Title

Elucidating the role of p75NTR on condylar cartilage of mice using transcriptomics analysis

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Elucidating the Role of p75NTR in Condylar Cartilage of Mice Using Transcriptomics Analysis

Sara Jahangiri

ABSTRACT

The temporomandibular joint (TMJ) is one of the most frequently used joints in the human body, acting like a sliding hinge to connect the jawbone to the skull. Temporomandibular joint disorders (TMD) are dysregulations of the TMJ structure and function, prevailing in about 31% of the US population. One of the contributing causes of TMD is the degeneration of matrices, which are mediated by chondrocytes in the TMJ cartilage. The TMJ cartilage is derived from mesenchymal cells that differentiate into chondrocytes, forming the core of the cartilage.

The p75 neurotrophin receptor ($p75^{NTR}$), a single membrane-spanning protein in the tumor necrosis factor receptor superfamily, is a low-affinity receptor capable of binding all neurotrophins. Previous studies have demonstrated the role of $p75^{NTR}$ in the regulation of the mouse alveolar bone development and mineralization potential of ectomesenchymal stem cells (EMSCs). Studies have shown that global $p75^{NTR}$ deletion in mice have caused the mice to lose the ability to produce TMJ cartilage, concluding that $p75^{NTR}$ is required for TMJ chondrocyte differentiation and proliferation. Further investigation is required to delineate the role of $p75^{NTR}$ in chondrogenesis and its downstream signaling pathways, with an emphasis on identifying the receptor's impact on cartilage-related genes and cellular mechanisms.

Understanding the various target cells and genes that exist in the TMJ will help further our study of the role of $p75^{NTR}$ in the TMJ and enhance our therapeutic advances of TMDs. This study utilized a comprehensive suite of molecular techniques across wildtype (WT) and p75NTR knock-out (KO) mice, including RNA sequencing, network analysis, Alcian Blue staining, and quantitative real time Polymerase Chain Reaction (qRT-PCR). We analyzed gene expression

related to the BDNF pathway, cartilage markers, and critical signaling pathways potentially modulated by p75NTR.

RNA sequencing and network analysis identified downregulation of BDNF pathway genes critical for protein folding and extracellular matrix organization in KO mice. Alcian Blue staining validated chondrogenic differences, revealing increased proteoglycan content in the presence of BDNF. qRT-PCR confirmed downregulation of most BDNF genes as well as cartilage marker genes, including Ihh, Aggrecan, Sox9, and types II collagen in KO mice, implying a pivotal role for p75NTR in cartilage synthesis and integrity. Furthermore, qRT-PCR results indicated a significant reduction in the expression of Akt, PI3k, and mTOR genes in KO mice, while Jun expression remained unchanged, suggesting a complex cascade of relationships downstream of p75NTR.

The findings highlight the significant role of p75NTR in the regulation of genes and pathways critical to cartilage formation. The downregulation of key signaling molecules associated with p75NTR suggests a broad involvement of this receptor in cellular homeostasis. The study provides valuable insights into the molecular effects of p75NTR absence, setting the stage for potential therapeutic interventions in cartilage repair and neurodegenerative disorders.

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LIST OF ABBREVIATIONS

BDNF: brain-derived neurotrophic factor

BMSCs: bone marrow derived mesenchymal stem cells

EMSC: ectomesenchymal stem cells

KO: knock-out mice

MCC: mandibular condylar chondrocytes

NGF: nerve growth factor

p75NTR: p75 neurotrophin receptor

qRT-PCR: quantitative Real Time Polymerase Chain Reaction

RNA-seq: RNA-sequencing

TMJ: tempo-mandibular joint

TMD: tempo-mandibular joint disorder

WT: wildtype mice

1. INTRODUCTION

1.1 Preface

The temporomandibular joint (TMJ) is one of the most frequently used joints in the human body, acting like a sliding hinge to connect the jawbone to the skull. Temporomandibular joint disorders (TMD) are dysregulations of the TMJ structure and function, prevailing in about 31% of the US population (1). Some symptoms of TMD include myofascial pain, stiff jaw and neck muscles, limited movement or locking of jaw, painful clicking or popping of jaw, and occlusal changes. One of the contributing causes of TMD is the degeneration of matrices, which are mediated by chondrocytes in the TMJ cartilage. The TMJ cartilage is derived from mesenchymal cells that differentiate into chondrocytes, forming the core of the cartilage. The cartilage develops four distinct zones that are made up of various cells, mediated by various cell-signaling pathways (2).

1.2 Cartilage zones

The fibrous zone, also known as the superficial zone or reserve zone, is located at the periphery of the growth plate, adjacent to the epiphyseal bone. Chondrocytes in this zone are relatively inactive and appear small and flattened. The ECM consists mainly of type II collagen and proteoglycans. The fibrous zone serves as a reservoir of chondrocytes that can be recruited for proliferation and differentiation when needed for bone growth.

The second zone, the proliferative zone, lies adjacent to the fibrous zone and is characterized by a distinct columnar arrangement of chondrocytes. Chondrocytes in the proliferative zone undergo active cell division, leading to the longitudinal growth of the bone. These cells are arranged in columns parallel to the long axis of the bone. The ECM contains type II collagen and

proteoglycans, along with factors such as Indian hedgehog (Ihh) that regulate chondrocyte proliferation and differentiation.

The mature zone, also known as the hypertrophic zone or zone of maturation and hypertrophy, lies adjacent to the proliferative zone. Chondrocytes in the mature zone undergo hypertrophy, characterized by an increase in cell size and the synthesis of type X collagen and alkaline phosphatase. Hypertrophic chondrocytes signal the transition from cartilage to bone formation. The ECM becomes calcified, facilitating the invasion of blood vessels and osteoblasts for the formation of trabecular bone.

The hypertrophic zone is the innermost region of the growth plate, adjacent to the metaphysis or primary spongiosa. Chondrocytes in the hypertrophic zone continue to enlarge and undergo apoptosis. This process creates channels within the cartilage matrix, allowing for the invasion of blood vessels and osteoprogenitor cells. The calcified cartilage matrix serves as a scaffold for bone formation, ultimately leading to the replacement of cartilage with bone tissue in the process of endochondral ossification.

1.3 P75NTR mediated signaling

The p75 neurotrophin receptor ($p75^{NTR}$) is a low-affinity single receptor of all neurotrophins, including the nerve growth factor (NGF), and encodes a transmembrane glycoprotein that belongs to the tumor necrosis factor receptor (TNFR) superfamily (3). The p75 gene is located on chromosome 17q21-22 in humans and is composed of multiple exons that undergo alternative splicing to generate various isoforms. The primary isoform, p75NTR, consists of an extracellular domain, a single transmembrane domain, and an intracellular domain. The extracellular domain contains cysteine-rich repeats responsible for ligand binding, while the

intracellular domain harbors motifs implicated in signal transduction and protein-protein interactions. p75 engages in complex signaling pathways that modulate cellular responses to extracellular cues, including neurotrophins and other ligands. Upon ligand binding, p75 can activate downstream signaling cascades, such as the NF-κB pathway, the JNK pathway, and the RhoA pathway, leading to diverse cellular outcomes such as cell survival, apoptosis, neurite outgrowth, and synaptic plasticity (4). Moreover, p75 can interact with other cell surface receptors, including Trk receptors and integrins, to regulate cell adhesion, migration, and survival (5).

NGF is widely recognized as the most extensively studied neurotrophin, primarily acknowledged for its pivotal role in the embryonic development of the nervous system and the survival and differentiation of neuronal cells (6, 7). However, recent research has unveiled unexpected functions of NGF in various cell types beyond nerve cells, thus expanding our understanding of its roles. Studies have revealed that NGF and its receptors are involved in processes such as inflammation, bone resorption, and the acceleration of wound healing in mouse skin. Additionally, it has been demonstrated that NGF treatment can influence the fate of mesenchymal stem cells (MSCs), directing them towards neural lineages when exposed to specific conditions, and NGF secreted by MSCs can enhance the differentiation of neural stem cells (8, 9). Intriguingly, new studies suggests that NGF also plays a role in promoting bone formation and osteogenic differentiation (10, 11, 12). While lowaffinity receptor, p75NTR, is known to modulate signaling through the TrkA receptor, it is becoming more known that NGF binding to p75NTR mediates its own distinct signaling pathways and cellular events, such as activation of Rac GTPase and c-Jun N-terminal kinase (JNK) signaling in mouse oligodendrocytes (13).

Activation of p75NTR by NGF initiates downstream signaling pathways through its cytoplasmic death domain (DD). This domain interacts with several intracellular adaptor proteins, including TRAF6 (tumor necrosis factor receptor-associated factor 6), NRAGE (neurotrophin receptor-interacting MAGE homologue), and NRIF (neurotrophin receptor-interacting factor) (14). These interactions lead to the activation of signaling cascades such as the NF-κB pathway, the JNK pathway, and the RhoA pathway. The NF-κB pathway is involved in cell survival, proliferation, and immune responses. Activation of NF-κB promotes the expression of anti-apoptotic genes and pro-inflammatory cytokines (15). The JNK pathway regulates cell death, neurite outgrowth, and synaptic plasticity. JNK activation can induce apoptosis or promote cell survival, depending on cellular context and crosstalk with other signaling pathways (16, 17). The RhoA pathway influences cytoskeletal dynamics, cell migration, and neurite outgrowth (18). Activation of RhoA can lead to changes in cell morphology and motility through the regulation of actin cytoskeleton rearrangements. There is also evidence of interactions between p75NTR pathway with the Akt/Pi3k pathway (19). Upon activation by neurotrophin ligands, p75NTR can directly interact with and recruit Pi3k to the cell membrane. Binding of p75NTR to Pi3k leads to activation of Pi3k enzymatic activity, resulting in the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3), a key second messenger in the PI3K/Akt signaling pathway. Signaling pathways mediated by neutrophins binding to p75NTR and Trk receptors and their downstream cascades are shown in Fig 1 (20). Further examination needs to be made to understand the direct relationship between p75NTR pathway with downstream pathways such as Akt/Pi3k, JNK and NF-kB pathways.

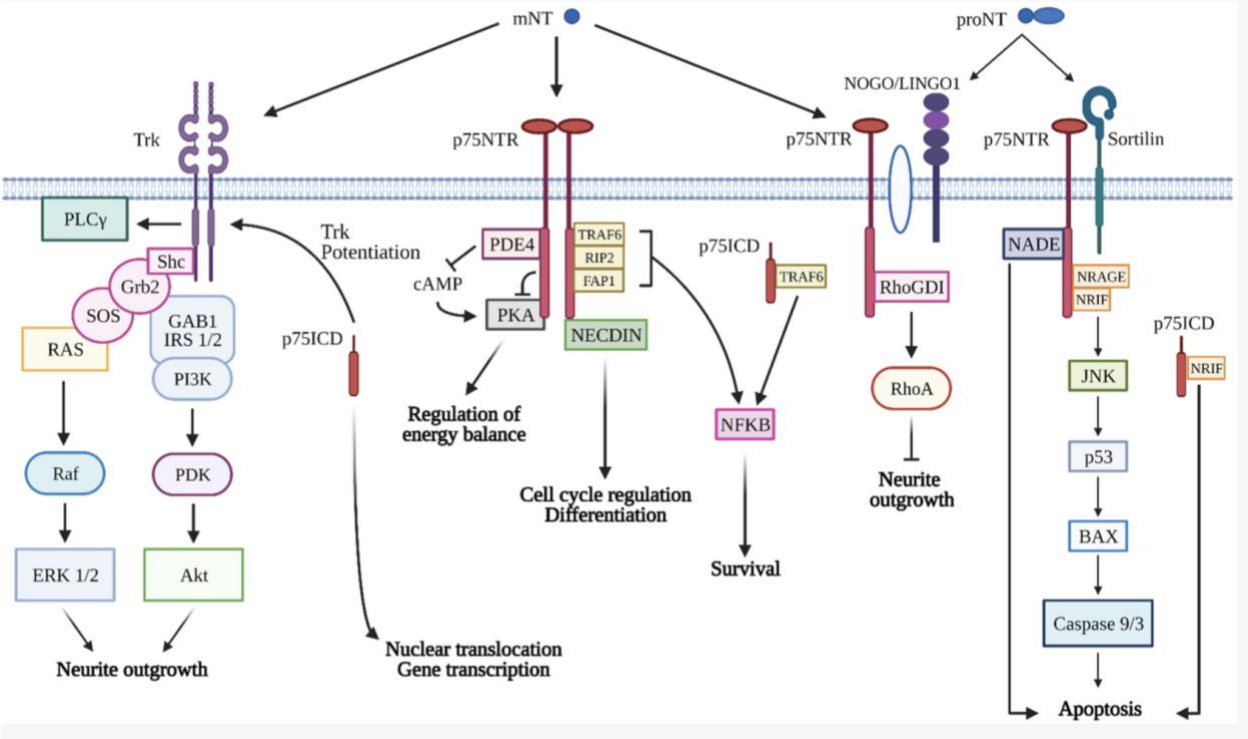

Figure 1. Signaling pathways mediated by neurotrophins' binding to their receptors.

Brain-derived neurotrophic factor (BDNF), Neurotrophin-3 (NT-3), Neurotrophin-4/5 (NT-4/5) are the other 3 neurotrophins that have the ability to bind to the low-affinity neurotrophin receptor P75NTR as well as the Trk receptors. These three neurotrophins have different receptor specificities. BDNF primarily binds to the TrkB receptor with high affinity, although it can also interact with the p75 neurotrophin receptor (p75NTR) to modulate signaling. NT-3 exhibits high affinity for the TrkC receptor, although it can also bind to TrkA and TrkB receptors with lower affinity. Additionally, NT-3 interacts with p75NTR. Lastly, NT-4/5 preferentially binds to TrkB, similar to BDNF, but it can also interact with TrkC and p75NTR. While historically studied primarily in the context of the nervous system, BDNF and its receptors have been detected in non-neuronal tissues, including cartilage, suggesting potential roles

beyond neuronal functions (21). It is our goal to study the BDNF signaling pathway more in depth to further elucidate it's role in cartilage turnover.

p75NTR can also interact with other cell surface receptors, including Trk receptors (tyrosine kinase receptors for neurotrophins) and integrins, to modulate signaling outcomes. Co-receptor interactions can enhance or suppress p75NTR-mediated signaling, depending on the ligand context and cellular environment. Trk receptors, particularly TrkA, can form complexes with p75NTR, enhancing NGF-induced signaling and promoting cell survival (22). Conversely, interactions with other neurotrophin receptors, such as TrkB and TrkC, can modulate p75NTR-mediated effects on neuronal differentiation and synaptic plasticity

1.4 Previous studies

Previous studies performed by the Hong lab have demonstrated the role of $p75^{NTR}$ in the regulation of the mouse alveolar bone development and mineralization potential of ectomesenchymal stem cells (EMSCs) (23). EMSCs are the progenitor cells of craniofacial hard tissue including the maxilla, mandible, and tooth tissue except for enamel. Studies have shown that global $p75^{NTR}$ deletion in mice have caused the mice to lose the ability to produce TMJ cartilage. The p75NTR KO mice showed decreased mandibular bone volume (Figure 2.A), reduced condylar head volume (Figure 2.B), and reduced condylar disc volume and thickness (Figure 2.D, E, F). Histological analysis of the TMJ of the p75NTR KO mice was also performed. The Alcian blue staining, which stains cartilage cells in blue, showed fewer cartilage cells in KO mice compared to WT mice. The Lastly, they performed histological analysis of the TMJ of p75 KO and wiltype mice. The Safranin O stain also showed fewer cartilage cells in the

KO mice (Figure 2.G, H). These results conclude that p75^{NTR} has a strong chondrogenic potential and may be required for TMJ chondrocyte differentiation and proliferation. These phenotypic changes in condylar cartilage encouraged further investigation to explore the role of p75^{NTR} at cellular level and its effect on the gene expression of the numerous of cells that make up the TMJ cartilage.

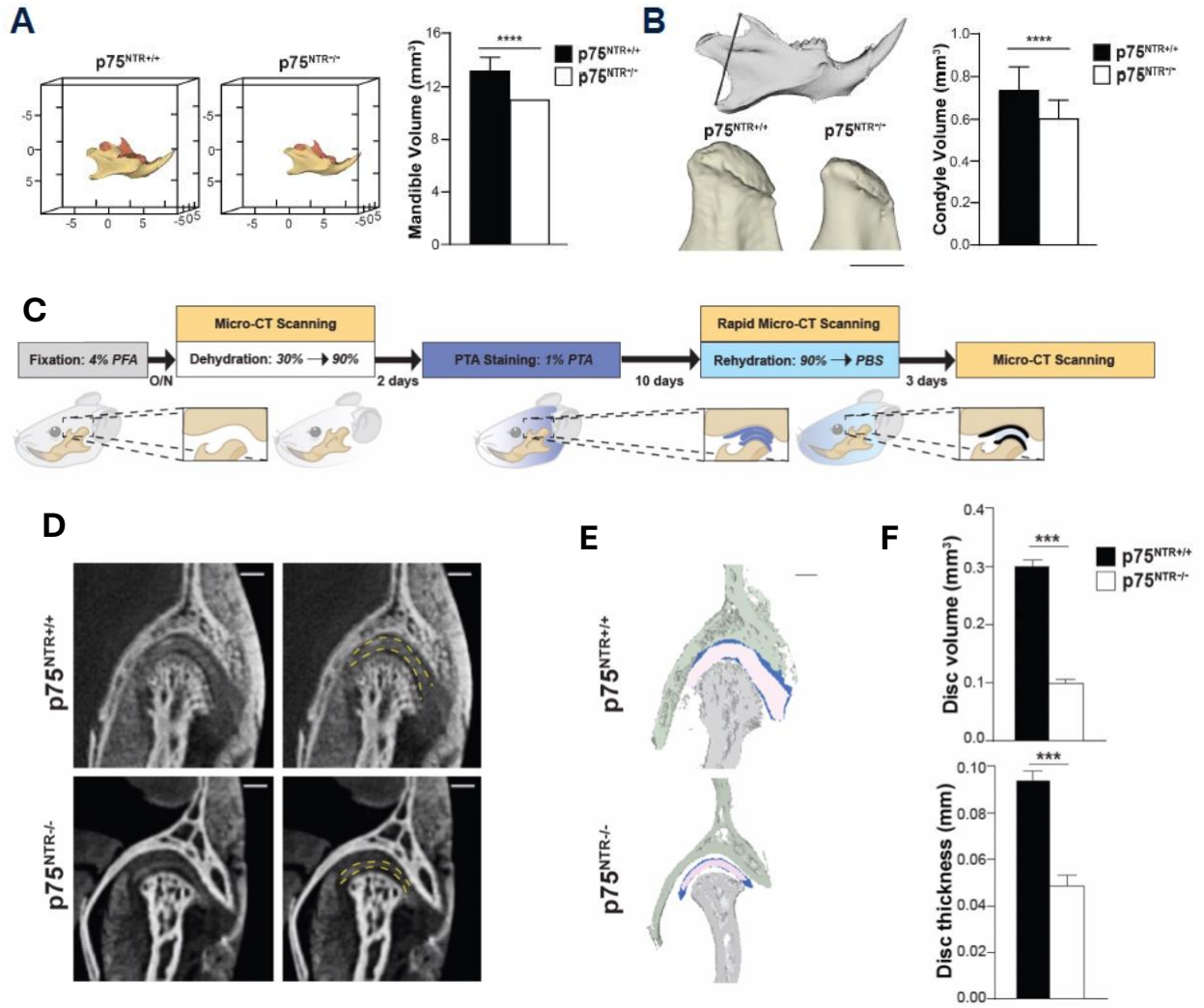

Figure 2a. A-H: Phenotypic study of mandibular condyle of 4-week-old p75^{NTR} KO mice.

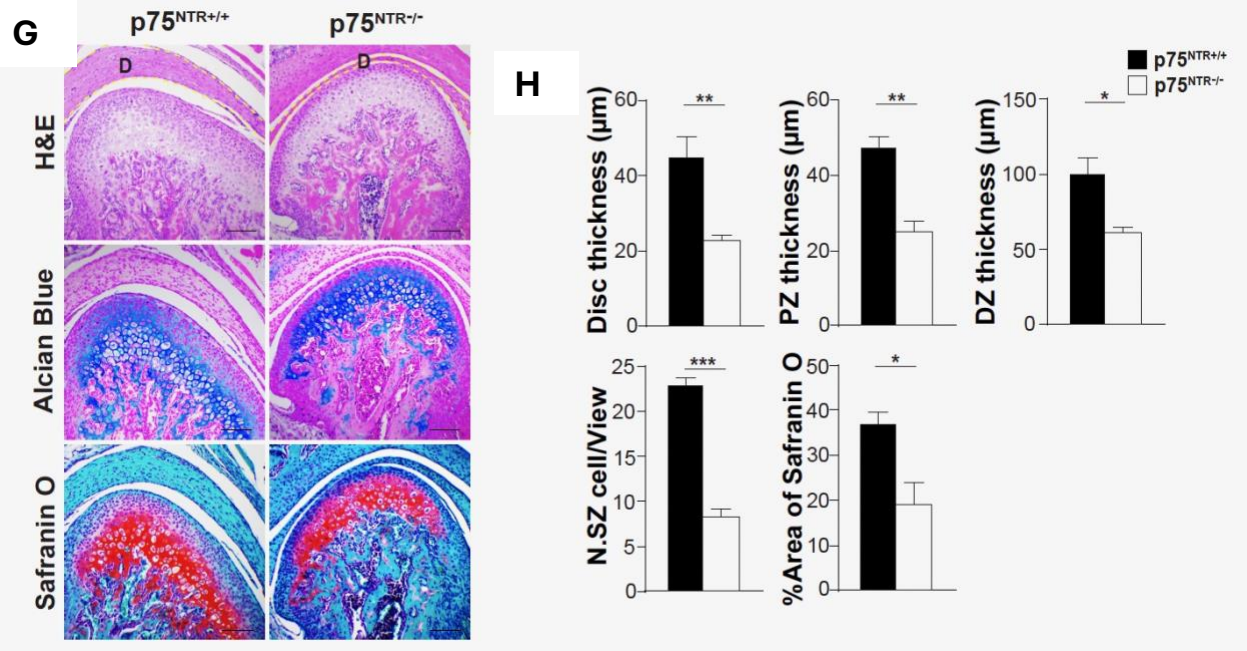

Figure 2b. A-H: Phenotypic study of mandibular condyle of 4-week-old $p75^{NTR}$ KO mice.

Understanding the various target cells and genes that exist in the TMJ will help further our study of the role of $p75^{NTR}$ in the TMJ and enhance our therapeutic advances of TMDs. RNA sequencing (RNA-seq) enabling comprehensive and quantitative analysis of gene expression profiles across diverse biological samples, describing the continuous variations in the transcriptome. Unlike traditional methods such as microarrays, RNA-seq offers several advantages, including unbiased detection of transcripts, single-nucleotide resolution, and the ability to quantify expression levels over a wide dynamic range. The workflow typically involves RNA extraction, library preparation, sequencing, and bioinformatics analysis. Key steps include mRNA enrichment, cDNA synthesis, adapter ligation, library amplification, and high-throughput sequencing. Sequencing reads are then mapped to a reference genome or transcriptome, and gene expression levels are quantified based on read counts or transcript abundance estimates. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) will be used on target genes in order to quantitatively measure the amount of a specific DNA target in our samples.

The goal of this study is to understand the role that $p75^{NTR}$ plays in the gene expression of TMJ cartilage in mice. We will study the bulk RNA sequencing of the isolated TMJ cartilage of $p75^{NTR-/-}$ (global knockout) mice to understand the quantitative gene expression relative to $p75^{NTR+/+}$ wildtype mice.

2. HYPOTHESIS

Hypothesis (Ha): p75NTR knockout (KO) mice will exhibit significant changes in the expression of cartilage marker genes and alterations in the Akt/PI3K/mTOR pathway components, as detected by RNA sequencing and qRT-PCR, compared to wildtype (WT) controls.

Null-hypothesis (H_0): p75NTR knockout (KO) mice will not exhibit significant changes in the expression of cartilage marker genes and alterations in the Akt/PI3K/mTOR pathway components, as detected by RNA sequencing and qRT-PCR, compared to wildtype (WT) controls.

3. SPECIFIC AIMS

AIM #1:

Comparing the transcriptomics of isolated TMJ cartilage of $p75^{NTR-/-}$ (global knockout) mice by RNA-seq.

AIM #2:

Elucidating p75NTR-mediated chondrogenic downstream pathways in TMJ cartilage of p75NTR KO and WT mice using qRT-PCR .

4. MATERIALS AND METHODS

4.1 Funding

The National Institutes of Health and the American Association of Orthodontics Foundation provided funding for the project, under the leadership of Dr. Christine Hong.

4.2 Sample size

4-week-old male mice were used in this project. 3 samples were p75NTR wildtype (WT) and 3 were global knock-out (KO) mice.

4.3 Mouse mandibular condylar chondrocyte (MCCs) Isolation

Mandibular condylar chondrocytes (MCCs) were isolated from mice by first dissection TMJ discs and condyles from C57BL/6J ages 4 weeks. TMJ condylar cartilages were then digested with 3 mg/ml collagenase type I (Sigma) and 4 mg/ml dispase II (Sigma) in 1X PBS for 3h at 37 °C. Single cell suspensions were cultured (5% CO_2, 37°C) in basal medium consisting of α-MEM (Gibco), 2 mmol/L glutamine (α-MEM-GlutaMax), supplemented with 20% fetal bovine serum, 100 U/ml penicillin/100 mg/ml streptomycin (1X Anti-Anti, Gibco), and 100 mmol/L 2-mercaptoethanol (Gibco) for 4–6 days. Cells were then detached with trypsin-EDTA (GIBCO) and plated at P1 for the in vitro experiments.

4.4 RNA sequencing (RNA-seq)

The condylar cartilage of P75NTR WT and KO mice were immersed in QIAzol Lysis Reagent (QIAGEN) and homogenized. Total RNA was extracted using RNeasy mini kit by first

centrifuging the harvested cartilage cells and discarding the supernatant. The cell pellet was then resuspended in lysis buffer (guanidine thiocyanate and B-mercaptoethanol) to lyse the cells and inhibit RNases. The lysate was then mixed and transferred to a gDNA Eliminator Spin Colum provided by the kit. The column was centrifuged to remove genomic DNA and other cellular debris, and then transferred to a new collecting tube containing ethanol to adjust the binding conditions for RNA. The RNA-containing flow-through was applied to an RNeasy spin column and centrigued. The RNA selectively binds to the silica membrane of the spin column. The RNeasy spin column was then washed with buffers from the kit to remove impurities. RNase-free elution buffer was added to elute the bound RNA. The concentration and purity of the extracted RNA was measured using a spectrophotometer. The extracted RNA was then frozen and stored in liquid nitrogen. Frozen RNA was sent to University of Connecticut, Core for RNA sequencing (RNA-seq). Fastq data was obtained and data analysis was performed using R studio, Enrichr and Cytoscape programs.

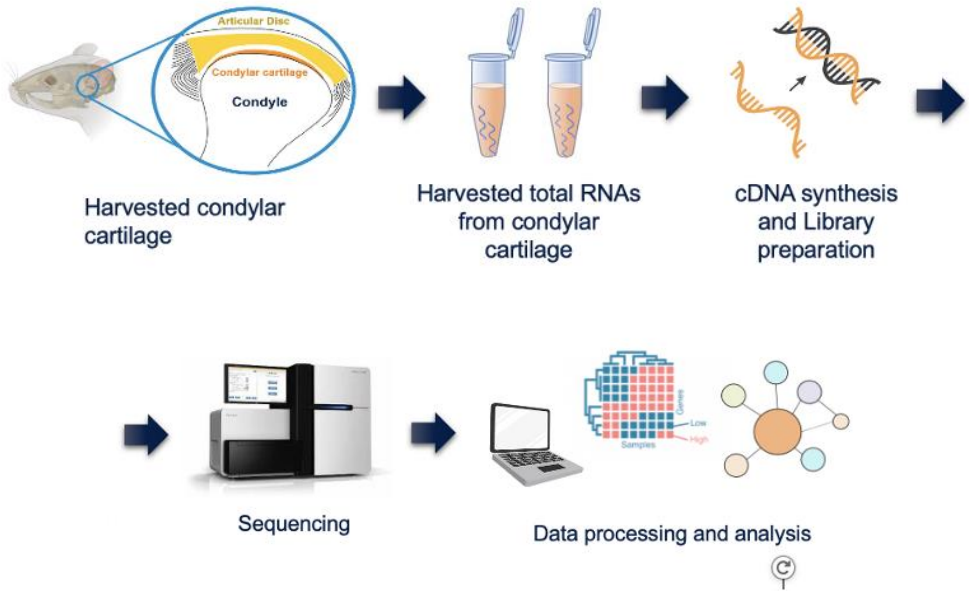

Figure 3. Flow of RNA sequencing.

4.5 Quantitative real time-polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from p75NTR WT and KO condylar cartilage of mice using QIAzol Lysis Reagent (QIAGEN). SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) was used to synthesize cDNAs from total RNA. PowerUp™ SYBR™ Green Master Mix for qPCR and the QuantStudio 3 Real-Time PCR system (Applied Biosystems) were used to perform the qRT-PCR reactions. Primers targeting the gene of interest were designed using Primer-BLAST, and qRT-PCR was conducted. To calculate the relative transcriptional expression, the Ct values of interested genes were normalized by average Ct values of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as ΔCt, and fold change in the relative transcriptional expression of interested genes was indicated with $2^{-\Delta\Delta Ct}$.

Listed below are the specific steps used to run qRT-PCR for the mRNA samples:

Materials

- cDNA of interested samples (diluted 10x with Nuclease free water after reverse transcription)
- 96 well PCR reaction plate and adhesive film for qRT-PCR
- PowerUp SYBR Green Master Mix (Applied Biosystems) 4℃, stock bottles are in the -20℃ freezer.
- Nuclease free water
- 10μM of Primers (Forward and Reverse for each)
- Ice

QuantStudio Preparation:

1. Turn on the QuantStudio 3 (Applied Biosystems)
2. In the New Experiment window, select template and open FAST_Power Up SYBR green 96well template folder
3. Add the name of the person performing the PCR, the experiment number, and the name of the primer to be used with the same plate this time, all after the characters originally displayed
4. Make the experimental plate design for 96well plate

   ∗ Use 3 wells per sample every time to measure since we take an average to know the exact value.

   Input sample and primer names in the window, respectively as image below.

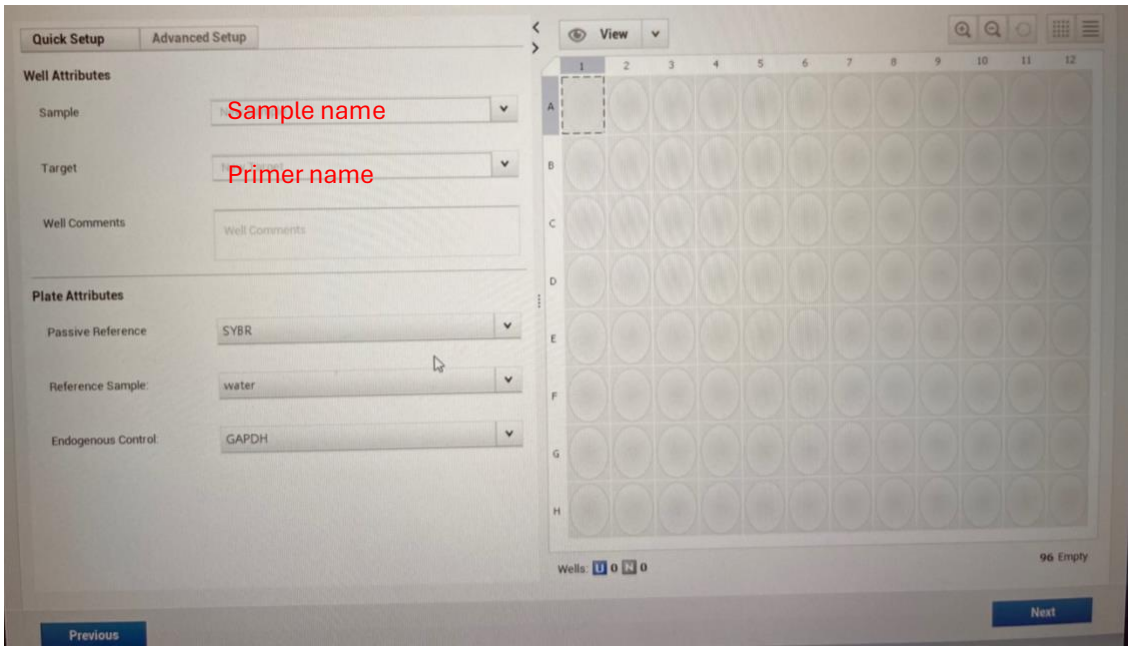

Figure 4. Example of QuantStudio Preparation.

5. Click the eject button in the upper right corner of the QuantStudio3 machine screen, place the prepared 96 well plate, and click the eject button again to close it.
6. Click the down arrow to the right of START RUN, and select the number 272326377
7. qRT-PCR will be started. FAST SYBR protocol will be finished around 44 min.
8. After qRT-PCR is done, click the analyze button and save the file

Sample Preparation:

1. Pipet 2 microliters of cDNA into a 96-well plate as designed
2. Mix Forward and Reverse primers, SYBR and water following the recipe.

	Volume (µl/well)
powerup SYBR green master mix (2X)	10
Forward primers(stock:10uM)	1
Reverse primers(stock:10uM)	1
Nuclease free water	6
cDNA templete (After RT, Dilute to 1/10 with RNAse/DNAse free water)	2
Total	20

3. Add 18µl of qRT-PCR mix to each wells as designed
4. Seal the top with the adhesive film. Do not make wrinkles on the wells.
5. Centrifuge for 10 sec
6. Set in QuantStudio at the appropriate time

4.6 Chondrogenesis on bone marrow derived mesenchymal stem cells (BMSCs) and Alcian Blue staining

Bone marrow derived mesenchymal stem cells were harvested from femurs and tibia of 4-week-old WT mice. The cells were cultured in a 24-well with chondrogenic induction media in the presence of various concentrations of BDNF or NGF with steps as followed: 1×10^5 cells were cultured in each well of 24-well tissue culture plate for differentiation. When the cultures were 80% confluent, the chondrogenic induction medium (DMEM high glucose medium containing 10% FBS, 0.1 µM dexamethasone, with 1% fetal bovine serum, 10 ng/ml transforming growth factor-β3, proline, sodium pyruvate, and 100 µg/ml ascorbic acid-2-phosphate) was used for differentiating the cells by changing every 3 days. On day 21 post treatment, the cells were stained with 1% Alcian Blue 8GX (Sigma-Aldrich, MA, USA) in 0.1N HCl for evaluating chondrogenic differentiation. ImageJ was used to measure Alcian Blue positive areas.

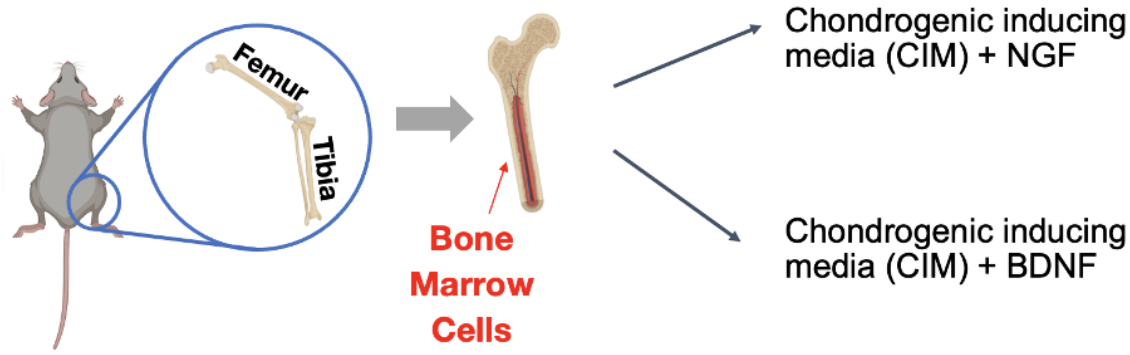

Figure 5. Depiction of BMSCs used for Alcian Blue staining.

5. RESULTS

5.1 Bulk RNA-Seq of 4-week-old p75NTR KO and WT condylar cartilage

Extracted RNA from p75NTR WT and KO mice was sent to University of Connecticut for RNA sequencing. Fastq data was obtained and data analysis was performed using R studio, Enrichr and cytoscape.

Differential expressed (DE) analysis was performed on the data received from qRT-PCR on the two groups of WT and P75NTR KO mice. A volcano plot was created to show the gene expression changes between the groups, based on DEseq2 with filter absolute log2foldchange greater than 0.5 and p-value less than 0.05 (Fig 6). The volcano plot shows a clear distinction between downregulated, upregulated and unchanged genes. We found 359 genes to be statistically significant (p value < 0.05) between the two groups. The upregulated genes are highlighted in red, while downregulated genes are highlighted in blue.

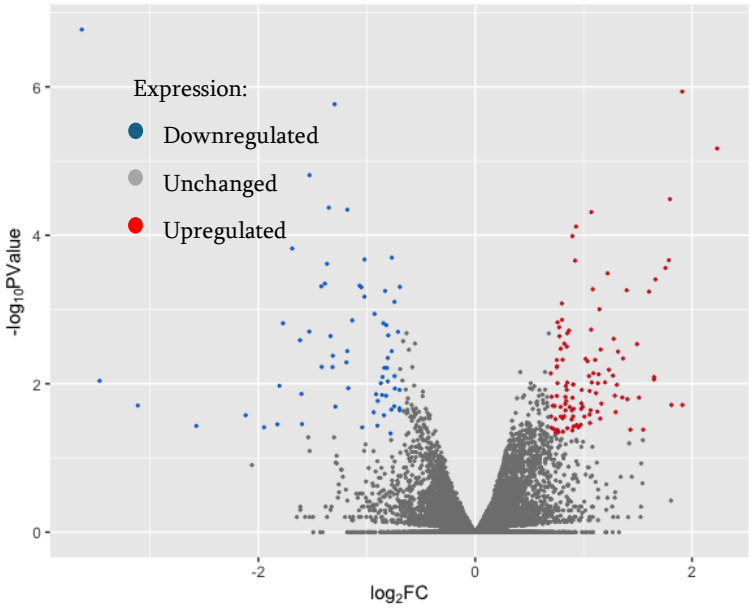

Figure 6. Volcano plot of male WT vs. KO found 359 significant genes (p-value<0.05).

A heatmap was created to visualize the gene expression patterns and identify clusters of coregulated genes in our samples (Fig 7). Each column represents a sample, 3 of which are WT mice and 3 are p75NTR KO mice. Each cell in the heatmap represents the expression level of a specific gene in a particular sample or condition. The cells colored in purple were the downregulated genes in each sample, while the cells colored in yellow were the upregulated genes. Cells in black were unchanged. We can clearly visualize a pattern distinguishing the WT and KO samples. For example, looking at the left half of the heatmap shows upregulation in WT samples and downregulation in KO samples for those sets of genes. This data confirms that p75NTR KO does have a substantial effect on overall gene expression levels, suggesting that p75NTR may be responsible for regulating these genes. Further analysis is required to understand what specific genes and pathways are mostly affected and what their roles are.

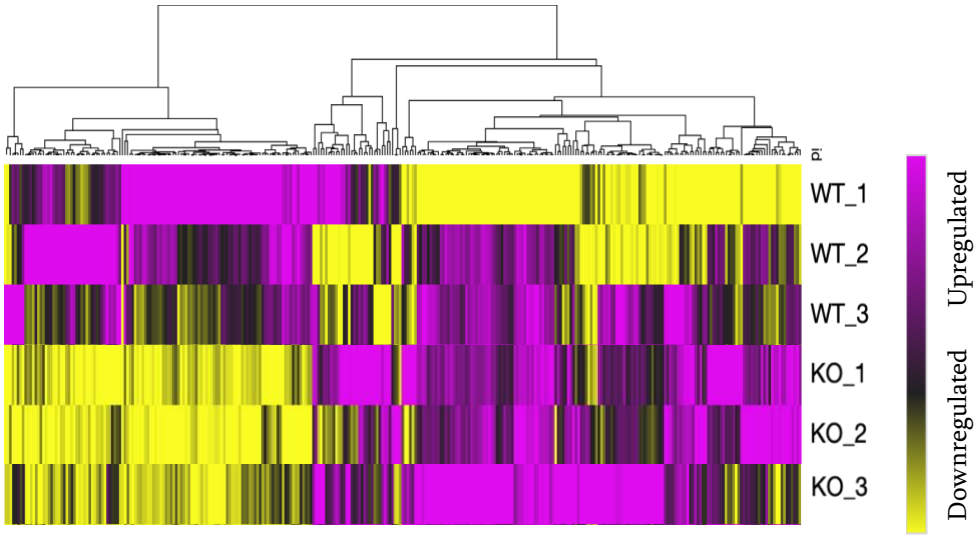

Figure 7. Heatmap of upregulated and downregulated genes in WT and KO mice.

We looked more specifically into the genes and pathways that were affected by taking the downregulated genes and sorting them into their specific pathways. Pathway analysis using BioPlanet 2019, Reactome 2022, and WikiPathway 2022 was performed on downregulated genes of p75NTR KO samples with the Enrichr program. After sorting the genes, we ranked each pathway by significance (Fig 8a-c). It is evident that the majority of genes that were downregulated are associated with pathways related to bone, cartilage, or ECM formation. The BDNF signaling pathway was the most significantly downregulated pathway according to the BioPlanet 2019 analysis. There are a total of 18 genes involved in the BDNA pathway. Network analysis of the 18 downregulated genes in the BDNF signaling pathway was completed to study the relationship between the 18 genes based on their expression patterns (Fig 9).

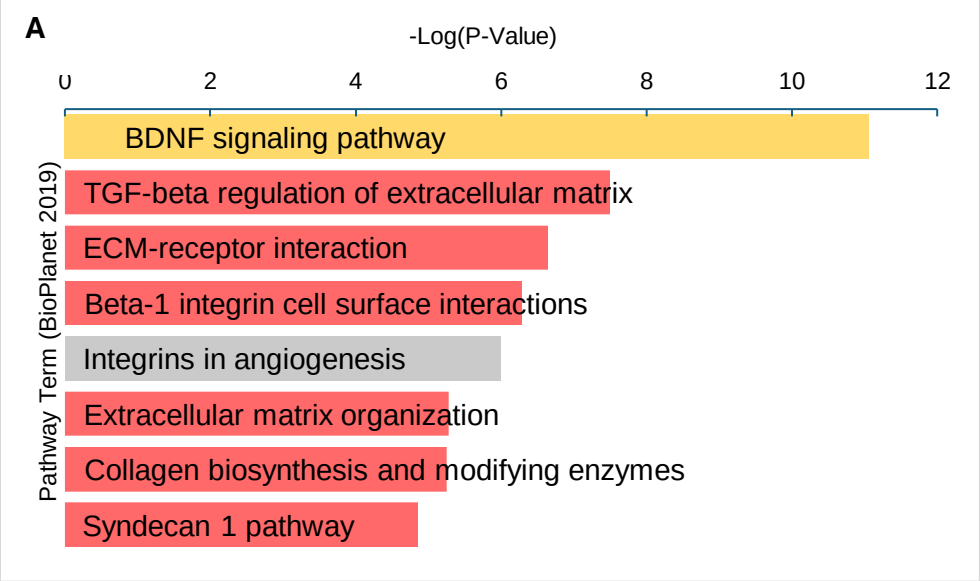

Figure 8a: Pathway analysis on KO downregulated genes using Enrichr.
A. Pathway analysis using BioPlanet 2019. Total of 18 genes were downregulated in the BDNF pathway.
B. Pathway analysis using Reactome 2022.
C. Pathway analysis using WikiPathway 2022.

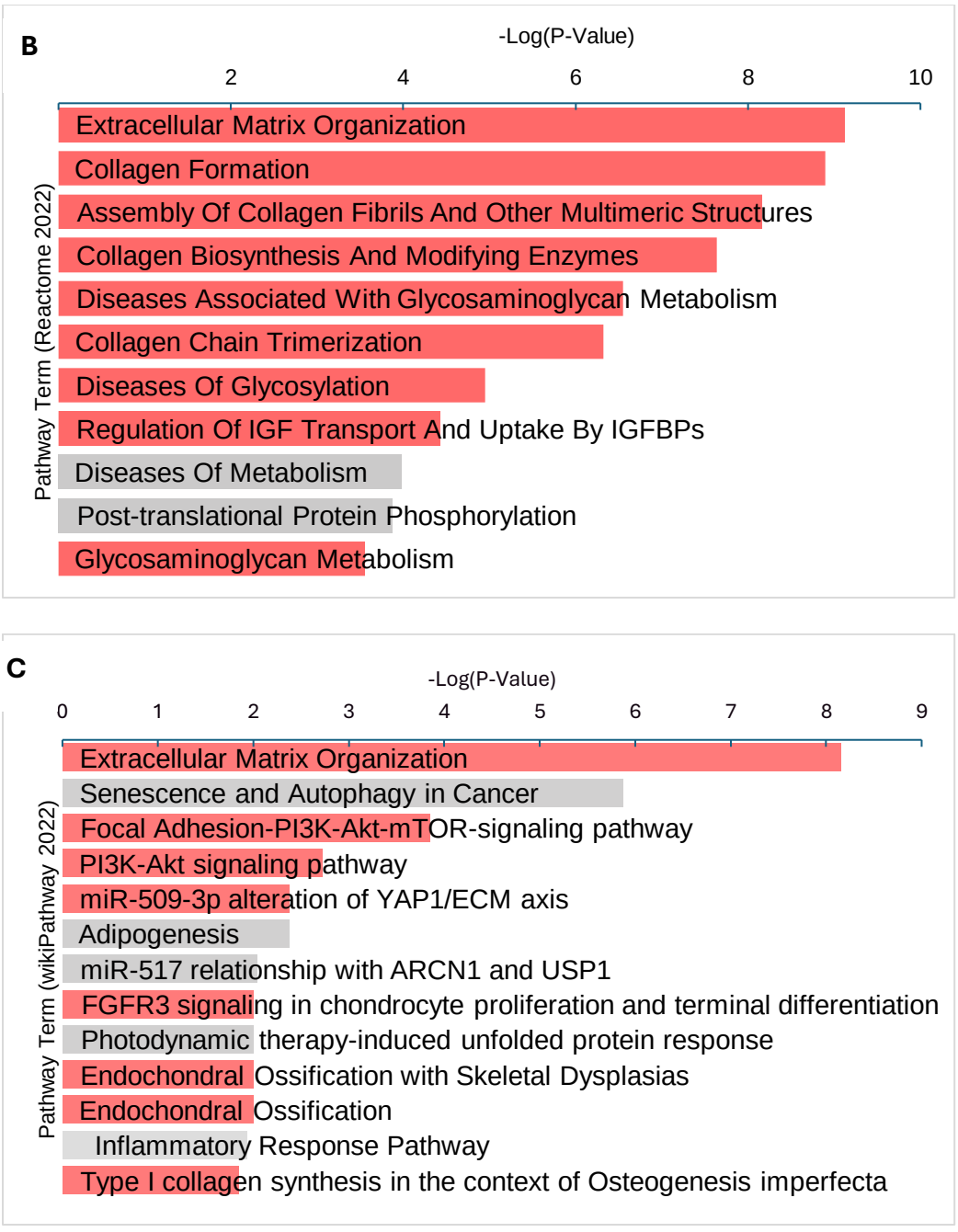

Figure 8b: Pathway analysis on KO downregulated genes using Enrichr.
A. Pathway analysis using BioPlanet 2019. Total of 18 genes were downregulated in the BDNF pathway.
B. Pathway analysis using Reactome 2022.
C. Pathway analysis using WikiPathway 2022.

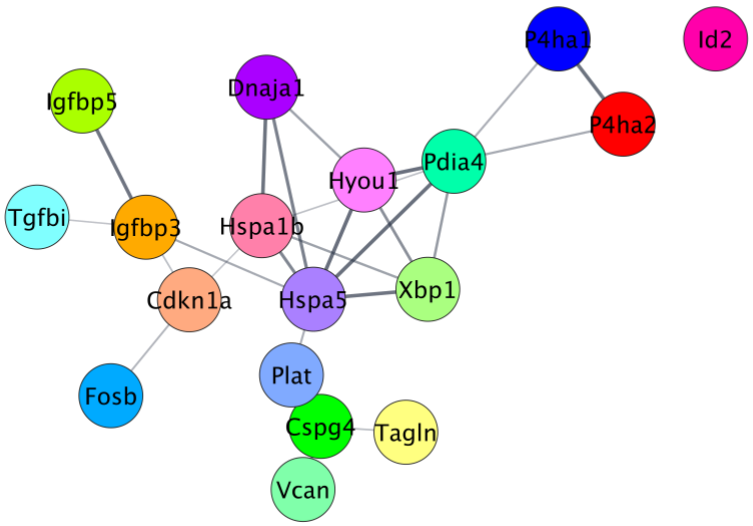

Figure 9: Network analysis of 18 downregulated genes in KOs listed in BDNF signaling pathway.

5.2 Chondrogenic differentiation with ligands for P75NTR

The RNA-seq data showed a strong correlation between p75NTR and bone/cartilage pathways. In order to verify this correlation, we wanted to figure out which neurotrophin is the main ligand for p75NTR involved in chondrogenesis. We used the bone marrow that we harvested from the mice and induced chondrogenesis in the presence of neurotrophins NGF and BDNF. After inducing chondrogenesis, we stained each well with Alcian blue, which turns cartilage dark blue (Fig 10). This lets us compare the amount of cartilage for each well. We found that the higher concentration of BDNF (50ng/mL) combined with the chondrogenic inducing medium (CIM) had the highest %area of cartilage cell development across the board. The increased cartilage development between BDNF and NGF was also statistically significant. This is an indicator that BDNF is likely the ligand responsible for p75NTR regulation of chondrogenesis.

CIM | CIM + NGF + 20ng/mL | CIM + NGF + 50ng/mL | CIM + BDNF + 20ng/mL | CIM + BDNF + 50ng/mL

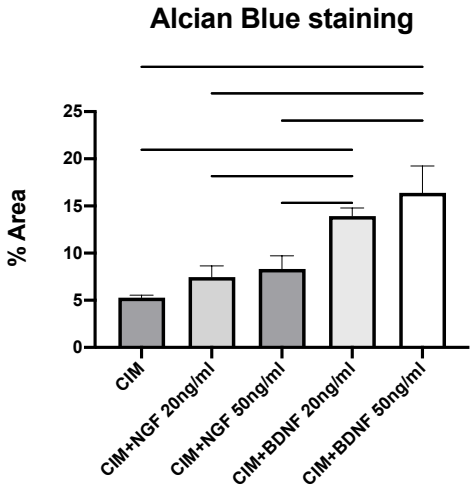

Figure 10: Alcian Blue is a standard stain used to quantify cartilage.

5.3 QRT-PCR analysis of relevant genes

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was used to confirm and quantify the downregulation of genes observed in the pathway analysis of the RNA-seq data. We were interested in studying 3 different categories of genes: those involved in the BDNF signaling pathway, well-known chondrogenic marker genes, and genes downstream of the p75NTR signaling pathway. The qRT-PCR data of candidate genes were then analyzed and visualized in

a bar graph using prism software (Fig 11). The genes that showed a statistically significant ($p<0.05$) between WT and KO samples were marked with *.

All 18 genes in the BDNF pathway were downregulated based on the RNA-seq results. The qRT-PCR confirmed that 12 of the 18 showed a statistically significant decrease in expression levels in the p75NTR KO group compared to the WT group. Furthermore, 8 of them are known to be directly involved in chondrogenesis: XBP1, CDKN1A, VCAN, P4HA2, ID2, and CSPG4.

We chose to study 5 known chondrogenic markers: Ihh, Aggrecan, Sox9, Col2a1, and Col10. From these genes, Ihh, Aggrecan, Sox0 and Col2a1 showed a statistically significant decrease of expression level in KO mice compared to WT mice. This correlation further elucidated the potential role that p75NTR plays in chondrogenesis. Lastly, we were interested to study the effects of p75NTR KO on downstream pathways and target genes. The qRT-PCR data portrayed a statistically significant decrease of Akt/Pi3k and mTOR genes in the KO sample, elucidating that p75NTR being knocked out directly decreases the expression levels of the Akt/Pi3k and mTOR pathways. Interestingly, no statistically significant difference was seen in the c-Jun pathway between the two groups.

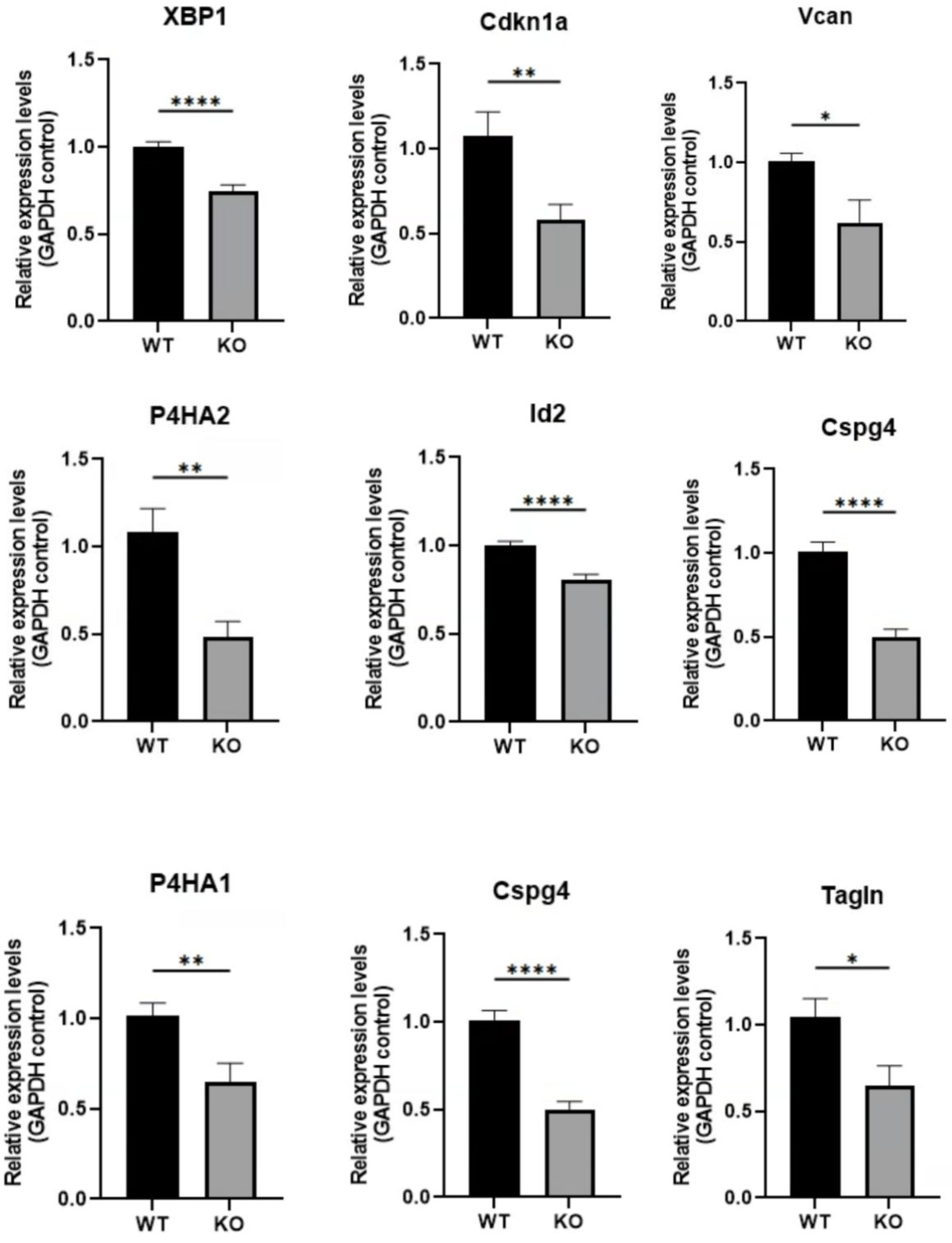

Figure 11a. Bar graphs showing qRT-PCR results of target genes in p75NTR KO and WT mice.

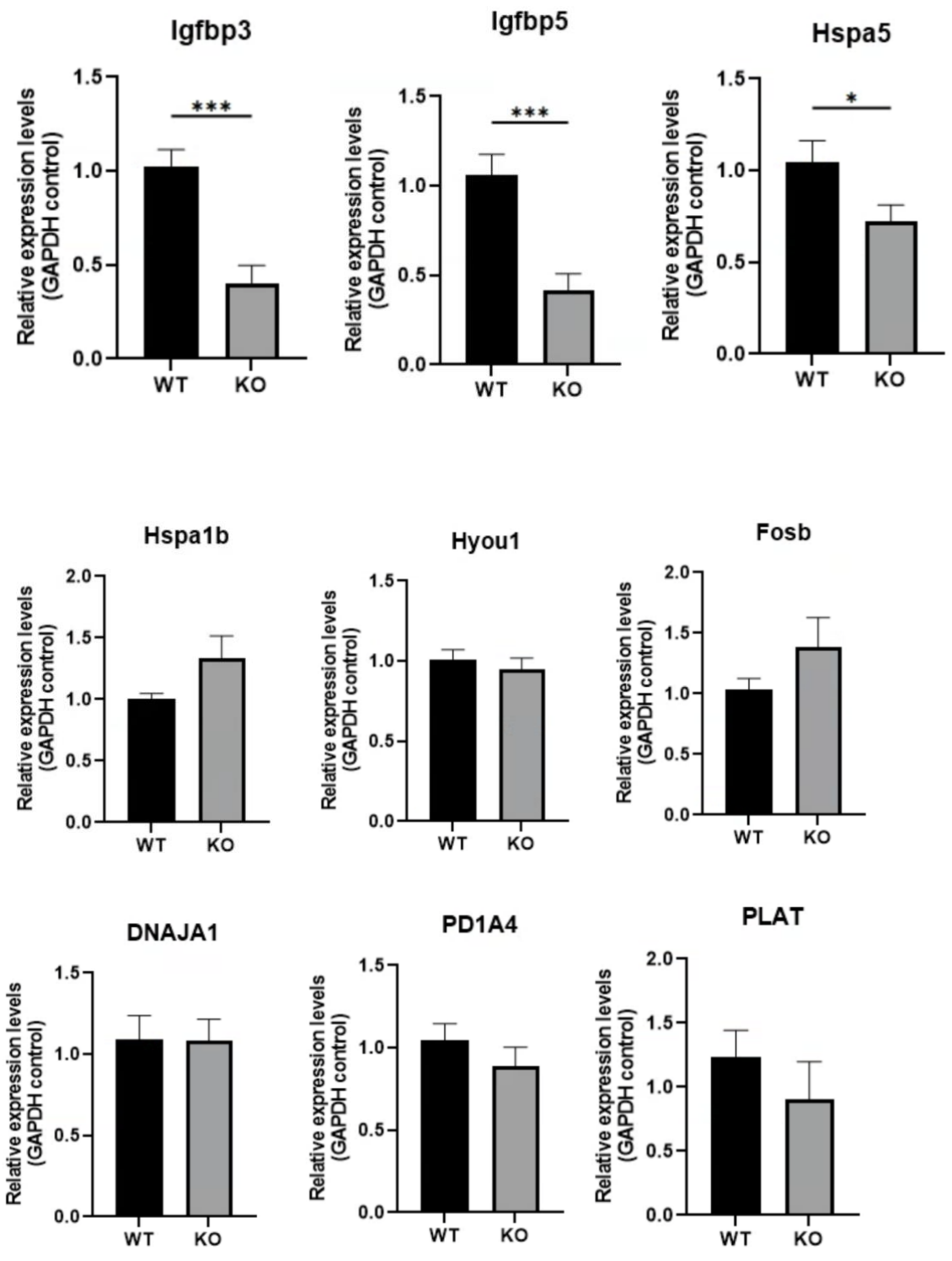

Figure 11b. Bar graphs showing qRT-PCR results of target genes in p75NTR KO and WT mice.

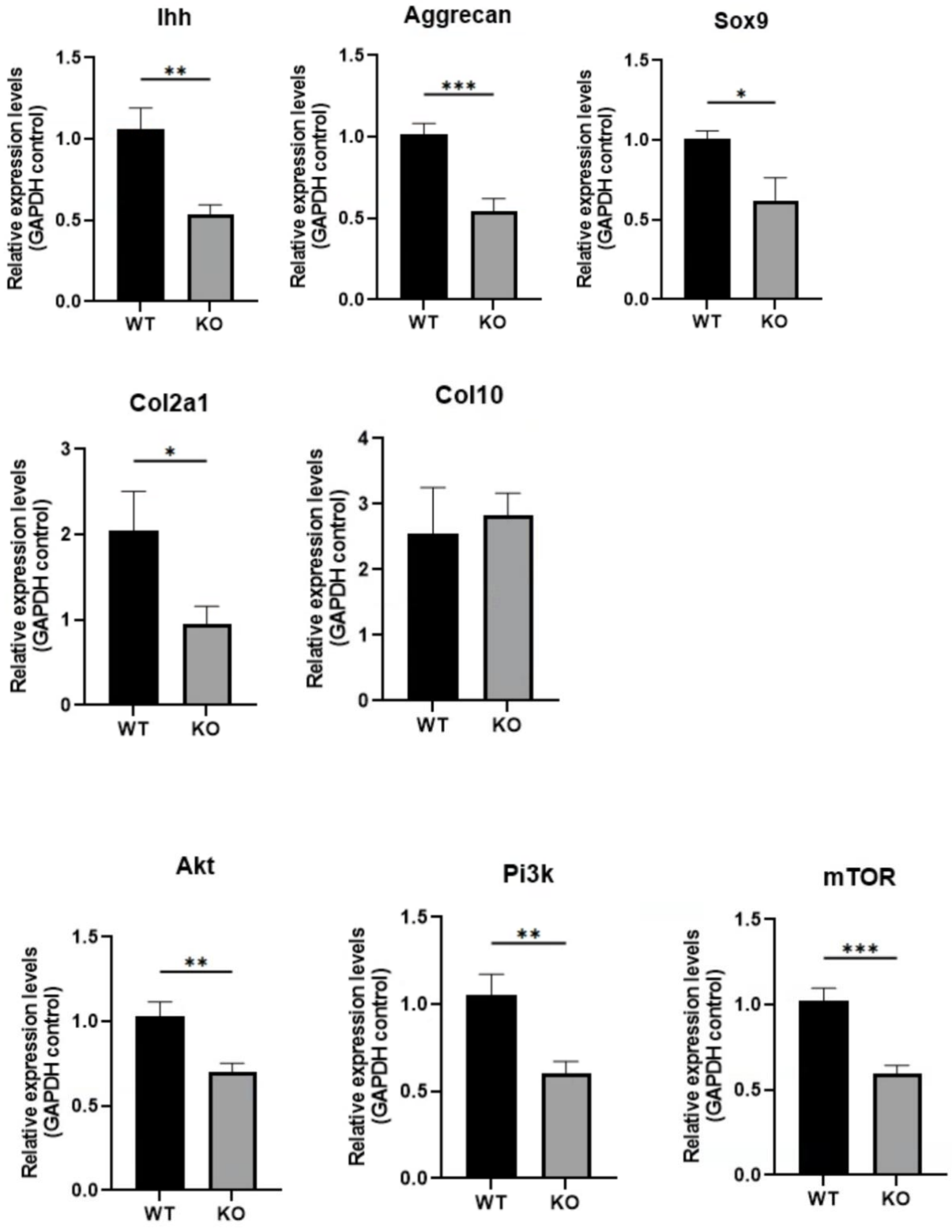

Figure 11c. Bar graphs showing qRT-PCR results of target genes in p75NTR KO and WT mice.

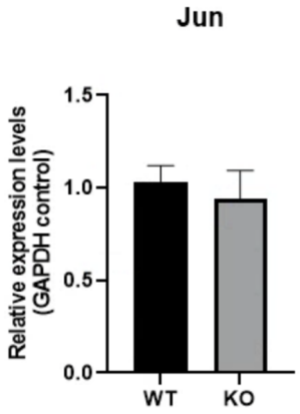

Figure 11d. Bar graphs showing qRT-PCR results of target genes in p75NTR KO and WT mice.

6. DISCUSSION

The p75 neurotrophin receptor (p75NTR), also known as the low-affinity nerve growth factor receptor, is a member of the tumor necrosis factor receptor superfamily. It binds to several neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), as well as their pro-neurotrophin forms. In the nervous system, the p75 neurotrophin receptor (p75NTR) plays a critical role by mediating the effects of neurotrophins, which are essential for neuron survival, growth, and apoptosis. It acts as a signaling receptor that can trigger cellular responses leading to growth, differentiation, or death of neural cells. p75NTR is involved in developmental processes, ensuring proper neuron density and connectivity, and it also participates in responses to injury, aiding in nerve regeneration and plasticity. Its function is complex, as it can promote both survival and apoptosis depending on the cellular context and the specific neurotrophins involved.

Although p75NTR is primarily associated with nervous system development and function, its role in chondrogenesis is currently being explored in literature. The Hong et al. lab showed phenotypic changes in mice condyle when p75NTR was knocked out. The p75NTR KO

mice showed decreased mandibular bone volume, reduced condylar head volume, and reduced condylar disc volume and thickness. Histological analysis of the TMJ also showed fewer cartilage cells in the p75NTR KO mice compared to WT mice. These phenotypic changes lead me to further question and explore the changes that occur genotypically when p75NTR is knocked out.

The RNA sequencing data from p75NTR knock-out and wildtype mice provided substantial insights into the molecular mechanisms potentially influenced by the p75NTR. Analysis via volcano plots and heat maps allowed for the identification of differentially expressed genes, which are crucial for understanding the specific pathways and cellular processes that may be disrupted or altered due to the absence of p75NTR.

Our volcano plot analysis highlighted several genes that were significantly upregulated or downregulated in the p75NTR knock-out mice compared to their wildtype counterparts (Fig 1). We found 359 genes to be statistically significant (p value < 0.05) between the two groups. The upregulated genes are highlighted in red, while downregulated genes are highlighted in blue. The upregulation of genes suggest a compensatory mechanism that might be attempting to counterbalance the lack of p75NTR. This is consistent with literature indicating p75NTR's role in promoting neuronal survival and regulating apoptosis through various pathways, including but not limited to the NF-kB and JNK pathways. We are particularly interested in the downregulated genes in order to better understand the specific downstream pathway of p75NTR.

The heat maps further provided a visual representation of gene expression patterns across multiple samples, illustrating the consistency of the genetic impact of the p75NTR deletion (Fig 2). By clustering similar expression patterns, heat maps facilitated an understanding of the consistency and variability of gene expression changes. This method was particularly useful in

identifying groups of genes that may work in concert, affecting neuronal development, survival, or other neurophysiological processes in the absence of p75NTR. Specifically, we can see that the lower left segment of the heatmap shows the clusters of genes that are all downregulated in the KO mice, compared to their upregulated counterparts in the WT mice. This visual depiction shows us the variability in the gene expression profiles between the two groups. Now that we understood that there is infact a clear difference between gene expression profiles of p75NTR KO mice to those of WT counterparts, we want to further understand what those specific genes and pathways are to better understand p75NTR's role in chondrogenesis.

We performed pathway analysis of the RNA-seq data using BioPlanet 2019, Reactome 2022, and WikiPathway 2022 databases, to provide a comprehensive overview of the genetic landscape differentially expressed between p75NTR KO and WT mice (Fig 3). The graphs indicate a the statistically significant downregulation of crucial biological pathways. All the pathways that are highlighted in red, making up the majority of the significant pathways, are pathways that have some sort of relationship to cartilage and collagen. This is extremely interesting given that there is not much literature showing the relationship of p75NTR, which is previously known for it's role in the nervous system, to cartilage and collagen.

The WikiPathway 2022 data highlighted the downregulation of the PI3K-Akt signaling pathway, which has known roles in cellular survival, growth, and metabolism. This observation correlates with the known interactions between neurotrophins and the PI3K-Akt pathway, mediated by p75NTR, further illustrating the extensive network influenced by this receptor. The downregulation of this pathway in KO mice may explain the molecular basis for observed phenotypic differences related to neural survival and apoptosis, as well as relationships with cartilage and chondrogenesis. Additionally, the BioPlanet 2019 database showed that the TGF-

beta pathway's regulation of the extracellular matrix and integrin cell surface interactions were notably downregulated, implicating p75NTR in broader cellular adhesion and migration processes.

The most profound downregulation in the BioPlanet 2019 database was observed within the BDNF signaling pathway. This is consistent with the expected role of p75NTR, as BDNF is one of its ligands. The attenuation of BDNF signaling in p75NTR KO mice suggests a pivotal role of this receptor in mediating BDNF's effects on neuronal survival and synaptic plasticity. The specific genes within the BDNF pathway are: XBP1, CDKN1A, TAGLN, IGFBP5, HSPA5, IGFBP3, PLAT, PDIA4, DNAJA1, VCAN, P4HA1, P4HA2, ID2, FOSB, HYOU1, CSPG4, TGFBI, and HSPA1B.

We performed network analysis of the BDNF pathway genes to understand the complex interaction landscape among the genes (Fig 4). Network analysis involves creating a network or map that illustrates how these entities interact with one another within a cell or across a biological system. These networks can help identify central nodes (key regulators or hubs) and pathways that are critical for certain biological functions, which can be important for understanding complex biological processes. Central nodes such as Hspa5, Xbp1, and Pdia4, which are intimately involved in protein folding and response to stress within the endoplasmic reticulum, appear to be key mediators. The prominence of these genes suggests a substantial impact of p75NTR signaling on the unfolded protein response, a crucial cellular process for maintaining protein homeostasis. Moreover, the network highlights the interaction between classical neurotrophic factors like BDNF and elements of the extracellular matrix such as Vcan and Tgfb1, bridging neurotrophic signaling with the structural and adhesive properties of neural tissue. Tgfb1 is a regulatory molecule that influences the production of the extracellular matrix,

including collagens, crucial for cartilage repair and homeostasis. P4ha1 and P4ha2 play a direct role in collagen maturation, a protein essential for cartilage integrity. This highlights a potential mechanistic link between BDNF signaling and cartilage biology, providing avenues for further research into cartilage-related diseases.

Building upon our network analysis, which suggested a link between BDNF signaling and cartilage biology, we progressed to a direct assessment of chondrogenesis via Alcian Blue staining (Fig 5). Alcian Blue preferentially binds to cartilage-specific proteoglycans, enabling the visualization of chondrogenesis and staining cartilage cells in blue. In our experimental setup, bone marrow harvested from mice was cultured in chondrogenic media supplemented with NGF and BDNF neurotrophins. The results from the Alcian Blue staining showed a gradation of blue intensity across samples, indicative of varying degrees of chondrogenesis. The most intense staining, corresponding to the highest proteoglycan content, suggests robust chondrogenic activity potentially modulated by BDNF. These findings imply a promoting role for BDNF in cartilage formation and maturation, aligning with the genetic evidence of BDNF's involvement in extracellular matrix organization and collagen synthesis pathways. This functional experiment complements our network analysis, providing a tangible link between BDNF signaling and chondrogenesis, and highlights the importance of BDNF in cartilage development and maintenance.

To substantiate the insights gleaned from the network analysis and to validate the RNA sequencing data, we employed quantitative Polymerase Chain Reaction (qRT-PCR) (Fig 6). This technique is highly sensitive and specific, providing the precision needed to quantify the expression of the 18 BDNF-related genes, downstream pathways that may be related to p75NTR, as well as well-known cartilaginous markers. qRT-PCR serves as a confirmatory step, ensuring

the downregulation observed was not due to experimental artifacts or false positives inherent in high-throughput sequencing methods. The qRT-PCR results presented here clearly corroborate our prior findings, showing significant downregulation in the expression of several key genes in p75NTR KO mice compared to WT controls. Notably, the genes Xbp1, Cdkn1a, and Vcan—important players in cellular stress response, cell cycle regulation, and extracellular matrix composition, respectively—exhibit decreased expression in the KO samples. This downregulation aligns with our earlier observations suggesting a potential disruption in protein homeostasis and extracellular matrix organization due to the lack of p75NTR. Further, the expression levels of collagen-modifying enzymes P4ha1 and P4ha2 are reduced in the KO samples, which may contribute to the altered cartilage matrix and its biomechanical properties, given their role in collagen stabilization. Additionally, Id2 and Cspg4, involved in cell differentiation and chondroitin sulfate proteoglycan synthesis, respectively, also show a decrease in expression, providing molecular evidence of impaired chondrogenesis pathways.

We also ran qRT-PCR on a list of key cartilage marker genes (Ihh, Aggrecan, Sox9, Col2a1 and Col10) to further study the relationship between p75NTR and collagen. We observed the downregulation of 4 of the 5 markers, portraying a strong correlation between p75NTR and collagen. Indian Hedgehog (Ihh), a pivotal growth factor in chondrocyte proliferation and differentiation, exhibited reduced expression in the KO group. This downregulation suggests a possible modulatory role of p75NTR in the Ihh signaling pathway, which could have downstream effects on the maintenance and development of cartilage. Aggrecan, a fundamental proteoglycan component of the cartilage matrix, also showed significantly lower expression levels in the KO mice. Aggrecan endows cartilage with its unique compressive properties, and its diminished expression implicates p75NTR in the structural

integrity and biomechanical functionality of cartilage. The transcription factor Sox9, essential for chondrocyte identity and cartilage formation, was similarly downregulated in the absence of p75NTR. This finding aligns with a potential deficit in the transcriptional machinery that drives early cartilage development steps, further supporting the hypothesis that p75NTR influences chondrocyte differentiation. The primary structural protein of cartilage, Type II collagen (Col2a1), also revealed a downregulated expression pattern in KO mice. As this collagen type imparts tensile strength to cartilage, its decreased expression may correlate with a weakened cartilaginous tissue predisposed to damage. Conversely, Type X collagen (Col10), associated with the maturation of chondrocytes in the growth plate, did not display significant changes in expression levels between the WT and KO groups. This non-significant difference may reflect a more complex role of p75NTR, possibly indicating its involvement varies across different chondrocyte maturation stages or cartilage zones.

Lastly, we ran qRT-PCR on Akt, PI3k, mTOR, and Jun genes to understand the relationship between p75NTR signaling pathway with these downstream pathways in order to discover new connections. The qRT-PCR data showed that Akt, PI3k and mTOR genes ere downregulation in p75NTR KO samples. These signaling pathways are key regulators of cell growth and metabolism, with direct implications for cartilage formation and maintenance. Akt stimulates the synthesis of matrix molecules, including types II and X collagen, essential for cartilage structure and function. PI3K activation leads to Akt signaling, promoting cell survival and proliferation, which are vital for chondrocyte function. p75NTR influences various neurotrophic factors that can activate the PI3K/Akt pathway, impacting cell survival and differentiation. Through this mechanism, p75NTR may indirectly participate in chondrogenesis by modulating the signals that govern the synthesis of extracellular matrix components,

including collagens. Furthermore, mTOR is a central regulator of protein synthesis, and its activation enhances the production of collagen and other extracellular matrix proteins, facilitating cartilage repair and regeneration. Activation of mTOR can result from AKT signaling, which is modulated by neurotrophins signaling through their receptors, including p75NTR. The expression level of Jun, a component of the AP-1 transcription factor involved in cell proliferation and apoptosis, remained unchanged. This could suggest that the downstream effects of p75NTR deletion do not significantly affect pathways involving Jun or that compensatory mechanisms are in place to maintain its expression levels.

The consistency between the RNA-seq data and qRT-PCR results strengthens the hypothesis that p75NTR, possibly through its interaction with BDNF, exerts significant influence on cartilage formation and maintenance. These findings suggest not only a crucial role for p75NTR in neural health but also extend its relevance to the proper development and function of cartilaginous tissue. This study sets a firm groundwork for future investigations into the therapeutic potential of BDNF and p75NTR modulation in cartilage-related diseases.

7. CONCLUSION

This study embarked on an exploration of the p75NTR role in cellular processes, with a particular focus on chondrogenesis. Our multifaceted approach encompassed RNA sequencing, network analysis, functional assays, and qRT-PCR validation to uncover the molecular underpinnings and downstream effects of p75NTR signaling.

Initial RNA sequencing and subsequent network analyses revealed a series of downregulated genes in the p75NTR KO mice, most notably genes within the BDNF pathway, implicating p75NTR in a variety of cellular functions, including those related to the endoplasmic reticulum stress response, collagen biosynthesis, and extracellular matrix organization.

Further experiments, including Alcian Blue staining portrayed BDNF's vital role in chondrogenesis. qRT-PCR validated cartilage marker genes such as Ihh, Aggrecan, and Sox9, underlined a connection between p75NTR and the maintenance of cartilage integrity. The downregulation of these genes in KO mice supported the hypothesis that p75NTR influences cartilage formation and extracellular matrix composition.

Our investigations into downstream signaling pathways of p75NTR using qRT-PCR to measure the expression of key molecules in the Akt/PI3K/mTOR pathway established a significant downregulation of Akt, PI3k, and mTOR in KO mice, reaffirming the role of p75NTR in these crucial cellular pathways. The lack of significant change in the Jun pathway gene expression suggested the complexity of the p75NTR downstream cascade.

Collectively, the data elucidate a multifaceted role of p75NTR, with impacts extending from neurotrophin signaling to extracellular matrix regulation and collagen biosynthesis. The study provides a nuanced understanding of how p75NTR's absence can lead to widespread changes in gene expression, disrupting processes fundamental to neural and cartilage health.

These insights pave the way for future research into the therapeutic potentials of modulating p75NTR and its associated pathways in neurodegenerative diseases and cartilage repair strategies.

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