Supporting Info

CRISPRai for Simultaneous Gene Activation and Inhibition to Promote Stem Cell Chondrogenesis and Calvarial Bone Regeneration

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Running Title: CRISPRai for gene regulation and bone formation

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Supplementary methods

Construction of reporter plasmid

To construct the reporter plasmid pmC-d2E, we PCR-amplified mCherry gene from pJZC78 (Addgene #62339), as well as SV40 poly A (pA) signal and SV40 promoter from pd2EGFP-N1 (Clontech). The three amplicons were fused together by overlap PCR and subcloned into TA vector (RBC Cloning Systems) to yield pTA-mCherry-pA-SV40p. PGK promoter was PCR-amplified from pPGK-Cre-bpA (Addgene #11543) and subcloned into pTA-mCherry-pA-SV40p to yield pTA-PGK-mCherry-pA-SV40p. Finally, d2EGFP-pA fragment was PCR-amplified from pd2EGFP-N1 and subcloned into pTA-PGK-mCherry-pA-SV40p to yield pmC-d2E.

To construct the control psgRNAa and psgRNAi, we performed inverse PCR by which the forward primer binds to the 3′ end of sgRNAa or sgRNAi scaffold while the reverse primer binds to the 5′ end of the hU6 promoter of the psgRNAa or psgRNAi, respectecvily. The resultant PCR amplicons was phosphorylated with T4 Polynucleotide Kinase (NEB) and ligated to yield psgRNAa (ϕ) and psgRNAi (ϕ) that lack the spacer sequence.

Western Blot

Cells cultured in 10-cm dishes were lysed with RIPA buffer (Merck) at 4°C for 30 min, and total protein was resolved on 12% polyacrylamide gel, transferred onto a nitrocellulose membrane and analyzed by Western blot following standard procedures. The primary antibodies were rabbit anti-FABP4 (GeneTex, 1:1000), mouse anti-C/EBPα (Santa Cruz Biotechnology, 1:500) and rabbit anti-GAPDH (Abcam, 1:10000). The secondary antibodies were goat anti-rabbit IgG-HRP (GeneTex) and goat anti-mouse IgG-HRP (Invitrogen). The membranes were visualized by Western Lightning Plus-ECL kit (Perkin Elmer) and a GeneGenome HR Scanner (Syngene). Semi-quantitation of bands were performed using the GeneTools software (Syngene).

Alcian blue staining and Oil Red O staining

Cells were fixed in 4% aqueous phosphate formaldehyde (Macron) at room temperature for 15 min. For GAG visualization, the cells were washed with deionized water and stained in 1% Alcian Blue solution (Sigma) at room temperature for 1 h. The cells were washed again with water and images were captured. For lipid droplet staining, the fixed cells were washed with water and incubated with 60% isopropanol at room temperature for 5 min. After removing isopropanol, the cells were stained in Oil Red O working solution (Sigma) at room temperature for 30 min. The cells were subsequently washed with water and imaged. Alternatively, the Oil Red O stain from the cells was extracted with 100% isopropanol and semi-quantified by reading the optical density at 450 nm (OD_{450}) using Multiskan EX (Thermo Scientific). Pure (100%) isopropanol was used as blank.

Supplementary Figures

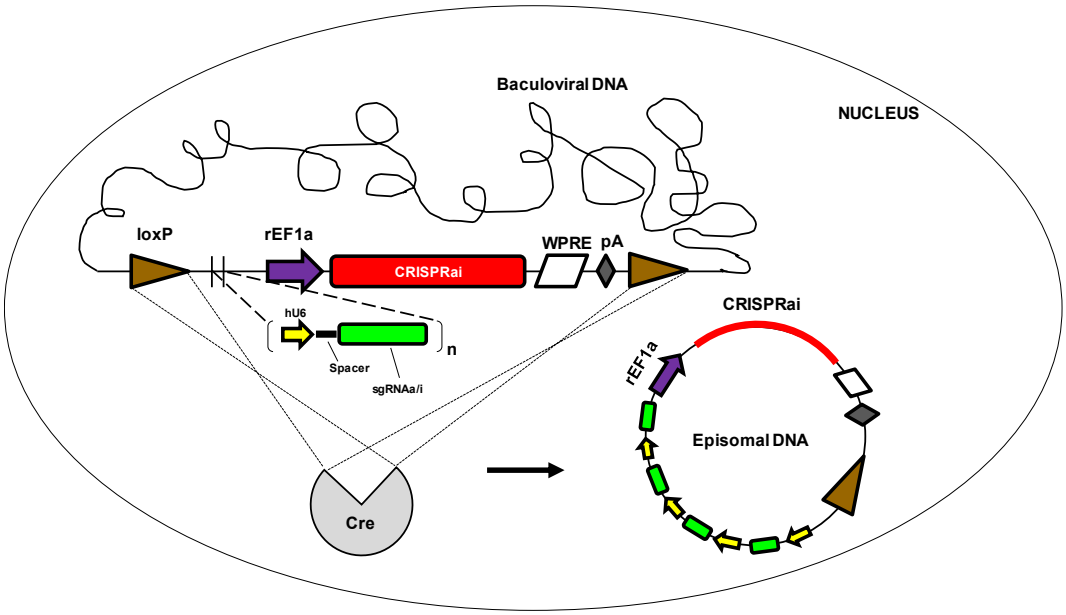

Figure S1. Schematic illustration of Cre/loxP-mediated DNA minicircle formation. Co-transduction of rBMSC with the baculovirus harboring the CRISPRai system (MOI 300) and Bac-Cre (MOI 100) allowed for the formation of the episomal DNA minicircle.

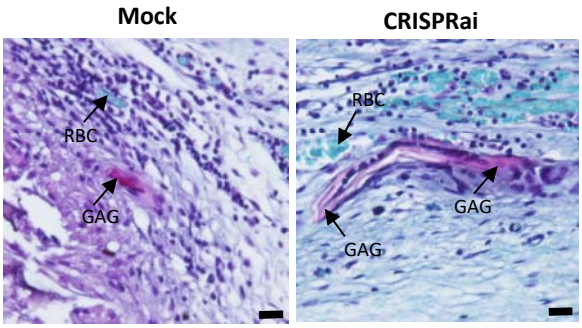

Figure S2. Endochondral ossification mediated by CRISPRai (*n*=6). Calvarial bone specimens harvested at W4 were fixed in 4% aqueous phosphate formaldehyde for 3 days followed by decalcification in Osteosoft® for 14 days. The decalcified bone specimens were subsequently dehydrated, embedded in wax and cut into 10-µm-thick-slides. The slides were rehydrated and stained with Hematoxylin followed by Fast Green solution and finally by Safranin O. The CRISPRai group exhibited the presence of cartilage template (magenta, GAG), suggesting that bone regeneration proceeded via endochondral ossification pathway. In contrast, the Mock group displayed poor formation of cartilage template with much smaller and less abundant island of GAG. Bar, 25 µm.

Supplementary Tables

Target gene	Spacer sequence (5' → 3')
PGK (NT1)	**G**CCCCGCAGCTCGCGTCGTG
PGK (T1)	**G**CTTCCATTGCTCAGCGGTGC
d2EGFP (NT2)	**G**AGGCTTTTGCAAAGATCGAG
d2EGFP (NT3)	GGGTGGTGCCCATCCTGGTC
Sox9	**G**AGAGAGCAAACTTACACACT
Sox9	**G**ATGGTGGGGTGAGGGGACTT
Sox9	GGGTTGGGTGACGAGACTCG
Sox9	**G**ACCTCGGACCAATCACGGCT
PPAR-γ	GCGACCGCCCCGCCTGGCCG
PPAR-γ	GCGGGCAATGTGCGTCCGTG
PPAR-γ	**G**CCATGCTCTGGGTCAACAGG
PPAR-γ	**G**TTACCTTGTGAAGTGCTCAT

Table S1. Spacer sequences used for targeting genes of interest designed from www.benchling.com. For mCherry activation, the PGK promoter sequence was subjected to calculation. For d2EGFP repression, the SV40 promoter and d2EGFP coding sequence were both screened for potential targeting position. For *Sox9* activation, the gene locus and its upstream sequences were retrieved from https://ncbi.nlm.nih.gov (NC_005109.4). Afterward, the window of −400 to −50 relative to *Sox9*'s transcription start site (TSS) was screened for potential targeting position. Similarly, for *PPAR-γ* repression, the gene locus was determined from the database (NC_005103.4). Since the *PPAR-γ* locus encodes for two major adipogenic regulators PPAR-γ1 and PPAR-γ2 with two distinct promoters, the window of −50 to +300 relative to the two TSSs was analyzed for potential targeting position. Bolded G was intentionally added to the 5′ end of the spacers where it was not present to facilitate transcription by U6 promoter.

Gene	Forward	Reverse
Acan	TGGCCTGCCTGACTTTAGTG	CCTGAACCACTGACGCTGAT
Col2a1	GAGACCTGAACTGGGCAGAC	GTTCCTGTCTCCGCCTTGAC
Fabp4	GGATGGAAAGTCGACCACCATA	TCACGCCTTTCATGACACATTC
C/ebpa	TCACTTGCAGTTCCAGATCG	TTGACCAAGGAGCTCTCAGG
Col10a1	TCCCAGGATTCCCTGGATCTAA	GCCCATTGAGGCCCTTAGTTA
Sox9	GTACCCGCACCTGCACAAC	TCCGCCTCCTCCACGAAG
PPAR-γ	CGCATTTTTCAAGGGTGCCA	TGGACACCATACTTGAGCAGAG
gapdh	AGACAGCCGCATCTTCTTGT	TGGACACCATACTTGAGCAGAG

Table S2. Primer sequences used for qRT-PCR