WNT16 Promotes Notochord Development and Improves Structure in the Intervertebral Disc

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INTRODUCTION: Intervertebral disc (IVD) degeneration is a major etiological factor in lower back pain which carries an estimated economic burden of at least $100 billion in treatment in the US and is the number one cause of job disability worldwide. IVD development centers on the notochord, a rod-like embryonic structure that contributes large, vacuole-filled cells to the nucleus pulposus (NP). *In vitro*, delivery of notochordal secreted factors to the IVD stimulates healthy matrix production and protects against catabolic cytokines in the IVD [1], making them compelling targets for IVD therapies. *WNT16* is a member of the *WNT* family of signaling proteins known for its role in mediating genetic influence on BMD and fracture risk. Recently, our team identified a role for *wnt16* in the notochord by showing that *wnt16* mutant zebrafish exhibit progressive defects in notochord expansion [2]. In parallel, our team has shown that in mice, *Wnt16* is the highest expressed in the IVD of the 19 *Wnt* ligands and that its expression is reduced during IVD degeneration induced by aging or compression [3]. Here, we used zebrafish and mouse models to test the hypothesis that WNT16 is a notochordal WNT signal necessary for vacuolated cell development, and whose overexpression protects the IVD from age-related degeneration.

METHODS: All animal studies were approved by an Institutional Animal Care and Use Committee (IACUC). Hybridization chain reaction RNA fluorescence *in situ* hybridization (RNA FISH) was performed using manufacturer's recommendations. Heterozygous *wnt16*$^{+/w1001}$ zebrafish [2] were crossed to create *wnt16*$^{-/-}$ mutants and wildtype (WT) controls. At 3 days post fertilization (dpf), animals were stained with 100µM BODIPY TR methyl ester and imaged live using confocal microscopy (WT n=6, *wnt16*$^{-/-}$ n=5). Vacuolated cells were manually segmented in 3D and shape descriptors were computed in MATLAB. For mouse studies, heterozygous male Tg(*Col1a1*-hWNT16) [4] were crossed to female Bl6 mice to generate heterozygous WNT16 overexpressing and WT offspring. Lumbar IVD and spinal segments were harvested at 8 months (WT n=5, WNT16 n=3) and 4 months (WT n=9, WNT16 n=7) of age. L1-3 were used for histology. Student's t-test was used for comparisons of two groups. For multi-factorial analysis, 2-way ANOVA was used followed by Sidak's multiple comparisons test.

RESULTS SECTION: We assessed the spatiotemporal expression of *wnt16* during zebrafish notochord development. At 1 dpf, staining for *wnt16* was observed mostly in the posterior half of the notochord. At 3 dpf, *wnt16* staining was localized to a subset of *col9a2*+ notochord sheath cells (specialized notochord cells that synthesize the notochord sheath) located at the ventral notochord midline (Fig 1A). To assess the role of *wnt16*, we performed 3D cell shape analysis in notochord vacuolated cells in *wnt16* mutants. Vacuolated cells in *wnt16*$^{-/-}$ mutants exhibited significantly reduced cell volume ($p<0.01$), with no significant difference in vacuolated cell number (Fig 1B, B'). Finally, we examined whether overexpression of WNT16 protects the IVD from age-related degeneration in mice. From Safranin O-stained images of the IVD (Fig 1C), degeneration scoring in the 8-month-old and the 4-month-old mice decreased in both WNT16 overexpression groups compared to controls, from 5 to 1.6 in the 8-month ($p<0.01$) and from 0.8 to 0.16 in the 4-month group ($p<0.05$). Furthermore, the IVD height increased from 183 µm to 284 µm ($p<0.01$) in the 8-month-old WNT16 overexpression group while there were no significant changes at 4 months of age (Fig 1D).

DISCUSSION: Our studies provide evidence that WNT16 is a notochordal signal whose overexpression promotes structure in the IVD. In regard to the role of *wnt16*, it appears to be required for vacuolated cell maturation (as evidenced by impaired size in *wnt16* mutants), but is dispensable for specification (since *wnt16* mutants have normal cell numbers). We also found that *wnt16* is dynamically expressed in the notochord, localizing to a subpopulation of notochord sheath cells immediately ventral to vacuolated cells. In our transgenic mouse studies, overexpression of WNT16 improved the structure of the IVD in the 8-month-old group by decreasing IVD degeneration score and increasing IVD height, a key characteristic of IVD degeneration, while only decreasing the degeneration score in the 4-month-old group. Thus, the benefit of overexpression of WNT16 appeared to be greater in the 8-month-old group compared to the 4-month-old group, which could be associated with the minimal expression of *Col1a1* in young-adult NP and its upregulation with aging in the NP. However, this requires further study. Taken together, WNT16 appears to be an important WNT signal for notochord development, and activating WNT16 signaling may improve structure in the aged IVD.

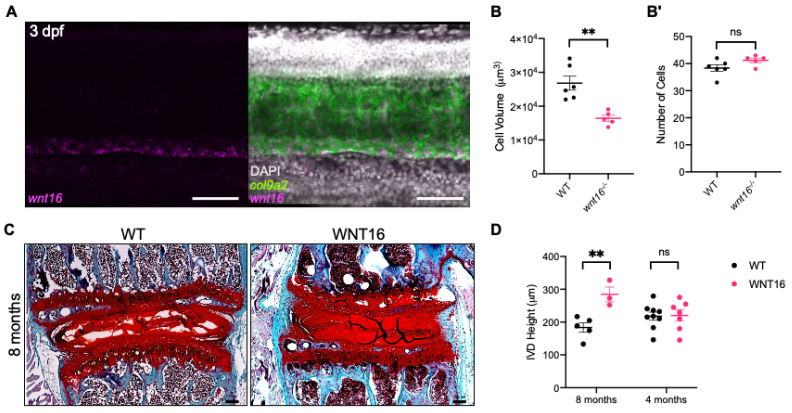

Fig 1. WNT16 is a notochordal WNT that promotes IVD structure. (A) HCR RNA in situ hybridization in 3 dpf wildtype zebrafish shows *wnt16* expression in the ventral notochord sheath cells (left). Notochord sheath cells are marked by expression of *col9a2* (right). Scale bar = 50 µm. (B-B') 3D analysis of notochord vacuolated cells comparing *wnt16*$^{-/-}$ zebrafish mutants and their wildtype siblings at 3 dpf shows decreased cell volume in *wnt16*$^{-/-}$ mutants (p-value = 0.02), but no significant difference in cell number (p-value = 0.1). (C) Sections of mouse IVD stained with Safranin O, taken from 8-month-old WNT16 overexpressing mice and their wildtype siblings. Scale bar = 100 µm. (D) IVD height increased significantly in the WNT16 overexpression group at 8 months (p-value = 0.0043), but not at 4 months (p-value = 0.99).

SIGNIFICANCE/CLINICAL RELEVANCE: Activating WNT16 signaling in the IVD of older individuals may serve as a therapeutic approach to IVD degeneration.

REFERENCES: [1] Erwin, et al. Arthritis Res Ther 2011. [2] Watson, Tang, et al. PLoS Genet 2022. [3] Holguin and Silva Sci Rep 2018. [4] Alam I, et al. Endocrinology 2016.

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