A *Wnk2* Mutation Associated With Familial Osteoarthritis Induces Bone Remodeling in a PTOA Mouse Model

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Introduction

Osteoarthritis (OA) is a debilitating disease affecting millions worldwide. Despite its prevalence, there are currently no effective disease-modifying therapies. The main obstacle in developing such therapies is a poor understanding of the mechanisms driving the development of OA. Our goal is to discover genes and molecular pathways in humans that are vulnerability points in the development of OA and generate mouse models with human disease alleles.

The osmolarity of the synovial joint changes in response to everyday use and injury and during the aging process. Chondrocytes of the joint need to sense and respond to osmotic changes to maintain cellular homeostasis, yet the genes and pathways mediating this response are unknown. We have analyzed the exomes of 151 families with multiple forms of OA and identified three novel independent rare coding variants in the With No Lysine (K) Kinase 2 (WNK2) gene associated (**Fig 1**). The WNK protein kinases are intracellular sensors that respond to hyperosmotic stress by regulating ion channel and signaling pathway activity.

Our previous *in vitro* studies showed that WNK2 is expressed in articular chondrocytes and upregulated in osteoarthritic human and mouse joints, where it plays a critical role in hyperosmotic stress response. The combination of increased WNK2 activity and osmotic stress triggers OA-associated gene expression. This effect is further enhanced by all WNK2 variants, with *WNK2*^{R2054Q} demonstrating the most pronounced activity, indicating the human mutations are gain-of-function alleles.

Here, we investigate how the *WNK2*^{R2054Q} human mutation contributes to OA *in vivo.* To do this, we generated a mouse model carrying the *Wnk2*^{R2054Q} mutation and induced OA through knee joint injury. Our findings indicate that WNK2 plays a central role in regulating joint homeostasis and *Wnk2*^{R2054Q} was sufficient to drive bone remodeling and molecular changes associated with OA progression. Based on these findings, we **propose** that inhibiting WNK2 activity could prevent OA. To test this, performed a drug screen to successfully discover and validate a specific WNK2 inhibitor. Future work will focus on testing these inhibitors *in vivo*.

Mutations in *WNK2* are associated with familial osteoarthritis (OA)

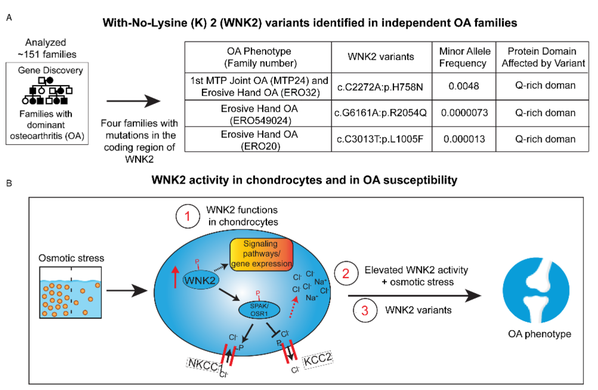

Figure 1: Identifying WNK2 as osteoarthritis (OA) susceptibility gene. (A) WNK2 variants identified in independent OA families. **(B)** Graphical abstract showing results of our previous *in vitro* study. Elevated WNK2 activity in combination with hyperosmotic stress induces OA-like gene expression. Expression of the WNK2 variants was also able to induce OA-like gene expression in the absence of hyperosmotic stress

We previously found that:
1. WNK2 signaling is central to the chondrocyte response to hyperosmotic stress.
2. Elevated WNK2 expression under osmotic stress induces OA-associated genes.
3. Familial OA-associated WNK2 variants amplify the pro-OA transcriptional response.

Results

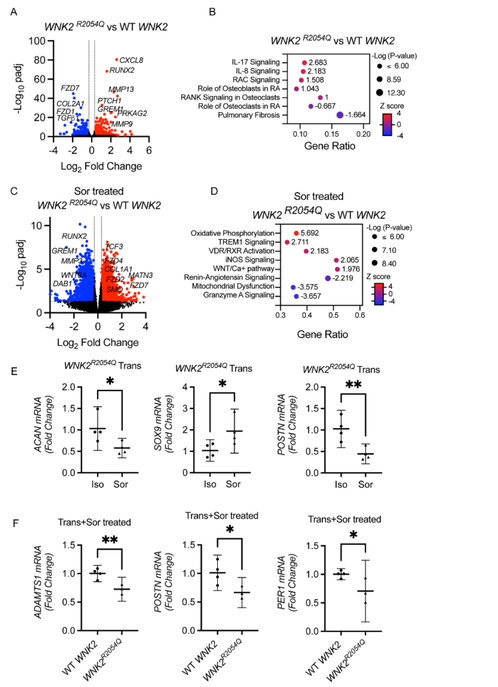

Figure 2: *WNK2* variants associated with familial OA are sufficient to induce a pro-OA transcriptional response. Comparative RNA-seq analysis of T/C-28a2 chondrocytes comparing *WNK2*^{R2054Q} vs WT WNK2-overexpressing cells. Volcano plots show significantly upregulated (red) or downregulated (blue) genes: **(A)** without osmotic stress, **(C)** with sorbitol (Sor-100mM) treatment. (B, D) Bubble plots display top KEGG pathways from differentially expressed genes, with the x-axis representing gene ratio and the y-axis listing pathways. Bubble color indicates pathway activation (red = increased, blue = decreased), with size reflecting -Log(P-value). **(E-F)** Primary human chondrocytes show similar transcriptional responses to *WNK2* variant overexpression or hyperosmotic stress as T/C-28a2 chondrocytes.

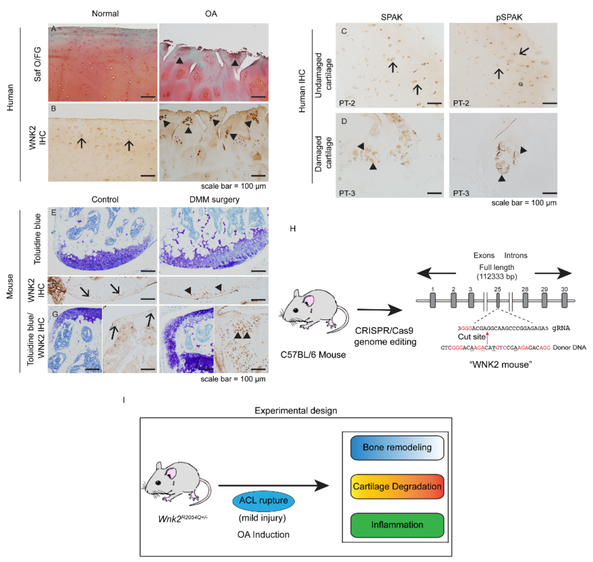

Figure 3: WNK2 expression is elevated in hypertrophic chondrocytes present in human osteoarthritic tissue and injured mouse knee joints. (A) Safranin O/Fast Green staining shows structural differences between healthy and OA cartilage. **(B)** Immunohistochemical staining reveals WNK2 expression in normal and OA cartilage of human **(B)** and mouse **(F)**. **(C-D)** SPAK and pSPAK (phosphorylated) expression is altered in damaged human cartilage. **(E and G)** Toluidine Blue staining of mouse cartilage shows changes in the medial femoral condyle **(E)** and osteophyte tissue **(G)** after injury. Arrows: normal chondrocytes, arrowheads: hypertrophic chondrocytes. Scale bar = 100µm. **(H)** Diagram illustrating the CRISPR/Cas9-based generation of the *Wnk2*^{R2054Q} mouse model. **(I)** Experimental design for OA induction and outcome measures for OA progression.

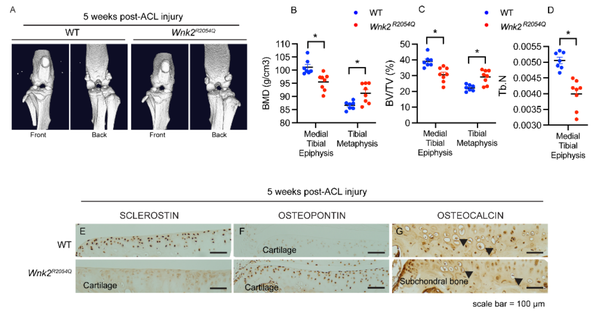

Figure 4: *Wnk2*^{R2054Q} is sufficient to induce bone remodeling in response ACL injury. (A) µCT images of the whole knee joint of injured WT and *Wnk2*^{R2054Q} mice (front and back views). Data indicating BMD **(B)** and BV/TV **(C)** and Tb.N **(D)** are altered in *Wnk2*^{R2054Q} mice. BMD-Bone Mineral Density; BV/TV-Bone volume to Total Volume; TB.N-Trabecular Number. **(E-G)** Immunohistochemical staining shows alterations in bone markers Sclerostin **(E)**, Osteopontin **(F)**, and Osteocalcin **(G)** in the knee joints of WT and *Wnk2*^{R2054Q} mice.

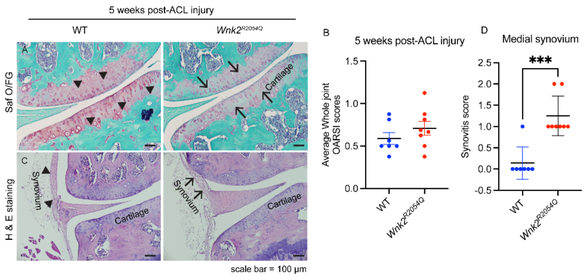

Figure 5. *WNK2*^{R2054Q} does not alter cartilage degradation but induced synovial inflammation in knee joints 5 weeks post-ACL injury. (A) Safranin O/Fast Green (Saf O/FG) stained knee joints of injured WT and *Wnk2*^{R2054Q} mice. **(B)** Average whole joint and maximal OARSI scores of knee joints of injured WT and *Wnk2*^{R2054Q} mice. **(C)** Hematoxylin and eosin (H&E) staining of knee joints after injury. **(D)** Synovitis score at medial synovium of knee joints of injured WT and *Wnk2*^{R2054Q} mice. Arrows indicate loss of proteoglycan content and increased inflammation, while arrowheads mark undegraded cartilage and normal inflammation in panels **A** and **C**. Scale bar = 100µm. Statistical significance (*p ≤ 0.05) by two-tailed unpaired t-test, n = 6-8.

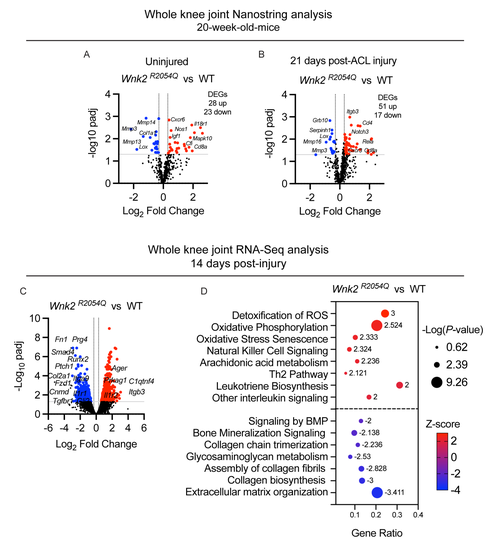

Figure 6. The *WNK2*^{R2054Q} mutation alters the early joint response to injury. (A-B) Nanostring analysis and **(C-D)** RNA-Seq analysis of whole knee joints of WT and *Wnk2*^{R2054Q} mice. Volcano plots of altered genes in *Wnk2*^{R2054Q} mice compared to WT mice: **(A)** uninjured, **(B** and **D)** post-injury. **(D)** Bubble plots indicate that many pathways associated with OA are downregulated in *Wnk2*^{R2054Q} mice compared to WT mice.

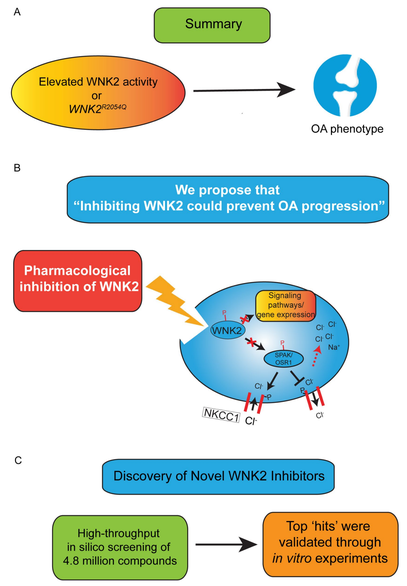

Figure 7. (A) Schematic overview summarizing key findings from *in vitro* and *in vivo* experiments. **(B)** Proposed model based on the findings. **(C)** Approach for the discovery of novel WNK2 inhibitors.

Figure 8. Identification of a novel chemical inhibitor of WNK2. (A) Diagram illustrating WNK2 activity. **(B-C)** Western blot analysis of pSPAK levels under isotonic (Top) (ISO; 300 mOsm) and sorbitol-induced hyperosmotic (Bottom) (Sor; 200 mM/500 mOsm) stress conditions. GAPDH was used as a loading control. **(B)** Initial screening of top-hit compounds showing the top 6 inhibitors. **(C)** Dose-dependent effect of M04 inhibitor on pSPAK levels. **(D)** XTT assay of WNK2 inhibitors showing dose-response curves for M04. X-axis: inhibitor concentration (µM), Y-axis: cell viability (%), with IC_{50} values in the inset.

Conclusions

- We identified WNK2 mutations linked to dominant forms of familial OA. All WNK2 variants show enhanced signaling activity, indicating they are gain-of-function mutations.
- We demonstrate *Wnk2*^{R2054Q} is sufficient to induce bone remodeling after ACL injury, affecting bone mineral density, BV/TV and reduced trabecular number. IHC analysis indicated decreased Sclerostin and Osteocalcin expression and increased Osteopontin expression in the joint.
- *Wnk2*^{R2054Q} disrupts the early joint response to injury, promoting OA by impairing ECM and bone remodeling, while increasing inflammation and oxidative stress. Targeting its effects on inflammation and ECM remodeling could offer therapeutic strategies to mitigate joint degradation and improve OA outcomes.
- We successfully discovered and validated a specific WNK2 inhibitor.
- In **conclusion**, we found *Wnk2*^{R2054Q} is sufficient to induce bone remodeling and molecular changes associated with OA development. Inhibiting WNK2 could prevent OA. Future studies will evaluate whether WNK2 inhibition mitigates OA-related joint damage in vivo using the discovered inhibitor compounds

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