INTRODUCTION
Deterioration of the intervertebral disc (IVD) is common in the elderly and has been shown to be one of the major causes for back pain. While studies have shown that age-related disc degeneration is characterized by enhanced apoptosis and loss of normal extracellular matrix composition in the intervertebral disc (IVD), mechanisms underlying the degeneration of the intervertebral disc remain poorly understood. Hedgehog (Hh) pathway is critically involved in the induction of scleroderm and development of skeletal system. Sonic hedgehog (Shh) plays a key role in maintaining the notochord structure and forming of the nucleus pulposus during development of intervertebral disc, whereas Indian hedgehog (Ihh) is essential for maintaining growth plate and articular surface during postnatal development. Due to embryonic lethality resulting from the deletion of the pathway, the roles of Hh pathway in postnatal intervertebral disc growth, maintenance and aging remain unknown.

In this current study, we utilized a Tamoxifen inducible Col2CreER mouse model which allows postnatal deletion of gene in the intervertebral disc to examine the role of Gli2, the major transcription factor induced by all Hh signaling in postnatal intervertebral disc growth and differentiation. We demonstrated that deletion of Gli2 postnatally led to a retardation and deformation of intervertebral disc. Further examination of Hh signaling in aged lumbar intervertebral disc demonstrated the potential link of hedgehog pathway in age-related degeneration of the intervertebral disc.

MATERIALS AND METHODS

Animal models: Gli2lacZ mice (kindly provided by Dr. Alexandra Joyner in Memorial Sloan-Kettering Cancer Center) were crossed with Col2CreER; Rosa26R mice to generate Gli2f/f; Col2CreER; Rosa26R triple transgenic mice. The incorporation of Rosa26R allows us to track the location of the mutant cells and further determine the deletion efficiency in intervertebral disc. In all experiments, Tamoxifen (TM) at a dose of 1mg/10g of body weight was injected ip every other day for a total of 3 times. Lumbar discs T2-4 were harvested at day 18, 1 month, and 2 months of age. MicroCT and histologic analyses were performed in both mutant and their respective control mice. Aged C57BL6 mice were purchased from National Cancer Institution.

X-Gal staining and In situ hybridization: 5-Bromo-4-chloro3-indolyl-β-D-galactopyranoside (X-gal) and antisense riboprobes against murine Col2α1, Coll, ColX, Ihh, MMP13, and MMP9 genes were synthesized. The sections were incubated in hybridization buffer (50% formamide; 0.3 M NaCl, 20 mM Tris HCl, 5 mM EDTA, 10% dextran sulfate, 0.02% Ficoll, 0.02% BSA, 0.02% polyvinyl pyrolidone, and 0.5 mg/ml yeast RNA) containing riboprobe at 10,000 cpm/μl. Hybridization was performed at 55°C overnight. Emulsion-dipped slides were exposed for about 7–14 days depending upon the intensity of the signals. For X-Gal staining, all slides were stained in X-gal solution (0.02% NP40, 10 mM EDTA, 0.02% glutaraldehyde, 0.05% X-gal and 2 mM MgCl2 in phosphate buffer, PH 7.5) for 24 hours. Beta-gal positive cells were visualized and photographed under light microscopy.

Immunohistochemical staining: Immunohistochemical staining was performed in representative tissue sections. The slides were probed with primary antibodies overnight, followed by biotinylated secondary antibody and streptavidin-conjugated HRP. Proliferation of the cells was examined using BrdU labeling. Samples were harvested at 6 hours after BrdU injection and sections were stained using BrdU staining kit. TUNEL assay was performed using DeadEnd Fluorometric TUNEL system (Promega) on freshly prepared paraffin-embedded sections.

RESULTS
Col2CreER-mediated postnatal deletion of Gli2 resulted in growth retardation and deformation of intervertebral disc. To determine the role of Gli2 in postnatal IVD growth and maintenance, we generated a Gli2lacZ;Col2CreER;Rosa26R mouse model, which permits deletion of Gli2 postnatally in annulus fibrosus, endplate and growth plate following treatment of Tamoxifen (TM). Deletion of Gli2 via Col2CreER led to rapid destruction of cartilaginous tissue in IVD just 3 days following TM injection at postnatal day 18. TUNEL assay showed a near 5-fold increase of apoptosis in endplate and a massive induction of apoptosis in hypochondrocytes in Gli2lacZ:Col2CreER mice, coincided with the severe loss of Col2α1, ColX and MMP13 gene expression as determined by in situ hybridization. In addition to enhanced apoptosis, BrdU labeling showed a near 3 fold reduction of proliferation in growth plate chondrocytes. Deletion of Gli2 further led to disorganization of annulus fibrosus, as evidenced by the loss of Col2α1 expression in the inner layer of annulus but aberrant induction of Col2α1 expression in the outer layer of the annulus. By 2 month of age, Gli2-mutant IVD displayed a nearly complete loss of cartilaginous tissue, disruption of endplate, rupture of annulus fibrosus, shrinkage and severe loss of nucleus pulposus, and fusion of vertebral bodies (Fig. 1).

Figure 1. Postnatal deletion of Gli2 in IVD leads to progressive destruction of intervertebral discs in mice. Gli2lacZ;Col2CreER and the control Gli2lacZ mice were treated with Tamoxifen at postnatal day 10. Lumbar discs T2-4 were harvested at day 18, 1 month, and 2 month of age. A-F) Histologic analyses demonstrate the progressive changes in IVD of Gli2lacZ;Col2CreER mice that lead to the distortion of annulus fibrosus, loss of nuclear pulposus, and destruction of endplate/growth plate cartilage. G-J) MicroCT analyses demonstrate osteophyte formation (red arrows) as well as destruction of IVD in Gli2lacZ;Col2CreER at 1 month (G and H) and 2 month (I and J) of age. K) LacZ staining demonstrates specific localization of Hh signaling as depicted by Ptc1-LacZ staining in annulus fibrosus (*) and proliferating chondrocytes (red arrow) in IVD disc. Some signals were also found in bone marrow. L) LacZ staining in Gli2lacZ;Col2CreER;Rosa26R mice demonstrates that Col2CreER mediates efficient gene recombination in annulus fibrosus, endplate and growth plate cartilage in IVD. M-N) TUNEL assay demonstrates marked induction of apoptosis in the end plate and hypertrophic growth plate of IVD in Gli2lacZ;Col2CreER mice on postnatal day 18. O-P) In-situ hybridization demonstrates aberrant Col2α1 gene expression in Gli2lacZ;Col2CreER IVD, as evidenced by disappearance of Col2α1 gene expression in the inner annulus but increased expression of the same gene in the outer annulus (indicated by arrow) on postnatal day 18.

DISCUSSION
Using Col2CreER mediated deletion of Gli2 in IVD, we demonstrated a critical role of Hh in postnatal growth and maintenance of IVD. Gli2 can be activated via both Ihh and Shh pathway. Based on the Ptc1-LacZ staining, we found that Hh signaling was active in both growth plate and annulus fibrosus, indicating a differential role of Shh and Ihh in maintaining the postnatal growth and differentiation of IVD. Further study will be needed to determine the role of each Hh ligand in postnatal maintenance of IVD. To examine the role of Hh pathway in age related disc degeneration, we examined the Hh signaling in lumbar disc from both young and aged mice. We found that the progressive loss of Hh signaling paralleled with aging and degeneration of IVD in mice. Further studies are necessary to establish the causal relationship of Hh pathway in age-related degeneration of IVD.

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