INTRODUCTION:
Degenerative disc disease afflicts 1 in 7 adults and is the leading cause of disability and back pain. However the molecular signals that guide the normal disc development, as well as the molecular signals involved in disc degeneration are not well defined. We have standardized a model system to study these processes during the postnatal development of mouse disc. The intervertebral disc (IVD) has three components: a central core of nucleus pulposus (NP) cells in a gelatinous matrix, a surrounding layer of fibrocartilaginous annulus fibrosus (AF) cells between adjacent vertebrae, and a mineralized end plate (EP) over the surfaces of adjacent vertebrae. NP cells are known to originate from embryonic notochord cells that act as important signaling center during embryogenesis. Notochord secretes sonic hedgehog (Shh), which is required for patterning of neural crest and somites. We hypothesize that NP cells continue to be an active signaling center during the postnatal stages, and are required for the maintenance of the AF and EP cells. To test this hypothesis we have established an in vitro system to culture neonatal mouse discs. The loss-of-function for Shh was studied using cyclopamine, a chemical inhibitor of hedgehog signaling. Understanding the molecular mechanism of disc growth will help design experiments to delineate what goes wrong during degeneration of the disc.

METHODS:
Lumbar discs from neonatal mice of postnatal age 1-4 days were dissected and cultured in DMEM Ham F-12 medium, either in the presence of fetal calf serum (FCS) or insulin-transferrin-sodium selenite (ITS) supplement, at 37°C in 5% CO2 for 2, 4, 6 and 8 days on Collagen IV coated cell culture inserts. To study the effect of loss of Shh 250 μM cyclopamine was added to the serum-free culture medium. 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 8 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.

RESULTS:
Immunostaining showed that the NP cell markers like cytokeratin 19, brachyury, and Shh were expressed at all the time points (Fig 1). And the absence of FCS did not affect the expression. In our previous study we showed that the disc cells are actively responding to several major signaling pathways during the early postnatal ages. Major cell signaling pathways including BMP and TGFβ were analyzed using antibodies against the activated cytoplasmic messengers: phospho-Smad 1, 5, & 8 and phospho-Smad 2 & 3 respectively. All the components of the disc responded to these signals produced by the NP cells. As the disc grows, there are changes in the matrix assembly, and this was monitored by immunostaining for the collagen I and collagen II expression in the AF cells. Expression of collagen’s I and II was detected till the end of the culture. Addition of cyclopamine to the cultured discs inhibited the hedgehog signaling, as monitored by using antibody for the down-stream intermediate Gli1, which was lost in the treated discs. The expression of NP marker brachyury was also lost following inhibition of hedgehog signaling. Immunostaining for CyclinD1, as cell-cycle marker was used to determine the effect of inhibition of hedgehog signaling on proliferation of the disc cells. It was observed that the number of Cyclin D1 decreased following cyclopamine treatment.

DISCUSSION:
Results show that the NP cells continue to express their characteristic molecular markers, and the disc continues to grow in vitro even in the absence of the serum. Small molecule inhibitor like cyclopamine is able to inhibit signaling pathways in the cultured discs. This provides an excellent model system to study the role of specific signals for disc maintenance by use of antagonists or inhibitors. With this knowledge we will design molecular treatments for disc injury aimed at regenerating (healing) the disc along the pathway it followed during its original postnatal development.

**Figure 1.** Immunostaining for cytokeratin 19 (A), brachyury (B), and Shh (C) in the neonatal mouse discs cultured for 5-days. Nuclei were counter-stained blue, and matrix was counter-stained green.