Characterization of putative Wnt3a-Inducible Enhancers

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Introduction and Background

The Wnt signaling pathway has been previously shown to play a major role in regulating bone metabolism and it is emerging as a target for the therapeutic intervention of bone thinning disorders such as osteoporosis. Several Wnt proteins have been shown to be expressed in bone and mutations in Wnt pathway members such as Wnt co-receptor Lrp5 and Wnt inhibitor Sost have been shown to be associated with low or high bone mass disorders, however, very little is known about specific roles played by different Wnt ligands in bone development, repair and remodeling. To identify downstream targets of Wnt signaling we sequence RNA synthesized by Wnt3a treated osteoblasts and found 214 genes to be significantly up-regulated by >2-fold in the presence of Wnt3a ligand. Computational analysis of ChipSeq data in the loci of these 214 genes predicted 163 putative Wnt-inducible enhancers. In this project, we sought to functionally validate these putative enhancer elements that may account for the Wnt3a-dependent activation of these 214 transcripts. Candidate enhancers were cloned, transfected into osteoblast-like cell lines, and examined for transgene expression in the presence and absence of recombinant Wnt3a protein. Preliminary data suggests that several noncoding elements may have enhancer properties both in the absence or presence of Wnt3a, *in vitro*.

- Osteoblasts are bone cells responsible for bone formation.
- Wnt describes a family of highly conserved proteins that regulate cell proliferation, fate specification, polarity, and migration.
- Wnt signal stimulates several intra-cellular signal transduction cascades, including the canonical or Wnt/β-catenin dependent pathway.

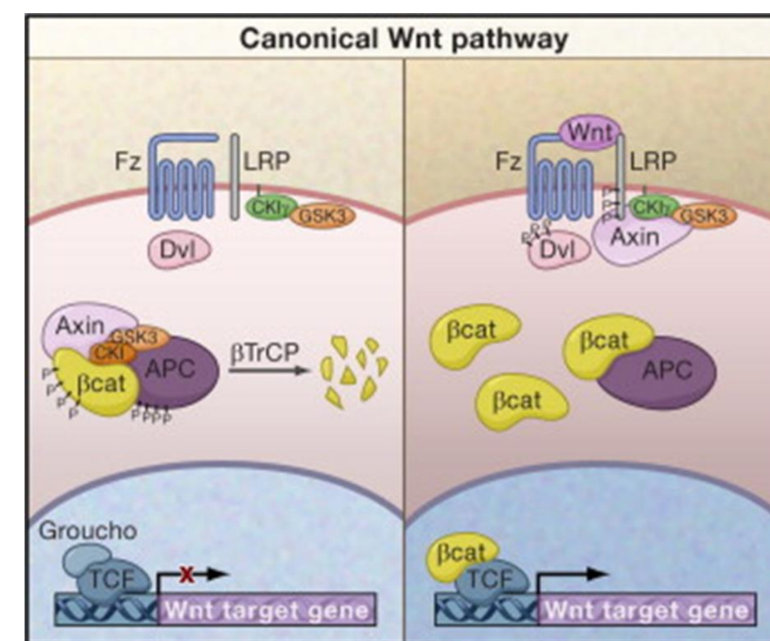

Figure 1. When Wnt receptor complexes are not bound by ligands, β-catenin is targeted for rapid destruction and transcription of Wnt target genes is inhibited. Once bound by Wnt, a co-receptor complex activates the canonical signaling pathway stabilizing β-catenin and promotes the transcription of Wnt target genes.

- Enhancers are distal regulatory elements that bind transcription factors and increase the rate of transcription of genes.
- 20 putative enhancer regions were selected based on close proximity to a gene up-regulated by Wnt3a administration, the presence of TCF/LEF binding sites, DNAse hypersensitivity, as well as methylation and acetylation markers.

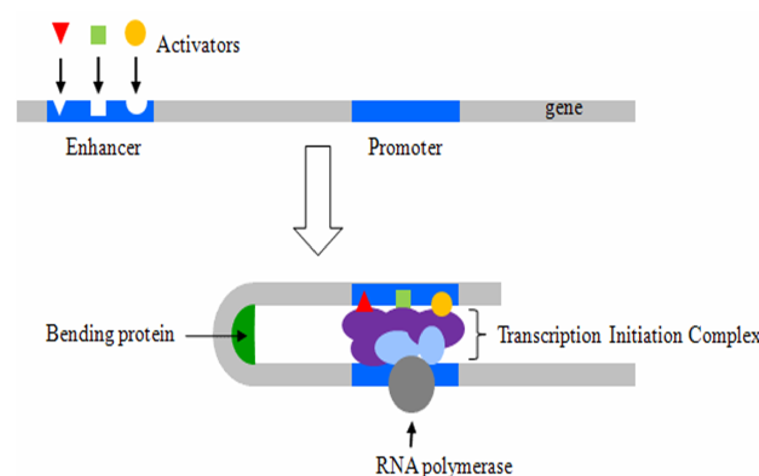

Figure 2. Function and location of enhancers on a chromosome

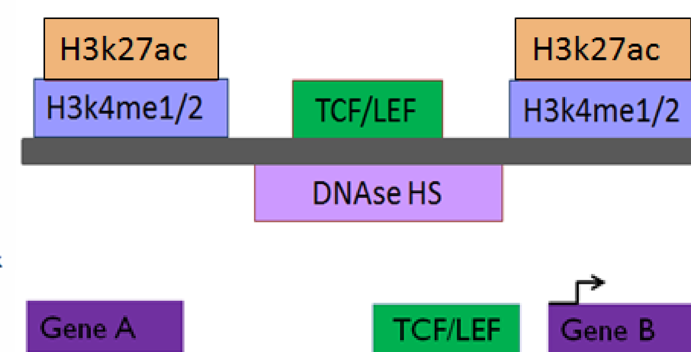

Figure 3. Selection criteria for Wnt inducible enhancers

Methods

1 Create enhancer-expression constructs

- Design primers and PCR amplify putative enhancers
- Clone enhancers into expression plasmids
- Identify correct inserts using restriction enzyme digests

2 Determine if enhancers are Wnt inducible

- Prep DNA for transfection
- Grow cells for transfection while learning aseptic tissue culture techniques
- Transform UMR cells with enhancer clones
- Treat transfected clones with Wnt3a
- Quantify transgene expression via luciferase reporter gene
- Determine if transfected clones are Wnt3a inducible

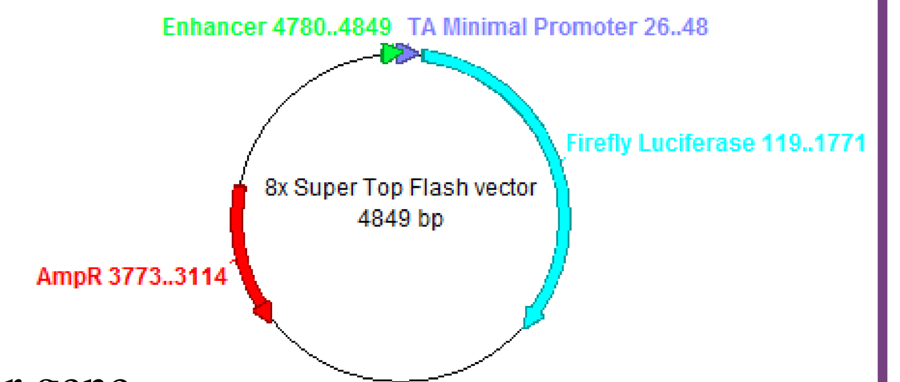

Figure 4. Topflash plasmid

Results

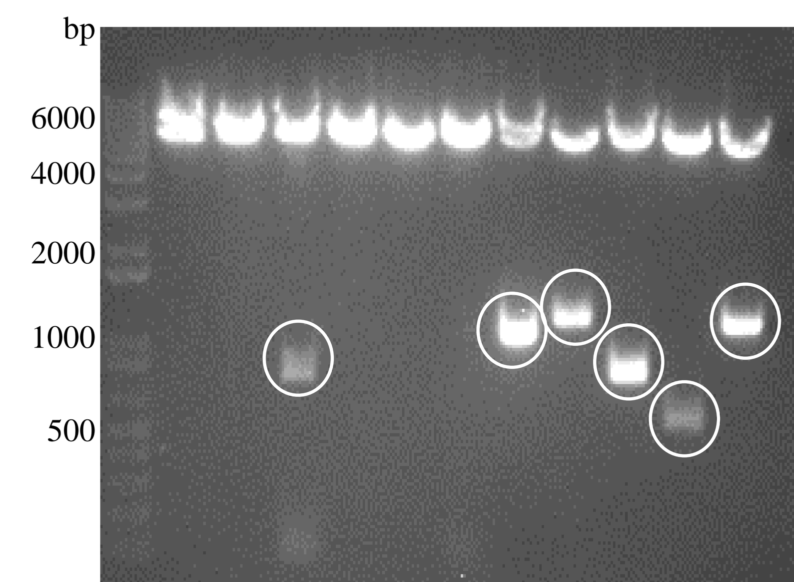

Figure 5. Gel electrophoresis for diagnostic digest shows positive screening results after cloning with the appropriate enhancer inserts. First lane shows a 1KB+ molecular weight ladder as a reference for DNA fragment size. Large molecular weight bands show the vector backbone and circled bands show successful inserts.

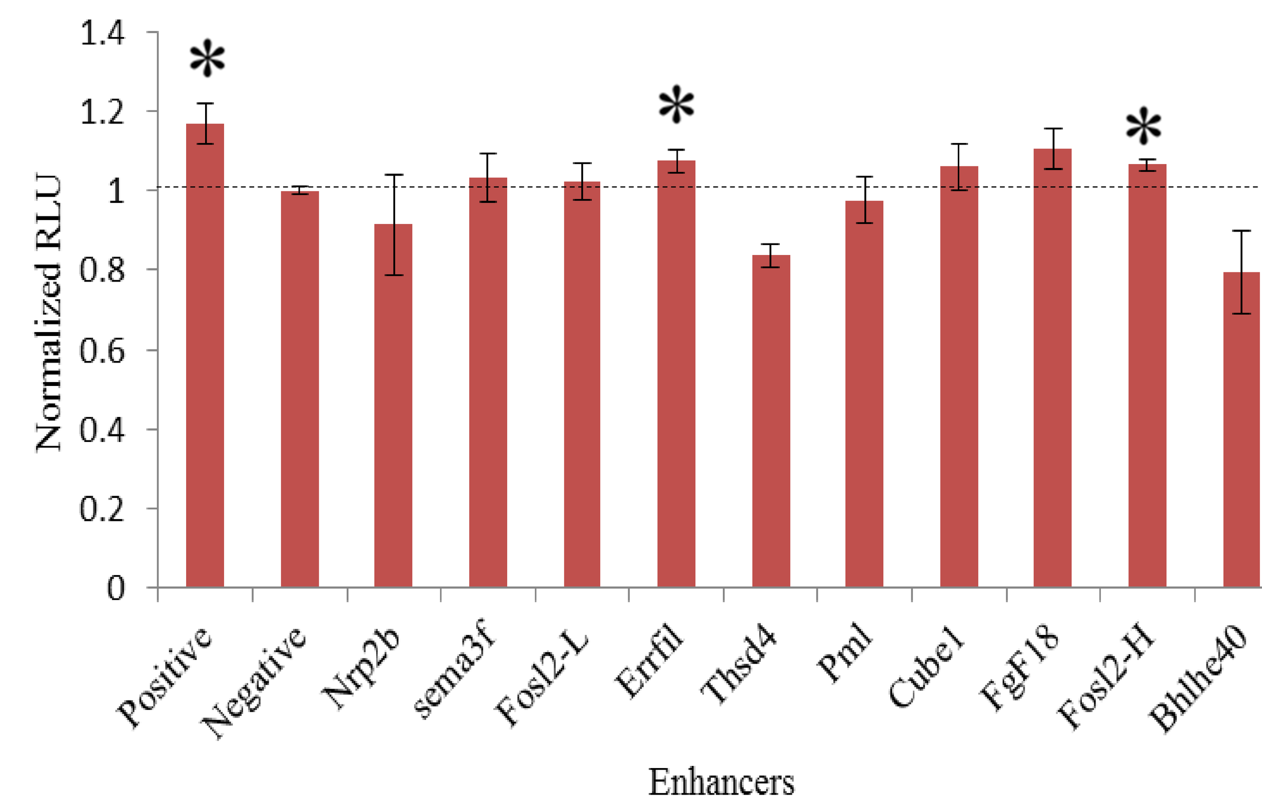

Figure 6. Expression level in relative light units of reporter gene driven by enhancer constructs in the absence of Wnt3a. Significant increase in expression shows enhancers are active regardless of Wnt3a activation. When compared to the expression level of the negative control which has no TCF/LEF binding sites, three enhancers showed significant expression levels without added recombinant Wnt3a protein. These enhancers include the positive control (p-value=0.0047), Errfil (p-value=0.0131), and Fosl2-H (p-value=0.0023).

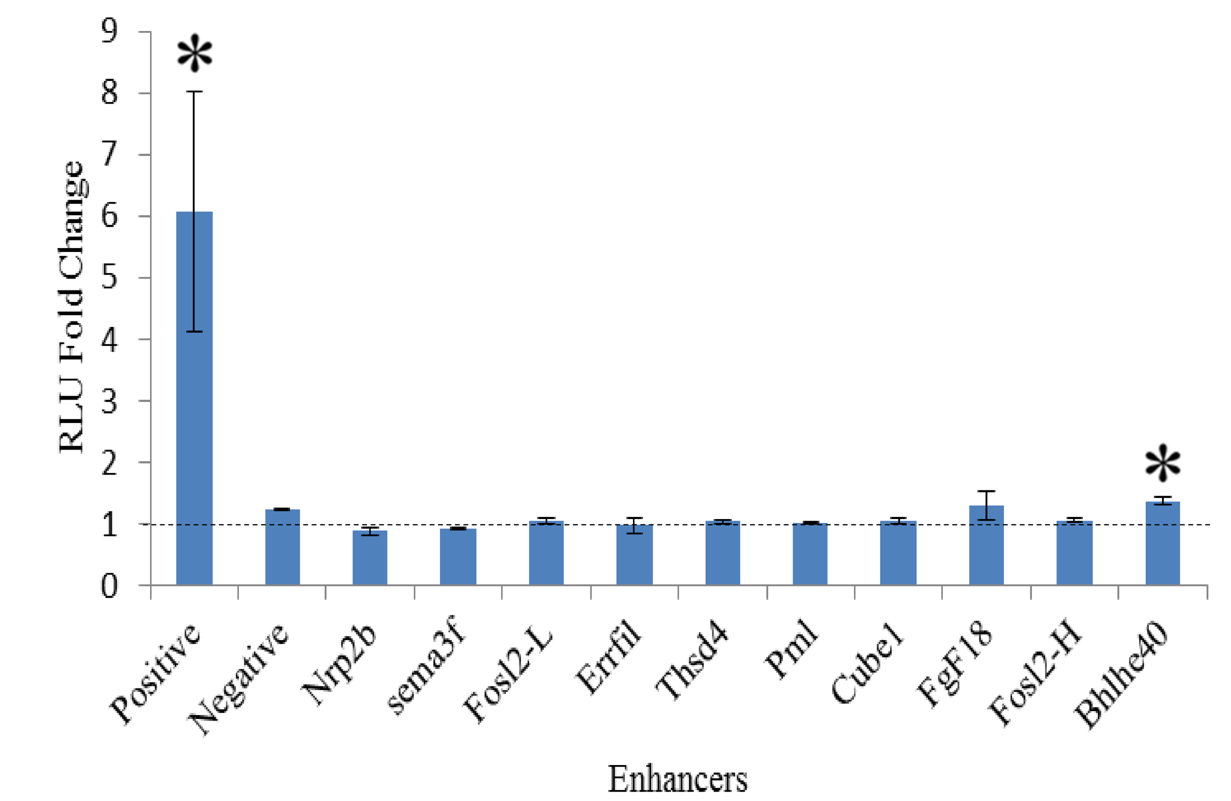

Figure 7. Expression level in relative light units of reporter gene driven by enhancer constructs in the presence of Wnt3a. Comparing stimulated and unstimulated expression driven by enhancers. Increase fold change indicates enhancers are active due to Wnt3a activation. The Topflash plasmid positive control showed significant up-regulation of transgenic expression in the presence of Wnt3a protein (p-value=0.0106). This is in concurrence with its seven TCF/LEF binding sites which greatly increase the amount of Wnt-signaling. Bhlhe40 (p-value=0.0098) also experienced a significant increase in transgenic expression.

Conclusions and Next Steps

Through diagnostic digests using the appropriate restriction enzymes, we were able to identify and isolate the correct inserts, properly transfect them into UMR host cells, and quantify transgene expression through a luciferase reporter gene. Cloning enhancers into expression plasmids was successful, and the preliminary data from this experiment showed that there was at least one enhancer that significantly up-regulated transgenic expression in the presence of Wnt3a. The Topflash plasmid positive control has seven TCF/LEF binding sites which aided in significantly up-regulating expression in the presence of Wnt3a (p-value=0.0106). The Bhlhe40 enhancer also showed a significant increase in transgenic expression (p-value=0.0098). To increase the reproducibility of this experiment, future studies might include plating a higher concentration of cells for transfection and letting them transfect for a longer period of time. These validated enhancers further our understanding of transcriptional regulation by Wnt signaling, and will be used to improve conditions for validation of more putative enhancers in the future. These findings will also be used to examine relationships between Wnt-inducible enhancers and predicted phenotypes such as bone formation. These Wnt inducible enhancers may have additional effects in humans and may be sites for developmental control.

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