Applying Immunohistochemistry & Reverse Transcription PCR to Intervertebral Disc Degeneration in an Animal Model

Sprague Dawley Rat (*Rattus norvegicus*)

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07/29/05

Motivation

- Back pain: the number-one cause of disability in workers under age 45*
- $50 billion annually is spent in direct connection to back pain*
- Little is known about the causes
  - Disc degeneration a possible suspect
  - More information = better treatment
- Model degeneration in the rat
- Objective: develop preliminary study by applying IHC and RT-PCR

* American Chiropractic Society

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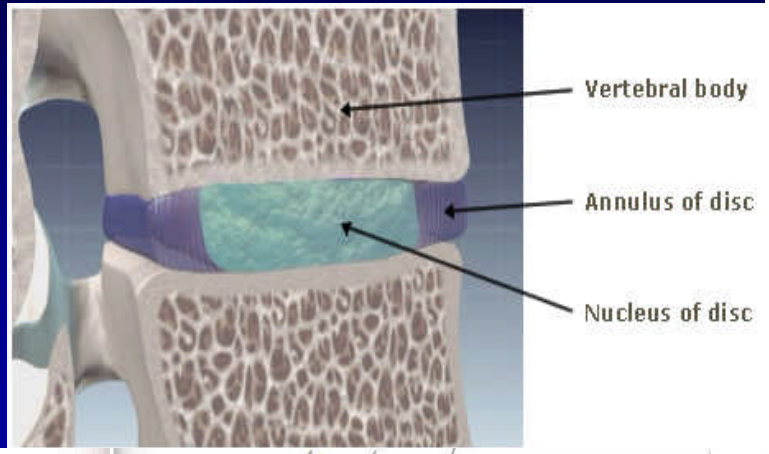
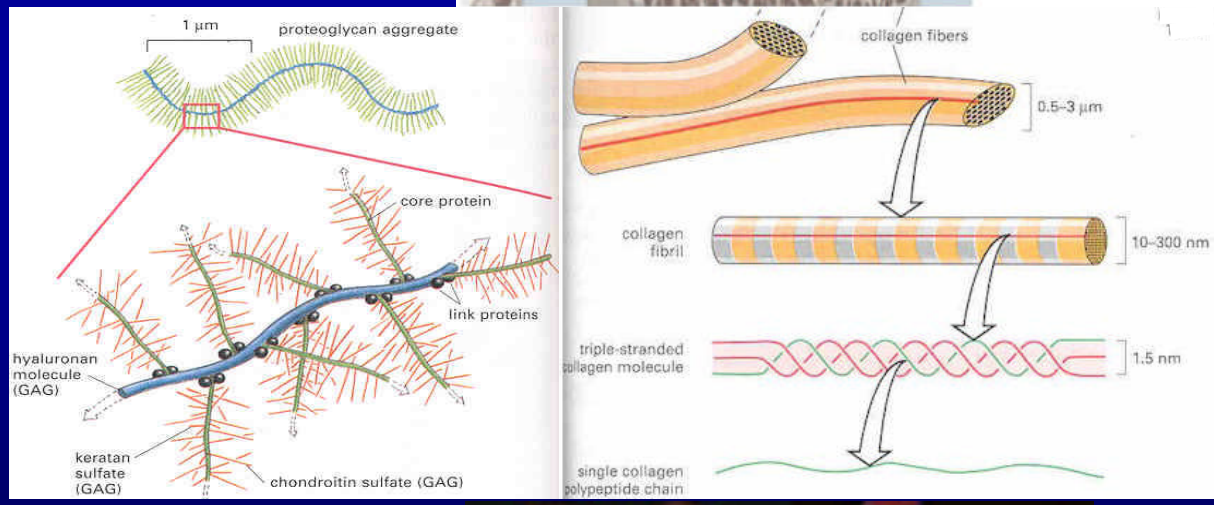
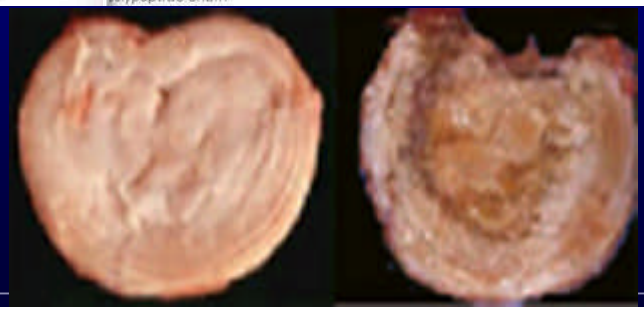
Background

- **Intervertebral disc composed of annulus fibrosis and nucleus pulposus**
- **Extracellular matrix controls disc function: components of interest**
  - **Nucleus: proteoglycan**
  - **Annulus: collagen**
  - **Various other proteins, enzymes, inhibitors…**
- **This matrix changes with degeneration**







Normal (left) and Degenerate (right) disc

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Immunohistochemistry

- **Definition**
  - **Microscopic localization of specific antigens in tissues by staining with antibodies labeled with visible material**
- **Current Objective**
  - **Developing procedures**
  - **Creating baseline data on healthy discs**
- **Future Plan**
  - **Understand changes as disc degenerates in: various types of collagen, proteoglycan, and enzymes and their inhibitors**

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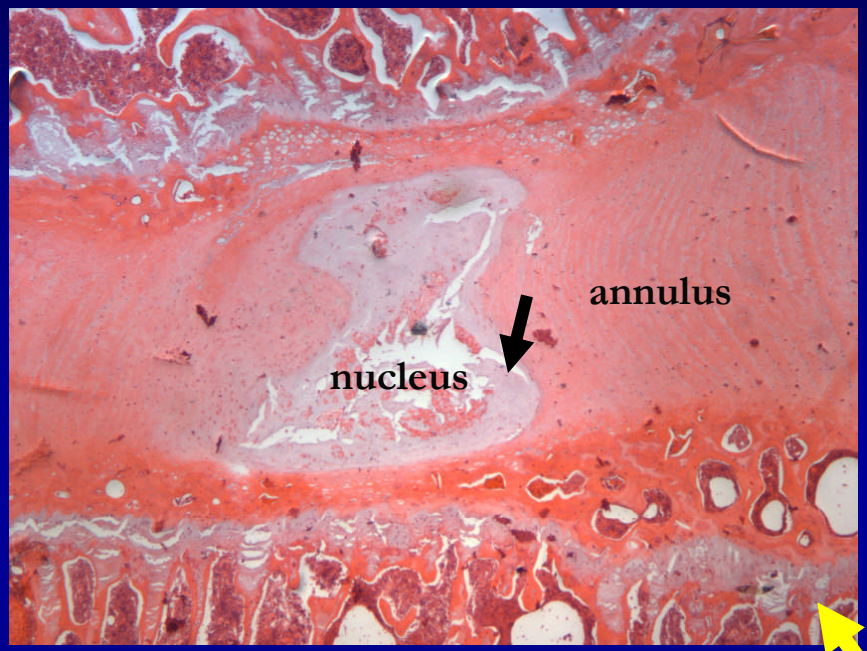
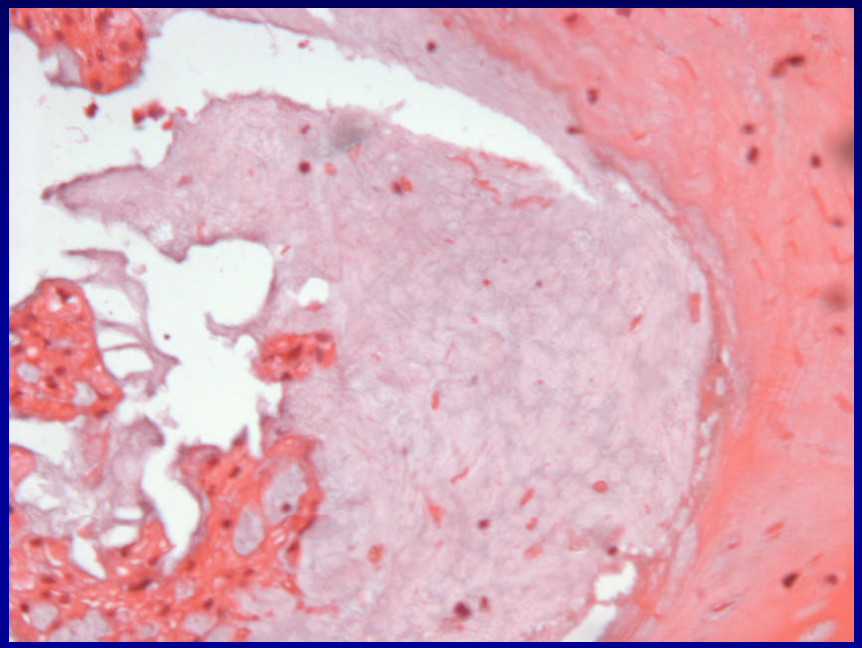
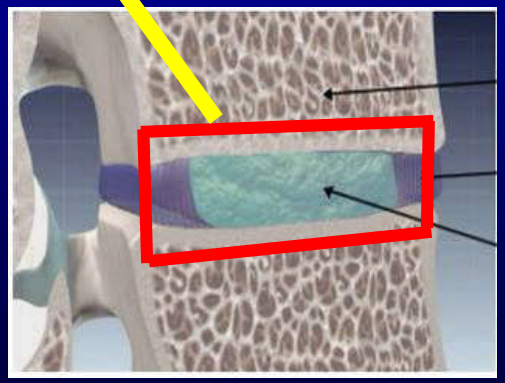
Basic Histology: Hematoxylin & Eosin





- Sagittal section
- Stains nuclei blue and tissues red
- Structure of disc apparent
- Break-up of nucleus a problem



- Higher magnification of nucleus
- Cells are sparse

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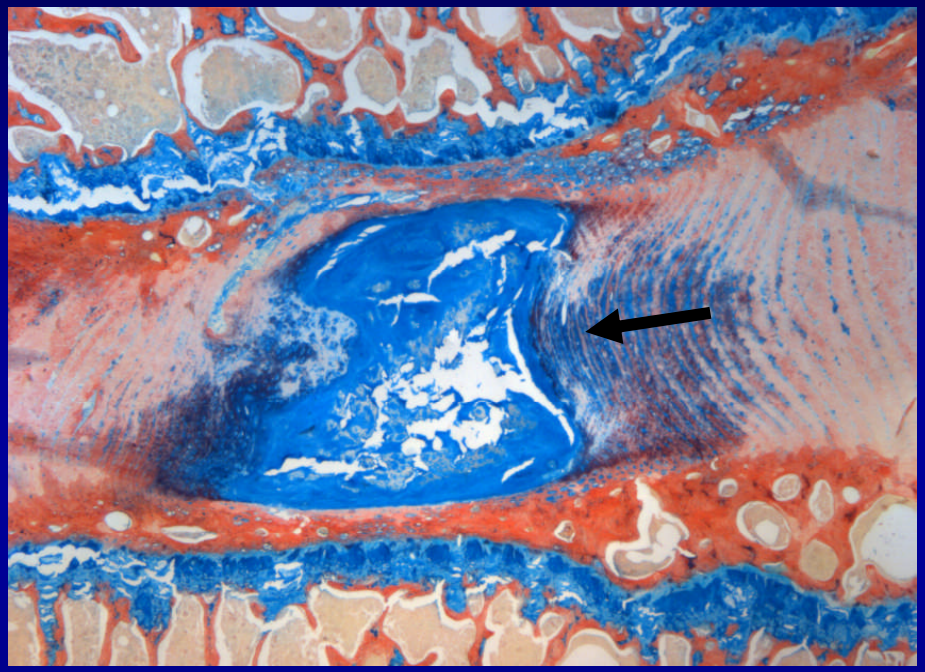
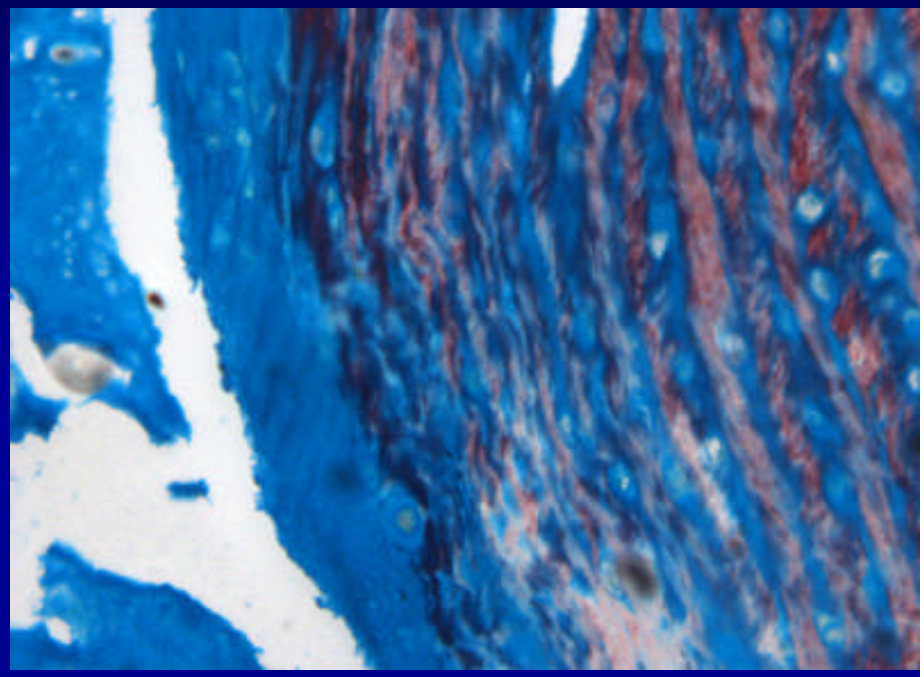
Basic Histology: Alcian Blue & Picrosirius Red





- Stains proteoglycan blue and collagen red
- Distribution of the two apparent

- Transition from nucleus to annulus is clear
  - Nucleus: no visible collagen, even stain
  - Inner Annulus: some collagen, disorganized fibers
  - Outer Annulus: more collagen, organized fibers

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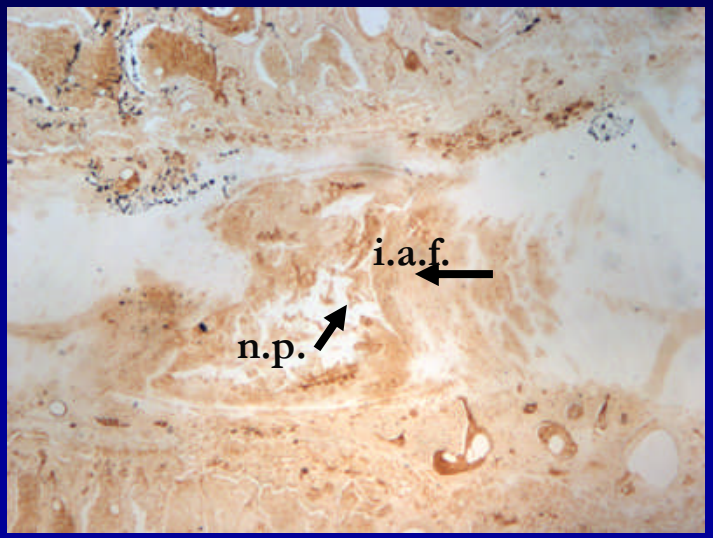
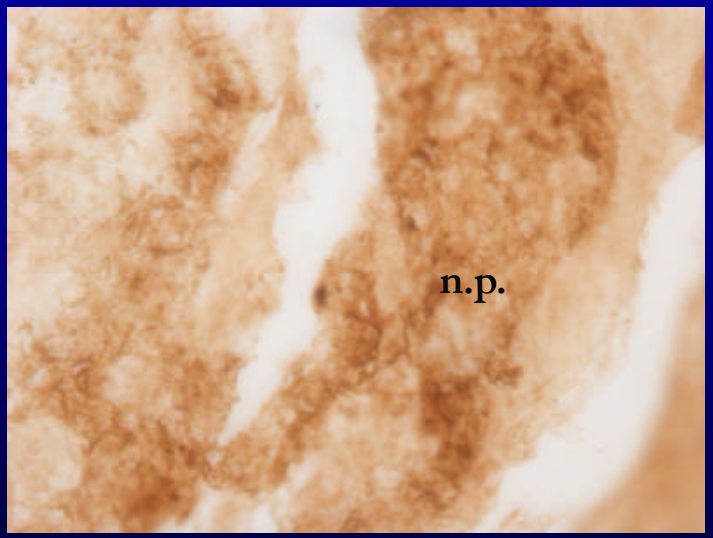
IHC Results: Collagen I





- Most problematic stain
- Literature suggests light staining in outer annulus, none in inner annulus, nucleus
- Background effects significant
  - Reagents becoming trapped in tissue- thickness?
  - Possible cross-reactivity

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IHC Results: Collagen II

- Significantly more successful stain
- Literature suggests Collagen II concentrated in inner annulus, some in nucleus
- No staining in nucleus, likely due to low overall concentration

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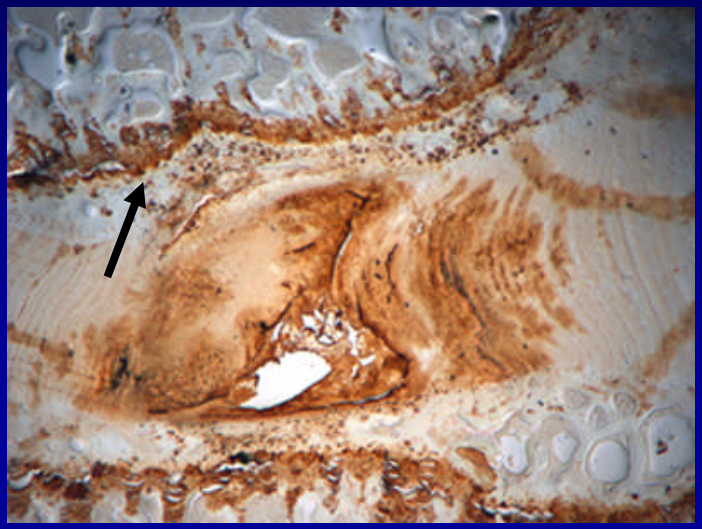
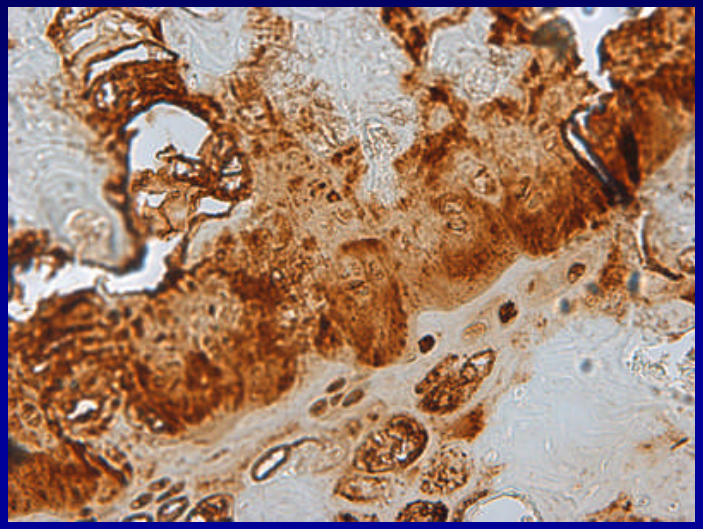
IHC Results: Aggrecan





- Corresponds to Alcian Blue stain
- Literature also suggests aggrecan concentrated in nucleus, inner annulus, endplates
- Staining highly vivid, again, likely background effects
  - More color = less detail
  - Thick tissue sample traps reagents

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IHC: Recommendations

- Goal to localize at cellular level, background must be minimized
- Possible changes:
  - Concentration
  - Time of exposure/wash
  - Thickness of section
    - Currently: 7 µm
    - Want: 5 µm
- Next step: back to histology

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Gene Expression by RT-PCR

- **Definition**
  - the process by which a gene's coded information is translated into the structures present and operating in the cell (either proteins or RNAs)
- **Current Objective**
  - Develop a set of protocols to extract information from a sample
  - Apply to baseline discs
- **Future Plan**
  - Apply to both further baseline and degenerate disc material

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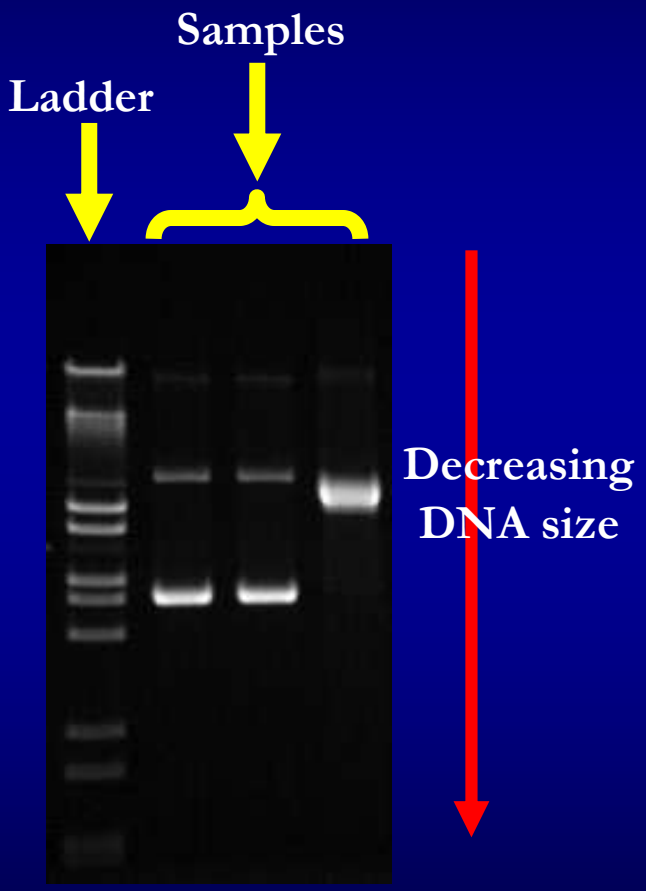
PCR: Interpreting Results

- Gel electrophoresis separates DNA fragments by size (number of base pairs in gene)
  - Potential is induced over the length of the gel, DNA is charged
  - Larger segments (higher molecular weight) do not travel as far as smaller fragments
  - Allows identification of DNA present
- Leftmost column is ladder
  - specifies size of product



http://www.cgeservice.com

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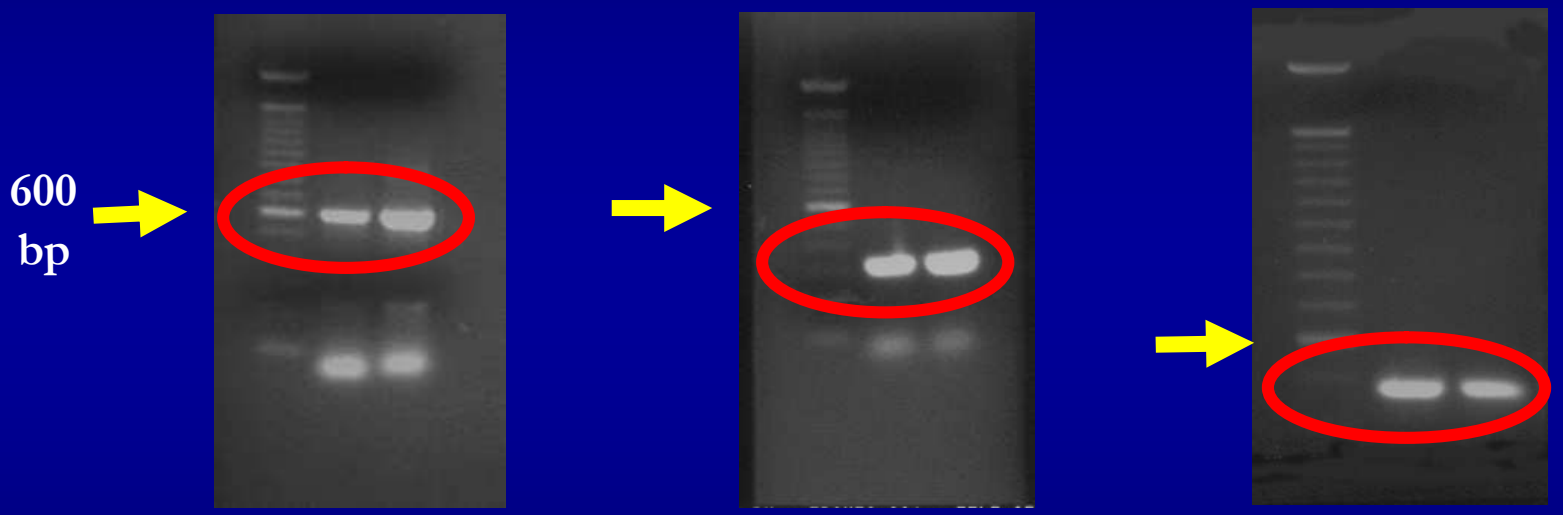
RT-PCR Results

- **The two columns to the right are products from L2 and L3 levels of spine, respectively:**

Collagen I (599 bp) Aggrecan (322 bp) Fibronectin (481 bp)



- **Collagen I: several bands- different primer needed, overamplified**
- **Aggrecan: overamplified**
- **Fibronectin (adhesion protein increases with degeneration): expression in healthy disc significant**

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RT-PCR: Recommendations

- Split the nucleus from the annulus
- Apply this protocol to more proteins and enzymes
- PCR should be quantified
  - Include a control sample for each sample of interest when doing PCR
  - Control will amplify a gene always present in the disc + not affected by degeneration
  - Measure the intensity of the band for the desired gene, normalize it by control

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Conclusions

Work conducted this summer has shown that immunohistochemistry and RT-PCR are powerful tools which can be applied to understanding the intervertebral disc. There is, however, still a significant amount of background work which must be done before an actual study can be devised.

A deeper understanding of the intervertebral disc gained through such a study will hopefully lead to better back pain treatments and relief.

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Thank You

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National Science Foundation
WHERE DISCOVERIES BEGIN