Human genetics to drugs: Using familial osteoarthritis to identify novel pathways for drug discovery and validation

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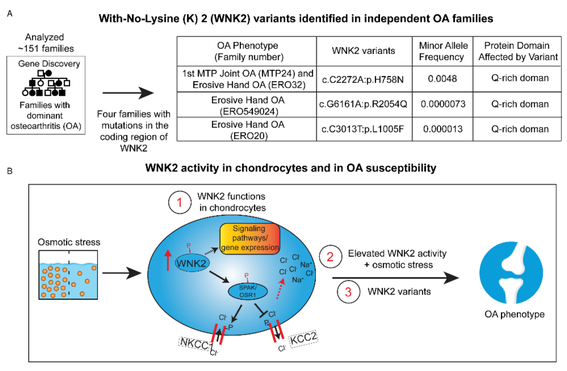
Introduction

Osteoarthritis (OA) is a common joint disease characterized by abnormal remodeling of joint tissue. Despite our understanding of the molecular and cellular processes that contribute to OA susceptibility, we lack bona fide genetic targets for drug development. To discover promising drug targets, our lab has taken a unique approach to identify genes and pathways that contribute to OA susceptibility in humans. We study many unrelated families with dominant inherited forms of OA to identify susceptibility alleles that have strong determinate effects. Our goal is to discover human OA susceptibility genes, generate mouse models with human disease alleles, and discover novel therapeutic compounds.

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 published data demonstrated that WNK2 mediates the response of chondrocytes to hyperosmotic stress, and the combination of elevated WNK2 expression and hyperosmotic stress promotes an OA-associated transcriptional response. Further, the expression of the WNK2 variants in the absence of hyperosmotic stress is sufficient to promote expression of pathways associated with OA, a response that is further amplified under conditions of hyperosmotic stress. To test the role of WNK2 in vivo, we generated a mouse model harboring the human *Wnk2*^{R2054Q} allele and a Wnk2 null (*Wnk2*$^{-/-}$) mouse. Our in vivo work indicated that *Wnk2*^{R2054Q} accelerates OA development in an injury model through the enhancement of proinflammatory signaling pathways. The goal of our work is to discover and validate novel inhibitors of WNK2.

Mutations in *WNK2* are associated with familial osteoarthritis



OA Phenotype (Family number)	WNK2 variants	Minor Allele Frequency	Protein Domain Affected by Variant
1st MTP Joint OA (MTP24) and Erosive Hand OA (ERO32)	c.C2272A:p.H758N	0.0048	Q-rich doman
Erosive Hand OA (ERO5549024)	c.G6161A:p.R2054Q	0.0000073	Q-rich doman
Erosive Hand OA (ERO20)	c.C3013T:p.L1005F	0.000013	Q-rich doman

Figure 1: Identifying WNK2 as an 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

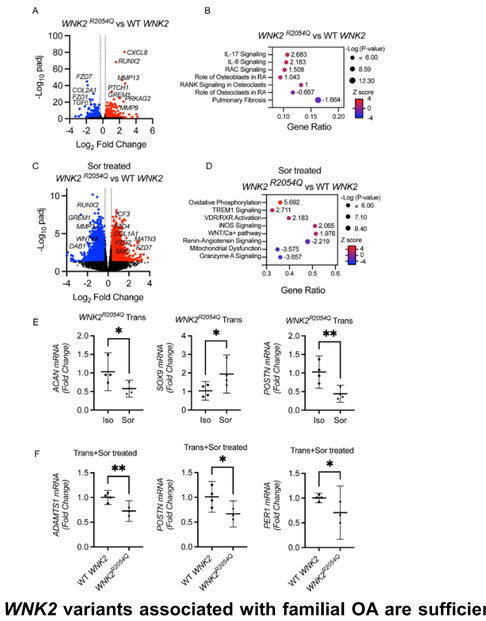


Figure 2: *WNK2* variants associated with familial OA are sufficient to induce a pro-OA transcriptional response. Comparative RNA-seq analysis of T/C-28a2 chondrocytes comparing *WNK2*^{R2054Q} vs WT WNK2-overexpressing cells. Volcano plots show significantly upregulated (red) or downregulated (blue) 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.

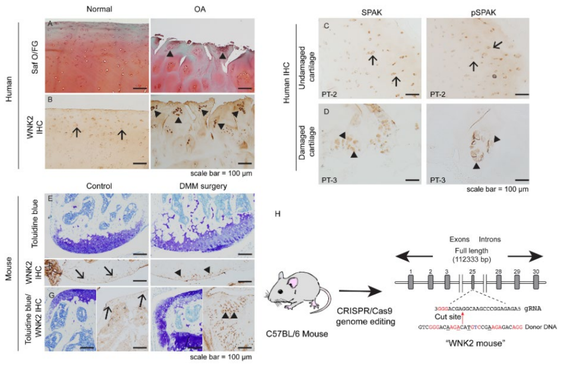


Figure 3: WNK2 expression is elevated in hypertrophic chondrocytes present in human osteoarthritic tissue and injured mouse knee joints. (A) Safranin O/Fast Green staining shows structural differences between healthy and OA cartilage. **(B)** Immunohistochemical staining reveals WNK2 expression in normal 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.

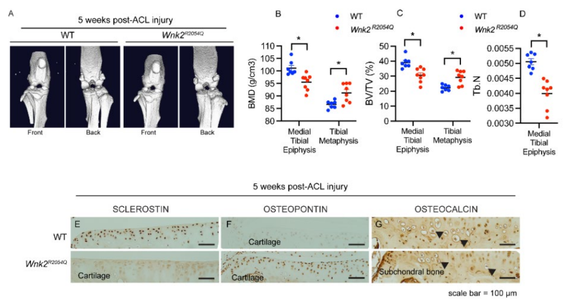


Figure 4: *Wnk2*^{R2054Q} is sufficient to induce bone remodeling in response ACL injury. (A) µCT images of the whole knee joint of injured WT and *Wnk2*^{R2054Q} mice (front and back views). Data indicating BMD **(B)** and BV/TV **(C)** and Tb.N **(D)** are altered in *Wnk2*^{R2054Q} mice. BMD-Bone 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.

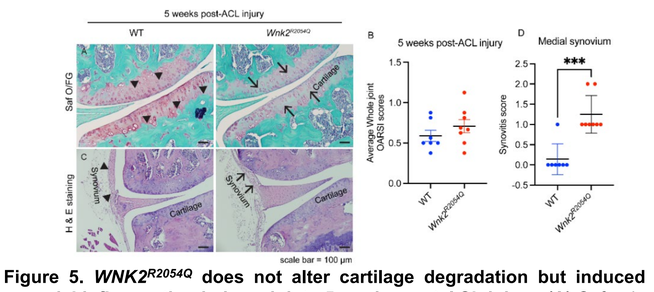
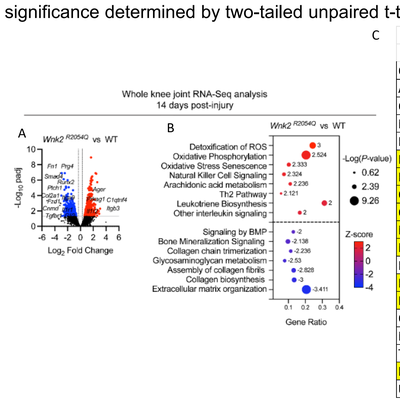


Figure 5. *WNK2*^{R2054Q} does not alter cartilage degradation but induced synovial inflammation in knee joints 5 weeks post-ACL injury. (A) Safranin O/Fast Green (Saf O/FG) stained knee joints of injured WT and *Wnk2*^{R2054Q} mice. **(B)** Average whole joint and maximal OARSI scores of knee joints 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 determined by two-tailed unpaired t-test, n = 6-8.



C

	WNK2R2054Q vs WT	
	14 days post-DMM-Mouse	
Gene	log2FoldChange	padj
ARNTL	-0.11786943	0.898999624
CLOCK	-0.296708884	0.545692933
NPAS2	NA	NA
PER1	NA	NA
PER2	-1.503000428	0.002624268
PER3	-1.379689398	0.000253245
CRY1	-0.216991122	0.739295815
CRY2	-0.148133718	0.791537054
NR1D1	-1.264567727	0.026959111
NR1D2	-1.416365906	6.30E-05
RORA	-0.281424246	0.59858587
RORC	-2.052682053	0.010948909
DBP	-1.521580452	0.000925521
CIART	NA	NA
NFIL3	NA	NA
TEF	NA	NA
HLF	-1.677296484	0.002423023
USP2	NA	NA

Figure 6. The *WNK2*^{R2054Q} mutation alters the early joint response to injury. RNA-Seq analysis of whole knee joints of WT and *Wnk2*^{R2054Q} mice 14-days post DMM. **(A)** Volcano plots of altered genes in *Wnk2*^{R2054Q} mice compared to WT mice. **(B)** Bubble plots indicate that many pathways associated with OA are downregulated in *Wnk2*^{R2054Q} mice compared to WT mice. **(C)** Clock gene expression is disrupted in the knee joints of *Wnk2*^{R2054Q} mice. Yellow highlights indicate *CLOCK* genes that are significantly altered in *Wnk2*^{R2054Q} mice.

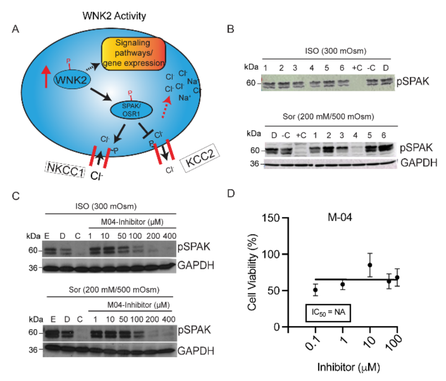


Figure 7. Identification of a novel chemical inhibitor of WNK2. (A) Diagram illustrating WNK2 activity. **(B-C)** Western blot analysis of pSPAK levels under isotonic (Top) (ISO; 300 mOsm) and sorbitol-induced hyperosmotic (Bottom) (Sor; 200 mM/500 mOsm) stress conditions. GAPDH was used as a loading 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.

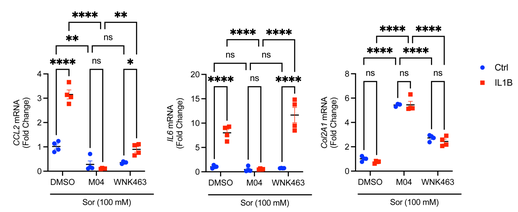


Figure 8. M04 inhibits IL1β mediated proinflammatory gene expression in chondrocytes and promotes COL2A1 expression. RT-qPCR analysis of control and IL1β stimulated T/C-28a2 chondrocytes. IL1β induces a proinflammatory response in control chondrocytes and M04 treatment inhibits this response, while upregulating COL2A1 gene expression. This effect is more pronounced in M04 vs WNK463(a pan-WNK1-4 inhibitor) treated cells, indicating some WNK activity is needed in chondrocytes.

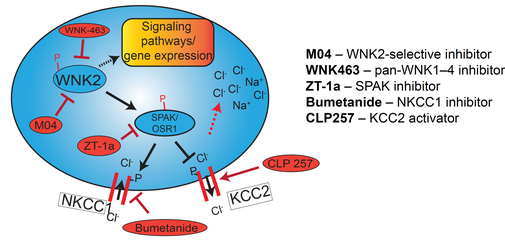


Figure 9. Determining what pathway WNK2/M04 functions through to inhibit IL1β mediated proinflammatory gene expression. Primary chondrocytes we treated with IL1β and the following compounds: M04 – WNK2 inhibitor; WNK463 pan-WNK1-4 inhibitor; ZT-1a – SPAK inhibitor; Bumetanide – NKCC1 inhibitor; CLP257 – KCC2 activator. Cells were treated for 24 hours and RNA was isolated for RNA-seq analysis.

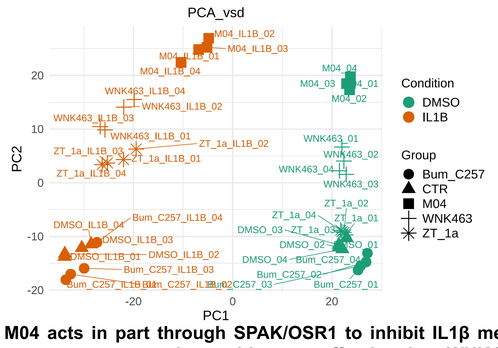


Figure 10. M04 acts in part through SPAK/OSR1 to inhibit IL1β mediated proinflammatory gene expression and is more effective than WNK463. PC analysis of RNA-Seq analysis of primary chondrocytes treated with IL1β and the following compounds: M04 – WNK2 inhibitor; WNK463 pan-WNK1-4 inhibitor; ZT-1a – SPAK inhibitor; Bumetanide – NKCC1 inhibitor; CLP257 – KCC2 activator.

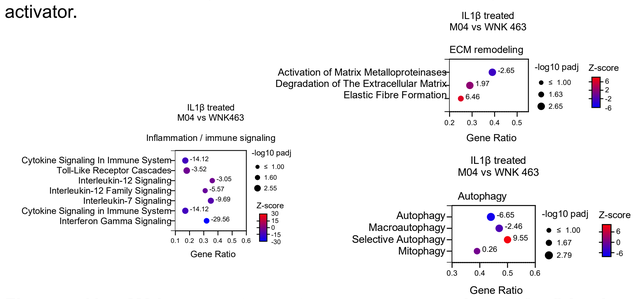


Figure 11. M04 promotes stronger suppression of OA-relevant inflammatory and immune pathways and reduced MMP-associated matrix remodeling compared with WNK463. Bubble plots indicating significantly altered pathways in M04 vs WNK463 in IL1β treated chondrocytes.

Conclusions

- We identified WNK2 mutations linked to dominant forms of familial OA. All WNK2 variants 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.
- WNK2/M04 inhibition in primary chondrocytes treated with IL1β reduces proinflammatory gene expression through SPAK-dependent and independent pathways.
- 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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