INTRODUCTION:
Intervertebral disc degeneration is a major cause of back pain and associated disorders, accounting for a huge financial loss due to missed days at work as well as medical bills. Current treatments aim towards management of pain rather than the pathogenesis of the disease. Regeneration of the diseased disc would be an ideal treatment option, however it has not yet been successful due to the fact that the normal process of intervertebral disc growth and development is poorly understood. Once the normal process of disc growth and development is dissected at the molecular levels, it will help in designing approaches towards regeneration of the disc tissue. One of the most important features of the disc is its gelatinous core, which results from secretion of large amounts of proteoglycans and extracellular matrix molecules by the nucleus pulposus (NP) cells. Regeneration of disc is associated with the loss of this gelatinous core. The NP cells, which originate from the embryonic notochord, secrete major signaling ligands like sonic hedgehog (Shh), which is an important signaling molecule during embryogenesis. We have previously shown that the components of disc like annulus (AF) and end plate (EP) cells express patched-1 receptor and show active Shh signaling. We hypothesize that NP cells continue to be an active signaling center during the postnatal stages, and Shh is an important signaling molecule required for the maintenance of the NP, AF and EP cells. To test this hypothesis we have established an in vitro system to culture combined with transgenic mice model to study the molecular control of disc growth and maintenance during the postnatal development of mouse disc.

METHODS:
Lumbar discs were cultured from P4 (postnatal day 4) mice on type IV collagen coated inserted in serum-free DMEM Ham F-12 medium supplemented with insulin-transferrin-sodium selenite (ITS), at 37°C in 5% CO₂ for 2-5 days. To study the effect of blockade of Shh signaling pathways 250 µM cyclopamine (hedgehog inhibitor) was added to the culture medium. The discs were pulse with BrdU towards the end of culture to study the effects on cells proliferation. At the end of the culture the discs were removed, washed three times in buffered saline and snap frozen in OCT molds. Using the doxycycline induced ROSA rTA, TetO-cre, Shh floxed mice the specificity of the effects of blockade of Shh signaling was confirmed. The tamoxifen induced ShhCreER² mice were used to study the effects of Shh that are mediated through its downstream target Sox9. 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-iodine™. Imaging was carried out using confocal microscope.

RESULTS:
Immunostaining for Gli1, the downstream mediator of Shh signaling confirmed the loss of Shh signaling in the disc. We also observed loss of markers of NP cells like Brachyury, and Sox9. BrdU incorporation study showed loss of cell proliferation following blockade of Shh signaling. Dramatic effects were observed on the expression of extracellular matrix molecules like collagens and proteoglycans. In vivo studies using Shh floxed mice confirmed our observations. These effects were reversed following addition of rShh to the cyclopamine treated discs, showing the specificity of the response. Loss of Sox9 in the ShhCreER² had very similar affects as that following loss of Shh signaling, like reduced BrdU incorporation, and loss of extracellular matrix markers. These results suggest that Shh may mediate its affects through Sox9 to maintain disc growth and development. However, loss of Sox9 had no affect on the expression of Shh and its signaling intermediate Gli1, suggesting the Sox9 acts downstream of Shh signaling in the NP cells.

DISCUSSION:
Results show that the Shh is required for maintenance of NP markers, and also the disc growth, both in vitro and in vivo. Our study also shows that disc culture system is a useful tool combined with small molecule inhibitors to study the effects of blockade of major 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.

SIGNIFICANCE:
Understanding the molecular mechanism of disc growth will help design experiments to delineate what goes wrong during degeneration of the disc, and provide crucial information to develop approaches for its regeneration.