The Wnt1^{G177C} mutation impairs mechanically induced bone formation through nuclear receptor 4a2

Mubashir Ahmad[1], Melanie Haffner-Luntzer[1], Astrid Schoppa[1], Timur A. Yorgan[2], Michael Amling[2], Thorsten Schinke[2], Anita Ignatius[1]

[1]Institute of Orthopedic Research and Biomechanics, University Medical Center Ulm, 89081 Ulm, Germany
[2]Department of Osteology and Biomechanics, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany
Email of Presenting Author: ahmad.mubashir@uni-ulm.de

Disclosures: MHL: ORS ISFR section office

INTRODUCTION: Mechanical loading triggers bone formation. While the precise mechanism driving this phenomenon remains elusive, accumulating evidence suggests the indispensable role of the Wnt signaling pathway in facilitating the anabolic response. In this study, our objective was to investigate the effects of the *Wnt1*^{G177C} mutation on mechanically induced bone formation, a mutation which leads to osteogenesis imperfecta type XV in humans. To this end, we combined *in vivo* and *in vitro* experiments using homozygous *Wnt1*^{G177C} mice and osteoblast cultures, respectively, to unravel the signaling cascades through which the *Wnt1*^{G177C} mutation influences bone formation induced by mechanical stimuli.

METHODS: The experimental procedures for *in vivo* mechanical loading of the mouse ulna were conducted with approval from the animal care committee of Baden-Württemberg (Regierungspräsidium Tübingen, No.1566). For *ex vivo* calibration of the loading protocol, strain gauges were affixed to the lateral surface of the ulnae in 12-week-old female mice of both the *Wnt1*$^{+/+}$ and homozygous *Wnt1*^{G177C} groups (n=6). The right ulnae were positioned in the loading apparatus to measure the load needed for a peak surface strain of 2000 µstrain. Cyclic axial compression of the right ulnae was conducted, applying a frequency of 2 Hz and a maximum load of 2.0 N and 0.5 N in 12-week-old female *Wnt1*$^{+/+}$ and *Wnt1*^{G177C} mice, respectively. Using this experimental setup, *in vivo* loading was performed on the ulnae of 12-week-old female *Wnt1*$^{+/+}$ and *Wnt1*^{G177C} mice (n=4) during anesthesia over three consecutive days, each for one minute. The left ulnae were utilized as non-loaded controls. For micro-computed tomography (µCT) and RNAseq analysis, the mice were euthanized on day 16 and day 4, respectively. The ulnae were subjected to µCT using Skyscan 1172 with an 8 µm resolution. A 1 mm thick section of the mid-diaphyseal cortical bone, starting 3 mm distally from the ulnae head, was designated as the region of interest. Regarding RNAseq analysis, the ulnae were cleared of the surrounding tissue and centrifuged (12500 rpm; 1 min) to remove bone marrow. The subsequent steps included RNA isolation using the RNeasy kit, evaluation of RNA concentration and integrity through 2100 Bioanalyzer and RNA 6000 Nano kit. RNA sequencing using Illumina technology was carried out by Novogene. Gene expression quantification was achieved by read count method. Principle component analysis (PCA) was implemented to determine sample variability and repeatability using RStudio with pcaExplorer. The overall distribution of differentially expressed genes (DEGs) was illustrated using volcano plots. Gene ontology (GO) analysis of DEGs was performed using Metascape. Furthermore, mechanical stimulation experiments were conducted on primary murine osteoblasts derived from the long bones of 12-week-old female *Wnt1*$^{+/+}$ and *Wnt1*^{G177C} mice (n=5). The cells were seeded onto Flexcell glass slides at a density of 20,000 cells/cm^2. Cells were either left untreated (static control) or mechanically stimulated by laminar fluid flow (LFF) applying a shear stress of 10 dynes/cm^2 for 1h using the Flexcell streamer system. RNA samples were collected immediately (0h) after LFF. The RNA isolation from primary osteoblasts involved extraction using RNeasy kit, cDNA synthesis using 1 µg RNA, followed by qPCR on the QuantStudio 5 system. The relative mRNA concentrations, normalized to beta-2 microglobulin, were calculated using the ΔΔCT method.

RESULTS: To elucidate the functional implications of the *Wnt1* mutation (*Wnt1*^{G177C}) in the bone's anabolic response to loading, we exposed the ulnae of 12-week-old female mice from both *Wnt1*$^{+/+}$ and *Wnt1*^{G177C} groups to mechanical loading (Fig. 1A). µCT analysis revealed that ulnae loading substantially increased cortical thickness in *Wnt1*$^{+/+}$ control mice, whereas this effect was attenuated in *Wnt1*^{G177C} mutant mice (Fig. 1B-C). RNAseq analysis revealed 1704 DEGs influenced by the *Wnt1*^{G177C} mutation during mechanical loading (Fig. 1D). By subjecting these DEGs to Metascape analysis, we identified various pathways, involved in skeletal system development, extracellular matrix organization, blood vessel development, regulation of neuron projection development, and mesenchyme development, that were modulated. To discern which of these DEGs were specifically governed by osteoblasts, we compared them with datasets from RNAseq performed on primary osteoblasts, where *Wnt1* was deleted using siRNA, followed by LFF-induced stimulation. This analysis highlighted 16 common gene, with one consistently demonstrating dependence on both *Wnt1*^{G177C} mutation and stimulation, was nuclear receptor subfamily 4, group A, member 2 (*Nr4a2*) (Fig. 1E). Depletion of *NR4A2* in human mesenchymal stem cells (MSCs) confirmed its positive role in promoting osteoblast differentiation. These findings demonstrate that *Wnt1*^{G177C} mutation impairs mechanical loading-induced bone formation through modulation of *Nr4a2* expression.

DISCUSSION: Our research delved into the effects of the clinically relevant *Wnt1*^{G177C} mutation on bone response to mechanical loading in mice. Ulnae loading triggered a substantial increase in cortical thickness in *Wnt1*$^{+/+}$ control mice, whereas this response was compromised in mice harboring the *Wnt1*^{G177C} mutation. Analysis using RNAseq revealed 1704 DEGs influenced by the *Wnt1*^{G177C} mutation during mechanical loading. Further investigation pinpointed *Nr4a2* as a key gene affected by both the mutation and loading, with depletion of *NR4A2* confirmed it promoting role on osteoblast differentiation. This study underscores the role of *Wnt1*^{G177C} mutation in disrupting bone formation prompted by mechanical loading through modulation of *Nr4a2* expression.

SIGNIFICANCE: Our study highlights the significant impact of the clinically relevant *Wnt1*^{G177C} mutation on impairing the bone's ability to respond to mechanical loading, leading to decreased cortical thickness. Through the identification of molecular pathways and the key involvement of *Nr4a2*, this research provides valuable insights into the complex mechanisms governing bone formation in response to mechanical stimuli. These findings offer potential avenues for therapeutic interventions targeting these pathways.

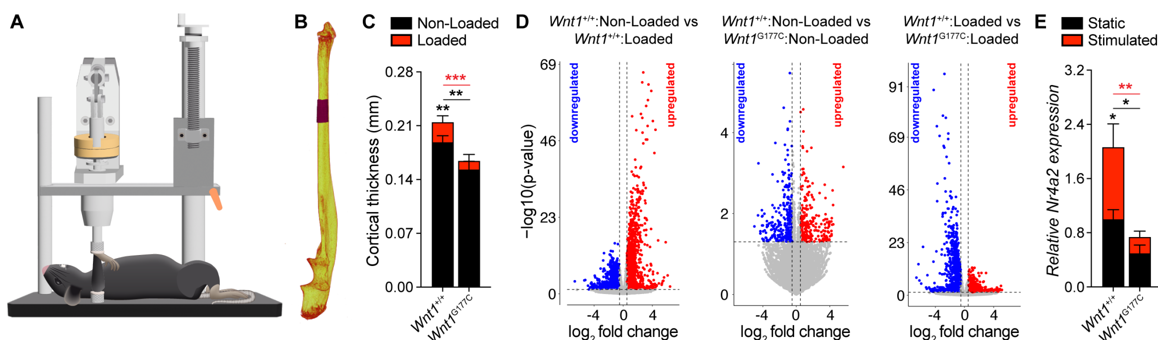

Fig. 1: Impact of the *Wnt1*^{G177C} loss-of-function mutation on mechanically induced bone formation via modulation of *Nr4a2* expression. (A) Illustration depicting the methodology of ulnae loading in mice with *Wnt1*$^{+/+}$ and *Wnt1*^{G177C} genotypes. (B) Representative micro-computed tomography (µCT) image of mouse ulnae, highlighting the 3 mm distal segement selected for subsequent µCT analysis post-loading. (C) Cortical thickness assessed by µCT analysis in female mice with *Wnt1*$^{+/+}$ and *Wnt1*^{G177C} genotypes (n=4/group) following ulnae loading (day 16). (D) Volcano plots illustrating the differentially expressed genes (DEGs) with upregulated genes shown in red and downregulated genes shown in blue. DEGs significance criteria: log2 fold change (log2FC) ≤ -0.5 and ≥ 0.5; p-value < 0.05) (n=4/group).Non-DEGs are depicted in grey. (E) qPCR analysis of *Nr4a2* expression (0h after LFF) in primary murine osteoblasts isolated from mice with *Wnt1*$^{+/+}$ and *Wnt1*^{G177C} genotypes (n=5/group).