Regulation of major cell signaling pathways by Shh in neonatal intervertebral disc

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Degenerative disc disease affects 1 in 7 adults and is a major cause of lower back pain. Current treatments for the disc disease aim towards management of pain rather than the treatment of the pathogenesis of the disease. One of the reasons for this is the limited understanding of the normal disc growth and development during the postnatal stages, which is important for designing approaches for disc regeneration. It is well established that the cross-talk between major cell signaling pathways regulates the growth and maintenance of all kinds of cells and tissues. We have previously shown using a mouse model system that the major cell signaling pathways are active in all the components of the disc during postnatal stages. The nucleus pulposus (NP), which originate from the embryonic notochord, were shown to secrete major signaling ligands like sonic hedgehog (Shh), BMPs and Wnts, while all the components like annulus (AF) and end plate (EP) cells responded to these signals. However, the role of these signaling pathways in disc growth and maintenance is not well understood. We hypothesize that Shh is the key signaling pathway that regulates major signaling pathways in postnatal disc and the cross talk between these signaling pathways regulate the growth and development of the disc. To test this hypothesis we have established an in vitro system to culture to study the molecular control of disc growth and maintenance during the postnatal development of mouse disc.

METHODS:
Lumbar discs from P4 (postnatal day 4) mice were dissected and cultured on type IV collagen coated inserted in serum-free DMEM Ham F-12 medium supplemented with insulin-transferin-selenium selenite (ITS), at 37°C in 5% CO₂ for 2-5 days. To study the effect of blockade of Shh signaling pathway 250 µM cyclopamine (hedgehog inhibitor) was added to the culture medium. Similarly, small molecule inhibitor LDN-193189 for BMP blockade, and XAV939 for Wnt blockade was used. Effects of Wnt signaling stimulator BIO were also studied. At the end of the culture the discs were removed, washed three times in buffered saline and snap frozen in OCT molds. Cryosections were collected at 6 µm thickness. Immunostaining was carried out using specific primary antibodies. Cy5 conjugated secondary antibodies were used for signal detection, and nuclei were counter stained with POPO3-iodide™. Imaging was carried out using confocal microscope.

RESULTS:
The blockade of Shh signaling in the cultured discs was confirmed by loss of Gli1 expression, the downstream mediator of Shh signaling following cyclopamine treatment. We also observed up-regulation of expression of phospho-Smad 1, 5, and 8 following Shh blockade suggesting higher levels of BMP signaling. Immunostaining for phospho-Smad 2 and 3, showed down-regulation of TGFβ signaling following Shh signaling blockade. Using discs cultured from TOPGAL mice, which is a Wnt signaling reporter mice, we observed up-regulation in β-gal staining, suggesting more Wnt signaling following Shh blockade. This suggests that Shh signaling is the major cell signaling pathway in the NP cells and regulates other major cells signaling pathways. Next, in order to study the cross-talk between the major cells signaling pathways, with respective to Shh signaling, we used the specific small molecule inhibitors of specific signaling pathway. BMP signaling blockade was established by culturing P4 discs for 3 days in 1 µM LDN-193189. Loss of phospho-Smad 1, 5 and 8 was observed by immunostaining confirming the blockade of BMP signaling. To study the effect of blockade of BMP signaling on Shh signaling, immunostaining for Gli1 was carried out. We observed up-regulation in the levels of nuclear Gli1 suggesting the BMP signaling has an inhibitory affects on Shh signaling in the disc. We also studied the effects of blockade of Wnt signaling, on active Shh signaling. Treatment of discs with 600 µM of the Wnt signaling inhibitor XAV939 resulted in loss of Gli1 expression. While treatment of discs with 30 µM of Wnt signaling activator BIO resulted in up-regulation of Gli1 expression. These results suggest that Wnt signaling is required for active Shh signaling in postnatal discs. The blockade and over-activation of Wnt signaling was confirmed by immunostaining for β-catenin, the downstream mediator of canonical Wnt signaling.

DISCUSSION:
Results show that Shh signaling has inhibitory affects on BMP and Wnt signaling. BMP signaling in turn inhibits Shh signaling. Suggesting that these two signaling pathways keep a check on each other. Our results also show that Shh signaling may be inhibiting Wnt signaling, while active Wnt signaling is required Shh signaling. This also shows that the role of major cell signaling pathways in postnatal disc growth and development can be studied using specific antagonists and agonists in vitro culture system. Delineating role of these major cell-signaling pathway in postnatal disc will be very provide very crucial information about molecular control of postnatal disc growth and maintenance.

SIGNIFICANCE:
Understanding the cross-talk between major cell signaling pathways, and the role of individual cell signaling pathway in the growth and maintenance of postnatal disc growth and development will help researchers develop strategies to re-grow the discs in the way it was originally formed.

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