing Rosa26-mT/mG mice with Pf4-cre⁺ mice. Littermate Pf4-cre⁻; Rosa26-mT/mG mice were served as negative controls.

DT injection and irradiation.

Adult Pf4-cre⁺; Rosa26-mT/mG mice were injected with DT (at the dose of 50 ng/g body weight) every two days. Two weeks after first injection, these mice were used for subsequent analysis. In addition, adult Pf4-cre⁺; Rosa26-mT/mG mice were subjected to 6.5 Gy and 3.5 Gy irradiation at day 1 and day 14, respectively. Four weeks after first irradiation, these mice were used for subsequent analysis.

Preparation of macrophages, fibroblasts and OBs.

BM macrophages were labeled with anti-mouse CD11b (M1/70; Biolegend) and F4/80 (BM8; Biolegend) antibodies.
BM-specific fibroblasts were isolated as described [1]. Briefly, BM cells were flushed from femur and tibia of mice. Bone marrow Mononuclear (BMM) cells were then isolated using Lympholyte-M (Cedarlane, Hornby, Canada) gradient centrifugation, and resuspended in RPMI-1640 (Hyclone, Logan, Utah, USA) medium supplemented with penicillin/streptomycin, glutamine, minimum essential medium and sodium pyruvate. Subsequently, BM stromal cells were obtained after the adhesion of BMM cells to polystyrene flasks and cultured in DMEM medium (Hyclone) containing 10% fetal bovine serum (FBS; Hyclone). Fibroblasts were purified from BM stromal cells by MACS using anti-fibroblast marker (sc-73355, Santa cruz, Rat IgG) in combination with anti-Rat IgG MicroBeads (Miltenyi Biotec), followed by flow cytometric analysis.

BM-derived mature OBs were isolated as described [2]. BM cells were flushed from the femur and tibia of mice. Cells were centrifuged at 300g for 10 min and re-suspended in 200 μL of ice-cold buffer (Dulbecco’s phosphate buffered saline without Ca\(^{2+}\) and Mg\(^{2+}\), with 0.5% bovine serum albumin and 2 mM EDTA). The mature OBs (ALP\(^+\) cells) were purified by MACS using anti-ALP antibody (ab108337, Rabbit IgG, Abcam) in combination with anti-Rabbit IgG MicroBeads (Miltenyi Biotec), followed by flow cytometric analysis.

Reference

[1] Frassanito MA, Rao L, Moschetta M, Ria R, Di Marzo L, De Luisi A, et al. Bone marrow fibroblasts parallel multiple myeloma progression in patients and mice: in vitro and in vivo studies. Leukemia. 2014; 28: 904-16.

[2] Li D, Liu J, Guo B, Liang C, Dang L, Lu C, et al. Osteoclast-derived exosomal miR-214-3p inhibits osteoblastic bone formation. Nat Commun. 2016; 7: 10872.
Figure S1

Pf4-Cre is ectopic recombined at a low-level in macrophages, fibroblasts and osteoblasts under normal conditions and in the context of DT injection or irradiation. (A-C) Flow cytometric analysis of...
the percentage of Tomato-expressing cells in (A) macrophages (CD11b⁺, F4-80⁺), (B) fibroblasts and (C) osteoblasts obtained from the BM of adult Pf4-cre⁺; Rosa26-mT/mG mice after DT injection or irradiation (Vehicle 1 vs DT, vehicle 2 vs irradiation). Pf4-cre⁻; Rosa26-mT/mG littermates were served as negative controls. Data are representative of three independent experiments. (D) Scheme for DT administration to Pf4-cre⁺;iDTR and Pf4-cre⁻;iDTR mice.
**Figure S2**

MKs promote OBs proliferation, but have no significant effect on ECs proliferation in vitro. (A) The purity of megakaryocytes on 9th day after culture was about 90.6%, which was determined by flow cytometry according to the expressions of CD41 and CD42b. (B) Proliferation of OBs in indirect culture with or without MKs-CM for 5 days (n=6 per group). (C) Proliferation of ECs in indirect culture with or without MKs-CM for 5 days (n=6 per group). Data are shown as mean ± SD. **P < 0.01. ns, no significant.

For all panels in this figure, data are representative of three independent experiments.
TGF-β1 secreted by MKs promotes the proliferation and differentiation of osteoblasts in vitro and bone formation in vivo. (A) The concentrations of TGF-β1, VEGF, BMP6, IGF-1, PDGF-BB, CXCl12, BMP2, TGF-β2, TGF-β3 and BMP4 in MKs-CM determined by ELISA (n=6 per group). (B) Differentiation of OBs in culture in the presence of MKs-CM and indicated individual neutralizing antibody (Ab) or IgG. Quantification of the activity of alkaline phosphatase (left) and (right) on day 7 (n=6 per group). (C) The concentration of TGF-β in the BM of TGF-β1MKΔ/Δ and TGF-β1fl/fl mice, determined by ELISA (n=6 mice per group). (D) Proliferation of OBs in culture with MKs-CM from TGF-β1MKΔ/Δ and
TGF-β1^fl/fl^ mice for 5 days (n=6 per group). (E) Relative mRNA level of osteorix and type I collagen during differentiation of OBs treated without or with MKs-CM from TGF-β1^MKΔ/Δ^ and TGF-β1^fl/fl^ mice for 4 days and 14 days (n=6 per group). (F) Quantitative Micro-CT analysis of BMD, BV/TV, Tb.N, Tb.Th, Tb.Sp and Ct.Th of femur from TGF-β1^MKΔ/Δ^ and TGF-β1^fl/fl^ mice (n=6 mice per group). (G) Quantitative biomechanical analysis of femur (Load of peak and stiffness) from TGF-β1^MKΔ/Δ^ and TGF-β1^fl/fl^ mice (n=6 mice per group). (H) Bone marrow osteocalcin concentrations by ELISA from TGF-β1^MKΔ/Δ^ and TGF-β1^fl/fl^ mice (n=6 mice per group). (I) The quantification of osteocalcin^+^ cells on the surfaces of TB and EB from TGF-β1^MKΔ/Δ^ and TGF-β1^fl/fl^ mice (n=6 mice per group). Data are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. ns, no significant. (Student’s t-test). For all panels in this figure, data are representative of three independent experiments.
Figure S4
TGF-β1 secreted by MKs alleviates radioactive osteoporosis in mice by promoting bone formation. (A) Quantitative Micro-CT analysis of the trabecular bone fraction (Tb.N, Tb.Th and Tb.Sp) of femur from sham or irradiated mice 2 months after treated with MKs or TPO (n=6 mice per group). (B) The values of bone histomorphometry parameters (MAR, BFR) at the distal femur metaphysis from sham or irradiated mice 2 months after treated with MKs or TPO (n=6 mice per group). (C) Quantitative biomechanical analysis of femur (Load of peak and stiffness) from sham or irradiated mice 2 months after treated with MKs or TPO (n=6 mice per group). (D) Representative images of immunostaining of type I collagen on distal femur metaphysis from sham or irradiated mice 2 months after treated with MKs or TPO (n=6 mice per group). Scale bar, 100 µm. (E) Representative images of immunostaining of masson staining on distal femur metaphysis from sham or irradiated mice 2 months after treated with MKs or TPO (n=6 mice per group). Scale bar, 100 µm. (F) Representative images of Emen (red) and Ki67 (green) immunostaining of proliferating endothelial cells from sham or irradiated mice 2 months after treated with MKs or TPO (n=6 mice per group). Scale bar, 100 µm. (G) The quantification of CD31 hi Emen hi cells in the BM of sham or irradiated mice 2 months after treated with MKs or TPO (n=6 mice per group). (H) Quantitative Micro-CT analysis of BMD, BV/TV, Tb.N, Tb.Th, Tb.Sp and Ct.Th of femur from TGF-β1MK∆/∆ mice with or without radioactive bone injury 2 months after treated TPO (n=6 mice per group). (I) VEGF concentrations in bone marrow of TGF-β1MK∆/∆ mice with or without radioactive bone injury 2 months after treated with TPO (n=6 mice per group). (J) Angiography-based quantification of vessel volume and surface area from TGF-β1MK∆/∆ mice with or without radioactive bone injury 2 months after treated with TPO (n=6 mice per group). (K) Quantification of CD31 hi Emen hi immunostaining of femur from TGF-β1MK∆/∆ mice with or without radioactive bone injury 2 months after treated with TPO (n=6 mice per group). Data are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. ns, no significant. (Student’s t-test). For all panels in this figure, data are representative of three independent experiments.
Figure S5

MKs can repair DNA damage and reduce apoptosis of OBs by secreting TGF-β1. (A) Representative images of cleaved-caspase-3 immunostaining of calvariae from sham or irradiation 24 hours after treated with MKs or MKs+TGF-β inhibitor (n=6 per group). Scale bar, 100 µm. Inh, inhibitor. (B) Flow cytometric analysis of the apoptosis of OBs in control, MKs-CM plus vehicle, MKs-CM plus TGF-β inhibitor, MKs-CM (from TGF-β1fl/fl mice) and MKs-CM (from TGF-β1MK∆/∆ mice) groups 24 hours after 12 Gy irradiation (n=6 per group). (C) Calvariae harvested from neonatal mouse pups were irradiated and treated with MKs in growth medium with or without TGF-β inhibitor. Calvariae were harvested and followed by γ-H2AX staining after 12 hours. The percentage of apoptotic OBs in calvariae was quantified (n=6 mice per group). Scale bar, 100 µm. Dashed lines outline bone surface. Inh, inhibitor. Data are shown as mean ± SD. **P < 0.01, ***P < 0.001. ns, no significant. (Student’s t-test). For all panels in this figure, data are representative of three independent experiments.
Table S1

Primer sequences

| Gene        | Forward Primer                      | Reverse Primer                      |
|-------------|-------------------------------------|-------------------------------------|
| Osterix     | GGAAAGGAGGCCACAAAGAAGC              | CCCCTTAGGCACTAGGAGC                 |
| Type I collagen | GCTCCTTTAGGGGCACCT                | ATGGGGACCCCTTACGAGGCA               |
| Xrcc2       | ATGTGTAGGCAGACTTTGCAGA             | CATCAGCAAAACAGTTGGTTT               |
| Rapa1       | CAGTTCGCCAGTGAGACTGAAG             | GCTGGTCATAGAAGGGAGTAGAC             |
| Xrcc3       | CGAATTACTGCTGGTTAAGGA              | CCCGAAGGTGTAGAGAGGCA                |
| Rad51       | CGGGAGGTGGTGTTATCC                 | CCGGCACATCTTGGTTATTTGT              |
| Brca1       | CTCTTGGTGAAGATTTCGTTG              | GAGTGGCACAAGAGTTGGGAA               |
| Xrcc1       | AGCCAGGACTCGACCCATT                | CAAAGGCGAGCCATCTATT                |
| Rad54       | CGGGTGCTACGAGTCTTCC                | GATGGATTGCAAAGGACCAT                |
| Rpa2        | GAGTCCGAGCCACGATATT                | CCTGTGAAATCTCGACATCTCCA             |
| Xrcc5       | ATGGCGTGTCGGTTAATAAG               | CCGTGTCCTGGAGAAACAGTGTC             |
| Xrcc6       | ATGTCAAGTGGAGTCTTAC                | TCGCTGTATTAGTCTTACTGT               |
| 53bp1       | GGGAAGCAGATGAGCCCTA                | GGAAGGTGTCAGATAGACAG               |
| Prkdc       | AAACCTGTTCAGAGCTTTCG               | TAAAGGCCAACATCTGCT                 |
| Lig4        | ATGGCCTTCTCACAACACTTCAC            | TTTTCGTGCAGTCTTACCTTT              |
| Nheg1       | TGCCATGGTTACAACCTTGC              | AACCGTGCTTGGTGATAGACA               |
| GAPDH       | CCTCGTCCCGTAGACAAAATG              | TCTCCACTTTGCGACTGCAA               |