Variant-to-gene mapping at BMD GWAS loci using promoter Capture 4C-sequencing identifies RUNX1 effector genes regulating bone formation

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Introduction: Bone mineral density (BMD) is a strong surrogate measure of bone strength and a strong predictor of fracture risk. BMD is a complex trait which is regulated by various factors including behavioral, environmental, and genetic factors. Genetic factors contribute to 60-80% risk to an individual with osteoporosis, which is primarily characterized by loss of BMD. Recent BMD genome wide association studies (GWAS) using 426,824 individuals, identified 518 genome wide significant loci associated with eBMD. This study replicated several known loci associated with BMD in prior GWAS and meta-analysis reports. Interestingly, all of these genetic studies reported multiple single nucleotide polymorphism (SNPs) in RUNX1 gene associated with changes in BMD. Although GWAS is an effective strategy to dissect the genetic basis of complex traits and diseases, it identifies only the sentinel SNPs with strongest association in a particular genomic locus, which may not be actual causal variants due to the presence of other SNPs in linkage disequilibrium (LD). Almost ~90% of GWAS associated SNPs are non-coding, residing in either intronic or intergenic regions, indicating that their impact on phenotype is primarily via gene regulation. The reported BMD GWAS-SNPs (rs13046645, rs2834676, and rs2834694) in RUNX1 gene also lies in the intronic region which further raises the question how these noncoding variants affect the regulation of BMD by RUNX1? The noncoding nature of these SNPs in RUNX1 region further suggests that regulatory elements maybe affected which may regulate another effector gene in the neighborhood to control the function of RUNX1 and thus affect the reported changes in BMD. Therefore, present study was undertaken to functionally establish the role of RUNX1 intronic region harboring BMD GWAS loci for skeletal differentiation and bone formation. We used human induced pluripotent stem cells (iPSCs) as an experimental model to determine the functional requirements of regulatory regions containing RUNX1 GWAS variants for the bone formation.

Methods: We used CRISPR/Cas9 mediated genome editing approach to generate mutant iPSCs (Δ-iPSC) which lack RUNX1 intronic region harboring BMD-GWAS loci. Sendai viral based reprogramming method was used for the generation of ΔRUNX1-iPSC using our well-established iPSC lines derived from human skin fibroblast (BD1-4-iPSCs). The exact location of the deletions was determined using several criteria including genetic associations with BMD, linkage disequilibrium data for these SNPs from 1000 Genomes Project, ChIPSeq data from Roadmap Epigenome for H1-MSCs and osteoblasts, and mammalian conservation. Based on linkage disequilibrium, these regions encompassed ~55kb and ~70kb at positions chr21:36282225- 36337655 and chr21:36759535-36828803, which were denoted as Δ1-RUNX1-iPSCs and Δ2- RUNX1-iPSCs respectively. They do not overlap any coding portions or potential splice sites of the RUNX1 gene. We generated both homozygous and heterozygous mutant clones lacking both and single allele respectively. Multiple clones for each deletion were generated Δ1-RUNX1-Homozygous (#9, #11), Δ1-RUNX1-Heterozygous (#46), Δ2-RUNX1-Homozygous (#13, #27), Δ1-RUNX1-Heterozygous (#42). Differentiation of iPSC into osteoblast require an intermediate population which we termed as mesenchymal progenitor cells or mesenchymal stem like cells (iMSCs). The differentiation of iPSCs into iMSCs was performed using our established direct plating method. The characterization of the MSC like feature was performed using gene expression and flowcytometric analysis of cell surface markers. Osteogenesis of iMSCs was induced by culturing cells in osteogenic medium consisting of DMEM supplemented with 1mM sodium pyruvate, 0.1μM dexamethasone, 50 μg/ml ascorbic acid 2-phosphate, 10mM β-glycerophosphate, 10% FBS and 1X penicillin/streptomycin for 21 days. At end of 21 days culture, differentiation potential was determined by gene expression analysis of osteogenic genes and Alizarin Red staining. To determine the *in vivo* osteogenic potential of these iMSCs, we used our established calvariae bone defect model using NOD-SCID mice. The iMSCs were encapsulated in 4% PEG-4MAL hydrogel and implanted to defect size at time of surgery and at 4-, and 8-week after surgery. To next determine how the intronic region of RUNX1 locus regulate bone formation, we analyze the interaction of these GWAS regions with long distance effector genes via long-range promoter interactions mapping using circularized chromosome conformation capture (4C)-sequencing approach. 4C-sequencing was performed using our established method using the NextSeq550. Genomic viewpoint primers were designed using 4C-seq primer db and chromatin was digested with the combination NlaIII/DpnII. The interactions between wild type and RUNX1 deletion mutant lines were assessed using PeakC and significant interactions were represented using the UCSC interact track format.

Results: Our data showed that CRISPR/Cas9 mediated genome editing approach in human skin fibroblast (BD1-4-iPSCs) iPSCs for the deletion of RUNX1 intronic regions harboring BMD GWAS loci (rs13046645, rs2834676, and rs2834694) resulted in generation of various RUNX1 mutant iPSC lines. Characterization of these iPSC lines showed that homozygous and heterozygous mutant iPSCs (ΔRUNX1-iPSC) exhibited all features of pluripotency as determined by morphological, gene expression and immunofluorescence staining for the stemness markers suggesting that deletion of RUNX1 intronic region in mutant iPSC clones did not affect their pluripotency. Our results further showed that these mutant iPSCs can efficiently differentiated into mesenchymal progenitors (iMSCs) and exhibited similar mesenchymal surface marker expression and comparable phenotypic features *in vitro*. Interestingly, differentiation of iMSCs into osteoblasts demonstrated that deletion of RUNX1 intronic regions (Δ1 or Δ2) induced osteogenic potential in a dose dependent manner *in vitro*. Alizarin red staining and expression assay for osteogenic genes such as *RUNX2, OSX, OCN* demonstrate that homozygous deletion of Δ1 or Δ2 exhibited enhanced osteogenic potential as compared to heterozygous deletion or wild type clones. These results suggest that RUNX1 intronic region harboring BMD-GWAS loci contains regulatory sequences which control the genes involved in osteogenic differentiation of MSCs. We next assessed *in vivo* osteogenic commitment of these mutant lines using sub critical-sized (2.3 mm) parietal bone defect model in mice. We implanted wild type and mutant homozygous iMSCs to defect site and osteogenic capacity was determined at 12-week post-surgery by quantifying using volume of bone regenerated (BV/TV) in within the cranial defect using μCT analysis. In line with the *in vitro* findings, our results showed that as compared to wild type iMSCs, Δ2 RUNX1-iMSCs exhibited 22% more bone regeneration in cranial defect (**Fig. 1A**). Furthermore, Δ1 RUNX1-iMSCs also showed increased bone regeneration but did not reach statistical significance. Together, these findings suggest that intronic region in Δ2 RUNX1-iMSCs contains regulatory sequences which may influence another effector genes regulating osteoblast commitment. To determine the role of regulatory sequences with RUNX1-GWAS loci and to identify their effector genes, we used variant-to-gene mapping approach using 4C-sequencing in wild type and mutant iMSCs. Sequencing run had >67 M reads, and analysis was performed using PeakC single analysis where significant interactions were determined by difference from background and ratio of observed to expected reads. Following analysis with PeakC, the wild type-iMSCs showed multiple significant interactions occurring at each viewpoint which specifically indicate interactions of rs1304665 (Viewpoint 2) and rs2834694 (Viewpoint 4) with the SETD4 promoter. The Δ2-Runx1 deletion mutant, the interaction of viewpoint 2 and rs1304665 were removed and contact of the SETD4 promoter with rs1304665 was suggested by the change in span of contacts with the SETD4 promoter (Viewpoint 6), lacking rs1304665 as a target (**Fig. 1B**). We next confirmed the reliability of identified effector genes using gene expression in both homozygous and heterozygous mutant iPSC lines which confirmed SETD4 as probable effector gene for RUNX1 GWAS loci.

Discussion: Here, we carry out variant to gene mapping in human mesenchymal progenitor cells employing a promoter Capture-C method to characterize the significance of non-coding GWAS loci near RUNX1 intronic region, known to be associated with adult BMD. By intersecting our Capture-C and gene expression data, we observed contacts between candidate causal variants (rs1304665, RUNX1 SNP) and putative effector target genes such as SETD4. We further showed that presence of regulatory sequence near this SNPs regulate osteogenesis and promote bone formation in calvarial defect model.

Significance: Our results Our results established a novel functional genomics pipeline for variant-to-gene mapping using iPSCs as an experimental model and demonstrated the importance of long-range chromatin interactions mapping for identifying functional targets of disease loci.

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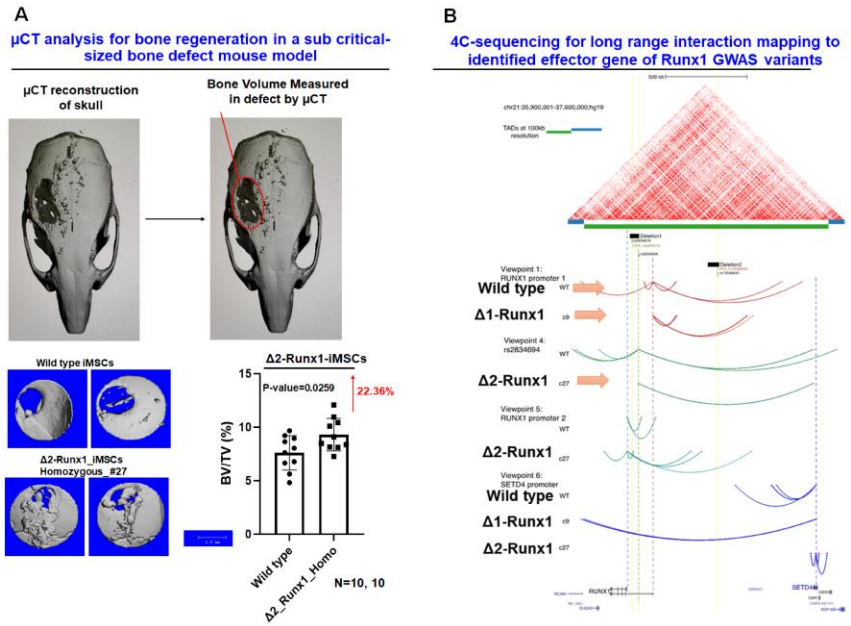

A. Mutant RUNX1 (Δ2)-iMSCs encapsulated in a PEG hydrogel stimulates bone regeneration in a sub critical-sized bone defect mouse model; **B**. Long range interaction mapping using 4C-sequencing showed interactions RUNX1 GWAS loci with various effector genes.