Role Of Circulating Signals In The Control Of Postnatal Intervertebral Disc Growth And Differentiation

Sarah Loh¹, Eric Mahoney², Christopher Wylie², Chitra L. Dahia, M.Sc., Ph.D.¹.
¹Hospital for Special Surgery, New York, NY, USA, ²Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA.

Disclosures:  S. Loh: None. E. Mahoney: None. C. Wylie: None. C.L. Dahia: None.

Introduction: Intervertebral discs are fibrocartilagenous joints that connect adjacent vertebrae, and resist tension and compression forces of body weight and movements. Degeneration of the disc, due to injury or aging causes neurological symptoms, including lower back pain, which afflict almost 80% of the population. However, current treatments are palliative and prone to failure. The ideal treatment would be to stimulate disc regeneration using the signals that control the normal process of their formation, and postnatal growth and differentiation. Degenerative disc disorder is associated with loss of its central core; nucleus pulposus (NP) cells and its markers. NP cells are postnatal notochord, and continue to act as a signaling center to maintain postnatal disc including surrounding annulus fibrosus (AF). We have established the mouse disc organ culture system as a model to identify these signals, as well as possibilities for their use in disc regeneration. Using this system previous study showed that sonic hedgehog (Shh) produced by the NP is both necessary and sufficient for postnatal cell proliferation and disc differentitation. Recently study has shown the existence of a signaling loop between Wnt and Shh signaling during disc growth. At the end of the growth period, both signaling pathways are observed to be down-regulated, coincident with reduced expression of disc differentiation markers.

Methods: Lumbar intervertebral discs were dissected from postnatal day five (P5) old mice and cultured for two (P5t2) to eight (P5t8) days either in presence of different concentration of fetal bovine serum (FBS) obtained from different sources, or minimal media supplemented with insulin-transferrin-sodium selenite (ITS) using mouse disc organ culture system. In order to determine the effects on cell proliferation, the discs were pulsed with BrdU 20 hrs before the termination of culture. At the end of culture, the discs were washed in phosphate buffered saline, and either snap frozen for cyrosectioning, or dissected to isolate the NP and AF cells for RNA isolation followed by qPCR analysis. Histological analysis was carried out using HnE staining. Cell proliferation was determined by immunostaining for BrdU, followed by microscopy and calculating the percentage of proliferating cells. Changes in NP cells were determined by immunostaining for its marker Shh. The effect on cells viability was determined by TUNEL staining followed by microscopy to quantify the percentage of cell death. qPCR analysis using TaqMan probes was used to determine the affects of circulating factors present in the serum on on Shh targets like Gli1, Ptc1, Wnt target like Axin2, and disc differentiation markers like collagens and proteoglycans. GAPDH and B2m were used as reference genes and normalization of the qPCR data.

Results: The discs looked similar after two days in cultured in all the groups studied. However, after culturing for eight days, the NP cells in the discs cultured in the minimal medium were rounded up and aggregated in the center of the disc space. The NP of the discs cultured in very low (2.5%) of FBS were also aggregated together, while the NP cells of the discs cultured in the presence of higher concentration (10%) of FBS could retain their normal reticular structure for longer culture periods. As
loss of response to Shh signaling has previously shown to cause the same phenotype, we looked for the expression of Shh by immunostaining. Shh expression was not changed much after two days of culture in all the groups. However, Shh expression was dramatically reduced in the NP cells of the discs cultured in minimal medium compared to those from 10% FBS treated group (Fig. 1). Active state of Shh signaling was studied by looking for its downstream targets Gli1 and Ptch1 by qPCR. A dose response increase in the expression of these Shh targets was observed in the FBS treated group compared to control at the eight-day culture period. BrdU cell proliferation assay showed continued cell proliferation in the NP cells of 10% FBS treated P5t8 discs, while no proliferating cells were observed in the control group. TUNEL staining showed a large percentage of NP cells were dying in the control group. qPCR analysis for collagen1a1, collagen2a1 and aggrecan showed higher expression in the FBS treated discs. The FBS treated discs also showed high expression Axin2, a Wnt target, suggesting active Wnt signaling.

**Discussion:** In this work, we show that systemic signals control growth and differentiation of the mouse disc as well as maintenance of Shh and Wnt signaling. Using the disc organ culture method, we show that FBS enhances cell proliferation and expression of differentiation markers of neonatal disc. These changes were not only observed in NP cells, but also in the surrounding AF cells, suggesting that the nucleus pulposus is a signaling center in the postnatal disc.

**Significance:** We conclude that circulating signals present during rapid growth phase, control disc growth and differentiation, and identification of these signals could be used to reactivation of aged or injured discs.

![Figure 1](image_url)

**Figure 1.** Fluorescence microscopy image of NP cells from discs cultured in ITS for two days, or ITS for eight days or 10%FBS for eight days. The cryosections of the discs were immunostained for Shh and counterstained with DAPI to show the nuclei. The images of NP cells were captured using the same laser settings using a Nikon Eclipse microscope using a 20x lens. These are representative images from experiment with five discs were cultured in each group. The experiment was repeated three times.