IN-SITU TISSUE ENGINEERING OF THE INTERVERTEBRAL DISC

by

Pang-ning Teng

BS, Washington University in Saint Louis, 2001

Submitted to the Graduate Faculty of

the School of Engineering in partial fulfillment

of the requirements for the degree of

Master of Science in Bioengineering

University of Pittsburgh

2003

UNIVERSITY OF PITTSBURGH

SCHOOL OF ENGINEERING

This thesis was presented

by

Pang-ning Teng

It was defended on

October 30, 2003

and approved by

James D. Kang, M.D., Associate Professor, Department of Orthopaedic Surgery

Rebecca K. Studer, Ph.D., Associate Professor, Department of Orthopaedic Surgery

Richard R. Koepsel, Ph.D., Associate Professor, Department of Bioengineering

Thesis Advisor: Lars G. Gilbertson, Ph.D., Associate Professor, Department of Bioengineering

ABSTRACT

IN-SITU TISSUE ENGINEERING OF THE INTERVERTEBRAL DISC

Pang-ning Teng, MS

University of Pittsburgh, 2003

A possible approach to stimulate proteoglycan and collagen synthesis for treating intervertebral disc degeneration (IDD) is introduction of growth factors. The objective of Part I of this study was to screen the effect of human recombinant bone morphogenetic protein (BMP)-2 and BMP-12 on nucleus pulposus (NP) cells and to investigate the effect of Ad/BMP-12 on NP and anulus fibrosus (AF) cells. Cells were isolated from degenerated human discs and cultured in monolayer. RhBMP-2 (25, 50, 100, 200, 300, ng/ml) and rhBMP-12 (25, 50, 100 ng/ml) stimulated NP cells in serumless media (1% ITS) for 2 days. Ad/BMP-12 (50, 100, 150 MOI) transduced NP and AF cells, then pellets (150,000 cells/pellet) were formed and incubated in serumless media (1% ITS) for 6 days. Proteoglycan, collagen, and non-collagenous protein synthesis were measured. RhBMP-2 had a more substantial effect on upregulating matrix synthesis than rhBMP-12. Ad/BMP-12 significantly increased matrix synthesis. Total DNA content was increased pellets stimulated by Ad/BMP-12 when compared to control. The

increase in matrix synthesis was attributed to both an increase in cell number and in matrix synthesis per cell.

Intervertebral disc (IVD) is the largest avascular organ in the body, it has been suggested that lack of nutrition may be one of the cause of IDD. The goals of the second part of this study were to develop a rabbit disc organ culture method to study the effect of FBS concentration in disc metabolism and to attempt gene therapy in the organ culture. Twenty-seven rabbit lumber intervertebral discs were harvested and cultured for 2 weeks in serumless media (1% ITS) or F-12/DMEM (5%, 10%, 15% FBS). NP and AF wet weight, dry weight, % hydration, glycosaminoglycans (GAG), DNA, and lactate content were measured. In 25 discs, $6x10^6$ PFU of Ad/lacZ or Ad/Luciferase was injected into the NP 2 days after the culture to determine the effect of gene transfer. Histology and viability staining of 10 discs were used to show cell morphology and viability. Due to low cell viability, we did not observe successful gene transfer in the organ culture. NP and AF lactate content and AF DNA content were significantly higher in the 15% FBS group than serumless group.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

In the summer of 2001 I came to the University of Pittsburgh and began my journey in Bioengineering. I worked in the Spine group and learned how to conduct research and prepare for conferences and publications. Lars G. Gilbertson, Ph.D. taught me so much with such patience. I have learned about the intervertebral disc from him as well as how to be a good scientist. James D. Kang, M.D., Rebecca K. Studer, Ph.D., Christopher Niyibizi, Ph.D., and Richard R. Koepsel, Ph.D. guided me expertly through my experiments and gave me invaluable suggestions. I appreciate assistance from Molly Vogt, Ph.D. of Department of Orthopaedic Surgery and Jie Wang, M.S. of Department of Statistics in statistical analysis. Simon Watkins, Ph.D. and Glen Papworth, Ph.D. of Center for Biological Imaging provided 2-photon laser microscopy. Paul D. Robbins, Ph.D. of Department of Molecular Genetics and Biochemistry provided viral vectors used in this study.

During my two years at the Ferguson Laboratory, I made many friends from around the world and they assisted and accompanied me. I want to thank Diana Lin for being such a great friend and I owe much to Sang-Ho Ahn, M.D., Ph.D., Satoshi Sobajima, M.D., Ph.D., and Helga I. Georgescu, B.S. for their help. I also benefited from Brandi Day, B.S., Ross Green, and Pang-yu Tengs' help. I am particularly grateful to my parents Yao-Kung Teng and Jing-Ru Wang for giving me the opportunity to study in the US and my boyfriend Terry T. Lin for all his support. I will always remember my teachers and friends in Pittsburgh.

LIST OF ABBREVIATIONS

Ad =	Adenovirus
AF =	Anulus Fibrosus
BMP =	Bone Morphogenetic Protein
EGF =	Epidermal Growth Factor
FBS =	Fetal Bovine Serum
GAG =	Glycosaminoglycans
H&E =	Haematoxylin & Eosin
IDD =	Intervertebral Disc Degeneration
IGF-1 =	Insulin-Like Growth Factor-1
ITS =	Insulin Transferrin Selenium
NP =	Nucleus Pulposus
OP-1 =	Osteogenic Protein-1
PDGF =	Platelet-Derived Growth Factor
PEG =	Polyethylene Glycol
PEI =	Polyethylenimine
PG =	Proteoglycan
PLL =	Poly-L-Lysine

P/S =	Penicillin and Streptomycin
Rh =	Recombinant Human
TGF β-1 =	Transforming Growth Factor Beta-1
TZI =	Transition Zone

1.0 INTRODUCTION

1.1 Intervertebral Disc Degeneration (IDD)

During the natural aging process, the intervertebral disc undergoes degeneration.[1] IDD can be described as a number of mechanical and biological changes including water loss from the nucleus pulposus, tears within the annulus, disc height loss, disc protrusion, and disc herniation.[2] IDD is a major factor in low back pain, however its etiology and pathophysiology are not well understood.[3]

In healthy discs, the nucleus region of the disc is rich in proteoglycans, molecules that can attract water. Degenerated discs have less proteoglycans in the nucleus. This leads to dehydration. The loss of disc height in disc degeneration is due in part to loss of water content. These biochemical changes alter the mechanical function of the disc.[4,5]

Primary disc degeneration is another term to denote the mechanical and biological changes in the disc accompanying aging. Intervertebral discs can also undergo faster changes than the natural aging process, such as degeneration in discs adjacent to a long fusion or degeneration in discs following annulus injury. These changes are termed secondary disc degeneration. Other factors associated with secondary disc degeneration include smoking, obesity, and high mechanical spinal loads.[6,7]

1.1.1 Impact of IDD

Back pain causes substantial health and economic impact on the society. It affects a large proportion of the adult population. About 80% of adults will experience at least one episode of back pain during adulthood.[8] IDD has been implicated as a major factor in low back pain.[2] IDD first appears in males in their twenties and women in their thirties. By the age of 30, around 30% of the discs show noticeable degenerative changes.[9] By the age of 50, 97% of the discs show signs of degeneration.[1] Back pain is the 2nd most frequent reason for a physician visit, the 3rd most frequent reason for a surgical procedure, and 5th most frequent cause of hospitalization.[10] It can limit one's physical and social activities due to increased pain and it reduce a person's independence and quality of life.

Annual costs from back pain are estimated to be more than $50 billion and could be as high as $100 billion annually in the United States.[11] Direct health costs come from the diagnosis and treatment of musculoskeletal diseases and trauma. The indirect economic losses include work day loss, reduced productivity, and activity limitations due to musculoskeletal impairments and disability.[12] Back pain is the most common cause of activity limitation in persons less than 45 years of age.[13] An annual average of 175.8 million restricted activity days are caused by spine or back impairments[14].

1.1.2 Current Treatment Paradigms for IDD

There is currently no cure for intervertebral disc degeneration. Primary disc degeneration is a consequence of natural aging, therefore viewed as inevitable and unpreventable with the limited technology now available. Once the disc begins to degenerate, it is difficult to stop or reverse the process with current treatments. There are conservative treatments and operative or invasive treatment. While primary disc degeneration cannot be prevented, secondary disc degeneration may be minimized by decreasing smoking, maintaining healthy body weight, avoiding high mechanical spinal loads, and avoiding spinal fusion if possible.

Some of the more common therapeutic conservative techniques include bed rest or controlled physical activity, drug therapy with anti-inflammatory agents, trigger-point injection, epidural steroid injection, medication, traction, spinal manipulation, spinal bracing, physical therapy, back school, and exercise.[15] Some of these techniques might relieve the symptoms of IDD but they do not directly treat the disc. From the evidence available, controlled physical activity appears to be more effective than other conservative treatments. However, the objective support of efficacy of these techniques is lacking due to the difficulty in performing prospective double-blind studies.[16]

The current clinical options for treating IDD are limited and they are highly invasive. Operative treatments include chemonucleolysis, discectomy, and spinal fusion. Chemonucleolysis is the injection of chymopapain to decrease the intradiscal pressure to release nerve root pressure. However the efficacy is questionable and can induce severe complications such as paralysis.[17] Discectomy involves removing some or all portions of the disc and it usually

requires additional fusion surgery to restore stability of the spinal segments. Spine fusion is one of the most performed spine surgeries. More than 250,000 fusions are performed each year in the US.[18] Fusion eliminates the movement across an intervertebral motion segment by bony union.[19] The goals of these operative treatments are to decompress neural elements, stabilize unstable spinal levels, or correct deformity.[20] The outcome can vary and it may increase the secondary disc degeneration in the adjacent discs. As with conservative techniques, these surgical procedures cannot cure or regenerate the degenerated disc. Also as with any other surgical procedure, there are risks of complications. There remains a need for less-invasive, safer, and more effective treatments for IDD.

1.1.3 Potential Future Treatments

Scientists are making advancements in developing future treatments for IDD. Current research includes organ transplantation and tissue engineering. Options for intervertebral disc transplantation include autografts, allografts, and xenografts.[19] Tissue engineers are also testing novel techniques to directly treat the disc by nucleus pulposus implantation, cell implantation, matrix substitution, and cell-seeded scaffolds.[21,22] Notochordal cells and stem cell are potential candidates for cell implantation.[23,24] Many tissue engineering techniques can be used in conjunction with growth factors or gene therapy. In-situ tissue engineering methods that are under investigation include growth factor and intradiscal gene therapy.

One possible minimally invasive treatment for disc disease is the introduction of therapeutic amounts of growth factor proteins to the disc. Growth factors are extracellular

polypeptide-signaling molecules that can stimulate cells to grow or proliferate. They have been tested in animal models and have shown the ability to enhance tissue repair. In-vitro studies have demonstrated that addition of human transforming growth factor TGF-beta 1 to canine disc tissue in culture stimulated in-vitro proteoglycan synthesis, suggesting that TGF-beta 1 might be useful for the treatment of IDD.[25] However, direct injection of growth factors into a disc has the limitation of a short half-life. Disc degeneration is chronic and it appears to require a sustained delivery of exogenous growth factors to the disc.

Gene therapy can potentially provide a mechanism by which intervertebral disc cells might produce an encoded-for growth factor (or other gene product) continuously. There are many candidate genes that researchers are testing such as TGF beta-1, IGF-1, PDGF, EGF, OP-1, and BMP-2. In-vivo study in a rabbit model of intradiscal gene therapy using adenovirus with TFG beta-1 has shown positive results in upregulating matrix synthesis.[26] Human disc cells from degenerated discs are genetically modifiable in-vitro by adenovirus-mediated gene transfer.[27] One potential clinical application of intradiscal gene therapy would be to inject therapeutic genes into a degenerated disc with the intent of regenerating the disc constituents (collagens and proteoglycans) to improve biological and mechanical function. Another application, more along the lines of prevention than treatment, would involve injection of genes into a disc adjacent to a spinal fusion. Here the intent would be to modulate the biological activity within the disc to enable the disc to better withstand the abnormal mechanical loads that presumably occur following fusion. With gene therapy, there is a possibility to delay or even prevent disc degeneration (Figure 1).

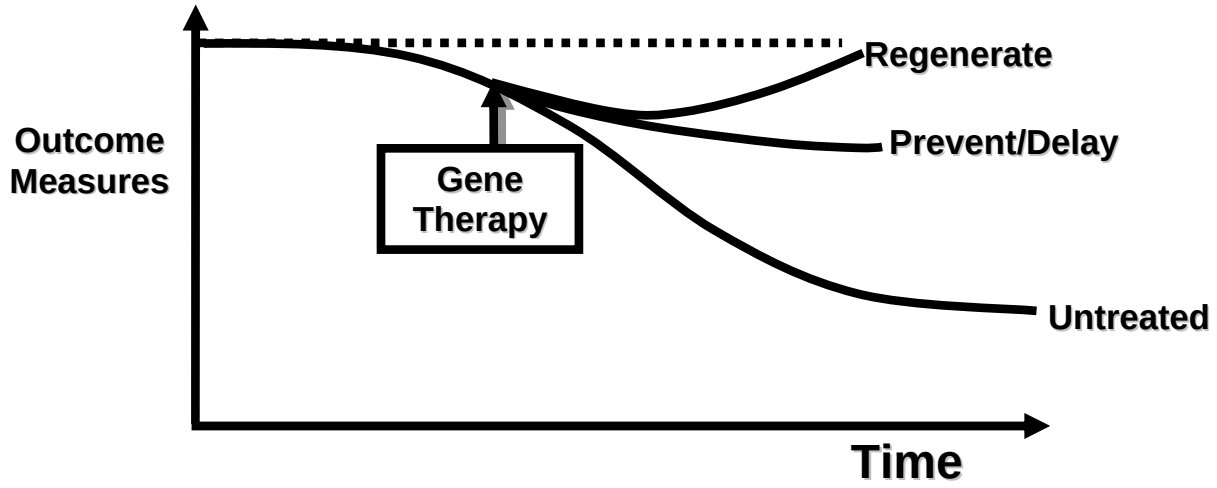


Figure 1 Potential regeneration ability of gene therapy

1.1.4 Therapeutic Potential of Ad/BMP-2 and Ad/BMP 12

The introduction of viral vectors carrying growth factor BMP-2 and BMP-12 genes to the degenerated intervertebral disc can potentially stimulate matrix synthesis. BMP-2 induces formation of cartilage, bone, and the connective tissues associated with the skeleton.[28,29] BMP-2 also has been reported to increase proteoglycan synthesis in rat intervertebral disc cells in-vitro.[30] BMP-12 plays crucial roles in the formation or induction of various tissue and organ during development.[31,32,33,34,35,36] Recently, BMP-12 has been reported to stimulate formation of tendon and cartilage-like tissue, suggesting its potential for reparative function in IDD.[37,38] Unlike BMP-2, BMP-12 does not stimulate bone matrix synthesis.[39] BMP-12 may have the potential to promote proteoglycan as well as collagen synthesis in the disc.

1.1.5 Importance of Nutrition

The disc is the largest avascular organ in the body and it is suggested that lack of nutrition may be one of the cause for disc degeneration.[40] Solute transport into the intervertebral disc depends on diffusion and fluid flow.[41] The two main routes for supply of nutrition and removal of small waste products in the disc are diffusion through blood vessels adjacent to 1) the annulus fibrosus and 2) the hyaline cartilage endplates (Figure 2).[31,42] Outer annulus can receive nutrients from the blood vessels in the soft tissues around the annulus periphery, and 10% of the endplate surface area is immediately adjacent to the vascular capillaries. However, the inner annulus and the nucleus pulposus rely on the diffusion of their nutrient supply over long and precarious path.[43] The endplates are impermeable to large molecular weight molecules, and with age, the endplates become remodeled and calcified restricting nutrient supply.[44]

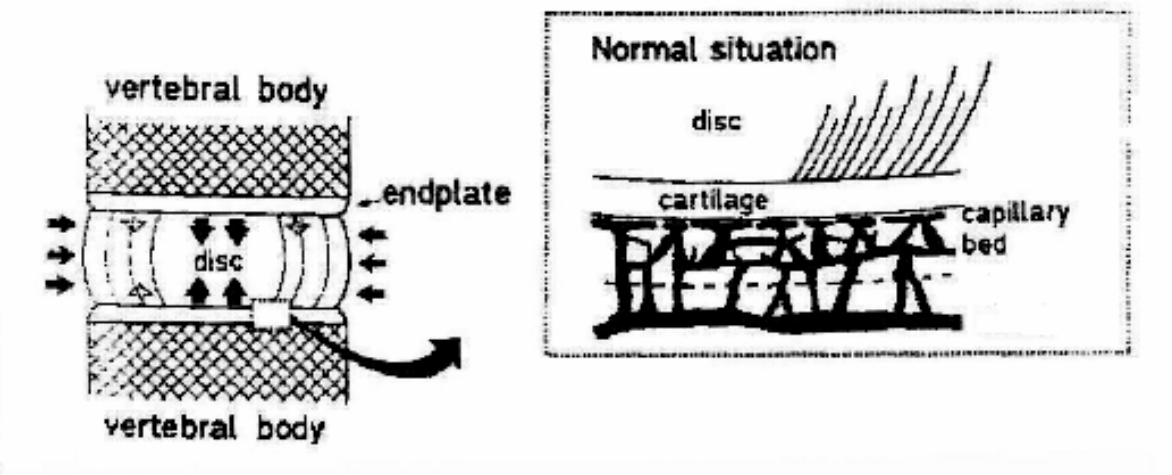


Figure 2 Nutrition routes into the intervertebral disc, adapted from Holm, 1990

The mechanical function of the disc depends on the disc matrix produced by disc cells. With sufficient nutrition in the disc, cells will continue producing proteoglycans that can draw in water to hydrate the disc. When nutrition in the disc is restricted, cell viability may decrease in the nucleus and thus decrease matrix synthesis and water content. Insufficient nutrition and inefficient removal of metabolic waste products can affect disc health and cell viability.[30]

Motion of the disc with long-term exercise can positively affect transport in the disc. Fluid expression during compression and the fluid imbibitions due to relaxation can facilitate the transport of larger molecules with lower diffusion coefficients. Small molecules have high rates of diffusion and will not be affected as much by fluid flow. Short-term exercises that produce about 5% of expansion and compression of the disc height has not been shown to change the movement of small molecules.[45,46,47] Prolonged exercise, immobilization due to fusion, vibration, and smoking are some of the factors that may also influence disc nutrition by disturbing the circulation and transport of the nutrients into the disc.[48]

Glucose concentration and oxygen tension is low in the center of the nucleus. Due to low oxygen in the nucleus, part of the glucose is metabolized through the anaerobic pathway. Lactate is produced in this pathway and it can lower the pH level in the disc. Lactate accumulation in the nucleus is not well understood, however the acidic environment can enhance some degradative enzymes which could affect disc health.[32,49]

Without adequate nutrition, cell number can decrease and gene therapy may not have the desired effect in treating the IDD. To achieve gene transfer, it is essential to have viable cells to produce the desired growth factors and synthesize matrixes. Due to the importance of disc

nutrition, further research in growth factor and gene therapy that is mindful of the nutritional aspects of the disc is needed.

2.0 SPECIFIC AIMS

2.1 Part I: Growth Factor and Gene Therapy Study in Cell Culture

- Determine the dose-dependent effects of rhBMP-2 and rhBMP-12 on matrix synthesis (PG, collagen, non-collagenous protein synthesis) in NP monolayer culture
- Determine the dose-dependent effects of rhBMP-12 on matrix synthesis in AF monolayer culture
- Determine the effects of adenovirus mediated delivery of BMP-12 cDNA on matrix synthesis in AF and NP pellets culture

2.2 Part II: Disc Nutrition Study in Organ Culture

- Develop rabbit disc organ culture method
- Study the effect of FBS concentration on disc metabolism in organ culture
- Analyze disc wet weight, dry weight, % hydration, DNA, GAG, and lactate content in the AF and NP of freshly harvested as well as cultured discs
- Examine cell morphology and cell viability in organ culture
- Attempt marker gene transfer (Ad/lacZ and Ad/Luciferase)

3.0 BACKGROUND

3.1 Disc Structure and Composition

The human spine consists of 33 vertebrae: 7 cervical vertebrae, 12 thoracic vertebrae, 5 lumbar vertebrae, 5 sacral vertebrae, and 4 coccyxgeal vertebrae. An intervertebral disc is between each of the intervertebral bodies, except for C1-2, second sacral and Coccyxgeal vertebrae. Intervertebral discs make up about one fourth of the length of the vertebral column above the sacrum.[31] The disc has a gelatinous nucleus pulposus (NP) at the center region surrounded by a laminated, fibrous annulus fibrosus (AF). The nucleus occupies about 40% of the disc's cross-sectional area. [50] The annulus is composed of lamellae with collagen fibers of each layer runs at 30 degrees angles to each other. The cartilaginous endplates locate between the bone and the disc.[12] Figure 3 illustrates the spine and different views of the intervertebral disc.

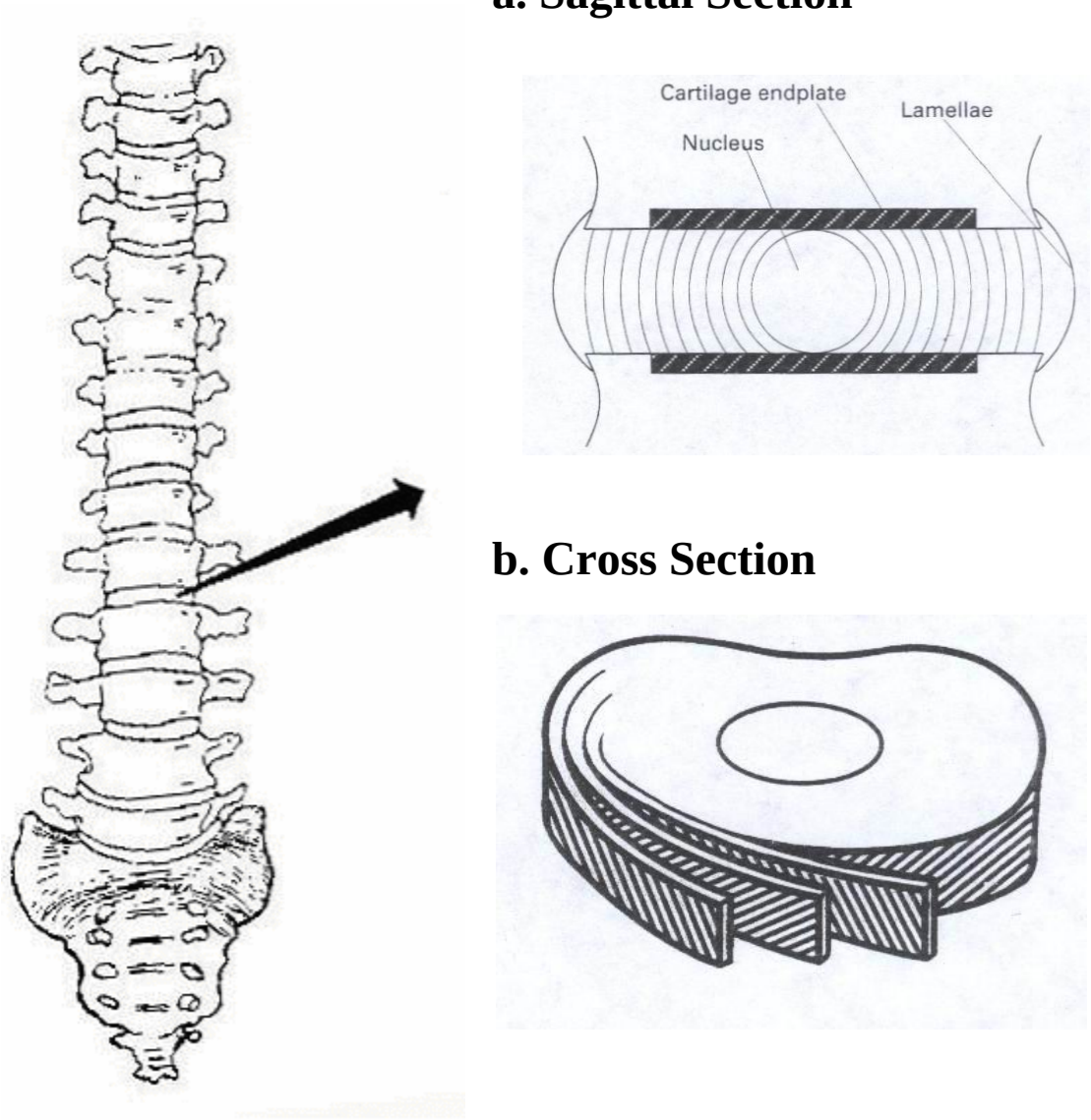


Figure 3 Schematic view of the spine and intervertebral disc, adapted from Holm 1990 (a), Corrigan 1998 (b,c)

A disc is mostly composed of proteoglycans, collagen, and water. A healthy NP is rich in proteoglycans and type II collagen, and has high water content. The AF contains type I collagen. The water content of the NP is 90% at birth, decreases to about 80% at maturity and decreases further to 70% or less in old age.[41] Water content of the AF remains relatively constant at 70% throughout its lifetime.

Proteoglycans play an important role in the disc because they are they are negatively charged and thus highly hydrophilic. They can draw water into the disc by creating an imbalance of ion concentrations between the inside and the outside of the disc.[51,52] Proteoglycans account for 50% of the NP dry weight and 10-20% of the AF dry weight. The structure of the proteoglycan consists of a protein core and carbohydrate that bond to the core protein covalently. The most common carbohydrate, or glycosaminoglycan (GAG) chains, are chondroitin sulfate and keratin sulfate in the disc. Other proteoglycans include versican, decorin, biglycan, fibromodulin, and lumican.[53]

Another important protein in the disc is collagen. Collagen provides the structural integrity and mechanical strength of the disc. Collagen accounts for 65-70% of the AF dry weight and 15-25% of the NP dry weight. The AF has 60% of type I and 40% of type II collagen. The outer annulus is almost all type I collagen, and the percentage of type II collagen increases toward the center. The inner annulus-nucleus interface is almost all type II collagen. Collagen molecules are linked by cross links formed on hydroxylysine residues along the collagen molecule. The cross-linking protects the collagen framework from mechanical disruption and provides strength and stability.[54] There are other proteins besides collagen. Theses non-collagenous proteins include matrillins, core proteins on which GAGs are attached, fibromodulin, and enzymes involved in collagen processing and crosslinking, etc. These non-

collagenous proteins play an important role in extracellular matrix organization. Some of these proteins bind to collagen and participate in fibrillogenesis and matrix assembly.

For human discs, cells take up about 5% of the matrix volume. The cellularity is 4,000 cells/mm^3 for NP, 9,000 cells/mm^3 for AF[33]. Table 1 shows the disc composition in human and rabbit discs.

Table 1 Disc composition in human and rabbit discs

Human Disc Composition • Disc Hydration: 3-5 g/g dry wt, 1-2 g/g dry wt outer AF, 2-3 g/g dry wt inner AF • GAG Content: 0.15-0.35 g/gdry wt NP, 0.05 g/g dry wt outer AF, 0.1-0.15 g/g dry wt inner AF • Collagen Content: 0.15-0.25 g/g dry wt NP, 0.5-0.6 g/g dry wt outer AF, 0.15-0.3 g/g dry wt inner AF • Lactate: 1-2 mmol/g wet wt NP near endplate, 10-15 mmol/g wet wt NP near center • pH: 6.8-7.4 Healthy, 6.6-7.5 Degenerated • Oxygen Concentration: 4-10 mmHG in central NP
Rabbit Disc Composition • Disc Hydration: 77% in NP • DNA Content: 10 μg/ NP, 4.1 μg/AF • GAG Content: 1987 mmol GAG/disc, 320 mmol of chondroitin sulfate/mg dry wet in NP, 240 mmol of chondroitin sulfate/mg dry wt in AF • PG Synthesis: 10-27.1x10^{-6} mmol of incorporated sulfate/hr/g dry wt • Lactate: 14.4 mmol/g wet wt in whole disc

3.2 Disc Metabolism

Metabolism is the totality of the physical and chemical processes occurring in a living organism, which may be partitioned into anabolism and catabolism. Catabolism, or degradation, is the breakdown of complex molecules to simple molecules to reuse their components and/or to generate energy. Anabolism, or biosynthesis, is the synthesis of complex molecules from simple molecules and it usually requires energy.[55] Living cells require energy to make small molecules such as amino acids and nucleotides as well as large molecules such as DNA, RNA, and proteins. Energy is also required for basic cellular mechanisms such as transport of ions and metabolites. Energy is stored and transferred through adenosine triphosphate (ATP). The hydrolysis of ATP gives 7.3 kcal/mol. A disc cell's carbon and energy source comes from glucose. In the cell, glucose goes through glycolysis and becomes pyruvate. There are two pathways to break down pyruvate: aerobic and anaerobic respiration (Figure 4).[56]

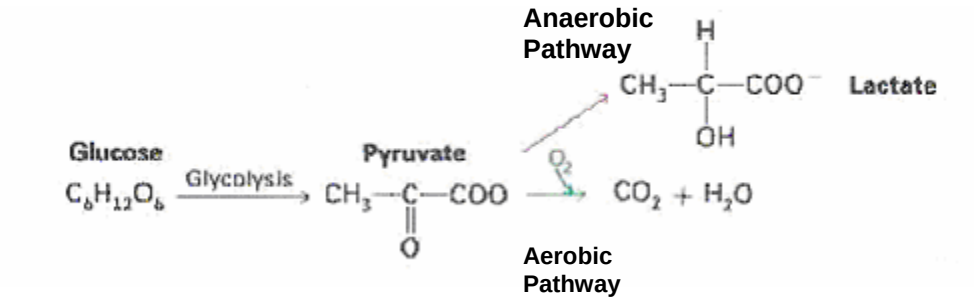


Figure 4 Glycolysis and breakdown of pyruvate, adapted from Voet, 2002

Oxygen tension can be as low as 1% in the center of the disc.[57] Due to the low oxygen tension within the disc, 98.5% of the glucose is metabolized through anaerobic pathway producing lactate.[49] Lactic acid production is dependent on oxygen tension levels. When oxygen tension decreases from 10% to 1%, lactic acid production doubled.[57] Lactate accumulation in the disc can cause a lower pH and affect the health of disc cells.

1.5% of the glucose is metabolized through the aerobic path way in the center of the disc. When cells use the aerobic pathway of glycolysis, it goes through oxidative phosphorylation. Study by Ishihara and Urban have suggested that an important fraction of the energy production of the disc comes from oxidative phosphorylation.[57] In the outer area of the disc, oxygen tension is higher and energy production from oxidative phosphorylation increases. In the aerobic pathway, complete oxidation of a mole of glucose gives rise to 38-mol adenosine triphosphate (ATP), whereas anaerobic glycolysis produces only 2-mol ATP. The equations below show that the anaerobic pathway is 19 times less efficient than the aerobic pathway.[46]

Anaerobic glycolysis:

$$C_6H_{12}O_6 + 2\ ADP + 2\ P_i \longrightarrow 2\ lactate + 2\ H^+ + 2\ H_2O + 2\ ATP$$

Aerobic metabolism of glucose:

$$C_6H_{12}O_6 + 38\ ADP + 38\ P_i + 6\ O_2 \longrightarrow 6\ CO_2 + 44\ H_2O + 38\ ATP$$

3.3 Gene Transfer

Gene therapy is the introduction of a gene to specific tissues and cells to allow gene expression of the inserted gene product.[58] Successful gene therapy for IDD should direct disc cells to produce more proteoglycan and collagen. Ultimately, one of the goals of the gene therapy is to give the disc more water content in the nucleus and to regain or maintain its ability to withstand mechanical loads. With this in mind, the gene of interest is chosen based on its potential ability to stimulate matrix synthesis.

There are many ways one can deliver the gene of interest into the cells, and some methods are more effective than others. Besides the method of delivery, sufficient amounts and duration of gene expression also determine if successful gene transfer can be achieved. Two main approaches for gene delivery are viral and non-viral vectors. Viral systems have been shown to be more successful than the non-viral systems currently available.[59] Viral vectors include retrovirus, adenovirus, adeno-associated virus. Some of the non-viral transfection agents are liposomes, PLL, and PEI.[60,61] In this study we have chosen adenovirus as our gene delivery vehicle because of its effectiveness in gene delivery.[62] We have shown upregulated matrix synthesis in disc cells treated with Ad/TGF-β1 in previous studies.[27] In this study we have chosen Ad/BMP-12 gene to investigate its potential to stimulate matrix synthesis in disc cells. There is a risk of immune responses in viral gene therapy. However, our group had previously shown that the disc provides an immune-privileged environment for adenoviral gene therapy because of its avascular structure.

3.4 Culture Systems in Disc Metabolism Study

Culture systems to study the disc metabolism include cell, tissue, and organ culture. Most studies are done in cells or tissues from small animals because large animal studies are expensive and human material is difficult to obtain. In human material, the cellular activity is low from discs taken postmortem.[63]

In tissue culture, the key issue is maintenance of PG in the tissue. Thompson et al cultured canine disc tissue in serumless medium to study the effect of growth factors on matrix synthesis.[26] In their culture, they observed low PG content for 3 to 4 days and the culture was maintained for up to 8 days. Bayliss et al cultured disc tissue in a dialysis bag with polyethylene glycol (PEG) and were able to maintain PG in the disc for 6 hours.[64] However, the daily replacement of medium in the dialysis bag increased the contamination risk and exposure to PEG, which is toxic to the tissue.

To minimize loss of PG in the NP, Chiba et al developed an organ culture method to study the metabolism of the rabbit disc.[65] They have cultured discs up to 1 month and observed a decrease in tissue dry weight, DNA, as well as PG. Takegami, Masuda, and An are continuing to develop the organ culture method to study disc metabolism using intact intervertebral discs. Their disc culture contains disc and endplates. The authors did not indicate in the abstract that intervertebral bodies were kept with the disc in the culture. Discs were injected with OP-1 and maintained in the culture for 2 weeks.[66]

3.5 Summary of Literature

- **Part I: Growth Factor and Gene Therapy Study in Cell Culture**

BMP-2 was shown to increase proteoglycan synthesis in rat intervertebral discs.[30] Studies have shown that BMP-12 stimulates formation of tendon-like tissue in-vivo, suggesting its potential for reparative function in AF cells.[37,38] Although there have been some reports showing the potential of gene delivery in NP cells, no reports were found showing virus mediated gene therapy to increase matrix synthesis in human AF cells from degenerated discs.

- **Part II: Disc Nutrition Study in Organ Culture**

Research on the nutrition of the intervertebral disc began in the late 1920's.[40] However disc nutrition is still poorly understood because it is difficult to maintain the integrity of the tissue in-vitro. Studies had been done in NP culture, but PG comes out of the tissue very quickly.[25] In order to maintain PG in the disc, whole organ culture was developed. There are currently ongoing nutrition studies on the effect of OP-1 stimulation using whole organ culture by Takegami et al.[66] No study was found on the effect of gene therapy in a disc organ culture.

3.6 Overview of Proposed Study

All of the experiment protocols were approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee at the University of Pittsburgh.

- **Part I: Growth Factor and Gene Therapy Study in Cell Culture**

The effect of rhBMP-2 and rhBMP-12 on matrix synthesis will be examined in the NP monolayer culture. Although the half life of growth factors is short, the study will be necessary to screen their effect on disc cells. Proteoglycan, collagen, and non-collagenous protein synthesis will be measured. Different doses of the growth factors will be used to compare the effect of rhBMP-2 and rhBMP-12. After screening the effect of these growth factors, we will use adenovirus for gene transfer of Ad/BMP-12 to determine its efficacy. Ad/BMP-12 will be administered on AF and NP cells in a pellet culture to study its effect on matrix synthesis. The differences between AF and NP cells' response to Ad/BMP-12 will be examined. Figure 5 shows the project design of Part I of the study of the effect of growth factor and gene therapy on matrix synthesis of human disc cells.

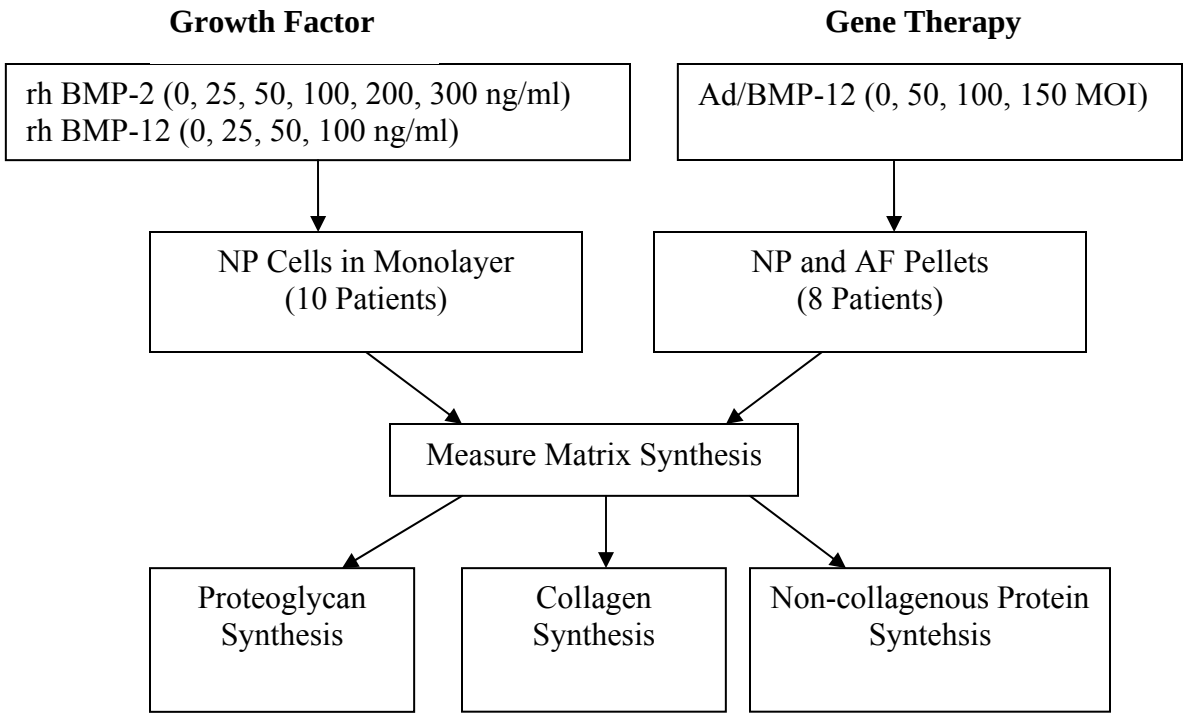


Figure 5 Project design of Part I: Growth Factor and Gene Therapy Study in Cell Culture

- **Part II: Disc Nutrition Study in Organ Culture**

A method for organ culture of rabbit intervertebral disc will be developed in Part II of the study. Effects of different concentrations of nutrient on the metabolism of the disc in the culture will be determined. Whole rabbit discs will be incubated in medium containing different percentage of fetal bovine serum (FBS). Disc hydration, GAG, DNA content, lactic acid will be measured. Histology of cultured and non-cultured discs will be used to show cell morphology. Cell viability of cultured discs will be investigated. BMP-2 content in the tissue of non-cultured discs will also be measured to establish baseline composition data. Then Ad/lacZ and Ad/Luciferase will be injected into the discs to attempt gene transfer. Figure 6 shows the project design of Part II of the study on disc nutrition in organ culture.

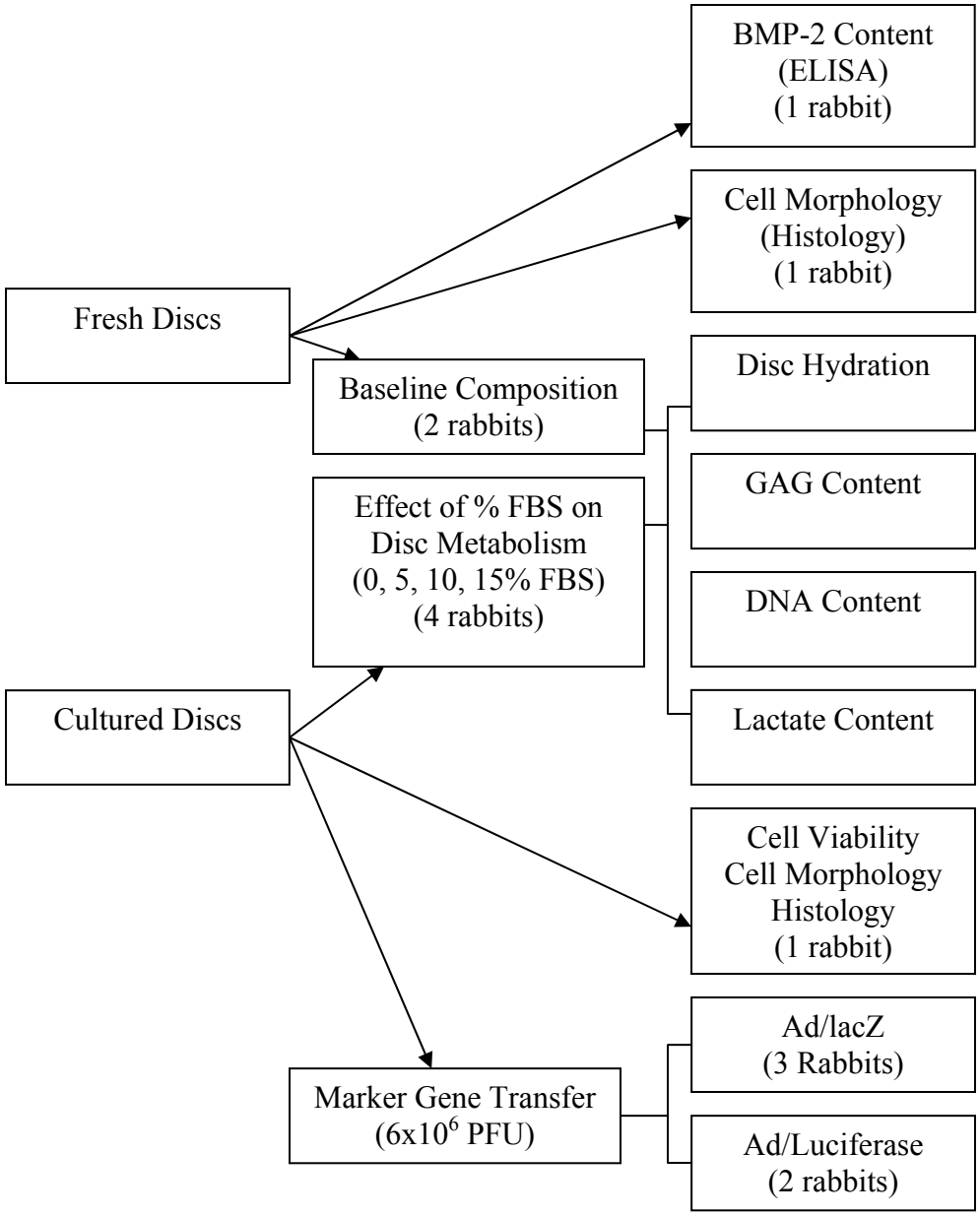


Figure 6 Project design of Part II: Disc Nutrition Study in Organ Culture

4.0 METHODS

4.1 Part I: Growth Factor and Gene Therapy Study in Cell Culture

4.1.1 Disc Cell Isolation

AF and NP tissues from degenerated discs were harvested from 18 patients during surgical disc procedures performed for herniated disc and spinal stenosis. The specimens were rinsed with saline three times in a Petri dish. Tissues were minced in a sterile conical glass tube into small fragments of approximately $2mm^3$ volumes each in a sterile conical glass tube. Tissues were digested in Ham's F-12 medium (F-12, GIBCO-BRL, Grand Island, NY) with 1% penicillin/streptomycin (P/S), 5% FBS (fetal bovine serum, GIBCO-BRL, Grand Island, NY), and 0.2% pronase (Calbiochem, La Jolla, CA) for 90 minutes. Cells were washed with 20ml of saline 3 times and then incubated in 0.01% collagenase type II (Sigma, St. Louis, MO) overnight at 37°C dry incubator under gentle agitation in a filter glass. Cells were isolated through a sterile nylon mesh (75μm pore size) and centrifuged at 2000 RPM for 5 min. Cells were resuspended in Ham's F-12 with 10% FBS and 1% P/S seeded in monolayer and cultured with F-12 medium (containing 1% PS, 50μg/ml L-ascorbic acid) in a 5% CO_2 incubator until confluency.[67] Depending on amount of tissue received, confluency was typically attained within 2 to 3 weeks.

4.1.2 rhBMP-2 and rhBMP-12 Stimulation in Monolayer Cell Culture

NP cells harvested from 10 patients (cervical and lumbar discs) were processed as described in Section 4.1 and incubated in serumless media with 1% ITS+ (premix) for 2 days with BMP-2 (25, 50, 100, 200, 300 ng/ml) or BMP-12 (25,50,100 ng). Cells were then incubated with ^{35}S or ^{3}H-proline to measure matrix synthesis as described in Sections 4.4 and 4.5, respectively. Experiments were performed in triplicate and the data was normalized by cell number. Cells were trypsinized and centrifuged at 2,000 rpm for 5 min. Then cells were resuspended and cell solution was pipetted onto each side of the hemocytometer. A manual counter was used to count the cells in the grids under the microscope. Only live cells were counted.

4.1.3 Ad/BMP-12 Transduction in 3D Pellet Culture

NP and AF cells were isolated from 8 patients (cervical and lumbar discs) as described in Section 4.1. Monolayer cells were incubated with Adeno/BMP-12 (0,50,100,150 MOI) for 6 hrs. Knowing the number of cells and multiplicity of infection (MOI), the amount of virus needed in plaque forming unit (PFU) was calculated using this equations below:

$$MOI = \frac{PFU}{Cell}$$

After Ad/BMP-12 transduction, monolayer cells were trypsinized and made into pellets by centrifuging at 2000 RPM for 5 minutes. A hemocytometer was used to count the cells as described in Section 4.1.2 and each pellet was made with approximately 150,000 cells. Therefore 0, $7.5x10^5$, $1.5x10^6$, $2.25x10^7$ PFU per pellet was used for 0, 50, 100, 150 MOI respectively. Pellets were incubated in serumless media with 1% ITS+ (premix) for 6 days. Experiments were performed in triplicate and data was normalized by DNA content as described in Section 4.3.2.1.3.

Viral vectors were provided by the Paul D. Robbins, PhD of Department of Molecular Genetics and Biochemistry. In the adenoviral vector construction, each recombinant adenoviral vector originated from replication-deficient Type 5 adenovirus lacking E1 and E3 loci. Complementary DNAs were cloned into the E1 region, and expression was driven by a human cytomegalovirus (CMV) promoter. Hight-titer suspensions of recombinant adenovirus were prepared by amplification in confluent monolayers of transformed human embryonic kidney 293 cells. Titers were estimated from the optical density at 260 nm and standard plaque assay.[27]

4.1.4 ^{35}S-Sulfate Incorporation

Cells or pellets were incubated in Newman Tytell seamless medium containing ^{35}S-sulfate (10 μCi/ml) for 7 hours. The medium was replaced by Guanidine Hydrochloride (GuHCl) and samples were digested by shaking in the cold room (-20^{o}C) for 48 hours. Then 200μl of medium and extract from each sample were applied to chromatography on Sephadex G-25 in PD-10 columns (Pharmacia Biotech, Uppsala. Sweden) equilibrated and eluted with 4M GuHCl

containing 0.05M Tris and 0.05 M Na2SO4. The 1 mL fractions were collected in scintillation vials mixed with 7 mL of scintillation fluid (Ultima Gold, Packard Mariden, CT) and counted by a liquid scintillation counter (Packard=1990 TR, Mariden, CT).[68]

4.1.5 ^{3}H-Proline Incorporation

Cells or pellets were incubated with serumless media containing 50 μg/ml ascorbic acid, 50 μg/ml β-amino-propionitrile, and 10 μCi of ^{3}H-proline/ml, then incubated at 37°C for 24 hours. After incubation, the medium was collected and the samples were treated with homogenizing buffer (0.5ml/well) and subjected to three freeze/thaw cycles at –80˚ C for 2 hrs to homogenize the cell extracts 2 times. The samples were then stirred with magnetic micro stirring bars (Cat. No. 14-511-67, Fisher Scientific, Fair Lawn, New Jersey) at 4˚ C for overnight, and then the medium and cell layer were combined and relative collagen synthesis was determined by ^{3}H-proline incorporation using a modified collagenase digestion method[69]. Briefly, a carrier protein (2 mg/ml of pepsin-solubilized bovine type I collagen) was added and the proteins were precipitated by addition of 10% trichloroacetic acid (TCA). The TCA-precipitated proteins were recovered by centrifugation at 13,000 rpm for 10 min at 4°C. The recovered protein pellets were resuspended in 1ml of 1mM proline solution, and washed with 5% TCA to remove the unbound isotope. This step was repeated three times. After repeated washing with TCA the pellets were resuspended in the collagenase buffer (5 mM $CaCl_2$ and 3 mM *N*-ethylmaleimide) and purified bacterial collagenase ABC form III (Advanced Biofactures, Lynbrook, NY) (20 U/ml). The pH was adjusted to 7, and pellets were incubated at 37°C for 2h. After incubation, the samples were

cooled down on ice, then TCA was added and the mixture was centrifuged for 10 min at 13,000 rpm. The collagenase-digestive proteins were separated from non-digested proteins by addition of 5% TCA followed by centrifugation at 13,000 rpm for 10 min. For radioactive scintillation counting for collagen synthesis, 1 ml of the supernatant was taken out and mixed with 6 ml of scintillation fluid. The pellets were redissolved in 0.2 M NaOH and aliquots of the pellets were subjected to radioactive counting to determine the level of noncollagenous protein synthesis. Data were normalized by Pico Green dsDNA quantitation. (Molecular Probes, Eugene, OR)

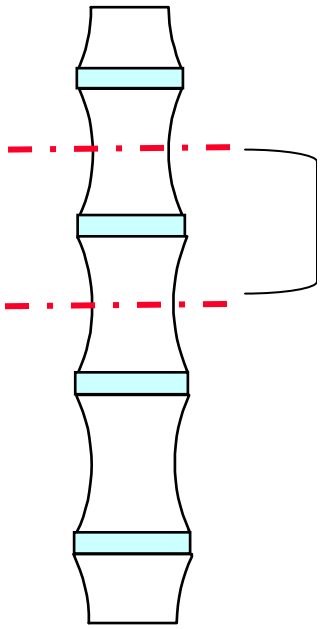
4.2 Part II: Disc Nutrition Study in Organ Culture

4.2.1 Organ Culture

Six skeletally mature New Zealand White rabbits (4-5 kg) were used in this study. After sacrifice and harvest of the spines, muscles were removed as quickly as possible. Using a bone cutter, the discs were cut in the center of vertebral body (Figure 7). Discs were kept moist with saline and bone marrow was flushed out using a 21G needle with saline containing 1% P/S. Discs were then washed with 10ml of saline with 1% P/S for 6 times. Five discs were harvested from each rabbit (L1-2, L2-3, L3-4, L4-5). Discs from 4 rabbits (1,2,3,4) were cultured for 2 weeks in media containing different % FBS. Discs from rabbit 1,2,3,4 were cultured in serumless media, 5%, 10%, or 15% FBS F12/DMEM respectively. Discs from 2 rabbits were not cultured to serve as fresh controls. Originally, we did not culture the disc with bone. We observed cracking of the endplate in many discs and NP came out of the disc during the culture.

When we left the vertebral bodies on the disc, we saw less swelling of the disc and it prevents cracking of the endplate.

a.



Intervertebral discs were separated by bone cutter by cutting at the middle point of the intervertebral bodies.

b.

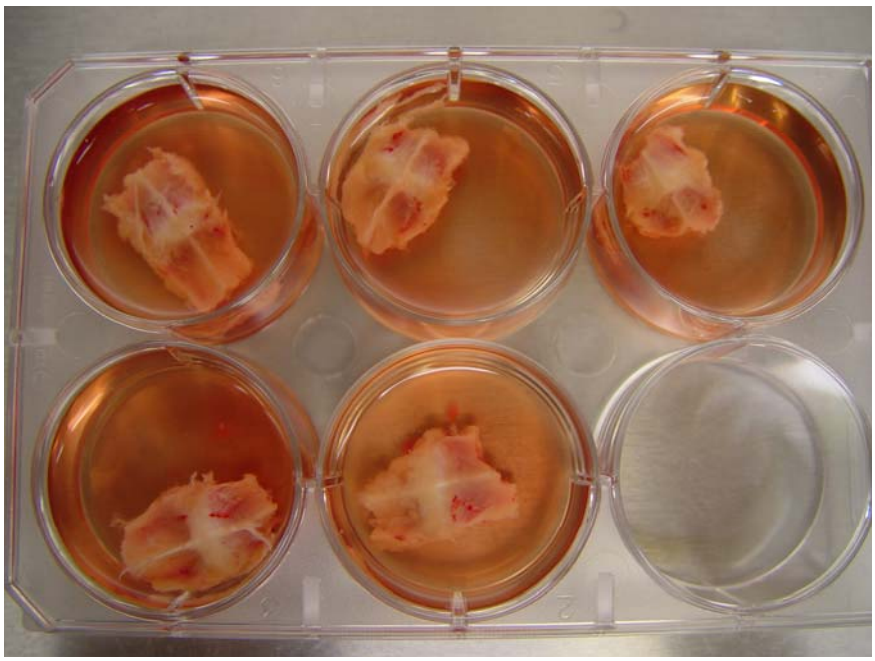


Figure 7 a) Bone-disc-bone sample b) Whole discs in culture

4.2.2 Gene Transfer

Twenty-five discs from 5 rabbits were harvested. Discs were first cultured in serumless media for 2 days after harvest. $6x10^6$ plaque forming units (PFU) of Ad/lacZ in saline, Ad/Luciferase in saline, or saline only was injected into the disc with a 30G needle (Figure 8). Three discs from one rabbit were injected with Ad/Luciferase. Two discs from the same rabbit were injected with saline as controls. Twenty discs from 4 rabbits were injected with Ad/lac-Z or saline control. Among the 20 discs, 4 culturing conditions (0%, 5%, 10%, 15% FBS) were used with 5 discs for each condition. The amount of virus stock solution needed (Z) per disc was calculated by the following equations:

$$X(\frac{Particles}{m\ell})x10^{-3}(\frac{m\ell}{\mu\ell})x10^{-2}(\frac{PFU}{Particles}) = Y(\frac{PFU}{\mu\ell})$$

$$\frac{6x10^6(PFU)}{Y(\frac{PUF}{\mu\ell})} = Z(\mu\ell)$$

X is the concentration of the virus stock solution in the unit of particles per ml and it is converted to Y in units of PFU. Z amount of virus stock solution was mixed with saline to make a total of 10µl of viral solution to inject into each disc. X varied with the batch of virus, therefore Z was different for different viruses. For example, X for the specific batch of Ad/lacZ that we used was $4.38x10^{12}$, Z was calculated to be 0.137 µl using the equations above. We multiplied Z by the number of discs that were going to be injected. Zx6 discs equaled 0.0.822 µl. To ensure accuracy, a larger amount of the virus stock solution was taken because we cannot pipette an amount smaller than 1 µl. It was best to use more than 5 µl, therefore we chose to take

5.48 of virus stock solution and mixed it with 394.52 µl of saline. When we take 10 µl of this virus solution, there would be 0.137 µl of virus stock and 9.863 µl of saline. After injection, the needle was left inside the disc for 20 seconds before slowly being withdrawn to minimize the amount of solution leaking out of the disc. The injection was carefully done in a sterile hood to minimize contamination. The discs were incubated in the media for one week before analyzing the effect of gene transfer.

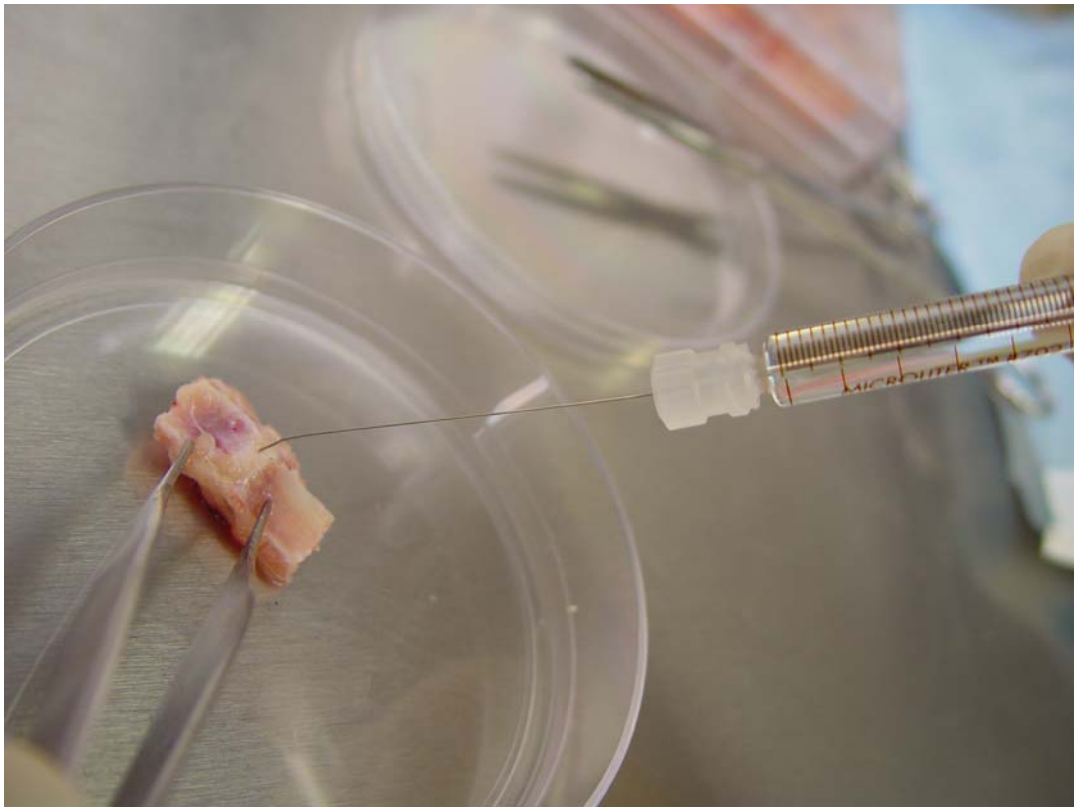


Figure 8 Injection of Ad/lacZ or Ad/Luciferase

4.2.3 Outcome Measures

4.2.3.1 Cells

4.2.3.1.1 Cell Morphology

Histology was used to observe cell morphology in fresh discs and cultured discs. Discs from one rabbit were non-cultured. Discs from another rabbit were cultured in serumless media for 2 days. There were a total of 9 discs, 5 fresh discs and 4 culutred discs. Discs were fixed in 10% buffered formalin for 3 days, processed using a tissue processor (Tissue-Tek VIP, Sakura Finetek USA, Torrance, CA), and dehydrated overnight. It was processed and dehydrated using 70% ETOH 45 min, 80% ETOH 45 min, 95% ETOH 45 min 2 times, 100% ETOH 2 times, xylene 45min 2 times, and then paraffin 4 hrs. The endplates were removed by a scalpel (#10 blade). Tissue was embedded in paraffin and 10μm thick cross sections were prepared using a microtome (Cut 4055, Olympus, Melville, NY) and stained with (H&E) Hematoxylin and Eosin. Hematoxylin stains for the nucleus and Eosin stains for plasma. The stain theory is based on the attractions of oppositely charged tissue and dye molecules. Hematoxylin binds to DNA, RNA, and acidic proteins in a cell because it is binds to negatively charged molecules.[70]

4.2.3.1.2 Cell Fate

Cell viability was detected with CMFDA (Molecular Probes, Eugene, OR) and propidium iodide (Calbiochem, San Diego, CA). Cell tracker CMFDA can diffuse into the live cell, where cytosolic esterases cleave the acetate groups and release a green fluorescent product. Propidium iodide can diffuse through the membrane of nonviable cells and bind to double-stranded nucleic acid and produce a red fluorescent product. Whole NP was taken out by a forceps and kept moist at all times. For AF and Transition Zone (TZ), samples from four different regions of the discs were harvested with a scalpel.(Figure 9) 1mm thick sections of AF, TZ, or the whole NP was incubated in 250µl of CMFDA and 250µl of prodidium iodide solution for 30min at room temperature. Samples were washed 2 times with PBS and fixed in 2% paraformaldehyde for 1 hour at room temperature. After washing for 2 times with PBS, samples were stored in PBS at 4^{o}C before imaging with 2-photon laser scanning microscope (IX 70, Olympus, Melville, NY) or fluorescent microscope (Eclipse E 800, Nikon, Melville, NY).

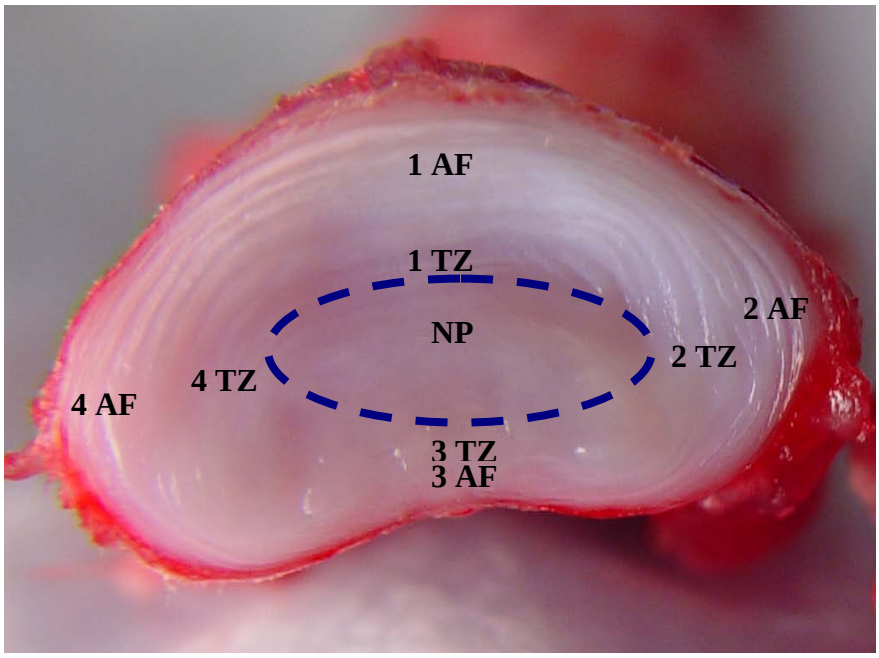


Figure 9 AF and TZ were obtained from four regions of the disc, whole NP was taken for staining

4.2.3.1.3 DNA Content

Tissue of AF and NP were harvested and AF was cut in smaller pieces of approximately $2mm^3$ in volume. Tissue was dried using Savant Speedvac (Savant Instruments, Farmingdale, NY) for 2 hours. Then tissue was digested in papain buffer (50mM phosphate buffer, 5 mM EDTA, 5mM cysteine HCl, papain 2.5 mg/ml) overnight at 65^oC. Then DNA content was analyzed using Pico Green dsDNA Quantitation Reagent and Kits (Molecular Probes, Eugene, OR). A standard curve for dsDNA was generated from 10ng/ml to 1000 ng/ml. Samples were diluted in TE buffer and 100 µl of the diluted samples were pippetted into a 96 well plate. Then 100µl of Pico

Green solution was added to the samples and standards and samples were run using the fluorometer with 485 nm excitation filter and 528 nm emission filter.

4.2.3.2 Biologic Activity of Cells

4.2.3.2.1 Growth Factor Production

Five discs from one rabbit was used to determine the BMP-2 level in a non-cultured disc. ELISA (R&D systems Quantikine BMP-2 Immunoassay, catalog Number DBP200) was done for both media and tissue. BMP-2 in the tissue was extracted by 2M Guanidine-HCL with protease inhibitors (0.05 M Tris, 5mM EDTA, 5mM Benzamidine, 5mM N-ethylmaleimide, 2mM PMSF) overnight, stirred in the cold room. Samples were then centrifuged and the supernatant was diluted so that the final concentration was less than 0.06 M prior to the assay.

This assay used the quantitative sandwich enzyme immunoassay technique.[71] A monoclonal antibody specific for BMP-2 was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any BMP-2 present was bound by the immobilized antibody. After washing away any unbounded substances, an enzyme-linked monoclonal antibody specific for BMP-2 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of BMP-2 bound in the initial step. The color development was stopped and the intensity of the color was measured.

4.2.3.2.2 lacZ Marker Gene Expression

To evaluate lacZ transgene expression qualitively, beta-galactosidase expression was analyzed using the X-Gal histochemical staining technique.[72] A total of 15 Discs from 3 rabbits were cultured in serumless medium for 2 days, injected with Ad/LacZ or saline control, and cultured for 1 more week. The discs were frozen in liquid nitrogen and 8um thick transverse serial cryostat sections were obtained. The sections were rinsed in PBS and fixed in 0.5% glutaraldehyde at 4^{o}C for 10min. Then sections were rinsed twice in PBS with 1mM MgCl2. The sections were then incubated in X-gal substrate overnight at 37^{o}C. X-gal substrate contains 20X KC (0.82 g K3Fe, 1.05 g K4Fe, 25ml PBS), 1M $MgCl_2$, 40mg/ml X-gal in DMSO, and PBS. The blue X-Gal reaction product was used to indicate expression of the lacZ product beta-galactosidase.

4.2.3.2.3 Luciferase Marker Gene Expression

Luciferase transgene expression was quantified using a standard luciferase assay kit (Promega). Ten discs from 2 rabbits were cultured in serumless medium for 2 days, injected with 6x10^{6} PFU Ad/luciferase or saline control, and then cultured for 1 week in 10% FBS. The NP was harvested and homogenized with 200µl of 1X cell culture lysis buffer by vortexing for 15 seconds. The samples were centrifuged at 13,000 RPM for 1 minute at 4^{o}C, and the supernatant was

transferred to a new tube. Luciferase assay reagent was mixed with cell extract and light production was measured with a luminometer and expressed in relative light units (RLU).

4.2.3.3 Extracellular Matrix (ECM) Composition

4.2.3.3.1 GAG Content

This assay measured the GAG content in the medium as well as the digested tissue. Tissue was digested by the method described in Section 4.8.2. This assay determines the change in absorption spectrum of the dye 1,9 dimethylmethylene blue (DMB) when it is complexed with sulfated GAG.[73] It gives a quantitative determination of total GAG content in a sample of proteoglycans. The standard curve was given by condroitin-6-sulfate (Sigma, Cat #C-4384). 40µl of samples and standards (0 to 60 µg/ml) were pipetted into a 96 well plate. Then 250µl of DMB reagent was added into each well. The plate was read by the plate reader within 5 minutes at 540 and 595 nm.

4.2.3.3.2 Water Content

NP and AF of the discs were harvested with a scalpel (#10 blade) and immediately weighed on pre-weighed vials using a digital balance (AE 200, Mettler Instument Corp., Hightstown, NJ) to obtain wet weight. Then tissue was dried using Savant Speedvac (Savant Instruments,

Farmingdale, NY) for 2 hours and the dry weight was measured. Percent hydration was then calculated by the following equation:

$$\frac{WetWeight - DryWeight}{WetWeight} x100\% = \%Hydration$$

4.2.3.3.3 Lactic Acid Content

The UV-method was used to determine lactic acid content in the media and digested tissue. (Cat. No. 0139084, R-Biopharm, Marshall, MI) Tissue was digested by the method described in Section 4.8.2 DNA content. 0.015 ml of digested sample solution, 0.15 ml of solution 1, 0.03 ml of solution2, 3 µl of solution 3, and 0.135 ml of distilled water were mixed by vortexing. Mixed solution was let stand for 5 min, and then absorbances of the samples A1 were read using the plate reader at 365nm. 0.003 ml of solution 4 was then added to the mixture. After 30 min, absorbances A2 absorbances were read at 365nm. Delta A was obtained by subtracting A1 from A2. Concentration of NADH (c) was calculated by the following eqation:

$$DeltaA = A2 - A1$$

$$c = \frac{2.018}{ExDeltaA}$$

The measured amount of NADH is the same amount of the L-lactic acid in the sample. E equals 3.4 at 365nm. L-Lactic acid (L-lactate) is oxidized to pyruvate by nicotinamide-adenine dinucleotide (NAD) in the presence of L-lactate dehydrogenase (L-LDH). By trapping pyruvate in a subsequent reaction catalyzed by the enzyme glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate, the equilibrium can be displaced in favor of pyruvate and NADH.

4.3 Statistical Analysis

The data from Part I: Growth Factor and Gene Therapy Study in Cell Culture were normalized to cell count or DNA content. Mean, standard deviation, and p value of student t-test was calculated. We hypothesized that rhBMP-2, rhBMP-12, and Ad/BMP-12 will increase matrix synthesis of disc cells. For Part II: Disc Nutrition Study in Organ Culture
, disc nutrition study in organ culture, one way analysis of covariance (ANCOVA) in SAS (Cary, NC) was utilized. Due to small sample size, assumption of no inter-rabbit effect was made. However, intra-rabbit effects of different disc levels and unbalanced data due to missing level were considered. We hypothesized that with increasing %FBS in the media, GAG, DNA, and lactate will increase. The list below shows the outcome measures and data normalization:

Part I: Growth Factor and Gene Therapy Study in Cell Culture

1. In the growth factor study, mean and standard deviation of proteoglycan, collagen, and non-collagenous protein synthesis were calculated. Data were normalized to cell count. P value was set at less than 0.05 to test the significance of the data.
2. In the Ad/BMP-12 study, mean and standard deviation of proteoglycan, collagen, non-collagenous protein synthesis were calculated. Data were normalized to DNA content. P value was set at less than 0.05 to test the significance of the data.

Part II: Disc Nutrition Study in Organ Culture

1. ANCOVA was used to determine the significance of the data for GAG, DNA and lactate content. P value was set at less than 0.05.
2. Mean and standard deviation of disc wet and dry weight, % hydration, GAG, DNA, and lactate content were calculated. GAG, DNA and lactate content were normalized to the dry weight of the tissue.
3. The correlation between GAG, DNA, and lactate was determined by Pearson correlation coefficients.

5.0 RESULTS

5.1 Part I: Growth Factor and Gene Therapy Study in Cell Culture

5.1.1 Effect of rhBMP-2 and rhMBP-12 on NP monolayer

In this dose dependent study, we investigated the effect of different doses of rhBMP-2 and rhBMP-12 on matrix synthesis of NP cells. RhBMP-2 significantly upregulated matrix synthesis for all doeses (25-300 ng/ml). We observed a dose-dependent response for proteoglycan, collagen and non-collagenous protein synthesis. Among the range of doses that we studied, maximal matrix synthesis by NP cells was observed at concentrations of 200 and 300 ng/ml of BMP-2. The matrix synthesis from 200 ng/ml and 300 ng/ml rhBMP-2 groups were not significantly different ($p>0.05$). The mean and standard deviation of proteoglycan, collagen, and non-collagenous protein synthesis were 386±178%, 310±201%, and 245±113% of control respectively ($p<0.01$) in NP monolayer incubated with 300 ng/ml of rhBMP-2.

RhBMP-12 also upregulating matrix synthesis of NP cells. We investigated a shorter range of doses of rhBMP-12 (25-100ng/ml) than that of rhMBP-2 (25-300 ng/ml) because we did not observe higher matrix synthesis at higher does in a preliminary study. We did not see a dose-dependent response for matrix synthesis. There is a significant increase in proteoglycan, collagen, and non-collagenous protein synthesis. However, matrix synthesis from 25, 50, 100 ng/ml groups were not significantly different ($p>0.05$). The mean and standard deviation of

proteoglycan, collagen, and non-collagenous protein synthesis were 158±49%, 138±42%, and 147±32% of control respectively ($p<0.01$) in NP monolayer incubated with 100 ng/ml of rhMBP-12.

In the range of doses that we have tested, rhBMP-2 showed a more substantial effect on upregulating matrix synthesis than rhBMP-12 (Figures 10-12). Raw data is shown in Appendix A.

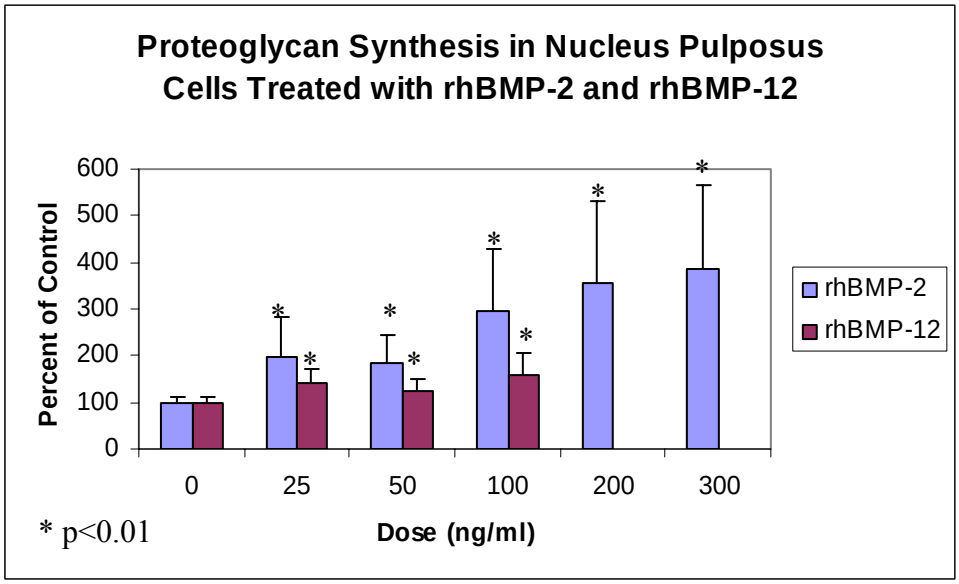


Figure 10 PG synthesis in NP treated with rhBMP-2 and rhBMP-12

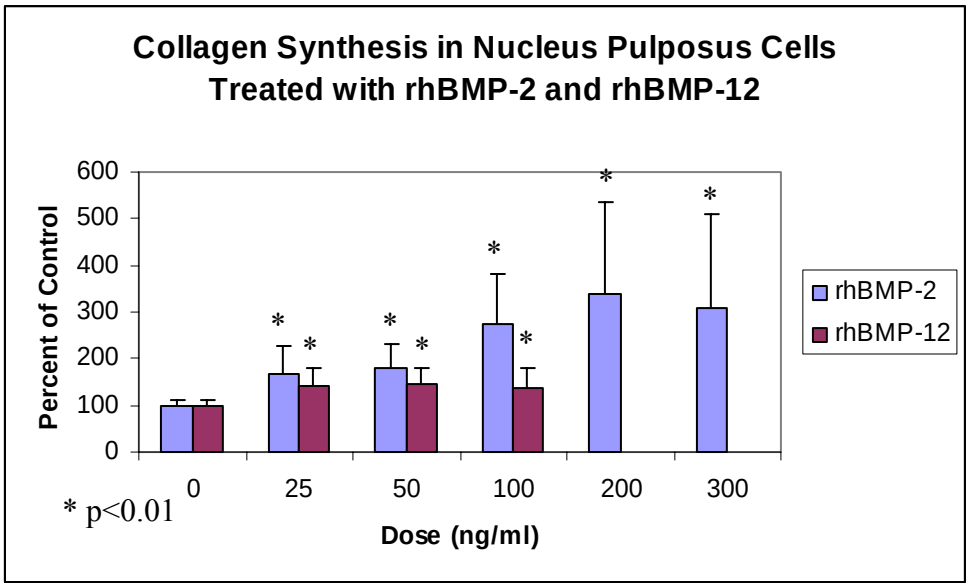


Figure 11 Collagen synthesis in NP treated with rhBMP-2 and rhBMP-12

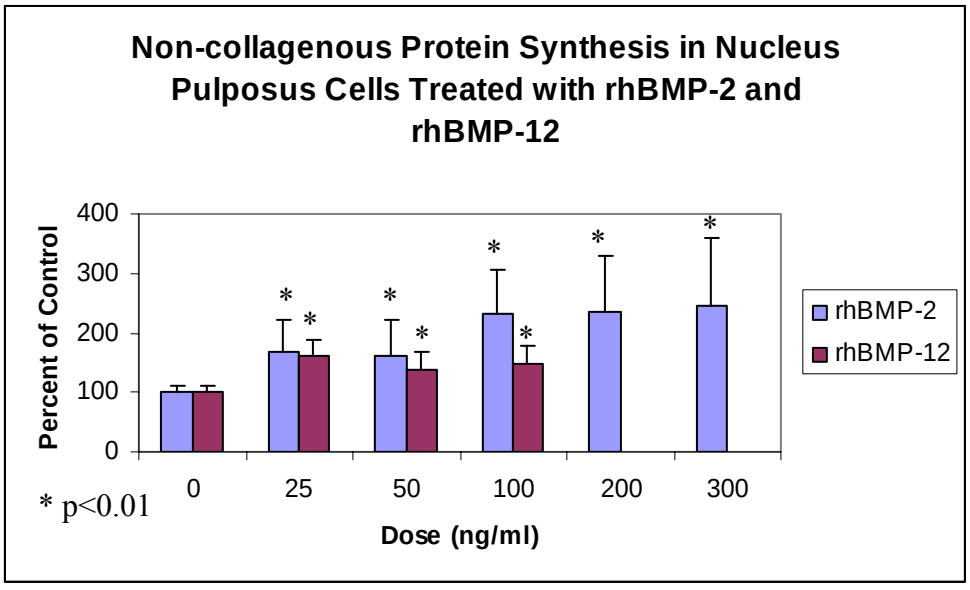


Figure 12 Non-collagenous protein synthesis in NP treated with rhBMP-2 and rh-BMP12

5.1.2 Effect of rhBMP-12 on AF monolayer

In annulus fibrosus, rhBMP-12 at 25 and 50 ng/ml demonstrated significant upregulation in only non-collagenous protein synthesis as shown in Figure 15 ($p<0.01$). For AF cells treated with 50 ng/ml of rhBMP-12, mean and standard deviation of non-collagenous protein synthesis was 127±30% of control. No significant increase of PG or collagen synthesis was observed (Figure 13,14). In a preliminary study, we have also treated AF cells with rhBMP-2 (25, 50, 100 ng/ml). Collagen synthesis was significantly increased at rhBMP-2 at 100 ng/ml rhBMP-2 with mean and standard deviation of 121±36% of control. Non-collagenous protein synthesis was significantly increased at rhBMP-2 at 25 and 100 ng/ml. For AF cells treated with 100ng/ml of rhBMP-2, the mean and standard deviation of non-collagenous protein synthesis was 151±56% of control. We observed no significant increase of PG synthesis. Preliminary results of rhBMP-2 on AF culture monolayer are shown in the Appendix B.

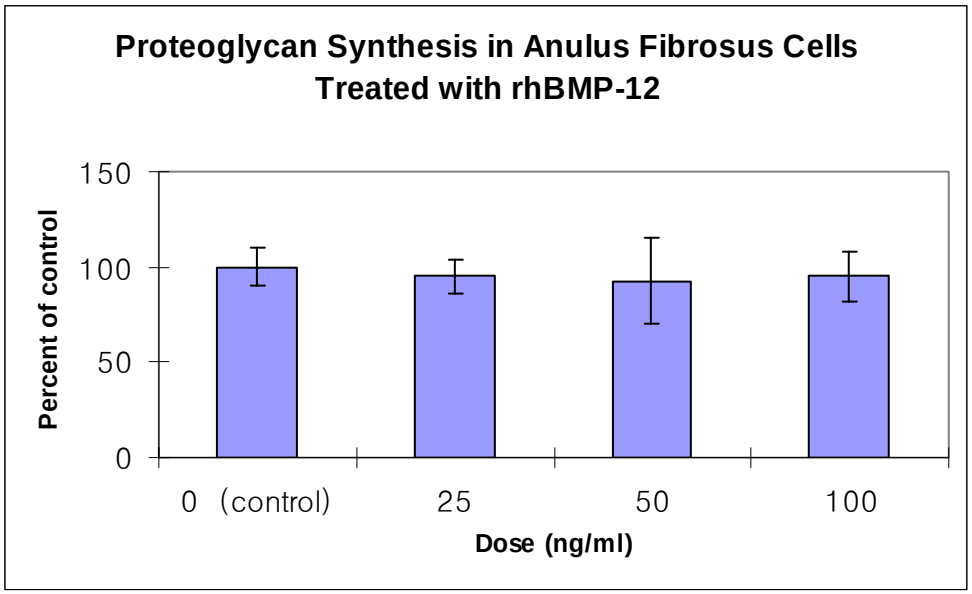


Figure 13 PG synthesis in AF treated with rhBMP12

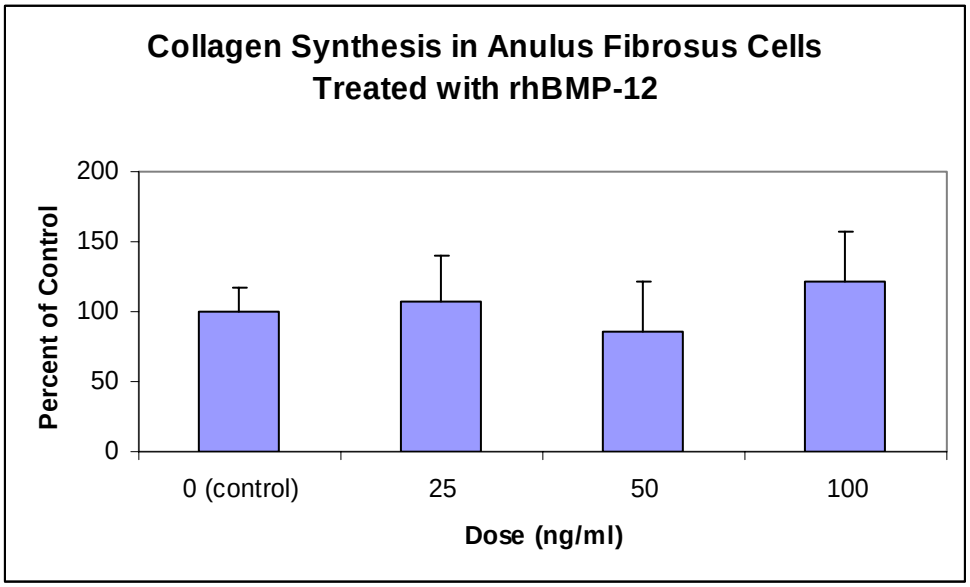


Figure 14 Collagen synthesis in AF treated with rhBMP-12

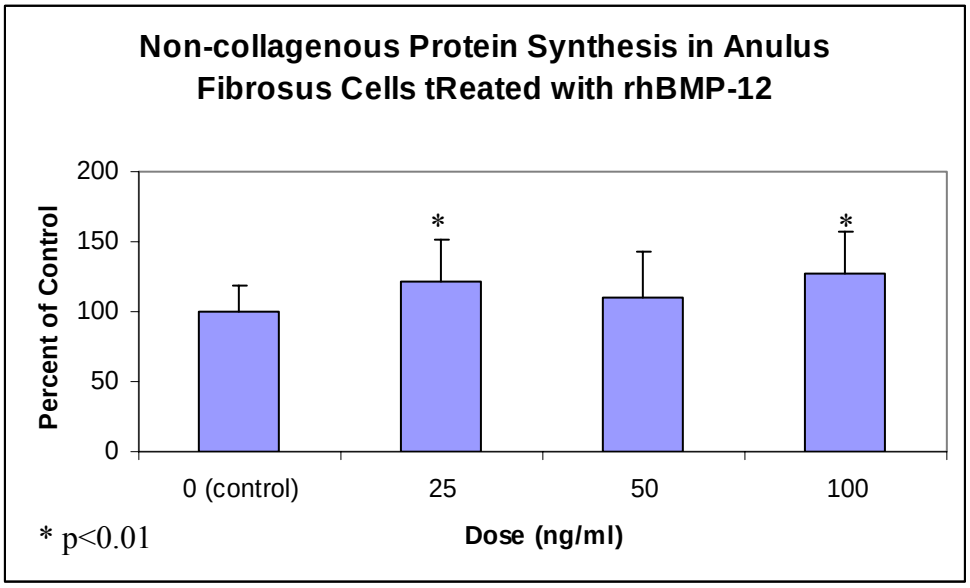


Figure 15 Non-collagen protein synthesis in AF treated with rhBMP-12

5.1.3 Effect of Ad/BMP-12 on AF and NP pellets

Newly synthesized PG, collagen, and non-collagenous protein in AF and NP pellets treated with Ad/BMP-12 were significantly increased when compared to controls before normalization by DNA content for all doses($p<0.01$). In the range of doses we investigated (50-150 MOI), we observed highest PG synthesis in AF pellets at 50 MOI. PG synthesis at 50 MOI is significantly higher than 100 and 150 MOI. For collagen and non-collagenous protein synthesis in AF pellets, there were no significant differences between different doses. In AF pellets treated with 50 MOI of Ad/BMP-12, the mean and standard deviation of PG, collagen and non-collagenous synthesis were 336±37%, 269±89%, and 222±9% of control respectively before normalization by DNA content.

For NP pellets treated with Ad/BMP-12, PG, collagen, and non-collagenous protein synthesis were not significant between different doses. In NP pellets treated with 50 MOI of Ad/BMP-12, the mean and standard deviation of PG, collagen and non-collagenous synthesis were 388±31%, 371±46%, and 282±36% of control respectively before normalization by DNA content.

For both AF and NP pellets treated with Ad/BMP-12, total DNA content significantly increased ($p<0.01$). Total DNA content of AF pellets treated with Ad/BMP-12 were not significantly different between different doses. For AF pellets treated with 100 MOI of Ad/BMP-12, the mean and standard deviation of DNA content was 208±16% of control. Total DNA content of NP pellets treated with Ad/BMP-12 were not significantly different between different doses. For NP pellets treated with 100 MOI of Ad/BMP-12, the mean and standard deviation of DNA content was 216±50% of control. After DNA normalization, PG, collagen, and non-collagenous protein synthesis in AF and NP pellets treated with Ad/BMP-12 (50,100,150 MOI) were significantly increased when compared to controls except for non-collagenous protein synthesis in AF pellets.

Results of AF pellets before and after DNA normalization are shown in Figures 16-21. Results of NP pellets before and after normalization are shown in Figure 22-26. Raw data for NP and AF pellets are included in the Appendix C and D respectively.

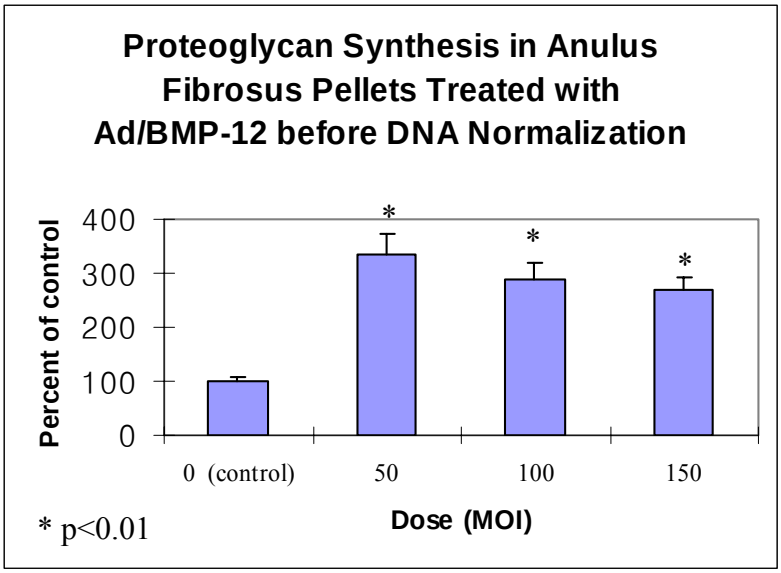


Figure 16 PG synthesis in AF treated with Ad/BMP-12 (before DNA normalization)

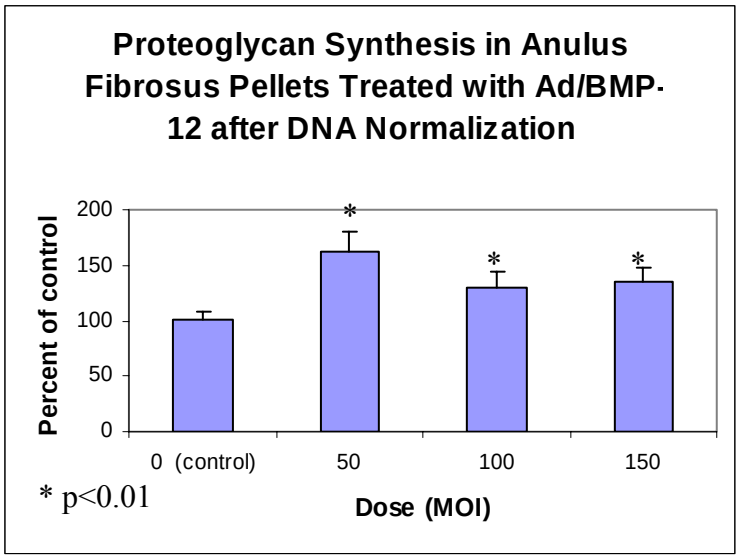


Figure 17 PG synthesis in AF treated with Ad/BMP-12 (after DNA normalization)

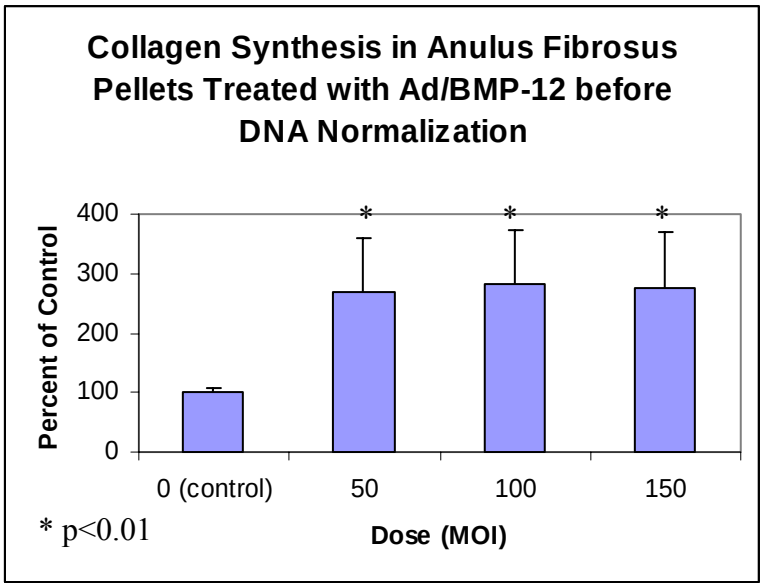


Figure 18 Collagen synthesis in AF (before DNA normalization)

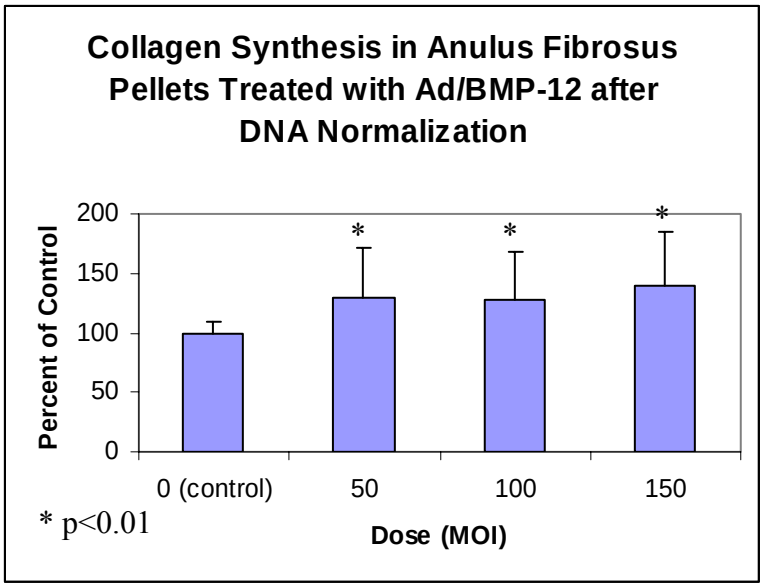


Figure 19 Collagen synthesis in AF (after DNA normalization)

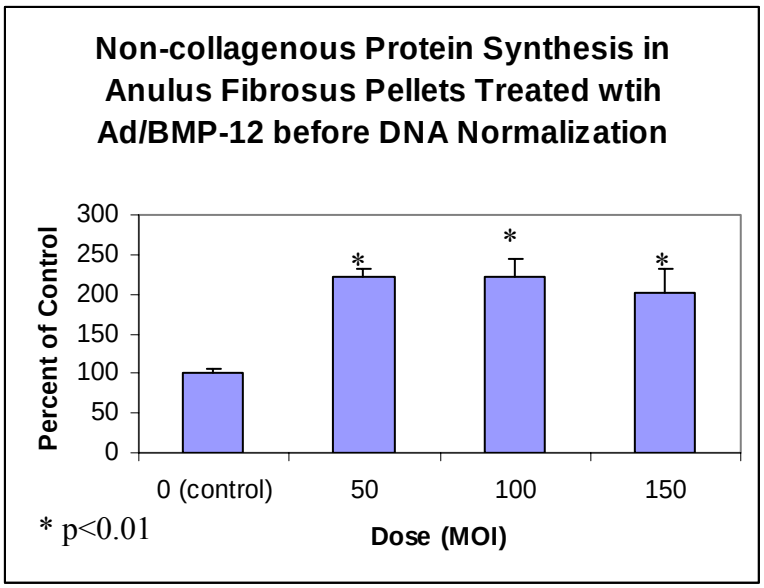


Figure 20 Non-collagenous protein synthesis in AF (before DNA normalization)

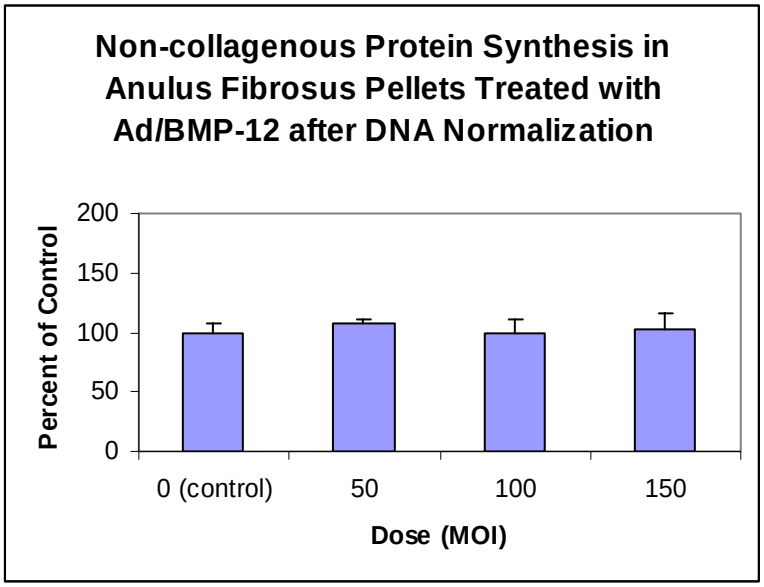


Figure 21 Non-collagenous protein synthesis in AF (after DNA normalization)

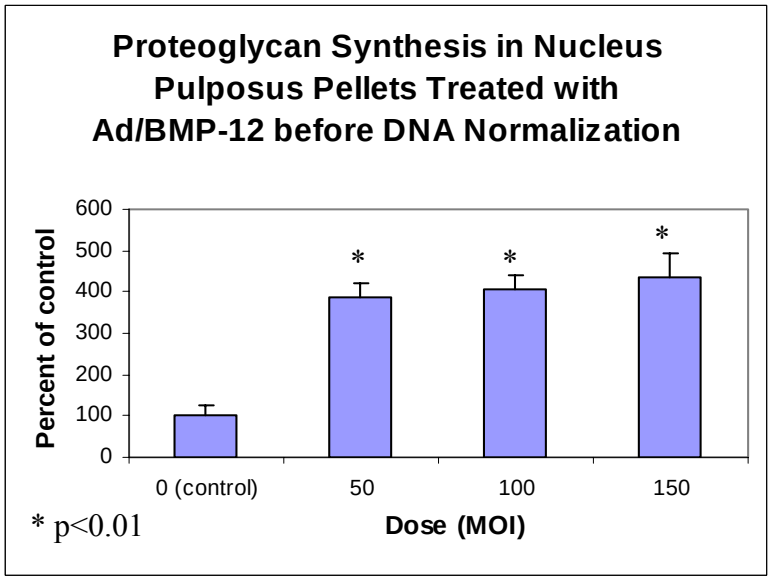


Figure 22 PG synthesis in NP treated with Ad/BMP-12 (before DNA normalization)

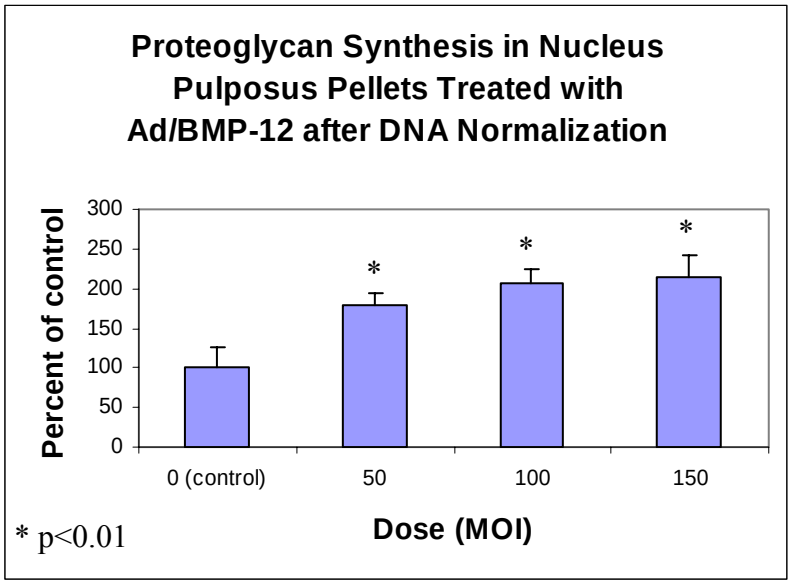


Figure 23 PG synthesis in NP treated with Ad/BMP-12 (after DNA normalization)

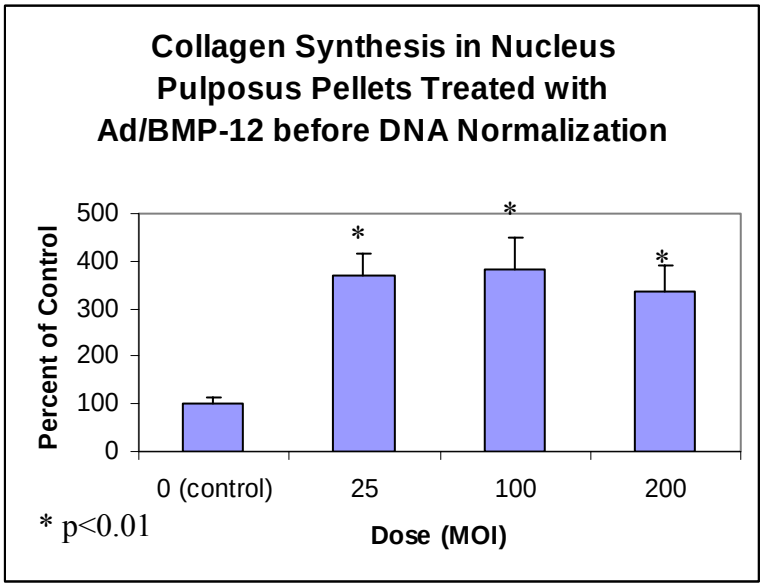


Figure 24 Collagen synthesis in NP treated with Ad/BMP-12 (before DNA normalization)

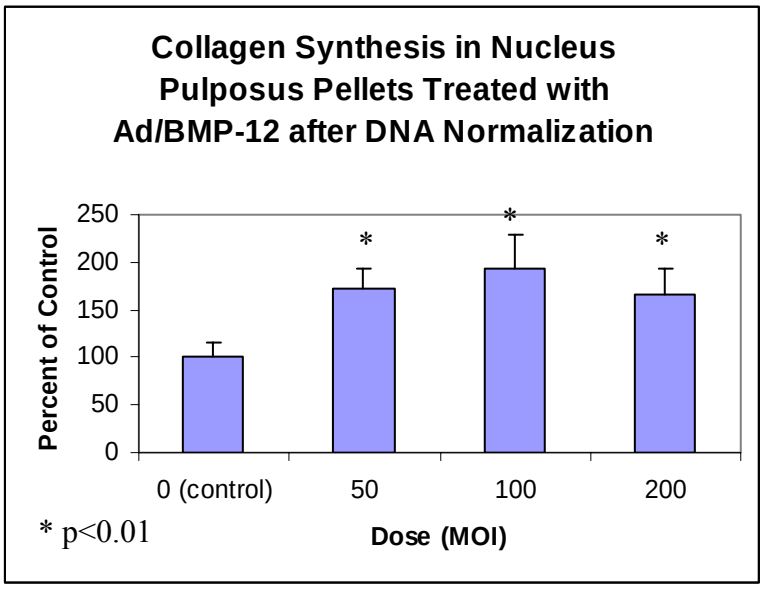


Figure 25 Collagen synthesis in NP treated with Ad/BMP-12 (after DNA normalization)

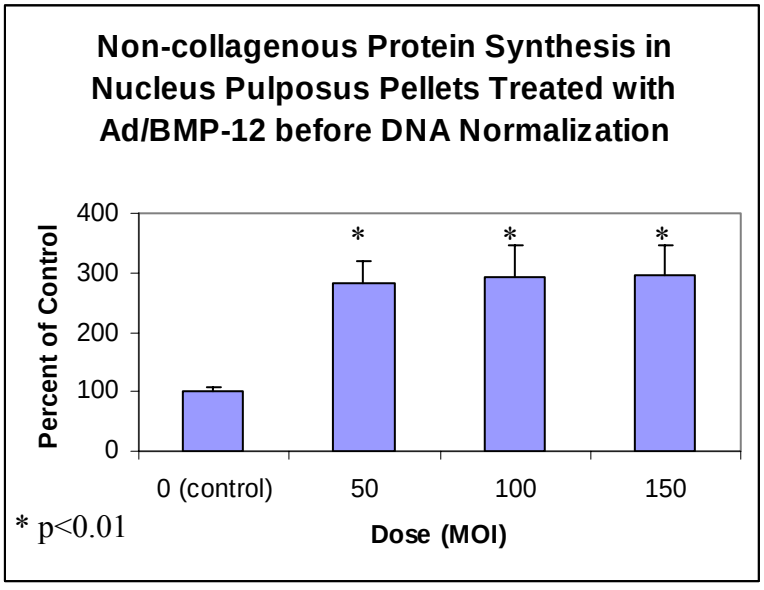


Figure 26 Non-collagenous protein synthesis in NP treated with Ad/BMP-12 (before DNA normalization)

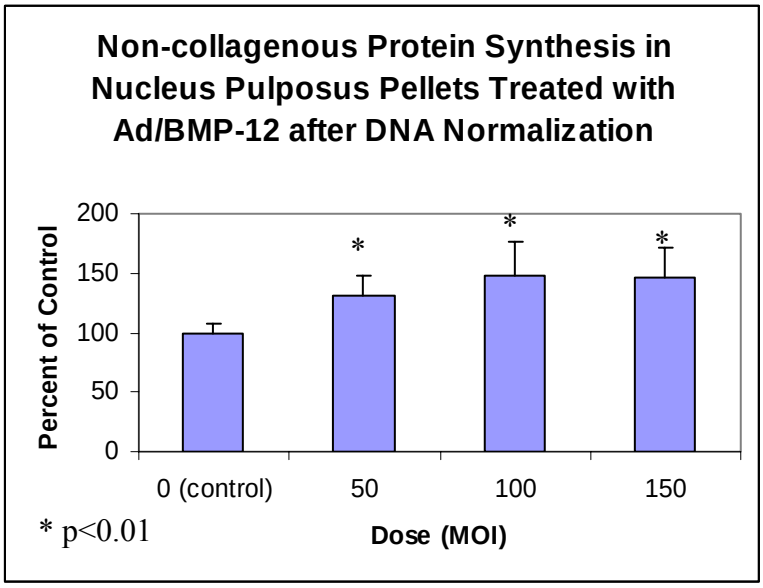


Figure 27 Non-collagenous protein synthesis in NP treated with Ad/BMP-12 (after DNA normalization)

Mean and standard deviation of matrix synthesis of AF and NP pellets treated with 50 MOI of Ad/BMP-12 before and after normalization by DNA content is described in Table 2. Matrix synthesis of AF and NP pellets treated with 50 MOI of Ad/BMP-12 were significantly higher than control except for non-collagenous protein synthesis in AF pellets after DNA normalization.

Table 2 Matrix synthesis of AF and NP pellets treated with Ad/BMP-12 before and after DNA normalization (shown in percent of control)

Matrix Synthesis	AF (before normalization)	AF (after normalization)	NP (before normalization)	NP (after normalization)
Collagen	269±89%	129±43%	371±46%	172±21%
Non-collagenous Protein	222±9%	107±5%	282±36%	131±17%
Proteoglycan	336±37%	161±19%	388±31%	180±14%

5.2 Part II: Disc Nutrition Study in Organ Culture

5.2.1 General Observations

The AF had layered lamellas and AF cells had flat cell shapes. AF matrix was organized and fibers of different layers were oriented at different angles giving the tissue a layered appearance. Cells near the periphery of the disc were very flattened and difficult to see (lower half of Figure 28A, B). Between AF and NP, cells in the transition zone were scattered singly or in small groups in the dense fibrous matrix (upper part of Figure 28A, B). The morphology of cells in AF and TZ region did not appear to be different between fresh and cultured discs. The fibrous matrix was loose in the NP region when compared to AF. (Figure 29A, B) The NP cells had round cell shapes. After 2 days of culture in serumless media, NP cells appeared to be separated by PG matrix into multiple cell islands. (Figure 29B)

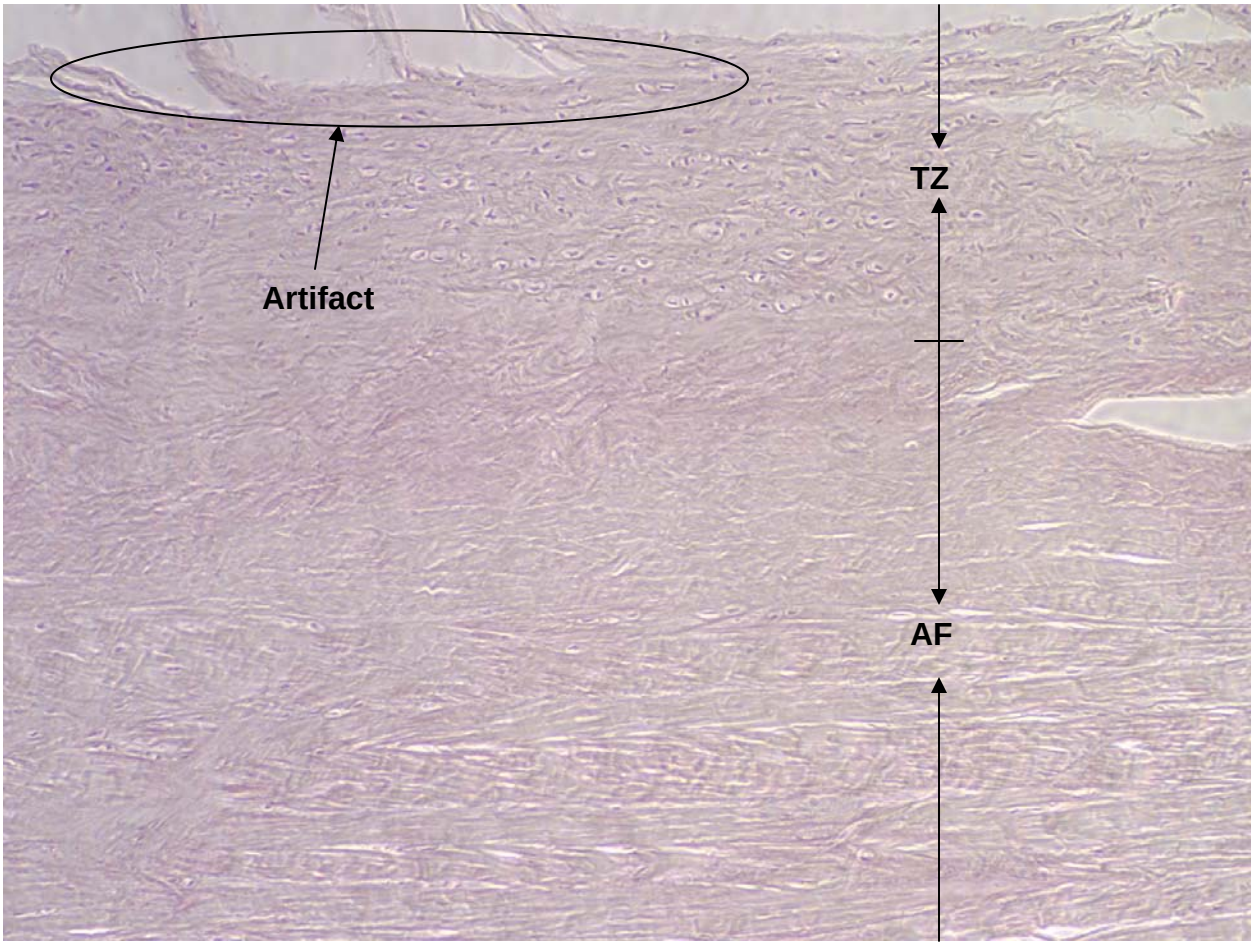


Figure 28A H&E staining of AF and TZ region of fresh disc (200x)

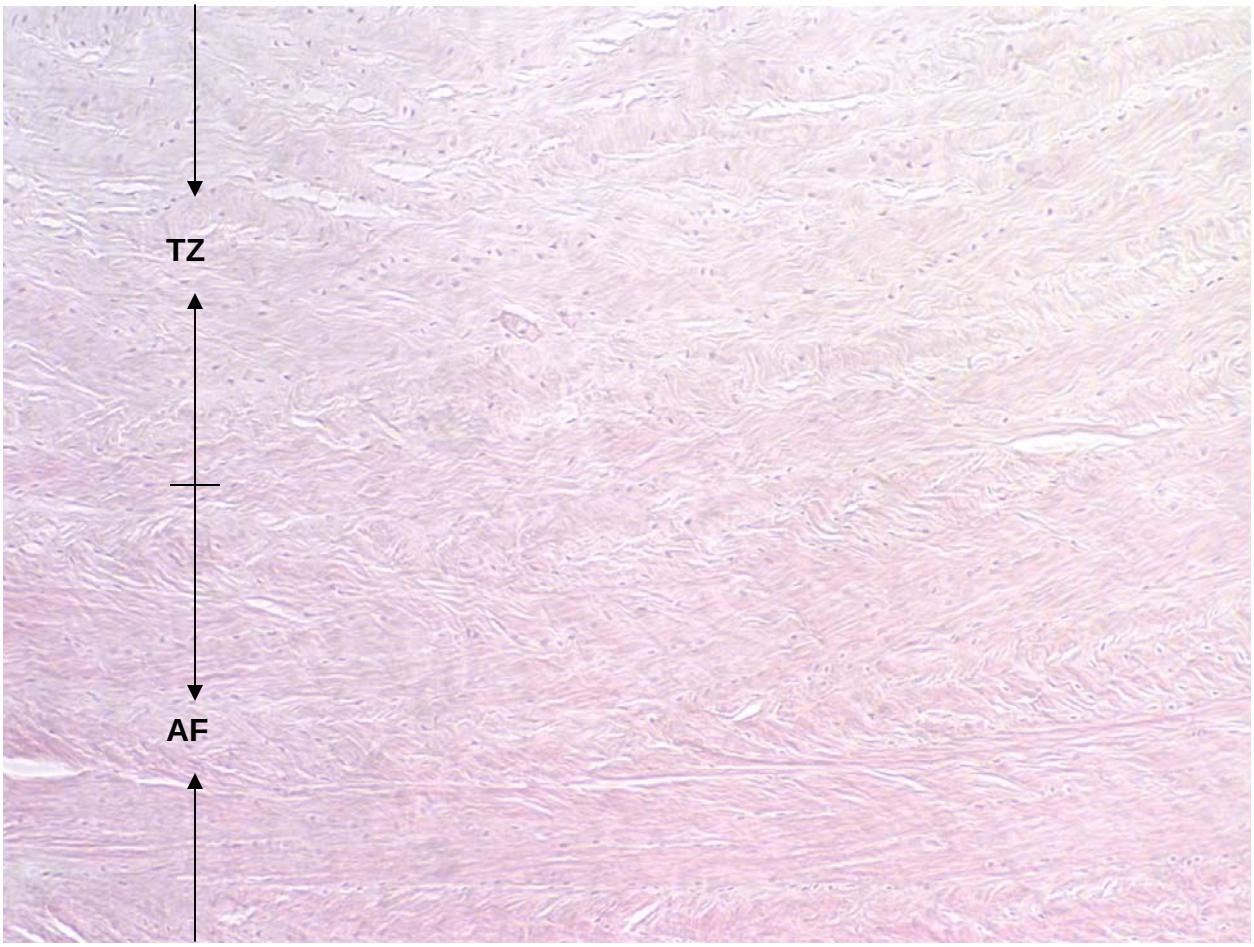


Figure 28B H&E staining of AF and TZ region in disc cultured for 2 days in serumless media (200x)

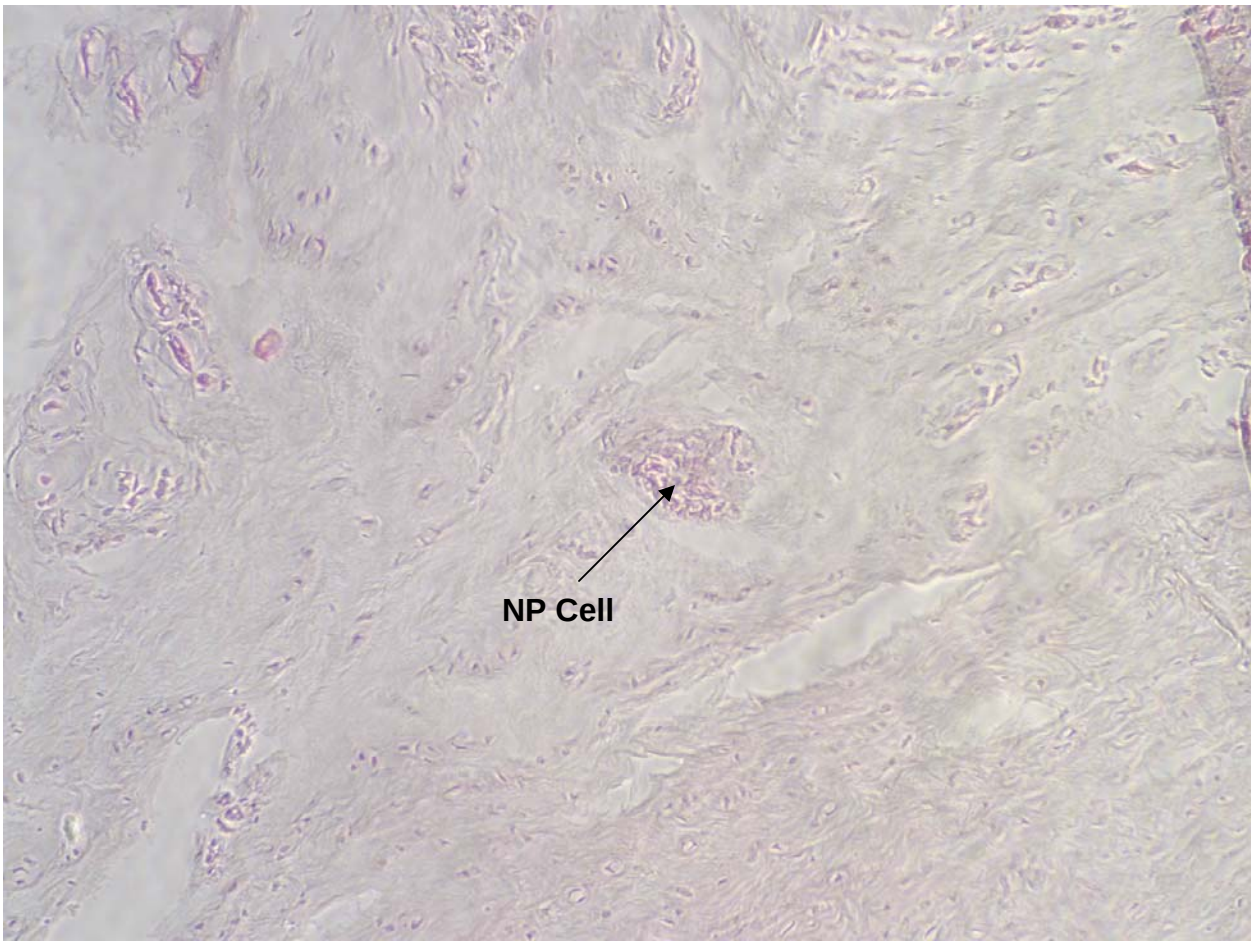


Figure 29A H&E staining of NP region in fresh disc (200x)

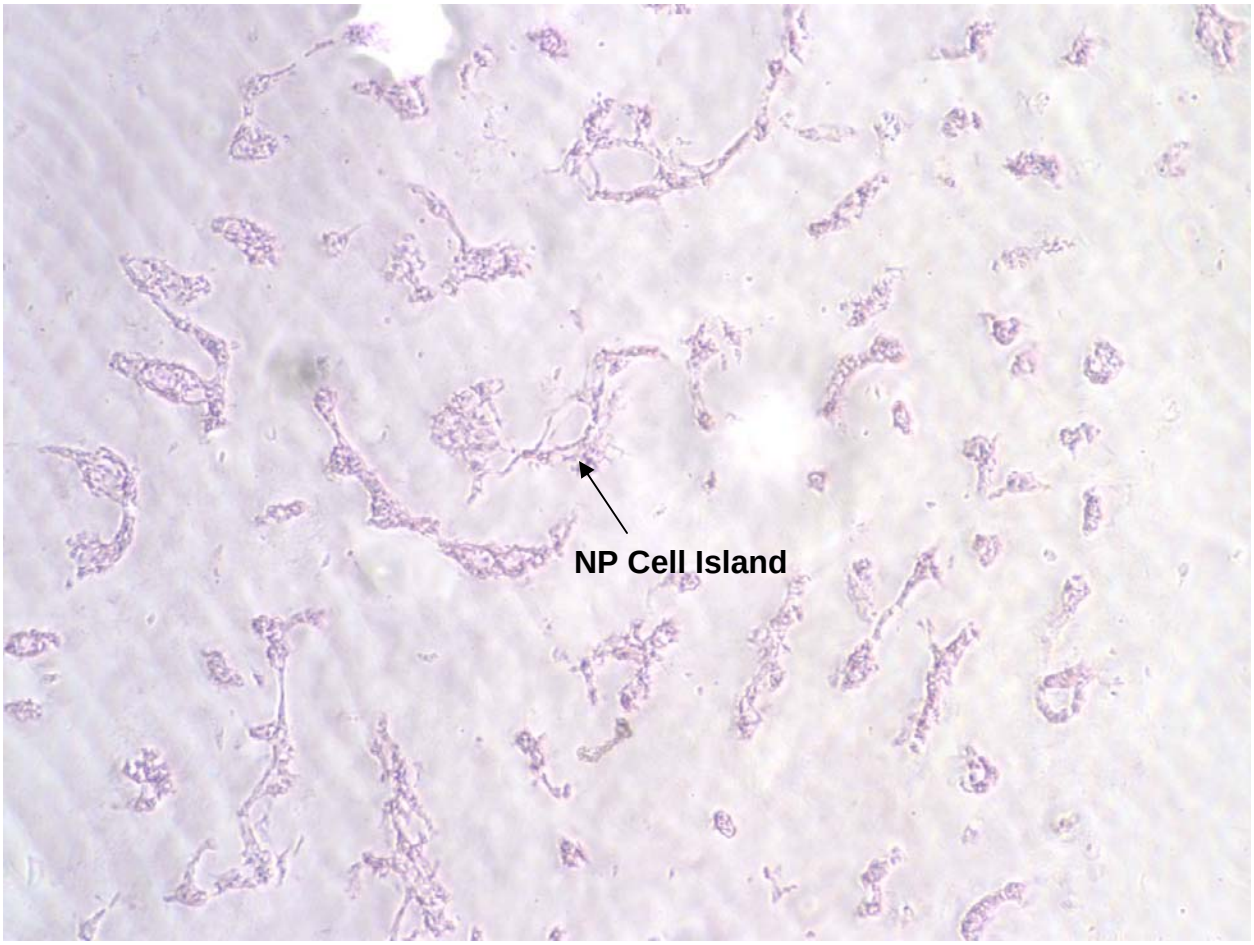
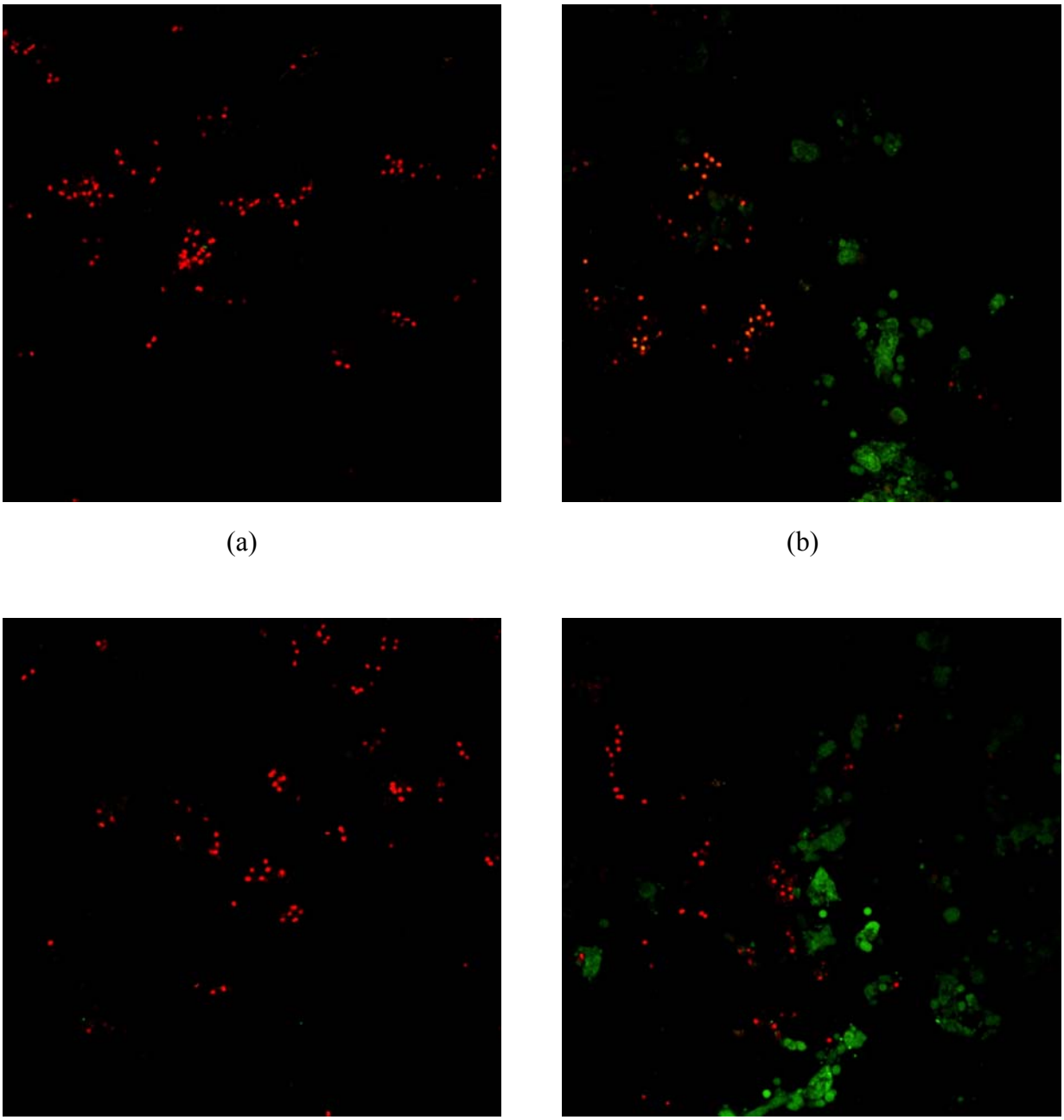


Figure 29B H&E staining of NP region in disc cultured for 2 days in serumless media (200x)

In the NP cultured after 2 dyas in serumless media, approximately 75% of the cells were nonviable (Figure 30). The red staining shows nonviable cells while the green staining represents viable cells. The 2 photon microscopy showed clear images of live and dead cells in the NP at 4 random locations (a,b,c,d). We observed both live and dead cells in the AF and TZ regions after one week of culture in serumless, 5%, 10%, and 15% FBS media. We were not able to see the difference in cell viability from different culture condition by this staining method. Also, the staining method was not sensitive enough to test whether cell viability of AF and TZ from 1,2,3,4 regions of the disc were different.



(a) (b)

(c) (d)

Figure 30 Cell viability staining in 4 random locations (a,b,c,d) of NP in 2 day culture in serumless media (2 photon microscopy)

5.2.2 Outcome Measures

5.2.2.1 Disc Metabolism

- **NP Composition**

The mean and standard deviation of wet weight and dry weight of NP were 25±5 mg and 4±1 mg respectively for fresh discs. Wet weight and dry weight of NP of fresh discs and discs cultured for 2 weeks were not significantly different. The mean and standard deviation of % hydration of the NP was 85%±2% for fresh discs. % hydration of NP of fresh discs and discs cultured for 2 weeks were not significantly different. Other values of the outcome measures are listed in Table 3. Results of cultured discs showed no significant changes in the GAG and DNA content in NP for different concentration of FBS (Figure 31 and 32). DNA content of discs in the 15% FBS group was significantly higher than other % FBS groups. Lactate content increased significantly with increasing % FBS ($p<0.05$). The mean and standard deviation of GAG content of the fresh discs was 621±51 μg/mg dry weight, which was not significantly different than the cultured discs. The DNA and lactate content of the 15% FBS group were closest to the fresh disc group when compared to other % FBS groups (Figure 32 and 33). GAG, DNA, and lactate data did not correlate with each other. Raw data is included in Appendix E.

Table 3 Mean and standard deviation of outcome measures of fresh and cultured (2 weeks) NP

NP	Wet wt	Dry wt	% Hydration	GAG	DNA	Lactate
	(mg)	(mg)		(ug/mg)	(ng/mg)	(mg/mg)
Fresh	25±5.0	4±1.0	85±2.0	621± 51	1691±562	3.4±2.8
Serumless	31±1.0	5±0.3	84±0.5	616± 75	1367±105	1.1±0.2
5% FBS	27±1.0	5±1.0	82±3.0	526± 64	1466±157	1.4±1.8
10% FBS	32±3.0	6±0.5	82±3.0	581±110	1260±224	3.1±1.6
15% FBS	25±1.0	4.5±0.5	83±2.0	492± 52	1761±164	3.1±3.6

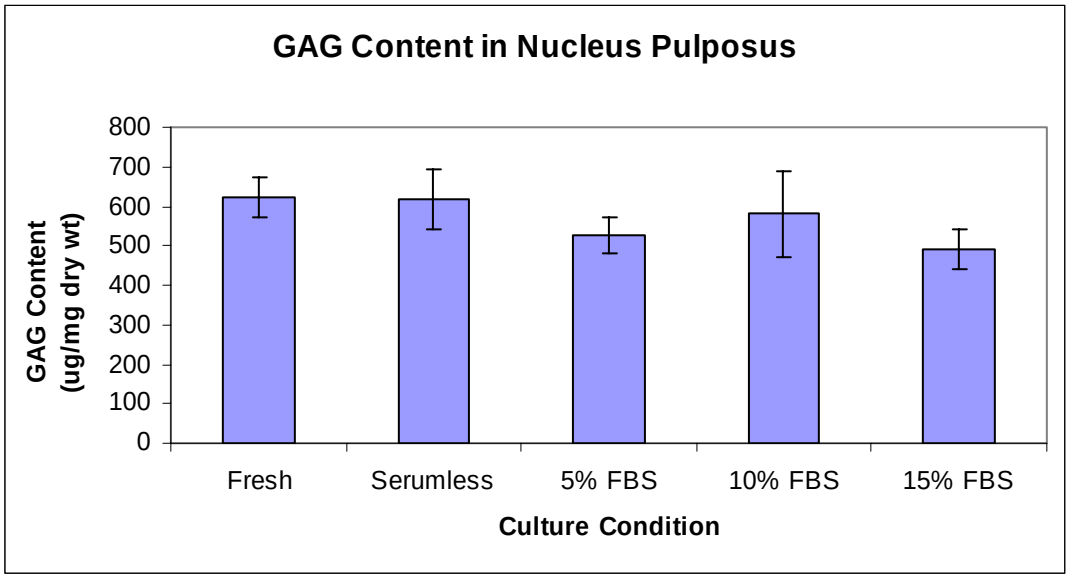


Figure 31 GAG content of NP in fresh and cultured (2 weeks) discs

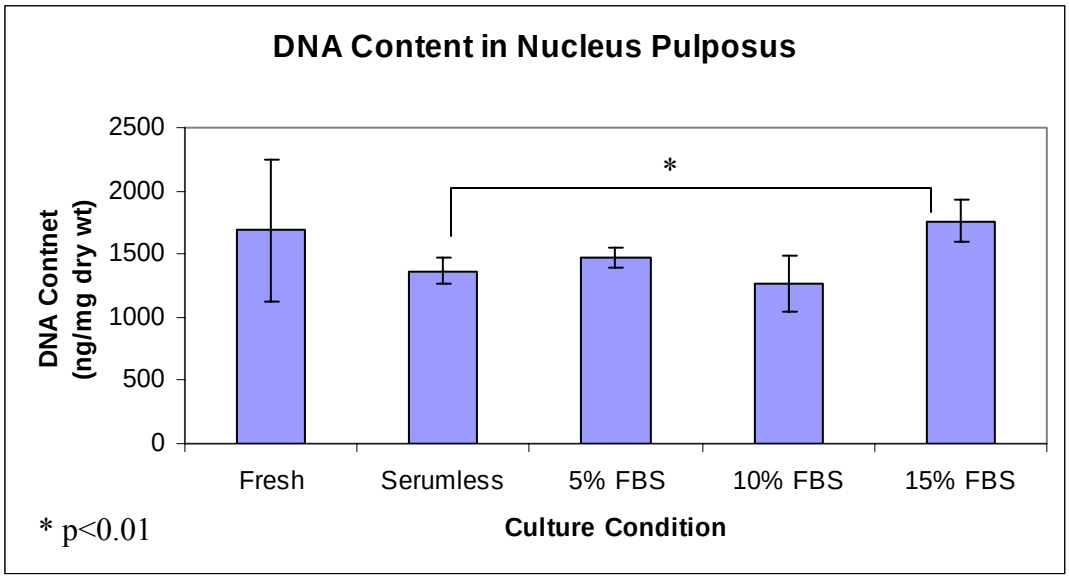


Figure 32 DNA content of NP in fresh and cultured (2 weeks) discs

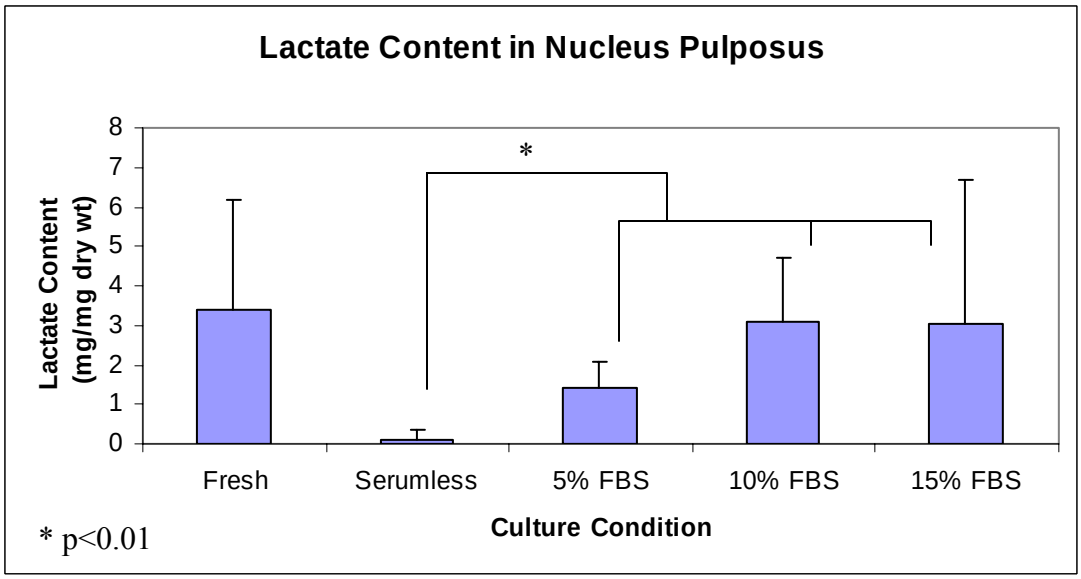


Figure 33 Lactate content in NP of fresh and cultured discs

- **AF Composition**

The mean and standard deviation of wet weight and dry weight of AF were 88±24 mg and 27±8 mg respectively for fresh discs. Wet weight and dry weight of AF of fresh discs and discs cultured for 2 weeks were not significantly different. The mean and standard deviation of % Hydration of the AF was 70±8% for fresh discs. % hydration of NP of fresh discs and discs cultured for 2 weeks were not significantly different. Other values of the outcome measures are listed in Table 4. For AF after culture, the GAG content showed no significant difference between different % FBS group (Figure 34). For both DNA and lactate content, there were significant increases with increasing % FBS (Figure 35, 36). The mean and standard deviation of GAG content of the fresh discs was 227±90 ug/mg dry weight and the value is not significantly different than cultured discs. The average DNA and lactate content of the cultured discs were higher than those in the fresh disc. DNA and lactate data correlated with each other with Pearson correlation coefficient of 0.631. ($p<0.01$) There were no correlation observed between GAG and DNA or GAG and lactate. Raw data is included in Appendix E.

Table 4 Mean and standard deviation of outcome measures of fresh and cultured AF

AF	Wet wt (mg)	Dry wt (mg)	% Hydration	GAG (ug/mg)	DNA (ng/mg)	Lactate (mg/mg)
Fresh	88±24.0	27±8.0	70±3.0	227±90	218±49	1.6±0.8
Serumless	99±3.0	30±2.0	70±2.0	200±30	443±121	1.1± 0.2
5% FBS	78±14.0	24±5.0	69±1.0	204±45	791±80	2.8± 0.7
10% FBS	81±16.0	26±6.0	70±1.0	255±31	776±105	3.4± 1.0
15% FBS	84±21.0	26±9.0	70±2.0	208±22	818±81	3.3± 1.5

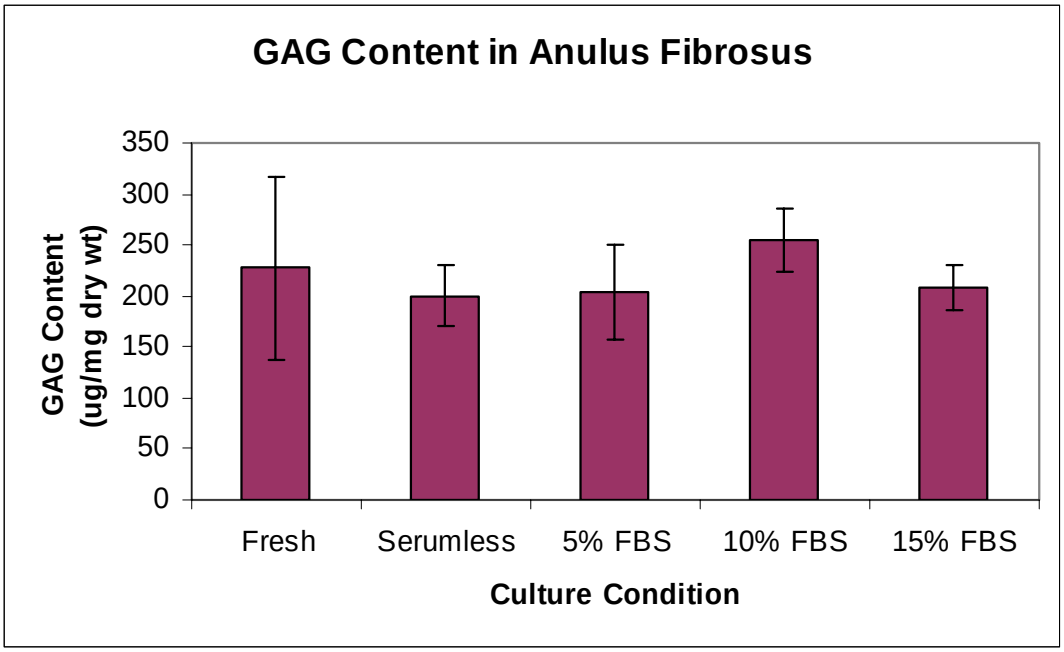


Figure 34 GAG content in AF of fresh and cultured discs

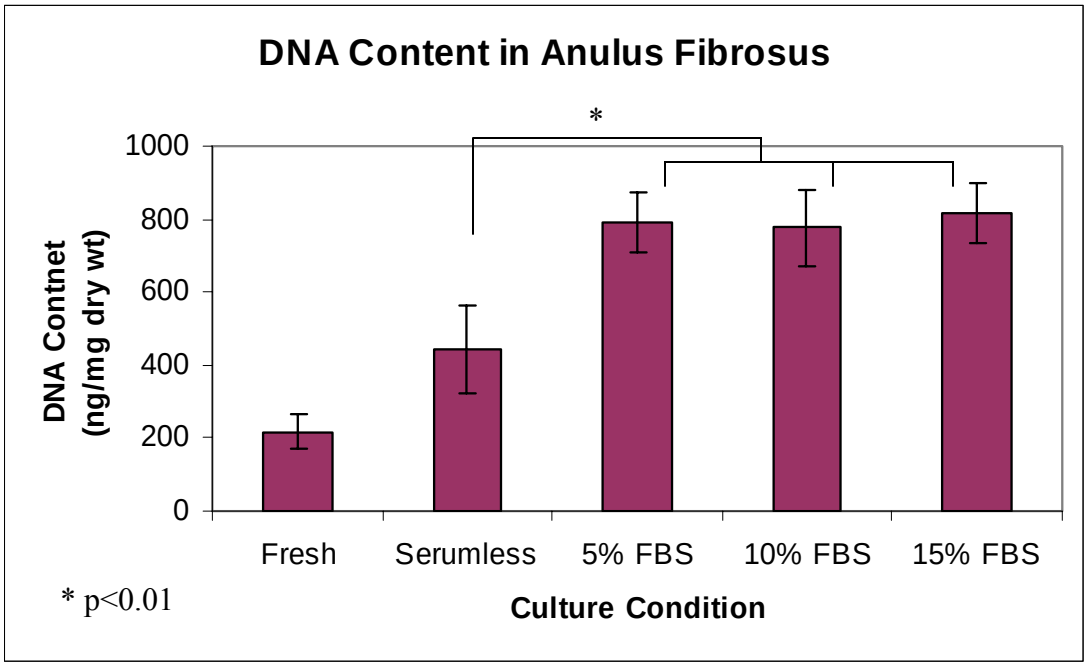


Figure 35 DNA content in AF of fresh and cultured discs

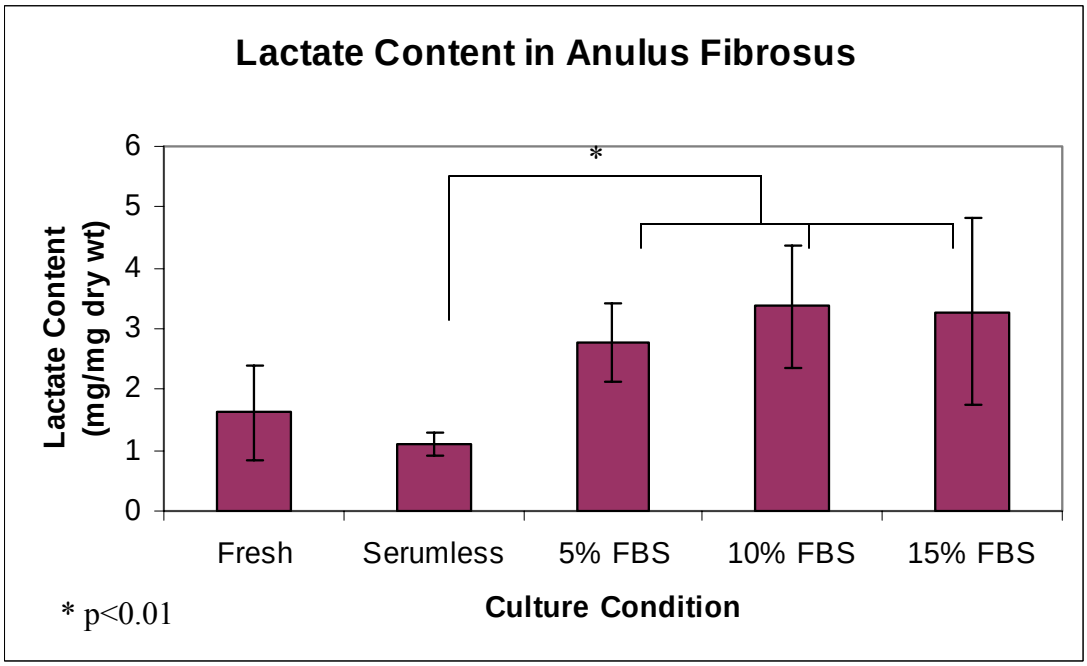


Figure 36 Lactate content of AF in fresh and cultured discs

- **Data from Culture Media**

Media were collected and GAG and lactate leached out of the disc were measured (Table 5). Total GAG in the media ranged 88 to 144 μg/mg, which was about 14% of the total GAG produced (tissue + media). Total lactate in the media ranged 1.2 to 2.2 mg/mg, which was around 27% of the total Lactate produced (tissue + media). Figure 37 and 38 illustrates the distribution of GAG and lactate in the media and tissue.

Table 5 GAG and lactate in the media and tissue

GAG (ug/mg)	Tissue+Media	Total Media
serumless	903	88
5% FBS	863	132
10% FBS	963	137
15% FBS	844	144

Lactate (μg/mg)	Tissue+Media	Total Media
serumless	2.4	1.2
5% FBS	6.2	2.0
10% FBS	8.5	1.8
15% FBS	8.6	2.2

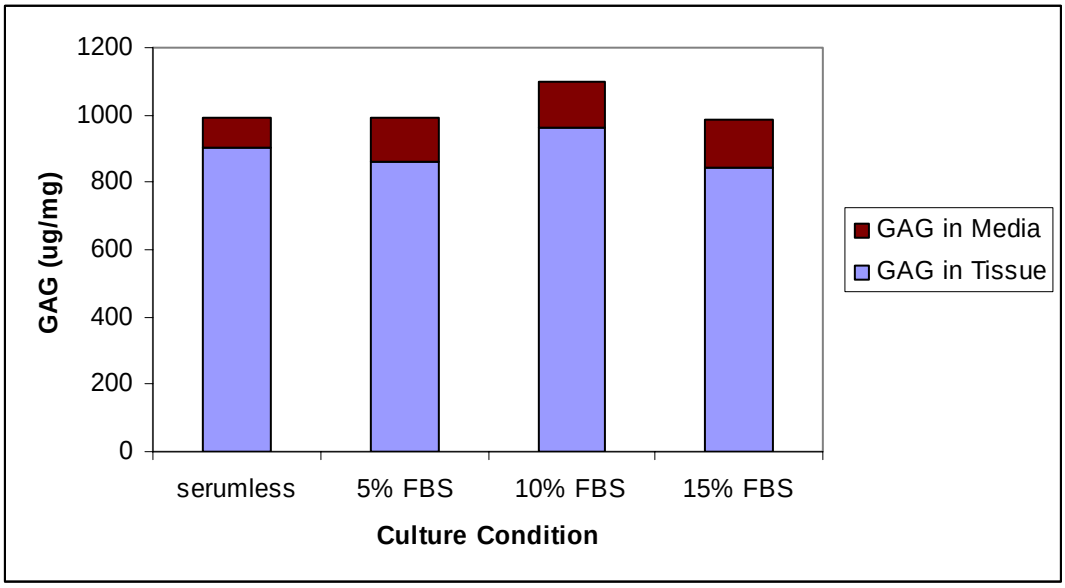


Figure 37 GAG in the culture media and tissue

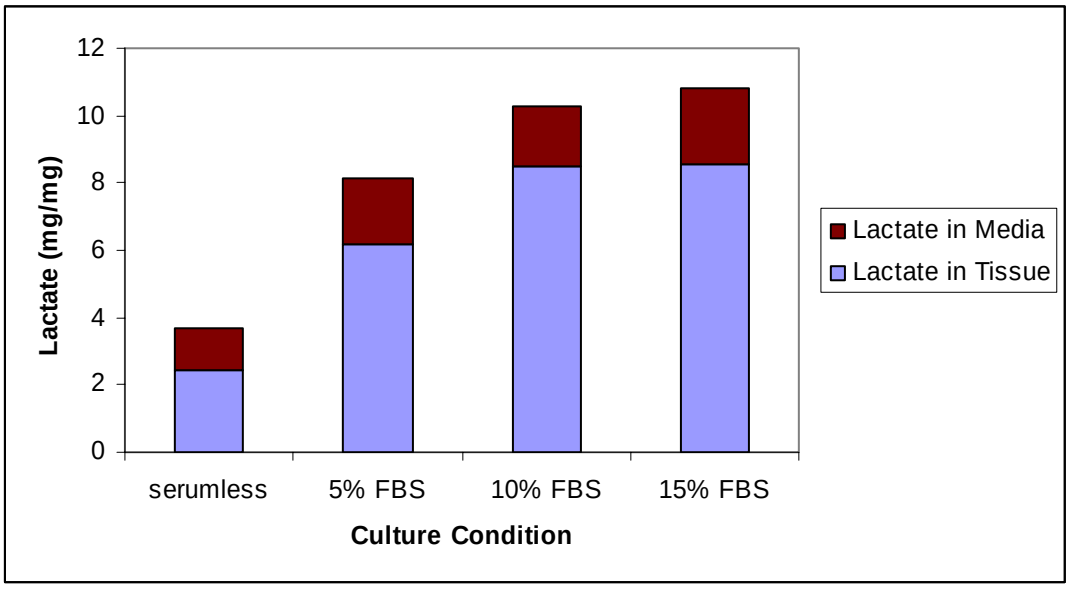


Figure 38 Lactate in the culture media and tissue

5.2.2.2 BMP-2 Measurement

Baseline data of BMP-2 in fresh discs were obtained from digested tissue using ELISA. 5 discs from one rabbit were used. The mean and standard deviation of BMP-2 in NP and AF were 52±20 and 44±12 pg/mg wet weight respectively.

5.2.2.3 Marker Gene Transfer

- **Ad/lacZ**

Fifteen discs from 3 rabbits were harvested and cultured for 2 days in serumless media. Then they were injected with Ad/lacZ. Discs were cultured for 1 more week in media containing different % FBS (serumless, 5%, 10%, 10% FBS). No blue staining was observed after X-gal staining in any of the group (Figure 39).

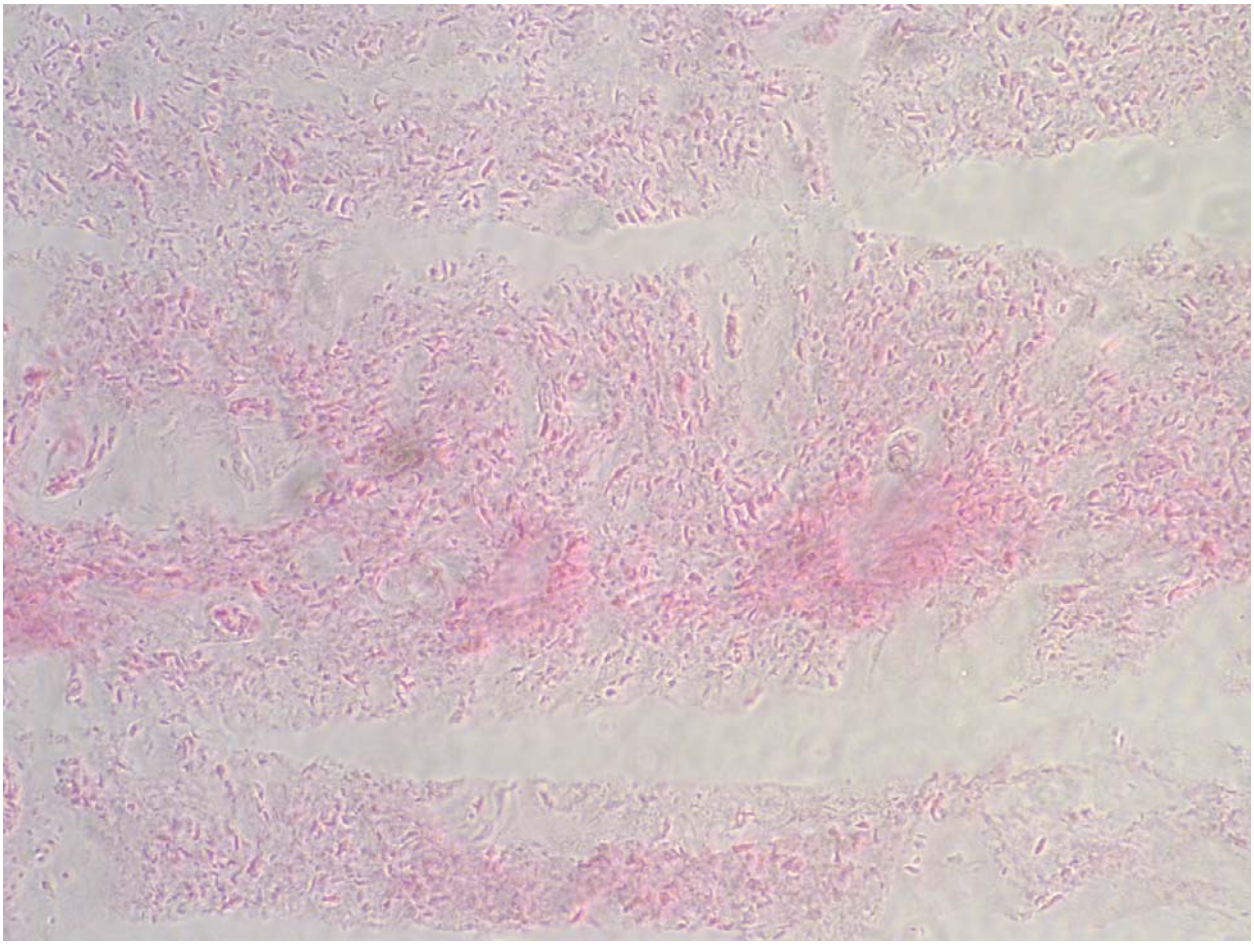


Figure 39 Negative lacZ result after X-gal staining of NP (200x)

- **Ad/Luciferase**

We observed negative from discs injected with Ad/Luciferase. The luciferase activity in Ad/Luciferase group was under 1000 RLU which showed no successful gene transfer (Table 6).

Table 6 Luciferase activity in three experimental groups: positive control, saline control, and Ad/Luciferase

Positive Control (3ng of Luciferace)	Saline Control	Ad/Luciferase
1288890 RLU/s	17.3 RLU/s	87.7 RLU/s

6.0 DISCUSSION

6.1 Comparison with Published Data

6.1.1 Part I: Growth Factor and Gene Therapy Study in Cell Culture

Our results of rhBMP-2 increases matrix production agrees with study by Yoon T.S, Kim S.K., and Li J. on "The effect of bone morphogenietic protein-2 on rat intervertebral disc cells in-vitro" (2003). However, AF and TZ of rat disc were used in Yoon's study while we used human NP cells. In both studies, a dose dependent response was observed. No published data were found on the effect of rhBMP-12 or Ad/BMP-12 on disc cells.

6.1.2 Part II: Disc Nutrition Study in Organ Culture

Compare to Kazuhiro Chiba's study[74] with adolescent rabbit discs with endplates, our results of dry weight and DNA of AF had similar average values. Their NP dry weight was lower compared to our result. This could be due to different culture condition and difference in rabbit age. Chiba reported mean and standard deviation of dry weight of NP and AF to be 1.5±0.5 and 20±5 mg/disc after two weeks of culture. In Chiba's study, they showed DNA of NP and AF to

be 4±2 and 12.5±2.5 μg/disc after one week of culture. Chiba saw a decrease in NP dry weight after two weeks of cultured when compared to that of discs cultured for one week. In our study, we measured dry weight of NP in fresh discs and did not see a significant decrease after two weeks of culture. Chiba did not report data of fresh discs therefore we can not compare their results with ours.

In our study we measured the GAG leached out of from the disc to the media. The amount of GAG leached out during the two weeks culture was a small fraction when compared to GAG in the disc. In Thompson's study with canine disc tissue[26], they reported disc swelling and loss of tissue proteoglycan after three or four days of culture. In our model, the bone-disc-bone structure of whole disc culture reduced disc swelling and GAG leaching problem.

6.2 New Findings

6.2.1 Part I: Growth Factor and Gene Therapy Study in Cell Culture

In monolayer culture, rhBMP-2 appeared to be more effective in upregulating matrix synthesis than rhBMP-12. Optimal doses for rhBMP-2 and rhBMP-12 were 200ng/ml and 25ng/ml respectively.

Ad/BMP-12 upregulated matrix synthesis in both NP and AF pellets. Table 7 shows the distribution of increase in matrix synthesis due to increase in cell number and matrix produced per cell. For AF, collagen and non-collagenous protein synthesis was increased largely due to cell proliferation. Increased proteoglycan synthesis was due to both increase in cell number and

cell production with equal importance. For NP, proteoglycan and collagen synthesis was upregulated more as a result of increased matrix produced per cell. Increase of non-collagenous protein synthesis in NP pellets was attributed equally to cell proliferation and increase in cell production.

Table 7 Distribution of percent increase of matrix synthesis of AF and NP

Cell Type	Matrix Synthesis	% of Increase Attributed to Increase in Cell Number	% of Increase Attributed to Increase in Matrix Production Per Cell
AF	Proteoglycan	48%	52%
	Collagen	67%	33%
	Non-Collagenous Protein	88%	12%
NP	Proteoglycan	31%	69%
	Collagen	34%	65%
	Non-Collagenous Protein	53%	47%

6.2.2 Part II: Disc Nutrition Study in Organ Culture

The effect of varying FBS concentration was demonstrated. No effect on GAG and % hydration was observed in the organ culture for NP and AF. Higher DNA and lactate content was observed in higher % FBS for NP and AF. In AF, DNA and lactate content data correlate with each other. The correlation between AF DNA and lactate means that the cell metabolism depends on amount

of cells. When %FBS increased, we observed an increase in DNA as well as an increase in lactate. When there is more DNA, there are more cells. Therefore more glucose is needed and they are utilized and producing more lactate. Originally, half of the cultured discs was injected with Ad/BMP-2 ($6x10^6$ PFU) one week days after the culture, and then it was cultured for another week. The other half was injected with 10 μl of saline as control. Appendix E showed disc levels injected with saline or Ad/BMP-2. However, no significant result was observed between the two groups. Therefore, we combined the data.

6.2.3 Energy Cost of Gene Therapy: A Theoretical Analysis

In Part I of the study on gene therapy, we observed matrix synthesis of disc cells increased after treated with Ad/BMP-12. Pellets were cultured in serumless media, which were minimal nutrients for cell survival. We did not observe any negative effects of gene transfer on disc cells such as cell death. Total DNA content of pellets increased after treated with Ad/BMP-12, and matrix synthesis per cell also increased. If the energy cost of gene transfer was high, then cells should not have sufficient energy to survive or even proliferate. Therefore, Part I of the study showed that successful gene transfer did not affect cell viability, implying that the energy cost of gene transfer is low compared to energy cost of basic cell functions.

In Part II of the study on organ culture, we could not transduce the disc cells due to low cell viability. In this case, the cell death could be due to lack of nutrients in the NP. The cell death in NP was not caused by gene transfer. If we have sufficient nutrients in the disc for cells to survive, then we should be able to transduce the cells.

6.3 Limitations

6.3.1 Part I: Growth Factor and Gene Therapy Study in Cell Culture

The rhBMP-2, rhBMP-12, and Ad/BMP-12 study was done in an in-vitro environment. We do not know if we can achieve the same result in the in-vivo condition. The optimal dose found in the in-vitro setting will be different than that in the in-vivo condition. Therefore, the next step should be to investigate the effect of Ad/BMP-12 in an animal model. In the growth factor study, we only tested rhBMP-2 up to 300 ng/ml and rhBMP-12 to 100 ng/ml. It was not practical to test too many different concentrations. Therefore, we cannot conclude what the optimal dose of rhBMP-2 and rhBMP-12 is. To do so, a wider range of doses should be studied. We might see higher matrix synthesis at higher doses or toxic effects when doses are too high. Another limitation of the study is that we assumed all well plates of cells or pellets responded similarly to the stimuli. Different well plates of cells or different pellets were used for various assays such as cell count, DNA, PG, collagen, and non-collagenous protein synthesis. The best and most accurate way to normalize our data is to use the same well plate or pellet for measurement of matrix synthesis and cell count or DNA content. However, it was not possible to conduct different assays on the same sample.

6.3.2 Part II: Disc Nutrition Study in Organ Culture

In the organ culture study, healthy rabbit discs were chosen for the animal model. The results will be different from the degenerated human discs. Also, the study is in-vitro, therefore there is no fluid flow in the disc culture from movement as in the in-vivo condition.

In the statistical analysis, the assumption of that discs from different rabbit was the same was made due to small sample size. We did not have sufficient number of rabbit per experiment group to test whether there was an inter-rabbit effect. However, we did investigate and found that intra-rabbit effect was very small on disc weight, % hydration, DNA, and lactate when compared to the effect of different % FBS. Therefore we can neglect differences between discs from different levels.

The main limitation of the current culturing method is the difficulty in maintaining the cell viability in the NP and the inability to transduce cells in the culture. In order to study the effect of gene transfer on disc metabolism, successful gene transfer needs to be achieved in the organ culture. Other culture method such as mono-layer or pellet culture can be considered to investigate the effect of gene transfer on cell metabolism.

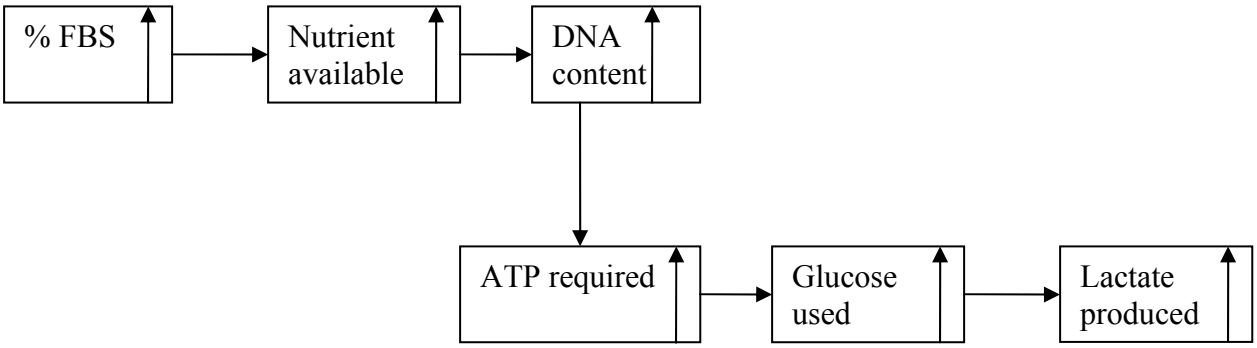
6.4 Conclusions

6.4.1 Part I: Growth Factor and Gene Therapy Study in Cell Culture

RhBMP-2 and rhBMP-12 both upregulated proteoglycan, collagen, and non-collagenous protein synthesis by NP cells in monolayer culture, although rhBMP-2 demonstrated a more substantial anabolic effect on matrix production. We observed a dose dependent response to rhBMP-2 in NP cells. RhBMP-12 demonstrated the potential for matrix synthesis in NP cells. RhBMP-12 upregulated PG, collagen and non-collagenous protein synthesis in NP cells. In AF cells, only non-collagenous protein synthesis was increased after treating with rhBMP-12. Ad/BMP-12 increased AF cell proliferation and matrix synthesis per cell, which resulted in an overall increase in total matrix production. PG, collagen, and non-collagenous protein synthesis increased in AF pellets treated with Ad/BMP-12. The increased collagen synthesis may help maintain the structural integrity and mechanical strength of the disc. We observed increase in cell number in pellets treated with Ad/BMP-12. It is an exiting possibility to combine Ad/BMP-12 with adeno-virus carrying other growth factor genes to treat IDD.

6.4.2 Part II: Disc Nutrition Study in Organ Culture

In the organ culture, % FBS in the medium did not significantly affect % hydration and GAG content of NP or AF during the two weeks duration of the study. In NP, DNA content was higher in the 15% FBS group and the lactate content increased with increasing % FBS. In AF, DNA and lactate both increased with increasing % FBS. There is a positive correlation between the DNA and lactate in AF. The flow chart below shows possible relations between DNA and lactate content with increasing % FBS:



From the result of administering Ad/lacZ and Ad/Luciferase, we did not achieve successful gene therapy in the organ culture possibly due to low cell viability.

6.5 Future Directions

6.5.1 Part I: Growth Factor and Gene Therapy Study in Cell Culture

There is an urgent need for less-invasive therapies for IDD that aim to treat or prevent the disease. In order to provide more treatment options for patients and to advance our knowledge in medicine, there is a need for basic science research. In this study, we have demonstrated the potential of Ad/BMP-12 to upregulate matrix synthesis of cells from degenerated discs in an in-vitro setting. The next step will be to study the effects of Ad/BMP12 in an in-vivo setting in an animal model of IDD.

6.5.2 Part II: Disc Nutrition Study in Organ Culture

Further studies should focus on attempts to achieve successful gene therapy in the organ culture as well as to study the effect of gene therapy on disc nutrition in other in-vitro culture such as monolayer or pellet culture. One possible reason for unsuccessful gene transfer was low cell viability. To address this issue, different culture method, culture medium, and time of injection needs to be reconsidered. Also, applying mechanical loads to discs to mimic in-vivo condition may improve disc nutrition in the organ culture.

An alternative to using organ culture to study effect of gene therapy is the use of other culture method such as mono-layer or pellet culture. Future research on finding the lowest

concentration of glucose to increase matrix synthesis by gene transfer can be helpful in understanding the energy demand of gene transfer. Another area of interest is to investigate the oxygen consumption of cells with and without gene transfer. This study will allow us to determine if the oxygen demand increases when cells are producing more matrixes after gene transfer. Due to the great cost and suffering of back pain, extensive research is needed to further improve our understanding in effects of potential treatments such as gene therapy in order to successfully treat or prevent the IDD and improve the quality of life.

APPENDICES

Appendix A Part I: Growth Factor Study in NP Cell Culture

Table 8 Effect of rhBMP-2 on PG in NP monolayer

Proteoglycan Synthesis in NP						
Group	control	BMP-2 25	BMP-2 50	BMP-2 100	BMP-2 200	BMP-2 300
Sample	104	116	103	131	584	402
	95	105	110	150	446	555
	100	132	91	115	549	526
	113	174	185	270	614	707
	102	201	140	302	503	691
	85	169	183	277	586	577
	98	240	215	260	515	550
	102	207	324	254	630	646
	121	347	178	439	204	260
	99	277	242	340	192	251
	79	280	236	349	190	253
	91	201	227	474	200	237
	86	171	286	402	206	240
	123	198	174	417	192	244
	94	166	151	450	241	245
	104	143	139	316	171	182
	106	146	154	404	204	260
	96	145	128	395	194	267
	92	131	159	484	202	197
	96	138	125	391	216	236
	107	160	134	409	468	468
	94	144	160	494	505	505
	93	138	139	157		
	94	129	301	175		
	102	425	232	140		
	124	275	236	182		
	104	395		169		
	93			163		
	99			172		
	103			147		
	98			148		
				141		
				468		
				505		

Table 8 (continued)

	control	BMP-2 25	BMP-2 50	BMP-2 100	BMP-2 200	BMP-2 300
Mean	100	198	183	297	355	386
S.D.	10	84	62	132	178	178

Table 9 Effect of rhBMP-12 on PG synthesis in NP monolayer

Proteoglycan Synthesis in NP			
Group	BMP-12 25	BMP-12 50	BMP-12 100
Sample	89	131	91
	78	99	89
	73	112	90
	150	147	125
	140	120	141
	178	134	113
	179	162	128
	165	171	139
	150	128	123
	145	84	160
	126	75	106
	156	85	125
	110	114	112
	90	114	135
	87	113	152
	146	131	227
	154	142	214
	148	118	229
	169	139	212
	181	153	233
	161	121	223
	152	165	214
	171	117	218
	164	145	176
	137	122	196
	131	129	111
	134	105	196
		123	135

	BMP-12 25	BMP-12 50	BMP-12 100
Mean	139	125	158
S.D.	32	24	49

Average Proteoglycan synthesis in NP for control is 140 pico mol S per sample

Table 10 Effect of rhBMP-2 on collagen synthesis in NP monolayer

Collagen Content in NP						
Group	control	BMP-2 25	BMP-2 50	BMP-2 100	BMP-2 200	BMP-2 300
Sample	89	226	225	284	526	639
	104	250	198	341	542	592
	102	277	289	330	490	377
	105	278	270	306	549	533
	92	139	217	283	786	569
	101	172	224	291	584	575
	110	160	210	296	573	661
	97	152	189	355	611	581
	116	115	141	236	184	235
	97	112	100	197	232	209
	99	170	161	142	154	163
	88	131	90	151	244	173
	76	169	180	293	181	160
	108	139	190	181	172	125
	104	126	164	169	160	122
	111	130	180	204	217	106
	91	168	192	486	175	122
	94	144	120	307	98	111
	93	122	164	334	134	101
	123	104	165	540	116	120
	121	94	128	366	241	203
	78	117	118	513	372	313
	101	149	185	301	357	301
	110	118	136	132	413	348
	123	165	114	138		
	131	251	222	182		
	97	236	259	145		
	87	288	232	147		
	88	196	171	148		
	81			224		
	97			191		
	87			230		
	116			238		
	99			367		
	100			353		
	84			408		

	control	BMP-2 25	BMP-2 50	BMP-2 100	BMP-2 200	BMP-2 300
Mean	100	169	180	272	338	310
S.D.	13	57	50	108	198	201

Table 11 Effect of rhBMP-12 on collagen synthesis in NP monolayer

Collagen Synthesis in NP			
Group	BMP-12 25	BMP-12 50	BMP-12 100
Sample	132	123	156
	149	112	154
	195	115	170
	156	117	135
	151	179	149
	126	142	169
	139	174	170
	156	155	156
	109	168	149
	130	124	124
	149	146	143
	96	153	100
	136	163	118
	145	229	108
	198	192	179
	110	183	63
	134	107	171
	249	124	231
	74	68	83
	166	174	107
	178	70	75
	63	183	76
	153	186	86
	150	139	116
	155	155	101
	116	138	166
	145	133	213
	136	109	155
			183

	BMP-12 25	BMP-12 50	BMP-12 100
Mean	143	145	138
S.D.	37	37	42

Average collagen synthesis in NP for control is 2159 counts per minute (CPM)

Table 12 Effect of rhBMP-2 on non-collagenous protein synthesis in NP monolayer

Non-collagenous protein synthesis in NP						
Group	control	BMP-2 25	BMP-2 50	BMP-2 100	BMP-2 200	BMP-2 300
Sample	89	226	225	284	340	321
	104	250	198	341	279	452
	102	277	289	330	308	189
	105	278	270	306	269	304
	92	139	217	283	358	426
	101	172	224	291	306	338
	110	160	210	296	303	333
	97	152	189	355	306	341
	116	143	129	176	136	170
	107	131	83	228	167	147
	101	162	116	192	139	134
	75	131	93	178	151	114
	96	203	188	190	150	132
	106	163	157	149	139	123
	115	147	127	181	141	126
	83	150	143	219	149	148
	88	110	108	218	166	147
	82	111	116	251	162	172
	99	121	103	246	72	163
	84	130	97	260	241	246
	107	128	109	278	394	402
	107	119	112	233	323	330
	132	126	113	270	378	386
	75	143	121	104		
	101	109	122	139		
	92	208	195	140		
	105	215	221	170		
	99	257	245	148		
	89	238	195	121		
	116			104		
	108			135		
	114			224		
	118			367		
	96			300		
	98			352		
	88					

	control	BMP-2 25	BMP-2 50	BMP-2 100	BMP-2 200	BMP-2 300
Mean	100	169	163	230	234	245
S.D.	12	53	58	76	96	113

Table 13 Effect of rhBMP-12 on non-collagenous protein synthesis in NP monolayer

Non-collagenous protein synthesis in NP			
Group	BMP-12 25	BMP-12 50	BMP-12 100
Sample	132	123	156
	149	112	154
	195	115	170
	156	117	135
	151	179	149
	126	142	169
	139	174	170
	156	155	156
	137	177	137
	144	167	116
	150	156	116
	129	141	80
	216	135	100
	172	181	90
	190	168	177
	129	203	57
	175	96	171
	164	91	168
	177	121	179
	176	98	159
	135	137	187
	185	105	172
	142	92	176
	184	107	157
	202	135	151
	154	136	142
	195	172	177
	192	135	134
			162

	BMP-12 25	BMP-12 50	BMP-12 100
Mean	163	138	147
S.D.	26	31	32

Average non-collagenenous protein synthesis in NP for control is 5981 CPM

Appendix B Part I: Growth Factor Study in AF Cell Culture

Table 14 Effect of RhBMP-2 and rhBMP-12 on PG synthesis in AF monolayer

Proteoglycan synthesis in AF							
Group	control	BMP2 25	BMP2-50	BMP2-100	BMP12-25	BMP12-50	BMP12-100
Sample	126	121	94	95	103	97	102
	93	92	88	100	101	95	108
	81	88	119	106	98	109	91
	121	113	116	118	88	98	104
	88	81	87	85	104	100	95
	90	106	97	97	109	102	101
	100	100	131	103	94	96	134
	96	105	85	84	104	119	76
	113	95	85	113	103	86	91
	91				97	87	88
	96				91	75	96
	115				101	73	95
	86				89	72	94
	100				98	75	89
	98				95	73	104
	96				110	110	120
	114				80	115	75
	88					120	
	97					125	

	control	BMP2 25	BMP2-50	BMP2-100	BMP12-25	BMP12-50	BMP12-100
mean	100	100	100	100	98	96	98
sd	13	13	17	12	8	17	14

Average Proteoglycan synthesis in AF for control is 289.38 pico mol S per sample

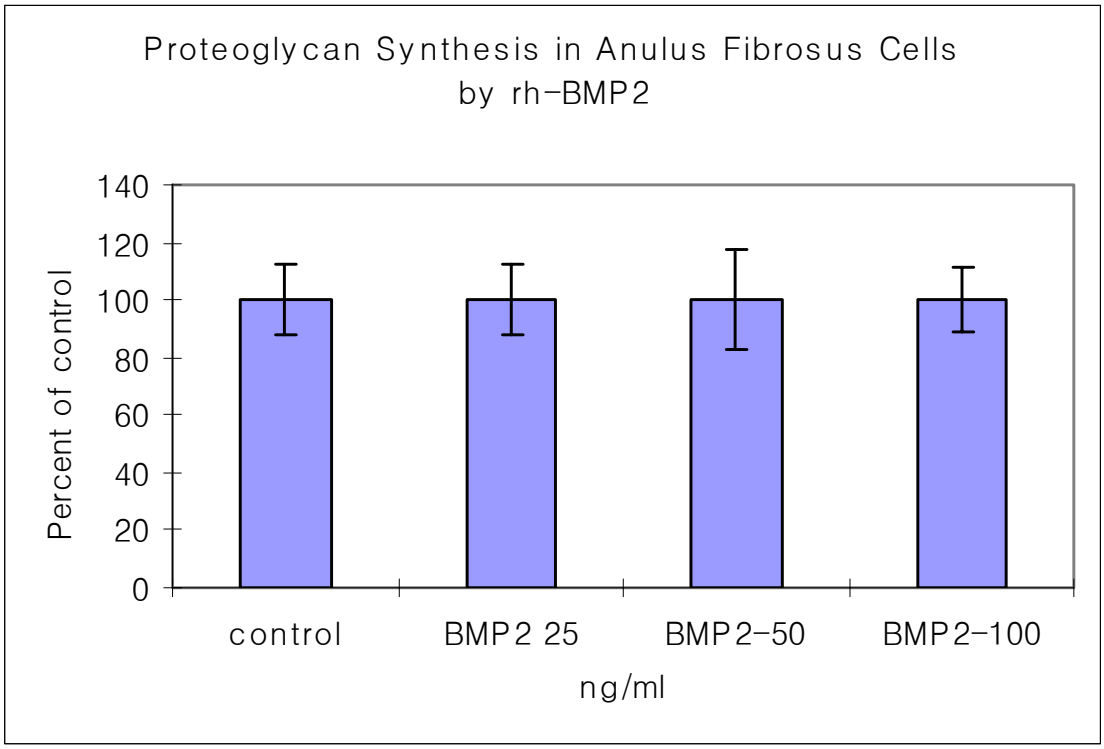


Figure 40 PG synthesis in AF treated with rhBMP-2

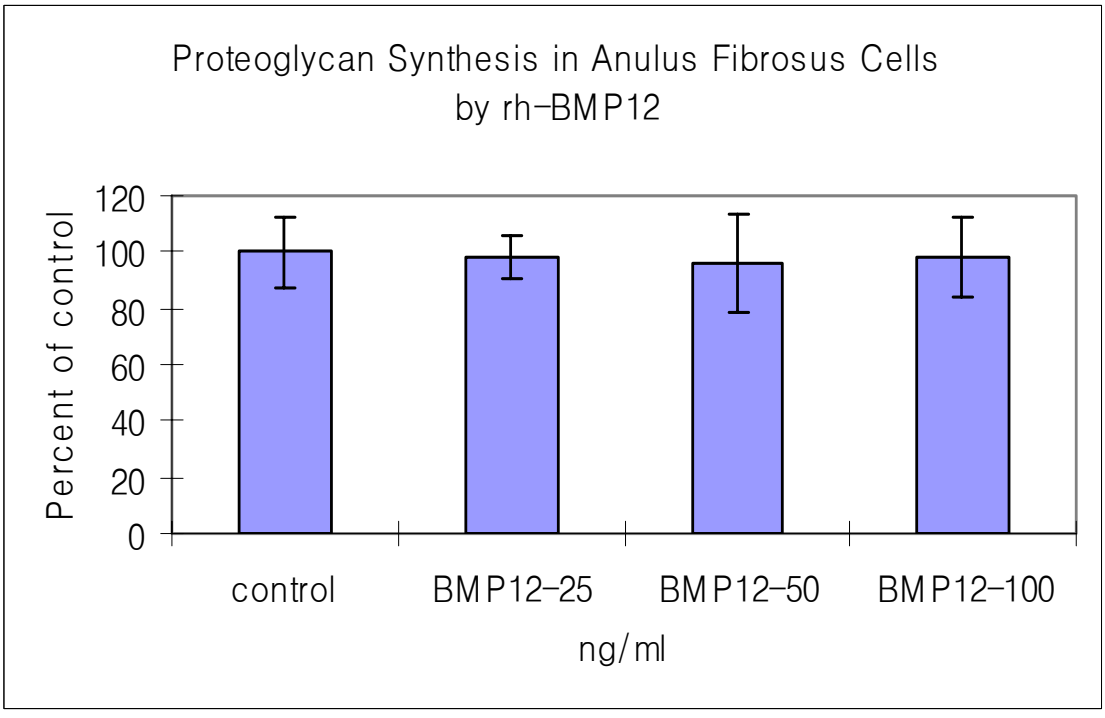


Figure 41 PG synthesis in AF treated with rhBMP-12

Table 15 Effect of rhBMP-2 and rhBMP-12 on collagen synthesis in AF monolayer

Collagen Synthesis in AF (% of control)							
Group	control	BMP2-25	BMP2-50	BMP2-100	BMP12-25	BMP12-50	BMP12-100
Samples	102	119	72	192	135	59	146
	94	63	86	123	57	62	52
	85	55	59	106	91	88	113
	119	155	111	115	139	59	88
	125	81	108	101	135	55	79
	70	84	39	105	91	73	85
	93	94	34	91	69	48	110
	113	111	59	60	59	103	75
	115	134	148	131	148	114	122
	111	122	81	120	130	111	152
	118	123	120	125	130	135	105
	56	153	115	184	158	135	126
	114				112	97	104
	118				99	111	80
	101				109	101	82
	99				101	99	104
	105				101	109	112
	89				91	109	104
	97				109	93	91
	95				103	67	114
	81				88		

	control	BMP2-25	BMP2-50	BMP2-100	BMP12-25	BMP12-50	BMP12-100
Mean	100	108	86	121	108	91	102
S.D.	17	33	35	36	28	26	24

Average collagen synthesis in AF for control is 11989.3 CPM

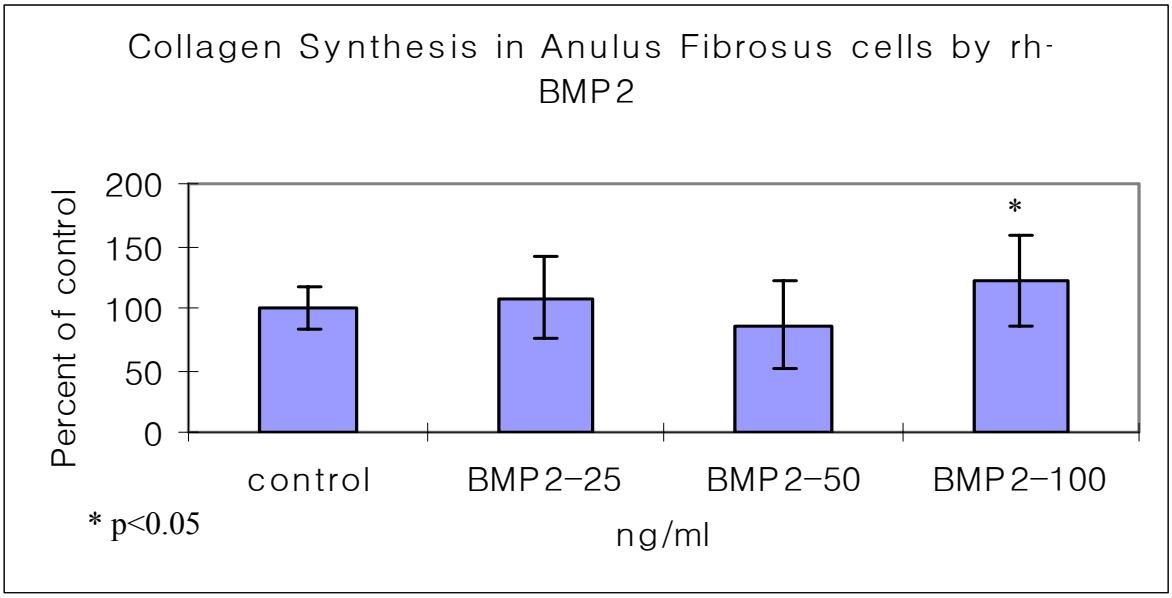


Figure 42 Collagen synthesis in AF by rhBMP-2

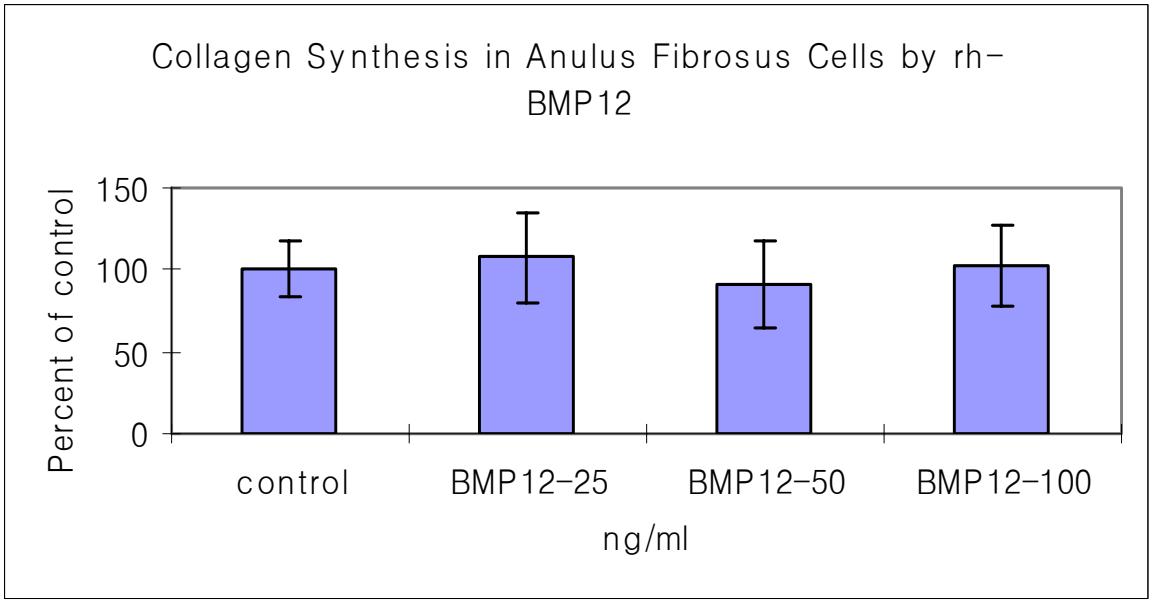


Figure 43 Collagen synthesis in AF by rhBMP-12

Table 16 Effect of rhBMP-2 and rhBMP-12 on non-collagenour protein synthesis in AF monolayer

Non-collagenenous Protein Synthesis in AF (percent of control)							
Group	control	BMP2-25	BMP2-50	BMP2-100	BMP12-25	BMP12-50	BMP12-100
Samples	100	138	77	165	137	53	133
	96	47	85	114	62	65	128
	84	44	55	89	88	72	93
	121	120	95	111	123	60	70
	109	96	161	195	154	104	161
	90	178	45	147	127	77	119
	73	159	43	247	111	116	180
	128	195	65	257	201	127	79
	101	124	121	105	152	127	111
	133	122	86	111	125	96	143
	109	116	126	121	136	118	107
	57	128	124	155	142	117	113
	90				87	107	119
	98				114	107	139
	118				115	124	123
	92				101	112	111
	97				123	102	121
	91				133	136	156
	96				102	149	129
	118				109	159	136
						185	193

	control	BMP2-25	BMP2-50	BMP2-100	BMP12-25	BMP12-50	BMP12-100
Ave	100	122	90	151	122	110	127
s.d.	18	45	37	56	29	33	30

Average non-collagenenous protein synthesis in AF for control is 15224.7 CPM

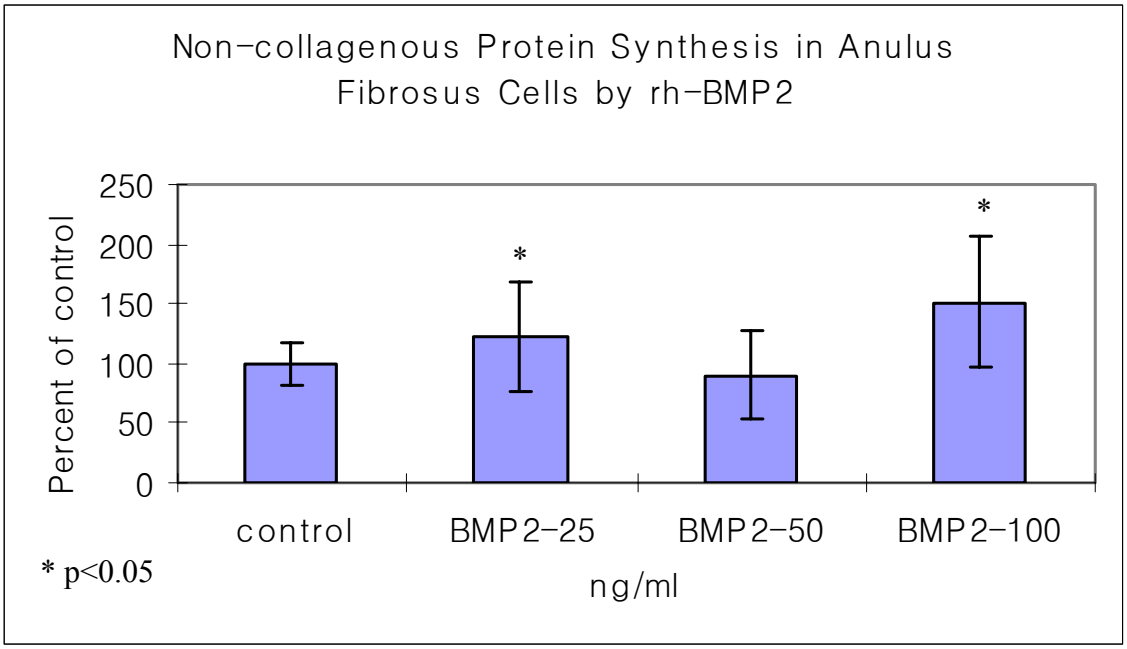


Figure 44 Non-collagenous protein synthesis in AF by rhBMP-2

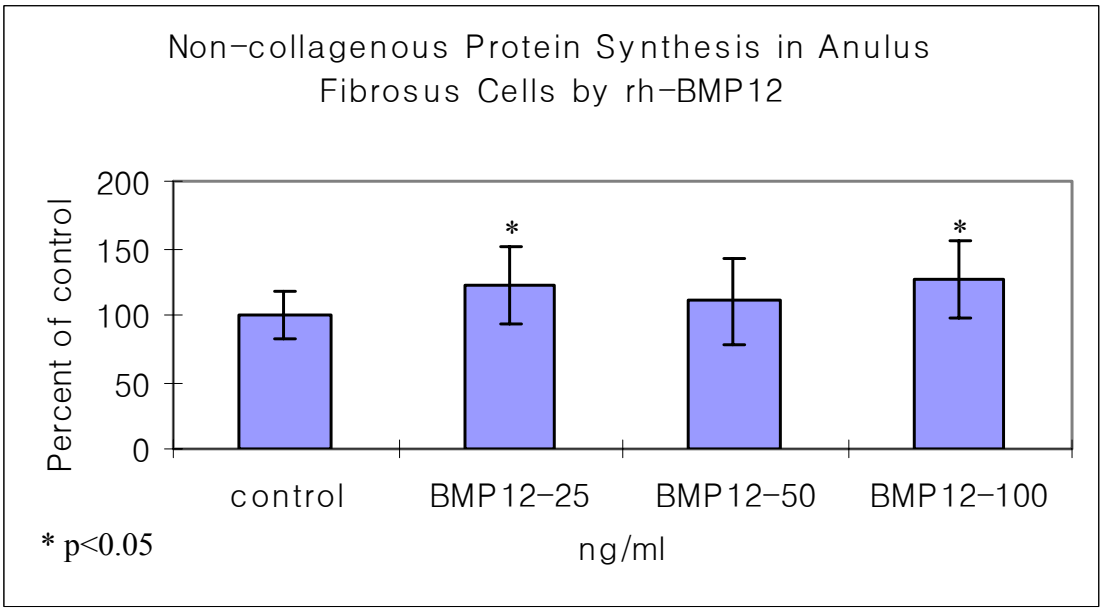


Figure 45 Non-collagenous protein synthesis in AF by rhBMP-12

Appendix C Part I: Gene Therapy Study in NP Pellet Culture

Table 17 Effect of Ad/BMP-12 on DNA content in NP pellets

DNA Content in NP				
Group	control	50MOI of BMP-12	100MOI of BMP-12	150MOI of BMP-12
Sample	87	201	186	195
	94	314	215	233
	107	218	218	198
	109	185	182	181
	106	184	182	182
	97	192	200	227

Concentration	control	50MOI of BMP-12	100MOI of BMP-12	150MOI of BMP-12
Mean	100	216	197	203
S.D.	8	50	16	22

Average DNA content in NP is 84.4 ng/pellet

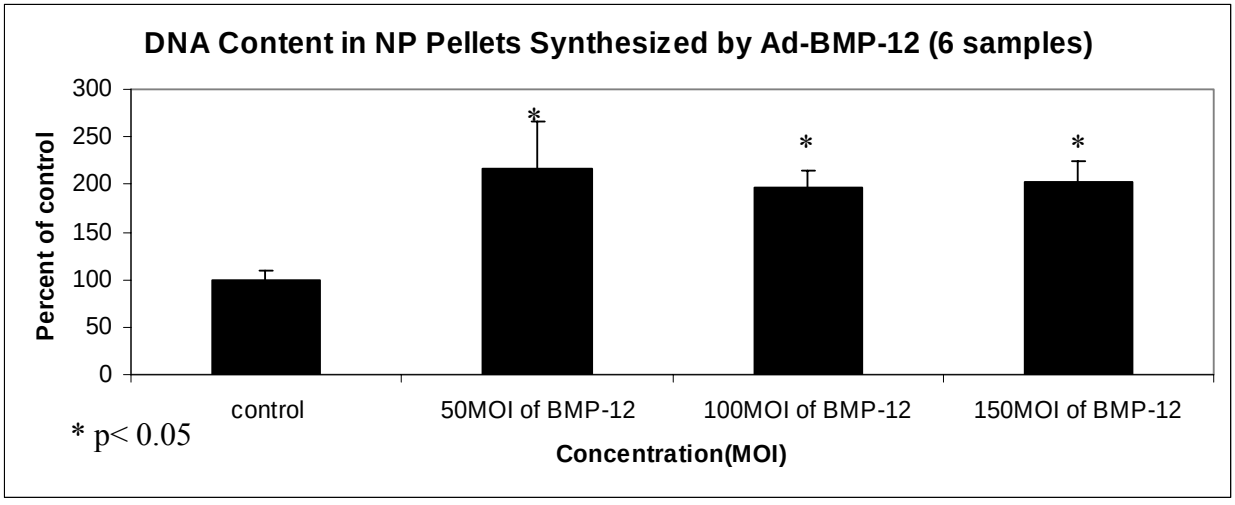


Figure 46 DNA content in NP pellets by Ad/BMP-12

Table 18 Effect of Ad/BMP-12 on PG synthesis in NP pellet (before normalization)

PG synthesis in NP pellet				
	Not Normalized by DNA Content			
Group	control	BMP12 50	BMP12 100	BMP12 150
Sample	79	383	354	461
	88	349	394	354
	98	398	419	501
	92	379	411	401
	102	433	450	453
	149			
	84			
	142			
	86			
	82			

	control	BMP12 50	BMP12 100	BMP12 150
Mean	100	388	406	434
S.D.	25	31	35	57

Table 19 Effect of Ad/BMP-12 on PG synthesis in NP pellet (after normalization)

PG synthesis in NP pellet				
	Normalized by DNA Content			
Group	control	BMP12 50	BMP12 100	BMP12 150
Sample	79	177	180	228
	88	162	200	175
	98	184	213	248
	92	175	209	198
	102	200	228	224
	149			
	84			
	142			
	86			
	82			

	control	BMP12 50	BMP12 100	BMP12 150
Mean	100	180	206	215
S.D.	25	14	18	28

Averaged PG for NP control is 55.95 pico mol S per pellet

Table 20 Effect of Ad/BMP-12 on collagen synthesis in NP pellet (before normalization)

Collagen Content before Normalization				
Group	control	BMP-12 50	BMP-12 100	BMP-12 150
Sample	80	305	430	340
	116	295	315	365
	103	431	360	293
	105	347	377	302
	113	401	437	349
	103	322	359	360
	78	347	384	223
	92	391	561	389
	71	394	358	405
	98	398	348	338
	105	416	340	
	87	409	318	
	125			
	92			
	106			
	119			
	94			
	106			
	86			
	123			

	control	BMP-12 50	BMP-12 100	BMP-12 150
Mean	100	371	382	336
S.D.	15	46	68	53

Table 21 Effect of Ad/BMP-12 on collagen synthesis in NP pellet (after normalization)

Collagen Content after Normalization				
Group	control	BMP-12 50	BMP-12 100	BMP-12 150
Sample	80	141	218	168
	116	137	160	181
	103	199	183	145
	105	160	192	149
	113	185	222	173
	103	149	182	178
	78	161	195	110
	92	181	285	193
	71	182	182	200
	98	184	177	167
	105	193	173	
	87	189	161	
	125			
	92			
	106			
	119			
	94			
	106			
	86			
	123			

	control	BMP-12 50	BMP-12 100	BMP-12 150
Mean	100	172	194	166
S.D.	15	21	34	26

Average collagen of NP control is 790.55 CPM

Table 22 Effect of Ad/BMP-12 on non-collagen synthesis in NP pellet (before normalization)

Non-collagenous protein before normalization				
Group	control	BMP-12 50	BMP-12 100	BMP-12 150
Sample	94	274	248	260
	101	288	225	296
	98	285	281	238
	92	255	264	286
	98	293	284	290
	93	268	297	286
	97	303	306	239
	101	373	448	330
	105	303	323	310
	103	230	275	411
	106	258	264	
	95	254	284	
	104			
	98			
	105			
	98			
	92			
	126			
	95			
	99			

		BMP-12 50	BMP-12 100	BMP-12 150
Mean	100	282	292	295
S.D.	8	36	56	50

Table 23 Effect of Ad/BMP-12 on non-collagen synthesis in NP pellet (after normalization)

Non-collagenous protein after normalizaion				
Group	control	BMP-12 50	BMP-12 100	BMP-12 150
Sample	94	127	126	129
	101	134	114	146
	98	132	142	118
	92	118	134	142
	98	136	144	144
	93	124	151	142
	97	140	155	118
	101	173	227	164
	105	140	164	153
	103	107	140	203
	106	120	134	
	95	118	144	
	104			
	98			
	105			
	98			
	92			
	126			
	95			
	99			

		BMP-12 50	BMP-12 100	BMP-12 150
Mean	100	131	148	146
S.D.	8	17	28	25

Average non-collagenous protein of NP control is 4686.9 CPM

Appendix D Part I: Gene Therapy Study in AF Pellet Culture

Table 24 Effect of Ad/BMP-12 on DNA content of AF pellets

Mean DNA content of AF pellets				
	control	BMP-12 50	BMP-12 100	BMP-12 150
Mean (ng DNA /pellet)	62	129	138	123

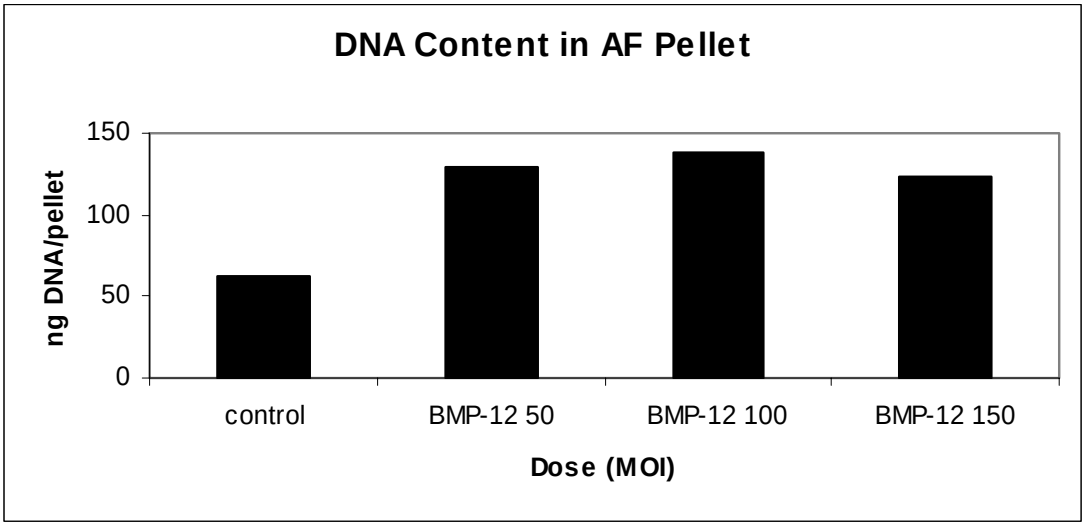


Figure 42 DNA content of AF treated with Ad/BMP-12

Table 25 Effect of Ad/BMP-12 on PG synthesis in AF pellets (before normalization)

PG in AF pellets before DNA normalization				
Group	control	BMP-12 50	BMP-12 100	BMP-12 150
Sample	84	417	272	276
	101	327	347	235
	96	307	275	263
	107	324	273	267
	100	317	256	260
		323	302	310

	control	BMP-12 50	BMP-12 100	BMP-12 150
Mean	100	336	287	269
S.D.	8	37	30	22

Table 26 Effect of Ad/BMP-12 on PG synthesis in AF pellets (after normalization)

PG in AF after DNA normalization				
Group	control	BMP-12 50	BMP-12 100	BMP-12 150
Sample	84	201	123	139
	101	157	156	119
	96	148	124	133
	107	156	123	135
	100	153	115	131
		155	136	156

	control	BMP-12 50	BMP-12 100	BMP-12 150
Mean	100	161	129	136
S.D.	8	19	15	12

Average PG for AF control is 55.94 pico mol S per pellet

Table 27 Effect of Ad/BMP-12 on collagen synthesis in AF pellets (before normalization)

Collagen Content in AF before DNA normalization				
Group	control	BMP-12 50	BMP-12 100	BMP-12 150
Sample	105	437	150	412
	109	243	441	365
	86	232	148	412
	106	293	303	321
	94	220	291	251
	100	189	274	221
			369	187
			323	136
			281	305
			245	253
				176
				276

	control	BMP-12 50	BMP-12 100	BMP-12 150
Mean	100	269	282	276
S.D.	9	89	89	90

Table 28 Effect of Ad/BMP-12 on collagen synthesis in AF pellets (before normalization)

Collagen Content in AF after DNA normalization				
Group	control	BMP-12 50	BMP-12 100	BMP-12 150
Sample	105	210	68	207
	109	117	199	183
	86	112	66	207
	106	141	137	162
	94	106	131	126
	100	91	123	111
			166	94
			145	68
			127	153
			110	127
				88
				139

	control	BMP-12 50	BMP-12 100	BMP-12 150
Mean	100	129	127	139
S.D.	9	43	40	45

Average collagen synthesis in control AF is 1284 CPM

Table 29 Effect of Ad/BMP-12 on non-collagen synthesis in AF pellets (before normalization)

Non-collagenous protein in AF before DNA normalization				
Group	control	BMP-12 50	BMP-12 100	BMP-12 150
Sample	90	237	184	213
	97	226	249	225
	109	224	185	226
	103	211	203	231
	100	224	232	190
		213	223	159
			232	168
			246	
			234	

	control	BMP-12 50	BMP-12 100	BMP-12 150
Mean	100	222	221	202
S.D.	7	9	25	29

Table 30 Effect of Ad/BMP-12 on non-collagen synthesis in AF pellets (after normalization)

Non-collagenous protein in AF after DNA normalization				
Group	control	BMP-12 50	BMP-12 100	BMP-12 150
Sample	90	114	83	108
	97	109	112	114
	109	108	84	114
	103	102	91	117
	100	108	105	96
		102	101	81
			104	85
			111	
			106	

	control	BMP-12 50	BMP-12 100	BMP-12 150
Mean	100	107	100	102
S.D.	7	5	11	15

Average non-collagenous synthesis in control AF is 5730.8 CPM

Appendix E Part II: Disc Nutrition Study in Organ Culture (NP Outcome Measures)

Table 31 Outcome measures of NP in fresh and cultured discs (2 week culture)

NP	Disc Level	Wet wt	Dry wt	% Hydration	GAG	DNA	Lactate
		mg	mg		ug/mg	ng/mg	mg/mg
fresh	L1-L2	30.7	4.3	86	548	1284	3.3
	L2-L3	17.9	3	83	663	1407	0.6
	L3-L4	26.1	3.5	87	679	1671	4.6
	L4-L5	28.3	5.2	82	621	1040	1.6
	L5-L6	26.6	4.5	83	670	1069	0.3
	L1-L2	30.6	4.7	85	553	1958	5.2
	L2-L3	19.7	2.6	87	606	2297	2.7
	L3-L4	16.6	2.2	87	585	2702	9.4
	L4-L5	26.2	4.4	83	663	1789	2.8
serumless	L1-L2*	30.9	4.8	84	559	1504	0.0
	L2-L3	29.1	4.6	84	541	1409	0.0
	L3-L4*	29.7	5	83	612	1291	0.0
	L4-L5	32.1	5.5	83	639	1236	0.5
	L5-L6*	30.8	5	84	730	1397	0.0
5% FBS	L1-L2	27.6	4.4	84	520	1495	3.6
	L2-L3*	28.2	4.8	83	440	1463	0.0
	L3-L4	26.8	4.9	82	589	1646	2.1
	L5-L6*	26	5.8	78	555	1263	0.0
10% FBS	L1-L2*	32.6	5.1	84	530	1591	1.4
	L2-L3	33.3	5.2	84	467	1391	5.6
	L3-L4*	32.5	5.6	83	760	1128	3.6
	L4-L5	33.1	6.1	82	585	1125	2.2
	L5-L6*	27.3	6	78	563	1066	2.7
15% FBS	L1-L2	25.2	3.9	85	535	1916	0.0
	L2-L3*	26.3	4.3	84	419	1820	7.2
	L3-L4	23.7	4.6	81	494	1778	5.0
	L4-L5*	25.8	5.2	80	521	1531	0.0

* Injected with Ad/BMP-2 ($6x10^6$ PFU) one week after culture, then cultured for one more week

Appendix F Part II: Disc Nutrition Study in Organ Culture (AF Outcome Measures)

Table 32 Outcome measures of AF in fresh and cultured discs (2 week culture)

AF	Disc Level	Wet wt	Dry wt	% Hydration	GAG	DNA	Lactate
		mg	mg				
fresh	L1-L2	88.8	22.2	75	226	198	0.1
	L2-L3	70.2	19.9	72	194	122	3.0
	L3-L4	99.1	30.8	69	265	289	1.9
	L4-L5	94.9	30.4	68	193	248	1.7
	L5-L6	136	42.4	69	228	214	1.4
	L1-L2	100.2	34.5	66	146	184	1.0
	L2-L3	53.7	16.8	69	155	243	2.0
	L3-L4	80	24.1	70	195	208	1.7
	L4-L5	69.9	18.9	73	446	259	1.8
serumless	L1-L2	94.2	27.9	70	150	238	1.2
	L2-L3	102.8	28.6	72	201	451	1.3
	L3-L4	96.8	30.1	69	211	462	1.1
	L4-L5	98.9	30.9	69	228	545	1.1
	L5-L6	100.5	32.4	68	211	519	0.8
5% FBS	L1-L2	66.2	20.9	68	134	796	3.3
	L2-L3	66.1	19.7	70	223	703	3.4
	L3-L4	87.8	27	69	228	895	2.1
	L5-L6	92.8	29.9	68	231	771	2.3
10% FBS	L1-L2	65.7	19.2	71	247	823	3.6
	L2-L3	64.9	19.2	70	210	751	4.7
	L3-L4	87.5	25.8	71	251	935	3.8
	L4-L5	103.8	32.5	69	289	670	2.5
	L5-L6	84.5	26.2	69	278	703	2.2
15% FBS	L1-L2	62.4	17.8	71	215	806	1.8
	L2-L3	71.1	19.6	72	186	714	5.4
	L3-L4	93.3	30.3	68	236	908	3.3
	L4-L5	108.6	36.1	67	196	844	2.7

BIBLIOGRAPHY

BIBLIOGRAPHY

[1] Lipson S.J.: "Aging Versus Degeneration of the Intervertebral Disc." The Lumbar Spine, Ed by JN Weinstein and SW Wiesel. Philadelphia, W.B. Saunders Company, (1990), pp. 261-265.

[2] Giot B., Fessler R., Molecular Biology of Degenerative Disc Disease, Neurosurgery, Vol. 47(5), (2000), pp. 1034-1040.

[3] Antoniou J., Goudsouzian N., Terrence F., Winterbottom N., Steffen T., Poole A.R., Aebi M., Alini M. The Human Lumbar Endplate: Evidence of Changes in Biosynthesis and Denaturation of the Extracellular Matrix with Growth, Maturation, Aging, and Degeneration, Spine, Vol. 21(10), (1996), pp. 1153-1161.

[4] Kang J.D., Stefanovic-Racic M., McIntyre L.A., Georgescu H.I., Evans C.H., Toward a Biochemical Understanding of Human Intervertebral Disc Degeneration and Herniation: Contributions of Nitric Oxide, Interleukin-6, and Prostaglandin E2, Spine, Vol. 21, (1996), pp. 1065-1073)

[5] Lipson S.J., Muir H., 1980 Volvo Award in Basic Science: Proteoglycans in Experimental Intervertebral Disc Degeneration, Spine, Vol. 6, (1981), pp194-210.

[6] Garfin S.R., Herkowitz, "The Intervertebral Disc: Disc Disease – Does It Exist?." The Lumbar Spine, Ed by JN Weinstein and SW Wiesel, Philadelphia, W.B. Saunders Company, (1990), pp. 369-380.

[7] Nachemson A.L., Jonsson E., Neck and Back Pain: The Scientific Evidence of Causes, Diagnosis, and Treatment, Philadelphia, Lippincott Williams and Wilkins, (2000), pp. 79-126.

[8] Deyo R.A., Cherkin D., Conrad D, Volinn E., Cost, controversy, crisis: Low back pain and the health of the public. Annu Rev Public Health , Vol. 12, (1991), pp. 14156.

[9] Oshima H, Ishihara H, Urban JPG, Tsuji H. The Use of Coccygeal Discs to Study Intervertebral Disc Metabolism. Journal of Orthopaedic Research, Vol. 11,(1993); 11:332-338

[10] Musculoskeletal Conditions in the United States by American Academy of Orthopaedic Surgeons (AAOS), (1999).

[11] Frymoyer J.W., Cats-Baril W.L. An overview of the incidences and costs of low back pain. Orthop Clin North Am, Vol. 22, (1991), pp. 26371.

[12] Kelsey JL, Mundt DF, Golden AL: "Epidemiology of low back pain." The Lumbar Spine and Back Pain, Ed by Jayson MIV, Churchill Livingsone, (1992) , pp537-549.

[13] Andersson G.B.J.: "Epidemiology", Essentials of the Spine. Ed. Weinstin JN, Rydevik BL, Sonntag VKH, New York, Raven Press, (1995).

[14] Praemer A., Furner S., Rice D.P.: Musculoskeletal conditions in the United States. Illinois: American Academy of Orthopaedic Surgeons, (1992), pp 23-33.

[15] Boden S.D., Wiesel S.W., Wolfe R.M.: "Standardized Approaches to the Diagnosis and Treatment of Low Back Pain and Multiply Operated Low Back Patients", Low Back Pain, Ed. By Wolfe R.M., Borenstein D.G., Wiesel S.W, Matthew Bender & Company, Inc., (2000), pp 93-157.

[16] Borenstein D.G., Wiesel S.W., Boden S.D.: "Medical Diagnosis and Comprehensive Management", Low Back Pain, WB Saunders Company, (1995), pp595-650.

[17] Boden S.D., Wiesel S.W., Laws E.R., Rothman R.H.: The Aging Spine: Essentials of Pathophysiology, Diagnosis, and Treatment, W.B. Saunders Company, (1991), pp 3-38, 167-189.

[18] Spinal Fusions in the United States by American Academy of Orthopaedic Surgeons (AAOS), (1999)

[19] Boden S.D.: Fusion: Biology of Lumbar Spine Fusion and Bone Graft Materials, Ed by JN Weinstein and SW Wiesel. Philadelphia, W.B. Saunders Company, (1996), pp 1284-1306.

[20] Esses S.I., Textbook of Spinal Disorders, J.B. Lippincott Company, (1995), pp135-172.

[21] Gruber H.E., Leslie K.P., Ingram J.A., Autologous Intervertebral Disc Cell Implantation: A Model Using Psammomys Obesus, the Sand Rat, Spine, Vol. 27, (2002), pp. 1626-33.

[22] Hutton W.C., Meisel H.J., Akamaru T., Autologous Disc Chondrocyte Transplantation for Repair of Acute Disc Herniation (Abstract), North American Spine Society, 16th Annual Meeting, Seattle, WA; 2001.

[23] Okuma J., Mochida J., Nishimura K, Reinsertion of Stimulated Nucleus Pulposus Cells Retards Intervertebral Disc Degeneration: an In-vitro and in-vivo Experimental Study. J Orthop Res, Vol. 18, (2000), pp988-97.

[24] Aguiar D.J., Johnson S.L., Oegema T.R., Notochordal Cells Interact with Nucleus Pulposus Cells: Regulation of Proteoglycan Synthesis. Exp Cell Res, Vol. 246, (1999), pp. 129-37.

[25] Thompson J.P., Oegema T.R. Jr., Bradford D.S., "Stimulation of mature canine intervertebral disc by growth factors", Spine, Vol. 16, (1991), pp. 253-260.

[26] Nishida K., Kang J.D., Gilbertson L.G., Moon S.H., Suh J.K., Vogt M.T., Robbins P.D., Evans C.H., "Modulation of the Biologic Activity of the Rabbit Intervertebral Disc by Gene Therapy: An In Vivo Study of Adenovirus-Mediated Transfer of the Human Transforming Growth Factor beta-1 Encoding Gene", Spine, Vol. 24, (1999), pp. 2419-2415.

[27] Moon S.H., Gilbertson L.G., Nishida K., Knaub M., Muzzonigro T., Robbins P.D., Evans C.H., Kang J.D., Spine, Vol. 25, (2000); 25, pp. 2573-2579.

[28] Hecht B.P., Fischgrund J.S., Herkowitz H.N, "The use of recombinant human bone morphogenetic protein 2 (rhBMP-2) to promote spinal fusion in a nonhuman primate anterior interbody fusion model", Spine, Vol. 24, (1999), pp. 629-36.

[29] Wozney J.M., Rosen V., "Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair", Clin Orthop, Vol. 346, (1998), pp. 26-37.

[30] Yoon T.S., Kim S.K., Li J., "The effect of bone morphogenetic protein-2 on rat intervertebral disc cells in vitro", Spine, Vol. 18, (2003), pp. 1773-80.

[31] Davidson A.J., Postlethwait J.H., Yan Y.L., "Isolation of zebrafish gdf7 and comparative genetic mapping of genes belonging to the growth/differentiation factor 5, 6, 7 subgroup of the TGF-beta superfamily", Genome Res, Vol. 9, (1999), pp. 121-9.

[32] Fu S.C., Wong Y.P., Chan B.P., "The roles of bone morphogenetic protein (BMP) 12 in stimulating the proliferation and matrix production of human patellar tendon fibroblasts", Life Sci, Vol. 72, (2003), pp. 2965-74.

[33] Furuya K., Nifuji A., Rosen V. "Effects of GDF7/BMP12 on proliferation and alkaline phosphatase expression in rat osteoblastic osteosarcoma ROS 17/2.8 cells", J Cell Biochem, Vol. 72, (1999), pp. 177-80.

[34] Lee K.J., Mendelsohn M., Jessell T.M., "Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord", Genes Dev, Vol. 12, (1998), pp. 3394-407.

[35] Settle S., Marker P., Gurley K., "The BMP family member Gdf7 is required for seminal vesicle growth, branching morphogenesis, and cytodifferentiation", Dev Biol, Vol. 234, (2001), pp. 138-50.

[36] Watakabe A., Fujita H., Hayashi M., "Growth/differentiation factor 7 is preferentially expressed in the primary motor area of the monkey neocortex", J Neurochem, Vol. 76, (2001), pp. 1455-64.

[37] Lou J., Tu Y., Burns M., "BMP-12 gene transfer augmentation of lacerated tendon repair", J Orthop Res, Vol. 19, (2001), pp. 1199-202.

[38] Wolfman N.M., Hattersley G., Cox K., "Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF-beta gene family", J Clin Invest, Vol. 100, (1997), pp. 321-30.

[39] Lou J., Tu Y., Ludwig F.J., "Effect of bone morphogenetic protein-12 gene transfer on mesenchymal progenitor cells", Clin Orthop, Vol. 369, (1999), pp. 333-9.

[40] Maroudas A., Stockwell R.A., Nachemson A., Urban J., "Factors involved in the nutrition of the human lumbar intervertebral disc: cellularity and diffusion of glucose in vitro", J. Anat. , Vol. 120, 1, (1975), pp.113-130.

[41] Holm, S.H.: "Nutritionof the Intervertebral Disc", The Lumbar Spine, Ed by JN Weinstein and SW Wiesel. Philadelphia, W.B. Saunders Company, (1990), pp 244-259.

[42] Oegema T.R.: "Recent Advances in Understanding the Biochemistry of the Intervertebral Disc", Disorders of the Lumbar Spine, Ed by Yizhar Floman. Aspen Publishers Inc. and Freund Publishing House Ltd., (1990), pp53-71.

[43] Horner H.A.M., Urban, J.P.G., "Effect of Nutrient Supply on the Viability of Cells from the Nucleus Pulpsus of the Intervertebral Disc", Spine, (2001), Vol. 26, pp. 2543-2549.

[44] Bernick S., Cailliet R., "Vertebral End Plate Changes with Aging of Human Vertebrate", Spine, (1982), Vol. 7, pp. 97-102.

[45] Holm S., Nachemson A., "Variations in the nutrition of the canine intervertebral disc induced by motion", Spine, (1983), Vol. 8, pp. 866-874.

[46] Holm S., Selstam G., "Oxygen tension alterations in the intervertebral disc as a response to changes in the arterial blood", Upsala J Med Sci, (1982), Vol. 87, pp. 163-174.

[47] Urban J.P.G., Holm S., Maroudas A., Nachemson A., "Nutrition of the intervertebral disc: An in vivo study of solute transport". Clin Orthop, (1977), Vol. 129, pp. 101-114.

[48] Holm S.H.: "Nutritional and Pathophysiologic Aspects of the Lumbar Intervertebral Disc", The Lumbar Spine, Ed by JN Weinstein and SW Wiesel. Philadelphia, W.B. Saunders Company, (1996), pp 285-309.

[49] Holm S., Maroudas A., Urban J.P.G., Selstam G., Nachemson A., "Nutritionof the intervertebral disc: Solute transport and metabolism", Conn Tiss Res, (1981), Vol. 8, pp. 101-110.

[50] Corrigan B., Maitland G.D., Vertebral Muskeletal Disorders, Reed Educational and Professional Publishing Ltd, (1998), pp1-17.

[51] Urban J.P.G., Maroudas A.: The Measurement of Fixed Charged Density in the Intervertebral Disc. Biochim Biophys Acta , Vol. 586, (1988), pp. 166-178.

[52] Urban J.P.G., MacMullin J.F., "Swelling Pressure of the Intervertebral Disc: Influence of Proteoglycan and Collagen Contents", Biorheology, Vol. 22, (1985), pp. 145.

[53] Johnston B., Bayliss M.T. The Large Proteoglycans of the Human Intervertebral Disc: Changes in their Biosynthesis and Structure with Age, Topography, and Pathology, Spine, Vol. 20, (1995), pp. 674-684.

[54] Urban J., Holm S.H., Lipson S.J.: "Biochemistry", The Lumbar Spine, Ed by JN Weinstein and SW Wiesel. Philadelphia, W.B. Saunders Company, (1990), pp 231-243.

[55] Voet D., Voet J.G., Pratt G., "Overview of Metabolism", Fundamentals of Biochemistry, John Wiley & Sons, Inc., (2002), pp354-359.

[56] Voet D., Voet J.G., Pratt G., "Overview of Glycolysis", Fundamentals of Biochemistry, John Wiley & Sons, Inc., (2002), pp383-406.

[57] Ishihara H., Urban J.P.G., Effects of Low Oxygen Concentrations and Metabolic Inhibitors on Proteoglycan and Protein Synthesis Rates in the Intervertebral Disc, Journal of Orthopaedic Research, Vol. 17, (1999), pp. 829-35.

[58] Hannallah D., Peterson B., Liebermann J.R., Fu F.H., Huard J., Gene Therapy in Orthopaedic Surgery, JBJS, Vol. 84-A(6), (2002), pp. 1046-1061.

[59] Anderson W.F., "Reviews: Human Gene Therapy", Nature, Vol. 392, (1998), pp. 25-27.

[60] Li S., Huang L., Nonviral Gene Therapy: Promises and Challenges, Gene Therapy, Vol. 7, (2000), pp. 31-34.

[61] Kwoh D.Y., Coffin C.C., Lollo C.P., Stabilization of Poly-L-Lysine/DNA Polyplexes for In-vivo Gene Delivery to the Liver, Biochimica et Biophysica Acta, Vol. 1444(2), (1999), pp. 171-190.

[62] Nishida K., Kang J.D., Suh J.K., Robbins P.D., Evans C.H., Gilbertson L.G., Adenovirus-Mediated Gene Transfer to Nucleus Pulposus Cells: Implications for the Treatment of Intervertebral Disc Degeneration, Spine, Vol. 23, (1998), pp. 2437-43.

[63] Oshima H., Ishihara H., Urban J.P.G., Tsuji H., "The Use of Coccygeal Discs to Study Intervertebral Disc Metabolism", Journal of Orthopaedic Research, Vol. 11, (1993), pp. 332-338.

[64] Bayliss M.T., Urban J.P., Johnstone B., Holm S., In-vitro Method for Measuring Synthesis Rates in the Intervertebral Disc. J. Orthop Res., Vol. 4, (1986), pp10-17.

[65] Chiba K., Andersson G., Masuda K., Momohara S., Williams J.M., Thonar E., A New Culture System to study the Metabolism of the Intervertebral Disc In-Vitro, Spine, Vol. 23(17), (1998), pp. 1821-1827.

[66] Takegami K., Masude K., An H., A Novel Culture System for the Intervertebral Disc: an Organ with Endplates (Abstract), Orthopaedic Research Society, 47th Annual Meeting, San Francisco, CA, (2001).

[67] Chelberg M.K., Banks G.M., Geiger D.F., Oegema T.R., "Identification of heterogenous cell populations in normal human intervertebral disc", J Anat, Vol. 186, (1995), pp. 43-53.

[68] Frisbie D.D., Kawack C.E., Trotter G.W., McIlwraith C.W., The Assessment of Chondrocyte Proteoglycan Metabolism using Molecular Sieve Column Chromatography as Compared to Three Commonly Utilized Techniques. Osteoarthritis and Cartilage, Vol. 6, (1998), pp. 137-145.

[69] Suzuki K., Oyama M., Faulcon L., "In-vivo Expression of Human Growth Hormone by Genetically Modified Murine Bone Marrow Stromal Cells and its Effect on the Cells In-vitro", Cell Transplant, Vol. 9, (2000), pp. 319-27.

[70] Alberts B., Bray D., Lewis J., Raff M., Roberts K., Watson J., Molecular Biology of the Cell, Garland Publishing, (1994), p 143.

[71] Asai D.J., Wilson L., Matsudaira P.: Methods in Cell Biology, Antibodies in Cell Biology, Academic Press, (1993), Vol. 37.

[72] Sanes J.R., Rubenstein J.L.R., Nicolas J.F., Use of a recombinant Retrovirus to Study Post-Implantation Cell Lineage in Mouse Embryos., EMBO J, Vol. 5, (1986), pp. 133-42.

[73] Farndale R.W., Buttle D.J., Barrett A.J., Improved Quantitation and Discrimination of Sulphated Glycosaminoglycans by use of Dimethylmethylene Blue, Biochimica et Biophysica Acta, Vol. 883, (1986), pp. 173-177.

[74] Chiba K., Andersson G., Masuda K., Momohara S., Williams J., Thonar E., "A New Culture System to Study the Metabolism of the Intervertebral Disc In-Vitro", Spine, Vol. 23, (1998), pp. 1821-1827.