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Supplemental Information

Coactivation of Endogenous *Wnt10b* and *Foxc2* by CRISPR Activation Enhances BMSC Osteogenesis and Promotes Calvarial Bone Regeneration

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Supporting Info

Supplemental methods

Isolation and culture of BMSCs

To isolate BMSCs, bone marrow of 4-week-old Sprague Dawley (SD) rats (Lesco Biotech, Taiwan) was flushed out of bilateral rat tibias and femora using α-MEM medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, 100 U/mL streptomycin and 4 ng/mL bFGF (Lo, et al., 2017). All cells were cultured for two days and non-adherent cells were discarded. The adherent cells were passaged 3-5 times and the resultant BMSCs were used for subsequent experiments.

Alizarin Red staining and calcium deposition

Rat BMSCs were cultured with osteoinduction medium for 14 days and stained by Alizarin Red S (A5533, Sigma-Aldrich), which chelates with calcium to form an Alizarin red-calcium complex (red color). The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min. After fixation, the cells were washed 3 times with deionized water and then stained with 40 mM Alizarin Red S (pH 4.2) for 10 min, followed by 3 PBS washes and microscopic observation.

To quantify the Alizarin red-calcium complex, the Alizarin Red S stain was extracted using 10% acetic acid, by 30 min incubation at room temperature with shaking. After incubation, the sample was heated to 85℃ for 10 min and placed on ice for 5 min. After extraction, the sample was neutralized with 10% ammonium hydroxide and assayed using a spectrophotometer to measure optical density at 405 nm (OD_{405}).

Alternatively, the cells were washed 3 times with PBS, followed by incubation with 0.6 N HCl overnight. The supernatant was collected the next day for calcium phosphate deposition quantification using the CALCIUM liquicolor Complete Test kit (HUMAN Inc.). The calcium deposition is expressed as mg/dl.

Oil Red O staining and quantification

Rat BMSCs were cultured in adipoinduction medium for 14 days and stained by Oil Red O. After removing the medium, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min. After fixation, the cells were washed 2 times with deionized water and then incubated with isopropanol (60 %) at room temperature for 5 min. After incubation and isopropanol removal, the cells were stained with Oil Red O solution (dissolved in 60 % isopropanol, 1.8 mg/ml) for 10 min, followed by 5 washes with deionized water, and microscopic observation. For quantification, the cells were washed 3 times with 60% isopropanol for 5 min with gentle shaking, followed by wash with 100% isopropanol for 5 min to extract Oil Red O stain. The optical density at 500 nm (OD_{500}) of the samples was assayed using a spectrophotometer.

Histological and immunohistochemical staining

After μCT scanning, calvarial bone specimens were removed from the rats and immersed in Osteosoft® (Merck) for 15-20 days for complete decalcification, and dehydrated in a series of graded concentration of ethanol from 70% to 100%. After embedding in paraffin and coronal sectioning (thickness=10 μm), the sections were stained with hematoxylin and eosin (H&E). Alternatively, rehydrated sections were subjected to trypsin treatment for 1 h at 37 ℃ for antigen retrieval, followed by blocking in 5% skim milk and immunohistochemical staining. The primary antibodies were rabbit anti-OPN (1:200, Abcam) and mouse anti-BSP (1:200, Abcam). The secondary antibodies were goat anti-rabbit IgG-HRP (1:5000, GeneTex) and goat anti-mouse IgG-HRP (1:5000, Invitrogen). All of the samples were developed with 3,3'-Diaminobenzidine (DAB, Sigma) and counterstained with Gill's Hematoxylin (Dako). The histochemical staining of tartrate-resistant acid phosphatase (TRAP) was performed using leukocyte acid phosphatase kit (Sigma).

Supplemental Tables

Table. S1. Spacer (targeting) sequences for Wnt10b and Foxc2*.

Target gene	Distance to TSS	Spacer sequence (5'→3')
Wnt10b	-74	GGGGAGCGACTGCGTTCTCC
Wnt10b	-95	GTGTGTGTGCACGCTTTGGA
Wnt10b	-394	GCACTTTCGGTGGACAAACG
Foxc2	-97	GATGATTGGTGCAAACTTC
Foxc2	-162	GTCTTAGAGCCGACGGATTC
Foxc2	-219	GGTTAACTTGAGCTGGGGTT

*The 20-nt spacer sequences were designed using a guide RNA design tool CRISPR-ERA (crispr-era.stanford.edu/). The sequences with the highest efficacy and specificity scores were chosen for construction of sgRNA. TSS, transcription start site.

Table. S2. Primer sequences used for qRT-PCR

Gene	Forward	Reverse
Wnt10b	CCTGCACCTGAACCGCTGGA	CTAAGGGCGGAGGCCGAGAC
Foxc2	CGCTCTTACGCGCCCTACCA	TCTTCTCCGGCGCGTTCTGG
Runx2	GCTTCTCCAACCCACGAATG	GAACTGATAGGACGCTGACGA
OPN	CTGCCAGCACACAAGCAGAC	TCTGTGGCATCGGGATACTG
OCN	CTCTGTCTCTCTGACCTCACAG	CAGGTCCTAAATAGTGATACCG
OSX	ATGGCGTCCTCTCTGCTTG	TGAAAGGTCAGCGTATGGCTT
PPARγ	CGCATTTTTCAAGGGTGCCA	TGGACACCATACTTGAGCAGA
C/EBP-α	TCACTTGCAGTTCCAGATCG	TTGACCAAGGAGCTCTCAGG
FABP4	GGATGGAAAGTCGACCACCATA	TCACGCCTTTCATGACACATTC

Supplemental Figures

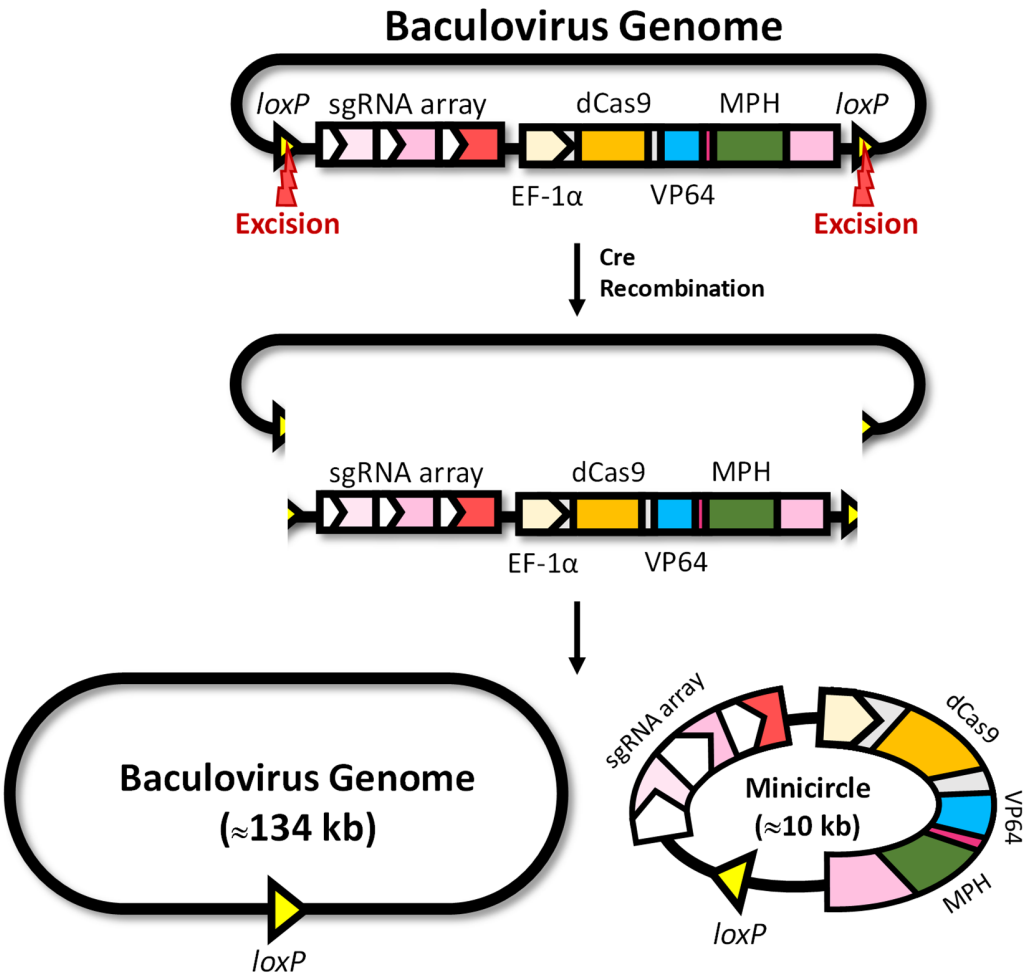

Fig. S1. Schematic illustration of Bac-CRISPRa system. The all-in-one hybrid baculovirus genome carried the entire SAM-based CRISPRa system which was flanked by two loxP sequences. Co-transduction of cells with Bac-Cre and Bac-CRISPRa led to Cre expression. Cre recognized the two loxP sequences and excised the CRISPRa system off baculovirus genome, leading to recirculation of the CRISPRa system into a DNA minicircle (≈10 kb). The DNA minicircle was tremendously smaller than the baculovirus genome (≈134 kb), devoid of bacterial sequences and able to persist in the cells for a longer period of time to prolong the expression of the CRISPRa system.

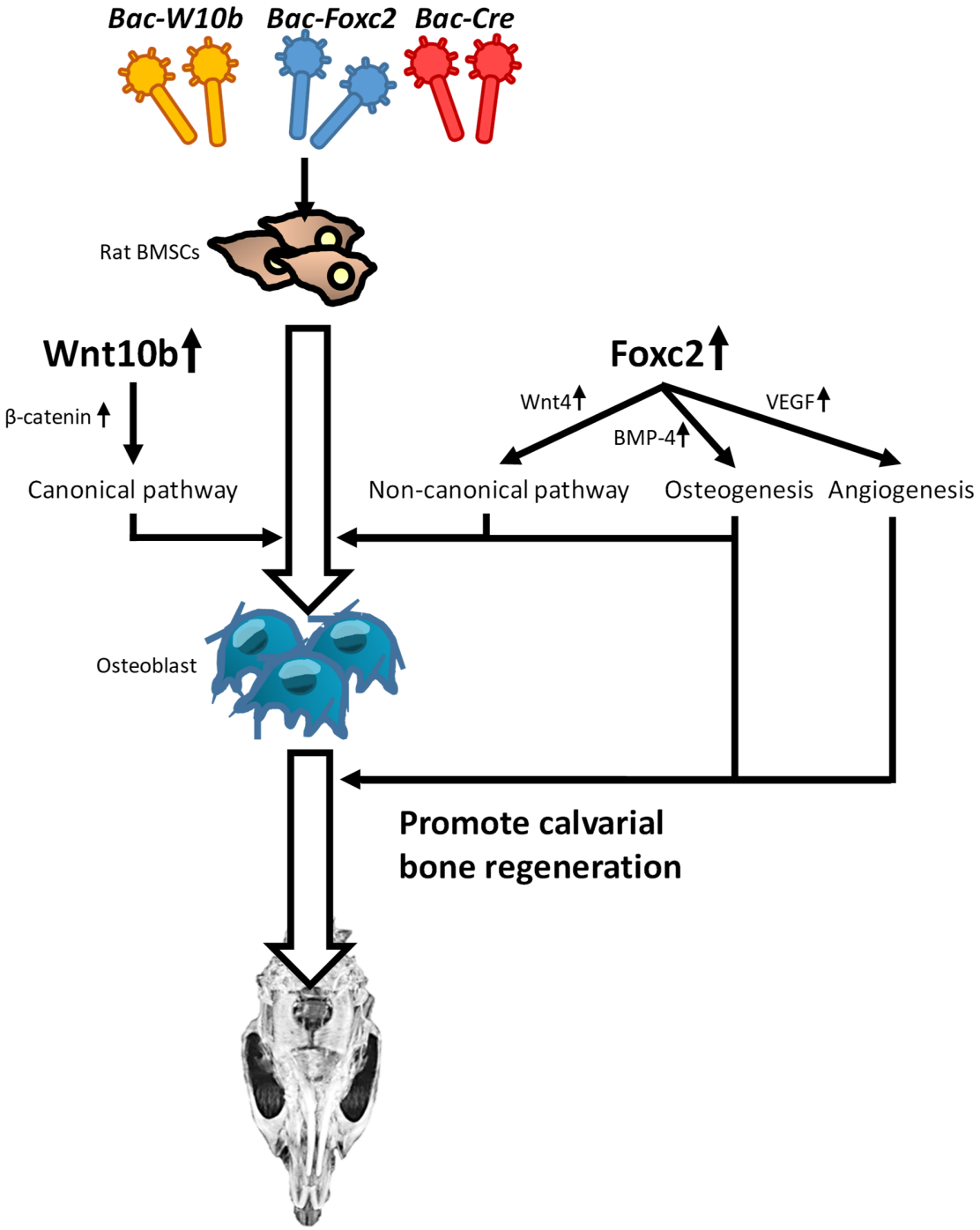

Fig. S2. CRISPRa system promoted osteogenesis through canonical and non-canonical Wnt pathways in rat BMSCs. After rat BMSCS were co-transduced with the hybrid Bac-CRISPRa system, the CRISPRa system activated Wnt10b and Foxc2 expression and promote canonical and non-canonical Wnt pathways to enhance the osteogenic differentiation and inhibited adipogenic differentiation. Foxc2 activation also enhanced BMP-4 and VEGF expression to promote *in vivo* healing.