INTRODUCTION:
Gene transfer can effect therapeutic changes in IVD cells. Prior to clinical application, however, its risk/benefit profile must be thoroughly characterized. Although we have found intradiscal gene transfer to be safe when appropriately dosed and delivered, our group is exploring the potential side effects of excessively dosed or misdirected gene therapy in order to define its safety profile. A pilot study from our lab revealed the deleterious dose-dependent effects of Ad/TGF-ß1 when delivered to the epidural space via a window created in the L5-L6 ligamentum flavum in 19 skeletally mature NZW rabbits. The animals were grouped as follows: 4 Ad/LacZ rabbits, 6 Ad/TGF-ß1 rabbits, 5 Ad/BMP-2 rabbits all receiving a viral load of 10^8 PFU, and 4 Ad/BMP-2 rabbits at 10^6 PFU (standard dose used for intradiscal injections in prior studies).

METHODS:
The following protocol was approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee at the University of Pittsburgh. Adenoviral/gene constructs (Ad/TGF-ß1, Ad/BMP-2, or Ad/LacZ) were delivered to the epidural space via a window created in the L5-L6 ligamentum flavum in 19 skeletally mature NZW rabbits. The animals were grouped as follows: 4 Ad/LacZ rabbits, 6 Ad/TGF-ß1 rabbits, 5 Ad/BMP-2 rabbits all receiving a viral load of 10^8 PFU, and 4 Ad/BMP-2 rabbits at 10^6 PFU (standard dose used for intradiscal injections in prior studies).

Following the procedure, animals were observed daily by vet staff and the investigators for neurologic deficits and overall clinical wellbeing (feeding behavior, bowel movements, and activity level). They were sacrificed 6 weeks after gene delivery. Spinal cords including intact thecal sacs were harvested and analyzed histologically by a neuropathologist blinded to the intervention. Ad/LacZ specimens were stained with X-Gal to determine vector distribution patterns. Study animals with significant local or systemic toxicity prior to the scheduled date of sacrifice were euthanized and underwent a post-mortem autopsy performed by vet staff. Furthermore, plasma and CSF samples were collected from these toxic rabbits for ELISA quantification of transgene production (i.e. recombinant human TGF-ß1 or BMP-2).

RESULTS:
Ad/LacZ rabbits did not demonstrate any clinical signs of systemic toxicity or neurologic deficits. Histologic evaluation of their thecal sacs revealed normal meninges and spinal cords with very mild, focal epidural inflammation. X-gal staining revealed successful transgene uptake in the meninges, dorsal root ganglia, and cord parenchyma.

Three of the six Ad/TGF-ß1 rabbits exhibited clinical manifestations of toxicity. Two developed sepsis with bilateral lower extremity flaccid paralysis and complete sensory loss at weeks 1 and 2 respectively. The third experienced lower extremity paresthesia as evidenced by self-mutilation of its hind-paws at week 6. The other three Ad/TGF-ß1 rabbits did well clinically until sacrifice at week 6. Histologic evaluation revealed dramatic fibrotic thickening of the meninges with lymphocytic infiltration in all but one rabbit. Furthermore, the toxic, paralyzed rabbit sacrificed at week 1 demonstrated lymphocytic inflammation in its cord parenchyma (both gray and white matter). Plasma samples obtained from the three toxic rabbits exhibited high levels of rhTGF-ß1 as compared to control values.

The four rabbits with Ad/BMP-2 at 10^8 PFU had no signs of systemic or local toxicity. Their meninges and spinal cords were normal except for mild, focal epidural inflammation.

In contrast, the five Ad/BMP-2 rabbits at 10^8 PFU did not fare as well. Two animals were found deceased at weeks 1 and 2, respectively. They were clinically well prior to their sudden demise. Because of post-mortem autolysis, their organs, including thecal sacs, were not salvageable for histologic analysis. Two others developed lower extremity paresthesia as evidenced by self-mutilation at week 5. The last animal did well clinically until sacrifice at week 6. Plasma and CSF samples obtained from the two animals with paresthesia demonstrated high levels of rhBMP-2 as compared to control values. Histologic examination of the paresthesia rabbits revealed diffuse lymphocytic inflammation within the gray matter and central canal as well as subacute infarction of focal regions within the dorsal spinal cord. Additionally, one of the rabbits with paresthesia demonstrated chondrogenic metaplasia with adjacent lymphocytic inflammation within the epidural space. Histology for the healthy Ad/BMP-2 rabbit was normal except for mild focal epidural inflammation.

DISCUSSION:
Several conclusions can be drawn from the results obtained in this study. First, the pattern of X-Gal staining supports the assertion made by previous investigators that adenoviral vectors are capable of penetrating the blood brain barrier from the epidural space into the CNS, resulting in gene uptake and expression by cells of the dura as well as those of dorsal root ganglia and spinal cord. Moreover, adenoviral immunogenicity is a well known observation, and in our study, focal epidural inflammation was evident in all 19 animals. However, toxic meningeval/spinal inflammation and systemic illness were seen only with Ad/TGF-ß1 and Ad/BMP-2 (cytokine growth factors) and not with Ad/LacZ (reporter gene). It appears significant local and systemic inflammation is due to a synergy between adenoviral immunogenicity and the potential toxic effects of these cytokine growth factors.

So what are the toxic effects of these growth factors when they are continuously produced by genetically modified cells within the dura and CNS? The reported effects of TGF-ß1 are controversial. However, several studies have identified detrimental effects. Over-expression of TGF-ß1 can increase the susceptibility of the CNS to immune cell infiltration and autoimmune diseases, as well as increase the resulting neurologic impairment. Furthermore, the cytokine is thought to promote scar tissue formation within the CNS via the activation of glial cells following neuronal injury. These observations seem to explain our histologic findings of meningeal thickening/inflammation and cord inflammation. The resulting neurologic deficits were likely due to these changes.

As for BMP-2, extensive safety investigations with the protein demonstrated no systemic or local toxicity. An animal study demonstrated direct contact between rhBMP-2 and dura does not result in local toxic effects within the neural elements and did not induce intradural bone formation. Furthermore, daily intravenous injections of rhBMP-2 into animals for 28 consecutive days failed to identify any systemic toxic effects. The lack of systemic toxicity has been postulated to be the result of the protein’s rapid systemic clearance (half life 10-15 minutes), which results in very little systemic exposure. Until this study, nothing was known about the toxic effects of continuous local and systemic exposure to rhBMP-2. It appears from our data that continuous local production of rhBMP-2 can result in chondrogenic metaplasia and dramatic spinal cord inflammation and even infarction. The study animals subsequently developed lower extremity paresthesia. Moreover, continuous levels of systemic rhBMP-2 seemingly resulted in the rapid demise of two animals.

These local and systemic toxic effects of BMP-2 were observed only in those rabbits that received the higher viral load of 10^8 PFU. In contrast, animals that were given the lower dose of 10^6 PFU did not exhibit detrimental effects. A dose threshold apparently exists beyond which toxicity ensues.

These findings underscore the concept that successful clinical translation of intradiscal gene therapy will depend on rigorous safety studies to characterize vector systems and genes of interest. Our group will continue to investigate its safety profile.