GLOBAL DELETION OF *SOST* INCREASES INTERVERTEBRAL DISC HYDRATION BUT MAY TRIGGER COMPENSATION

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For mom and dad.

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LIST OF ABBREVIATIONS

μCT	micro-computed tomography
AF	Annulus Fibrosus
Bglap	Osteocalcin
CEPs	Cartilaginous Endplates
CLCs	Chondrocyte-like cells
Col2	Collagen type 2
ColX	Collagen type 10
Dkk1	Dickkopf WNT Signaling Pathway Inhibitor 1
ECM	Extracellular Matrix
Foxa2	Forkhead box a2
Gdf5	growth differential factor 5
HSP	Heat Shock Protein
IHC	Immunohistochemistry
IVD	Intervertebral Disc
LBP	Lower back pain
LRP	low-density lipoprotein receptor-related protein
MRI	Magnetic Resonance Imaging
NCs	Notochordal Cells
NP	Nucleus Pulposus
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
Opg	Osteoprotegrin
qPCR	quantitative Polymerase Chain Reaction
RANKL	receptor activator of Nfκb ligand
Sclerostin	protein product of Sost

sFRP4	Selected frizzled-related protein 4
Shh	sonic hedgehog
Sost KO/Sost-/-	Sost Knockout
Sp7/Osx	Osterix
Wnt	Wingless-related integration site
WT	Wild-type

ABSTRACT

Kroon, Tori M.S.B.M.E., Purdue University, May 2020. Global Deletion of *Sost* Increases Intervertebral Disc Hydration But May Trigger Compensation. Major Professor: Nilsson Holguin.

Intervertebral discs (IVD) degenerate earlier than many other musculoskeletal tissues and will continue to degenerate with aging. IVD degeneration affects up to 80 percent of the adult population and is a major contributing factor to low back pain. Anti-sclerostin antibody is an FDA-approved treatment for osteoporosis in postmenopausal women at high-risk for fracture and, as a systemic stimulant of the Wnt/LRP5/β-Catenin signaling pathway, may impact the IVD. Stabilization of β-Catenin in the IVD increases Wnt signaling and is anabolic to the extracellular matrix (ECM), while deletion of β-catenin or LRP5 decreases Wnt signaling and is catabolic to the ECM. Here, we hypothesized that a reduction of Sost would stimulate ECM anabolism. Lumbar and caudal (tail) IVD and vertebrae of Sost KO and WT (wild-type) mice (n=8 each) were harvested at 16 weeks of age and tested by MRI, histology, immunohistochemistry, Western Blot, qPCR, and microCT. Compared to WT, Sost KO reduced sclerostin protein and Sost gene expression. Next, Sost KO increased the hydration of the IVD and the proteoglycan stain in the nucleus pulposus and decreased the expression of genes associated with IVD degeneration, e.g., heat shock proteins. However, deletion of Sost was compensated by less unphosphorylated (active) β-Catenin protein in the cell nucleus, upregulation of Wnt signaling inhibitors Dkk1 and sFRP4, and catabolic ECM gene expression. Consequently, notochordal and early chondrocyte-like cells (CLCs) were replaced by mature CLCs. Overall, Sost deletion increased hydration and proteoglycan protein content, but activated a

compensatory suppression of Wnt signaling that may trigger chondrogenesis and may potentially be iatrogenic to the IVD in the long-term.

1. INTRODUCTION

Assessing, diagnosing, and treating intervertebral disc (IVD) degeneration is important because they degenerate earlier than any other musculoskeletal tissue [1] and is a major etiological factor of lower back pain (LBP). [2] LBP is the number one cause of job disability worldwide [3] and the total cost related to treatments exceeds $100 billion per year in the US. [4] IVD degeneration is a multifaceted disease as aging, loading, and genetics are all common factors of onset and progression. IVDs are responsible for the spine's flexibility, mobility, and protection. They absorb the everyday shock and load experienced when we walk or jump and keep the spinal cord from injury. Because IVD degeneration is a progressive disease, once an IVD begins to degenerate, there is little that can be done to stop or attempt to reverse the degeneration. [5] While not deadly, IVD degeneration can lead to other serious chronic conditions that can impair a person's quality of life. An area that has been heavily studied as an intervention in treatment is modulation of the Wnt signaling pathway, as my research will emphasize, for it is known that proper regulations of the Wnt signaling pathway is required for IVD development and regulation. [6]

1.1 Diagnosis

1.1.1 X-ray

X-rays are a form of electromagnetic radiation and contain a high amount of energy that allows it to pass through most objects. Medical x-rays are used to generate an image of a structure. X-rays will be emitted and travel through the body and hit an x-ray detector on the other side of a patient, and results are called a radiograph.

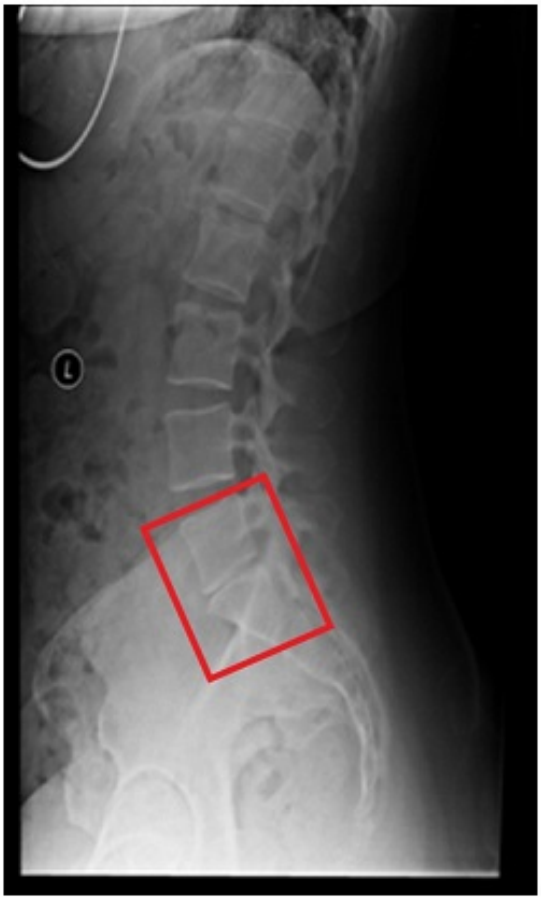


Fig. 1.1 The image is an X-ray taken of the lumbar portion of a human spine. X-rays are more often used to discount other potential diagnoses rather than IVD degeneration itself. Other disease can include scoliosis (curvature of the spine), fractures, or bony protrusions. This X-ray shows the narrowing of the disc space between L5 and S1 (highlighted), and loss of IVD height is a characterization of IVD degeneration. [7]

To obtain a radiographic image, an object or structure that is to be imaged is placed between the x-ray source and the x-ray detector. When the x-rays are emitted, they are absorbed by different in different amounts by different tissues in the body depending on the radiographical density and the atomic number, the amount of protons in an atom's nucleus, present in the structure. The higher the atomic number, the more radiographically dense a structure is, meaning it will have a high amount of contrast compared to the black background of a radiograph. [8] Bones are able to absorb high amounts of x-rays thus appearing white it on the radiograph. In contrast, less radiographically dense structures such as muscles and fat tissue will appear darker since they do not absorb as much of the x-rays. X-rays can be used as treatment in LBP to see if there are any immediate abnormalities in the spine such as; fractures or breaks, arthritis, spondylolisthesis (vertebral dislocation or slipping), tumors, bony abnormalities, or curvature of the spine (Fig. 1.1) [9]

1.1.2 Magnetic Resonance Imaging

MRI, or magnetic resonance imaging, uses the natural magnetic properties of the body to produce an image that is highly detailed. The MRI uses the hydrogen nucleus to help produce an image. [10] Normally, the hydrogen proton has a north and south pole, behaving similar to a magnet, and spins with their axes randomly aligned in the body. When exposed to a strong magnetic field, i.e. the MRI scanner, the hydrogen proton axes all line up with each other. The uniform aligned creates a vector oriented along the axis of the scanner. When additional energy (in the form of a radio wave) is added to the field, i.e. the scanner is turned on, the magnetic vector is deflected. The radio wave that gets emitted is dependent upon the hydrogen nuclei and the strength of the magnetic field, or the scanner. When the radio frequency source is turned off, the magnetic vector returns to its original position while emitting a signal. These signals are what is used to create an image. The intensity of the signal received is plotted upon a grey scale and through series of emissions, the cross-sectional image is

built. Multiple transmitted radiofrequencies can be used to help distinguish tissues from each other or emphasize abnormalities. This occurs because different tissues relax at different rates when the radiofrequency pulse is turned off. Most diseases reveal themselves due to the increase in water retention, so MRIs are used to detect this change. [11] T2 relaxation time measurements correlate with IVD tissue water content, [12] and because key features of IVD degeneration including NP size, proteoglycan content, and hydration, [13] MRIs are used as a clinical measurement in assessing and diagnosing IVD degeneration (Fig. 1.2).

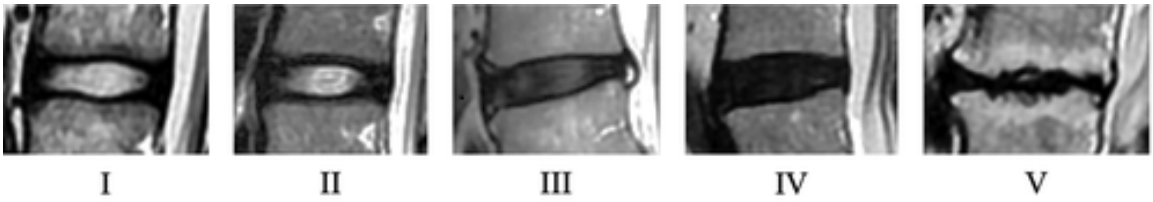


Fig. 1.2 The figure represents different variations of degeneration in the IVD from MRI. Image I represents a healthy IVD; it is highly hydrated indicated by the homogeneity of the white color of the IVD and high amount of signal intensity, there is clear distinction between NP and AF, and a normal IVD height. II is still a relatively healthy IVD, the color of the IVD is slightly inhomogeneous but still maintains a high amount of signal intensity. There still remains a clear distinction between NP and AF and normal IVD height. III is a degenerated IVD with inhomogeneous coloring appearing grey, an intermediate amount of signal intensity, indicating a slight loss of hydration. The distinction between NP and AF is unclear and the IVD height could be normal or slightly decreased. IV is a more degenerated IVD with nonhomogeneous appearing grey or black in color and an intermediate or hypointense signal. There is no distinction between NP and AF, and a decrease in IVD height. V is a severely degenerated IVD. This is indicated by the black color of the IVD and a lost signal intensity. There is no distinction of NP and AF and a complete collapse of IVD height. [14]

1.2 Cost Associated with LBP

The overall cost of treating back pain and costs related to treatment is on average around $100 billion per year in America. [4] Factors included in treatment costs range from noninvasive imaging and treatment options to invasive treatments. Two

thirds of the cost incurred of treating back pain is due to lost wages and decreased productivity. [15] It is estimated, on average, that one individual will spend up to $6100 on medical expenditures associated with LBP in a single year, with 10% of that population incurring a much higher bill due to more extensive work and treatments. [16]

Imaging is used to initially diagnose and asses back pain. With the use of either an X-ray or an MRI/CT scan, this is the first step taken in diagnosis and treatment and is noninvasive. These are used to see if there are any alarming physical disruptions in the spine itself. An x-ray of the spine can cost on average around $100-$450 while an MRI or CT can range from $1000-$2000. [17] After analyzing images, clinicians will start with the palliative treatment options. This can include, rest, over the counter NSAIDs (nonsteroidal anti-inflammatory drugs), or physical therapy. Some clinics or pharmacies will charge around $20 for a one-month supply of generic over the counter medication to help manage inflammation due to lower back pain, and depending on how long a patient will experience LBP and on health insurance coverages, this cost can increase very quickly. If NSAIDs alone do not help, physical therapy is also prescribed along with administration of medication. A single session of physical therapy can cost anywhere between $50-$350, depending on health care providers and insurance, and is often prescribed 2-3 times a week for at least 4-6 weeks to try and measure any progress that can occur. If pain still isn't alleviated, the next minimally invasive step is an injection. While different types of injections are used for pain treatment and management, each injections can cost up to $600, and for most patients this can be more than a one-time cost. [18] If pain insists, since these treatments do not assess the physical and morphological changes associated with a degenerated IVD, invasive surgery is typically the next and last step in trying to treat LBP. Depending on level of degeneration present, there are several different surgical options; a discectomy or microdiscectomy, which can cost $20,000-$50,000 or a spinal fusion which is estimated to cost $80,000-$150,000, both of which are what is most clinically seen. [19] After surgery, during the process of recovery which can

last 6 months up to a year, physical therapy is required to regain spinal motion and strength, only adding to the cost an individual will incur due to LBP.

1.3 Treatment Options

After analyzing images, there are different paths clinicians may to take treatment wise starting with the most minimally invasive option first to try to ensure patients' safety.

1.3.1 Non-invasive options

The first treatment step taken is usually prescribed drugs to help with pain management or alleviation. Common pain medications prescribed include; acetaminophen, NSAIDs, narcotics, or muscle relaxants. Most of these just deal with the inflammation present which could be the source of pain. Each medication has its benefits, limitations, and risks associated. Because most LBP is more than mild inflammation or a tight muscle, physical therapy is often prescribed concomitantly. Physical therapy begins with an initial evaluation to try and deduce what could be causing the pain. This could be either lack of mobility and flexibility, or weak muscles, improper firing of muscles, or compensation. At the end of a physical therapy regime, a re-evaluation is done by the physical therapist to assess progress and possible improvement, followed by a second appointment with the doctor to discuss further options.

1.3.2 Invasive Options

If there is no progress or symptoms continue to worsen, additional scans will be needed typically leading to more invasive treatments. These invasive treatment options can include injections or surgeries.

Injections

There are several different kinds of injections given to help with back pain, but none of which are an actual cure, just something used for pain management and alleviation. A hyaluronic acid injection is used to provide lubrication to the site of pain, typically a joint, to mimic the synovial fluid that is naturally occurring to help alleviate stiffness and aid with movement. A cortisone or steroid injection provides the site with an anti-inflammatory drug to reduce swelling and irritation. More spine specific, epidurals can be given. Epidural means "around the spinal cord" which means the corticosteroid is delivered to the epidural space to try and provide symptomatic relief to a larger area. Lastly, there is a nerve block. This involves delivering an anesthetic, typically lidocaine, to the area around a specific nerve in hopes of alleviating pain. Injections are typically given to help ease enough pain to have the patient start with a physical therapy program.

Surgery

Because these treatment options are not assessing the physical and morphological changes associated with a degenerated IVD, if LBP persists or continues to worsen an invasive surgery is typically the next and last step in treatment. Depending on the varying level of degeneration seen in the back, there are several different surgical options. There were nearly 300,000 discectomies performed each year in the U.S. and in 2018, there were over 352,000 interbody fusion surgeries performed. [20]

Discectomy

A discectomy involves removing portions of the spine and muscle that are blocking the affected IVD to gain access. The surgeon begins by going through the back and removing a portion of the lamina, the flattened portion of the vertebra that forms the backside, or posterior, of the spinal canal that covers the spinal cord. The spinal nerves are retracted to one side and depending on the level of severity of degeneration in the affected IVD, either a portion or the entire IVD is removed. Once removed, the

surgeon returns the surrounding muscles and bones back into their proper positions. A microdiscectomy has the same end goal, but instead of it being an open procedure, this technique uses a small skin incision to be as minimally invasive as possible. (Fig. 1.3).

Additionally, they will tunnel through the musculature in the back and use special tools to allow the surgeon to see and operate in a smaller space. A microdiscectomy will often cause less disruption in the back and may decrease recovery time. [21] Although this surgery is considered to be very safe and successful, there are risks. There is a possibility of permanent nerve root damage, scar tissue formation, infection, injury to adjacent discs causing mechanical instability, all of which could require a revision surgery. [22] Most often times, the alternative surgery to discectomies, or the secondary revision surgery, is fusion.

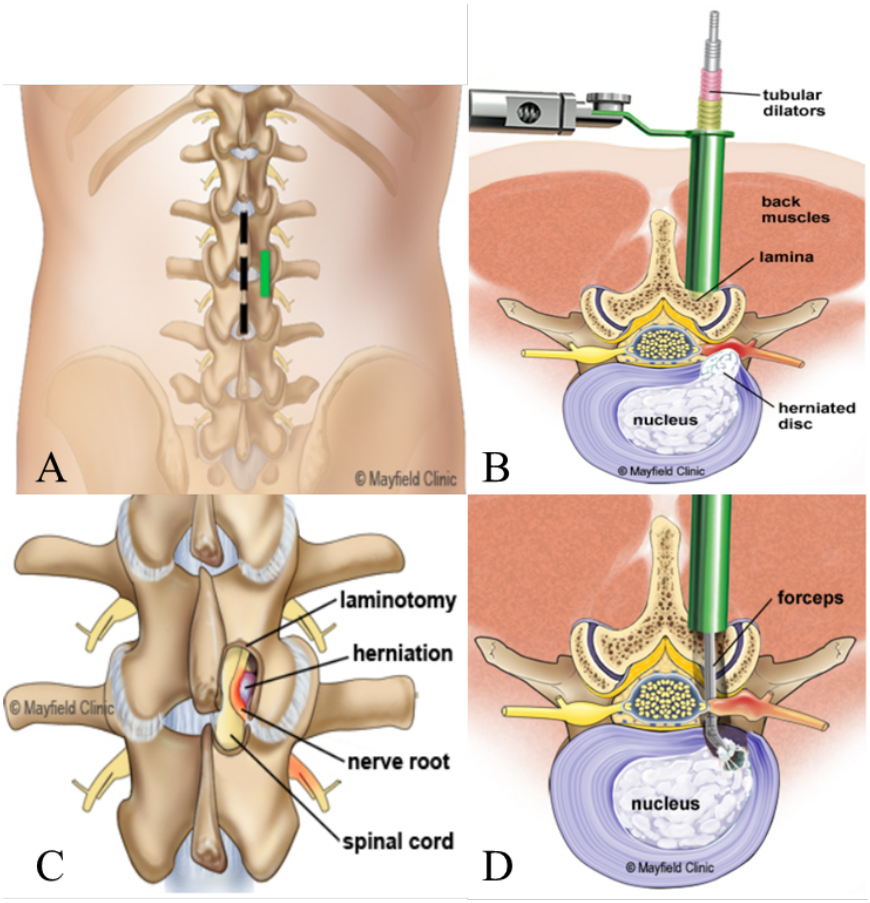


Fig. 1.3 The figure below displays the steps involved in a microdiscectomy. A) The black lines indicate the 1-2-inch incision made for an open discectomy, where the next step would be for the surgeon to retract the muscles overlaying the injured vertebra to one side to gain access to the IVD. The green line indicates the small stab entry for a minimally invasive microdiscectomy made in the skin near the midline. B) A series of tubular dilators, or retractors, increasing in size, are used to gradually separate muscles to create a tunnel to gain access to injured IVD. C) A laminotomy is performed. This involved creating a small opening in the lamina by removing a small portion of the bone above and below the spinal nerve. This can be done unilaterally, on one side, or bilaterally, on both sides, or on multiple vertebrae. The nerve root and spinal cord can be gently retracted to one side to expose the injured IVD. D) Once there is full access to the damage IVD, the surgeon will use forceps to remove the IVD material compressing the nerve root. The last step involves removing the muscle retractors and repairing the muscles and skin with sutures or staples for closure. [21]

Fusion

In fusion, the surgeon will open the back posteriorly, and move the necessary muscles to reach the affected IVD. The surgeon will completely remove the affected IVD from the disc space. Once it is cleared out, they will implant a bone graft between the two vertebrae to facilitate fusion and help promote bone healing. After the graft is placed, surgeons may add a plate or screws for additional stability and support to the spine (Fig. 1.4). [23]

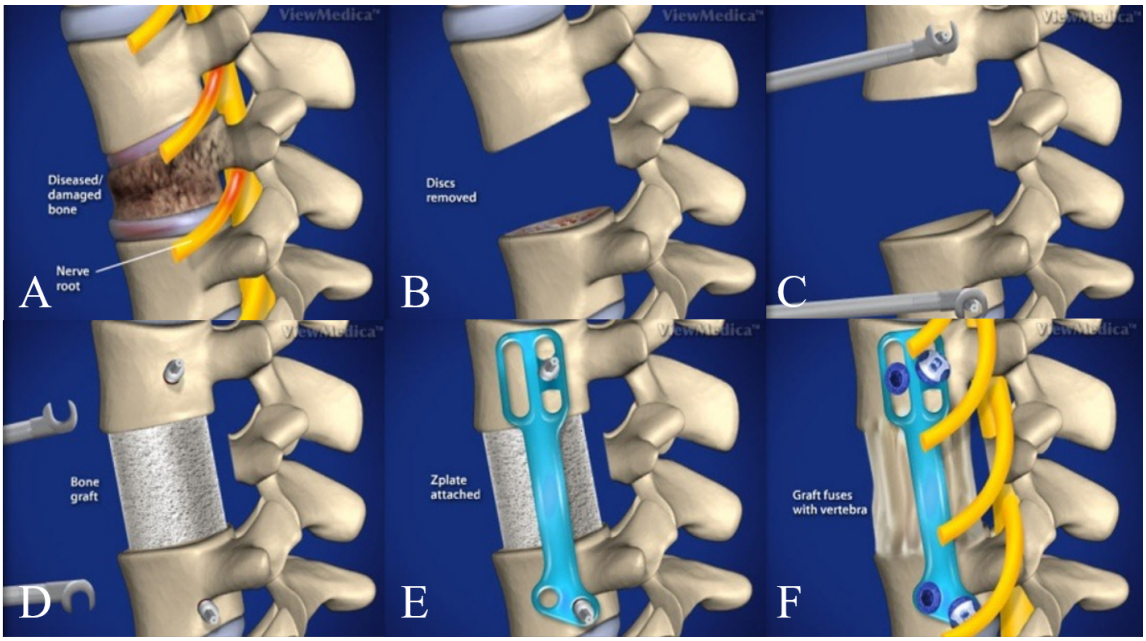


Fig. 1.4 Steps of spinal fusion. A) The surgeon will make an incision in the left side of the patient to gain access to the spinal column near affected vertebrae. B) The diseased or damaged vertebrae is removed as well as the IVDs above and below. The bone surfaces are cleared for the bone graft. C) Bolts are inserted in the upper and lower vertebrae, and with the help of tools, the surgeon will open the vertebral space and correct the curvature of the spinal cord. D) One the space is opened the bone graft is implanted. The vertebral space is closed to secure the bone graft in place. E) A metal plate is placed over the bolts in both the upper and lower vertebrae and screwed into place. This is used to ensure the plate does not move. F) With time, the graft will begin to fuse with the vertebrae forming a single solid bone fragment.

Once the surgery is complete, because this procedure is so involved and invasive, hospitalization is required and can last anywhere from 2 days to one week. Patients are given anti-inflammatory drugs to reduce pain and swelling, as well as a rigorous physical therapy regime to regain proper mechanical function and motion. Recovery from fusion could take as long as six months to one year to fully heal. [24]

While both of these methods seem to permanently fix the problem, they also delay further degeneration. With discectomies, removal of certain parts of the disc will cause strain to the back since the full disc structure is not present this will cause uneven loading throughout the rest of the back causing a new location of pain later in life. In a 2018 study, that patients that underwent a discectomy were almost 3 times more likely to undergo a lumbar fusion. [22] With fusion, since the two vertebrae are permanently stuck together, there is too much stiffness in the affected area. This causes the back to be abnormally loaded which then can later affect other intervertebral discs causing more degeneration throughout the spine. Additionally, the rods and screws can cause nerve irritation and excess inflammation. These surgeries require extensive bone and muscle work that could lead to more pain and revision surgeries down the road.

1.4 Anatomy

To better understand IVD degeneration it is critical to know the components and functions of each part, to help create an understanding of what happens during degeneration and what changes occur as a result. The IVD is an important feature in the normal functioning spine. It is a system of fibrocartilage and serves as the principle joint between two vertebrae in the spinal column. [25, 25] The primary function is mechanical, acting as a shock absorber between vertebrae transmitting load from body weight and muscular activity. [26] IVDs are approximately 7-10 mm in height and 4 cm in diameter in the lumbar, [27] and are composed of three distinct parts; the nucleus pulposus (NP), annulus fibrosus (AF), and two cartilaginous endplates

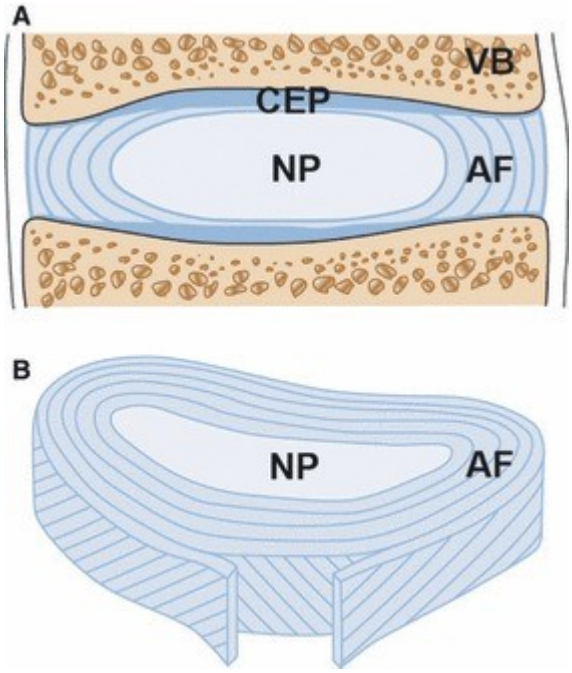


Fig. 1.5 A schematic representation of the IVD. A) The image shows all components of the IVD within the spine. The central NP, surrounded by the AF, encased by two CEPs one above and one below the NP/AF, with a vertebral body (VB) one above and below the IVD. B) The main portion of the IVD, the AF formed by the lamellae highlighting the different angular orientations of each different lamellae encasing the NP. [30]

(CEPs) (Fig. 1.5A). [28] Tissues within the IVD vary from NP to AF. There is more type II collagen and proteoglycans in the NP and more type I collagen in the AF. [29]

1.4.1 Nucleus Pulposus

The NP is the central compartment of the IVD (Fig 1.5B). The main function of the NP is to act as the shock absorber for the spine. Due to the proteoglycan, there is a high amount of water content and hydration which causes the NP to act like a pressurized fluid, allowing it to absorb impact and distribute the force equally across the CEPs protecting the spine from injury. [30] It is a network of randomly oriented type II collagen fibers and radially arranged elastin fibers embedded in a proteoglycan rich-gelatinous matrix, primarily comprised of water, collagen II, proteoglycans, notochordal cells, and at low density, chondrocyte-like cells (CLCs). [26, 29, 31, 32] There has been made a clear distinction between NP tissue and hyaline cartilage, as there is a higher ratio of proteoglycan (Fig. 1.7D) to collagen in the NP, 27:1, whereas in hyaline cartilage structures, such as the CEP, the ratio is only 2:1. [33]

The NP cells also vary in levels in the ratio of collagen and aggrecan and has also been used to potentially distinguish between chondrogenic and "IVD-like" differentiations of mesenchymal stem cells (MSC). Studies have assessed notochordal cells (NCs) to understand their phenotype in order to describe the initial state of NP cells before maturation, since NCs are lost with aging in humans. [34] NCs in the NP are classified as large cells, being approximately 30-40 μm in diameter (Fig. 6G). They commonly appear as clusters of cells with intracellular vacuoles composing of at least 25% of the cell area. [34] There is an increasing amount of evidence that suggests that NP cells derive from NCs that are functional during embryogenesis, and is thought to be due to the similar phenotype. It has been found that Cytokeratin-8, -18, -19, and brachyury (T-box gene), are required for notochordal cell differentiation and all expressed in both NC and NP cells. [35–37] Also observed, there was reduced gene expression in NCs of specific ECM markers such as collagen I, biglycan, and TIMP-1, a metalloproteinase inhibitor, compared to mature NP cells. There was also no difference in chondrogenic-like genes such as collagen II, Sox-9, and aggrecan between NCs and mature NP cells. The expression of these three molecules is what distinguishes chondrocytes from all other cell types. Since they both have similar expression profiles, mature NP cells can be considered "notochordal-like" cells. [38] Although, mature NP cells are smaller than NCs, having a diameter of around 10 μm in diameter while lacking an intracellular vacuole. [34] It is also important to note that in both healthy and degenerated IVDs, chondrocyte markers Sox-9 and collagen II were still present. [39]

1.4.2 Aggrecan

Aggrecan, the major and most abundant proteoglycan in the IVD, interacts with hyaluronan to form large aggregates. The purpose of its function is to maintain water retention which resists compressive loads. The core protein of aggrecan contain large numbers of chondroitin sulfate and keratin sulfate chains that aid in producing the os-

motic gradient. The sulfated GAG chains create a highly negative fixed charge within the matrix, and these molecules can bind electrostatically to polar water molecules contributing to the hydration of the NP. [40] The concentration of the negative charge will determine the concentration of positive extracellular molecules needed to keep the ion charge in equilibrium. Small positive molecules such as cations, are attracted to the GAG chains while negative ones are slightly repelled. [41] The osmotic gradient produced from the balance between the positive and negative charges are what attribute to the swelling pressure of the IVD, therefore responsible for the function of the NP.

1.4.3 Annulus Fibrosus

The AF is made of 15-25 highly organized, collagen-rich concentric rings, or lamellae (Fig. 1.6B), that wrap around the NP keeping it pressurized (Fig. 1.6A). [26, 29] The function of the AF is to work in tension to withstand the compressive forces from the NP (Fig. 1.6 [42]). This is to make sure the NP stays encapsulated and protected. The collagen fibers within each layer lie parallel to each other (Fig. 1.7E), angling between adjacent vertebrae at 65-70 degrees to the vertical. [43] But the collagen fibers in each successive layer lie perpendicular to each other. The alternation in alignment of fibers is what give the AF its compressive strength and its ability to withstand applied forces from almost any direction. [44]

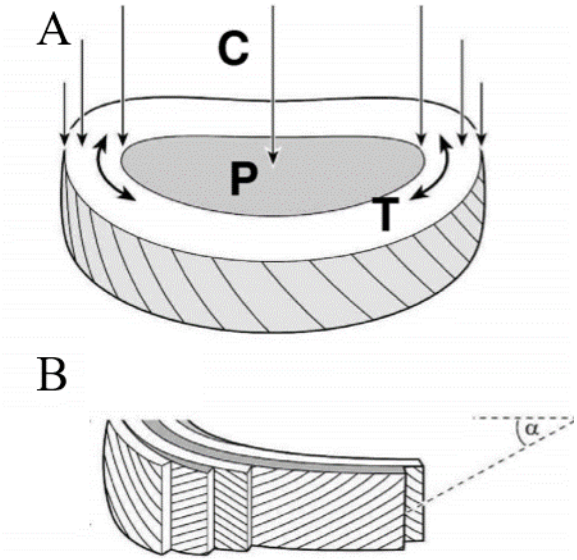


Fig. 1.6 Structure and function of the IVD. A) Upper spinal loading or compression (C) will create hydrostatic pressure (P) in the NP. The pressure within the NP will cause tension (T) within the AF to keep it in place and protected. B) Highlights the alternating orientations of the collagen fibers in the lamellae of the AF contributing to its strength. α=$\sim$30°

The AF is made of water, collagen I, proteoglycans, non-collagenous proteins, and chondrocyte-like cells (CLCs). [29] Proteoglycans, in particular GAGs, are responsible not just for hydration like the NP, but the water-binding ability enables the tissue to undergo rapid deformation due to loading. [45] Composition in the AF also varies by location. On the outer perimeter of the AF, elongated and thin fibroblast-like cells can be found, while there are more rounded CLCs toward the inner part of the AF (Fig. 1.7F). [46] The proportion of collagen I and collagen II also vary upon location, collagen I increasing from the inner to the outer AF, while collagen II does the opposite, increasing in levels from outer to inner. [47] As we move from AF to NP a "transition zone" is apparent. This area is where NP and AF meet and cell type and ECM composition change to go from one structure to the other (Fig. 1.7C).

1.4.4 Cartilage Endplates

Encasing the AF and NP are the cartilaginous end plates or vertebral end plates, located above and below each disc at the interface where it would meet the bony vertebral body. [29] The end plates, composed of hyaline cartilage and fibrocartilage, form a thin layer that covers the area of the vertebral body. The end plates are

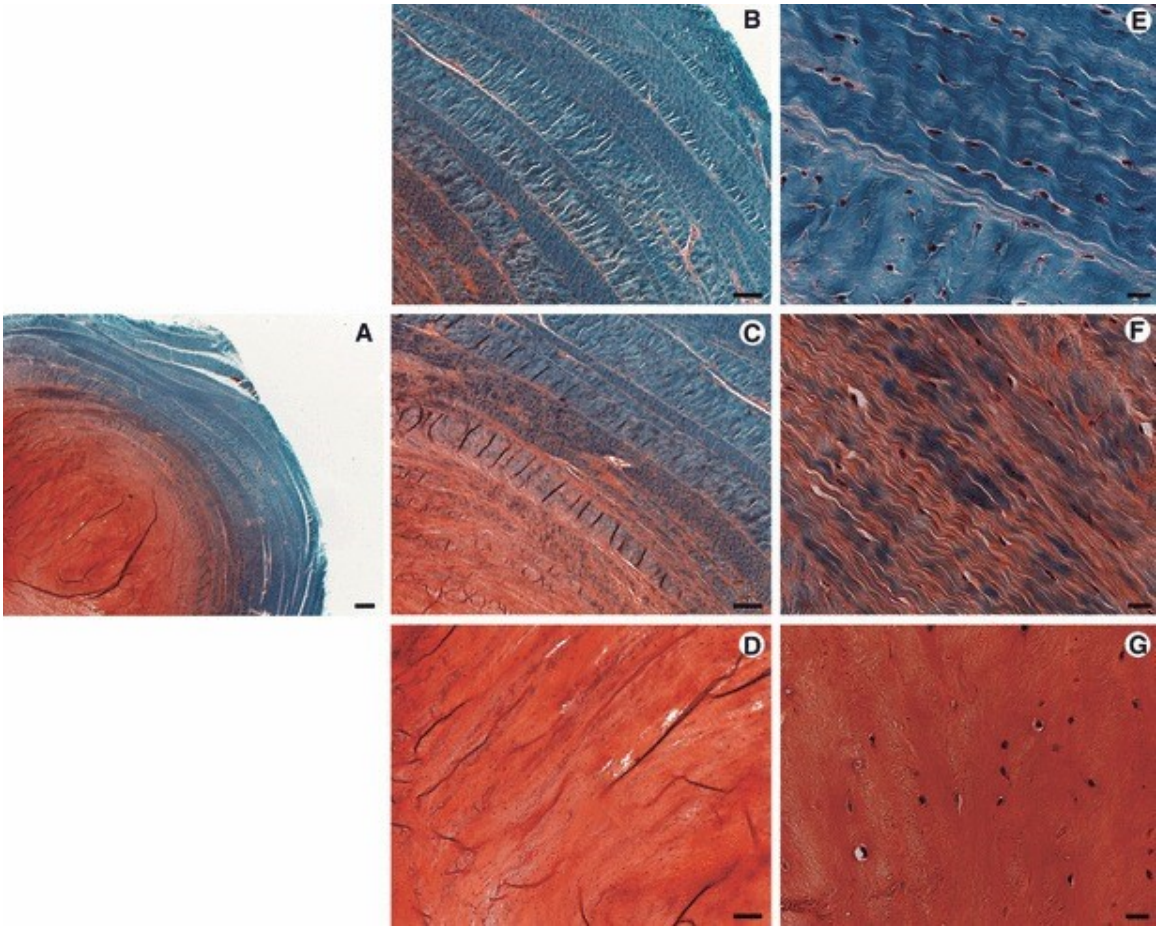


Fig. 1.7 The histological images taken of the IVD are Safranin O-Fast-green staining. This is of the tail from a 6 month onld bovie calf showing the differences in cellular phenotype between the different structures of the IVD. A) The iamge is of the outer portion of the IVD showing the AF in blue, rich in collagen, and the central NP shown in red, rich in glycosaminoglycans (gags). B) The image details the the difference in structure between the concentric lamellae of the AF. E) A zoomed in image to show the cells of the lamellae following the orientation of each layer. The primary cell type in the AF are fibroblast-like, elongated in shape. C) The transistional zone between the AF and NP. Moving from the AF to the NP, there is a slight decrease in collagen with an increase in gags accompanied by a gradual loss of the lamellar structure. F) The cell phenotype also begins to shift slightly, while there are still mostly fibrotic like cells from th AF, there is also a small number of chondrocyte-like cells (CLCs) coming from the outer portion of the NP. D) The central NP is composed primarily of gags and red in color indicating high proteoglycan content. (G) A magnified image of the NP displaying the cell type. The cells of the NP are more rounded in shape and resemble condrocytes. Scale bars: 500 μm (A), 200 μm (B–D), 20 μm (E–G). [30]

attached strongly through the AF to the disc connecting via the region on the end plate called the outer articular area, but weakly attached, via the inner growth zone, to the vertebral bodies. [44] It is believed that the end plates are critical for maintaining the health and function of the intervertebral disc. Because intervertebral discs do not have a direct blood supply, end plates must be strong enough to prevent fracture in the vertebra, while also being porous enough to facilitate transportation of blood, oxygen, and nutrients between the disc cells and vertebral capillaries. [48] Like the AF, at the embryonic stage, Wnt signaling activity is apparent and weakens with maturation. Wnt signaling and β-catenin levels play an important role in function and development of the CEP chondrocytes.

2. ROLE OF WNT SIGNALING IN IVD DEGENERATION

2.1 Characteristics of IVD Degeneration

Many things will change due to IVD degeneration. Tissue changes in the NP and AF will compromise the function of the IVD. As the IVD degenerates the tissue will lose hydration leading to changes in the biomechanical properties of the IVD as well as leading to alterations in overall composition of the structure. IVD cells have previously been called "chondrocyte-like" cells or "IVD chondrocytes" but have since been categorized and described in more detail. There are some physical similarities between different cell types within the IVD but there are distinct differences that have been identified. [30] It is known that modulation of Wnt signaling is required for proper development and organization for the IVD, but the level of Wnt signaling also changes with degeneration. Early IVD degeneration is related to the downregulation of the Wnt signaling pathway. [49]

2.1.1 Tissue Level Changes in the NP

The main function of the NP is to be the shock absorber of the spine. In healthy conditions, the NP generates high osmotic pressure contributing to its function as well as to the hydration of the structure, overall IVD height, and mechanical integrity. But as the IVD degenerates, the NP loses hydration causing a multitude of cascading effects.

Aggrecan is known for contributing to the ability to bind to water to increase hydration to a structure, and with degeneration, the amount of aggrecan production is decreased when compared to non-degenerated IVDs. [39] Aging also causes a loss of aggrecan as it is found to be in high abundance at a young age but decreases with

time to a low level. [45] Due to the loss in aggrecan , because it contributed to the function of the IVD, the NP no longer acts in hydrostatic pressure. [50] leaving the AF and CEPs susceptible to the blunt of the force in the spine. Not only is there less water in the structure, there is increased permeability. Compared to a healthy IVD, a loaded degenerated IVD will lose water quicker leading to loss in IVD height and abnormal loading. [51]

The structure of the NP also shows physical changes. A healthy NP would present as a single central mass with a high number of cells. With degeneration, the cell number decreases and is accompanied with fragmentation of the single mass into smaller "honeycomb" like clusters and is dispersed throughout the NP. As degeneration worsens, the area once highly populated with cells gets replaced by matrix, until the NP "structure" is mineralized or there is a complete loss of the NP compartment. [52] Fragmentation leads to desiccation within the NP [26], and in addition to the loss of hydration, the NP will become more fibrotic [53] and loses it's 'gel-like' nature becoming stiffer, and losing its transparent appearance. [41] Cleft and tears may also appear with increasing amount of degeneration, where severe damage to the NP can be characterized by the longest cleft being greater than 50% of the NP compartment. [52]

2.1.2 Tissue Level Changes in the AF

Like previously mentioned, the dehydration in the NP portion has other consequences in other parts of the IVD. Decreased hydration in the NP causes a loss of IVD height and forces the AF to resist mechanical compression directly. [5] In healthy conditions, the AF acts in tension to keep the NP contained and protected, but in a degenerated state the AF now must act in compression and is much weaker. This direct force to the AF causes tears or fissures and promotes the degeneration process. [41] Annular tears can be categorized into three different classifications (Fig. 2.1 [42, 54, 55]). There are circumferential or delamination which are a result of shear

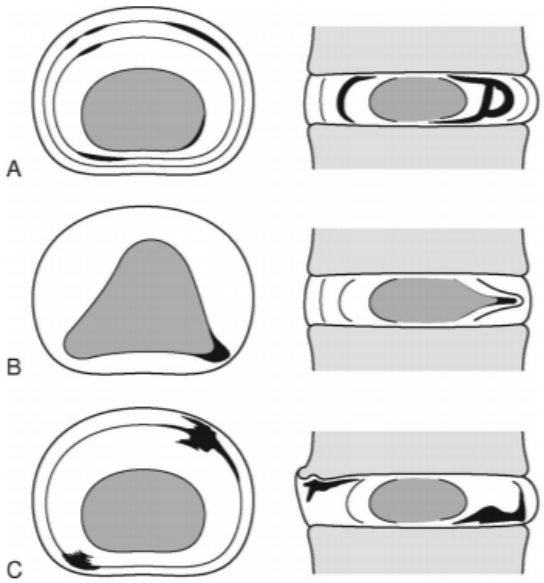


Fig. 2.1 3 common types of AF tears seen in degeneration. A) Circumferential cleft or delamination occuring between the lamellae of the AF. B) Radial fissures result when the AF develops a tear from the NP extending out toward the periphery. C) Peripheral rim lesion which is a result from injury to the edge of the IVD which can result in detachment from the CEP.

stress between the laminae of the AF, peripheral rim tears which typically present in the anterior portion of the AF, or radial fissures, which are typically in the posterior or posterolateral portion of the periphery of the AF. [56]

The physical appearance of the AF also changes with degeneration. A healthy AF would have a complete and concentric lamellar structure surrounding the NP. Slight alterations in structure including a serpentine-like or widened AF structure would indicate slight degeneration, reversal of lamellae intruding into the NP would indicate increased degeneration, followed by a loss of definition in lamellae or increased protrusion into the AF, and complete degeneration can be categorized as a mineralized matrix or loss of AF structure.

A common physical characteristic of degeneration is the loss of the border between the AF and NP. The "transition zone" boundary between the two structures becomes blurred and loses its clear anatomical border due to changes in both NP and AF structure and worsens with increasing degeneration (Fig. 2.2).

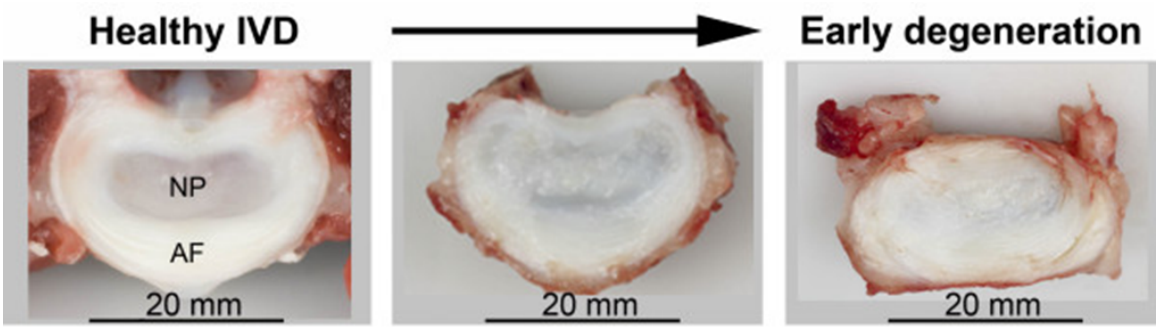


Fig. 2.2 The image shows the macroscopic degeneration process and the phenotype transition that occurs in the IVD from left to right. The healthy IVD (left) has a clear, distinct border between NP and AF. As degeneration occurs, the distinction between NP and AF is less apparent (middle). The degenerated IVD (right) has no distinction between NP and AF with the NP being mineralized. [57]

2.1.3 Cellular Changes in the NP

Aging and IVD degeneration lead to degradation of the ECM [58] by many mechanisms, one of which is a cellular phenotype switch. [34] CLCs are found in the NP along with NCs. CLCs in the IVD resemble articular chondrocytes by similar transcriptional expression, but are phenotypically distinct from NCs. [36, 59] With degeneration there is a loss of NCs and is associated in the early stages of disease development and chondrogenesis. [60] IVD degeneration activates the differentiation of pre-hypertrophic CLCs and deactivates Wnt signaling. [61] Therefore aging and IVD degeneration induce the disappearance of NCs [62] accompanied by a concomitant increase of CLCs (Fig. 2.3 [57]) by either NC apoptosis or CLC differentiation of pre-hypertrophic CLCs. [63]

A decrease in Shh drives the differentiation of NP cells to transition and differentiate to CLCs, also accompanied by a cell phenotype transition by NP cells fusing together. [64] NCs are able to stimulate GAG and aggrecan core protein synthesis, increasing proteoglycan content contributing to the health and maintenance of the NP. [65] While both NCs and CLCs express Sox-9, in degenerated IVDs aggrecan expression from cells and Collagen II expression decrease. [39]

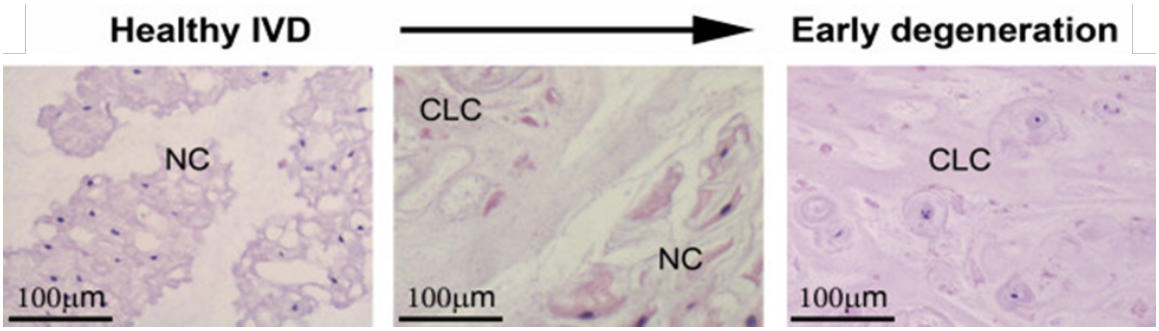


Fig. 2.3 As degeneration occurs, a cellular switch also occurs in the NP. The primary cell type, notochordal cells (NCs), found in a healthy NP (left). A cellular transition also occurs (middle) where both NCs and CLCs are present in the NP. The primary cell type found in degenerated IVDs are CLCs (right).

2.1.4 Cellular Changes in the AF

The AF contains cells that have been related to "fibroblast-like" cells but differ from the typical fibroblast cells since the matrix it produces is different from fibrous cartilage, tendons, and ligaments. [30] While it is a fact that AF cells do not have a chondrocytic phenotype, the AF does not undergo as drastic of changes with degeneration as the NP does. In the AF, the number of cells decreases with increased time and stress. [66] Additionally, with aging, the inner annulus will transition from collagen II being replaced by collagen I. Collagen X, a known marker for hypertrophic chondrocytes, has been found to increase in degenerated cells of the outer AF only, potentially indicating a cellular conversion to hypertrophic chondrocytes. [67]

2.1.5 Inflammation

Cytokines are produced by inflammation in response to injury. They are a key characteristic of IVD degeneration, as they increase as a response to degeneration but they are also secreted by the IVD cells themself. [68] Such factors found in the IVD are different Interleukin (IL) factors and tumor necrosis factor (TNF)-α.

IL-1 is naturally occurring in the IVD and is responsible for maintaining ECM homeostasis [69] and degenerated IVDs spontaneously produce more inflammatory IL-1. Several other studies have published finding that corroborate that production of IL-6 is related to IVD degeneration. [70–72]

Increased expression of TNF-α also leads to increased inflammation resulting in progressive IVD degeneration. It is suggested that Wnt signaling regulates TNF-α levels and together form a positive feedback loop in NP cells. [73] Activation of the Wnt signaling pathway will increase TNF-α expression potentially causing degeneration of the NP.

In the case of IVD degeneration, with the increase secretion of inflammatory cytokines an imbalance begins to appear. These cytokines promote matrix degradation5 and drive changes in cellular phenotype. [68] The imbalance within the IVD due to cytokine production results in favor of catabolism leading to further IVD degeneration.

2.2 Causes of Degeneration

Despite a naturally low cell density, a healthy IVD is able to maintain its environment with the constant cycle that comes with matrix renewal, keeping the balance of slow matrix turnover in synthesis and degradation. [30] Degeneration can be defined by the biochemical changes and mechanical compromise of the IVD. [57] There is a progressive loss of ECM constituents, such as aggrecan, proteoglycan, and collagen resulting in the boundary between the NP and AF to disappear. The degeneration process leads to weakening of the IVD and the inability to perform properly. There is a catabolic shift where the NP and AF begin to produce more matrix degrading enzymes instead of ECM components. The negative alterations in the ECM allow the IVD to behave unfavorably, leading to mechanical dysfunction, inflammation, and cellular death. [74] Additionally, with degeneration there is a cellular phenotype switch. The NP is primarily composed of notochordal cells (NCs), while the AF is

primarily chondrocyte-like cells (CLCs), [57] and as degeneration progresses, the NCs will differentiate into CLCs [30] altering the environment of the IVD, compromising its function. Because degeneration is progressive, once it starts it will lead to cascading effects, leaving irreversible damage. (Fig. 8)

2.2.1 Aging

There are many different factors that can cause or attribute to IVD degeneration. Aging is one of the most common causes of degeneration as around 85% of the population will shows signs of IVD degeneration by the age of 50. [75] With aging, tissues lose the ability to self-repair and produce necessary components and maintain the proper environment needed for healthy IVDs. [41] With aging, the IVD will lose hydration causing the disc to thin or lose its height (Fig 11). When the disc is thinner, the distance between the bony vertebrae in the spine is smaller and the disc is therefore less effective as a cushion, making the IVD prone to injury. Along with aging is seen a loss in proteoglycan, which can be considered the most significant characteristic in degeneration. [76] Proteoglycan fragmentation increases with age and is accompanied by loss of hydration, particularly in the NP. [58] Proteoglycans, such as aggrecan, are crucial to maintaining hydration in the IVD. Because the gag chains are negatively charged, this gives the aggrecan molecules a high affinity to bind to polar water molecules. Additionally due to their negativity, proteoglycan molecules will repel each other when compressed, and being confined by the AF, this helps the structure maintain its shape and contribute to its ability to absorb shock. [77] However, during IVD degeneration proteoglycan molecules will degrade with smaller fragments diffusing out. This loss results in decreased hydration and a drop in osmotic pressure leading to a mechanical compromise of the IVD. [78] When loaded there is a decrease in height and a rapid loss of fluid. Loading under this scenario leads to improper stress concentrations along the annulus fibrosus and end plates which can result in a tear in the AF or rupture in the NP. Loss of proteoglycans influence the movement

of molecules into and out of the IVD. Aggrecan would normally prevent movement of large molecules like serum proteins and cytokines through the matrix. [79] With a decrease in proteoglycans there is a decrease in aggrecan, allowing for the movement of growth factor complexes and cytokines into the IVD affecting cellular behavior, changing the matrix, and potentially the progression of degradation. [26]

With aging and proteoglycan loss, in the AF there is a tendency for the fine collagen II fibers to be replaced by collagen I fibers as the transitional portion between the two structures, the NP and AF, disappears, and for more of collagen II throughout the cell to become more coarse making the IVD more stiff. Reduced ECM production is also a result of aging and increases and strengthens crosslinks between collagen molecules and fibrils. [80] Increased cross-linking inhibits proper matrix turnover and repair and encourages the retention of already damaged molecules potentially leading to reduced tissue strength. [81]

Aging also has effects on the Wnt signaling pathway in the IVD. The level of Wnt signaling activity decreases with age. [82] It is known that Wnt signaling is required for embryonic development and organization of the IVD, and it has been noted that cell proliferation corresponds to the amount of active Wnt signaling in the IVD. [83] Meaning, as aging occurs, the amount of Wnt signaling decreases and as a result there is less cell proliferation in both the NP and AF of the IVD.

2.2.2 Injury

Another cause of IVD degeneration can be excessive mechanical loading or acute trauma. [74] In the field, there are currently two different hypotheses on how injury can contribute to IVD degeneration. One thought is in the case of overload. A demanding mechanical environment will produce localized trauma to the IVD which impede the healing process due to the nature of the slow turnover rate of IVD tissues. [84] Microtrauma will begin to accumulate and weaken the IVD making it more susceptible to further injury. Because the IVD is slow to self-repair, since it is an avascular

structure, the accumulation of micro-damage and further mechanical loading will outpace the repair process leading into a vicious cycle of further injury leading to degeneration. [85] The second hypothesis suggests that a hypomobile, or immobilized, scenario can induce negative changes to the surrounding tissues in the spine leading to weakness and degeneration in the IVD. This could potentially be due to reduced stimulus for the ECM to respond to, leading to less production. Another thought is that there is altered nutrient transport to the IVD allowing for degeneration, since the IVD is not supplied with proper nutrition. [85]

Fissures and tears can develop in the AF as a result of injury or weakened structure. The tears will begin to induce the inflammatory response, bringing along inflammatory cytokines as well as matrix degrading enzymes. This can also potentially lead to other IVD injuries such as herniation. IVD herniation has been associated with disruption in the AF. [86] IVD herniation is believed to be a combination effect from mechanical compression and an increase in inflammatory chemokines. [87] One of the main functions of the AF is to keep the NP pressurized and in place since the AF is now compromised this can allow unbalanced loading on the NP which can cause it to herniate and become displaced from the intervertebral space. Not only is the IVD mechanically and structurally compromised, herniation can also lead to more painful scenarios such as nerve compression. When the NP protrudes through the AF, it can compress the spinal cord nerves causing pain associated with nerve compression or spinal cord dysfunction, otherwise known as myelopathy. [87]

2.3 Wnt Signaling and its Potential Role in IVD Degeneration

While there are several pathways to investigate, a core component to my project is the Wnt signaling pathway. It is a pathway that not only regulates critical aspects of cell fate such as proliferation, homeostasis, and migration during embryonic development [88, 89] but it is found to be more active in different parts of the IVD at different developmental stages. [6]

The Wnt signaling pathway can be further separated into two different pathways; canonical and non-canonical. The canonical pathway dealing with Wnt and β-catenin while the non-canonical can be subdivided into the Wnt/Ca2+ and planar cell polarity (PCP) pathway. [90] For the purpose of this project, the canonical wnt signaling pathway will be highlighted since we studied the interaction of β-catenin and its role in IVD health. The Wnt/β-catenin pathway is known to serve as an anabolic pathway for bone, increasing its formation, [91] but has emerged as a potential therapeutic for the IVD as an anabolic pathway. [82]

2.3.1 Active Wnt Signaling

When Wnt is active, it can bind to its cellular receptor complex composed of Frizzled (Fz) family of seven transmembrane, serpentine receptors and LRP5/6 which leads to the inhibition of the destruction complex (Fig. 2.4). [92] Upon binding to the Fz complex, a signal is transduced to the cytoplasmic phosphoprotein Dvl. [93] This allows for a myriad of steps. The signal induces transmembrane translocation of Axin (along with the remainder of the destruction complex) to the region of the membrane near the Fz and LRP receptors. This allows for Dvl phosphorylation and activation which inhibits GSK3 (part of the destruction complex), preventing β-catenin from phosphorylizing as well as β-TrCP from incorporating into the destruction complex, thereby preventing it from ubiquitinating β-catenin. [94,95] Since β-catenin cannot be ubiquitinated, it is not sent to the proteasome to be degraded. Instead, this allows for cytoplasmic accumulation and stabilization of β-catenin leading to translocation into the nucleus of the cell. Once inside the nucleus, β-catenin will directly displace Groucho from TCF/Lef [96] and acts as a transcriptional co-activator with DNA bound TCF/Lef leading to the transcription of Wnt related factors. [94,95,97] Previous data shows activating and increasing Wnt signaling by β-catenin stabilization to be beneficial to the IVD. There has been shown to be an increase in NP cell proliferation

and differentiation, [98] increase aggrecan synthesis, and a decrease in aggrecanase Adamts5 expression. [99]

2.3.2 Inactive Wnt Signaling

In the inactive state, β-catenin is subjected to constant synthesis and destruction (Fig. 2.4) [95] The cytoplasmic β-catenin gets degraded by the destruction complex, consisting of Axin, casein kinase 1α (CK1α), glycogen synthesis kinase 3 (GSK3), adenomatosis polyposis coli (APC), protein phosphate 2A (PP2A), Disheveled (Dvl), and beta-transducin repeat containing protein (β-TrCP). [94,100] Axin plays a pivotal role in the destruction complex as it is the main site of interaction for many of the components in the destruction complex. [95] When bound to the destruction complex, β-catenin gets phosphorylated by CK1α and GSK3, and is then targeted by β-TrCP to be ubiquitinated and sent to the proteasome for degradation, [95] resulting in low levels of cellular β-catenin and a decrease in Wnt signaling. In the inactive state, in the nucleus TCF is inhibited by Groucho, which prevents it from binding to DNA and transcribing Wnt related genes. [101] Previous data on decreased wnt singling and β-catenin levels show a differentiation of mesenchymal stem cells from osteoblastogenesis to chondrogenesis. [102] For the IVD, there is accumulation of chondrocyte-like cells in the NP opposed to notochordal cells and ECM catabolism. [61, 99]

2.3.3 Anti-sclerostin and Other Pharmacological Activators of Wnt Signaling

Anti-sclerostin

Romosozumab is an anti-sclerostin monoclonal antibody that has recently been FDA approved for the treatment of osteoporosis in postmenopausal women. [103] Osteoporosis, a skeletal disease, [104] is often caused by the imbalance in osteoblast and osteoclast activity. Osteoblasts are bone cells responsible for secreting matrix, or

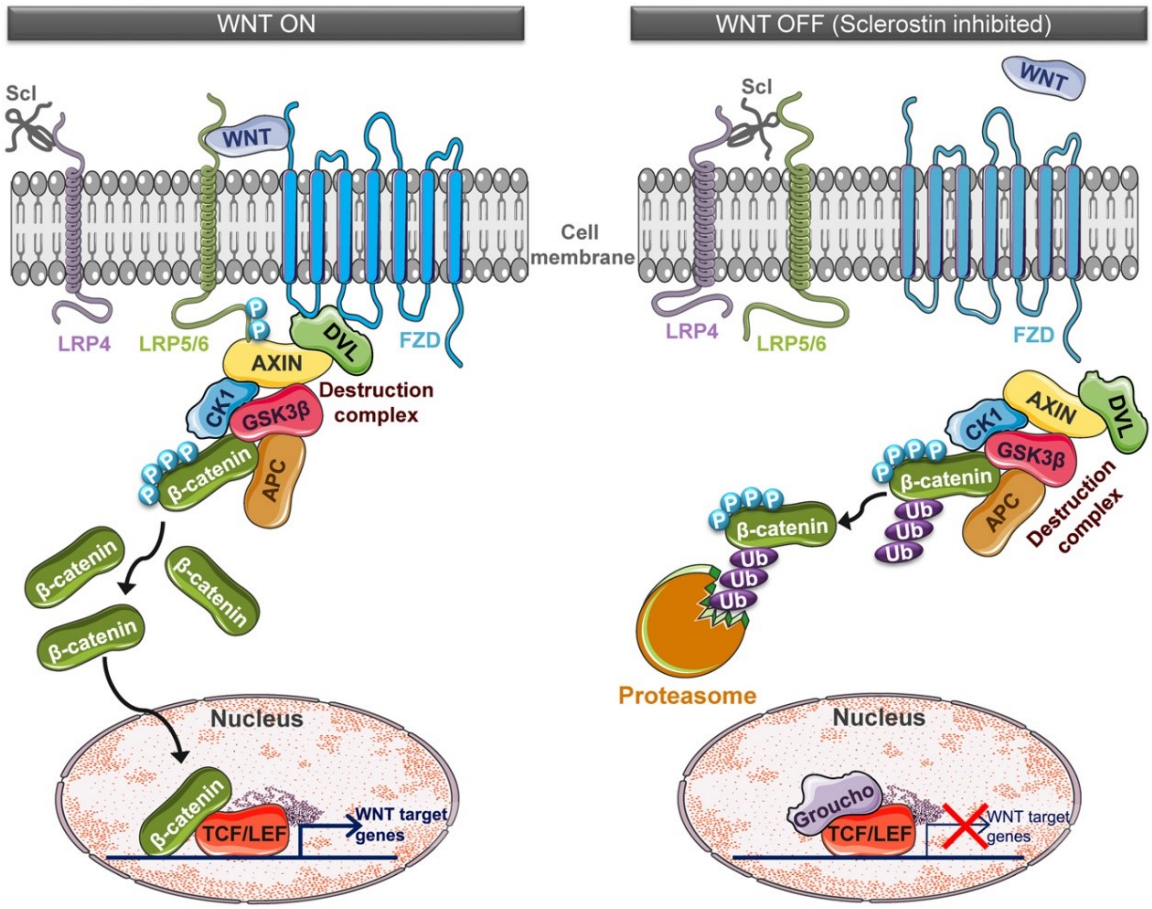


Fig. 2.4 The image below [95] shows the Wnt signaling pathway when Wnt is active or not inhibited (left) and when Wnt is inhibited by Sclerostin (right). Left) Wnt is free to bind to LRP5/6 and the Frizzled receptor analogous to the Sost KO model. This allows for the destruction complex to attach intracellularly and allow β-catenin to accumulate in the cytosol. The excess β-catenin will translocate to the nucleus of the cell and interfere with TCF/LEF transcription leading to increased Wnt signaling. Right) Sclerostin is present inhibiting the Wnt signaling pathway. The destruction complex will phosphorylate and ubiquitinate β-catenin and is sent to the proteasome to be degraded. This results in decreased Wnt signaling.

osteoid, that later get mineralized and become new bone. [105] While osteoclasts are bone cells that are responsible for absorbing and degrading said matrix. [106] But, anti-sclerostin antibodies target the Wnt signaling pathway, simultaneously inducing osteoblast activity, thereby increasing bone formation, while also decreasing bone resorption activity in osteoblasts by increasing Osteoprotegrin (Opg). [107]

This drug works by selectively targeting and inhibiting sclerostin which is a known inhibitor of the Wnt signaling pathway. When the glycoprotein sclerostin, encoded by the gene Sost, binds to the LRP5/6 and Frizzled co-receptors on the surface of the osteoblast, the canonical Wnt signaling pathway is inhibited. [103,108] Previously described, sclerostin is secreted by osteocytes, but is also expressed in the IVD. [109] By introducing anti-sclerostin, this will prevent β-catenin from binding to the destruction complex, allowing for the activation of the Wnt signaling pathway, resulting in translocation of β-catenin from the cytosol to the nucleus of the cells, leading to targeted gene transcription. In the case of osteoblasts, increased Wnt signaling leads to osteoblastic differentiation and proliferation, resulting in increased bone formation. But in the case of the IVD, studies have shown modulation of Wnt signaling to be both anabolic and catabolic to the IVD, so further in depth investigation is needed. [6, 49, 99]

Lithium Chloride

Lithium chloride (LiCl) has been found to inhibit GSK3, the enzyme that phosphorylates cytoplasmic β-catenin for ubiquitination and degradation, [110] this allows for β-catenin to translocate to the nucleus and increase the level and activity of Wnt signaling. It was revealed that LiCl slowed NP cell proliferation by inducing cell cycle arrest in the G2/M phase. [111] This portion of the cell cycle serves as a check point which allows the cell to repair damage of DNA, ensuring the accuracy of the replicated DNA, before entering mitosis. [111] If the cell cannot enter mitosis, it cannot divide and proliferate. Moreover, with the use of LiCl, there was an accompanied increase in β-gal levels which is associated with cell senescence. An increase in β-gal is associate with an increase in degeneration in IVDs, [112] additionally, it was found that an increase in nuclear β-catenin is linked to cellular senescence in the IVD. 123 Cellular senescence can lead to the inability in proper ECM metabolism maintenance further leading to IVD degeneration. Cellular senescence is also followed by the in-

duction of apoptosis. This study used LiCl to activate the Wnt signaling pathway in combination of modulating levels on β-catenin to understand the effects it has on IVD health. Further studies need to be done to understand how crucial the role of LiCl is in the modulation or activation of the pathway in order to be seen as a potential therapeutic.

2.3.4 Genetic Approaches to Regulation of Wnt Signaling

Regulation of β-Catenin

β-Catenin is a key transcriptional factor responsible for signal transduction to the nucleus triggering transcription of Wnt specific genes responsible for cell fate, [113] therefore modulation will result in different outcomes.

Deletion

One study induced a conditional knockout of β-Catenin from the growth plate and CEPs of the IVD and found that β-Catenin is necessary for the maintenance of the CEPs. It was also apparent that the deletion promoted the bone formation between the growth plate and CEP. [6] Another study showed with NP specific that β-Catenin deletion, Wnt signaling was reduced accompanied by a reduction in notochordal marker Brachyury, proliferation marker CCND1, and an increase in Osx expression matching the expression profile of a degenerated IVD. ECM alterations were also apparent as aggrecan expression decreased and Adamts5 increased. [99] Taken together, deletion of β-Catenin reduces Wnt signaling and could potentially be harmful to the IVD.

Stabilization

Stabilization of β-catenin has been shown to be anabolic to the matrix of the NP, marked by an increase in aggrecan expression and a decrease in matrix degrad-

ing genes such as Adamts5 and MMP13. [99] Notochordal marker Brachyury was increased and Wnt related markers Axin2, CCND1, and DKK1 were upregulated toward activation. In addition, Wnt ligands 16, 10b, 5a, 11, and 9a were all upregulated in response to β-Catenin stabilization. Results from gene expression indicate stabilization of β-Catenin increases Wnt signaling and is beneficial to the composition of the IVD. [99] Further, stabilization of β-Catenin was also tested as a protective mechanism in the IVD. Tails were compressed and results found the WT group had a decreased amount of β-Catenin after loading, and the β-Catenin stabilization group had increased levels after loading. Further, aggrecan expression was decreased in the WT group and unchanged in the β-Catenin stabilization group after loading. [99] This suggests that stabilization of β-Catenin is anabolic to the IVD but can also serve as a protective mechanism when loaded which reveals β-Catenin's role as a potential therapeutic in the IVD.

Consequences of Overactivation of Wnt Signaling

Overactivation of β-catenin can cause severe IVD degeneration. β-catenin protein is upregulated and activated in degenerated IVDs, [114] and is upregulated in NCs in early IVD degeneration. [115] Changes seen in conditionally activating β-catenin included a severe loss in proteoglycan in the NP and AF and degradation marked by an increase in ECM catabolic biomarkers Adamts5 and MMP13. [114] There is disorganization in the lamellae of the AF due to increased cell proliferation, disruption of the growth plate contributed to loss of hypertrophic chondrocytes and proteoglycan content, and subsequent osteophyte formation. [6, 116] These data suggest that overactivation increases Wnt signaling to a point where it is no longer beneficial to the IVD.

Regulation of LRP5

LRP5/6 is a receptor in the Wnt signaling pathway when activated leads to the stabilization of cytoplasmic β-catenin. When Wnt is present, GSK3 activity is blocked thereby allowing for β-catenin levels to increase in the cytoplasm and nucleus, increasing Wnt signaling activity. [117]

Several studies have explored the effects of modulating LRP5 in the bone as it is expressed by cells in the osteoblastic lineage but not by osteoclasts, so very limited studies have been done in relation to the IVD. Loss of function mutation, or conditional KOs in LRP5 results in a dramatic reduction in skeletal mass and low bone formation rates. [118] While gain of function mutations in LRP5 have been shown to increase bone mass. [119] Changes in LRP5/6 interfere with the Wnt signaling pathway altering bone formation rates.

One study found a relationship between LRP5, Wnt signaling and IVD degeneration. [61] While Wnt signaling decreases with aging and compression, the cell membrane receptor for LRP5 does as well. [118] Targeting Osx expressing cells in the IVD of young-adult mice, deletion of LRP5 induced IVD degeneration by inactivating Wnt signaling in the tail and lumbar. In the tail of the KO, Wnt signaling was reduced by 60% in the NP, while in the lumbar, Wnt signaling was reduced by 95% in the NP and 40% in the AF.

These data indicate that LRP5 plays a role in modulation of Wnt signaling where its deletion is associated with decreased activity leading to a reduction in bone formation and an increase in IVD degeneration. This suggests that regulation is important to normal function and could be explored as a potential therapeutic for IVD degeneration.

Sost KO

Sost, is an inhibitor of the Wnt signaling pathway. [120] When Sost is present, it will bind to the LRP5/LRP6 receptor causing a cascade of intracellular signaling

disrupting the Wnt-induced Frizzled-LRP complex formation, [121, 122] ultimately resulting in a decrease in Wnt signaling. [73] Deletion of Sost is known to increase bone formation by reducing sclerostin mediated Wnt antagonism, allowing Wnt signaling activity to increase, but the impact of Sost regulation in the IVD is yet to be determined. [121]

Sost KO Effects in Bone

Osteocytes are a key regulator in the canonical pathway. Osteocytes secrete the protein Sclerostin, encoded by the SOST gene [116] which is expressed by mature osteocytes but not early osteocytes or osteoblasts. [123] The deletion of Sost drives increased bone formation on all surfaces, promoting the survival of osteocytes and osteoblasts, while inhibiting osteoclast function by increasing Opg. Opg is a decoy receptor for receptor activator of Nfκb ligand (RANKL). With increased Opg, instead of binding to RANK on the surface of osteoblasts, RANKL will bind to Opg, limiting the formation, activation, and survival of osteoclasts. [116] This allows for an increase in BMD in both sexes of mice and improved structural properties in cortical and trabecular bone. Increased bone strength can be attributed to the changes in bone composition, as sclerostin deficient bones have increased proteoglycan content, lower crystallinity, and reduced ECM mineralization. [124]

Sost KO Effects in Cartilage

Sost has also been proposed to play a role in maintaining the integrity of healthy cartilage, [125] but have some conflicting data. Chan et al demonstrated that increased SOST in chondrocytes may protect against osteoarthritic cartilage degradation, [126] while Bouaziz et al revealed that loss of SOST promoted osteoarthritis using a SOST KO model. [127] Although some conflicting data exists in cartilage, Sost KO models have shown benefits in bone. Since SOST is a key regulator of the Wnt signaling pathway, and Wnt signaling is essential to IVD development and main-

tenance, investigating a Sost KO model in the IVD could lead to a novel therapeutic approach to IVD degeneration.

2.4 Conclusion to Literature Review

Wnt signaling plays a pivotal role in embryonic development and is key in development and organization of the NP and AF in the IVD. It is active at different levels at different times within the IVD so modulation and regulation are key in IVD health. While there are many things that can target the Wnt signaling pathway, not many have been studied in depth or long enough to know if there are long term affects, or if they directly interact with the IVD.

Wnt signaling in the IVD has been heavily researched, and with recent FDA approval, an anti-sclerostin antibody has been administered to post-menopausal women who are at high risk for vertebral fracture, but systemic application of a Wnt signaling upregulator can affect other musculoskeletal tissues. While there are "treatments" or modalities that can help with the pain associated with IVD degeneration and LBP, none have aimed at trying to be preventative or aid in reversal of the disease. Exploration in the relationship between Wnt signaling and Sclerostin could lead to a more targeted and specific treatment of IVD degeneration.

3. DELETION OF WNT INHIBITOR

3.1 Introduction

The purpose of this experiment is to study the effects of a genetic SOST KO mouse model and what the implications are on the IVD. Motivation for this study includes the FDA approved bone anabolic for post-menopausal women who are at high risk of vertebral fracture. [107,128] The drug is an anti-sclerostin antibody which works analogously to our genetic SOST KO model. Sclerostin (encoded by SOST) is an inhibitor of the Wnt/β-catenin signaling pathway and global suppression of Sclerostin by systemic injection or genetic ablation promotes bone formation. [116] But, since IVDs have also been found to secrete Sclerostin, it is unclear how or if the beneficial suppression of sclerostin in the bone would have implications on the IVD, or if alterations are a direct results of deletion from the IVD. While back pain is not noted with the anti-sclerostin-antibody administration, 142 the lack of back pain does not preclude the induction of IVD degeneration, which can occur in asymptomatic patients. [129]

3.1.1 Sclerostin and Wnt Signaling

Osteocytes are a major source of sclerostin, [108, 130] but IVD cells also express SOST/Sclerostin and modulation of Wnt signaling in the IVD impacts cell fate and the extracellular matrix (ECM). [6, 109, 114, 131] Inactivation of Wnt signaling shifts differentiation from osteoblasts to early chondrocytes while activation in early chondrocytes triggers hypertrophic chondrocyte maturation. [132] In the IVD, development requires Wnt signaling, [6] and maturation is characterized by a progressive loss. [6, 82] Signaling activity is found in the AF and endplates at the embryonic

stage and decreases with age. But in the NP, signaling is low during the embryonic stage and increases with aging. [6] With excessive upregulation, the growth plate of the IVD is severely degenerated with a loss of proteoglycan content, loss of hypertrophic chondrocytes, and structural disruption. AF cells undergo slight degeneration resulting in disorganization but induce an anabolic reaction with removal of signaling. And NP cells experience a decrease in proteoglycan content with Wnt signaling activation. [6] The activation of Wnt signaling can affect different parts of the IVD and those changes can cause cascading effects on the rest of the IVD.

3.1.2 Aim

The aim of this study was to determine if Wnt signaling is increased or decreased as a result of a global deletion of SOST and the resulting effects on the IVD. We hypothesize that a global deletion of SOST will increase Wnt signaling and decrease IVD degeneration from natural aging. We tested the hypothesis by quantifying hydration in the IVD by MRI, β-Catenin protein expression by Western Blot, phenotypic changes by histology, select protein expression by immunohistochemistry (IHC), gene expression by qPCR, and bone changes by microCT (Fig. 14). IVD desiccation is key feature of IVD degeneration, [133] that deletion of SOST improved upon in the IVD. Consequently, increased water content may have reduced the need for other ECM components and cellular signaling. For instance, SOST KO reduced the gene expression of aggrecan and notochordal cell specific markers Foxa2 and gdf5, but upregulated mature chondrocyte-like cell markers Osterix and Bglap, suggesting a cellular phenotype transition from notochordal cells to mature chondrocyte-like cells. Chondrogenic differentiation requires reduced Wnt signaling and we found that the IVD induced a compensatory reaction to SOST deletion by increasing Wnt signaling inhibitors and reducing active β-catenin in the cell nucleus. Overall, global genetic deletion of SOST contributed to mild IVD degeneration by instigating a compensatory breakdown of some ECM components and silencing cellular mechanisms, but

increased proteoglycan and imbibed the IVD, which could serve to prevent severe IVD degeneration and its consequences.

3.2 Methods

3.2.1 Mice

Male *Sost* KO mice and their wild-type (WT) littermates (n=5/group) were on C57Bl/6 background and have been previously described. [134] Mice were housed in a 12-hour light/dark cycle, fed standard chow, and all experiments were performed with prior IACUC approval. Mice were aged to 16 weeks of age and lumbar and caudal (tail) spinal sections were harvested. This was done by identifying spinal landmarks of both spinal portions and harvesting them from the mice for further separation for the following tests (Fig. 3.1). PCR genotyping was done on IVD tissue to confirm homozygous *Sost-/-*.

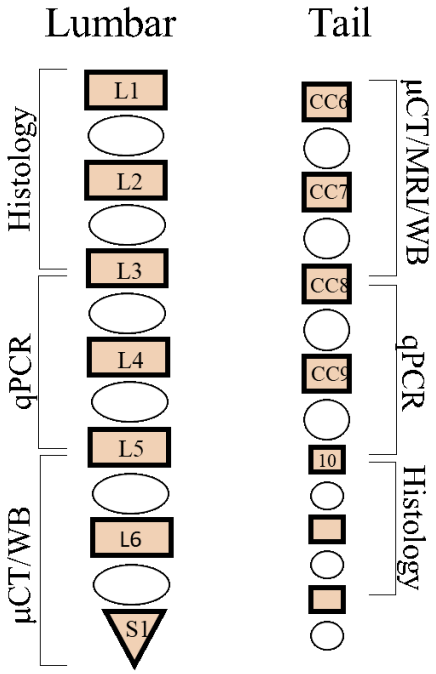


Fig. 3.1 The graphic above represents how the lumbar and caudal (tail) spine sections were separated for testing. Motion segment (bone-disc-bone) L1-L3 were used for histology, two IVD between L3-L5 used for qPCR, L6 for μCT imaging and analyzation, and two IVD between L5 and S1 for Western Blots. For the tail, CC6-CC8 were used for μCT, MRI, and Western Blot, IVDs between CC8-CC10 used for qPCR, and CC10-12 used for histology.

3.2.2 Histology and Immunohistochemistry

WT and Sost IVD were run in one batch. L1-3 and CC10-11 were fixed in 15 mL of 10% formalin on a rocker for 24 hours and kept in 70% ethanol until embedding in paraffin and sectioning (5 μm). Safranin-o/fast green counterstain images were analyzed by 4 independent observers and then averaged for IVD degeneration scoring. [52, 61] In short, the NP, AF, and boundary between the two structures were scored based on structural properties and added for a total IVD score between 0-14, with increasing scores denoting greater IVD degeneration. Proteoglycan content in NP was estimated as the amount of staining per area. Alcian blue staining was the counter stain for the IHC staining of Osterix (#22552, Abcam) and Collagen 2 (II-II6B3-c, DSHB). For both Osterix and Collagen 2 quantification in the NP, positive cells were counted and compared to the total number of cells that stained brown for the protein and blue for the cell nuclei. For Osterix quantification in the AF, the percent of the area stained brown was measured and compared.

3.2.3 MRI

Motion segment CC6-7 was submerged and wrapped in 1x PBS-soaked gauze overnight in a 4°C freezer until imaged. The MRI machine used was the Bruker BioSpin 9.4 T using a 400 nm slice thickness for 2D imaging. The motion segment was imaged in a sagittal orientation using a 0.052 x 0.052 mm voxel resolution in the x-y direction and a 0.4 nm voxel resolution in the z direction taking 16 averages/slice. Two samples were placed, one on top of the other, inside of a glass tube to remain upright, and two glass tubes were placed, separated by foam composite, inside of a 15mL tube to ensure samples would not move while being imaged. ImageJ (NIH) was used to analyze the images. Area and MRI intensity of the IVD were determined and multiplied to estimate the hydration content of the IVD.

3.2.4 Micro-computed Tomography

Motion segments L6-S1 and CC6-7 were harvested and submerged in 1x PBS prior to imaging. Specimens were imaged using the Bruker SkyScan 1272 Micro-CT at a resolution of 8 micrometers. Images of motion segment were contoured around the periosteal and the endosteal of the bone. For the trabecular analysis, the growth plate was used as a landmark and trabecular bone analysis consisted of the next 30 consecutive images (slices). [135] For cortical analysis, the longitudinal center of the bone was identified and 15 images above and below were analyzed using the Bruker CTan64 MicroCT software. [16] The reason behind these ROI is due to the quick variation between trabecular and cortical bone in the small vertebral structure of mice. Parameters measured include; BV/TV (bone volume/tissue volume), cross-sectional thickness, trabecular number (Tb.N), trabecular thickness (Tb.Th), etc., using a lower threshold of 60 and upper threshold of 225 for analysis.

3.2.5 Western Blots

IVDs between CC6-8 were isolated for whole cell lysate and cytoplasmic and nuclear separation Western Blots. IVDs were minced in ice-cold phosphate buffered saline (PBS; Fisher) containing 2% fetal bovine serum (FBS; Atlanta biologicals) and protease inhibitor PMSF (Sigma). Two tail IVDs from a single animal, per isolation method, were homogenized using a Tissue Tearor (BioSpec Products). Whole cell lysate was generated using diluted 1x cell lysis buffer (Cell Signaling) supplemented with PMSF. Fractionation of the nuclear protein was performed according to the Pierce cytoplasmic and nuclear extraction kit instructions (Sigma). Samples were run on an SDS-Page gel (BioRad) and transferred to PVDF membrane (BioRad). Blots were probed for anti-β-catenin (unphosphorylated; Cell Signaling) and subsequently an HRP-tagged anti-Rabbit secondary antibody (#7074S, Cell Signaling). Whole cell lysates were normalized to HRP-tagged Actin antibody (#A3854, Sigma Aldrich) and nuclear fractions to HRP-tagged Histone H3 (#12648, Cell Signaling). All blots were

developed using Immobilon Luminata Forte (Sigma) and images collected digitally with the Amersham Imager 600 (GE Healthcare). Densitometry quantification was conducted on ImageJ to enumerate relative protein values between groups.

3.2.6 QPCR

L3-5 and CC8-10 IVDs were harvested separately, frozen in liquid nitrogen, hand-pulverized using a metal mortar and pestle, then suspended in TRIZOL (Ambion) until completing the following. [61] RNA isolation and purification steps were followed (RNeasy mini kit, Qaigen) and RNA concentration was quantified (Nanodrop). CDNA was synthesized (iScript, Biorad) from 400 ng of total RNA for the following Taqman probes (Life Technologies); aggrecan (`Mm00565794_ m1`), keratin8 (`Mm04209403_ g1`), dmp1 (`Mm01208363_ m1`), Sost (`Mm00470479_m1`), adamts5 (`Mm00478620_m1`), collagen1 (`Mm00801666_g1`), collagen2 (`Mm01309565_m1`), osterix (`Mm04209856_m1`), β-catenin (`Mm01350387_g1`), serpina1a (`Mm02748447_g1`), serpina1c (`Mm04207703_mH`), serpina1d (`Mm00842095_mH`), sosdc1 (`Mm03024258_s1`), foxa2 (`Mm01976556_s1`), axin2 (`Mm00443610_m1`), sFRP4 (`Mm00840104_m1`), gdf5 (`Mm00433564_m`), Wnt16 (`Mm00446420_m1`), Dkk1 (`Mm00438422_m1`), Wnt3a (`Mm00437337_m1`). Relative gene expression was normalized to IPO8 (`Mm01255158_m1`) for each group and then experimental values (Sost KO) were normalized to the average of the WT value ($2^{-\Delta\Delta CT}$)). Gene expression properties that did not differ between groups (Table 3.1).

Table 3.1 The table below displays genes that were tested in qPCR but relative expression was not different between WT and KOs. Values in KO column are expressed as mean, standard deviation. Keratin 8 is a marker for notochordal markers, Serpin A1A, A1C+, and D relate to inflammation, Sostdc1, Axin2, Wnt 16, and Wnt3a are involved with the Wnt signaling pathway.

Gene	WT	Sost KO	P-val
Keratin 8	1	0.50, 0.50	0.26
SerpinA1A	1	0.93, 0.45	0.80
SerpinA1C+	1	0.72, 0.09	0.06
SerpinA1D	1	0.52, 0.19	0.11
Sostdc1	1	1.86, 0.71	0.11
Axin 2	1	1.38, 0.75	0.38
Wnt 16	1	1.79, 1.46	0.36
Wnt 3a	undetermined	undetermined	N/A

3.2.7 Statistics

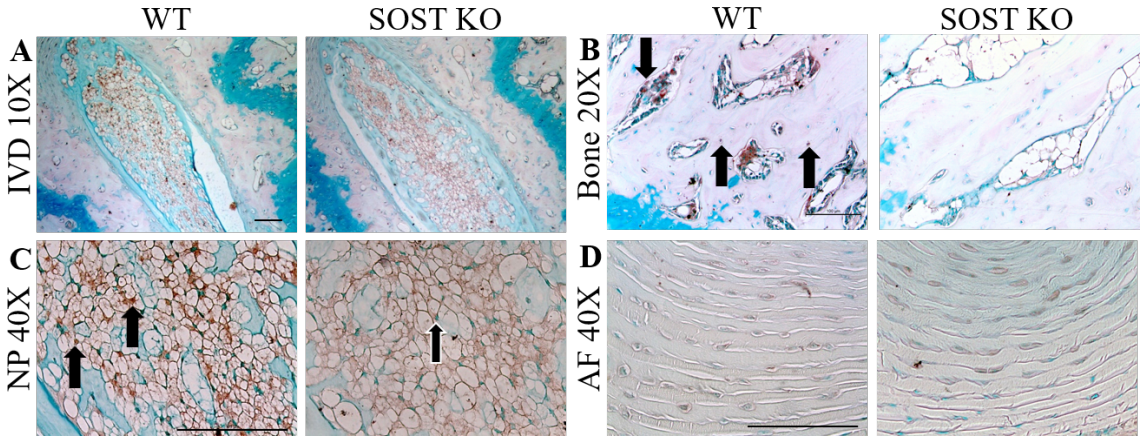
A three-way ANOVA (section, level, and region) was used for histological grading and analyzed using SPSS (IBM Version 25), and a Gabriel's post-hoc test was used due to small sample sizes. Correlations associated IVD degeneration to osterix expression in the NP and to bone cross-sectional thickness. All other outcomes were compared by a Student's T-test. Data were expressed as mean +/- standard deviation. For all tests, a p-value of ¡ 0.05 was considered significant.

3.3 Results

3.3.1 Corroboration of Global *Sost*/Sclerostin Deletion

To demonstrate efficacy of the mouse model, immunohistochemistry staining of Sclerostin was performed as well as qPCR (Fig. 3.2) for corroboration. Qualitatively from IHC staining of the IVD (Fig. 3.2 A-D), there are more positively stained cells (brown dots), indicating sclerostin expression, in the WT, and more diffuse staining

in the KO, indicating less Sclerostin expression. Because osteocytes are known to secrete sclerostin, from the images of bone, in the WT, there are more brown stains indicating sclerostin is expressed by osetocytes and osteoblasts while in the KO there is essentially no sclerostin staining. (Fig. 3.2B).



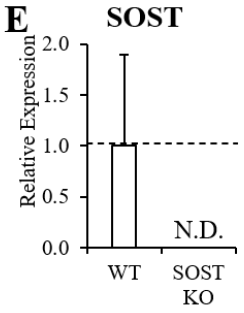


Fig. 3.2

Fig. 3.2 Qualitative images of sclerostin staining of the WT and SOST KO IVD and Sost gene expression from qPCR. A) 10X magnification image of IVD showing Sclerostin staining of the IVD, with the KO appearing to have less staining. B) 10X magnification image of the vertebrae in the WT and KO, with more intense brown staining in the WT indicating more osteocytes. C) 40X magnification of the NP, the WT having more defined brown staining while the KO has less sclerostin staining indicating there is less sclerostin expression in the KO. D) 40X magnification image of the AF, resembling the NP, the WT has more brown stained cells in the AF than the NP, again indicating less Sclerostin expression in the AF. E) qPCR of Sost with the KO being normalized and compared to the WT. Sost gene expression was not able to be detected (N.D. – not detected). Scale bars represent 100 μm. n=5/group

Further, qPCR analysis from IVDs of the WT and KO found Sost gene expression was not detectible in the KO (Fig. 3.2E). Lastly, from microCT imaging (Table 3.2), bone parameters increased in the KO as expected.

Table 3.2 The table displays different bone parameters measured between the WT and KO of the tail and the lumbar. Values displayed as mean, standard deviation. * indicates significance of $p<0.05$. n=5/group

Parameter	Tail		Lumbar	
	WT	KO	WT	KO
Trabecular BV/TV (%)	49.36%	53.52%	25.26%	45.74%
Cortical Thickness (μm)	148, 15	172, 12 *	-	-
Tb.N	5.52, 0.30	5.84, 0.14	6.11, 0.50	6.45, 0.76
Tb.Th (μm)	0.089, 0.01	0.091, 0.01	0.069, 0.01	0.071, 0.01

3.3.2 Deletion of *Sost* Increased IVD Hydration and Proteoglycan Staining

MRI measures hydration content by intensity or brightness of an image. Brightness and intensity can be compared qualitatively (Fig. 3.3A) and the brighter and more intense an image, the more hydration in the structure. Quantitatively, relative water content was estimated by multiplying the area and intensity measured (Table 3.3) and Sost KO increased the level of hydration by 71.6% in the KO IVDs (Fig. 3.3B). Deletion of Sost increased proteoglycan staining. From Safranin-o and Fast Green counterstain images (Fig. 3.4A), proteoglycan staining was measured by the amount, or percent, of red staining present in NP area. While not different in the lumbar, with deletion of Sost, proteoglycan staining significantly increased by 137% in the KO (Fig. 3.4B). Amount of proteoglycan and water content are correlated, [76, 136] meaning the increase in proteoglycan staining in the KO could help explain the increase in hydration from the MRI in the KO.

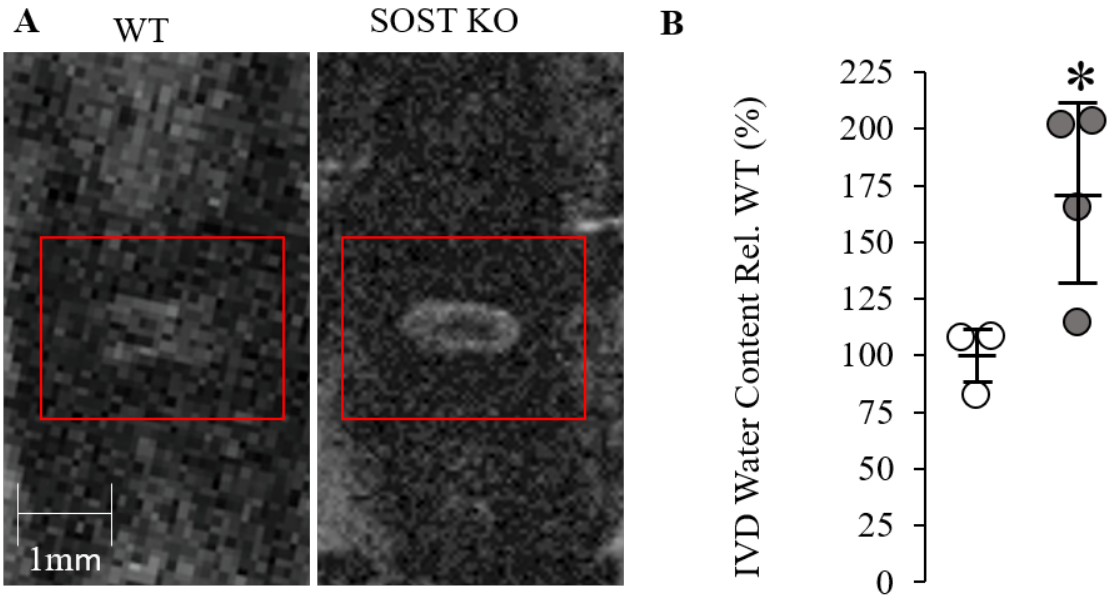


Fig. 3.3 Using a 9.4T MRI, motion segments were imaged and relative quantifications were measured using ImageJ. A) MRI of WT and SOST KO highlighting the IVD. MRI measures hydration content by intensity or brightness of an image. From the images the Sost KO image is brighter than the WT indicating more brightness. B) The graph represents the quantitative measure of the MRIs from the WT (white dots) and KO (grey dots) IVDs using the area multiplied by intensity, indicating increased hydration in the KO. *$p<0.05$ Scale bar is 1 mm. WT n=3, KO n=4.

Table 3.3 The table below shows measurements from MRI of each sample. Area and Intensity were measured using ImageJ and multiplied together to obtain the Estimated Water Content value. N=3 for WT and N=4 for Sost KO, this is due to imaging limitations.

Animal	Area (mm^2)	Intensity	Estimated Water Content
WT 1	0.32	956	301.12
WT 2	0.32	1235	395.20
WT 3	0.32	1004	391.56
WT AVGV	**0.34 +/- 0.04**	**1065 +/- 149.2**	**362.63 +/- 52.29**
SOST KO 1	0.65	1145	739.67
SOST KO 2	0.64	1155	733.43
SOST KO 3	0.34	1225	416.50
SOST KO 4	0.40	1501	600.40
SOST KO AVG	**0.51 +-/ 0.16**	**1256.5 +/- 166.8**	**622.50 +/- 151.61**

3.3.3 Deletion of *Sost* Induced Mild IVD Degeneration

Despite the increase in hydration and proteoglycan staining, deletion of Sost mildly increased IVD degeneration score. IVD Degeneration scores were characterized between AF, NP, and boundary using Safranin-o images (Fig. 3.4A). There was an interaction (*) between KO, part, and level indicating the KO increased IVD degeneration scores increase in the KO group when compared to the WT group. (Fig. 3.4C). Using a Gabriel's post-hoc test due to small sample sizes, there was also a main effect between parts (#). This means when only looking at parts of the IVD, the NP degeneration score is different from the boundary degeneration score. Together, this suggests that the NP of the KO group degenerates the most. To elucidate an increase in degeneration, form IHC, there was a decrease in Col 2 protein expression (Fig. 3.5A,B), an increase in Osterix protein expression in the NP (Fig. 3.5C,D), and a decrease in Osterix protein expression in the AF of the KO (Fig. 3.5E,F). For Col 2, the fraction of positively stained cells (brown cells) decreased by 49.3% in the KO

(Fig. 3.5B). The fraction of positively stained cells for Osterix in the NP increased by 65.9% in the KO (Fig. 3.5D). Lastly, for the AF, the percentage of the area stained brown when normalized to the WT decreased by 53% in the KO (Fig. 3.5F). These findings could hint toward a cellular phenotype switch accompanied by degeneration.

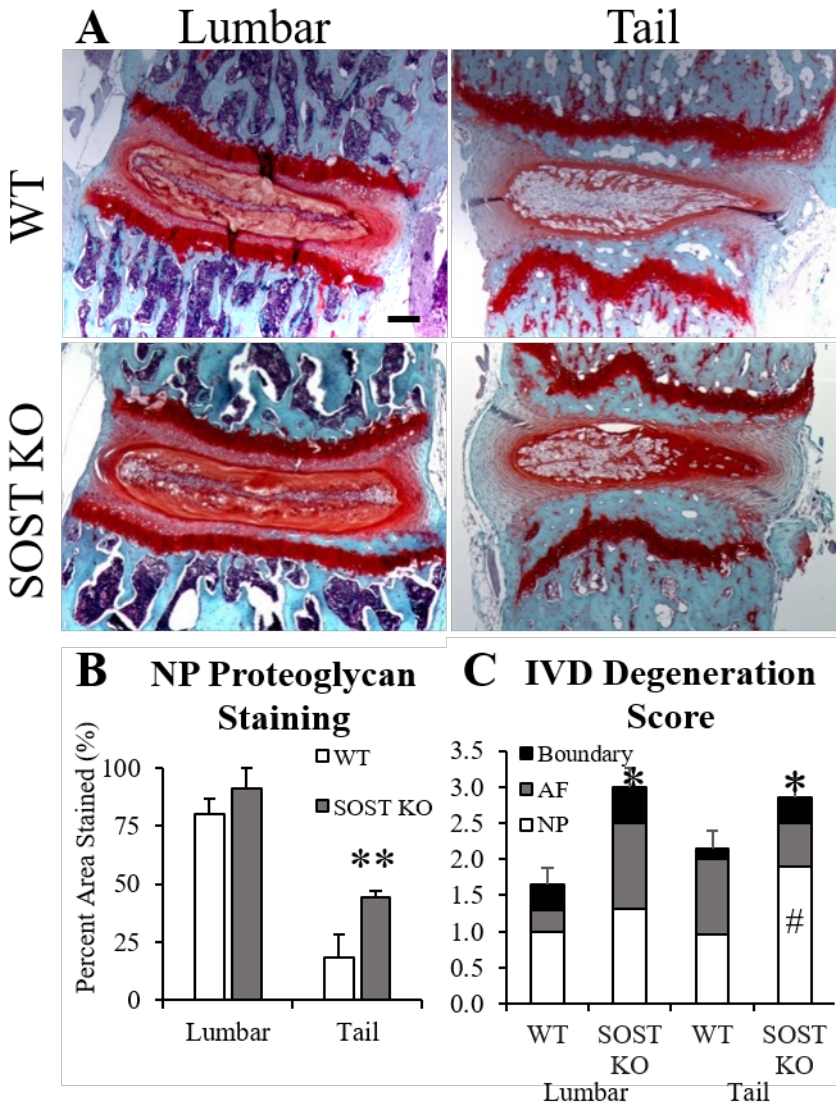


Fig. 3.4 A) Safranin-O and Fast Green counter stain images of WT and Sost KO lumbar and tail IVD. Red staining indicating proteoglycan content. Scale bar is 100 μm. B) Quantitative measurement of proteoglycan content of lumbar and tail IVD of WT and KO **p<0.01. C) IVD Degeneration scores using NP, AF, and boundary structure for grading between lumbar and tail and WT and KO. * indicating main effect between groups, both KO groups received a higher score than their WT counterpart, # indicating an interaction between KO, part, and level. Grading scale is 0-14. n=5/
group

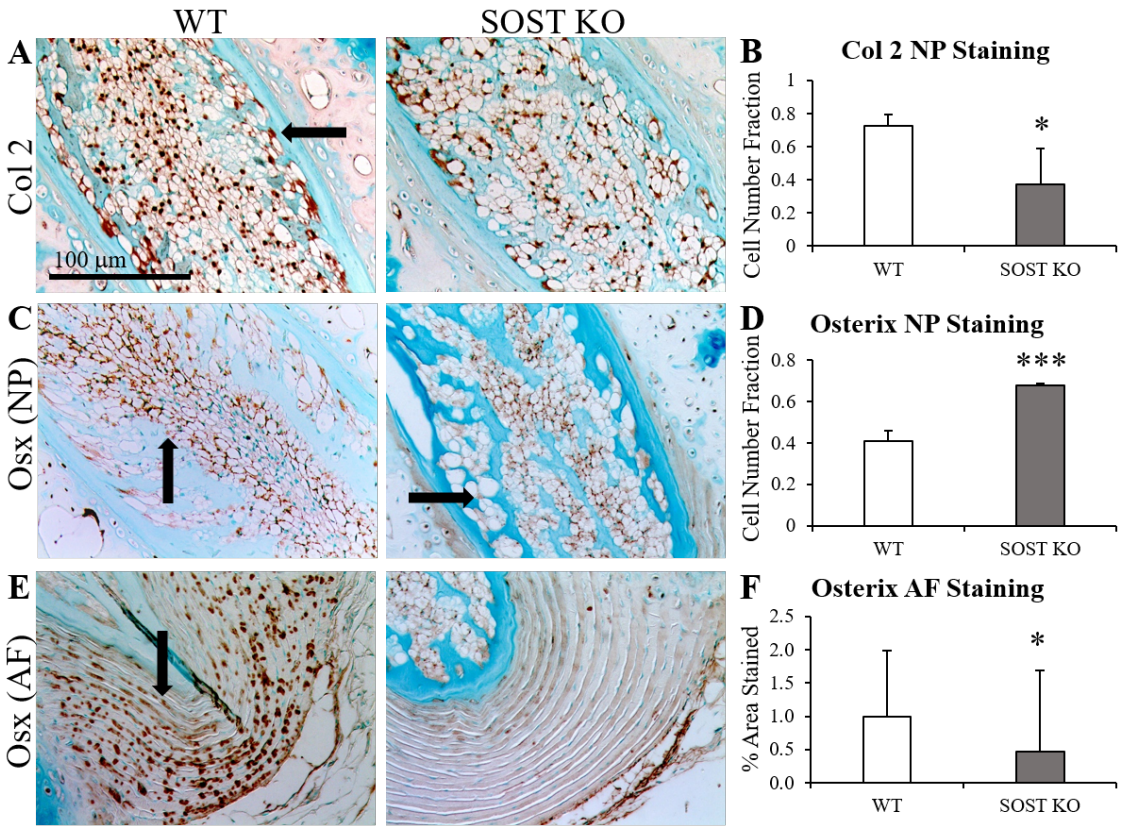


Fig. 3.5 A) Collagen 2 staining of the NP of the WT and KO. B) Quantitative measurement of the fraction of the number of positively (brown) stained cells for collagen 2. C) Osterix staining of the NP of the WT and KO. D) Quantitative measurement of the fraction of the number of positively (brown) stained cells for Osterix. E) Osterix staining of the AF of WT and KO. (F) Quantitative measurement of the % area stained positive for Osterix. *$p<0.05$ Scale bar is 100 μm. Arrow indicating positively stained cell. n=5/group

3.3.4 *Sost* Deletion Alters Matrix Composition in the IVD and Shifts Cellular Phenotype Expression

QPCR was run on WT and KO tails (Fig. 3.6). Alongside the decrease in Col 2 protein expression from IHC, deletion of Sost also decreased expression of genes related to matrix composition and anabolism (Fig. 3.6A). Aggrecan, related to proteoglycan, decreased 1.7-fold in the KO. Adamts5, an aggrecanase, was significantly increased 1.6-fold in the KO. Early chondrocyte-like cell markers Collagen II decreased in the KO by 1.6-fold (Fig. 3.6A) also corroborated by IHC. Further, Sost deletion decreased ColX, a marker for hypertrophic chondrocytes, 2-fold (Fig. 3.6A). Though these data confound the increase in hydration and proteoglycan content, the changes in matrix related gene expression could be due to the decrease in heat shock protein expression (Fig. 3.6B). Sost deletion decreased notochordal and early chondrocyte-like cell expression and increased mature chondrocyte-like cell expression again hinting toward a cell phenotype switch aforementioned with IHC staining. For notochordal cell markers Foxa2 and Gdf5, Sost deletion significantly decreased expression by 2.3-fold and 3-fold respectively (Fig. 3.6C). Concomitantly, deletion of Sost increased mature chondrocyte-like cell markers Sp7 (Osterix)3.7-fold in corroboration with IHC, as well as Bglap (Osteocalcin) 2.4-fold respectively (Fig. 3.6C). Taken together, these data indicate a cellular phenotype switch toward one of a more mature and degenerated expression profile.

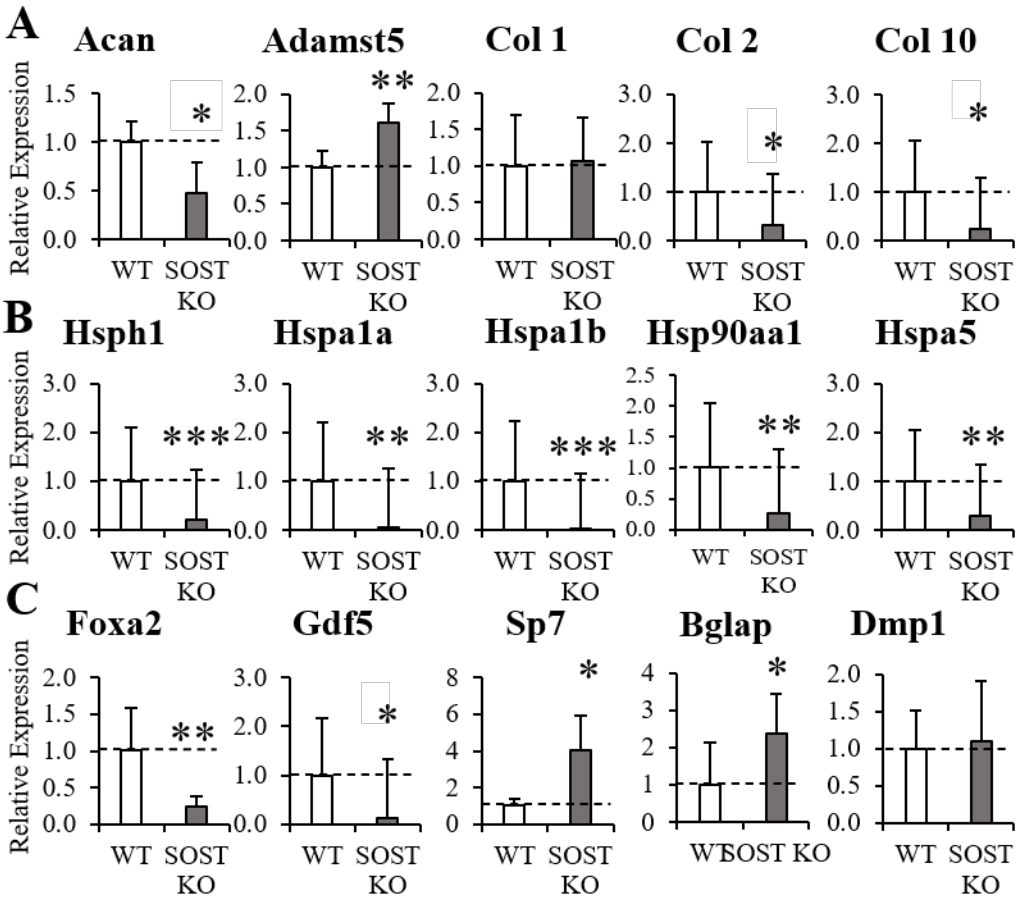


Fig. 3.6 Gene expression of ECM, Wnt signaling, heat-shock proteins, and cell type-specific markers in WT and Sost KO IVD. A) Genes related to matrix metabolism; Aggrecan, Adamst5, Collagen I, Collagen II, and Collagen X C) Heat Shock Protein genes D) Genes related to cellular phenotypes; Foxa2, Gdf5, Osterix, Osteocalcin, Dentin-Matrix Protein *p<0.05 **p<0.01 ***p<0.001 n=5/group.

3.3.5 *Sost* Deletion Results in a Wnt Signaling Compensations in the IVD

It is known that with degeneration and aging there is a loss or reduction of Wnt signaling, [6, 82] and previous data has shown that stabilization of β-Catenin has shown to be beneficial to the IVD by ECM anabolism while deletion of β-Catenin showed ECM catabolism. [137] Contradicting results from MRI, histology, and IHC, lead us to further investigate the level of Wnt signaling present by quantifying the amount of active β-Catenin in the cells. From western blots, *Sost* deletion decreased the amount of active β-Catenin protein expression in the cell nuclei of the KO (Fig. 3.7B), meaning there was less Wnt signaling activity. But, the amount of active β-catenin is unchanged in the cytoplasmic separation (Fig. 3.7C) and whole cell lysate Western Blot (Fig. 3.7D) which could explain unchanged levels of β-catenin from qPCR (Fig. 3.7A). Markers of downstream regulators of Wnt signaling increase in the KO when compared to the WT. From qPCR, there was a 9.7-fold increase in Dkk1 expression and a 3.45-fold increase in Sfrp4 expression (Fig. 3.7A), both of which are negative regulators of Wnt signaling. This change in Wnt signaling level may attribute to the phenotype switch, which is to be further discussed.

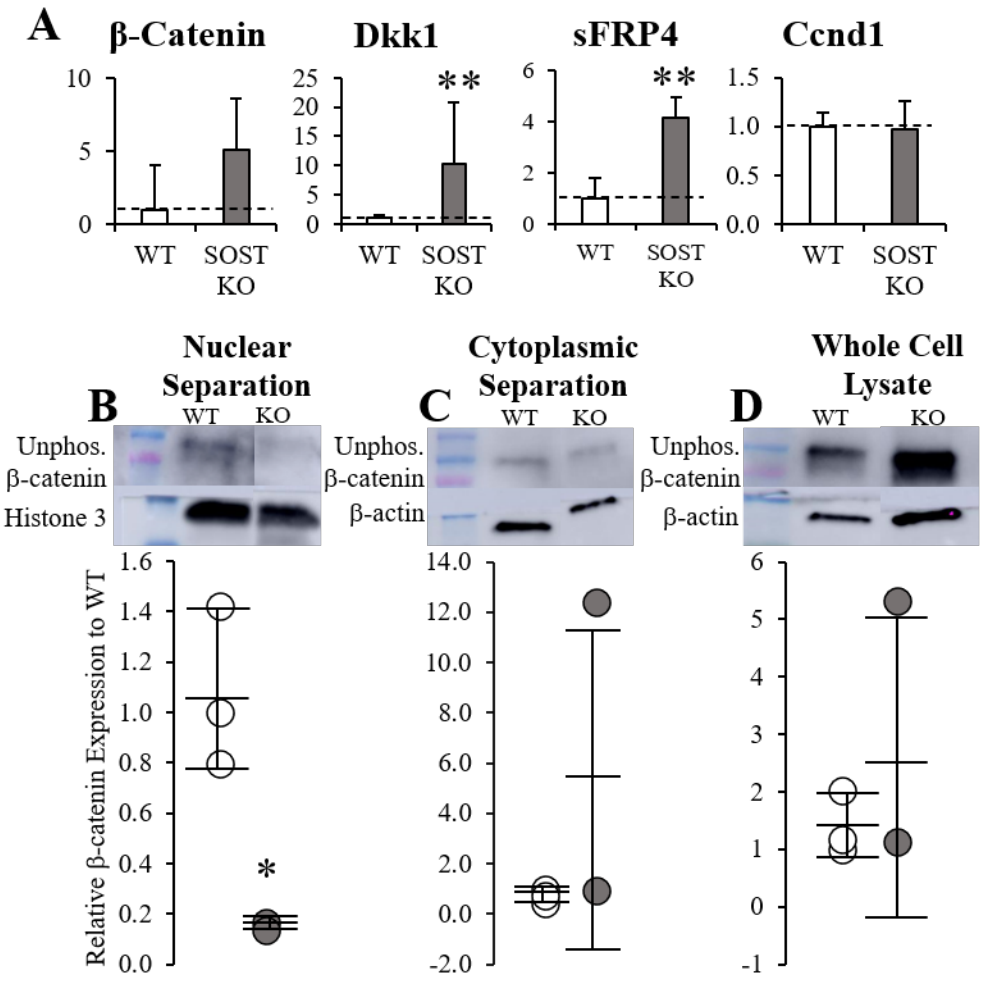


Fig. 3.7 Regulators of the Wnt signaling pathway and Western Blot data. A)Genes involved in the Wnt signaling pathway, β-catenin is unchanged between the WT (white circles) and KO (grey circles), increased Dkk1 and sFRP4 (downstream regulators of the Wnt patheway) expression in the KO, and unchanged expression of Ccnd1. B) Nuclear separation western blot shows decreased amount of active β-catenin in the cell nuclei of the KO, using Histone 3 as control. C) Cytoplasmic separation western blot shows unchanged amount of active β-catenin in the cytoplasm, using β-actin as control. D) Whole cell lysate western blot shows unchanged amount of active β-catenin using β-actin as control. *p<0.05 **p<0.01 Unphosphorylated (active) β-catenin at 92 kDa, Histone 3 at 17 kDa, β-actin at 92 kDa. n=3/group

3.3.6 *Sost* KO Increases Bone Structure

With deletions of sclerostin, bone changes occur while not significantly altering body mass. In the tail, vertebral trabecular bone volume/tissue volume (BV/TV) increases by 80.6% in the KO (Table 3.2). Vertebral cortical thickness increased by 16.2% in the KO. Cortical bone volume (BV) increases 15.9%. In lumbar trabecular bone, tissue surface increases by 9.6%, bone surface increased by 24.8% but there was not a significant change in BV/TV.

3.3.7 Correlations

IVD degeneration is correlated to osterix expression and bone structure. A moderately strong positive correlation exists between tail degeneration score and percent of the NP stained by Osterix, meaning the more the NP is stained by Osterix positive cells, the more likely it is to be degenerated (Fig. 3.8A). While a moderately strong negative relationship between cross sectional thickness and tail NP degeneration, meaning the thicker the cross-sectional area, the more likely the IVD is degenerated (Fig. 3.8B).

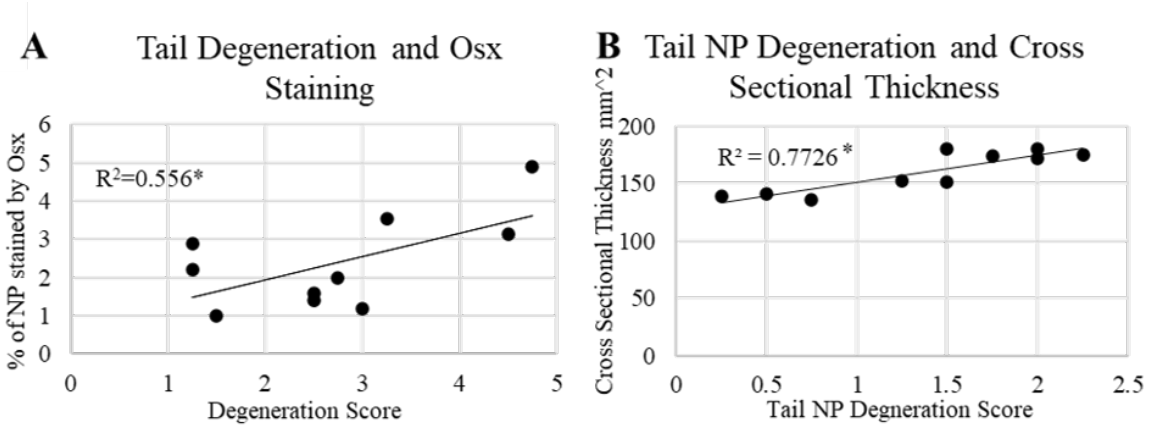


Fig. 3.8 Correlations exist between tail IVD degeneration scores and Osx staining and vertebral cross-sectional thickness. A) With more of the tail staining positive for Osx it was found that the degeneration score increases as well. This, correlates increasing Tail IVD Degeneration Scores with increasing Osx staining. B) With an increasing thickness of the vertebral cross-section the score of the NP in the tail increases. This, correlates increasing Tail IVD Degeneration Scores with increased cross-sectional thickness.

3.4 Research Discussion

3.4.1 Global Deletion of *Sost* Improved the IVD

We hypothesized that global deletion of Sost would stimulate ECM anabolism of the IVD by increasing Wnt signaling. Osteocytes and osteoblasts are the dominant Sclerostin-expressing cells and loss of sclerostin in these cells induces bone formation, [138] but cells of the IVD also express *Sost*/Sclerostin. [73] Further, IVD-specific upregulation and downregulation of Wnt signaling is anabolic and catabolic to the ECM, respectively, and similarly consequential to the mechanics of the IVD. [3]

Here, alongside increasing vertebral bone structure, [116] global Sost deletion increased hydrophilic proteoglycan in the nucleus pulposus and imbibed the IVD measured by high-resolution magnetic resonance imaging (MRI). Consequently, while the increase in water content improved the ECM, that may have reduced the need for other ECM components and cellular signaling. The benefits to the ECM were ac-

companied by a cellular phenotype switch consistent with IVD degeneration and a compensatory loss of Wnt signaling.

Early notochordal cells of the nucleus pulposus as distinguished by nucleus pulposus-specific (FoxA2 [139], Gdf5 [140]) and early chondrocyte markers (Col2 [140]) were replaced by CLC cells as indicated by Osterix/Sp7 expression. [61] Overall, these data suggest that suppression of Sost/Sclerostin may improve IVD desiccation, which is a key feature of IVD degeneration, [133] but caution must be applied to avoid compensatory chondrogenesis.

3.4.2 *Sost* KO Increased IVD Hydration

Wnt signaling may attenuate IVD degeneration by promoting ECM anabolism and hydration and extracellular regulation of Wnt signaling has pharmacological potential. IVD degeneration is characterized by a loss of IVD height from proteoglycan breakdown and dehydration. [58, 141–143] MRIs are used in the clinic to assess and diagnose IVD pathology in patients experiencing back pain [144–146] and the signal intensity of the IVD from T2-weighted MRI represents the water content. [137] Here, deletion of Sost increased water content of the IVD and was corroborated by greater histological staining for proteoglycan in the nucleus pulposus by Safranin-O. Similarly, genetic stabilization of β-catenin in the nucleus pulposus increases Wnt signaling, proteoglycan staining, and aggrecan gene expression. [99] These data suggest that Sost, in competition with Wnt ligands, binds to an LRP to inhibit Wnt/β-catenin signaling in the IVD. We find that deletion of β-catenin [99] and LRP5 [61] in the IVD similarly reduce Wnt signaling.

However, few IVD cells express Sost and deletion of Sost is strongly compensated by upregulation of other Wnt signaling inhibitors (Dkk1 [147] and sFrp4). Therefore, upregulation of Wnt ligands may be a more potent and consistent method to activate Wnt signaling and stimulate IVD regeneration. For instance, genetic stabilization of β-catenin increases the mRNA expression of Wnt ligands 5a, 9a, 10b, 11 and 16. [99]

Further, overexpression of Wnt3a, 5a and 11 in nucleus pulposus cells from herniated IVD patients stimulates aggrecan expression and glycosaminoglycan content. [148]

In addition to ECM anabolism, Sost KO suppressed the gene expression of mechanoresponsive heat shock proteins which serve to regulate the IVD in response to environmental stresses, [149] and are increased with IVD degeneration [150] and herniation. [151] Potentially, the IVD cells did not require to be as mechanoresponsive because of the elevated hydration. Reduction of *Sost*/sclerostin improved a key clinical feature of the IVD, e.g., water content, and these data implies that other extracellular agents may similarly stimulate Wnt signaling in the IVD.

3.4.3 Deletion of *Sost* Shifted Cellular Phenotype

Despite imbibition of the IVD, deletion of Sost shifted the cellular phenotype populating the IVD from notochordal cells to CLCs, a cellular shift inconsistent with the increased Wnt signaling expected from deletion of *Sost*. Wnt signaling is a driver of cell fate, [115, 152]proliferation and homeostasis. [88, 89] Here, we find that *Sost* KO reduced the gene and protein expression of markers of notochordal cells (FoxA2 [139] and Gdf5 [153–156]) and of early chondrocytes (Col2 [157] and Col10 [64]), but increased the gene and protein expression of markers of CLCs (Osterix [158] and BGLAP (Osteocalcin)). We have previously targeted Wnt signaling in the IVD and similarly note opposing regulation of notochordal cell and CLC markers.62,113 In contrast to the expected regulation by *Sost* KO, these studies [61,99] demonstrate that reduced Wnt signaling reduce markers of notochordal cells and increase markers of CLCs, because notochordal cells require differentiation factors Wnt signaling, FoxA2 and gdf5. [159–161]

3.4.4 *Sost* Deletion Triggers Wnt Signaling Compensation

We determined regulation of intracellular and extracellular constituents and targets of canonical Wnt signaling. Gene expression of *Sost* was not detected in Sost

KO IVD, but gene expression for Wnt signaling transcription factor β-catenin and downstream target CCND1 was not significantly different from WT IVD. Secondly, the cell nuclear fraction of unphosphorylated (active) β-catenin was less in the Sost KO IVD compared to WT IVD. Lastly, Wnt signaling inhibitors Dkk1 and sFRP4 were highly upregulated in Sost KO IVD. These results suggest that Wnt signaling is downregulated in *Sost* KO IVD by a compensatory mechanism. Dkk1is a downstream target of Wnt signaling, [147] which may be upregulated to prevent over activation of Wnt signaling. Compensation of Wnt signaling-related agents can occur in bone [162, 163] and IVD. For instance, genetic deletion of β-Catenin following 1 week of tamoxifen dosing is ephemeral and ineffective over the long-term, as noted by upregulation of β-Catenin and affirmed by an increase of CCND1 gene expression. [99] The transcriptional promotion of ECM degradation in Sost KO IVD is further indicative of a transcriptional decrease in Wnt signaling, [61, 99] which may have occurred to maintain homeostasis in already healthy IVD. It is unclear if this compensation would occur in the physiological case of a degenerated IVD or an osteoporotic-like phenotype.

3.4.5 Limitation

We would like to address some of the limitations of the study. First, anti-sclerostin antibody is currently available to postmenopausal women at great risk for fracture, but the young-adult, male mice in the current study correspond to humans aged 20-30 years. [164] Further, while the current study was conducted using male mice, Sost deletion may similarly impact the IVD of females because Wnt signaling is anabolic in female IVD [99] and bone formation and bone strength show results are similar between male and female mice. [116] However, the impact of estrogen-deficiency and aging on the responsiveness of the IVD to deletion of Sost is unclear and may be impaired. [99]

Secondly, QPCR was performed on entire IVDs and the effect of *Sost* KO could not be differentiated by gene expression, but key outcomes were discerned by immunohistochemistry staining. For instance, Sost KO increased the gene expression for Osterix (Sp7) in the IVD and the number of cells expressing Osterix protein in the NP, whereas the fewer cells expressed Osterix in the AF. Thirdly, there may be interactive effects in the global KO between the IVD and neighboring tissues that express *Sost*, e.g., bone.

Lastly, notwithstanding the issue of homogenized NP and AF tissue, we find that nuclear protein expression of active β-catenin was less in *Sost* KO IVD than WT IVD, suggesting that persistent Wnt signaling is impaired with deletion of *Sost*. Therefore, future studies using anti-Sclerostin antibody to regulate Wnt signaling may need to clarify the mechanism preventing activation of Wnt signaling.

3.4.6 Conclusion

Deletion of *Sost* increased IVD ECM anabolism and hydration, but endogenous compensation of Wnt signaling may have also increased catabolic ECM markers and promoted a cellular phenotypic switch toward chondrogenesis. Together, these data show that the musculoskeletal benefits of anti-sclerostin-antibody romosozumab (Evenity) may extend beyond bone to improve key features of the IVD.

4. DISCUSSION

4.1 Experimental Discussion, Limitations, & Recommendations

4.1.1 Summary

Our experiment yielded promising results. We showed that modulation of the Wnt signaling pathway via the deletion of *Sost* could be beneficial to the IVD, although a global KO could be excessive. The initial reaction to *Sost* deletion was an increase in Wnt signaling. This can be explained by the increase in hydration found form MRI and the increase in proteoglycan content, the main component that contributes to maintaining hydration. This data matches previous experiments with modulation of β-catenin and increased proteoglycan production. Holguin and Silva found with β-catenin stabilization in the IVD, Wnt signaling was increased as well as aggrecan anabolism. Wnt signaling related genes, such as notochordal marker T, Axin2, CCND1, and DKK1 were upregulated toward activation, and Wnt ligand markers 16, 10b, 5a, 11, and 9a were increased. Genes related to ECM anabolism were also increased toward production. Aggrecan expression increased while aggrecanase marker Adamts5 and MMP13 were decreased. [99] Together this suggests that early activation of Wnt signaling is beneficial to IVD health.

4.1.2 Limitations

While there were benefits, we also found some potential drawbacks. Because we used a conditional KO model, *Sost* deletion was constantly occurring. We found a cellular phenotype switch that could be triggered by a compensation of Wnt signaling. The initial reaction to *Sost* deletion was an increase in Wnt signaling, providing benefits to the IVD but only up to a certain point. Due to the constant *Sost* deletion,

following a model, this should cause excess β-catenin in the nucleus of the cells, but from Western Blots we found there to be a decrease in nuclear amounts of β-catenin indicating the IVD shifted and compensated to cause a decrease in Wnt signaling. Our data also matches previous data where deletion of β-catenin increased aggrecan catabolism, marked by a decrease in aggrecan expression and an increase in aggrecanase marker Adamts5. [99] Additionally, downstream regulators, or inhibitors of Wnt signaling, Dkk1 and sFRP4, were upregulated from qPCR.

Because our model was not targeted to the IVD, we also can't be sure if every change that occurred in the IVD was a direct result from the KO or if it's a result from changes in bone or the nutrition pathway. A known effect from *Sost* deletion is an increase in bone formation. The increase in bone could interfere with the nutritional pathway of the IVD. Since the IVD is an avascular structure it relies on the diffusion through the endplates for oxygen and glucose supply to maintain function. But, if this pathway is compromised, the IVD would no longer be have access to proper nutrition or be able to maintain its environment potentially leading to IVD degeneration.

4.1.3 Discussion

As previously discussed, there are some drawbacks to increasing Wnt signaling. The canonical Wnt pathway and the proinflammatory cytokine TNF-α share an intricate and complex relationship, and both play a role in homeostasis and development. But, TNF-α is highly expressed in degenerated IVDs and form a positive feedback look with Wnt signaling in the NP. When TNF-α increases this causes a an increase in Wnt signaling indicating Wnt signaling is regulated by the release of proinflammatory cytokine TNF-α. [73] Even though this is the case, we do not believe this to be a major concern.

While we targeted Wnt signaling and β-catenin via a genetic knockout model, there are other ways to do so. Shh signaling from the NP cells is required for the growth and differentiation of both NP and AF cells, and for ECM component syn-

thesis. [165] Wnt signaling is also essential for proper organization and development of the IVD [6] and activation of Wnt signaling can be beneficial. [99] Both Shh and Wnt signaling are down regulated with aging. [111] While the underlying mechanism is unknown, it has been found that a feedback loop exists between Shh signaling and Wnt signaling, where inhibition of Shh signaling causes an increase in Wnt signaling. [98] Not only do they influence the activity of each other, but Shh and Wnt singling pathways have been shown to work in parallel with each other to control cell cycle progression. [166] In the case of the IVD Wnt and Shh expression is only specific to NP cells but all components of the IVD respond, suggesting modulation of these pathways in the NP will still affect other IVD components. [165] Winkler et al showed that age related changes in the NP can be partially reversed by re-activation of either pathway, [98] suggesting that either direct or indirect modulation of Wnt signaling is beneficial to the IVD and in combination with Shh could be a potential therapeutic for IVD degeneration, but until the mechanism for their interaction is uncovered, further investigation is needed

Caveolins are integrated plasma membrane proteins that function as complex signaling regulators whose activity is dependent on cellular context. [167] Caveolin-1 is required for notochordal development [168] and regulates canonical Wnt signaling by; recruiting β-catenin to the caveolin membrane inhibiting GSK3 from ubiquitinating it thereby preventing β-catenin proteasomal degradation, [169] or after being triggered by Wnt3a, internalizes LRP6, activating the Wnt signaling pathway. [170] Caveolin-1 protein expression is almost exclusively in the NC but decreases with increasing NP degeneration. But other researchers have found increased expression of caveolin-1 to be associated with NP senescence [171] and subsequent IVD degeneration. But caveolin is also known to be involved in cellular processes such as stem cell proliferation and regulation. [172] And deletion of caveolin-1 coincides with onset of early IVD degeneration and decreased NP health. [49] Because the nature of caveolin-1 and its interactions depend on cellular context and the stage of degeneration present, fur-

ther exploration is needed to understand the complex interaction of caveolin-1, Wnt signaling, and IVD degeneration.

Aging and injury both increase and induce IVD degeneration, [61] and subsequently the level and activity of Wnt signaling decreases. Disrupting Wnt signaling and β-catenin during development also deteriorates the IVD. [6] Winkler showed in 1 year old mice, that re-activation of Wnt signaling or Shh signaling in older IVDs can re-activate the molecular markers that are lost with aging in the IVD. [98] In regards to injury, moderate mechanical forces can potentially protect the IVD from degeneration, [173] while excessive mechanical forces change the balance of ECM homeostasis toward catabolism. Holguin and Silva showed that IVDs with elevated Wnt signaling and β-catenin expression can partially protect the IVD from tail compression. [99] Taken together, this suggests that activating Wnt signaling can potentially reverse or protect the IVD from degeneration.

4.1.4 Compensation

From our results, we believe the IVD is trying to maintain homeostasis by decreasing Wnt signaling to correct the over-reaction of the initial response to *Sost* deletion, but the underlying mechanism behind what is driving the switch in Wnt signaling levels is unknown and needs to be investigate further. Other studies have explained the similar phenomena of compensation.

Witcher et al reported deletion of Dkk1 is ineffective at increasing bone when sclerostin is present suggesting sclerostin increases to compensate for potential effects from dkk1 inhibition. But when *Sost* is deleted, inhibition of dkk1 was highly anabolic. Together, this indicated dkk1 and sclerostin could exhibit mutual compensatory regulations, meaning one molecule can become highly expressive when the other is selectively suppressed. [163]

Another study reported compensation specifically in the IVD. Holguin and Silva found with short term deletion of β-catenin there were negative effects such as a

decrease in β-catenin, aggrecan, and CCND1 with an increase in Adamts5 showing anti-anabolic changes to the ECM. But after 11 weeks many of the negative consequences were regulated, β-catenin was increased as well as aggrecan and CCND1 while Adamts5 decreased. [99]

4.1.5 Potential Model

In our study, it seems like there are two halves to two different stories. At some point, Wnt signaling is elevated and is beneficial, but at a different point, Wnt signaling is decreased and its harmful to the IVD.

Explaining a potential model may help describe what is happening in the IVD (Fig. 4.1).

As an initial reaction to *Sost* deletion, Wnt signaling is increased. This is because Sclerostin is no longer present to inhibit Wnt signaling, and this was beneficial to a certain point, there was an increase in IVD hydration and increased proteoglycan staining.

But because our model is a global genetic KO model, deletion of Sclerostin and SOST is prolonged, they are always gone, so Wnt signaling is constantly upregulated.

But, to try and prevent catabolic changes potentially resulting from an overactivation of wnt signaling like previously mentioned, other mechanisms in the IVD are trying to compensate to potentially shift Wnt signaling activity. There was a shift to chondrogenesis, transcriptional promotion of anti-anabolic gene expression, with a decrease in heat shock protein expression, and an increase in Wnt inhibitors in the KO, and these are indicative of a transcriptional decrease in Wnt signaling. This may have occurred to try and maintain homeostasis or prevent catabolic changes from overactivation. These compensatory changes results in an IVD that is highly hydrated despite cells being chondrogenic.

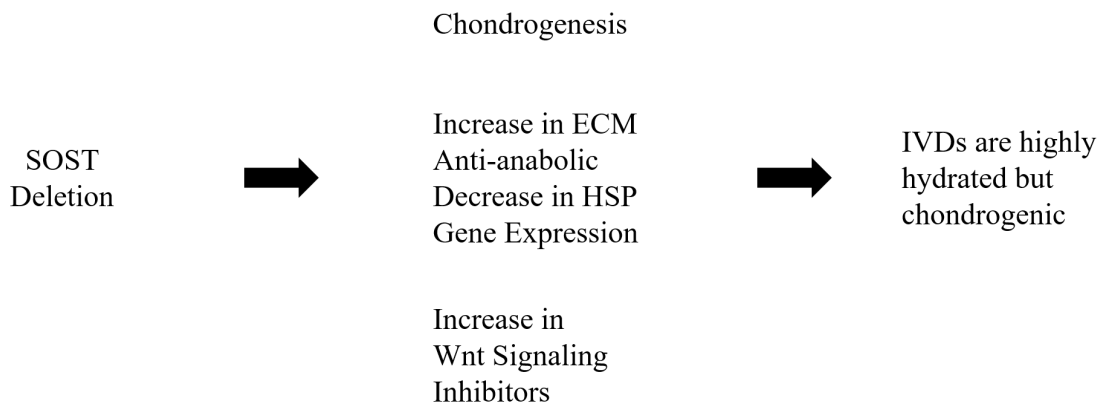


Fig. 4.1 The figure above depicts a potential model of the overall results due to gloabal genetic *Sost* deletion. Although there were positive effects i.e. increased hydration and proteoglycan, as a result of constant *Sost* deletion, there was increased chondrogenesis, increased anti-anabolic ECM gene expression with decreased HSP gene expression, and increased expression of Wnt signaling inhibitors. This ultimately resulted in IVDs that were highly hydrated but chondrogenic.

4.1.6 Future Studies

Although minor conflicting data appears from different levels of Wnt signaling, we believe brief activation to be beneficial. Moving forward, we intend to do a future study that relies specifically on targeting the just IVD to ensure effects from a *Sost* deletion are due to changes in the IVD and not effects from changes in surrounding tissues. Additionally, we would like to do a time dependent study to monitor the level of Wnt signaling activity and to see when the compensational shift occurs and potentially what causes it. We believe anti-sclerostin could be a targeted therapeutic by preventing and reversing mild or severe IVD degeneration.

4.2 Closing

IVD degeneration is a critical problem that affects many people worldwide. Associated with lower back pain, it the number one cause of job disability worldwide. There are no treatment options between prescription anti-inflammatories and surgery.

This is where our study comes in. From our study, we showed that a *Sost* KO increases proteoglycan and hydration and because of this, it can potentially serve as a therapeutic for treatment or prevention of IVD degeneration.

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