Novel-Unidentified Targets for Driving Osteogenesis in ASCs Using a Genome-Wide CRISPRa Screen

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Bone Healing

- Bone is the 2nd most commonly transplanted tissue [1].
- There are 500,000 bone defect repairs annually in the U.S [2].
- There are 450,000 spinal fusion procedures each year in the U.S. [3].
- 5-10% of bone fractures do not heal properly [4].

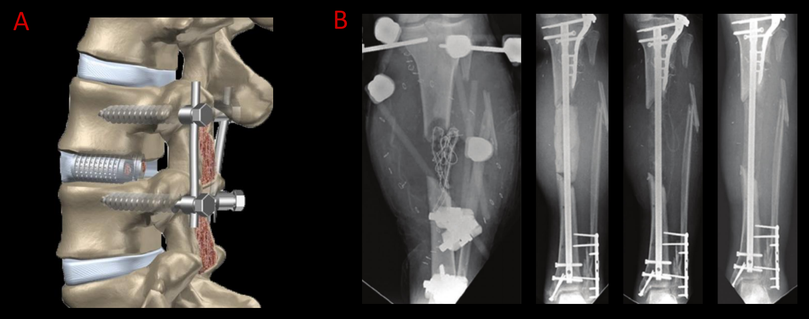

Figure 1: Facilitated bone healing is necessary in many different scenarios, including (A) spinal fusion procedures and (B) critical-size defect repairs.

CRISPR-Guided Gene Modulation and Osteogenesis

- CRISPR-guided systems are capable of robustly modulating gene expression.
- With VPR tethered to deactivated Cas9 (dCas9), it acts like a synthetic transcription factor which greatly increases target gene expression (Figure 2).
- Adipose-derived stem cells (ASCs) are multipotent cells capable of osteogenesis.
- Osteocalcin (OCN) is a late-stage marker of osteogenic differentiation.
- Utilizing an osteogenic reporter cell line based on OCN promoter activity and upregulating every protein-coding gene in the genome individually, we can identify novel gene upregulation targets that promote osteogenesis.

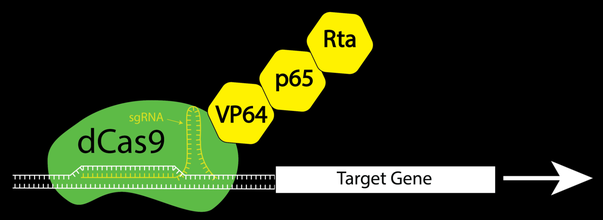

Figure 2: The dCas9-VPR fusion protein forms a complex with gene-specific sgRNAs and then binds tightly to genomic DNA at the designed locus in the promoter of the target gene. The VPR effector molecule then recruits transcription machinery to increase expression of the target gene.

Research Hypothesis

Using a genome-wide CRISPR-activation screen, we can identify novel gene upregulation targets for promoting osteogenesis in ASCs.

Methods

1. ASCs are transduced with lentiviruses containing the dCas9-VPR and OCN-dTomato expression cassettes to create the osteogenic reporter cell line (Figure 3).
2. Antibiotic selection and FACS are used to create a homogenous population of transduced cells (Figure 3B).
3. The reporter cell line is tested by comparing cells grown in proliferative and osteogenic conditions and observing dTomato expression by flow cytometry.
4. A CRISPRa plasmid library containing 5 sgRNAs targeting each protein-coding gene is co-transfected into HEK293T cells with viral packaging plasmids to produce a lentiviral library (Figure 4A,B).
5. Viral titer is determined by transducing ASCs with serial dilutions of the stock virus and observing transduced cells by flow cytometry.
6. Reporter ASCs are transduced with the lentiviral library at an MOI of 0.3 to ensure that each cell receives only a single sgRNA.
7. Cells undergo FACS to select for transduced cells (TagBFP+) and are then cultured for 3 weeks.

A. CRISPRa System:

B. Osteocalcin Reporter:

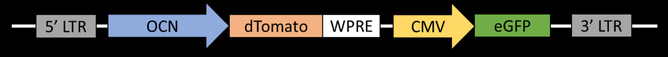

Figure 3: Expression cassettes for (A) the CRISPRa system with the sequences for the dCas9-VPR fusion protein, and (B) the osteocalcin reporter with the dTomato fluorophore driven by the OCN promoter sequence.

Methods (continued)

8. The cells undergo FACS again to select for dTomato+ (top 10%) and dTomato- (bottom 50%) cells.
9. Cells are harvested and gDNA is isolated
10. Protospacers are amplified and Next-gen sequencing (NGS) adapters are attached using PCR.
11. sgRNAs present within the samples are identified and quantified by NGS.
12. sgRNAs enriched in the dTomato+ group as compared to the dTomato- group are selected using the MAGeCK algorithm [5].

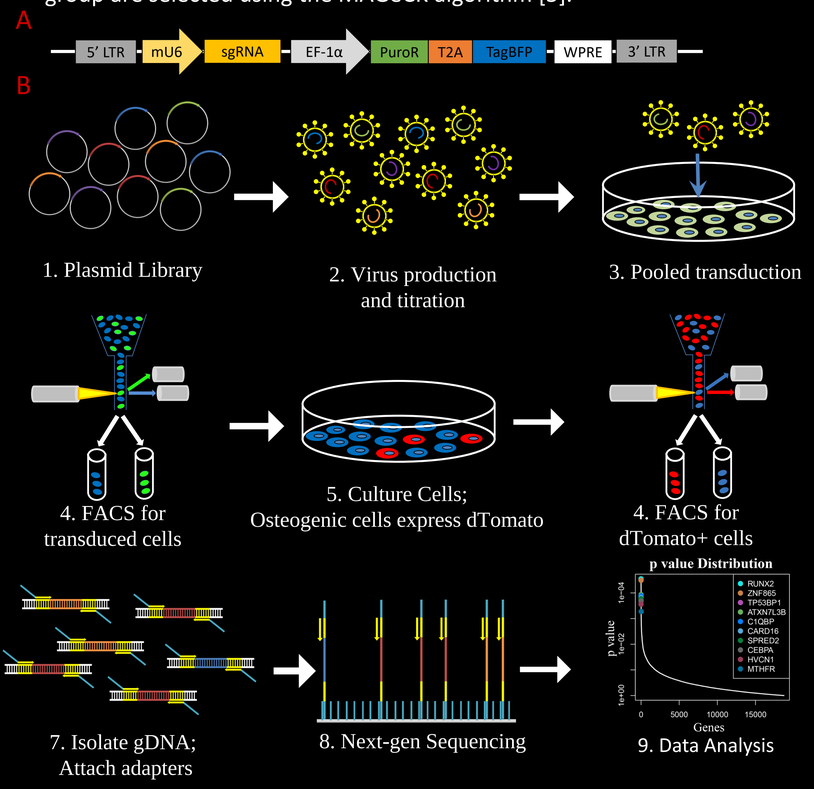

Figure 4: (A) Expression cassette of the CRISPRa guide plasmid library showing constitutive expression of the target-specific sgRNA and TagBFP for FACS. **(B)** Workflow schematic of the CRISPRa screen for identifying upregulation targets that improve cell survival in a low-pH medium.

12. Top-performing sgRNAs from the screen are selected for validation.
13. Selected guides are cloned into new vectors individually (Figure 5).
14. Lentiviruses containing individual sgRNA expression cassettes are produced and ASCs are transduced.
15. Osteogenic cell lines are validated for improved osteogenesis in monolayer by assaying alkaline phosphatase (ALP) activity at 7 days.
16. Cells are further validated in 3D cultures on type-I collagen sponges in osteogenic medium for 4 weeks with improved osteogenesis demonstrated by alizarin red staining for calcium deposition, and micro-CT imaging.

The Osteocalcin Reporter Identifies Osteogenic Cells

- After 3 weeks of culture, cells grown under osteogenic conditions with BMP2 dosing expressed higher levels of dTomato (Figure 5A,B)
- After the screen, the percentage of cells above the dTomato threshold increased from 10% to 12.9% (Figure 5C,D).

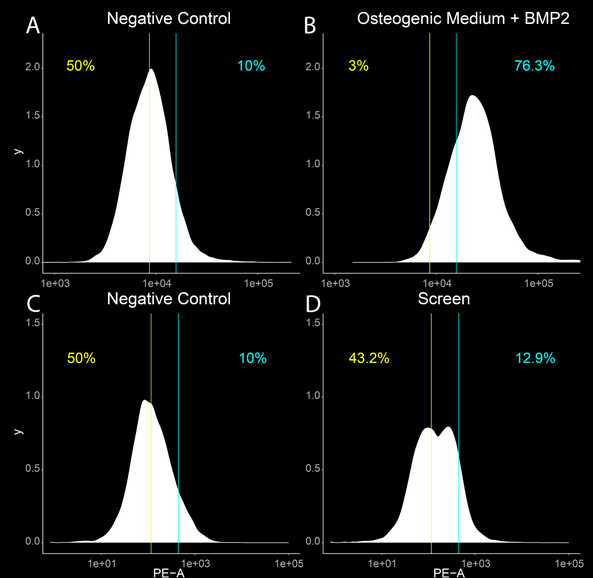

Figure 5: Flow cytometry data for dTomato expression in (A) negative control vs (B) positive control during OCN reporter validation and FACS data for (C) negative control vs (D) genome-wide screen sample during the screen.

The CRISPRa Screen Identified Novel Osteogenic Targets

- The screen identified 248 gene targets enriched in the dTomato+ cells and 1176 gene targets enriched in the dTomato- cells (Figure 6A)
- The MAGeCK algorithm identified top gene targets enriched in the dTomato+ cells (Figure 6B)

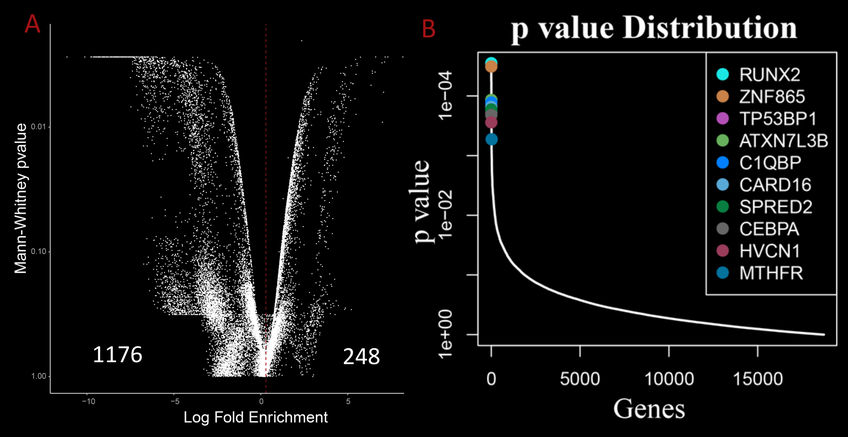

Figure 6: (A) Volcano plot showing Mann-Whitney p-value vs Log Fold Enrichment in the dTomato+ cells. (B) Gene ranking results from the MAGeCK selection method showing some of the top results based on p-value.

Osteogenic Upregulation Targets Are Validated Individually

- 6 of the 10 targets identified by the MAGeCK algorithm had increased ALP activity in monolayer cultures (Figure 7A).
- SPRED2 upregulation increased mineralization in ASCs grown in 3D cultures as shown by micro-CT imaging and alizarin red staining. (Figure 7B-D).

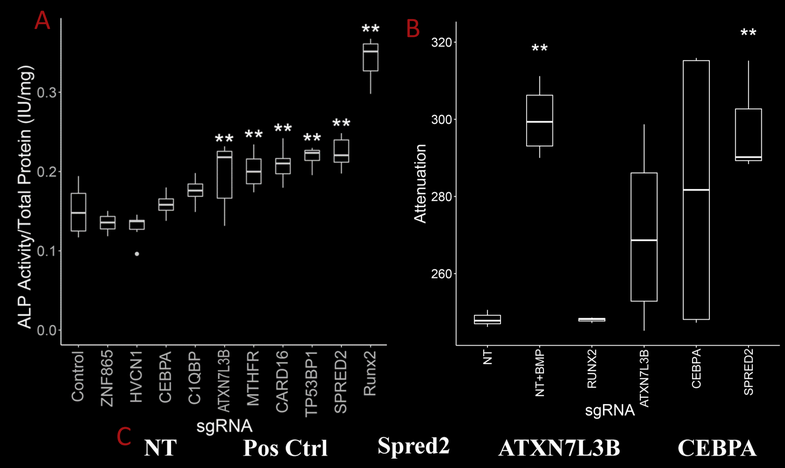

Figure 7: (A) ALP activity after one week of culture in standard growth medium in monolayer. (B) Micro-CT quantification and (C) images of selected cell lines after 4 weeks of culture on type-I collagen gels in osteogenic medium. (D) Alizarin red and H&E staining of histological sections from the 3D cultures. ** denotes p-value < 0.05 by one-way ANOVA with Dunnett's post hoc test

Discussion

- The CRISPRa screen identified several gene upregulation targets that were shown to improve osteogenesis in ASCs.
- Some of the identified targets, including Runx2, showed increased ALP activity in monolayer but did not improve mineralization in the more-challenging 3D cultures.
- The top-performing upregulation target, SPRED2, showed increased ALP activity in monolayer and increased mineralization in 3D culture.

References

[1] Baldwin, P. et al. J. Orthop. Trauma (2019). [2] Amini, A. R., Laurencin, C. T. & Nukavarapu, S. P. (2012). [3] Deyo, R. A. The Spine Journal (2015). [4] Ball, A. N. et al. J. Orthop. Res. (2018). [5] Li, W. *et al. Genome Biol.* (2014).